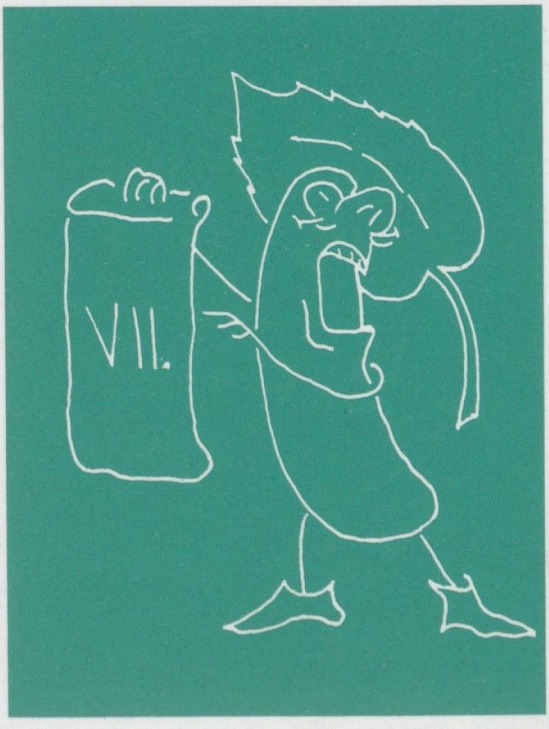
PROCEEDINGS OF THE
7th INTERNATIONAL CONFERENCE
ON PLANT PATHOGENIC BACTERIA
BUDAPEST, HUNGARY, JUNE 11–16, 1989

PLANT PATHOGENIC BACTERIA

PART A

EDITED BY
Z. KLEMENT



AKADÉMIAI KIADÓ, BUDAPEST

PLANT PATHOGENIC
BACTERIA

PARTICIPANTS
OF THE 7TH INTERNATIONAL CONFERENCE ON PLANT
PATHOGENIC BACTERIA



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Z. KLEMENT

Plant Protection Institute
of the Hungarian Academy of Sciences
Budapest, Hungary

AKADÉMIAI KIADÓ, BUDAPEST 1990

MTA
KIK 0 00003 74745 2

679472

The 7th International Conference on Plant Pathogenic Bacteria was sponsored by the International Society of Plant Pathology, Bacteria Section and Hungarian Society of Agricultural Sciences

MAGYAR TUDOMÁNYOS AKADÉMIA KÖNYVTÁRA

ISBN 963 05 5871 8 Parts A–B
ISBN 963 05 5872 6 Part A

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Printed in Hungary
by Akadémiai Kiadó és Nyomda Vállalat, Budapest

M. TUD. AKADÉMIA KÖNYVTÁRA
Könyvleltár 11850/19 90. sz.

PREFACE

The Organizing Committee and the Hungarian colleagues felt it a great privilege that the 7th International Conference on Plant Pathogenic Bacteria was held in Budapest, June 11-16. I think it was a great pleasure for all of us to see each other again and to establish good personal contacts with the new generation of phytobacteriologists from all over the world.

As far as I can remember, more than 25 years ago when the first meeting of phytobacteriologists was organized by Lelliot in Harpenden England, the number of participants was only 35 or 40 from a few countries. At the 7th international meeting more than 230 scientists were present from 42 countries: from East to West and from North to South. The increased number of participants shows the worldwide interest in phytobacteriology.

Just before the beginning of our conference I received the shocking news from Bob Goodman that our dear friend and colleague Prof. Mortimer Starr died on April 29 at 72 years of age, of cancer. Mortimer Starr a prolific thinker, experimenter and worker was a legend in phytopathogenic bacteria, was well known. I remember he was one of the first bacteriologists who visited us in Hungary in 1962 and later in 1979. He also intended to participate in our conference but unfortunately he could not be with us any more. The members of the conference stood in silence to mourn his passing away.

This conference was organized into 9 sessions as: Host-parasite relationship; Control; Biocontrol; Epidemiology; Genetics and Molecular Biology; Identification; Erwinias; Agro-

bacterium and Serology. Altogether 142 papers for oral presentation and 91 posters were presented. In the Second Circular of the conference we asked the opinion of contributors about the grouping of the lectures of the sessions. More than 60% of participants wanted to group lectures according to main topics. However in the case of soft rot bacteria we used another system ranging lectures from identification to genetics in the frame of one session. This system is followed by this Proceedings.

The Organizing Committee hopes that the Proceedings of the 7th International Conference on Plant Pathogenic Bacteria will be useful to all research scientists who are interested in this exciting field. It is hoped that the time spent in Budapest of the participants was both fruitful and enjoyable.

Z. Klement
Chairman of the
Organizing Committee

ACKNOWLEDGEMENTS

The Organizing Committee of the 7th International Conference on Plant Pathogenic Bacteria gratefully acknowledges the financial support received from the following sources:

Hungarian Academy of Sciences
Hungarian Ministry of Agriculture and Food
Hungarian Microbiological Society
Hokko Chemical Industry Co., Ltd., Japan
International Society of Molecular Biology

Special appreciation is also extended to Iringo Kecskés for her activity as hostess of our Conference and for cop editing the Conference Proceedings. The assistance of Zsuzsa Szöllősi, Csilla Kertai, Csilla Magyarfalvi, Judit Pogány and Katalin Salánki in contributing to the success of the Conference is especially appreciated by the Organizing Committee.

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WELCOME ADDRESS

Ladies and Gentlemen!

It is an honour and a pleasure to welcome you to the 7th International Phytobacteriology Conference in Hungary. On behalf of the Hungarian Academy of Sciences, the Hungarian Plant Protection Society and the Plant Protection Research Institute I would like to welcome particularly our foreign guests who were kind enough to come to our country to share common interests discussing problems of phytobacteriology.

By way of introduction to this Conference I would like to dwell for a moment on the extremely important results achieved in the field of plant pathological bacteriology. I would like to stress that in addition to the practical aspects of controlling plant diseases caused by bacterial infections, phytobacteriology contributed a series of important results as regards host-pathogen interaction, mechanism of pathogenicity and/or virulence of bacterial pathogens, genetic aspects of infection caused by *Agrobacterium* spp., the role of ice-nucleation in frost damages to plant etc.

Regarding current topics in phytobacteriology, one might feel that plant pathological bacteriology has lost its separate identity. Indeed, we see some of our colleagues dealing with genetical, biochemical and molecular biological aspects of bacterial diseases, rather than crop-oriented plant pathology *sensu stricto*. Only a few among us can be regarded today as crop-oriented plant pathologists. But for all that, for myself,

I am happy to continue being called a plant pathologist while hailing molecular biologists and biotechnologists as fellow workers in the same field.

I am convinced that we still have a series of important basic problems to be solved in the future that are related both to practical disease control as well as to the compatibility questions between the host and the pathogen.

Another problem was and is continuously initiated by public-minded people. This is to find several control measures that are environmentally safe and sound. In the era of successful chemical plant protection this seems a challenge. I am afraid that the appropriate answer is still not at hand! How to maintain high yield and, at the same time, reduce chemical pesticide used in the field and the greenhouse? This remains to be solved in the future. We have sooner or later, to answer this question!

Dear guests and colleagues! Demonstrating results and changing ideas on problems mutually interesting for us certainly will increase scientific understanding during this Conference and will improve personal friendship between scientists from many different countries. We are proud to have you with us here in Hungary. I only hope that we can make you as happy in being here, as we are to receive you. Have a successful meeting and a pleasant time in our capital!

Professor Zoltán Király
Academician
Head of the Plant Protection Society

PLENARY LECTURES

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

NEW TRENDS IN PHYTOBACTERIOLOGY

A. KELMAN

Department of Plant Pathology
University of Wisconsin-Madison
Madison, Wisconsin, USA

It is a great pleasure and an honor to present the opening address at the Seventh International Conference on Phytopathogenic Bacteria. The Conference brings together the leading scientists in the world interested in basic and applied studies on the unique bacteria that have evolved mechanisms to invade and cause disease in plants. This meeting does have special significance since not only is it the first time that we have met in Hungary, it also occurs at an exciting time politically when new opportunities have arisen for improved communication among scientists in countries that have very different political systems. In this hospitable environment in Budapest we are confident that the warm personal ties that already exist among us because of common scientific interests will be enhanced in the days ahead . We are very appreciative of the invitation from the Institute of Plant Protection of the Hungarian Academy of Sciences to come to Budapest and for the very excellent physical facilities and social functions that have been arranged on our behalf.

It is very appropriate for members of the Plant Protection Institute to serve as our hosts because scientists on their staff have been in the forefront of research in the area of phytobacteriogy for several decades. In preparation for this presentation I contacted a member of the research unit at the Institute for Scientific Information (ISI -publishers of Current Contents) and asked if they would kindly make a survey of the most highly cited papers in the field of phytobacteriology based on articles published in Phytpathology, the journal of the American Phytopathological Society. It was most interesting to learn from their study that Dr. Zoltan Klement, who is serving as Chairman of this Conference, was one ot the top ten most frequently cited authors in the field of plant pathology and phytobacteriology.

It is also worth noting that our conference is scheduled most appropriately in the year that has been designated as the Year of the Microscope to commemorate the 150th anniversary of the founding of the Royal Microscopical Society in England. At a Conference on phytobacteriology it is also important to remember our debt to this remarkable instrument and we can join in this recognition of the Year of the Microscope on behalf of the members of this Conference.

Over a century has passed since Robert Koch and Louis Pasteur first promulgated the concepts on which the field of phytobacteriology was to be developed. We are approaching now the date that marks the passage of one century since Erwin F. Smith, the father figure for our science, began his

seminal studies on bacterial diseases of plants. Recent progress in our science can be delineated by a variety of measuring devices; one involves the number and subject matter of papers given at annual meetings of a representative society. Thus, at the meetings of the American Phytopathological Society in the period from 1966 to 1981, the number of papers on bacteriological subjects increased from 17 to 106 (Table 1).

Table 1. Phytobacteriology papers presented at annual meetings of American Phytopathological Society

	1966	1971	1976	1981
Total Number of Papers	248	210	420	582
Total - Bacteriology	17	28	62	106
Percent - Bacteriology	6.9	13.3	14.8	18.2

This represents an almost three fold increase in the total number of papers in a 15 year period. This increase in numbers of papers continued during the next 7 year period, reaching a total of 131 at the last annual meeting in 1988 (Table 3). Similar increased interest in research on bacterial plant pathogens has been evident at the International Congresses on Plant Pathology. With respect to subject matter, it is interesting to note that there were no papers on genetics or molecular biology presented in 1966 and only 4% of the total presented in 1971 were concerned with molecular biology; however, the percentage in these areas increased to 12% of the total by 1981 (Table 2) and to 28% in 1988 (Table 4). Another interesting development has been the growth of interest in biological control involving bacteria as subjects for or agents of control. In 1966 and 1971 there were no papers on biological control (Table 2); however by 1988 the percentage had increased to 18% of the total in phytobacteriology (Table 4). The shifts in emphasis reflected in these tables are also manifest in the topics that will be presented in the course of this conference when contrasted with the subject matter of previous conferences.

Table 2. Percentage of papers in Phytobacteriology

Areas	1966	1971	1976	1981
Genetics	0	4	8	12
Physiology	47	39	39	30
Biological Control	0	0	6	12
Epidemiology	6	7	10	24

Table 3. Phytobacteriology papers presented at annual meetings of the American Phytopathological Society

	1983	1986	1988
Total Number of Papers	625	735	825
Total - Phytobacteriology	83	124	131
Percent - Phytobacteriology	13	17	15

Table 4. Percentage of papers in phytobacteriology

	1983	1986	1988
AREAS			
Genetics/ Molecular Biology	8	14	28
Physiology	16	14	22
Biological Control	16	17	18
Epidemiology	12	19	16

It has become obvious that there are great opportunities now to apply the powerful tools of molecular biology and genetic engineering in the resolution of a myriad of long standing problems. With the aid of R-DNA techniques we have the bright prospect of discovering the answers to the perplexing questions such as what is the biochemical and genetic nature of disease resistance and what are the basic mechanisms of pathogenesis. In addition the new techniques of bioengineering will make it possible to develop improved biocontrol agents, to introduce genes for insect and disease resistance into major crop plants, to determine the genetic basis for host specificity and virulence in plant pathogens and to improve greatly the techniques for rapid, reliable detection and identification of causal agents. Genetic engineering also holds the promise of enabling us to monitor the fate of modified organisms in the environment, and to analyze population dynamics of pathogens and non-pathogens with new-found precision (5).

One of the beneficial aspects of the advent of new approaches in biotechnology is the fact that there has been an influx of scientists from biochemistry, molecular biology, and genetics into the field of phytobacteriology. These young scientists are bringing new perspectives and levels of expertise into many departments of plant pathology and to research institutes as well as those companies established to develop new products for biocontrol agents or to engineer plants with disease resistance. Phytobacteriology stands at the threshold of a new era with the technology to advance our field in ways that few of us could have envisioned even one decade ago.

During these recent years we have also come to realize that the attitude of the public has changed in most developed and many developing countries with respect to scientists and risk/benefit balance for technological advances (4,5,7,10). This is perhaps best illustrated by the fact that there was much opposition to the release of genetically engineered strains of Pseudomonas syringae (minus the ice-nucleation gene). Concerns of ecologists as well as the strong opposition of a number of self-styled public interest groups raised public interest to the extent that the proposed field experiments received world-wide media coverage. Thus, the study on release of an ice-minus strain became the most highly publicized experiment in the history of biological sciences and called attention to the field of phytobacteriology in a most unexpected manner.

This leads to the recognition that we need to be more sensitive to societal and ethical questions about our research findings than we have ever been in the past. This is essential if we are to cope with many of the current misconceptions about hypothetical dangers that may be associated with the introduction into the environment of bacteria modified by the use of recombinant DNA techniques. Discussion of release of genetically engineered organisms into the environment has been cited in the press as a conflict between molecular biologists and ecologists. However, full recognition has not been given to the fact that the resolution of many of the introduction questions will depend on phytobacteriologists rather than ecologists or molecular biologists Thus, we urgently need to improve our ability to explain and interpret to the general public the nature of the art and science of plant pathology and phytobacteriology, in particular, as well as the potential gains to be derived from support of our research and its application. It is ironic that many of those groups most concerned about the dangers of the extensive use of pesticides also have been leading the battle to impose stringent and in some instances unwarranted regulations on the release of genetically modified organisms although these biocontrol agents provide the best hope for reducing pesticide pollution (8).

In addition to the factors associated with public perceptions of our science and its application, environmental changes of great import are taking place in the world in which we live. If we considered our planet earth to be a living entity and gave it a physical examination today, we would have to admit that many of its key vital organs are in a state of decline. Measurements of CO_2 and other gases, such as chlorofluorocarbons and methane, indicate that these compounds have increased every year since 1958 when careful measurements were first instituted. If the so-called "greenhouse effect " will become a reality and if in addition dramatic changes in rainfall and temperature occur, an entire new set of disease and insect problems may appear in large areas of the world. It is difficult at this stage to develop solid predictions for the future, but certainly the patterns of distribution and severity of bacterial diseases may be significantly altered. In view of the high sensitivity of bacterial pathogens to changes in temperature and moisture, particularly foliage pathogens, bacterial diseases may be affected more by some of the predicted climatic changes than diseases caused by other groups of pathogens.

One of the difficulties that we face in striving to insure the effectiveness of improved biological control methods is the lack of quantitative data on the ecology of soil-borne as well as foliage pathogens (8,9). Such information is also essential for a responsible approach to the development of rational regulations on release of genetically engineered

organisms. A number of elegant and sensitive techniques have been developed that have great promise in leading the way for other developments in this area. One of these involves the use of a strain of Pseudomonas fluorescens with the genetic marker known as "LacZY" which provides a means for detecting tiny populations of the bacterium in soil.

One of the exciting developments in recent years has been in the rapid advances that are being made in instruments that greatly expand our ability to examine the manner in which plants react to invasion by microorganisms and to analyze the chemical composition of cell walls and organelles in the tissues. Some of the major new types of equipment make it possible to examine tissues by differential interference contrast microscopy, magnetic resonance imaging, energy dispersive x-ray microanalysis, and Fourier Transform infrared spectroscopy (2,3). Few phytobacteriologists have reported on the use of these relatively sophisticated procedures although these techniques have a great potential for a range of new analytical approaches in studies on host/pathogen interactions (3). In particular magnetic resonance imaging provides a means of analyzing water status in tissues of stems, fruits and storage organs such as potato tubers without destruction of the tissue to be examined (1). We have used this equipment to determine whether there are defects in potato tubers that are not visible to the eye such as hollow heart, black spot and internal brown spot. By means of Fourier Transform infrared spectroscopy it is possible to "see" in a chemical sense inside plant material in a manner never possible previously (3).

Partly because of growing public opposition to the use of animals for teaching, as well as for biomedical research, and the difficulties associated with experiments on animals, most introductory courses in microbiology do not provide laboratory exercises for students that demonstrate Koch's Postulates. Many bacterial diseases of plants are ideal systems for use in such introductory courses (6). It is unfortunate that undergraduates are deprived of the thrill of rediscovering the conceptual basis for one of the most important advances that has been made in microbial and medical science. Those of us who work in universities should make the effort to convince our colleagues in microbiology that they should utilize bacterial diseases of plants to illustrate Koch's Postulates in introductory courses. This would be an effective means of attracting students to the prospect of future careers as researchers in phytobacteriology.

A potential model system for studies on host/pathogen relationships that warrants study by phytobacteriologists is the use of rapid-cycling brassicas that are suspectible to Xanthomonas campestris. The "Wisconsin Fast Plants" that were developed by Dr. Paul H. Williams (11) can be grown at a density of 880 plants/sq. meter; they flower in 15-18 days and cycle from seed to seed in five weeks. Thus, it is possible to obtain 10 generations in a given year. Various quantitative traits and markers for physiological characteristics are available for researchers. Rapid-cycling forms of Brassica rapa have many other advantages including the ability to be crossed with turnip and Chinese cabbage.

An important aid in the use of improved genetic techniques for the modification of plants and microbes is the availability of personal computers that place within the reach of all of us data recording and analytic systems with incredible capacity and power. We now have the ability with computer programs to develop improved methods for predicting the patterns of bacterial disease epidemics.

One of the major problems affecting the activity of international research groups has been the lack of funding for meetings on specific bacterial diseases and the need for continuing a rapid exchange of ideas and suggestions after conferences of this type. However, the lack of means for rapid communication no longer exists if we avail ourselves of the computer communication networks already in place or in the process of development. Scientists in other fields such as physics and meteorology have been able to undertake major cooperative research projects involving investigators in many countries. International research groups in these fields have been able to make major advances that could never have been completed by an individual scientist or a single laboratory group. International meetings and conferences facilitate the exchange of information and working groups have cooperated on specific programs. However, we have not as yet fully developed the mechanisms to enable close cooperation of a number of independent researchers working on one disease or research problem. If Erwin F. Smith were alive today, he might well ask why we still do not know the biochemical basis for disease resistance against a single bacterial disease.

The time is at hand for a new effort to enhance and stengthen cooperative research on a few selected diseases for which model systems are now available. As part of this approach, steps should be taken to establish communication networks for researchers on several major diseases. Perhaps the Committee on Bacteriology of ISPP that sponsors this conference should explore this possibility. At present, other than the Committee on Taxonomy, there is little activity or interaction at the international level to advance the establishment of multinational working groups in phytobacteriology in addition to the groups such as those interested in citrus canker, bacterial blight of rice, fireblight, and soft rot diseases. These groups schedule periodic meetings and conferences, but are not involved in active cooperative research projects at the level possible.

At the Vth International Conference on Plant Pathogenic Bacteria held in Cali, Colombia, in 1983 Dr. Anne Vidaver, at that time Chairperson of the Bacteriology Section of the International Society of Plant Pathology, opened the conference with a talk entitled "The Challenges in Plant Pathogenic Bacteriology in the Year 2000" (12). The clear vision of future developments that she presented at that time are as applicable six year later as they were at the time of her presentation. In the subjects covered in my discussion I have touched upon a number of these opportunities for future advances in phytobacteriology. However, in conclusion I wish to reiterate a point that was made in her talk. That concerns the responsibility that we have in guiding the younger members of our science. At a time when there is fierce competition for limited funds, there is often a tendency to limit our vision of research objectives to safe research proposals that are likely to obtain reportable results. There is a tendency to avoid the type of research that explores new areas in innovative or unconventional ways. We must encourage all of our colleagues to strive to think creatively and to be willing to accept the prospect that research ventures may fail. The solutions to the many complex problems that are still unresolved will not come to those who only try what is safe.

The prospects for future rapid advances in the field of phytobacteriology are the brightest that they have ever been. It has been a great pleasure to have had the opportunity to work during a period when the doors are opening wide to exciting research opportunities far beyond the dreams that many of us had when our careers were starting.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

RECENT TRENDS IN BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF BACTERIAL PLANT PATHOLOGY

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FLAGELLA

Flagellar activity is a key to infection and the report of Panopoulos and Schroth (1974) unequivocally stamps motility as a factor that can potentiate the intensity of pathogenesis. Using their calculation of flagellar-induced speed for a bacterial cell of 25 um/sec, makes their movement approximately 20 times faster than man.

CHEMOTAXIS

Chemotaxis has frequently been evaluated as a process whereby motile soil inhabiting and other plant pathogenic bacteria locate their hosts or infection sites expeditiously. Studies by Chet et al (1973) and by Raymundo and Ries (1980) reported Pseudomonas lachrymans and Erwinia amylovora were attracted to a number of organic compounds however, no causal relationship was established between chemotaxis and pathogenesis. An early study of Riker (1923) using the capillary tube assay, more recently popularized by Adler (1969) and initially devised by Pfeffer in 1888, revealed that A. tumefaciens was attracted to expressed tomato stem sap from which he concluded that a chemotactic stimulus might account for entrance of this pathogen into a wound. The likelihood of chemotaxis being causally related to tumorigenesis has recently been given additional support. Ashby (1988) from Shaw's laboratory have presented considerable evidence to suggest that acetosyringone, sinapinic acid and syringic acid at 10^{-7} M attract A. tumefaciens and engender directional movement. Further, Stachel (1985) and co-workers as well as Shaws' group have clearly established that these compounds were gene inducers that participated a priori in the transfer

process of T-DNA into the wounded host cell and its' eventual integration into the host genome, Parks et al., 1987. Ashby et al (1988) established that specific substitution of a benzene ring was necessary for optimum chemotaxis and vir induction. Bolton and co-workers (1986) established the key role of the 4' hydroxyl group in vir induction. For optimum plasmid-controlled chemotaxis and vir induction it seems that in addition to 4' hydroxyl group the 3' and 5' orthomethyl groups and a polar substituent at the 1' position are also important. Hence it would appear that vir genes function solely in T-DNA transfer, a process which is turned on by certain phenolic compounds. The receptor for the phenolic signal is according to Winans et al (1986) a cytoplasmic membrane vir A protein which interacts with the soluble vir G protein which promotes transcription of the remaining vir genes that complete the transfer, Zorreguieta et al., 1988.

ATTACHMENT

It is also clear now that for A. tumefaciens attachment is a necessary prelude to the transfer of bacterial DNA to the wounded host cell (Douglas et al., 1982). The bacterial genes that appear to control attachment are in the main chromosomal. The attachment sites on the surface of the host plant appear to be the cell wall per se, more specifically the pectinaceous components of the wall. Bacterial attachment is controlled as mentioned previously by 5 chromosomal loci chvA, chvB, exoC, cel and att. Basically, it is believed that chromosomal genes for a .1-2 glucan and cellulose biosynthesis are the contributors to attachment from the bacterial cell point of view.

A further specific and apparently unique feature of pathogenesis engendered by A. tumefaciens is the genetic information provided by the bacterial cell to its host the capacity to produce specific amino acids (opines) that only the pathogen can catabolize and use as a source of nitrogen and carbohydrate. Thus the pathogen programs the host cell for its' unique dietary requirements.

In passing it is interesting to note that several of the features associated with A. tumefaciens pathogenesis are integral to infection of legumes by Rhizobium sp. For example the acidic polysaccharide of R. trifolii interacts specifically with clover root hair surfaces and the lectin trifoliin A and plays a determining role in host specificity (Abe et al. 1984)

Although research is in progress concerning the role of newer toxins, ie., syringotoxin, tagetitoxin and others, improved biochemical techniques and equipment has permitted further definition of so-called older toxins. For example tabtoxin, which was first reported 72 years ago as a toxic metabolite of Pseudomonas syringae pv tabaci, is now known to be tabtoxinine .-lactam which is also produced by a number of closely related pseudomonads that induce analogous symptoms in tobacco, oats, beans, soybean. Although some uncertainty still attends this group of toxins as to whether tabtoxin or . lactam - tabtoxinine is the toxigenically active form the latter seems now to be the active configuration. It causes leaf chlorosis by inhibiting glutamine synthetase (GS) whereas tabtoxin is inactive in this regard. As a consequence of GS inhibition ammonia accumulates, which may potentiate bacterial growth in situ. The ammonia is also causally linked to the chlorosis and subsequent development of necrosis. The sequence of critical events has been illustrated more clearly by the research of Turner and Debbage, 1982.

A recent review by Kosuge and Yamada (1987) has made our concept of toxins a bit more complex by suggesting the IAA, though itself not a phytotoxin, is a virulence factor. For example it is IAA, synthesized by P. syringae pv. savastanoi, which causes gall formation in oleander and olive. Biosynthesis proceeds as determined by Kosuge and his associates by converting tryptophan to indole-3-acetamide which in turn is hydrolyzed to IAA. The process is identical to the sequence of IAA synthesis in plant cells that have been transformed by A. tumefaciens to develop tumorigenically.

An interesting series of studies have recently been productive in unravelling the role of the phytotoxins syringomycin (SR) and syringotoxin (ST) in disease development. Both toxins are small peptides produced by numerous strains of Pseudomonas syringae pv. syringae. However, SR is produced by isolates in a number of host species whereas ST is produced by isolates only in Citrus. The antibiotic qualities of SR have long been known, however, proof of its participation in disease development has been hampered by the inability to demonstrate its presence in infected tissue. The necrogenic feature of SR is a potent one, being active in plant tissue at 1 ug/ml, however, its actual participation in disease development was demonstrated by the development of SR^- or tox^- mutants of the pathogen. Gross and his associates (Xu & Gross 1988) developed mutants with as little

as 17% of the pathogenic potential of the wildtype strains. Although these mutants grew in host tissue at levels of 10^5- 10^7 CFU's/cherry fruit the wildtype strain engendered populations of 10^9 CFU/fruit. It was concluded that SR was not essential for pathogenesis but indeed contributed significantly to virulence by causing more extensive necrotic lesions to develop.

Tn5-induced mutants defective in the synthesis of ST have been isolated and characterized by Morgan and Chatterjee (1985). These ST^- mutants revealed alterations in the presence of two large proteins that are implicated in the biosynthesis of ST. The mutants contained truncated versions of two large proteins, presumed to be synthetases for ST synthesis. Morgan & Chatterjee observed that the appearance of the two presumptive synthetases parallels the kinetics of toxin accumulation during growth of the P. syringae pv. syringae. Thus a genetic model has defined, at least presumptively, a role for ST in symptom development.

With respect to the mode of action of syringomycin, its activity in planta must be interpolated from its effect on corn and yeast cells. Studies, conducted by Riedl and Takemoto (1987) suggests the SR induces a K^+ efflux from suspension cultures of corn and yeast. They have hypothesized that a transport protein in the plasma membrane of the suspension culture cells is activated allowing an electrogenic K^+ efflux and a simultaneous counter H^+ influx.

EXTRACELLULAR POLYSACCHARIDE (EPS)

A relationship of bacterial extracellular polysaccharide has long been surmised for both pathogenesis and virulence in a number of plant bacterium interactions. The clearest association is between A. tumefaciens and its numerous host species. An essential prelude to tumorigenesis is attachment of the bacterium to the host cell wall. This is believed to be at least in part mediated by a .1-2 glucan whose synthesis is largely chromosomally controlled Kamoun et al. 1988. There are at least 5 chromosomal loci that are involved in attachment that appear to mediate attachment via their influence on polysaccharide synthesis. Two of these chv A and chv B are linked and required for virulence according to O'Connell and Handelsman (1989). Strains carrying mutations at these loci are defective in plant attachment and are as a consequence avirulent. A third mutant exoC (succinoglycan negative), initially detected in Rhizobium meliloti was subsequently exposed as a locus on the chromosome of A. tumefaciens. These

mutants are unable to attach to plant cells in vitro and form tumors. We have in our laboratory developed some evidence that attachment is to some extent mediated by the Ti plasmid. This evidence suggests that the plasmid controls the rate at which attachment takes place and may reflect the chemotactic influence of either vir A or the chemoattractant per se (Pu and Goodman, unpublished). The precise role that attachment plays in the subsequent sequence of T-DNA transfer and transformation can only be surmised at this time. Electron micrographs of Tarbah & Goodman (1987) suggest that EPS may position bacteria for efficient nucleic acid transfer.

The subject of T-DNA per se transfer remains to this time an unanswered question. However, a series of EM's have suggested that release of the DNA in a protected form occurs upon lysis of the bacterium (Tarbah and Goodman, 1988). We have hypothesized that the lytic event is programmed by phenols such as acetosyringone that induce a series of morphologic changes to occur in the bacterial cell that conclude with lysis.

Closely linked to virulence, but not causally, is EPS produced by Erwinia amylovora and Psuedomonas solanacearum. It remains to be shown whether this association is other than fortuitous. But clearly, the loss of EPS by isolates of either pathogen usually results in a loss, or at least a diminution of, virulence, Fett et al, 1986.

SOFT-ROT ENZYMES

Research has continued unabated on the subject of tissue maceration by the so called soft-rot bacteria. Earlier studies by Bateman and his colleagues (1976) clearly established the pectin degrading enzymes as integral to this process. More specifically, it was all but conclusive that dissolution of the plant cell wall put degenerative stress on the plasmamembrane which was the ultimate cause of cell death. However, with the exposure of 4 pectin lyases synthesized by Erwinia chrysanthemi came the question, were all necessary and/or equally active? The results of Thurn et al (1987) established Pel E (of the quartet A,B,C, and E) to constitute a major portion of the total pectate lyase activity of E. chrysanthemi and that the homologous sequence of pel E occurs in all E. chrysanthemi strains. In addition Chatterjee noted that despite the similarity of an endo-mode of substrate cleavage by the Pel B,C,A and E enzymes,B and C produced oligimers of galacturonate, an unsaturated digalacturonate was the major end-product of Pel E and Pel A. With respect to the ability to induce maceration,

electrolyte loss and cell death, Pel E was the most active followed in decreasing order by Pel B and C. Pel A did not elicit any of these cell damaging reactions. It must still be contended that species and even varietal differences in the plant cell wall provide the raison d'etre for the array of pectin degrading enzymes and the cooperative actions of polygalacturonases, pectin-methyl esterases, proteases, phospholipases, cellulases and xylanases.

The story as depicted by Willis and co-workers (1987) suggest that Erwinia cerotovora pv carotovora produces 5 major Pels (1,2,3,4 and 5, one endo-Peh and one exo Peh (polygalacturonase). Two of these Pel enzymes are periplasmic and 3 are extracellular. It had earlier been postulated that E. c. pv carotovora produces an enzyme that causes membrane damage directly and the endo Peh 1 from Ecc may be that enzyme.

RESISTANCE, HR AND LOCALIZATION

I have left to the end my discussion of research trends concerning the subject of resistance, which I might suggest could be considered as la piece de'resistance. Prior to the cloning of the avirulence gene by Staskowicz and co-workers (1984) and the report by Lindgren et al. (1986) of the gene cluster that controls both pathogenicity and HR of P. syringae pv. phaseolicola, Klement (1982) had inferred that pathogenicity and HR were very closely linked phenomena and that the development of necroses in both susceptible and hypersensitive tobacco was similar in many respects. An important difference in necrogenesis was the number of bacterial cells necessary to induce plant cell collapse. In comparing P. s pv. pisi with P. s pv tabaci injected into tobacco mesophyll, the ratio was 1:50-100 to cause necrosis.

It is not my intention to summarize what we know about HR as of this moment nor justify its role as a resistance reaction. Rather, I should like to call to your attention some recent and exciting work on HR, essentially studies that have been conducted by Anton Novacky and his associates. Since its discovery by Klement, Farkas and Lovrekovich in 1964 (a quarter of a century ago) many of us have sought to expose the inducer of this very potent biological phenomenon. Specifically what does a single bacterial cell $1u^3$ have at its disposal that permits it to kill a plant cell $5x10^4$ times its volume? Death of the plant cell can occur within 3-6 hrs.

What can the target site be that reflects such a violent and rapid destructive outcome? For some time there has been visual and some biochemical evidence that membranes in general were affected. Recent studies by Keppler and Novacky (1987, 1989) presented evidence that lipid peroxidation initiated by O_2 (superoxide) radicals may be involved in altering plant cell membrane permeability in the bacteria- induced hypersensitive reaction.

More specifically, the unsaturated fatty acyl groups of membrane lipids may be attacked by O_2 OH and $^1O_2$ radicals and the resulting fatty acyl hydroperoxide molecules degenerate autocatalytically releasing organic radicals that propagate a chain reaction of lipid peroxidation. Keppler and Novacky have clearly shown that lipid peroxidation does occur during bacterially induced HR, which may account for alteration of the membrane lipids.

Both superoxides and hydroxyl radicals are ubiquitously produced by an array of enzymatic and nonenzymatic reactions. They are generally maintained at low levels in planta by superoxide dismutase and catalase and an array of antioxidants the scavengers of these cytotoxic radicals. The latter are best characterized by glutathione and ascorbate. Hence, a study by Keppler and Novacky specifically monitored lipoxygenase, superoxide dismutase and catalase during the ontogeny of HR in cucumber cotyledons. Briefly they were able to detect increases in lipoxygenase during the development of HR in cucumber induced by Pseudomonas syringae pv. pisi. Additionally they observed significant reduction of membrane alteration by the addition of superoxide dismutase.

However, even more recent evidence, to be presented later in this conference by Anton Novacky, indicates OH• radicals rather superoxides are the HR causes he observed to membranes.

Exciting work, as yet unpublished, from the laboratory of Stayton and Tomaki at the University of Wyoming in concert with Noel Keen suggests that the triggering molecule of HR in P.s syringae pv glycinea is a polysaccharide with a MW of about 10,000. We wait in anticipation.

Still awaiting further analysis is the increasingly frequent observation of bacterial pathogen localization in host tissue, first observed by Goodman et al. (1976) and later by Sequeira et al. (1977).

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

STRATEGIES FOR RESTRAINING BACTERIAL PLANT DISEASES

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1. BACTERICIDES

Effective bactericides for plant protection are urgently needed, as indicated by reviews of Egli and Sturm (1981) and Sekizawa and Wakabayashi (1989). The traditional bactericides used to control bacterial plant diseases are copper compounds. Nearly all bacterial plant diseases are affected by copper. The effect is protective and in no way curative. This makes frequent sprays necessary, so that copper may cause phytotoxic effects, such as russeting, discoloration and cracks, especially in fruits. The accumulation of copper in the soil can lead to a decline in plant vigor and inhibit the development of beneficial mycorrhiza, especially in acid soils (Graham et al. 1986).

However, effective and low-cost alternatives to copper are not available in many instances. Thus, copper-containing products were generally superior to other types of bactericides in reducing *Xanthomonas campestris* pv. *citri* (Mc Guire 1988). In order to reduce copper concentrations the preparation 'Copper-Count N' with 8% metallic copper was introduced. It was often more effective at reducing bacterial populations than compounds containing more than six times as much copper. However, the opposite results were also reported, e.g. a comparison of several copper preparations against the foliar blight phase of bacterial canker of tomatoes showed that Copper Count N was inferior to Kocide 101 or tribasic copper sulfate (Shoemaker and Echandi 1988). An ammoniacal copper sulfate preparation, called Copac E, gave satisfactory control of *Erwinia amylovora* and was well tolerated by numerous pear varieties (Mappes et al. 1983), but was phytotoxic for apples. The extensive use of copper has led to the appearance of copper tolerant bacterial strains e.g. *X. vesicatoria* in Mexico and U.S. (Adaskaveg and Hine 1985). Applications of a copper-mancozeb combination or Zineb are more effective than copper in controlling copper tolerant strains (Cox 1982; Marco and Stall 1983), however, Zinc tolerance has rapidly developed (Adaskaveg and Hine 1985).

Treatment of seeds with acidic bactericidal preparations has proven to be effective in several diseases. For instance, soaking of wheat seeds in warm acidified cupric acetate resulted in *Xanthomonas translucens*-free seed (Forster and Schaad 1988), 0.6% HCl or acetic acid is used to control *C. michiganensis* ssp. *michiganensis* in tomato seeds, Griesbach (1987) reported that a complete elimination of this pathogen from highly infected tomato seed could be achieved by soaking for one hour in 3% tartaric acid or 0.1% Nourseothrizin. The long known bactericidal effect of sodium hypochlorite to disinfest seeds was successfully used in our experiments against infestation of coriander seeds with pseudomonads (Toben unpubl.). However, a comparison of sodium hypochlorite with acid treatment (0.6 N HCl) of tomato seeds revealed a much better effect of acid to disinfest seeds of *C. michiganensis* (Dhanvantari 1988).

Some synthetic bactericides for use in plant protection have been developed, however, because these compounds are either not very effective or limited in the number of bacterial species which can be controlled, they have not gained broad practical importance.

Sankel (Fig. 1)
(Nickeldimethyldithiocarbamate) is applied against bacterial leaf blight in rice (*X. campestris* pv. *oryzae*), the control is 40 - 60%.

Bronopol (Fig. 2)
(2-Bromo-2-nitropropane-1,3-diol) is recommended as a dry seed dressing against *X. malvacearum*.

Phenazin (Fig. 3)
(phenazine 5-oxide) is confined to control of *X. oryzae*.

Tecloftalam (Fig. 4)
is also used against *X. oryzae*. The compound has a protective as well as a curative effect and inhibits the multiplication of the bacteria 'in planta'.

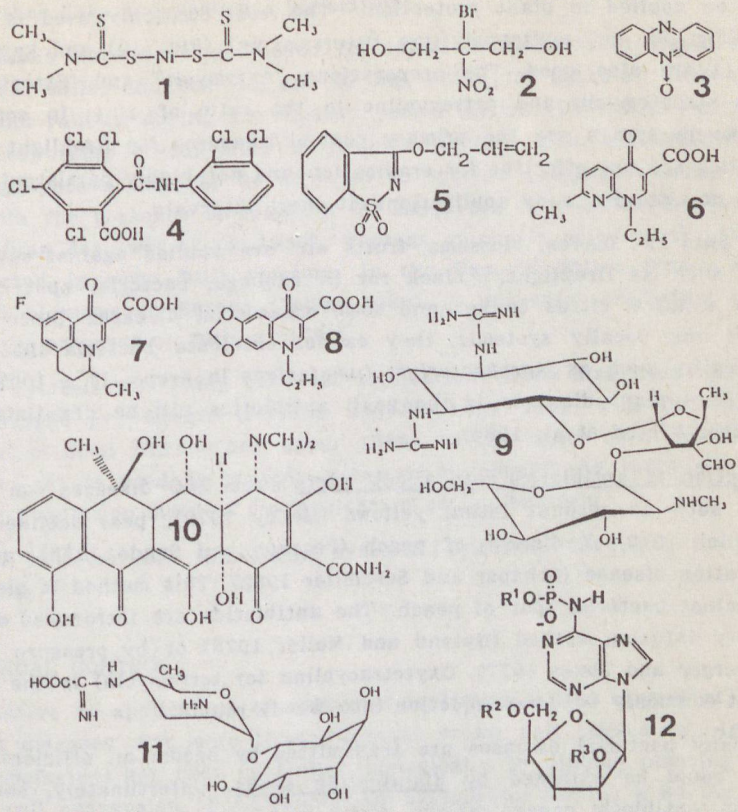
Oryzemate (= probenazole) (Fig. 5)
is used against several phytopathogenic bacteria (*X. oryzae*, *P. glumae*, *P. lachrymans*). Interestingly, it has no 'in vitro' antibacterial activity but changes the reaction of the plant tissue from compatible to incompatible.

Nalidixic acid, (Fig. 6)
a quinolone carboxylic acid, long known as a pharmaceutical with antibacterial activity (Lesher et al. 1962).

Flumequine (Fig. 7)
belongs to the same group of compounds and is effective against fireblight in the field.

Oxolinic acid (Sumitomo No. S-0208) (Fig. 8) is another derivative of quinolone carboxylic acid. It was effective against *E. carotovora* on chinese cabbage (Anonymous 1977) and potato (Kelman 1988), as well as against fireblight (Byrde 1988, personal communication). Oxolinic acid was recently registered as a seed bactericide against *Pseudomonas glumae* in Japan. Some curative systemic and persistent mode of action was reported (Anonymous 1977).

The CIBA-GEIGY product 78039, a halogenated quinoline-carboxylic acid, was withdrawn due to toxicology risks, although it had proved to be effective towards phytopathogenic bacteria of several genera (Vantomme et al. 1982, 1984).



Figs. 1-12: Bactericides and antibiotics: 1) sankel 2) bronopol 3) phenazine 4) tecloftalam 5) oryzemate 6) nalidixic acid 7) flumequine 8) oxolinic acid 9) streptomycin 10) oxytetracycline 11) kasugamycin 12) agrocin 84

2. ANTIBIOTICS

In several countries antibiotics are still used in plant protection against bacterial pathogens. Although introduced for plant protection in the 1950's, antibiotics have generally not been as effective for controlling bacterial plant diseases, as other chemicals have been in control of fungal diseases. This may be explained by several drawbacks of antibiotics:

1 - Non-persistance or instability,
2 - phytotoxic side-effects,
3 - high price,
4 - development of resistant bacterial populations.

Due to instability and phytotoxicity many antibiotics known from medicine cannot be applied in plant protection. The most commonly used is streptomycin (Fig. 9), but oxytetracycline (=terramycin) (Fig. 10) and kasugamycin (Fig. 11) are also used. The preparations "Agromycin" and "Streptocycline" contain streptomycin and tetracycline in the ratio of 10:1. In some areas streptomycin sprays are the primary control measures for fireblight. Because antibiotics are not effective for eradication and not highly persistent, disease control may require many applications at short intervals.

Sprays onto the leaves, blossoms, fruits etc. are applied against several diseases, such as fireblight, black rot in cabbage, bacterial spot of peach, tobacco wildfire, citrus canker and some ornamental diseases. Since antibiotics are only locally systemic, they cannot eradicate bacteria that survive systemically, such as *Agrobacterium tumefaciens* in grape. If a 100% control of bacterial plant diseases is the goal, antibiotics will be unsatisfactory in most cases (Thind et al. 1984).

By injection of antibiotics into woody parts some MLO-diseases can be controlled, such as coconut lethal yellows (McCoy 1972), pear decline (Nyland and Moller 1973), X-disease of peach (Pearson and Sands 1978), and apple proliferation disease (Schaper and Seemüller 1982). This method is also effective against bacterial spot of peach. The antibiotics are introduced either by a gravity infusion method (Nyland and Moller 1973) or by pressure injection (Rosenberger and Jones 1977). Oxytetracycline (or terramycin) is the antibiotic most commonly used for injection into woody plants.

Since many bacterial diseases are transmitted by seeds, an efficient disease control could be obtained by disinfesting seeds. Unfortunately, soaking of seeds in antibiotic concentrations which eliminate the bacteria is usually phytotoxic (Burr and Norelli 1989). Certain combinations of antibiotic and heat treatment may give the best results and have to be worked out for every disease (e.g. Kritzman and Ovadia, 1989).

A major problem in the use of antibiotics to control plant pathogenic bacteria is the development of pathogen resistance especially in the case of *Erwinia amylovora* (Moller et al. 1981). It must also be kept in mind that genetically stable and inheritable resistance can be transmitted among diverse groups of bacteria (Burr et al. 1988), including human pathogens. Although we know of no documented case, in which a human disease has become more difficult to control because the build-up of antibiotic resistant phytopathogenic bacteria, such processes may have happened unnoticed. In any case, the risk of the development of resistance points to the necessity for alternative strategies to control bacterial diseases.

3. FORECASTING SYSTEMS

One possibility to reduce chemical control measures, is the development of forecasting systems. Many bacterial diseases occur sporadically, they are severe in some years and of minor importance in others. In order to avoid unnecessary sprays we must be able to predict disease outbreaks on the basis of weather data and bacterial populations.

Several models have been developed for infection by *Erwinia amylovora* by Mills, Powell, Miller and Schroth, and Billing, which are based on temperature, humidity and rainfall during the blossom period (Zeller 1989). The model from Beer and coworkers (1984) tries to combine weather risk and orchard risk. Forecasting systems have to be adjusted to specific geographic areas. Thus, in California the presence of epiphytic *E. amylovora* in pear flowers could be predicted (van der Zwet et al. 1988), whereas epiphytic populations have not been reported in some fruit blossoms in the Eastern United States nor in ornamental blossoms in Europe (Zeller 1989). A universal predictive system was recently proposed by Billing (1989).

Predictive systems for timing chemical applications have also been developed for *P. syringae* pv. *tomato* (Jardine and Stephens 1987) and for bacterial brown spot of bean (Hirano and Upper 1989). In the latter case the tube ice nucleation test is used to provide a relatively simple and rapid means for estimating population sizes of ice-nucleation active bacteria.

In general, however, few forecasting systems have been developed for bacterial diseases.

4. BIOLOGICAL CONTROL

An alternative to application of chemicals is the biological control of bacterial plant diseases. For more than 10 years crown gall caused by *Agrobacterium tumefaciens* has been controlled biologically by dipping planting material in a cell suspension of *Agrobacterium radiobacter* strains K 84. As shown by Kerr (1980), this treatment results in a rather high level of disease control (between 80 - 100%). The effect is due to an antibiotic, agrocin 84 (Fig. 12). The synthesis of this antibiotic is encoded by a 47 kb plasmid. Because of the threat, that this plasmid might be transferred conjugatively to pathogenic agrobacteria, which then become insensitive to agrocin 84, a new biological control strain was constructed recently by Jones and Kerr (1989). The new strain, called K 1026, is unable to transfer its agrocin-84 plasmid to other agrobacteria and shall replace strain K 84 for biological control in the future.

Notwithstanding the very successful work of Kerr in biological control of crown gall, the development of biological control agents may be more complicated than the development of a new chemical control agent (Beer, 1989). A biological control program involves three living systems; each may vary in itself, interact with the environment and with the other two living

systems. Nevertheless, many examples of biological control of bacterial disease have been reported which cannot be listed here.

5. RESISTANCE BREEDING

The classical method to reduce losses by bacterial disease is breeding for resistance. It is impossible to list all bacterial plant diseases against which resistant cultivars have been bred. Only a few examples can be mentioned. Brinkerhoff and coworkers (1984) developed immunity to bacterial blight of cotton, *X. campestris* pv. *malvacearum*, by combining several single-gene resistance factors onto a polygenic resistant background. The segregation progeny after each backcross was screened with a mixture of virulent races of the pathogen. In experiments subsequently conducted in many cotton-growing areas of the world, this immunity remained stable for over 20 years.

Similarly, Mew and coworkers (Mew 1987) at the International Rice Research Institute were successful in breeding rice cultivars resistant to bacterial blight, *X. campestris* pv. *oryzae*. Other examples are resistance to *X. campestris* pv. *manihotis* (Lozano 1986), or bacterial diseases of bean (Coyne and Schuster 1979). It was stated by Lozano and Zeigler (1989) that both inoculation and evaluation methodologies are critical for a successful resistance screening programme. Sustainable and long-term advances can be achieved only through careful and precise development of techniques specifically designed for the pathosystem, the crop, the environment, and the economic and logistic facilities available. New methods in molecular genetics and cell suspension or tissue culture may help to speed up resistance breeding considerably.

6. PHYTOSANITARY MEASURES

Phytosanitary measures may be the most important means to restrain bacterial diseases. They include proper methods in agronomy up to highly sophisticated methods to detect bacteria in plants. Necessary methods in agronomy are:

- Manipulation of sowing dates,
- extensive inspection of fields and orchards and removal of diseased plants or parts of plants,
- proper irrigation and fertilization,
- ensure decomposition of plant debris, e.g. by completely covering plant residues by clean-plowing after harvest,
- planting of wind breaks to reduce the force with which driving rain can spread bacteria (*X. campestris* pv. *citri* in Argentina),
- in greenhouses: Control of humidity and temperature, disinfection after a crop has finished

The use of bacteria-free seeds or propagation material is effective in controlling many diseases. Very sophisticated methods are now available to detect bacteria in seeds, e.g. by the use of bacteriophages or serological

methods, as has been demonstrated by Schaad (1982, 1989), van Vuurde (1989), and others. Molecular cloning and colony hybridization methods can also be applied to detect phytopathogenic bacteria in plants, as was shown by Roth (1989). Although these complicated methods are very fascinating, they should only be applied if an improvement can be expected, for instance in sensitivity. This may not always be the case. We found that traditional detection of *P. syringae* pv. *phaseolicola* in bean seeds by soaking, streaking on semiselective media and confirmation of the results by an *E. coli*-bioassay for phaseolotoxin can be very sensitive (Jansing and Rudolph 1989), so that 2×10^1 bacterial cells in the soaking solution or 1 diseased bean seed within 50000 healthy seeds were detected.

With a combination of these methods it has been possible to eradicate pathogenic bacteria from certain geographic areas. An example is the ring rot disease of potatoes (*C. michiganensis* ssp. *sepedonicus*) which was eradicated from Denmark. For this purpose bacteria-free seed potatoes were obtained by propagation of plants from meristem cultures (Dinesen 1989).

Other examples of eradication are *E. chrysanthemi* in greenhouse carnations and Dahlia in the Netherlands, *P. solanacearum* in Sweden, as well as *Xanthomonas citri* in the U.S.A. - for a certain period (Maas-Geesteranus and Stall 1989).

7. CONCLUSION

Losses by bacterial plant diseases can only be reduced when several methods of control are combined, such as the application of bactericides and antibiotics - if possible with forecasting models -, use of biological control wherever feasible, as well as of disease resistant cultivars, and proper phytosanitary measures. The optimal combination of control measures has to be worked out for every bacterial plant disease within a limited geographical area.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

USE OF GENETIC AND MOLECULAR APPROACHES IN ELUCIDATING BACTERIAL PLANT PATHOGENICITY DETERMINANTS

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Plant pathogenic bacteria have the ability to invade and to multiply within specific host plants causing typical disease damage. This raises the following questions concerning the determinants for these properties :

- What are the biochemical functions required for pathogenicity ?
- What are the molecular bases for specificity of the pathogen towards particular host plants ?

Biochemical approaches have allowed the identification of compounds synthesized by the bacteria tentatively involved in disease development. In certain cases their role in this process has been clearly established. They include structural components such as exopolysaccharides (EPS), lipopolysaccharides, degradative enzymes (pectinases, cellulases, proteases ...), toxins and plant hormones.

In the last ten years the availability of molecular biology techniques facilitated the investigations and led to significant advances in the understanding of plant-bacteria interactions. This was primarily achieved in the case of crown gall caused by *Agrobacterium tumefaciens* and for *Rhizobium*-legume symbiosis, but more data are becoming available on other plant pathogenic bacteria. In this paper we do not intend to give an exhaustive view of the information which became available through the molecular approach but rather try to illustrate the contribution of these techniques to plant pathology.

1. TWO IMPORTANT ASPECTS OF THE MOLECULAR APPROACH

Two specific aspects of the molecular approach have widely contributed to its success in plant pathology.

- Once a biochemical function had been identified in an organism the best way to demonstrate its contribution to pathogenicity is to compare pathogenicity of two strains which differ from each other

only by the single character of interest. Molecular biology techniques offer the possibility to construct such strains which cannot be obtained directly from nature.

- Genetics combined with molecular biology is a means to detect functions important for pathogenicity independently of the knowledge of their biochemical nature. Mutants can be obtained which are detected only as being nonpathogenic. This is a way to reveal important pathogenicity determinants which might have previously been unsuspected or unidentified.

2. ROLE OF EPS IN PATHOGENICITY

For a long time EPS has been considered as a potential pathogenicity factor among plant pathogenic bacteria (Sequeira 1979). This role has been clearly established for *Erwinia stewartii* by Dolph *at al.* (1988). These authors have cloned a cluster of 5 genes (cps A-E.) controlling the synthesis of the bacterial capsule. Mutants have been obtained for each gene. All of them are impaired in EPS synthesis and have lost the ability to induce wilting of corn. In addition it has been shown that 4 of these genes (cps B-E) are also required for the induction of the water-soaked lesions which develop on corn leave parenchyma. These data suggest that EPS has two different functions which are required at different stages of the infectious process. Expression of *cps* genes has been shown to be under the control of two regulatory genes (*lon* and *res*) which are homologous to the ones of *E. coli* (Torres-Cabassa *et al.* 1987).

3. ROLE OF DEGRADATIVE ENZYMES

Plant pathogenic bacteria harbor degradative enzymes (including cellulases, pectinases, proteases etc.) which are active against certain structural components of the plant. Therefore they have been considered as potential pathogenicity determinants of these organisms. The possibility to individually abolish each of these functions led to important conclusions concerning their roles in pathogenesis.

3.1. *Erwinia chrysanthemi* pectate lyases

E. chrysanthemi causes maceration of infected plant tissues. This organism is known to have a high level of pectinolytic activity. Therefore these enzymes have been suspected to be major pathogenicity determinants (Collmer and Keen 1986).

Biochemical studies established that most *E. chrysanthemi* strains produce 5 different pectate lyases (PL) which are easily distinguished on the basis of their isoelectric properties (for a review see

Kotoujansky 1987). Each enzyme is encoded by a particular structural gene (*pel* A-E) which has been cloned. This allowed the construction of a set of mutants each one carrying a mutation which inactivates a single *pel* gene. All of them had retained the ability to macerate detached plant organs, but they differed in their ability to induce a systemic infection on their host. Whereas *pel*B or *pel*C deficient mutants did not differ significantly from the wild-type strain, *pel*A or *pel*D mutants were only slightly affected. In contrast the *pel*E mutant ,although it multiplied in the surroundings of the inoculation site, had lost the ability to systemically invade the host (Boccara *et al.* 1988).

These experiments demonstrate the differential effect of individual PL and the crucial importance of *the pel*E enzyme in pathogenicity.

3.2. Other degradative enzymes

A similar approach has shown that in *Xanthomonas campestris* pv. *campestris* and *Pseudomonas solanacearum* individual plant cell wall degradative enzymes have a minor role in pathogenicity since endoglucanase or polygalacturonase deficient mutants have been found to be only sightly impaired in aggressivity under the assay conditions used (Robert *et al* 1988, Schell *et al.* 1988, Gough *et al.* 1988, Dow *et al.* 1989). However it is important to keep in mind that these conditions might be very important in assaying pathogenicity since certain essential steps of the natural infection process can be by-passed using certain artifical inoculation procedures. This is exemplified in the case of *Xanthomonas campestris* protease deficient mutants which cannot be distinguished from the pathogenic wild-type parent after stem inoculation, but which failed to become systemic when inoculated in the parenchyma of wounded leaves suggesting that protease probably acts in facilitating the progression of the bacteria toward the vascular bundles of the plant (Tang *et al.*1987, Dow *et al.*1988).

4. IRON ASSIMILATION

As mentioned earlier, a genetic approach combined with molecular biology techniques has allowed the identification of pathogenicity factors which had not previously been suspected to play a role in plant-bacteria interactions. This has been exemplified with *E. chrysanthemi* mutants deficient for the iron assimilation system. A prerequiste for plant pathogenic bacteria is an ability to grow and multiply within the plant. This requires that the plant should provide the bacteria with all the essential nutrients. Certain of these nutrients might be present in limiting amounts in the plant and the plant pathogen might have to compete with the plant for such limiting factors.

In *E. chrysanthemi* certain spontaneous mutants resistant to phage and/or bacteriocin have been found to be avirulent. Biochemical characterization of these mutants revealed that they were affected for several different characters but that they were all deficient for the iron assimilation system

(Expert and Toussaint 1985). Further mutagenesis allowed the isolation of a set of mutants specifically affected in each of the steps of the iron assimilation process. All these mutants were shown to be unable to produce a systemic infection on *Saintpaulia*, although they had retained the ability to macerate detached leaves or potato slices (Enard *et al.* 1988). This suggests that, within the plant, *E. chrysanthemi* has to compete for iron with its host and demonstrates that the bacterial iron assimilation system is a pathogenicity determinant which permits active mobilization of this element to sustain bacterial growth and development of a normal infectious process.

5. *hrp* GENES

Random mutagenesis of the entire genome followed by the screening for nonpathogenic mutants led to the discovery of a class of mutants which are impaired both in the ability to cause disease symptoms on the homologous host and in the ability to induce a hypersensitive response on the heterologous host. Genes governing this property have been called *hrp* (for Hypersensitive Response and Pathogenicity) and have been found in *P. syringae* (Lindgren *et al.*1986), *P. solanacearum* (Boucher *et al.* 1985), *Erwinia amylovora* (Steinberger and Beer 1988) and in *Xanthomonas campestris* (Stall, these proceedings) and most have been shown to be clustered. In *P. syringae* and *P. solanacearum* these genes are organized in two clusters. It has been shown in these latter two systems that the major cluster of *hrp* genes spans over about 22 kb of DNA (Lindgren *et al.* 1986, Boucher *et al.* 1987) and that the corresponding DNA sequences are structurally conserved among each species but do not show any detectable homology (as detected by cross hybridization) with each other (Boucher *et al.* 1988, Lindgren *et al.* 1988). In addition, sequences homologous to *P. solanacearum hrp* genes have been found in *X. campestris* (Boucher *et al.* 1988). In the case of *P. syringae hrp* genes have been found to be functionally interchangeable among different pathovars of this species (Lindgren *et al* 1988) and introduction of *hrp* genes into the non pathogenic *P. fluorescens* has been shown to confer the HR inducing ability to the recipient strain (Huang *et al.* 1988).

These data demonstrate that pathogenicity and HR inducing ability share some common biochemical determinants which are probably required in the very early steps of plant-bacteria interactions. In addition they show that large groups of plant pathogenic bacteria, which induce different diseases on different hosts, probably share a certain number of common basic pathogenicity functions.

6. GENETIC DETERMINANTS FOR SPECIFICITY

A particular pathogen usually causes disease on a limited number of host plants. In addition a particular line or cultivar of a host plant differentially responds to inoculation with a collection of strains of an organism representative of a particular bacterial species or pathovar indicating the existence of an infra specific level of specificity among strains of a pathogen.

In 1955 Flor established for fungi the "gene for gene theory" which states that in a host plant resistance is expressed as the result of the confrontation of a dominant resistance gene of the plant with a pathogen which harbors a corresponding dominant avirulence gene (Flor 1971). The cloning of bacterial avirulence (*avr*) genes in the last five years has allowed the formal demonstration that the gene for gene theory also applies to bacteria such as *Xanthomonas campestris* and *P. syringae* (Gabriel *et al.*1986, Staskawicz *et al.* 1988).

More recent data have shown that *avr* genes may also be responsible for limiting the number of plant species a pathogen can attack. Gene *avr*D has been found in *P. syringae* pv. *tomato* which once introduced into *P. syringae* pv. *glycinea* makes this pathovar incompatible on certain soybean cultivars (Kobayashi *et al.* 1989). Similar data were obtained for *X. campestris* where an *avr* gene has been found in *X. campestris* pv. *vesicatoria* which confers incompatibility to *X. campestris* pv. *phaseoli* on certain bean cultivars (Whalen *et al.*1988).

On the other hand, gene(s) which extend the host range have also been reported. In *P. solanacearum* such genes have been cloned from a strain compatible for peanuts. When introduced into a strain specific for potato, this clone permitted the transconjugants to wilt peanut (Ma *et al.* 1988).

6. CONCLUSION

It is clear today that pathogenicity of bacteria is a complex property which requires the coordinated interaction of multiple individual biochemical determinants. It has been shown that certain of these determinants play an essential role since deficient mutants were obtained which did not induce any disease symptoms on their natural hosts. On the contrary a large number of other functions individually contribute to the degree of aggressivity of the pathogen, but are not individually absolutely essential for disease expression. It is of particular interest to note that among essential functions genes have been found which have a regulatory effect on the expression of several genes which individually modulate aggressivity (Daniels, these proceedings). This indicates that coordinated expression of individual pathogenicity and/or aggressivity determinants is essential for disease development. In *Agrobacterium tumefaciens* it has been shown that *vir* (virulence) genes are clustered and that expression of the entire cluster is controlled by a single regulatory mechanism which is plant dependent (Stachel and Nester 1986). It is clear that the search for such regulatory functions among other plant pathogenic bacteria will be of major importance may lead to the conception of new means for disease control based on interference of the expression of pathogenicity genes.

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SESSION 1

HOST-PARASITE RELATIONSHIP

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

OBSERVATIONS ON OLIVE AND OLEANDER LEAF INFECTION BY PSEUDOMONAS SYRINGAE PV. SAVASTANOI

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Pseudomonas syringae pv. *savastanoi* attacks olive and oleander plants and causes the formation of tumorous outgrowths on twigs and branches. Naturally contaminated olive and oleander leaves serve as a source of inoculum for the establishement of the disease (Ercolani, 1971). However, it is not known why knots occur so rarely in nature on olive leaves. On oleander, on the other hand, these symptoms are very common.

The purposes of this study were to compare the colonization of olive and oleander leaves by different strains of *P. s.* pv. *savastanoi* and to follow the infection process on leaves in the hope of determining possible leaf sites of entry.

MATERIALS AND METHODS

Bacterial strains and plant inoculation. Strains ITM603, ITM317, PVBa225 & PVBa206 from olive and NCPPB640 and ITM519 from oleander were used. In pathogenicity tests, made as previously described (Surico et al. 1985), the olive strains resulted virulent on olive (cv. Nocellara del Belice) and avirulent on oleander while the oleander strains induced knots on both host plants.

Inoculation was carried out by one of the following methods: i) 2-year-old plants were kept in a humid chamber for 12 h, sprayed with a bacterial suspension containing 10^7 colony-forming units per millilitre, and incubated in the humid chamber at 95% relative humidity and 26±2 °C. After 6 days

the plants were transferred to a greenhouse; ii) 4 to 8 10-µl droplets of a 10^7 bacterial suspension were deposited on the lower surface of olive and oleander leaves. The leaf tissues were then punctured with a fine needle. As in the previous case, plants were kept in the humid chamber for 6 days after inoculation and thereafter transferred to the greenhouse.

Scanning electron microscopy (SEM). Samples for SEM were collected at 24 h intervals during the first 8 days and thereafter every 5 days up to 60 days following inoculation. Tissue samples were fixed for 2 h with 3% glutaraldeyde and post-fixed overnight with 1% osmium tetroxide followed by dehydration in a series of increasing ethanol concentrations. The material was dried in a critical point drier under CO_2, mounted on specimen holders and coated with gold-palladium. The sections were examined in a 505 SEM.

RESULTS AND DISCUSSION

Immediately after the spraying of uninjured leaves bacteria were randomly dispersed on the lower leaf surface, though most of them soon disappeared. After 48 h a few bacteria could be observed on or near the stomata, in direct contact with the leaf surface and, particularly, on the shields of the peltate hairs which almost uniformly cover the lower olive leaf surfaces. Occasionally, single cells, presumed to be *P. s.* pv. *savastanoi*, were detected in the intercellular spaces below the epidermal cells.

Three to five days after contamination the bacteria had increased though they still appeared, singly or in colonies, almost exclusively on the shields of the hairs (olive) and in the stomatal pits (oleander). Later still, some cases became embedded in a slime matrix. Despite the bacterial presence, no symptoms had developed after 90 days.

A similar situation was observed on oleander leaves (Fig. 1B). In this case, preferable bacterial multiplication sites appeared to be the stomatal pits.

When mechanically injured leaves were sprayed with the oleander and olive strains, large numbers of bacteria were observed in the tissues after 24-48 h. Three to five days later, on olive leaves inoculated with oleander

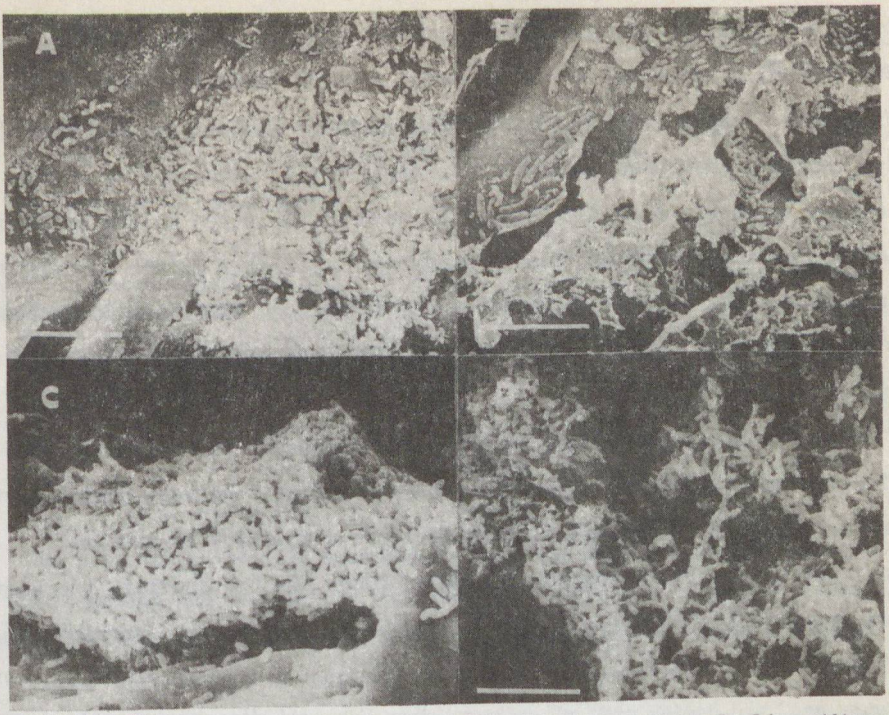


Fig. 1. *P. syringae* pv. *savastanoi* cells on the surface of olive (A) and oleander (B) leaf 10 days after inoculation. C and D, bacterial aggregates in intercellular spaces of olive (C) and oleander (D) tissues. Fig. 1C show fibrous material associated with bacterial colonies. Bars represent 10 µm.

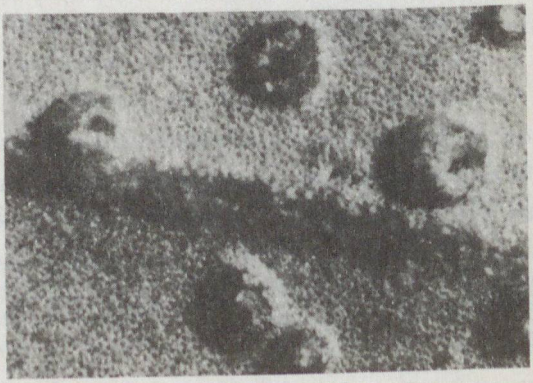


Fig. 2. Knots on leaf of olive in response to artificial inoculation with *P. syringae* pv. *savastanoi* strain NCPPB640.

strains, new tissues had appeared around the sites of inoculation. This tissue gradually increased in volume until a small knot was formed (Fig.2). Surprisingly, leaves inoculated with olive strains did not develop these knots; instead, they presented necrotic areas often surrounded by a chlorotic halo, probably due to indoleacetic acid accumulation in the sites of infection, or a very limited tissue overgrowth. Bacteria in the inoculated tissues were still alive 70 days after the infection. Fibrillar connections were often observed between bacteria (Fig. 1D) independently of the host origin of strains.

These results suggest that injuries are necessary for the oleander strains to incite knots on olive and oleander leaves. The very common knots observed in nature on oleander leaves could be simply due to bacteria moving systematically throughout the latex tubes. On the contrary the unexpected reaction of the olive leaves to inoculation with olive strains is still to be explained.

The author thanks Mrs E. Bruno for assistance on scanning electron microscopy and Mr A. Esposito for assistance with growing and inoculating plants. This work has been supported by a grant from the Italian Ministry of Education (Ministero della Pubblica Istruzione).

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

ULTRASTRUCTURAL STUDY ON THE COMPATIBILITY BETWEEN RICE LEAF VESSEL SYSTEM AND VARIOUS BACTERIA

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INTRODUCTION

The interaction between host and pathogen has been the particular subject of investigation and controversy related to the mechanism of host resistance, pathogenicity or host specificity of the pathogen. The relationship between host tissue and pathogenic bacteria has often been expressed by the words of compatibility/incompatibility and much progress in this regard has been made with different methods of approaches.

In an attempt to elucidate an intrinsic mechanism of the compatibility/incompatibility between host tissue and invaders, various bacteria different in pathogenicity were infiltrated into rice leaf parenchymatous tissue and ultrastructural investigation was made previously (Tsuno,K. & Wakimoto,S., 1983 ,1986,1988). The interactions observed in ultrastructure between rice leaf parenchymatous tissue and infiltrated bacteria were much different depending upon the kind of bacteria suggesting that the degree of compatibility between plant and bacterium was determined in consequence of some compatible and incompatible phenomena of interactions(Tsuno,K. & Wakimoto,S., 1988).

The objective of this study was to elucidate the phenomena related to compatibility between rice leaf vessel system and some bacteria different in pathogenicity.

MATERIALS, METHODS AND RESULTS

Bacteria used in this study were selected on the basis of their host and tissue specificity in pathogenicity. They were *X. campestris* pv. *oryzae* having specific pathogenicity to vessel systems of rice plant, *X. campestris* pv.

oryzicola specific to parenchyma of the same plant, and so on, as shown in Table 1. Besides these pathogenic bacteria, non-pathogenic bacterium (*P.fluorescens*) and non-biotic particles (Polystyrene latex, 0.804 μm in diameter) were also used as control. These materials suspended into sterilized distilled water at

Table 1. Pathogenicity of the bacteria used

Bacteria	Host plant	Compatible to the tissue of
X. campestris pv. *oryzae*	Rice	Vessel systems
X. campestris pv. *oryzicola*	Rice	Parenchyma
X. campestris pv. *campestris*	Crucifers	Vessel systems
X. campestris pv. *citri*	Citrus	Parenchyma
P. fluorescens	(a saprophytic bacterium)	
particles of Polystyrene latex	(non-biotic control)	

the concentration of ca.10^8cfu or particles/ml were introduced to the vessels of rice leaves through opening (ca. one mm width) wound made with a razor blade. At 3, 5 and 10 days after inoculation, small pieces (ca. 2mm x 3mm) of the samples were taken at 2 mm upper part from the inoculation site and subjected to prepare the specimens for electron microscopic observation.

(1)Interaction between *X. campestris* pv. *oryzae* and rice leaf vessel systems

Three days after inoculation, some of the vessels were filled with electron dense materials (EDM-v). Bacterial cells (B) were morphologically normal even in the EDM-v in the vessel (Fig.1). Bacteria multiplied gradually in the vessels to fill up and plug the vessel at 5 and 10 days after inoculation (Fig.2), inducing degeneration of rice cells around the vessel. EDM-v disappeared in vessel lumen at later stages of 5 and 10 days after inoculation.

(2)Interaction between *X. campestris* pv. *oryzicola* and rice leaf vessel systems

Ultrastructural features observed were almost the same as in the case of *X. campestris* pv. *oryzae* with only exceptions that EDM-v still remained in the vessel at 5 days after inoculation (Fig.3). Some of the bacterial cells, though they were not surrounded by EDM-v, were abnormal in shape (DB) at 10 days after inoculation (Fig.4).

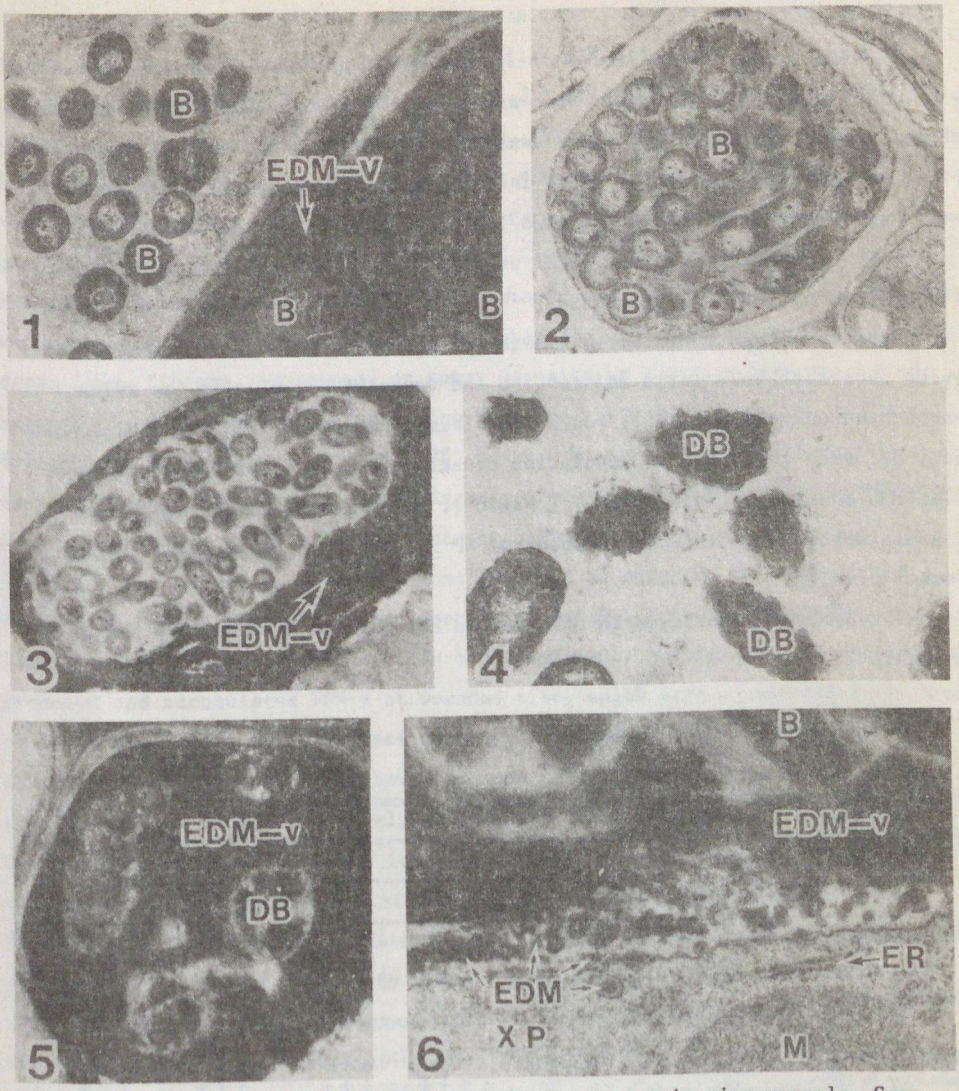


Fig.1 Bacterial cells (B) of *X. campestris* pv. *oryzae* in rice vessels. Some vessels were filled with electron dense materials (EDM-v) at 3 days after inoculation.
Fig.2 Multiplied cells of *X. campestris* pv. *oryzae* in rice vessel at 5 days after inoculation.
Fig.3 Cells of *X. campestris* pv. *oryzicola* decomposed EDM-v and multiplied in rice vessel at 5 days after inoculation.
Fig.4 Distorted cells (DB) of *X. campestris* pv. *oryzicola* not surrounded by EDM-v in rice vessel at 10 days after inoculation.
Fig.5 Distorted cells of *X. campestris* pv. *campestris* surrounded by EDM-v in rice vessel at 3 days after inoculation.
Fig.6 Production of electron dense materials (EDM) in a xylem parenchymatous cell (XP) and exudation of EDM toward the vessel.

(3) Interaction between *X. campestris* pv. *campestris*, *X. campestris* pv. *citri*, and *P. fluorescens* and rice leaf vessel systems

Most of the bacterial cells of *X. campestris* pv. *campestris*, *X. campestris* pv. *citri*, and *P. fluorescens* were always immobilized by EDM-v showing abnormal shapes in the vessel. Fig.5 shows denatured bacterial cells (DB) of *X.campestris* pv. *campestris* surrounded by EDM-v in a vessel. Although only a few bacterial cells were free from EDM-v, the majority of them were abnormal in shape.

Particles of Polystyrene latex used as a non-biotic control were also immobilized by EDM-v in the vessel.

Further ultrastructural investigation showed that EDM-v originated from xylem parenchymatous cells adjacent to the vessel (Fig.6). In the xylem parenchymatous cells (XP), rough endoplasmic reticulum (ER) and mitochondrion (M) were increased in population closely associating with production of electron dense materials (EDM) (Fig.6). EDM was once accumulatd in periplasmic spaces or developed vacuoles of XP and exudated into vessel through vessel pits.

The phenomena observed in relation to EDM-v in this study are summarized in Table 2.

Table 2. Interactions between rice vessel system and introduced various bacteria different in pathogenicity

Phenomena	Days after inoculation	*X. campestris* pv.				*P.fluorescens*	Polystyrene latex
		oryzae	*oryzicola*	*campestris*	*citri*		
Multiplication	10	++[a)]	+	−	−	−	−
Amount of EDM-v	3	+	++	+++	+++	+++	+++
	5	−	+	+++	+++	+++	+++
	10	−	−	+++	+++	+++	+++
Bacterial cells embedded in EDM-v	3	+	++	+++	+++	+++	+++
	10	−	−	+++	+++	+++	+++
Morphologically abnormal bacterial cells in EDM-v	3	±	+	+++	+++	+++	−
	10	−	+	+++	+++	+++	−
Morphologically abnormal bacterial cells not surrounded by EDM-v	3	−	−	±	±	±	−
	10	−	+	±	±	±	−

a) +: positive response (positive degree; +++> ++> +), −: negative response.

DISCUSSION

It has been reported that some deposits were found in the vessel of the plants infected with various bacteria (Wallis,F.M. *et al.*,1973; Hilton D.L. and Mollenhauer,H.H.1976; Goodman,R.N. and J.A.White,1981; Braun,E.J.,1982; Gardner,J.M. *et al.*,1983; Tyson,G.E. *et al.*,1985). In the citrus vessels infected with *Pseudomonas* spp. or Pierce's disease bacterium, fibrous and amorphous occlusions originated from host were reported to be observed non-specifically as a host defense mechanism (Gardner,J.M. *et al.*,1983). In the vessels of maize infected with *Erwinia stewartii*, in grapevine vessels infected with Pierce's disease bacterium and in cabbage vessels infected with *Xanthomonas campestris*, fibrous, amorphous, or gummy deposits or plugging materials which assumed to be of host origin were also reported (Wallis,F.M. *et al.*,1973; Mollenhauer,H. H. and D.L.Hilton,1976; Braun,E.J.,1982). Since these host-derived deposits in vessels of plants were produced more frequently in resistant plant than in susceptible one to restrict bacterial multiplication and translocation, the materials have been considered to be elicited by invaders and related to compatibility (Mollenhauer,H. H. and D.L.Hilton,1976; Braun,E.J., 1982; Gardner,J.M. *et al.*,1983). In our experiment, the rice vessel systems produced and accumulated EDM-v at wounded site, which were considered to be one of the non-specific healing reactions to protect vessel from invaders. EDM-v observed in rice leaves might have the same roles in defense mechanism of host plant as the materials previously reported by other workers. However, specific interactions between EDM-v and invaded pathogen were observed. *X. campestris* pv. *oryzae*, compatible to vessel systems of rice, readily decomposed EDM-v and invaded into the tissue. *X. campestris* pv. *oryzicola*, compatible to rice parenchymatous tissue but not to vessel, also decomposed EDM-v. However, some of the bacterial cells of *X. campestris* pv. *oryzicola* not surrounded by EDM-v showed abnormal shapes, suggesting that the bacteria encountered with some deleterious factors other than EDM-v in the vessel. These phenomena suggest that multi-phases exist in defense reaction of rice vessel systems against a bacterial invader. The bacteria non-pathogenic to rice plants and non-biotic particles could not pass EDM-v and immobilized. The possible defense mechanisms of rice leaf vessel systems against various bacteria were shown in Fig.7.

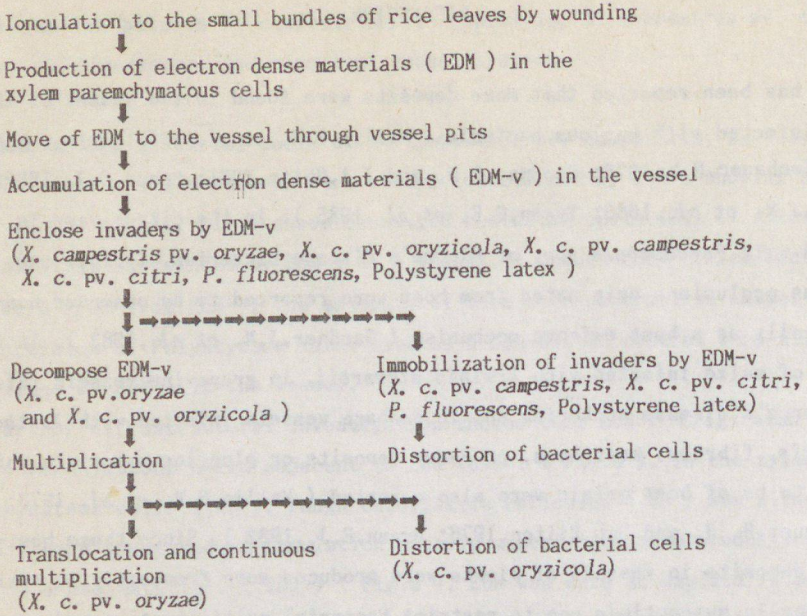


Fig.7 Host-bacteria interactions in rice leaf vessel systems

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

IMMUNOCYTOCHEMICAL LOCALIZATION OF HYDROXYPROLINE-RICH GLYCOPROTEINS IN BEAN AND MELON LEAVES CHALLENGED BY BACTERIA

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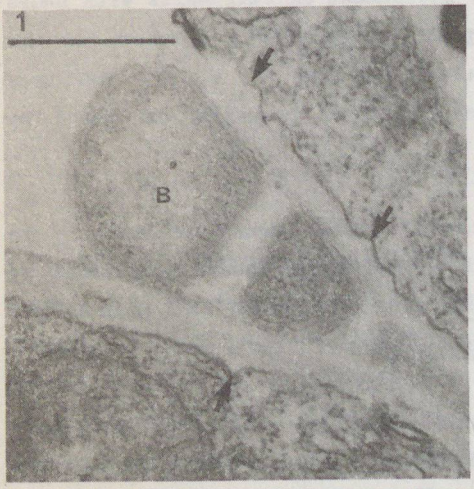
The resistance of plants to infection by pathogenic micro-organisms has been shown to involve the rapid induction of a series of genes, leading to the synthesis and accumulation of various plant secondary metabolites notably phytoalexins, hydrolytic enzymes, and also cell wall components such as hydroxyproline-rich glycoproteins (HRGPs) and lignin [1]. HRGPs (extensin) are normally present in small amounts in the cell walls of higher plants [2, 3]. The unusual, highly conserved structure of plant HRGPs has received much attention [4]. Enrichment of cell walls with HRGPs may prevent penetration by fungal hyphae and provide a barrier for the transfer of potentially toxic microbial metabolites [5, 6, 7]. Having a high pI, HRGPs may also inhibit the growth of bacteria and fungi by agglutination [8, 9].

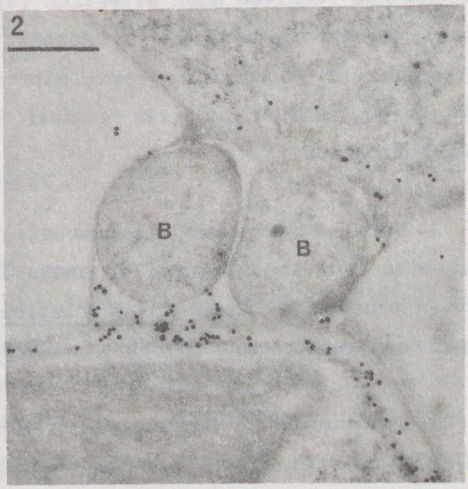
Here we describe the use of ultrastructural immunocytochemistry to examine the localization of HRGPs in bean and melon leaves challenged by the saprophyte Pseudomonas fluorescens and race 3 of the halo-blight pathogen Pseudomonas syringae pv. phaseolicola.

Suspensions of bacteria (5×10^8 ml^{-1}) in sterile distilled water were injected (with syringe and hypodermic needle) into expanded primary leaves of French bean (Phaseolus vulgaris) cv. Tendergreen and into leaves of cantaloupe melon (Cucumis melo) cv. Sweetheart at the 4-5 leaf stage. At intervals after inoculation, tissue from the site of infiltration or from the edge of lesions was examined. Interveinal tissue was excised and prepared for conventional electron microscopy (incorporating ruthenium red in the fixative [10]) or for immunocytochemistry as previously described [11] but using goat anti-rabbit 15 nm gold label and fixation for 16 h at

4°C. Antibodies were prepared against the extensin-like $HRGP_{2b}$ solubilized at low pH from melon callus homogenate [12]. The primary antibody used for immunocytochemistry was the rabbit anti-$HRGP_{2b}$ at 1:500 (v/v) dilution. Controls were, replacement of the primary IgG with either normal rabbit serum (Sigma) or rabbit anti-$HRGP_{2b}$ IgG saturated with purified antigen (melon $HRGP_{2b}$) as the primary antiserum. No gold labelling was observed on control sections.

No symptoms developed in bean or melon leaves inoculated with P. fluorescens and ultrastructural studies showed that the bacterium failed to multiply significantly [10]. Cells of P. fluorescens were encapsulated in a thin layer of amorphous material close to the plant cell walls (Fig. 1). The amount of encapsulating material increased with time after inoculation in bean [10] and also in melon. The pathogen P.s. pv. phaseolicola race 3 induced the hypersensitive reaction (HR) in both the non-host melon and the resistant bean cv. Tendergreen. Infiltrated tissue collapsed, becoming brown and desiccated within 30 h after inoculation and no further expansion of lesions occurred. Cells of P.s. pv. phaseolicola were not bound on to





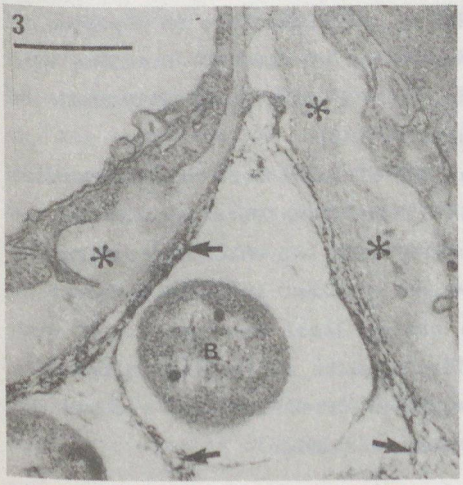
Figs. 1 and 2. Cells of P. fluorescens (B) attached to bean cell walls. In Fig. 1 (conventional preparation) note that the bacterial cell walls have been restricted to the same shape as the adjacent cell wall. Although the plant cell membrane is slightly convoluted (arrows) next to P. fluorescens no obvious papillae have been deposited (24 h after inoculation bar=0.5µm). Fig. 2 shows immunogold localization of HRGPs; note the accumulation of label within the plant cell wall and in the encapsulating material around bacteria trapped between the two mesophyll cells (10 h after inoculation, bar=0.5µm).

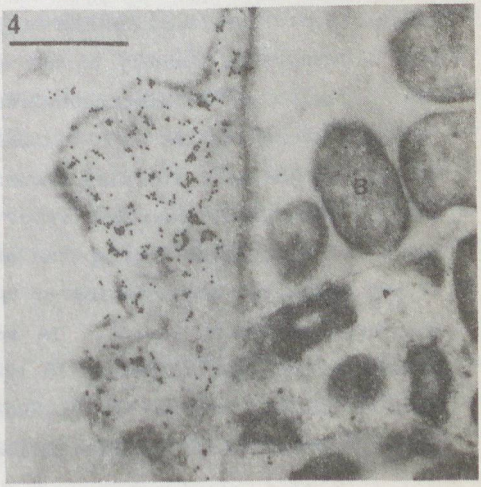
cell walls but formed small colonies in the intercellular spaces and produced fibrillar extracellular polysaccharides (EPS). An early response observed in adjacent plant cells was the deposition of paramural papillae (Fig. 3). Formation of these deposits was localized to the region of the cell wall in contact with the bacterial colony and preceded major changes in ultrastructure which followed irreversible membrane damage [10] leading to cytoplasmic disorganization during the HR (Fig. 5).

Immunogold labelling revealed the presence of HRGPs in the cell walls of uninfected melon and bean tissues; the density of labelling decreased with distance from the plant plasma membrane. Inoculation with either bacterium caused localized accumulation of HRGPs. The following four sites of accumulation were identified and are illustrated in Figs. 2, 4 and 5.

1) Labelling was observed in the material encapsulating the saprophyte (Fig. 2). This response was usually associated with an increase in label within the adjacent cell wall and between the wall and plasma membrane. The amount of labelled material increased with time after inoculation and was most marked after 24 h.

2) HRGPs were also located within paramural papillae (Fig. 4). Labelling was particularly marked in the large papillae formed in





Figs. 3 and 4. Papilla deposition in bean (Fig. 3) and melon cells (Fig. 4) adjacent to colonies of P.s. pv phaseolicola (B), 12 h after inoculation. Note that staining with ruthenium red reveals the accumulation of fibrillar EPS (arrows) around bacteria (Fig. 3, bar=0.5µm). Papillae (asterisks) are clearly visible in adjacent cells. Immunogold labelling (Fig. 4, bar=1µm) reveals the presence of HRGPs within the large papilla sectioned in the melon leaf

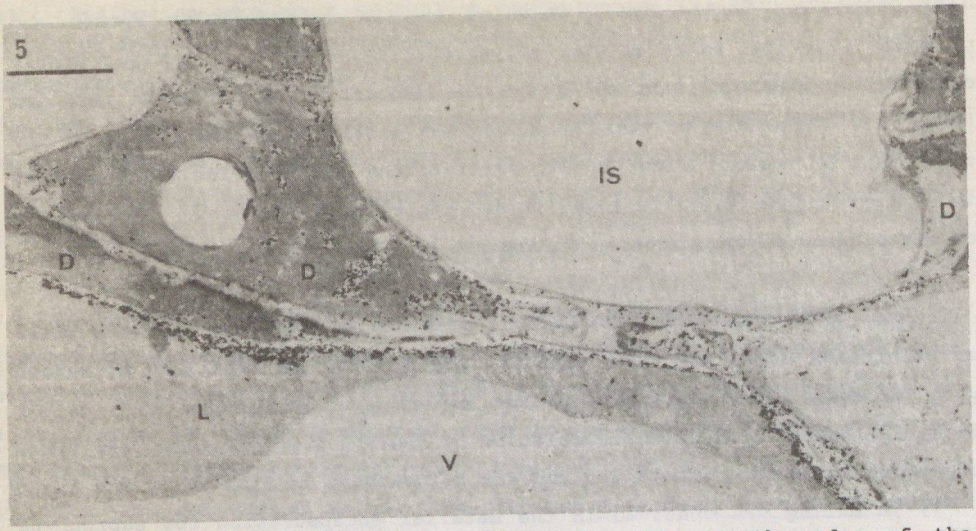


Fig. 5. Immunogold labelling of HRGPs in cells at the edge of the hypersensitive lesion in bean inoculated with P.s. pv. phaseolicola. Note the presence of label within the dead cells which have undergone the HR (D) and the association of dense aggregates of gold particles in the region between the wall and plasma membrane in the adjacent living cell (L). The remaining cytoplasm and vacuole (V) of this cell and the intercellular spaces (IS) are generally unlabelled (Bar=2µm).

responding cells in melon leaves and was apparent within 4 h after inoculation. The localization of HRGPs by gold labelling in papillae in bean corresponded closely to the accumulation of material staining with the periodic acid thiocarbohydrazide, silver proteinate procedure to localize carbohydrates with vicinal glycols [10].

3) and 4) The most striking accumulations of label were associated with the hypersensitive reaction (Fig. 5). Some HRGPs were present in the walls and disorganized cytoplasm of cells which had collapsed during the HR but localized accumulation of HRGPs was greatest in cells surrounding the hypersensitive lesion. In some cases, label was associated with widespread papilla formation in the living cells at the lesion boundary but, as illustrated in Fig. 5, accumulation often occurred without the obvious formation of papillae in both bean and melon.

Our results demonstrate the localized accumulation of HRGPs in tissue challenged by bacteria. It is important to consider the role that the glycoproteins may have in resistance to bacterial colonization. Concerning restriction of the saprophyte, HRGPs may have a primary role in the agglutination and immobilization of bacteria on plant cell walls. The mechanism for localized secretion of HRGPs into the intercellular space and the incorporation of the glycoprotein and possibly other components into encapsulating material remains to be determined. It seems unlikely that HRGP accumulation in papillae and in cells undergoing the HR can have a direct effect on bacterial multiplication, although changes in cell wall structure may restrict the transfer of potentially phytotoxic material. Phytoalexin accumulation may be of more significance for defense against phytopathogenic bacteria [13].

There may be different levels of control governing the accumulation of HRGPs at different sites within challenged leaves. Thus, the rapid formation of HRGPs in papillae may be controlled by activation of synthesis and accumulation from a preformed pool of the deglycosylated protein. Such a localized response may follow damage to the plasma membrane and be regulated by Ca^{++} influx as discussed for the deposition of callose [14]. Secretion of HRGPs as a component of the encapsulating material surrounding the saprophyte may involve a direct response to a bacterial product (elicitor) which may lead to transcription of new HRGP mRNA and also site-directed synthesis. The activity of such an elicitor may also be envisaged in the accumulation of HRGPs within cells undergoing hypersensitive collapse. However, HRGP deposition in live cells at the edge of the lesion was occurring at some distance from the bacterial colony and is more likely to be a response to an endogenous elicitor released from the dying plant cell [15].

The ability to localise HRGPs by immunocytochemical techniques applicable to electron microscopy makes their accumulation an attractive model for studies of signalling and response between plants and bacteria.

We wish to acknowledge support from the U.K. Agricultural and Food Research Council and Science and Engineering Research Council.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

BIOCHEMICAL ASPECTS OF THE INTERACTION BETWEEN PSEUDOMONAS SYRINGAE PV. PHASEOLICOLA AND HIGHER PLANTS

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Phaseolotoxin consists of a substituted L-ornithine, orn-P(O)(NH_2)-NH-SO_3H,(PNSOrn). the active principle of the toxin, and of L-alanyl-L-homoarginine, which has the function of a toxin vehicle in its transport to the plant cell (Rudolph and Stahmann 1966, Rudolph 1976, Mitchell 1976, Mitchell and Bieleski 1977, Moore et al. 1984). In sonic extracts from Pseudomonas syringae pv. phaseolicola (P. phaseolicola) we detected an amidinotransferase (AT) which catalyzes the biosynthesis of the phaseolotoxin precursors L-homoarginine and L-ornithine from L-arginine and L-lysine (Reuter 1989, Märkisch and Reuter 1989):

$$HOOC\text{-}CH(NH_2)\text{-}(CH_2)_3\text{-}NH\text{-}C(NH)\text{-}NH_2 + HOOC\text{-}CH(NH_2)\text{-}(CH_2)_4\text{-}NH_2 \rightarrow$$
$$HOOC\text{-}CH(NH_2)\text{-}(CH_2)_3\text{-}NH_2 + HOOC\text{-}CH(NH_2)\text{-}(CH_2)_4\text{-}NH\text{-}C(NH)\text{-}NH_2$$

The enzyme was purified 419-fold by affinity chromatography on homoarginine-Sepharose. It had an apparent mol. wt. of 200 000 and exhibited Michaelis-Menten kinetics with K_m 7.7 mM for arginine and K_m 4.2 mM for lysine.Ornithine competitively inhibited the enzyme with a K_i of 0.8 mM. Testing the connection between amidinotransferase activity and phaseolotoxin biosynthesis we found a growth coupled biosynthesis of phaseolotoxin; and in correspondence with this the cells already contained AT activity at the beginning of growth. The highest production rate of phaseolotoxin corresponded with the highest enzyme activity.
Cultures at different temperatures decreased their toxin

production with increasing growth temperature and also exhibited a decreased AT-activity with decreasing toxin production.

When microbes were grown on ribose, their enzyme activity was 60 % lower than when grown on glucose or glycerol. And toxin production was decreased to about 10 % when grown on ribose.

In order to check our hypothesis of a correlation between phaseolotoxin formation and AT activity, we examined different pathovarieties. We found that only those strains which were able to produce phaseolotoxin contained AT activity.

Furthermore, we testet the AT activity of the phaseolotoxin producing field strain (1321) and of mutants which produce different amounts of phaseolotoxin. At low toxin production, mutants exhibited a low enzyme activity and vice versa. These results show a close correlation between phaseolotoxin formation and AT activity which suggests that either AT activity is rate-limiting in phaseolotoxin biosynthesis, or several enzymes specifically concerned with phaseolotoxin biosynthesis are governed by the same metabolic controls, perhaps with the corresponding structural genes. Cloning of the genes involved in phaseolotoxin production, may give an answer to this question. Till now it is evident that AT is a prerequisite to phaseolotoxin formation.

Another prerequisite to phaseolotoxin formation is an ornithine carbamoyltransferase (OCT), which is insensitive to phaseolotoxin.

Since the results of Rudolph and Stahmann in 1966 and of Patil et al. in 1970 we have known that phaseolotoxin and PNSOrn are potent inhibitors of OCT, blocking the pathway from ornithine to citrulline, arginine and protein and accumulating ornithine. Thus phaseolotoxin may inhibit both the protein synthesis of its own producer and the biosynthesis of its toxin-precursor arginine as well. But P. phaseolicola is resistant to this selfproduced toxin. In previous publications (Jahn 1984, Jahn et al. 1985, 1987, 1989), searching for the molecular basis of this resistance we could

demonstrate the partial purification and characterization of OCT II, an isoenzyme of ornithine carbamoyltransferase which in contrast to OCT I cannot be inhibited by phaseolotoxin. Two distinct genes encord these enzymes with a close linkage between the gene encording the toxin-insensitive enzyme and genes involved in toxin biosynthesis (Quigley et al. 1985, Peet et al. 1986, Peet and Panopoulos 1987).

Moreover, we investigated the metabolism of carbohydrates against the background of the production of phaseolotoxin precursors and extracellular polysaccharides suspected to be decisive factors of virulence.

P. phaseolicola yields phaseolotoxin during growth on all C and energy sources used, in close relation to biomass formation without catabolite repression. C and energy sources which involve a high specific growth rate lead to a high production of phaseolotoxin. In dependence on the nature of C and energy source employed, P. phaseolicola transfers 2 to 6 % into biosynthetic products.

Among them phaseolotoxin is a minor component only: 6.5 % of the product mixture when grown on sucrose and only 1.0 % when grown on ribose. All of the substrates used yielded product mixtures composed of both high and low molecular compounds. In the high molecular fractions we found levan, alginate and lipopolysaccharides.

The natural C-source of P. phaseolicola in the intercellular liquid of the bean leaves is sucrose and hence sucrose has been established to constitute one of the best C-sources for the biosynthesis of both biomass and toxin (Schacke et al. 1989, Sauerstein et al. 1989).

We found two sucrose splitting enzymes in P. phaseolicola: a levan-sucrase and a sucrase. The levan-sucrase is present both extracellularly and intracellularly. It catalyzes the synthesis of the extracellular polysaccharide levan, which is supposed to be involved in the in vivo interaction with the host plant during pathogenesis. The enzyme synthesis is constitutively regulated, it seems to be independent of the carbon source employed. Only cells grown on sucrose contain

an additional sucrase. The two sucrose splitting enzymes differ in their pH and temperature optima as well as in their substrate specificities (Sauerstein et al. 1988).

Using radioactively labelled substances and investigating enzymes involved in their metabolism, we found the glucose and fructose produced to be metabolized via the 2-keto-3-desoxy-6-phospho-gluconic acid-, and hexose-monophosphate-(KDPG-, and HMP)pathways. The catabolic fructose-bisphosphate-pathway is blocked due to the absence of phosphofructokinase. On the other hand, the extracellular fructose is taken up through the phosphotransferase system, and the synthesized fructose-1P is transferred by fructose-1P kinase to fructose-1.6-bisphosphate which is catabolized by fructose-1.6-bisphosphate phosphatase via KDPG-pathway as well as by fructose-1.6-bisphosphate aldolase via glycolysis (Schacke et al. 1989).

Moreover, the phytopathogenic pseudomonad possesses an isomerase converting mannose into fructose. Since mannokinase and phosphomannose isomerase could not be detected, the isomerase and an inducible fructokinase will probably possess a function in the metabolism of mannose by this bacterium (Sauerstein et al. 1988), for instance, in connection with the biosynthesis of alginate, as a copolymer of mannuronic- and guluronic acid units. Both alginate and the related levan have been suspected to be decisive factors of virulence of P. phaseolicola (Fett et al. 1986, Gross and Rudolph 1987). Apparently, sucrose is not only a good carbon-source for the production of low molecular toxins but also of extracellular polysaccharides and other products which play a crucial role in the invasion of the plant cell tissue by the phytopathogenic microbe. On the other hand, different carbohydrates may be catabolized by quite different metabolic pathways such as KDPG-, HMP-pathway, glycolysis, thus making possible a channelling of the intermediates as precursors of toxin biosynthesis and of positive or negative effectors as well.

Summarizing our results we can postulate three levels of prerequisites for the virulence of Pseudomonas phaseolicola:

1. The regulation of toxin biosynthesis, e.g. by amidinotransferase.
2. The regulation of the biosynthesis of resistance factors against plant toxins or selfproduced toxins, e. g. the biosynthesis of the toxin insensitive isoenzyme of ornithine carbamoyltransferase.
3. The regulation of sucrose catabolism making possible a channelling of the intermediates as precursors of toxin biosynthesis, as positive or negative effectors of this biosynthesis and as precursors of extracellular polysaccharides suspected to be decisive factors of virulence.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

INTERACTIONS BETWEEN CELL WALL POLYMERS OF PHASEOLUS VULGARIS AND BACTERIAL CELL SURFACE POLYMERS OF PSEUDOMONAS SYRINGAE PV. PHASEOLICOLA

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Interactions between surface structures of plant and bacterial cells were assumed by several investigators (Keen and Holliday 1982, Sequeira 1984). It was observed, that a contact between bacterial cells and plant cell walls is necessary to induce the resistant reaction (Stall and Cook 1979). In bush bean leaves "agglutinins" were described, which specifically agglutinated non-pathogenic bacteria (Jasalavich and Anderson 1981), or weakly virulent bacteria were agglutinated stronger than highly virulent ones (Slusarenko and Wood 1983). When different bush bean cultivars were compared, a correlation between the degree of resistance to *P.s.*pv. *phaseolicola* and the intensity of agglutination *in vitro* was reported (El-Banoby and Rudolph 1980, 1981).

The goal of the here reported studies was to further characterize the role of agglutinin as a possible resistance factor of bush bean leaves (*Phaseolus vulgaris*) against *P.s.*pv. *phaseolicola*, to elucidate the role of bacterial polymers as possible virulence factors and to investigate the type of molecular interaction.

MATERIALS AND METHODS

The agglutinin was extracted from the first trifoliates of *Phaseolus vulgaris* cv. Red Kidney -RK- (susceptible), cv. Red Mexican -RM- (susceptible to race 1, resistant against race 2) and breeding line "02" (resistant against both races), as described by El Banoby and Rudolph (1981). The homogenate in 0.1 N HCl was centrifuged and the supernatant precipitated by 38% ammonium sulphate. The supernatant fraction contained the agglutinin.

For agglutination tests the following bacterial strains were used: *Pseudomonas syringae* pv. *phaseolicola*, race 1, isolates Ro-49-d and CH-21B and race 2 isolates FV-75-8a and N7a, *P.s.*pv. *pisi* 1447 and 105, *P.s.*pv. *tomato* 478 and 487, *P.s.*pv. *aptata* 1092 and 62, *P.s.*pv. *lachrymans* 82 and 1084, *P.s.*pv. *atrofaciens* 51R15 and 1440, *P. marginalis* 93 and 92, *P. andropogonis* 59, *P. putida* and *P. fluorescens*. The bacteria were harvested from 100 ml cultures grown in Keen and Williams (1971) medium. Extracellular polysaccharides (EPS) and lipopolysaccharides (LPS) were produced and prepared according to Gross and Rudolph (1987a and 1987b).

For the agglutination assay 10 µl agglutinin solutions in different dilutions were mixed with 10 µl of bacterial suspension (1.5 x 10^{10} cfu/ml) in Petri dishes. Agglutination was scored as degree of flocculation (scale 1 to 4) under the dissecting microscope.

Hexoses, uronic acids and protein contents were quantified according to Dubois et al. (1956), Bitter and Muir (1962) and Bradford (1976), respectively. Viscosity was measured with a falling ball viscosimeter (Haake, West-Germany).

RESULTS

The resistant cultivar "02" produced more agglutinin (twice as much) than the susceptible cultivar "RK" (tab. 1).

Tab. 1: Agglutinin production in leaves of three cultivars of *P. vulgaris* (average of 3 repetitions)

cultivar		mg aggl./g leaves (dry weight)
Red Kidney	(susceptible)	1.9
Red Mexican	(differential cv.)	2.2
02	(resistant)	3.9

Agglutinin production and agglutinating activity increased in all three cultivars with time after inoculation with *P.s.*pv. *phaseolicola* as well as after infiltrating the leaves with water by an atomizer. If bacteria were freed from extracellular polysaccharides (EPS) by washing in dilute saline, they were strongly agglutinated, in contrast to unwashed bacteria (tab. 2).

Tab. 2: Agglutination of *Pseudomonas* sp. by agglutinins of 3 cultivars of *P. vulgaris* (agglutination indices from 1 to 4)

	washed bacteria			unwashed bacteria		
cultivars	RK	RM	02	RK	RM	02
P.s.pv. phaseolicola						
RO	2	3.5	4	0	0	0.25
FV	2	2.5	3	0	0	0.25
N7a	0.5	1	1.5	0	0	0.5
P. fluorescens	0.5	0.5	1	0.25	0.5	0.25

In most cases the agglutinating activity of the resistant cultivars ("RM", "02") was higher than that of the susceptible cultivar "RK". The highly virulent strain N7a was always less agglutinated than the lower virulent strains. N7a produced the highest amount of EPS with the highest viscosity and alginate content (tab. 3).

Tab. 3: Production of extracellular polysaccharides (EPS) by *P.s.*pv. *phaseolicola* in batch culture

bacterial strains		EPS (mg/l)		viscosity**	alginate***
		gluconate*	saccharose*	(sec)	%
race 1	RO	108	670	14	30
	CH	189	450	14	30
race 2	FV	318	519	16	33
	N7a	1323	1248	22.5	86

* C-source in culture media
** in culture medium with saccharose
***100 % = commercial alginate from algae

Agglutinin of cv. 02 was also effective against strains of 5 other *P. syringae* pathovars as well as against *P. marginalis* and *P. andropogonis*. Several monomeric and polymeric sugars were tested for an inhibition of agglutination. Only sodium polygalacturonate and arabinogalactan inhibited agglutination of bacteria by plant extracts. Likewise, fractions from the bacterial envelope (EPS, alginate and levan) decreased agglutination of *P.s.*pv. *phaseolicola* and *P. putida* (fig. 1). Agglutination of bacteria disappeared after adding buffer solution of pH>5.

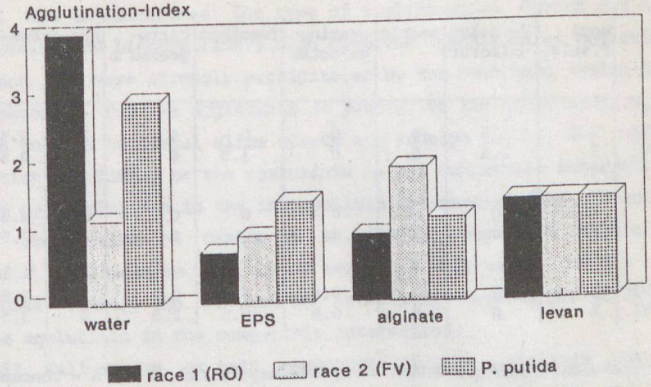


Fig. 1: Inhibition of agglutination by fractions of the bacterial surface

Not only intact bacteria, but also isolated fractions from the bacterial envelope (lipo-polysaccharides - LPS- and alginate) were strongly precipitated, especially by agglutinin of the resistant cv. "02" (fig. 2). Also the precipitation between LPS and agglutinin could be decreased by sodium polygalacturonate and EPS, or totally be dissolved by adding buffer of pH>4.

LPS=lipopolysaccharides;r=rough,s=smooth
alg=alginate
no reaction with levan

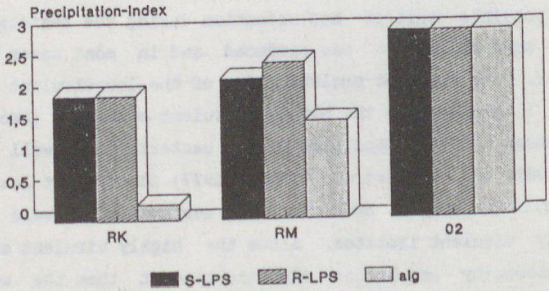


Fig. 2: Precipitation of fractions from the bacterial envelope with agglutinin of 3 cultivars of *P. vulgaris*

Bean agglutinin, pectin from citr s and 3 lectins showed agglutinating/precipitating activity with bacteria and LPS (tab. 4), whereas the strongly negative charged polysaccharides fucoidan and carrageenan L showed no reaction with LPS. Agglutinin from cv. "02" and pectin were more active against race-2-EPS with higher viscosity than against race-1-EPS.

Tab. 4: Agglutination of bacteria, and precipitation of bacterial lipopolysaccharides (LPS) and extracellular polysaccharides (EPS) with plant polymers

	aggl. P.vulg.	Na-poly-galactur. acid	pectin	arabino-galactan	fucoidan	carra-geenan L	PHA-P	PHA-M	Con A
P. phaseol.									
race 1 (CH)	2.5	1.5	4	0	0	4.5	2	0.5	4
race 2 (FV)	3	2.5	5	0	1.5	4	2.5	0.5	4
LPS									
rough	2.5	0	3	0	0	0	3.5	2.5	4.5
smooth	2.5	0	3	0	0	0	3.5	2.5	4.5
EPS									
race 1 (CH)	0.5	0	0.5	0.5	0.5	2.5	2	3.5	2
race 2 (FV)	2	0	5	0.5	0.5	2.5	3	3.5	3

PHA-P = Phytohaemagglutinin P, PHA-M = Phytohäemagglutinin M, Con A = Concanavalin A

A preliminary analysis of the agglutinin demonstrated hexoses and uronic acids to be the main components, with 2-4% protein. A separation by anion exchange chromatography (DEAE fractogel) with gradient elution revealed a neutral and an acidic polysaccharide. Both fractions had agglutinating activity, but the activity of the acidic fraction was higher. After hydrolysis and TLC analysis the typical plant cell wall sugars rhamnose, arabinose, galactose and galacturonic acid were detected in the acidic fraction, whereas galacturonic acid was missing in the neutral fraction.

DISCUSSION

The results suggest a possible role of agglutination during the resistant reaction. In the resistant cultivar more agglutinin was produced and in most cases the efficacy of agglutination was higher. The stronger agglutination of the low virulent isolates RO and CH (unpublished) (race 1) compared to the highly virulent strain N7 (race 2) may be due to quantitative differences in the composition of the bacterial cell wall envelopes. Also Slusarenko and Wood (1983) and Sequeira and Graham (1977) found, that less virulent isolates of *P.s.*pv. *phaseolicola* and *P. solanacearum*, respectively, were more strongly agglutinated than highly virulent isolates. Since the highly virulent strains produced more EPS with higher viscosity and higher algiante content than the weakly virulent strains, we suggest, that residual layers of EPS prevented the washed bacteria from *in vitro* agglutination by covering the bacterial surfaces. In ultrastructural studies of the hypersensitive reaction of the bean plant Brown and Mansfield (1988) also detected in the resistant plant more material originating from the plant cell wall within colonies of

*P.s.*pv. *phaseolicola* and on the outer surface of the host wall surface than in the susceptible plant. They also observed differences in form of EPS. A correlation between amount of EPS and agglutination was also found by Fett and Sequeira (1980) with *Xanthomonas campestris* pv. *glycines* and Bradshaw-Rouse et al. (1981) with strains of *Erwinia stewartii*. Since the agglutinating activity is not restricted to strains of *P.s.* pv. *phaseolicola*, agglutinin might also be involved in defense reactions towards non-bean-pathogenic bacteria.

The inhibition of agglutination by sodium polygalacturonate a d arabinogalactan as well as by bacterial EPS characterizes the type of agglutination. Duvick and Sequeira (1984) and Da Silva Romeiro and Lieberei(1987) also observed inhibition of agglutination by bacterial EPS. Since LPS were strongly precipitated by the bean leaf agglutinin, LPS may be the bacterial acceptor for the agglutinin *in planta*. On the other hand, alginate, a constituent of the soluble bacterial slime (Gross and Rudolph 1987b), may inhibit the bacterial agglutination by binding to the agglutinin in the compatible interaction, whereas it is precipitated by agglutinin in the incompatible interaction. These results support the ultrastructural observations of Graham et al. (1977), who found an interaction between purified LPS of *P. solanacearum* and tobacco mesophyll cell walls, and the observations of Takahashi and Doke (1984), who suggest an interaction between EPS of *X. campestris* pv. *citri* and citrus agglutinin in the compatible interaction.

The typical cell wall sugars as main components of the agglutinin were also found by other authors in agglutinin preparations of bush bean (Jasalavich and Anderson 1981), potato (Leach et al. 1982) and tobacco callus tissue cultures (Mellow and Helgeson 1982). We also observed agglutination of pseudomonads by pectic substances. Agglutination/precipitation disappeared, when buffer of pH>5 (4) was added (agglutinin solution = pH 5,5). LPS was not precipitated by the strongly negative charged polysaccharides fucoidan and carrageenan L. These facts indicate, that the agglutination/precipitation do not seem to be based on charge/charge interactions. Since agglutination/precipitation appear with agglutinin, which is rich in galacturonic acid, as well as with pectin, but not with arabinogalactan, galacturonic acid may be a decisive component of the agglutinin. A polysaccharide-polysaccharide interaction between LPS, EPS and agglutinin may play a part during the resistant reaction of leaves towards bacteria.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

EVIDENCE OF A CELLULAR SENSITIZED STATE IN THE PROTECTION OF TOBACCO LEAVES WITH PROTEIN-LIPOPOLYSACCHARIDE COMPLEXES

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INTRODUCTION

Hypersensitive confluent necrosis (HCN) caused by *Pseudomonas syringae* pv. *aptata* in tobacco leaves can be prevented by pretreatment with protein-lipopolysaccharide complexes (pr-LPS) (Mazzucchi *et al.*, 1988). A localized cellular reaction (LCR) can be observed starting from 4h after intercellular injection of pr-LPS complexes, which has a peak at 24h and is exhausted after 48h. When the state of induced protection begins to appear, 24h after pr-LPS treatment an intense vesiculation of the plasmalemma takes place. The connection between the prevention of HCN and induced protection is not clearly understood.

The protection induced by pr-LPS complexes is not due to preformed intercellular substances inhibiting bacterial growth (Minardi *et al.*, 1989) nor the result of structural and/or chemical alterations of the plant cell walls which prevent bacterial colonization of the tissue (Minardi *et al.*, 1988; Minardi unpublished). The prevention of HCN can be correlated to the activation of enzymes bound to the plasmalemma or the plant cell walls which create a sensitized state (Dean and Kúc, 1987).

This paper reports the results of experiments set up to test the sensitized state hypothesis. Protection was correlated to activation of the plasmalemma NADH-duroquinone reductase (NADH-DQR) and extracellular β-1,3 glucanase.

MATERIALS AND METHODS

Bacterial culture, pr-LPS complexes, leaf infiltration, fluid extraction and induced protection

The strain *Pseudomonas syringae* pv. *aptata* NCPPB 2665 and the methods according to Mazzucchi *et al.*(1988) were used.

Microsomal membrane preparation

The treated and control areas were homogenized in 2 ml g^{-1} fresh weight of ice-cold buffer A (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 10 mM 2-mercaptoethanol, 1 mM EDTA) with a Sorvall Omnimixer (30 sec. 210 V). The homogenate was filtered through a Miracloth and then centrifuged at 1,000 g for 10 min, 9,000 g for 20 min and 100,000 g for 60 min. The microsomal pellet (P 100K) was resuspended in buffer A and fractionated on a linear gradient of sucrose as described by De Luca *et al.* (1984). The proteins were determined for each fraction and the enzymatic markers of the different types of membrane were: Glucan synthetase II (GSII) as plasmalemma marker, Glucan synthetase I (GSI) as Golgi marker and Cytochrome c oxidase (CCO) as mitochondrial marker as described by Valenti *et al.* (1989).

Enzyme assay

NAD(P)H - DQR was assayed according to Guerrini *et al.* (1987) in a reaction mixture containing 50 mM Tricine-NaOH pH 8.0, 1 mM EDTA, 300 mM sucrose, 0.03% TRITON X100, 0.15 mM NADH or NADPH and 0.2 mM DQ.

The methods described by Pegg and Young (1981) were used to determine β-1,3 glucanase and chitinase activity. One unit of β-1,3 glucanase produced reducing groups equivalent to 100 µg glucose ml^{-1} reaction mixture in 1h at 30°C, pH 5.2; one unit of chitinase produced 100 µg N-acetyl-glucosamine ml^{-1} reaction mixture in 1h at 37°C, pH 5.2.

RESULTS AND DISCUSSION

In the microsomes of tissue pretreated with pr-LPS complexes (treated tissue) NADH and NADPH dehydrogenase activity was higher than in the tissue pretreated with water (control tissue) at 24h and 48h but not at 0h (fig.1.A). The NADH-DQR and NADPH-DQR activity in the treated tissue was approximately two and one and a half times that of the control. The microsomes at 24h, from the treated and the control tissue, were used to separate the different types of membrane at the isopycnic equilibrium in sucrose linear gradient (fig. 1.B). The activity of the two dehydrogenases was observed in different membranes but they were mainly associated with particles having plasmalemma properties (fig.2). In fact the peaks of the two activities were found at 37% of sucrose, coinciding with the GSII peak. In the plasmalemma fraction of the treated tissue the NADH-DQR had increased approximately five-fold as compared to the control whereas the NADPH-DQR remained the same (fig.2).

The plasmalemma NADH-DQR and NADPH-DQR have similar characteristics to DT-diaphorase described by Lind *et al.* (1982) (Guerrini *et al.*, 1987; Valenti *et al.*, 1989). In plant tissue quinone reduction may occur in a single step

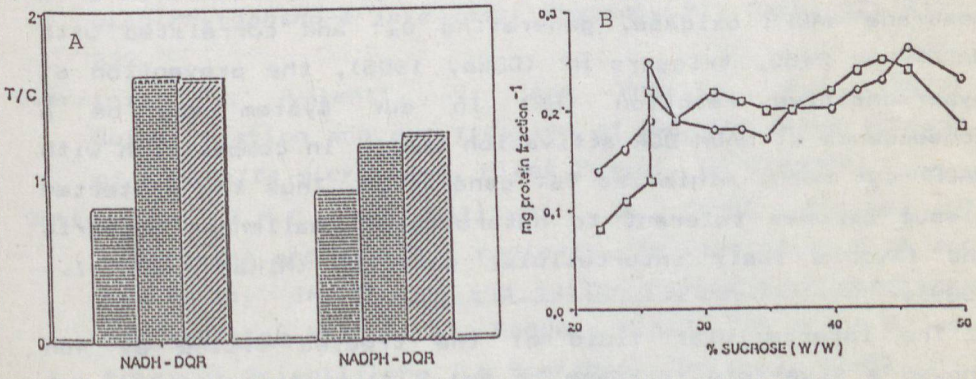


Fig.1: Relative variation of NADH-DQR and NADH-DQR activities (A) in microsomes from treated tissue as compared to control (T/C) at 0 time (▤), 24h (▩) and 48h (▨). Protein (B) in the linear sucrose gradients of 24h microsomal fractions from control (□) and pr-LPS treated tissue (○).

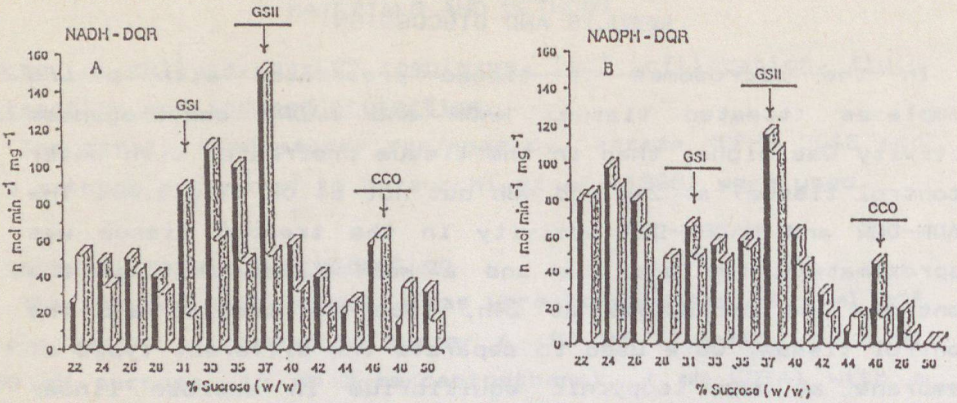


Fig.2: Linear sucrose gradients of 24h microsomal fractions from control (□) and pr-LPS treated tissue (■). Profile of NADH-DQR (A) and NADPH-DQR (B). Arrows indicate the peak centres of plasmalemma markers (GSI, GSII, CCO). Data expressed as specific activities.

(through DT-diaphorase type NAD(P)H-DQR) or in two steps (through NADPH-cyt P450) with the intermediate formation of the semiquinone radical involved in the generation of superoxide radicals ($O_2^{\cdot-}$) (Lind *et al.*, 1982). The $O_2^{\cdot-}$ are probably responsible for triggering the hypersensitive degeneration of the plasmalemma (Doke, 1985; Gutteridge and Halliwell, 1986), but in our system they do not have a direct lethal effect on the heterologous bacteria which induces it (Minardi and Mazzucchi, 1988). Assuming that activation of a membrane NADPH oxidase, generating $O_2^{\cdot-}$ and correlated with NADPH-cyt P450, triggers HR (Doke, 1985), the prevention of hypersensitive reaction (HR) in our system may be a consequence of NADH-DQR activation which, in competition with NADPH-cyt P450, minimizes $O_2^{\cdot-}$ generation. Thus the protected tissue becomes tolerant to heterologous challenger bacteria and favours their intercellular survival (Minardi *et al.*, 1989).

The intercellular fluid of the treated tissue at 48h showed a five-fold increase in β-1,3 glucanase activity but not in chitinase activity. The β-1,3 glucanase activity was 1.17 and 0.21 units in the intercellular fluid extracted from the treated and the control tissues. In fungi-plant systems there is a post-infection increase in both these enzymes

(Matta *et al.*, 1988; Boller and Mêtraux, 1988). In our system the non-activation of chitinase may be due to the absence of chitin in the pr-LPS elicitor complexes. Activation of β-1,3 glucanase in intercellular fluid of protected tissue may degrade the surface polysaccharides of challenger bacteria, leading to the rapid generation of signal molecules and thus conditioning the plant-bacteria recognition process (Mazzucchi *et al.*, 1988).

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
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EARLY INTERACTIONS BETWEEN BACTERIAL PATHOGENS AND PLANT CELL-SUSPENSIONS

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ABSTRACT

Previous work has demonstrated that addition of bacteria which induce a hypersensitive response (HR) will initiate a net uptake of extracellular H+ and net increase in extracellular K+ in tobacco cells. This response is preceded by a transient increase in lipid peroxidation and O_2^- production. The relationships between these early plant responses were studied using suspension-cultured tobacco cells and compatible, incompatible, and saprophytic bacteria. The bathing medium was monitored during a 6-hour period for pH, conductance, [K+] using ion chromatography, and active oxygen using chemiluminescence. In most cases a transient increase in chemiluminescence and pH occurred within the first few minutes after addition of the bacteria. After about 2 hours a more prolonged increase occurred with incompatible bacteria. The extent of the early transient or later prolonged response appears to depend on the host-pathogen compatibility.

INTRODUCTION

One of the earliest detectable events in the hypersensitive response of plants to bacteria involves the uptake of H+ from the extracellular environment and an efflux of K+ from the cell [1]. Studies using Tn5

*This work was supported in part by United States Department of Agriculture specific cooperative agreement #58-32U4-3-602 and United States Department of Agriculture Competitive Grants #87-CRCR-1-2304 and #88-37151-3694.

mutants of Pseudomonas syringae pv. syringae have confirmed the close association between this K+ efflux/H+ uptake and the hypersensitive response in tobacco [2]. We are currently studying the events which precede this K+/H+ response and believe that active oxygen may play an important role.

Immediately following the addition of bacteria to tobacco cell suspension cultures we observed concurrent transient increases in superoxide and lipid peroxidation [3]. Superoxide scavengers inhibited these increases and reduced the K+/H+ response. Recent studies of active oxygen using chemiluminescence supported these findings [4].

In this study these early events in tobacco were simultaneously monitored using a broader range of bacteria to compare incompatible, compatible and saprophytic interactions.

MATERIALS AND METHODS

Bacteria used in this study which cause a hypersensitive reaction on tobacco were Pseudomonas syringae pv. syringae wild-type (WT) and P. s. glycinea, races 4 and 6. Bacteria which did not cause a hypersensitive reaction included the compatible pathovar tabaci, a Tn5 mutant of pathovar syringae (B7) [3], and the saprophyte P. fluorescens.

Tobacco suspension cells (0.05g/ml) were bathed in assay medium containing 0.5 mM $CaCl_2$ and 0.5 mM K_2SO_4 in 0.5mM MES (2[N-morpholino] ethane sulfonic acid) buffer, pH 6.0, as previously described [1]. Bacteria were added for a final concentration of $1x10^8$/ml. The pH and conductivity of the suspension were measured directly by electrode. Aliquots of the suspension were assayed for K+ by ion chromatography.

Chemiluminescence measurements were carried out under identical conditions except that the assay medium contained 5.0 mM MES buffer, pH 5.8, to maintain constant pH. Luminol was added to samples and chemiluminescence was measured immediately in a scintillation counter [4].

Results are the average of 3 experiments with 2 replicates each. In most cases the data reflects the difference in response between the treatment and the control. Standard error bars are shown for WT and/or B7 and are representative of the other treatments.

Tobacco cells incubated with compatible, incompatible, or saprophytic bacteria showed a slight increase in H+ uptake immediately after bacterial inoculation (Figure 1A). Approximately 2 hours after inoculation a second, more prolonged H+ uptake occurred in treatments with HR-causing bacteria.

Increases in conductivity and [K+] of assay medium occurred after about 2 hr in HR-causing bacterial treatments while non HR-causing bacteria maintained levels close to buffer controls (Figure 1B). Treatments with B7 appeared to have less K+ efflux from tobacco cells than other non HR-causing bacteria (Figure 1B).

Measurements of chemiluminescence showed that there were two periods of increased active oxygen levels. The first occurred immediately after bacterial addition and appeared to be nonspecific, occurring in all bacterial treatments (Figure 2B). It was characterized by a very large response followed by a rapid decline in all treatments except the saprophyte P. fluorescens which remained high throughout the 6-hour period. This suggests that treatments with pathogens were able to neutralize the high active oxygen levels. A second period of increased active oxygen occurred in treatments with HR-causing bacteria. Chemiluminescence increased gradually after 2 hr, while treatments with non HR-causing bacteria decreased (Figure 3).

It was interesting to note that the initial increase in chemiluminescence (Figure 2B) followed the same pattern as the increase in the rate of H+ uptake (Figure 2A). This indicates that the slight H+ uptake which occurs when bacteria are added to tobacco cells may be due to scavenging of H+ by active oxygen species (such as superoxide) which are produced during this same period.

In the second response which initiates at about 2 hr with incompatible bacteria, there appears to be a reversal of the net K+/H+ transport in the cell. As we have reported previously [5], this may affect nutrient concentrations in the extracellular environment. We are continuing our investigation of events leading to the K+/H+ response, including those involved in active oxygen production and their possible role in signal transduction mechanisms.

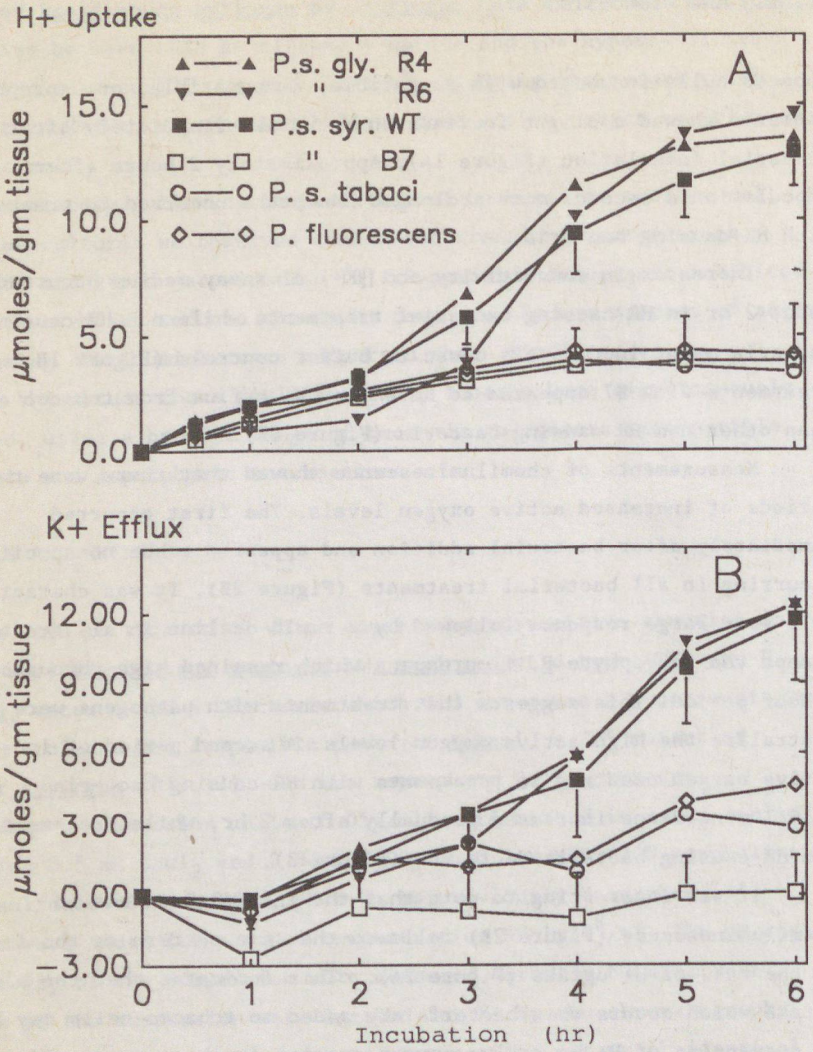


Figure 1. Change in H+ uptake and K+ efflux of tobacco suspension cells incubated with compatible, incompatible and saprophytic bacteria. A. H+ uptake was monitored by pH meter and calculated using titration curves. B. K+ efflux was determined by ion chromatography. Bacteria which result in a hypersensitive reaction on tobacco are represented by solid symbols.

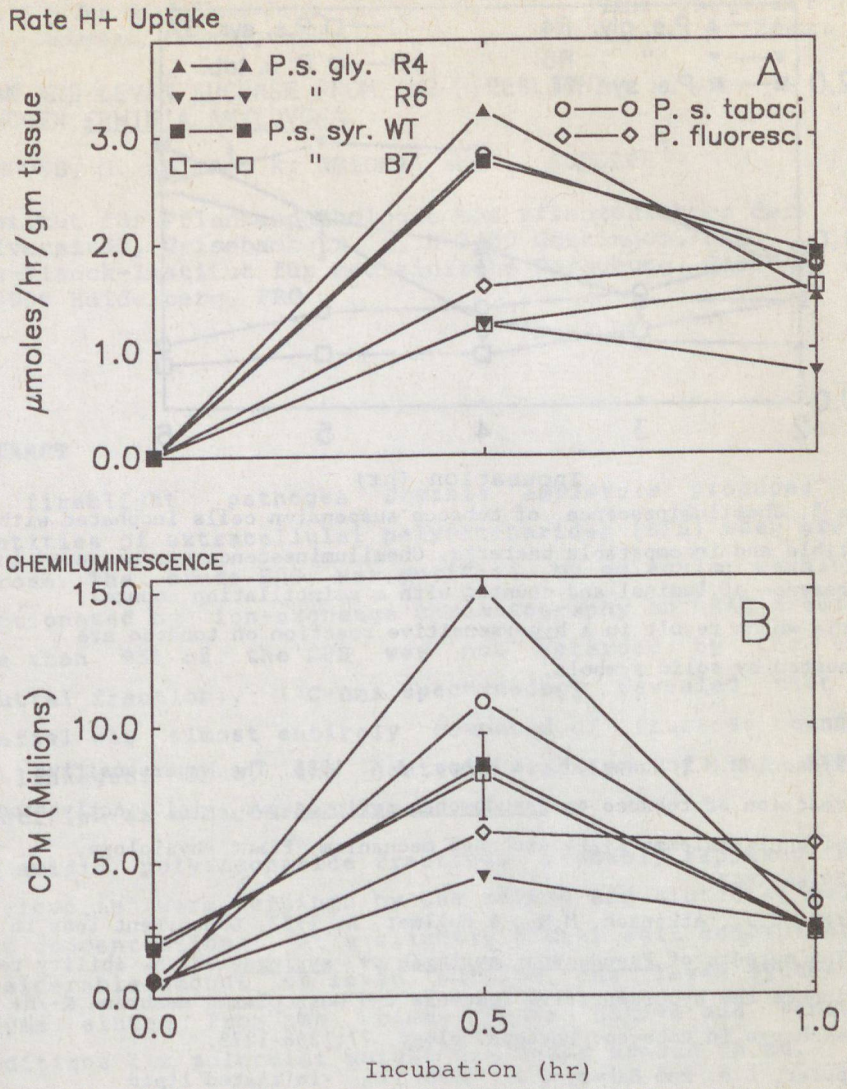


Figure 2. Comparison of initial rates of H+ uptake (A) and chemiluminescence (B) of tobacco suspension cells incubated with compatible, incompatible and saprophytic bacteria. Bacteria which result in a hypersensitive reaction on tobacco are represented by solid symbols.

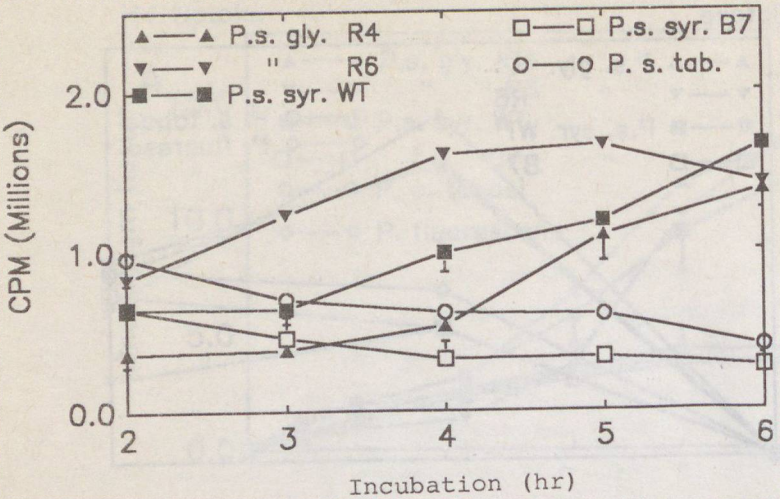


Figure 3. Chemiluminescence of tobacco suspension cells incubated with compatible and incompatible bacteria. Chemiluminescence was measured in the presence of luminol and counted with a scintillation counter. Bacteria which result in a hypersensitive reaction on tobacco are represented by solid symbols.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

LEVAN AND LEVAN SUCRASE FROM THE FIREBLIGHT PATHOGEN ERWINIA AMYLOVORA

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ABSTRACT

The fireblight pathogen *Erwinia amylovora* produces large quantities of extracellular polysaccharides (EPS) when grown on sucrose. The crude EPS was purified by molecular washing and fractionated by ion-exchange chromatography on DEAE fractogel. More than 95% of the EPS was not retarded by the column (neutral fraction). ^{13}C-nmr spectroscopy revealed that this material was almost entirely composed of fructose bound *via* 2,6-linkages. Thus, the neutral fraction of the EPS was identified as a bacterial levan.

Two acidic polysaccharide fractions probably representing the "amylovorin" were retained by the column and eluted at moderate salt concentrations. At a slightly higher salt concentration a considerable amount of levan sucrase, the levan synthesizing enzyme, eluted from the column. Under native and denaturing conditions its molecular weight was found around 60 kd.

INTRODUCTION

One of the severe fireblight symptoms is the formation of ooze at diseased parts of infected host plants, i.e. many species of the *Pomoideae*. Avirulent mutants of the causative bacterium *Erwinia amylovora* can be deficient in their ability to synthesise extracellular polysaccharide (STEINBERGER and BEER 1988). A 29 kb plasmid, common to *E. amylovora* (FALKENSTEIN *et.al.* 1988), affects ooze production on immature pear or on certain

media (FALKENSTEIN *et.al.* 1989). Besides the galactose-containing acidic polysaccharide (GOODMAN *et.al.* 1974), *E. amylovora* is also able to produce copious amounts of a fructan, when cultivated in an excess of sucrose (BENNETT and BILLING 1980). This feature has been utilized as a taxonomic criterion (SCHAAD 1980). We have investigated this type of polysaccharide with respect to its sugar linkage. We have also characterized the enzyme involved in fructan polymerization.

RESULTS

1. Synthesis and structure of levan

E. amylovora tolerates extremly high concentrations of sucrose (SCHAAD 1980). When grown in liquid medium with 100 g sucrose per litre, the bacteria converted more than 10% of the sugar into extracellular polysaccharide within two days, whereas their dry cell mass reached only about 1.8 g/l. The cell-free culture supernatant was concentrated by ultrafiltration and loaded on a DEAE-fractogel column. More than 95% of the EPS was recovered in the column pass through. After separation of the neutral polysaccharide, the column was further developed with a salt gradient, and the fractions obtained were assayed for total carbohydrate, protein, and levan sucrase activity. (GROSS and RUDOLPH 1987c) The latter was determined by incubating aliquots of the fractions in sucrose buffer. Fractions containing the enzyme soon developed a white opalescence due to the levan formed. The glucose liberated simultaneously was estimated enzymatically.

The neutral fraction not retarded by the column was recovered and subjected to ^{13}C-nmr spectroscopy. Authentical inulin and levan were used as reference standards. The signals obtained corresponded to a ß-2,6 linkage of the fructose residues. Thus, the neutral fraction of the EPS of *E. amylovora* was identified as bacterial levan.

2. Characterization of the levan sucrase

In order to polymerize the fructose residues of sucrose *E. amylovora* secrete an enzyme, which was enriched from the culture supernatant and applied to the DEAE-fractogel column as described above for levan. Levan sucrase (ß-2,6-fructan:D-glucose 6-fructosyltransferase, E.C. 2.4.1.10) eluted as a sharp, single peak and was characterized on SDS gels, by iso-electric focussing, and by gel filtration. In 0.1% SDS the enzyme was still active, which could easily be seen by swelling of the gel after incubation in a sucrose buffer. Levan sucrase migrated under partially denaturing conditions (0.1% SDS) with a protein size of 52 kd. The marker positions did not show a significant shift when run on PAGE in 0.1% SDS compared to a boiled sample in 1.0% SDS. We therefore assume that the position of the active levan sucrase in 0.1% SDS indicates its monomeric molecular weight. Separation by HPLC on TSK-gel columns revealed its native molecular weight around 60 kd eluting between ovalbumin and bovine serum albumin as markers. This indicates that levan sucrase is a monomeric protein in solution. On an IF-focussing gel the enzymatic activity focussed at a pH value of 4.3. Under other conditions we observed focussing at an even lower pH value.

When the cells were grown in medium with sorbitol or glucose instead of sucrose, levan sucrase was secreted at a similar level as in the presence of sucrose. The enzyme is therefore constitutively synthesized, and is not induced by sucrose.

CONCLUSIONS

The fireblight pathogen *E. amylovora* secretes an enzyme, levan sucrase, which polymerizes sucrose in the bacterial environment to levan, a ß-2.6 linked fructan. Constitutive expression of the enzyme suggests an important role in the life cycle of *E. amylovora*. On the other hand, levan is not synthesized by the bacterium on immature fruits. This implies the presence of sucrose during growth of *E. amylovora* in other parts of rosaceous plants.

The levan reported here is synthesized in addition to the galactose-containing acidic polysaccharide "amylovorin". It should be mentioned that also many pathovars of *Pseudomonas syringae* are capable of producing levan and an acidic polysaccharide (alginate; FETT *et.al.* 1986, GROSS and RUDOLPH 1987a). This may suggest similar modes of action of these different EPS types during pathogenesis.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

EFFECT OF THE EPS OF ERWINIA AMYLOVORA ON THE HOST METABOLISM OF CYDONIA OBLONGA

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ABSTRACT

Studies on the mode of action of the extracellular polysaccharide (EPS) of the fireblight pathogen Erwinia amylovora were undertaken in order to clarify if EPS has an essential function during symptom expression. As phenol metabolism plays an important role during the resistance reaction in many host parasite interactions,the total content of phenols, the qualitative changes of individual phenols and activity of phenoloxidase were estimated after EPS-treatment and infection in leaves of quince. From the results EPS has a light-reducing effect on the phenol metabolism in the plant, as for example a slight reduction in the phenoloxidase activity, in the total phenol content and of several phenols. However, in the inoculated leaves a drastic effect with to a marked reduction of the whole phenol metabolism was observed. The possible role of EPS during pathogenesis is discussed.

INTRODUCTION

Although the extracellular polysaccharides (EPS) in various bacterial host-pathogen combinations have been reported to play an important role in the development of symptoms or to have the properties of a virulence factor (Bennett and Billing, 1980), there are very few parameters currently known which could explain the mode of action of the EPS in the host plant. One of the most important mechanisms of the EPS is postulated to be the hindrance of contact between the bacteria and the plant cell wall, and thus the prevention of a rapid collapse of the

cell hypersensitivity reaction which otherwise results. The EPS from Erwinia amylovora would also appear to play an important role in the initial stages of pathogenesis (Goodman and White, 1981).

Initial physiological results indicate that EPS has an influence on the respiratory process: After the infiltration of different concentrations of the EPS into pear leaf tissue, a significant reduction in the use of oxygen could be established (Zeller, 1984). In addition the pale green colour of the leaves was maintained longer following the EPS treatment, whereas the control samples were nearly black, indicating a high accumulation of phenolic substances. Based on these observations it was concluded that the EPS has an inhibitory function on the resistance reaction of the host plant. For that reason we determined whether changes in the phenol metabolism, which is involved in the resistance reaction, could be established after an EPS treatment.

MATERIAL AND METHODS

EPS treatment and leaf sampling

EPS was obtained from the ooze from infected unripe pear fruit slices, cv. Bartlett, by the methods of Goodman et al. (1974) and Gerwe et al. (1987). This crude preparation was dialyzed for two days against double distilled water at 4°C, purified with Dowex 50 and 1, and stored in a desiccator over P_2O_5. Two-year-old container grown quince plants (Cydonia oblonga) were grown under glasshouse conditions (15-23°C) to a shoot length of 15-20 cm. The third and fourth leaves from the tip were removed and infiltrated under vacuum with 1.0 mg/ml EPS solution. For comparison, leaves were treated with 10^8 CFU/ml. Treatment with tap water served as a control. Infiltrated leaves were held in a humid chamber at 23°C for 72 h p. i., after which they were frozen in liquid nitrogen and ground to a fine powder. Each sample consisted of 10 leaves.

Extraction of phenols

Phenols were extracted as described by Rapp and Ziegler (1973). 0.25 g ground leaf material was homogenized by sonification for 5 min in 15 ml methanol and 0.1 g ascorbic acid. After filtration and evaporation to 2-3 ml, the residue was washed 3 x with 15 ml methanol, evaporated to dryness, resuspended in 5 ml methanol and stored at -20°C.

Determination of total phenol content, individual phenols and phenoloxidase activity (PPO)

Phenol content was determined according to Zeller and Brulez (1987). 0.1 ml of the methanol extract was pipetted into a 50 ml measuring flask and made up to volume with distilled water. 2.5 ml folinciocalteau reagent (Merck, 9001) and 7.5 ml 20% soda solution (Na_2CO_3dehydrated p. a.) were added and the colour development determined at 765 nm in a spectrophotometer (Beckman, model 25) against a reference curve with gallic acid. Individual phenols were determined by gas chromatography (GLC). They were first converted to their volatile derivatives by trimethyl silylation. For this purpose 1 ml methanol extract in micro-vials was dried under N_2(conducted over conc. $H_2SO_4$and P_2O_5). Silylation was carried out with 2.5 ml of a hexamethyldisilazan:trimethylchlorsilan:pyridin (2:1:5) mixture for 2 h at 70°C. Data of the GLC-analysis: gas chromatograph Perkin-Elmer 3920 HF with double FID; 2 glass columns in compensation, length 1.8 m, diameter 0.4 cm, filled with 4% siliconoil OC 17 on Chromosorb 6, AW-DMCS 880-100 mesh; carrier gas 80 ml He/min, fire gas H_2, synthetic air, temperature of the column oven 250°C, isotherm detector 300°C, injection block 300°C, volume injected 1µl. The activity of phenoloxidase was determined optically at 400 nm with a Beckman Photometer model 25 according to Zeller and Brulez (1987).

RESULTS

The following phenolic compounds could be identified by GLC to be present in quince leaves: o-cumaric acid, gallic acid, (+)-catechin, rutin, arbutin and chlorogenic acid. Rutin represented the most abundant compound with contents of more than

100 mg/g dried leaf material, the other phenolic compounds were present in the range of 2-40 mg/g (Fig. 1,2,3).

When the content of the individual phenolics was determined over a 72 h period after infiltration with EPS or after inoculation with the disease organism, it could be established that o-cumaric acid, rutin and (+)-catechin reacted similarly (Fig. 1,2). During the first 24 h p.i.a similar pattern compared with the water control was observed with these phenolics. After that period until the end of the experiment, their content in the EPS-treated sample was slightly lower. In the inoculated samples, however, their content was rapidly reduced nearly to zero (Fig. 1,2). The content of gallic acid and arbutin also showed a reduction following EPS treatment, however this was not so clearly demonstrated (Fig. 3). A determination of the total phenol content of the samples showed a pattern similar to that obtained with o-cumaric acid, (+)-catechin and rutin (Fig. 4). Like with them a slight reduction was recorded 24 h p. i. and the inoculation with bacteria resulted in a dramatic reduction. The phenoloxidase (PPO) activity recorded in the different samples gave a very similar picture (Fig. 5).

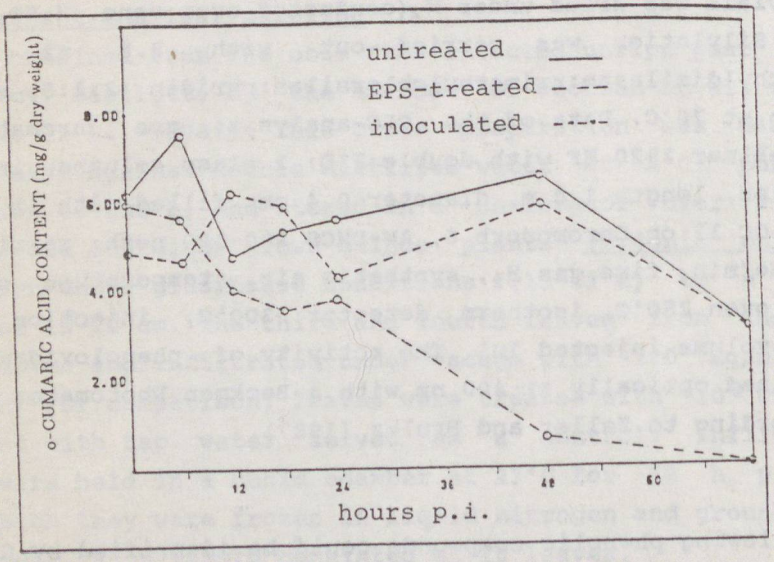


Fig.1 o-Cumaric acid content after EPS-treatment and inoculation with E. amylovora in leaves of Cydonia oblonga

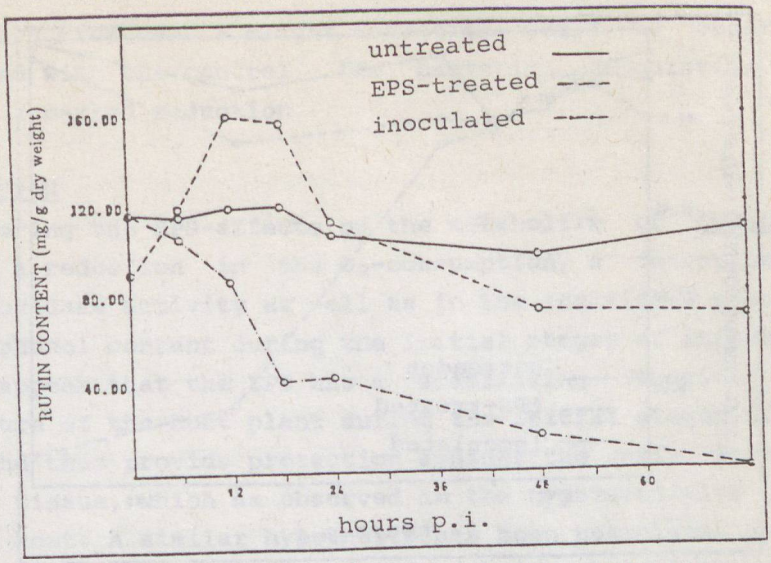


Fig.2 Rutin content after EPS-treatment and inoculation with E. amylovora in leaves of Cydonia oblonga

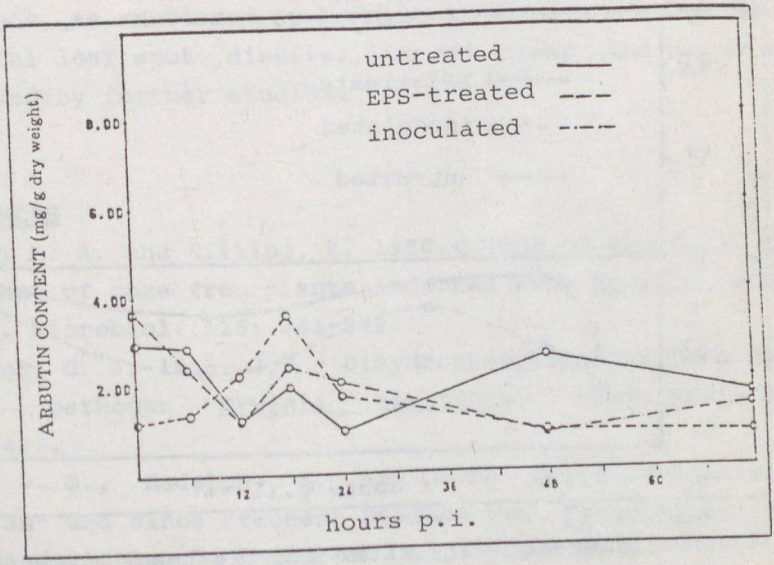


Fig.3 Arbutin content after EPS-treatment and inoculation with E. amylovora in leaves of Cydonia oblonga

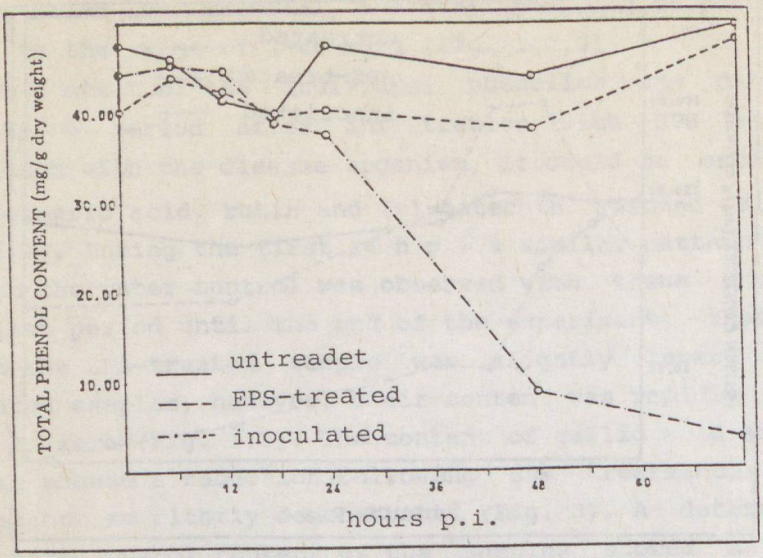


Fig.4 Total phenol content after EPS-treatment and inoculation with E. amylovora in leaves of Cydonia oblonga

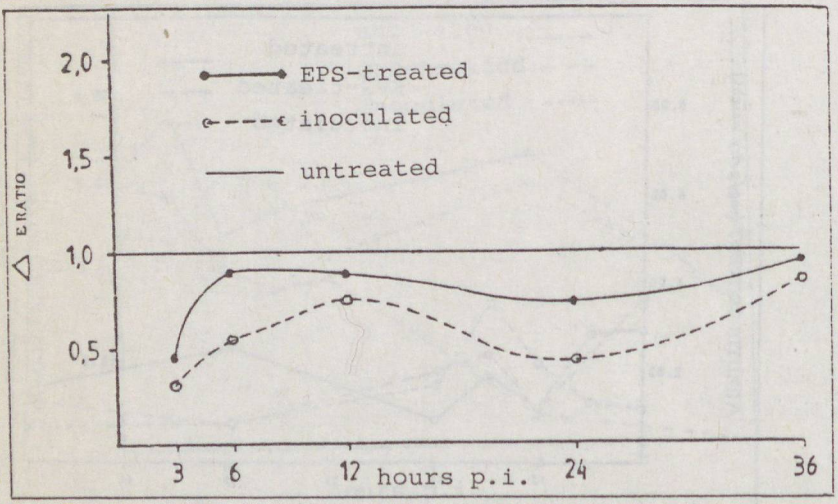


Fig.5 Phenoloxidase-activity after EPS-treatment and inoculation with E. amylovora in leaves of Cydonia oblonga

After EPS treatment a slight reduction could be observed as compared with the control. The bacterial inoculation resulted in a marked reduction.

DISCUSSION

Considering the EPS-effects on the metabolism of Cydonia oblonga, a reduction in the O_2-consumption, a reduction in the phenoloxidase activity as well as in the individual phenols and total phenol content during the initial stages of infection, it would appear that the EPS has a stabilizing function on the structure of the host plant during the initial stages of infection and thus provide protection against the early destruction of the tissue, which is observed in the hypersensitive reaction of the host. A similar hypothesis has been postulated by Feistner (1988) for the toxic compound dihydro-phenylalanine which he isolated from culture filtrates of E. amylovora; this he assumed to be an inhibitor of several enzymes of the aromatic pathway. The EPS effect would thus appear not to be a toxic component. If its primary role during the pathogenesis is to protect the pathogen against the production of phytoalexin-like compounds, as mentioned by Rudolph et al. (1989) for the EPS of bacterial leaf spot diseases, is not clear and it should be clarified by further studies.

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Acknowledgement: This work was sponsored by the Deutsche Forschungsgemeinschaft.

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

PROPERTIES OF A SYRINGOMYCIN-MACROMOLECULAR COMPLEX PRODUCED BY PSEUDOMONAS SYRINGAE PV. SYRINGAE

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ABSTRACT

Pseudomonas syringae pv. syringae strains produce syringomycin (SR) mainly as a high-molecular-weight complex. Its size as estimated by gel-filtration, is about $2x10^6$ D. The native SR-complex, when treated with several dissociating agent, seems to transform in another complex with a lower molecular weight.

The antibiotic activity of SR-complex on prokaryotic but not on eukaryotic microorganisms appeared differentiable from that of free SR.

INTRODUCTION

Syringomycin, which is produced by many pathogenic strains of Pseudomonas syringae pv. syringae van Hall, is generally isolated by treating bacterial cultures (cells and broth combined) with 50% acetone at pH2, removing the precipitate by centrifugation, reducing the volume about 20-fold and then extracting with water satured n-butanol. The active extract is then purified by cation-exchange chromatography[1] and afterwords by HPLC to obtain a group of structurally related substances[2].

By following a different procedure to process the supernatant of the bacterial cultures we observed that SR may be present in P.s. pv. syringae strain cultures in a macromolecular form[3,4].

Here, additional evidence is given that SR mainly occurs in culture as a high-molecular-weight complex.

MATERIAL AND METHODS

Five SR-producing strains from different hosts of P.s. pv. syringae were grown, as previously reported[4]. After 6-12 days of incubation, depending on the medium, the cultures were centrifuged and the supernatants were treated with ammonium sulphate (66% saturation). The ammonium sulphate precipitates (AS-P) were taken up in Tris-phosphate buffer (0.01 M, pH 6.5) and exhaustively dialysed against the same buffer. The antibiotic and phytotoxic activities of the AS-P were evaluated on G. candidum and by injecting the preparations into bean and tobacco leaves.

The AS-P from millet strain B359 was fractioned on A1.5 and A5 Bio-gel (Bio-Rad) columns eluted with Tris-HCl buffer (0.05 M, pH 5.5) additioned with NaCl (0.145 M). Gel filtration standards were applied to the columns as molecular size markers.

The AS-P was taken up in solution in acetate buffer (0.1 M, pH 4) and treated with several dissociating agents. In particular, the solutions were maintained for 5 minutes at 60°C and for 15 minutes in the presence of SDS(2%), EDTA(1%), dithiothreitol (DTT) (2.5%), triton X100 (1%), at pH2 and then the mixtures were passed through Amicon XM ultrafiltration membranes. The fractions which passed through as well as those retained by the membranes, were checked for activity against G. candidum. The fractions retained by the membranes were recovered by washing with distilled water.

The SR extraction and purification procedures used were those reported by Gross and DeVay (1977) with minor modification.

The HPLC conditions were those reported by Ballio et al. (1988).

RESULTS AND DISCUSSION

The AS-P from all the P. s. pv. syringae strains were biocidal toward G. candidum and phytotoxic on bean and tobacco leaves. When analysed by electrophoresis, AS-P did not contain

a band corresponding to free SR but instead exhibited several Coomassie-blue-stained bands with lesser electrophoretic mobilities than that of SR. Moreover, unstained gels, sliced and assayed using G. candidum, showed a broad zone of antibiotic activity corresponding to the part of the gel containing the Coomassie-blue-staining bands (Fig. 1).

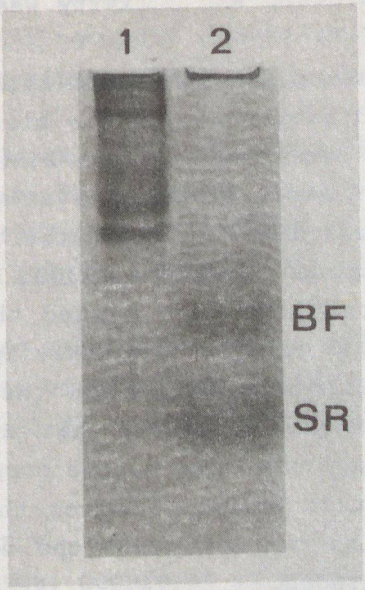


Fig. 1. Comparison of electrophoretic profile on gel polyacrylamide (15%) of AS-P from strain B3A (lane 1) and purified SR (lane 2). Basic fuchsin (BF) was added to SR sample as tracking dye.

Evidence that SR was the active component of AS-P was obtained by applying to AS-P the standard procedure for extraction and purification of SR. In fact, purified extracts analysed by electrophoresis showed a band corresponding to authentic SR. Moreover, the HPLC profile of SR from AS-P and its amino acid content were consistent with those of SR prepared in the usual manner.

When the AS-P from strain B359 was applied on Bio-gel A 1.5 and/or A 5 agarose columns, the bioactivity was eluted respectively with the void volume and co-eluted with Dextran Blue 2000 indicating a molecular weight of about 2×10^6 D for the SR-complex. Moreover, when the culture filtrate of the same strain

was applied on the Bio-gel A 5 column the bioactivity eluted with Dextran Blue 2000 and at the end of the run where free SR is eluted. This finding confirms that SR is present in culture filtrate mainly as a high-molecular-weight complex and in part as free SR.

When AS-P was passed through Amicon XM300 ultrafiltration membrane (300,000 D cutoff), the activity was retained in the membranes, as expected.

SR-complex in presence of high ionic strength and treated with urea seems not to dissociate since the bioactivity is still retained by the XM 300 membranes. On the other hand, when the AS-P solutions were heated, maintained at pH 2 and treated with SDS, EDTA, DTT, triton X100, the activity was present in the fractions passed through membrane XM300. Furthermore, when the latter active fractions were passed through an Amicon XM100 membrane (100,000 D cutoff), the activity was retained in the membrane. These findings confirmed the high molecular weight of the SR-complex and suggested that some of the dissociating agents used transformed the native complex into another one with a lower molecular weight. However the latter results must be interpreted with caution since the complex could interact with various surfaces.

The activity of the SR-complex and pure SR were comparable when assayed for their toxic effects on bean leaves and antibiotic activity against fungi and yeasts. On the other hand, the sensitivity of several bacterial species, including strains of P.s. pv. syringae to SR and SR-complex, was different.

In conclusion P.s. pv. syringae produces SR in culture mainly complexed with an unknown macromolecular component(s) and in part in the free form.

The occurrence or role of the SR-complex is not known but the fact that it differs from free SR in its toxicity to bacteria may be significant. Additional analyses of the association of SR with the high molecular fraction might help to explain the broad distribution of SR's "target" in plants and microorganisms.

Acknowledgements. The technical assistance of Mrs. S.L. Lonigro is gratefully aknowledged.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

RESISTANCE TO BACTERIALLY-INDUCED HYPERSENSITIVE NECROSIS IN TOBACCO PLANTS HAVING RESISTANCE TO PARAQUAT: A POSSIBILITY FOR THE ROLE OF ANTIOXIDANT SYSTEMS

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SUMMARY

Paraquat resistant (PR) tobaccos exhibit a protection from free radical mediated necrotic symptom expression (hypersensitive reaction) caused by Pseudomonas syringae pv. syringae. Our experimental results suggest that this protective effect is a consequence of enhanced antioxidant capacity of the PR plants. This conclusion also supports the idea that $O_2^{\cdot -}$ generation plays a primary role in the necrotic symptom expression during the hypersensitive reaction (HR).

INTRODUCTION

It has been proved earlier that oxyradical production (especially superoxide formation) plays a key role in the initiation of HR-related membrane damages (lipid peroxidation, decreases of double bond index in fatty acids of polar lipids and in the free sterol pool) both in attached tobacco leaves (Ádám et al., 1989) and in tobacco cell suspensions (Keppler and Baker, 1989). This rise in $O_2^{\cdot -}$ production was preceded by the initial increases in extracellular pH and K^+ concentration (Keppler and Baker, 1989). The latter events are probably the consequences of the activation of a K^+ efflux/H^+ influx exchange system across the plasmalemma (Atkinson et al., 1985).

Application of superoxide dismutase (SOD) and other free radical scavengers to the cells undergoing the hypersensitive necrotization, reduced the expression of symptoms (Keppler and

Novacky, 1987, Ádám et al., 1989, Keppler and Baker, 1989). It also has been established that the loss of HR-inducing capacity of an incompatible bacterium (due to Tn5 transposon mutagenesis) coincides with the loss of $O_2^{\cdot-}$ generating activity in tobacco plant tissue (Ádám et al., 1989, Keppler and Baker, 1989).

For better understanding of this phenomenon, we examined the development of bacterially-induced HR in paraquat resistant (PR) tobacco plants (Nicotiana tabacum cv. Samsun), which are in fact superoxide resistant. Paraquat acts as a competitor for electrons of the primary electron acceptor of photosystem I and induces a rise in superoxide formation (Stancliffe and Pirie, 1971). We concluded that PR, indeed $O_2^{\cdot-}$ resistant, tobaccos were protected from the damaging action of the oxy-radicals.

MATERIALS AND METHODS

Bacterial HR in PR and in the paraquat sensitive (PS) tobaccos were induced by infiltration of Pseudomonas syringae pv. syringae (10^6-10^9 cells ml^{-1}) into the leaves as we described earlier (Ádám et al., 1989). Seeds of PR Samsun tobacco (Furusawa and Miziguchi, 1988) were kindly supplied by Professor I. Furusawa, Kyoto University, Kyoto, Japan. Lipids from the leaf tissues were extracted according to Folch et al. (1957) and quantified as we described elsewhere (Ádám et al., 1989). Activity of superoxide dismutase (SOD) was determined in polyacrylamide slab gels (Ádám et al.,1989). Bacterial populations in leaf tissues were estimated by grinding 3 leaf discs (7 mm diameter) per treatment in 100 ul distilled water per disc. The homogenate was serially diluted and 100 ul of each dilution (two replicates per dilution) was spotted on nutrient agar plates. Colonies were counted after incubation for 48 hrs at 30° C.

RESULTS AND DISCUSSION

A comparison of the development of bacterially-induced tissue necrosis (HR) in PR and in PS (control) tobacco plants again demonstrated that $O_2^{\cdot-}$ generation plays an important role in the initiation of necrotization associated with the HR

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Table 1

Effect of paraquat resistance (PR) on the development of hypersensitive necrosis induced by Pseudomonas syringae pv. syringae in Samsum tobacco leaves at various concentrations of bacterial inocula

Bacterial concentration (bacteria ml^{-1})	PR tobacco	PS (control) tobacco
10^6	-	-
$5x10^6$	-	+-
10^7	-	++
$5x10^7$	+	+++
10^8	++	+++
10^9	+++	+++

+++ Confluent HR
++ Delayed development of confluent HR
+ Partial development of HR
+- Uncertain development of HR (sometimes no HR)
- No HR

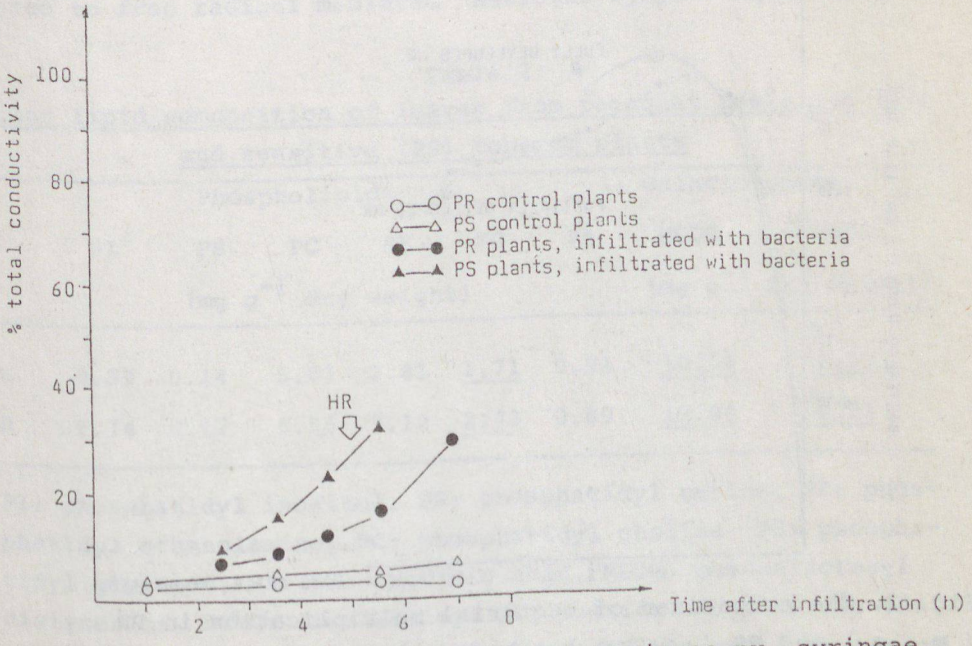


Fig. 1 Effect of infiltration with P. syringae pv. syringae (10^8 cells ml^{-1}) on the electrolyte leakage in paraquat resistant (PR) and sensitive (PS) tobacco plants.

(Table 1 and Fig. 1). Even at a higher concentration (10^8 cell ml^{-1}) a delay was experienced in the development of confluent HR (Table 1) which was also manifested in the ion leakage test (Fig. 1). The question emerged whether this effect was due to inhibiting free radical damages or also to the inhibition of bacterial multiplication. The investigation of this problem was facilitated by the use of a bacterial concentration (10^6 cells ml^{-1}) which did not cause early immediate necrotic symptoms for at least three days (Fig. 2). These results show that there was no difference between the multiplication rate of P. syringae pv. syringae in PS or in PR tobacco plants. This suggested that protection of PR plants from hypersensitive necrosis was associated with the lack of free radical mediated necrotic symptom expression rather than with the inhibition of bacteria.

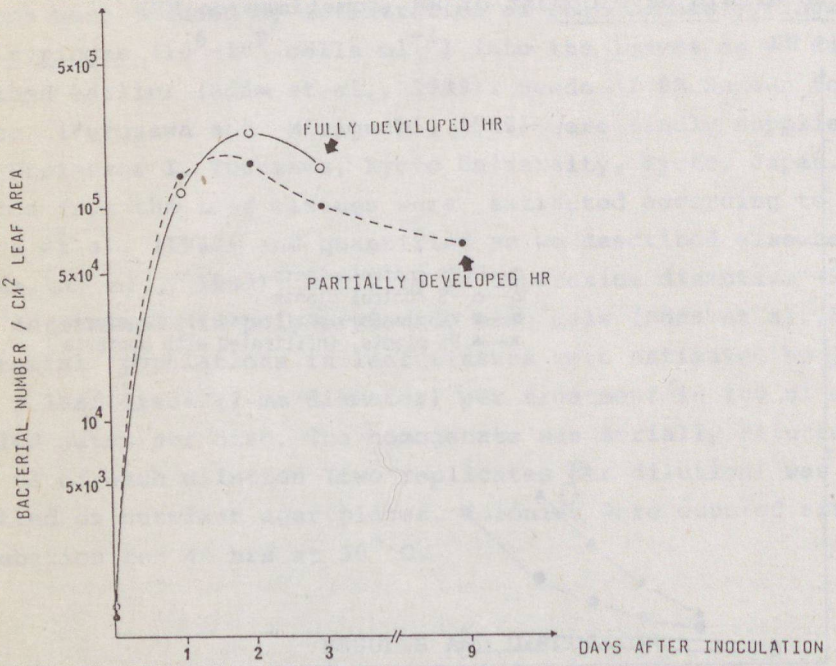


Fig. 2. The comparison of bacterial multiplication in PR (●---●) and PS (O——O) tobacco plants. Leaves were infiltrated with 10^6 cells ml^1 bacteria. Data are the means of two independent experiments.

This conclusion is also supported by some other experimental results. Furusawa and Mizuguchi (1988) reported that PR tobacco plants were resistant to the action of cercosporin, a pathotoxin that can generate $O_2^{\cdot -}$ only in light (Daub and Briggs, 1983). Shaaltiel and Gressel (1986) found higher superoxide dismutase (SOD) activity in chloroplasts of the paraquat resistant weed (Conyza canadensis) than in the paraquat sensitive control Conyza plants. We also detected higher SOD activity in total leaf extracts of PR tobacco (21.2 and 29.7 EU mg^{-1} protein for control PS and PR plants, respectively). Investigation of the polar lipid composition of PR tobacco leaves (Table 2) showed an increase in galactolipids (MGDG and DGDG) and in phosphatidyl glycerol content. These components are characteristic of chloroplast membranes. The enhanced antioxidant capacity of chloroplasts of PR Conyza plants was supposed to detoxify the paraquat mediated $O_2^{\cdot -}$ action (Shaaltiel and Gressel, 1986). Accordingly, a similar mechanism of detoxification could be the reason for resistance of PR tobacco to free radical mediated necrotic symptom expression (HR).

Table 2

Polar lipid composition of leaves from paraquat resistant (PR) and sensitive (PS) tobacco plants

	Phospholipids						Galactolipids	
	PI[a]	PS	PC	PE	PG	PA	MGDG	DGDG
	(mg g^{-1} dry weight)						(mg g^{-1} dry weight)	
PS	0.59	0.14	5.81	2.81	1.71	0.91	10.04	6.57
PR	0.74	0.17	5.96	3.12	2.43	0.89	12.98	7.93

[a]PI: phosphatidyl inositol, PS: phosphatidyl serine, PE: phosphatidyl ethanolamine, PC: phosphatidyl choline, PG: phosphatidyl glycerol, PA: phosphatidic acid, MGDG: monogalactosyl diglyceride, DGDG: digalactosyl diglyceride. Results are the means of two independent experiments with two replications.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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THE CRITICAL ROLE OF THE HYDROXYL RADICAL IN BACTERIA-INDUCED HYPERSENSITIVE REACTION AND PATHOGENESIS*

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The anionic superoxide radical (O_2^-) was suggested to be directly involved in lipid peroxidation and electrolyte leakage in plants during bacteria-induced hypersensitive reaction (HR) and pathogenesis in plant cells.

An increase in lipid peroxidation and electrolyte leakage appeared in leaves 3 hours after inoculation with bacteria (Keppler and Novacky, 1986; Ádám, et al.,1989 and Klotz et al. 1989) and this was found to be a precondition for the development of HR necrosis. Coincidentally with the increase in lipid peroxidation an increase in cell staining activity of the nitroblue tetrazolium(NBT)- Fe cytochrome c complex (inhibited by 100 µg/ml superoxide dismutase) was reported and taken as evidence for the direct involvement of superoxide radicals (Ádám et al., 1989). The NBT-staining reaction indicates the identity of the O_2^- radical generation site with the plasma membrane redox system.

Surprisingly a significant initial decrease in lipid peroxidation for both living and heat-killed compatible and incompatible bacteria was found in cucumber cotyledons (Keppler and Novacky, 1986). This was observed recently also during HR of cotton suspension culture cells induced by Pseudomonas syringae pv. pisi (Ben-Gweirif, 1988). The initial decrease in lipid peroxidation was accompanied by an immediate decrease in superoxide formation (Klotz et al.,unpublished), measured by the aerobic autoxidation of epinephrine (Misra and Fridovich, 1972). While 3 to 6 hr later the lipid peroxidation was restored in tissues inoculated with heat-killed incompatible, living and heat-killed compatible bacteria (Keppler and Novacky, 1986) or even increased above the water infiltrated control (Keppler and Novacky, 1986; Ádám, et al., 1989 and Klotz et al. unpublished). The level of superoxide was lower than that of the control. Epinephrine autoxidation developing only in the presence of plant tissue, was inhibited by 88% with 50 µg ml^{-1} exogenously applied superoxide dismutase. SOD is responsible for native enzymatic dismutation of superoxide and competes for superoxide with the cell wall peroxidase (the Haber-Weiss reaction) and during the assay with

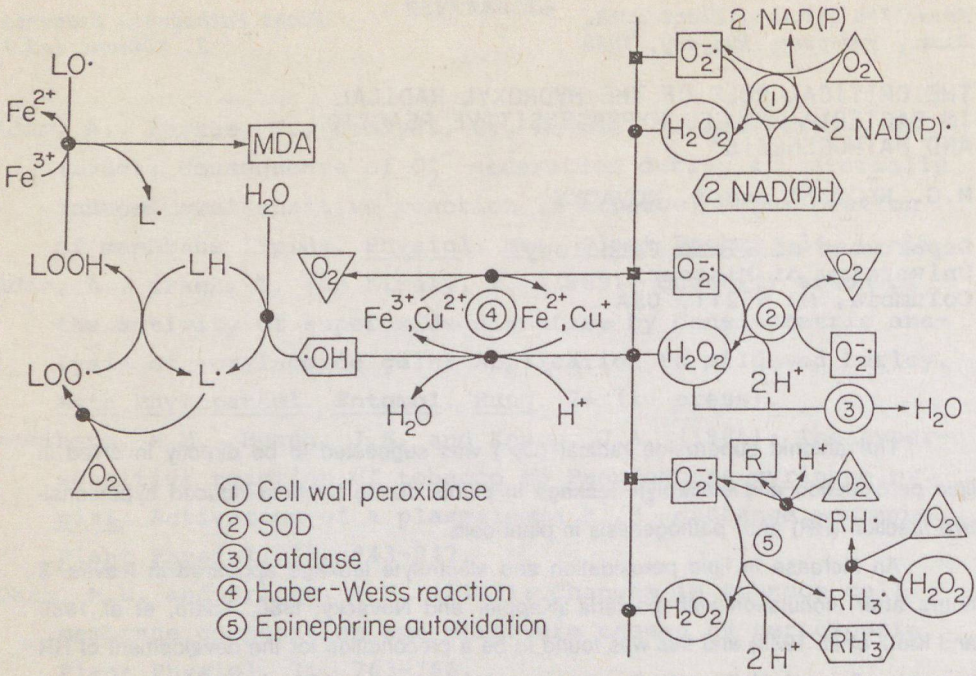


Fig.1. Model of reaction sequence leading to lipid peroxidation during HR

epinephrine (Fig. 1). This indicates that the epinephrine assay is more specific and sensitive to superoxide than the nitro blue tetrazolium staining technique.

In Fig. 2 a model is presented to explain the apparently contradictory results concerning lipid peroxidation and superoxide formation. The change in the level of hydroxyl radical is suggested to be the central event in bacteria-induced pathogenesis and hypersensitive reaction. After inoculation bacteria (compatible and incompatible) may quench the naturally *in planta* produced hydroxyl radicals. Some structural components of the bacterial envelope (lipopolysaccharide or protein) are assumed to function as a quencher for hydroxyl radicals.This hypothesis is supported by reports that D-mannitol and bovine serum albumin are efficient quenchers for the hydroxyl and not for the superoxide radical (Wolff et al., 1986). It was found already in 1976 by Mazzucchi and Pupillo that protein-lipopolysaccharide complexes prevent confluent HR and in 1977 by Király et al.that albumin prevents necrosis in the hypersensitive host both on tissue and cellular level. It may be suggested in addition that in the case of living incompatible (HR^+) bacteria, quenching of hydroxyl radicals might cause the release of the putative HR elicitor molecules found by Huang et al.(1989) and Somlyai et al. (1989).

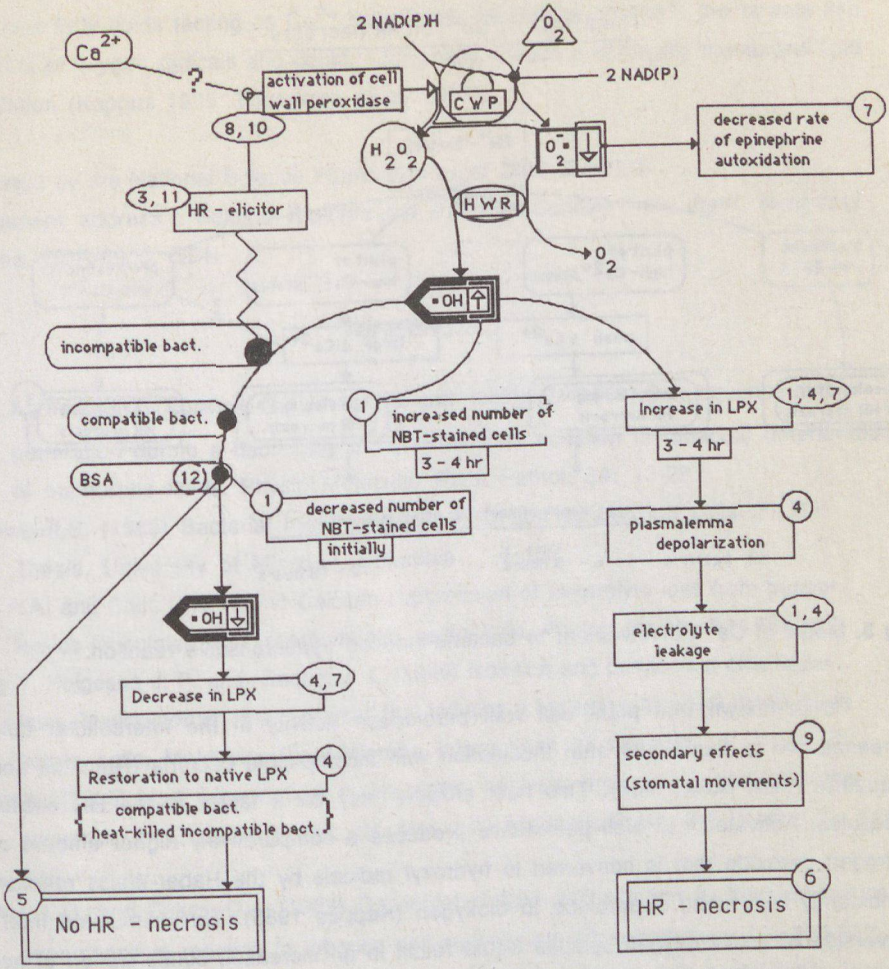


Fig. 2. Model of the role of ·OH in plant host/bacteria interactions. 1. Ádám et al.(1989) Keppler & Baker (1989);2. Cook & Stall (1971); 3. Huang et al. (1989); 4. Keppler & Novacky (1986); Pavlovkin et al. (1986) 5. Király et al. (1977), Mazzucchi & Pupillo (1976); 6..Klement (1982); 7. Klotz et al., unpublished; 8. Kunz (1986); Pike & Novacky (1988); 10. Rathmell & Sequeira (1974); Venere, (1980); 11. Somlyai et al. (1989); 12. Wolff et al. (1986)

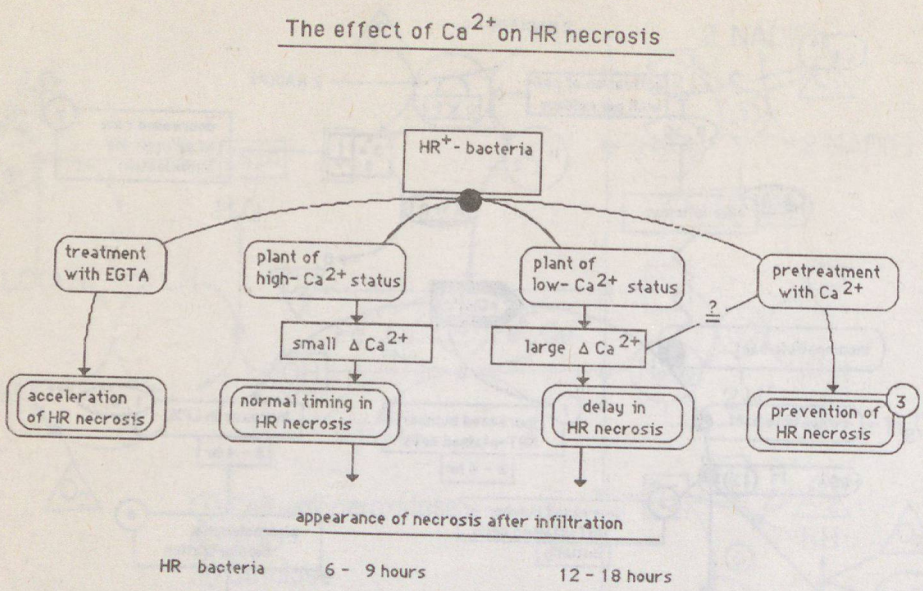


Fig 3. Model of Ca^{2+} involvement in bacteria-induced hypersensitive reaction.

It is known that plant cell wall peroxidase activity in the intercellular fluid increases two to four hours after inoculation with incompatible bacteria (Rathmell and Sequeira, 1974; Kunz, 1986). This host enzyme may be a target of the HR elicitor molecules. Activated cell wall peroxidase produces a comparatively higher amount of hydrogen peroxide that is converted to hydroxyl radicals by the Haber-Weiss reaction coincidently converting superoxide to dioxygen (Kappus 1985). This stimulated interconversion of active oxygen species would result in an increased concentration of hydroxyl radicals and a decreased concentration of superoxide compared to the basal level. The decrease in superoxide formation was detected by the epinephrine assay in the case of HR^+ bacteria (Klotz et al., unpublished). This increase in hydroxyl radicals after three to four hours increases lipid peroxidation above the control (Keppler and Novacky, 1986; Ádám et al.,1989; Ben-Gweirif, 1988 and Klotz et al, unpublished) and induces a sequence of events resulting in HR necrosis (Klement, 1982).

The release of cell wall peroxidase might be a Ca^{2+} (-calmodulin ?)- dependent process. This would explain that the Ca^{2+} status of the host plant is determining the timing of HR necrosis development (Fig. 3). In contrast, pretreatment with Ca^{2+} suppressed electrolyte leakage and HR necrosis (Cook and Stall, 1971) indicating that HR is regulated by both intracellular and extracellular membrane associated factors. Ca^{2+} is also known to activate plant lipid-degrading enzymes (Thompson et al. 1987). The re-

sulting free fatty acids (acting as Ca^{2+} ionophores, Serhan et al. 1981) are targets and quenchers of oxygen radicals and could, additionally, induce and amplify membrane lipid peroxidation (Kappus 1985; Thompson et al, 1987).

*Supported by the National Science Foundation grant DMB 8516038.

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Plant Pathogenic Bacteria Z. Klement (ed.)

AMINO ACID CATABOLISM IN XANTHOMONAS CAMPESTRIS PATHOGENESIS

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Different carboxylic acids were detected in broth cultures of *Xanthomonas campestris* pv. *manihotis* amended with amino acids. Each carboxylic acid was associated with a specific amino acid precursor : 3-methylthiopropionic (MTPA) and 3-methylthioacrylic acid were produced from methionine, phenylacetic acid from phenylalanine, tiglic acid from isoleucine, indolaçetic acid from tryptophane and isovaleric acid from leucine. These acids were catabolic homologues of MTPA which was produced by transamination and subsequent decarboxylation of methionine. All these acids reproduced typical blight symptom on bean, cassava, rice and tomato leaves. Intrinsic phytotoxicity was also expressed by buffered solution of the compound. Organic acid diversity and their further metabolisation are discussed for their potent involvement in the diversity of symptoms observed for diseases caused by xanthomonads.

INTRODUCTION

3-methylthiopropionic acid (MTPA) was identified as a blight inducing toxin in cassava bacterial blight caused by *Xanthomonas campestris* pv. *manihotis*. The toxin is produced as well in bacterial batch culture than in leaf lesions during pathogenesis (Perreaux et al., 1982; Perreaux et al., 1986). Its production is not restricted to the pv. *manihotis*, but also recognized for pv. *campestris* (Robeson & Cook, 1985), pv. *oryzae* (Noda et al., 1980), pv. *phaseoli* (Maraite & Snacken, 1987) and others (Maraite, unpublished data).

In batch culture, MTPA is accumulated only for methionine containing broth. Following investigations on the metabolism of this amino acid, a catabolic pathway leading to the production of MTPA, by transamination and subsequent decarboxylation of the methionine, was demonstrated (Ewbank & Maraite, 1989). Seeing the production of various low molecular weight carboxylic acids by *X.campestris* pv.*campestris* in relation to amino acid composition of the medium (Robeson & Cook, 1985), our purpose was to determine whether catabolic homologous of the MTPA could be produced *in vitro* by *X. campestris* pv. *manihotis*. Their phytotoxicity was further investigated.

MATERIALS AND METHODS

Bacterial cultures

All experiments were carried out with the strain HMB 23 (BM 12, ICPB XM 147) of *X. campestris* pv. *manihotis* . The strain was stored at room temperature on GYCA medium (Dye, 1962). Bacteria were grown for inoculum production in 50 ml GYS broth (5% glucose, 5% NaCl, 5% yeast extract, 0.5% $NH_4H_2PO_4$, 1.5% K_2HPO_4, 0.2% $MgCl_2.6H_2O$, percentages were expressed in terms of w/v) at 28 °C. Aliquots of 0.25 ml of this culture were used to inoculate various 50 ml broth medium in 250 ml Erlenmeyer flasks. The basal culture medium used had the following composition : 1% L-glutamic acid, 0.05% L-methionine, 5% sucrose, 3% $(NH_4)_2HPO_4$, 2% KH_2PO_4, 0.2% $MgCl_2.6H_2O$, 0.01% $FeSO_4.7H_2O$, 0.01% $MnSO_4.H_2O$). It was always complemented by one of the following L-amino acids at a concentration of 0.5% : methionine, leucine, isoleucine, phenylalanine or tryptophan. Cultures were incubated for 96 h at 28 °C on a gyratory shaker operating at 150 rpm. Growth was estimated by OD measurements at 600 ηm. At regular intervals during growth, 2 ml aliquots were aseptically sampled, bacteria were pelleted by centrifugation (10,000 g for 15 min) and supernatants were assessed for acidic metabolites.

HPLC analyis of acidic metabolites

Quantitative analysis of carboxylic acid was performed by ion pairing on a 25 cm long Alltech Rosil C18 column (Alltech Europe, B-9731 Eke, Belgium). The solvent system was water added with 0.85% (v/v) of Pic Reagent A (Waters trading preparation of tetrabutylammonium phosphate) and acetonitrile (92:8 v/v). Elution was isocratic with a flow rate of 1 ml min^{-1}. UV detection was performed routinely at 210 ηm or 254 ηm for phenylacetic acid (PAA) and 3-indolacetic acid (IAA) measurements. Samples were purified by liquid-solid extraction on Sep Pack C18 cartridge (Waters Associates Milford, Massachusetts 01757, USA). The cartridge was conditioned by two successive washings with 5 ml methanol and 5 ml H_3PO_4 10^{-2} M. After acidification to pH 2 with H_3PO_4 2 M, 2ml samples were passed through the Sep Pak. The cartridge was then washed with 1.5 ml H_3PO_4 10^{-2}M and acidic compounds of interest were eluted with 2ml of potassium phosphate buffer (20 mM, pH 7) and acetonitrile (80:20 (v/v)).

Bioassays

The phytotoxicity of acidic compounds was tested on detached leaflets of bean, tomato or cassava. Four little cross injuries were carried out at 1/3 and 2/3 of the leaflet height on both sides of the main vein. A 20 ml droplet of the solution to be tested was deposit on the injury and the leaflet incubated during 3 days in a moisture chamber at 18 °C. Effect on undetached leaves of rice plants was tested in a similar way. Solutions of MTPA, tiglic acid (TGA) and isovaleric acid (IVA) were prepared at concentrations ranging from 5 to 100 mM in distilled water or potassium phosphate buffer (200 mM, pH 7). Area of the blight lesion was measured with a MOP Videoplan planimeter (Kontron Electronic Group, Bresslauerstraβe, Munchen, Switzerland).

Because of their respective solubility, solutions of PAA were prepared up to 60 mM and solution of IAA up to 10 mM.

RESULTS

Bacterial growth and organic acid production from amino acid

The main difference between the growth curves according to the amino acid amendment was the lag time observed for the start of the exponential growth phase (Fig.1). The shortest lag was approximately 20 h in the case of leucine, while an additional 24 h was required for phenylalanine. The subsequent bacterial growth rates were quite similar and the maximal OD always approached 2.90 units.This observation resulted from the fact that N nutrient, principally introduced in the medium in the form of $(NH_4)_2HPO_4$, was the same limited nutrient factor for all broth.

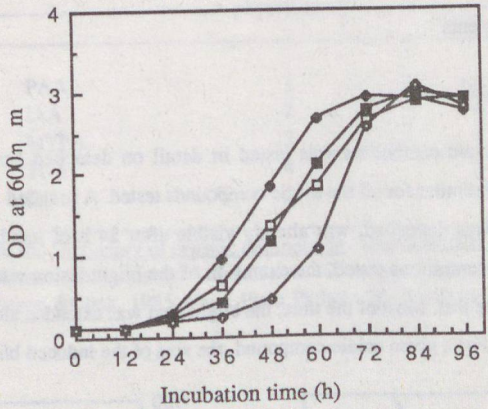


Fig.1: Growth curves of *X. campestris* pv. *manihotis* in the basal medium complemented with 0.5% (w/v) of either methionine (—□—), leucine (—●—), isoleucine (—■—), or phenylalanine (—◆—).

On a basal medium complemented with 500 $mg.l^{-1}$ of methionine, *X. campestris* pv. *manihotis* produced MTPA and MTAA which accumulated in the culture during growth. In terms of concentration, MTPA was by far the most important acid produced and it reached a maximum of 193 $mg.l^{-1}$ after 48 h. At the same time, MTAA concentration was only 33 $mg.l^{-1}$. The ratio between MTPA and MTAA remained constant during the various growth stages. Detection of MTPA and MTAA was strictly associated with the presence of methionine in the medium.

When methionine was substituted by another amino acid, the bacteria produced various organic acids identified by retention times comparison and co-chromatography with standards on ion pairing reverse phase HPLC. Each carboxylic acid was associated with a specific amino acid precursor; PAA was produced from phenylalanine, TGA from isoleucine, IVA from leucine and IAA from tryptophan. In all cases, the production kinetics of these acids were similar to the production kinetic of MTPA, concentration being

highest at the end of the logarithmic growth phase. However, the efficiencies of the amino acid transformation were smaller than for the production of MTPA from methionine (Table 1).

Table 1. Production of organic acids after 84 h incubation of *X. campestris* pv. *manihotis* in the basal medium complemented with various amino acid.

Precursor amino acid name	concentration (mg. l^{-1})	Bacterial growth OD 600 ηm [a]	Organic acid produced name	concentration [a] (mg. l^{-1})
Methionine	500	2.86	MTPA	193.6 ± 6.6
Methionine	500	2.86	MTAA	33.1 ± 0.6
Phenylalanine	500	3.04	PAA	35.3 ± 1.7
Tryptophan	500	2.72	IAA	33.1 ± 1.2
Isoleucine	500	2.98	TGA	17.8 ± 0.9
Leucine	500	2.90	IVA	trace only

[a] means of three experiments

Phytotoxicity of the organic acid

Phytotoxicity of the acidic catabolites was tested in detail on detached bean and cassava leaves. Appearance of the lesions was similar for all the acidic compounds tested. A rounded blight area, centered on the point where the droplet was deposited, was already visible after 24 h of incubation at 100% relative humidity. For the highest concentrations tested, the extension of the blight lesion was usually limited by the border and the main vein of the leaf. Most of the time, the blight area was extended along the secondary veins nearest the inoculation point. For a given acidic compound, the size of the induced blight area is proportional

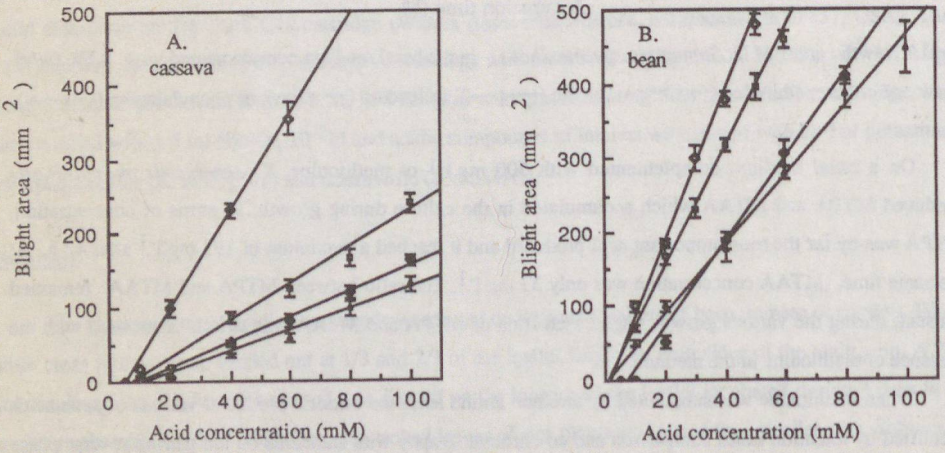


Fig.2: Blight area induced by different concentration of IVA (▲), TGA (◆), MTPA (■) or PAA (●) on detached leaves of cassava (A) or bean (B). Area were measured after 3 days of incubation in a moisture chamber. Each point on the graph was the mean of 6 repetitions.

to the concentration of the tested solution (Fig.2). Because of its weak solubility, phytotoxicity of IAA wasonly tested for concentrations of 5 and 10 mM. The blight area obtained for these concentrations of IAA were relatively large and approximately equal to those obtained with PAA at the same concentration. Intrinsic phytotoxicity decreased in the order : PAA, IAA, MTPA, TGA and TGA. Except for IAA, this classification was partially related to the pK_a value of the individual acids (Table 2). The same order was observed either on bean or on cassava, but the sensitivity of bean leaves was greater. The phytotoxicity of these acidic compounds was not specific and blight lesion were also induced on rice or tomato leaves. The sensitivity of the test was not affected by the use of phosphate buffered solution of the different acidic metabolites at pH 7.

Table 2. Relationship between the phytotoxicity of organic acids and their pK_a.

Organic acid name	Order of phytotoxicity	pK_a value
PAA	1	4.25 [a]
IAA	2	4.80 [a]
MTPA	3	4.42 [b]
TGA	4	5.02 [a]
IVA	5	4.77 [a]

[a] From the Dictionary of organic compounds, fourth edition, Eyre & Spottisswoode Publishers Ltd, London, 1965, five volumes, 3282pp.
[b] Robeson &Cook, 1985, Phys. Plant Pathol., 26, 219-230.

DISCUSSION

Although the chemical structures of the organic acids produced by *X.campestris* pv. *manihotis* were very different, all originated from amino acids. They could be produced by transamination of the amino acid and subsequent decarboxylation of the resulting 2-keto methylthiobutyric acid (KMBA),as demonstrated for the conversion of methionine into MTPA by *X. campestris* pv. *manihotis* (Ewbank & Maraite, 1989).

The phytotoxicity of the compounds seems not to be exclusively related to their acidic nature. Althought there was a partial relationship between the phytotoxicity and the pK_a of the various acids, the differences between the pK_a values appeared relatively small to explain the large differences observed in the blight area caused by the acidic compound. Moreover, the toxic effect of the different acid was not affected by the use of buffered solution. Lhoest et al. (1988) have reported on the other hand the synthesis of toxin-induced proteins by callus of *Oryzae sativa* subjected to MTPA. This modified gene expression was also obtained with neutralized solution of MTPA, but not with an HNO_3 acidified medium. Therefore it seems plausible that the phytotoxicity of organic acids derived from the amino acid metabolism was not only due to an increased activity of the hydrogen ion in the tissue.

Besides this intrinsic toxicity due the chemical structure of the molecules, a potent toxicity could be derived from further catabolic transformation of some of the acids or of their metabolic intermediates. From phenylalanine, Robeson and Cook (1985) have reported the production by *X. campestris* pv. *campestris*, of PAA, benzoic acid and m-hydroxybenzoic acid; transamination and decarboxilation appearing thus only as first steps of more intricate catabolic pathway. MTAA, also detected in broth culture of *X. campestris* pv. *campestris* (Robeson & Cook, 1985) and pv. *oryzae* (Noda et al., 1980), could originate from the dehydrogenation of MTPA. Ethylene and methanethiol production from the methionine 2-keto acid analogue KMBA was demonstrated for *E. coli* by Ince and Knowles (1986). Moreover several xanthomonads have increased production in presence of methionine (Swanson et al., 1979). KMBA, originated from the transamination of methionine, could therefore be involved in a dual metabolism leading to production of phytotoxins and to phytohormonal imbalance. The diversity of the phytotoxic compounds which could be produced directly by transamination and decarboxylation of amino acids or by a further catabolism proceeding from one of these steps, could explain the diversity of the symptoms (blight, yellowing, wilting, hyperplasia) observed in diseases caused by various xanthomonads.

ACKNOWLEDGEMENTS

Ewbank E. is "Aspirant" research worker of the FNRS. Work supported by an FDS grant of the Université Catholique de Louvain and FNRS grant 1.5.448.88F for HPLC equipment.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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Z. Klement (ed.)

ROLE OF OXYGENASES IN THE DEGRADATION OF PHENOLIC PROHIBITINS BY PSEUDOMONAS SOLANACEARUM

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INTRODUCTION

Secondary substances of plant origin notably phenols, flavonoids, stilbenes, tropolones, alkaloids etc., confer protection against parasitic microorganisms (Mahadevan, 1982). These substances are referred to as prohibitins and most of them inhibit bacteria, fungi and viruses. Despite the presence of prohibitins, a few microorganisms have developed mechanisms to circumvent them (Mahadevan, 1974; 1980; Boominathan et al., 1986). P. solanacearum is pathogenic to a variety of hosts (Bradbury, 1986). It produces catechol 1,2-dioxygenase in culture medium, which cleaves catechol a highly toxic phenol to simple organic acids (Arunakumari and Mahadevan, 1985). Boominathan and Mahadevan (1987) reported that P. solanacearum degraded catechin, an important component of wattle tannin, which is recalcitrant. This paper is a continuation of our earlier studies on the detoxification of phenolic prohibitions by P. solanacearum.

MATERIALS AND METHODS

P. solanacearum strain and its growth in culture media were described (Boominathan and Mahadevan, 1987). Filter sterilized aromatic substances were aseptically added to Dye's medium without carbon source (Dye, 1958). P. solanacearum 14 hr-old, grown on glycerol was inoculated and incubated in static condition at 30 ^{o}C. Aliquots of 5 ml culture were withdrawn at regular intervals and growth was monitored at 540 nm. For hydroxyquinol, viable cell count was made at predetermined intervals.

Oxidation of aromatic substances was studied in manometer (Mahadevan and Sridhar, 1987). Mode of ring cleavage was tested by Rothera's test (Mahadevan and Sridhar, 1987).

Preparation of cell free extract

Cells grown in liquid medium were washed twice in Phosphate buffer (0.025 M; PH 7.4) and suspended in the same buffer. The cells were disrupted by using MSE 150 Watt ultrasonic disintegrator at 4 amplitude for 5 minutes. The suspension was centrifuged at 20.000 x g for 45 minutes at $4^{o}C$ and the supernatant solution was used as crude enzyme. Cell-free extracts were prepared within 30 minutes after harvesting the cells and assayed immediately for enzyme activity. Protein in the extract was estimated (Lowry et al., 1951).

Assay for oxygenases

The reaction mixture for the oxygenase assay contained 1 ml of the substrate (10 umole), 0.5 ml of cell free extract and 1.5 ml of phosphate buffer (0.025 M, pH 7.2). Catechin, catechol, protocatechuate and hydroxyquinol were used as substrate for catechin oxygenase, catechol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase and hydroxyquinol 1,2-dioxygenase, respectively. Catechin oxygenase was assayed by measuring decrease in absorbance at 280 nm, the maximum for catechin (Sambandam, 1983). Catechol 1,2-dioxygenase was assayed according to Hegeman (1966) except that EDTA was omitted. Protocatechuate 3,4-dioxygenase was assayed by the method of Fugisawa and Hayashi (1968). The decrease in absorbance at 290 nm (maximum of catechin) and increase in absorbance at 285 nm (maximum for B-carboxy cis-cismuconic acid) were observed. Since hydroxyquinol underwent auto-oxidation in neutral and alkaline solutions, the OD change due to enzyme in the reaction mixture rapidly enhanced oxidation of hydroxyquinol. The decrease in absorbance at 285 nm (maximum for malylacetate) were observed. Auto-oxidation was substracted from the total value.

Specific activities of all the enzymes were expressed in terms of disappearance of umole of substrate per minute per mg of protein. To determine the sequential appearance of dioxygenases, cells maintained on catechin were inoculated onto Dye's medium containing catechin 5 mM as sole carbon source. After incubation, cells were harvested at predetermined intervals and assayed for the dioxygenases.

Intermediates of catechin degradation were identified from the culture filtrate by paper chromatography, thin-layer chromatography, U.V. spectra, mass spectra and HPLC.

P. solanacearum utilized catechin as sole carbon source. Growth occurred till 120th h of incubation and declined thereafter in 2 mM, 5 mM and 10 mM. However concentrations up to 30 mM decreased growth; 40 mM was inhibitory and 60 mM was toxic.

It cleaved catechin into phloroglucinol carboxylic acid and protocatechuic acid. Further degradation of phloroglucinol carboxylic acid proceeded through the formation of phloroglucinol, resorcinol and hydroxyquinol. Hydroxyquinol was finally ring cleaved via ortho pathway. Protocatechuic acid was directly cleaved via ortho pathway. But when accumulated it was decarboxylated to form catechol which was ring cleaved via ortho pathway (Fig. 1). Catechin oxygenase was involved in the cleavage of catechin into phloroglucinol carboxylic acid and protocatechuic acid.

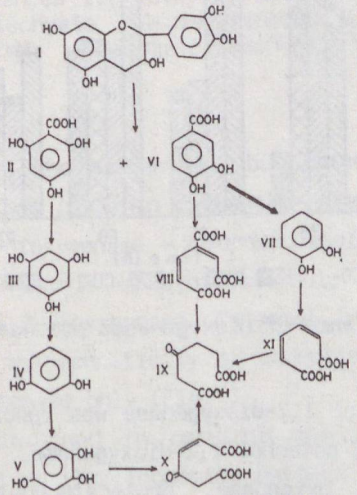


Fig. 1 Pathway for the degradation of catechin by Pseudomonas solanacearum (I-catechin; II-phloroglucinol carboxylic acid; III-phloroglucinol; IV-resorcinol; V-hydroxyquinol; VI-protocatechuic acid; VII-catechol; VIII-B-carboxy cis,cis-muconic acid; IX-B-ketoadipic acid; X-maleyl acetate; XI-Cis,cis-muconis acid.

Protocatechuate 3,4-dioxygenase, catechol 1,2-dioxygenase and hydroxyquinol 1,2-dioxygenase mediated the ring cleavage of protocatechuate, catechol and hydroxyquinol, respectively.

Sequential appearance of oxygenases

Levels of protocatechuate 3,4-dioxygenase, catechin oxygenase and catechol 1,2-dioxygenase were high in the early stages of growth. After 24 h incubation, protocatechuate 3,4-dioxygenase and catechol 1,2-dioxygenase activities were high. Cells contained moderate levels of catechin oxygenase. Hydroxyquinol 1,2-dioxygenase was comparatively low. Incubating the cultures for 36 h markedly increased the 4 dioxygenases. Catechin oxygenase, protocatechuate 3,4-dioxygenase and catechol 1,2-dioxygenase were high indicating the formation of protocatechuic acid and catechol.

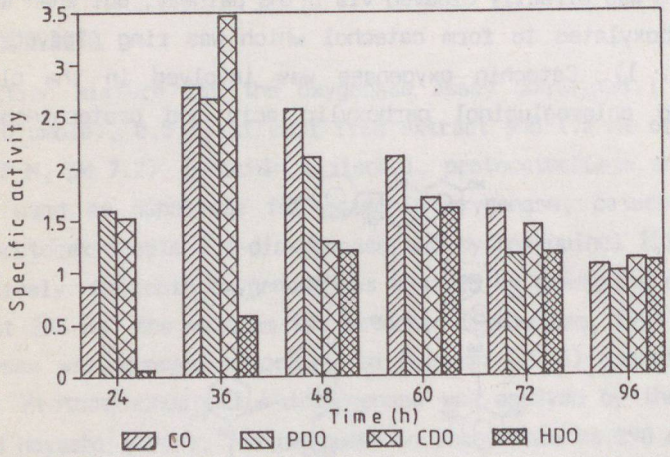


Fig. 2 Sequential appearance of dioxygenases produced by P. solanacearum.

Induction of hydroxyquinol 1,2-dioxygenase was recorded at 36 h. Generally cells had a high level of catechol 1,2-dioxygenase.

Levels of catechin oxygenase, protocatechuate 3,4-dioxygenase and catechol 1,2-dioxygenase declined at 48th h but hydroxyquinol 1,2-dioxygenase activity increased. When cells were incubated for 60 h, level of hydroxyquinol 1,2-dioxygenase was maximum.The delayed appearance of hydroxyquinol 1,2-dioxygenase indicates the time taken for the appearance of the substrate for the enzyme. By 96 h, all four oxygenase activities decreased.

Induction of oxygenases

Glycerol grown cells displaced little activity of any of the 4 oxygenases. The cells were inoculated onto liquid medium containing catechol (5 mM) or protocatechuate (5 mM) or catechol (2 mM) or hydroxyquinol (1 mM); incubated at prefixed interval and assayed for enzyme activity.

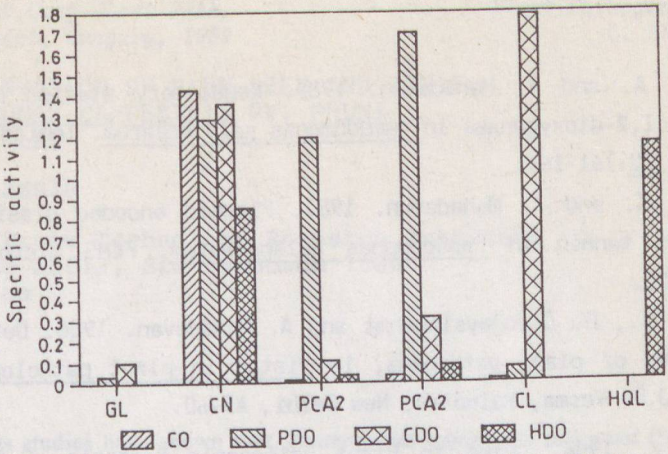


Fig. 3 Dioxygenase activities of cells maintained in glycerol and grown once in aromatic substances (CO-catechin oxygenase; CDO-catechol 1,2-dioxygenase; PDO-protocatechuate 3,4-dioxygenase; GL-glycerol; CN-catechin; PCA2-protocatechuate 2mM; PCA5-protocatechuate 5mM; CL-catechol; HQL-hydroxyquinol).

Catechin induced all the oxygenases, but catechin oxygenase was high. The order of induction was catechin oxygenase — catechol 1,2-dioxygenase — protocatechic acid 3,4-dioxygenase — hydroxyquinol 1,2-dioxygenase. Protocatechuate induced high levels of protocatechuic acid 3,4-dioxygenase. It also induced catechol 1,2-dioxygenase. Catechol induced only catechol 1,2-dioxygenase indicating the specificity of induction. Hydroxyquinol induced hydroxyquinol 1,2-dioxygenase only (Fig. 3).

When the cells maintained in catechin medium were used instead of glycerol grown cells, a similar induction pattern was recorded except that when the cells were grown in catechol, in addition to catechol 1,2-dioxygenase there was a significant level of protocatechuate 3,4-dioxygenase.

A search of phytopathogenic bacteria for the participation of detoxifying enzymes during pathogenicity would provide valuable information on the mechanism of specificity of phytopathogenic bacteria.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

SYSTEMIC INVASION OF PLUM SEED AND FRUIT BY XANTHOMONAS CAMPESTRIS PV. PRUNI THROUGH STALKS

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Previous studies have shown that *Xanthomonas campestris* pv. *pruni* (Smith)Dye moves systemically from infected plum leaves to shoots and other leaves to cause lesions on main and secondary veins. Both spring and summer canker development on plum are associated with systemic movement of *X. c.* pv. *pruni*. The possibility that *X. c.* pv. *pruni* also invades seeds and fruits systemically, was investigated by inoculating immature plum fruits through the stalks in an infected Golden King orchard. Many inoculated mature fruits developed unusual lesions extending from the exocarp to the endocarp. A few uninoculated, diseased fruits had similar lesions. The pathogen was isolated from both inoculated and uninoculated stalks and from seeds inside fruits. Scanning electron microscopy of inoculated stalks and mature fruits with unusual lesions showed that xylem elements of stalks, the testa (seed coat), stony endocarp, and mesocarp were filled with masses of *X. c.* pv. *pruni*. Bacterial colonies also occurred in parenchyma of fruit stalks, testa, mesocarp adjacent to the endocarp, diseased exocarp about three cell layers below the surface, and on the seed surface and adjacent inner surface of the endocarp. The pathogen was apparently absent from the starchy endosperm and surface of the diseased exocarp. The path of movement of *X. c.* pv. *pruni* into fruit stalks is presumed to be systemically from shoots. The prospect that *X. c.* pv. *pruni* is transmitted to seedlings through infected seeds of plum and other susceptible stone fruit species, is being investigated.

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

CHARACTERIZATION OF PLANT GENES WHOSE TRANSCRIPTS ACCUMULATE DURING THE HYPERSENSITIVE REACTION PROVOKED BY AN INCOMPATIBLE ISOLATE OF A PHYTOPATHOGENIC BACTERIUM, PSEUDOMONAS SOLANACEARUM

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INTRODUCTION

A hypersensitive reaction (HR) is developed by many plant species in response to infection by an incompatible (non-host or avirulent) pathogen. This plant response is characterized by a rapid cell death of the cells at the site of inoculation, which limits the invasion of adjacent tissues by the pathogen. The HR has been therefore associated with plant resistance.

Many physiological changes such as callous formation, synthesis of phenylpropanoids, electrolyte loss, lignification, variations in the respiration rates have been described during an HR. In addition, several genes whose function is known (PAL, CHS, CHI, CAD, HRGP...) and which may play a role in the development of this plant response are being studied extensively (Lamb et al., 1989). However, little is known on the molecular mechanisms leading to an HR. In order to have a better understanding of this plant response, we have been studying the HR of tobacco infiltrated with an incompatible isolate of Pseudomonas solanacearum. This bacterium, the causative agent of bacterial wilt was chosen because of our good knowledge of its genetics and the existence of various isolates and mutants able to induce various plant responses in tobacco (Boucher et al., 1985).

Indeed, a typical race I solate, K60, is compatible on tobacco leaves whereas an atypical race I isolate, GMI 1000, induces the development of a HR within 24 hours after infiltration. A deletion derivative of the letter isolate, GMI 1178, has been shown to be deleted of some genes involved in the HR and has no apparent effect on the inoculated leaves (Message et al., 1978). This mutant allows us to discriminate between molecular events specifically related to the HR and those induced by the physical damage caused by our inoculation procedure. This technique which consists in the

leaf detachment and the infiltration of the bacterial suspension under vacuo is quite stressful and produces changes in the plant mRNA activities.

1. mRNA ACTIVITIES OF TOBACCO LEAVES INFILTRATED WITH THE DIFFERENT *PSEUDOMONAS SOLANACEARUM* ISOLATES

In order to check that the forementioned *P. solanacearum* isolates have a differential effect on tobacco, total RNA from inoculated leaves was purified and translated in a rabbit reticulocyte lysate in the presence of ^{35}S L-Methionine. The polypeptides were then analyzed by 1D and 2D-SDS-PAGE (Ragueh *et al.*, in press). Within 6 hours after infiltration with the incompatible isolate, important changes in the translation products are already visible. At the time, both the compatible and GMI 1178 isolate have no detectable effect on the plant gene expression. It was also shown that the infiltration of bacteria per se provokes some non-specific changes in the polypeptide pattern. 13 hours after infiltrations, both the K60 and GMI 1000 isolates induce the appearance (or increase) of newly synthetized polypeptides. These results indicated clearly that the various *P. solanacearum* isolates have a differential effect on the plant genome expression.

2. ISOLATION AND CHARACTERIZATION OFcDNA CLONES WHOSE TRANSCRIPTS ACCUMULATE DURING THE HR

In order to isolate HR specific molecular probes, a cDNA library was constructed using mRNAs from leaves infiltrated with the GMI 1000 isolate 6 hours after infiltration. 15 cDNA clones whose transcripts accumulate during the HR were obtained by differential screening of this cDNA library using ^{32}P-labelled cDNAs prepared from mRNAs isolated from leaves infiltrated with the GMI 1000 or the GMI 1178 isolates (Marco *et al.*, in preparation). They were shown to belong to 6 genes (on gene Families) by cross hybridization. These cDNA clones do not encode salicylic acid induced P.R. proteins: indeed, no accumulation of mRNAs corresponding to these cDNA clones could be detected in tobacco leaves accumulation of mRNAs corresponding to these cDNA clones could be detected in tobacco leaves sprayed with this compound which is known to provoke the synthesis of several pathogenesis-related proteins (Van Loon, 1983).

3. KINETICS OF ACCUMULTATION OF mRNAs CORRESPONDING TO THE cDNA CLONES DURING COMPATIBLE AND INCOMPATIBLE INTERACTIONS

Total RNA leaves infiltrated with the K60, GMI 1000, GMI 1178 or water, was purified at different times after inoculation. The same amount was spotted on Genescreen Filters and hybridized with the ^{32}P-labelled inserts of the cDNA clones. The results of this experiment are shown in Fig. 1. They indicate that the cDNA clones can be classified into 2 groups: in group I, the maximum level of accumulation of mRNAs in the incompatible interaction was 3 to 7 times higher than the one obtained in the compatible interaction. No significant accumulation of mRNAs is detectable in leaves infiltrated with water or the GMI 1178 isolate (f). Consequently, the physical stress caused by our technique of inoculation does not induce the accumulation of mRNAs. Moreover, these results suggest strongly that both groups of genes corresponding to the cDNA clones are regulated in a different way. This was confirmed by the study of the influence of an elicitor preparation from Phytophthora infestans var. nicotianae, on the accumulation of transcripts corresponding to cDNA of both groups. Whereas mRNAs corresponding to cDNAs of group 2 accumulate in response to such a treatment, no difference in the steady-state levels of transcripts corresponding to cDNA clones of group I was detectable between treated and untreated cells.

4. TOBACCO SUSPENSION CULTURED CELLS RETAIN THE ABILITY TO RESPOND DIFFERENTIALLY TO COMPATIBLE AND INCOMPATIBLE ISOLATE OF P. SOLANACEARUM

Physiological studies by Atkinson (1985) suggested strongly that cultured cells respond differentially to compatible and incompatible isolate of P. solanacearum. The cDNA clones were used as molecular probes to repeat these experiments: tobacco cells were inoculated with the K60, GMI 1000, GMI 1178 or water. Total RNA was purified after different periods of incubation. The same amount was spotted on filters and hybridized to the various ^{32}P-labelled inserts. As shown in Fig. 2, the results obtained in the two systems (leaves and suspension-cultured cells) are very comparable. In particular, both groups of cDNA clones behave similarly. These results confirm the data obtained by Atkinson et al. and imply that at least in the early steps of the interaction with a pathogen, plant cells have conserved the potential to respond differentially to compatible and incompatible isolates of P. solanacearum. This system is, of course, much easier to

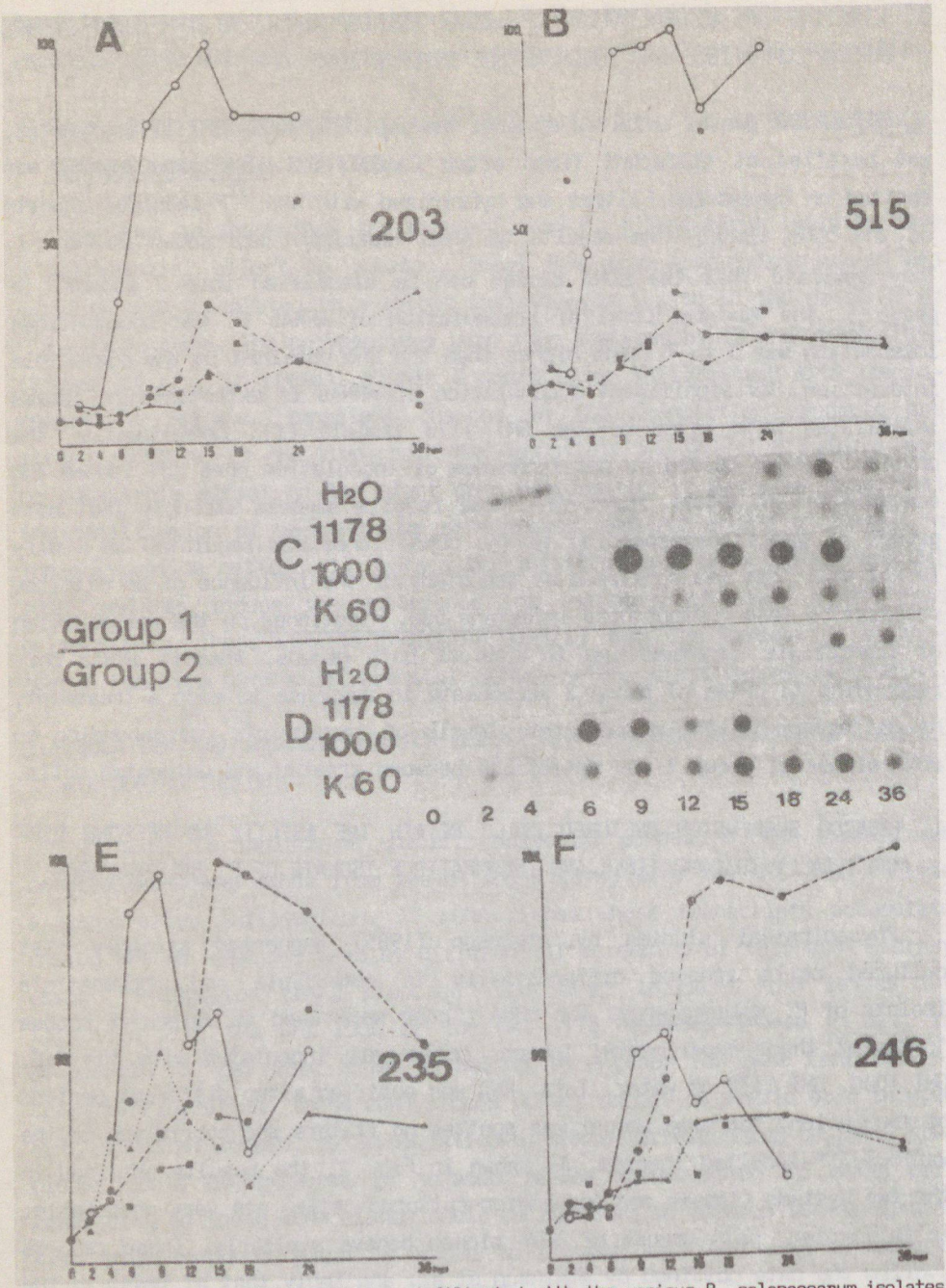


Fig. 1. 10 ug of total RNA from leaves infiltrated with the various P. solanacearum isolates or water were spotted on genescreen membranes and hybridized with the 32P-labelled inserts of the cDNA clones. After washing, the filters were exposed at -80°C. Panels C and D: Autoradiographs of filters after hybridization with cDNA clones 201 (Panel C) and 319 (D). Densitometer scanning of other autoradiographs obtained with cDNA clones 203 (A), 515 (B), 235 (E) and 246 (F).

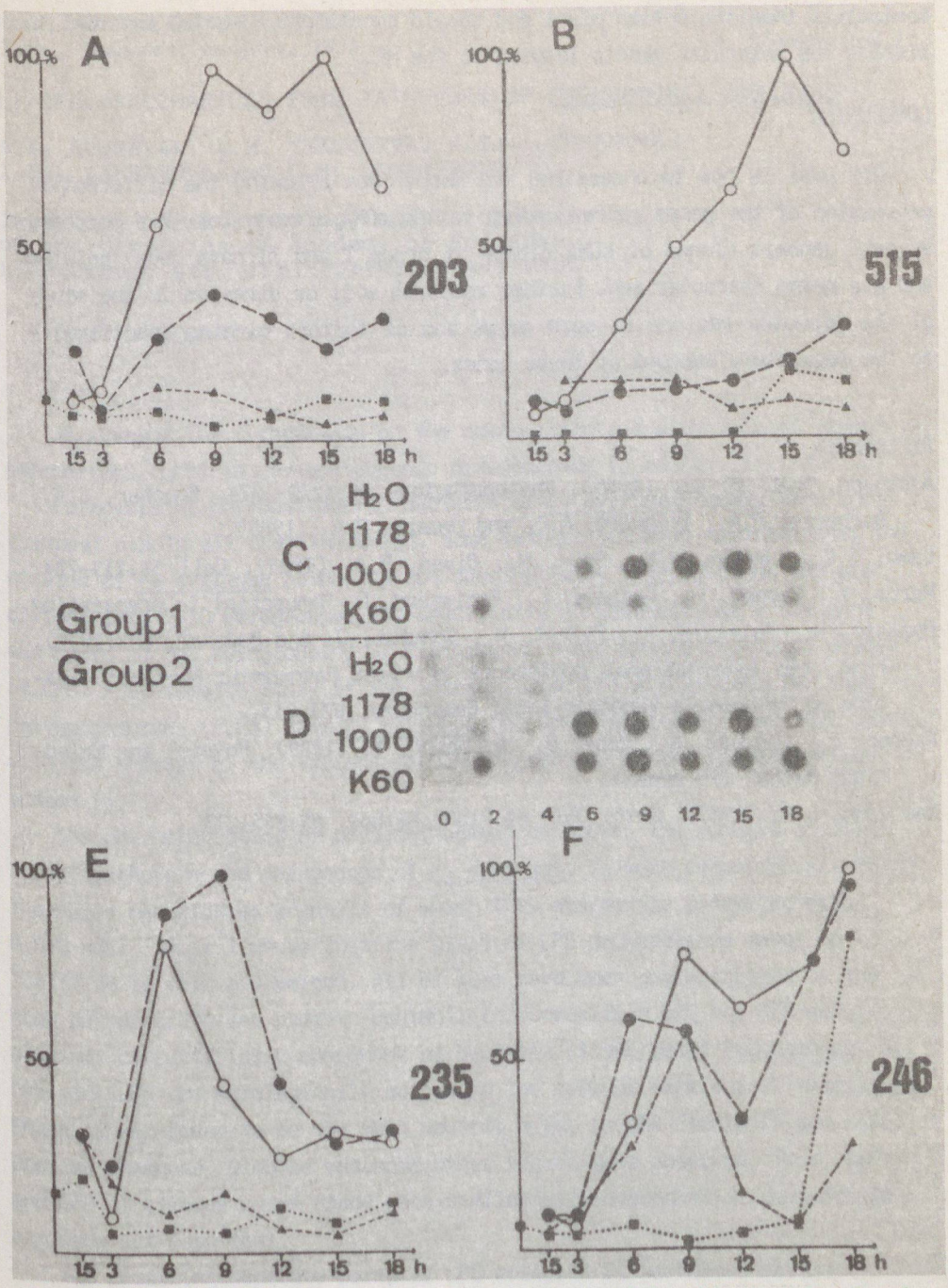


Fig. 2. 10 ug of total RNA isolated from cultured cells inoculated with the various P. solanacearum isolates or water were spotted on a genescreen membrane and hybridized with the purified 32P-labelled DNA inserts of our cDNA clones. After washing, filters were autoradiographed for 48 h at -80°C. Panels C and D: Autoradiographs of filters obtained after hybridization with the 32P-labelled inserts of the cDNA clones 203 (A) and 319 (D). Densitometer scanning of other autoradiographs obtained with cDNA clones 203 (A), 515 (B), 235 (E) and 246 (F).

manipulate than the entire plant and should constitute a useful material to dissect the molecular events leading to the HR.

CONCLUSION

Our goal is now to understand the mechanisms allowing the differential expression of the genes corresponding to the cDNA clones. For this purpose, several genomic clones of cDNA clones of group I and II have been isolated and are being characterized. Further research will be directed in the study of the promoter regions of such genes and of factors binding specifically to the regulatory regions of these genes.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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LIPOPOLYSACCHARIDES FROM PATHOVARS OF PSEUDOMONAS SYRINGAE

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At present the information on the composition and structure of lipopolysaccharides (LPS) in phytopathogenic pseudomonads is rather limited (1-3).

Pathovars of phytopathogenic bacteria of P. syringae damage many agricultural plants all over the world. Some authors 4-5) have reported that the strains of P. syringae pathovars are serologically heterogeneous and are divided into 9-10 serogroups. The specificity of thermostable o antigens was based on the serogrouping of Pastushenko and Simonovitch (5) by means of LPS. A systematic study of the intracellular LPS and exocellular lipopolysaccharides (ELPS) from P. syringae has been carried out for some years with the members of the various serological groups of the classification scheme (5).

The characteristics of phytopathogenic bacteria, the members of different pathovars and serogroups of P. syringae, is weak retention of LPS in the outer membrane as a result of which they are easily extracted with 0.85% NaCl. Table 1 shows that the yield of LPS preparations makes up 7.8-17.7% to cell dry weight. All of them have been characterized by the high carbohydrate and protein content. In composition LPS had KDO and α-alanine, characteristic component of bacteria of the genus Pseudomonas. All LPS and ELPS are serologically active in the various serological reactions. Rhamnose was found to be the main neutral sugar in LPS (Table 2) and ELPS. Besides rhamnose, glucose and some other sugars were observed. Thus, ELPS and LPS of strains under study were similar by monosaccharide composition but were not identical.

For obtaining separate parts of LPS molecule we performed acidic hydrolysis. As a result lipid A was separated. Gel filtration of carbohydrate of the molecule on column with sephadex G-50 resulted in obtaining

Table 1 Characteristics of LPS preparations

Species, pathovar, strain	Sero-group	Yield, % to dry weight of cells	Total carbo-hydrate	Pro-tein	KDO	α alanine	Serology activity in reaction of ring precipitation with antiserum	
			% to preparation weight				OH-	O-
P. syringae (P. holci) 8300	I	10.6	29.0	-	0.4	+	500000	100000
P. syringae (P. holci) 90a	II	9.5	50.0	1.6	1.8	+	1000000	500000
P. syringae pv. syringae P-55	III	8.4	37.0	-	0.5	+	1000000	500000
P. syringae pv. glycinae L-25	IV	9.3	15.0	3.8	+	+	100000	10000
P. syringae pv. atrofaciens 948	V	10.0	56.0	1.9	0.8	+	500000	50000
P. syringae pv. phaseolicola 120a	VI	7.8	29.5	2.6	0.4	+	100000	10000
P. syringae pv. tabaci 223	VII	17.7	35.0	0.9	0.7	+	100000	50000
P. syringae pv. tabaci 225	VIII	10.7	30.0	0.5	0.2	+	100000	50000

Table 2 Monosaccharide composition of LPS preparations neutral sugars - % to sum of batches at GlCh; X-sugar, heptose, aminosugars - % to dry weight of the preparation

Species, pathovar, strain	Serogroup	Rhamnose	Glucose	Galactose	Mannose	Xylose	Heptose	Fucose	X-sugar	Glucosamine	3-amino-D-fucose	Galactosamine
P. syringae (P. holci) 8300	I	90.8	7.3	tr	-	-	0.5	-	1.9	1.9	5.0	0.5
P. syringae (P. holci) 90a	II	96.8	tr	3.2	-	-	0.2	-	0.1	1.8	-	0.2
P. syringae pv. syringae P-55	III	69.0	6.5	5.0	-	-	0.3	16.7	2.9	2.5	-	1.0
P. syringae pv. glycinea L-25	IV	73.2	14.6	4.0	0.7	4.4	tr	-	2.9	4.5	-	0.5
P. syringae pv. atrofaciens 948	V	93.3	6.2	0.4	tr	-	1.6	-	tr	3.6	-	1.8
P. syringae pv. phaseolicola 120a	VI	64.7	6.4	3.5	0.3	8.7	tr	16.2	tr	3.9	-	1.5
P. syringae pv. tabaci 223	VII	88.7	11.3	-	-	-	-	-	tr	2.0	13.1	tr
P. syringae pv. tabaci 225	VIII	83.6	16.4	-	-	-	-	-	tr	7.9	-	0.6

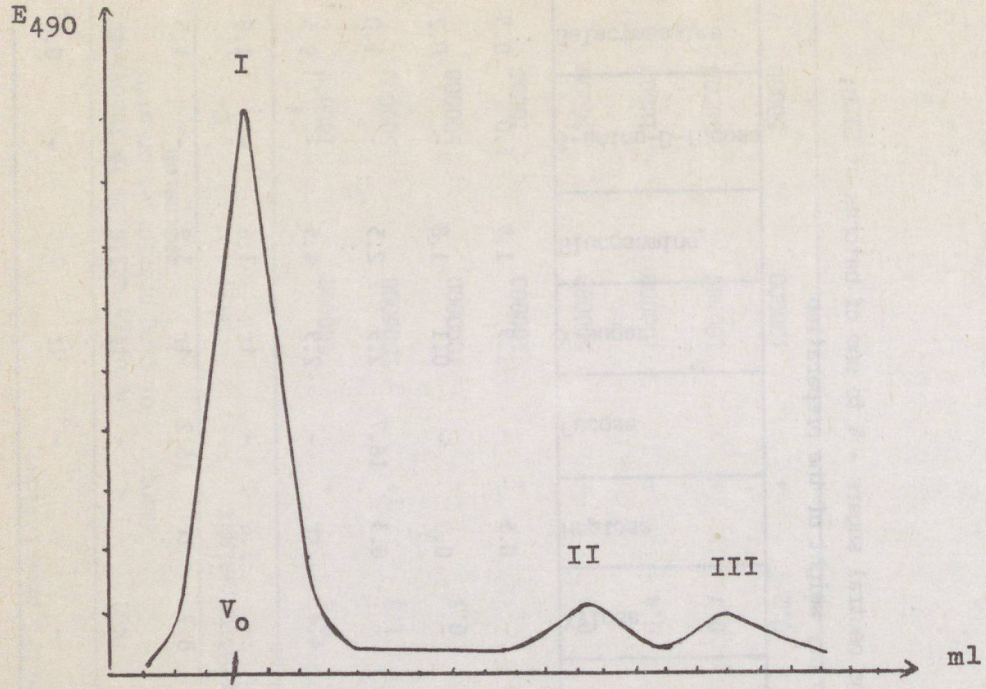


Fig. 1. Elution profile of degradated polysaccharide from P. syringae pv. phaseolicola 120a.

O-specific polysaccharide and core oligosaccharide. Figure 1 presents the elution profile of degraded polysaccharide of P. syringae pv. phaseolicola, strain 120a.

Irrespective of serogroup belonging of P. syringae parthovars, the character of the elution profiles was identical. Peak 1 is presented by highly molecular fraction of O-specific polysaccharide, peaks II and III - by the heterogeneous core oligosaccharide (low molecular fraction). High content of fraction 1 in strains shows the prevalence of molecule S-form in LPS.

Analysis of monosaccharide composition of core oligosaccharides (fraction 11) was performed with paper and gas liquid chromatography, ion-exchange chromatography. Our results demonstrated that rhamnose and glucose were the main neutral sugars in core composition. In core composition of all strains we found KDO, phosphorus and α-alanine and also unknown sugar. The component composition observed in core is characteristic for the core of bacteria of genus Pseudomonas. The obtained data show that on the level of the properties of the more conservative part os LPS molecule (core oligosaccharide) no correlation was found between the chemical composition

of LPS and adaptation of pathovars to host-plant, serogroup belonging to the strains.

Methods of methylation, degradation by Smith, ^{13}C- and ^{1}H-NMR-spectroscopy with effect of Overhauzer and without it chromatomass-spectroscopy, etc. helped to reveal structures of the repeating links of O-specific polysaccharides in some strains LPS. In the main chain of all polysaccharides examined, there was a similarity (by structure) in rhamnan built up with three or tetra-saccharide repeating links:

-3-α-L-Rha-(1-3)-α-L-Rha-(1-2)-α-L-Rha-(1-2)-α-L-Rha-(1-

-3-α-D-Rha-(1-3)-α-D-Rha-(1-2)-α-D-Rha-(1-2)-α-D-Rha-(1-

-3-α-D-Rha-(1-3)-α-D-Rha-(1-2)-α-D-Rha-(1-

A B C D

that confirmed pathovars belonging to one species. The members of the various serogroups differend either by the composition or site of sugar adhesion bound to the main chain as side branches in the positions A, B, C, D-chains. The members of serogroup VI, taken as example, and uniting P. syringae pathovars atrofaciesn, phaseolicola, P. Syringae (P. holci) showed no the correlation between chemical composition of LPS, the structure of O-specific polysaccharide and adaptation of pathovar to host-plant, the severity of the strain.

With the aim of determining the role of ELPS and LPS in the infectious process we performed the inoculation of the range of plants peculiar or not peculiar to particular pathovars: bean (pods), Sudan grass (into stem at stage of tube), tomatoes (green fruits on vegetating plants), tobacco leaves (by type of hypersensitive reaction), The plants were infected with twice-washed, living native cells, ELPS and LPS in the concentrations of 0.5-1.0 mg/ml. In experiments, P. syringae pv. syringae 281, pv. phaseolicola 120a, pv. pisi 7157, pv. tabaci 225, P. syringae (P. holci) 90a were used. It was noticed that unwashed cells induced greater damage of host-plants than pre-washed cells.

After injection into plant tissue the preparations of ELPS and LPS induced symptoms characteristic for the initial phases of plant infection by bacteria. These results show that LPS and ELPS intensify strain aggressiveness. It is likely that they facilitate invasion of bacteria into plant tissue even at the later stages of the infectious process.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

DIFFERENTIAL INTERACTIONS BETWEEN PSEUDOMONAS SYRINGAE AND DECIDUOUS FRUIT TREES

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INTRODUCTION

Deciduous fruit crop breeding programs in South Africa are primarily geared to supply cultivars that are adapted to local conditions. Emphasis is also placed on fruit quality. However, diseases caused by *Pseudomonas syringae*, particularly canker of stone fruit, pose a serious threat. This has to be taken into account in future programs. As a first step, this investigation examines the fate of selected strains of *P. syringae* in several plum and apple cultivars.

MATERIALS AND METHODS

Different strains of *P. syringae* were compared by inoculating [needle-injection; 10^8 colony-forming (CFU) units/ml as described by Norelli *et al.* (1987)] shoots of five plum and three apple cultivars in greenhouse tests. Results were recorded after 2 months.

Aggressiveness. Aggressiveness was measured by determining the length of canker-like symptoms developing on the current season's shoots.

Systemic spread. Leaves were removed and the shoot cut into 2-cm segments starting from the point of attachment to the main stem and extending 20 cm beyond the site of injection.

The number of CFU of the pathogen present in a sampling unit (i.e. leaves, petioles, or stem segments) was determined by dilution plating. The values were transformed to a 0-3 scale where 0 = nil, 1 = ≤10, 2 = >10-200, and 3 = >200 CFU per sampling unit. The Population Index (PI) of a host-pathogen combination was taken as the average value of the total scores of at least four individual shoots processed.

The identity of representative fluorescent bacterial isolates from shoots was confirmed to be *P. s.* pv. *syringae* or *P.* s. pv. *morsprunorum* by the GATTa tests (Latorre and Jones, 1979), ice-nucleation activity (Lindow *et al.*, 1978), production of syringomycin (Gross and De Vay, 1977), and the hypersensitive reaction on tobacco leaves (Klement, 1963).

RESULTS AND DISCUSSION

Two-way analysis of variance of the data indicated significant cultivar x strain interactions. Host-pathogen interactions varied more on plum than on apple. Six of the eight strains of *P. syringae* caused necrotic lesions exceeding 1% of the shoot length of one or more of the five plum cultivars. Four extremes were found: no growth of the pathogen and no symptoms; systemic spread but no symptoms; pathogen confined to lesion; extensive spread of pathogen beyond the lesion. Apple appears to be an inhospitable host. None of the mean values of lesions exceeded 1% of the length of apple shoots.

Evaluation and selection for bacterial canker resistance might be influenced by the aggressiveness of strains used in screening tests and the ability of these strains to spread systemically in the host. Our study indicates that symptomless trees could serve as important reservoirs for inoculum, especially if budwood is cut from these trees.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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ISOLATION OF BIOLOGICALLY ACTIVE COMPOUNDS FROM PSEUDOMONAS SYRINGAE PV. PAPULANS AND PSEUDOMONAS AMYGDALI

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The genus Pseudomonas includes several phytopathogenic species which induce disease symptoms on their hosts by producing phytotoxic or phytohormonal substances.[1]

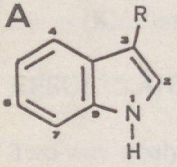
We previously reported that Pseudomonas syringae pv. savastanoi, the causal agent of olive and oleander knots, produces auxins and cytokinins, which are known to play a role in the pathogenesis of the bacterium.[2,3]

An investigation of the organic acid extracts of P.s. pv. savastanoi oleander strains made it possible to isolate and to identify the already reported indole-3-acetic acid (IAA, 1)[4], and a new IAA-lysine conjugate(Ac-IAA-lys, 2)[5]. In addition to IAA, indole-3-aldehyde(3)[6], but not Ac-IAA-lys, was isolated from olive strains indicating a different IAA metabolism for the two ecotypes. From the basic organic extracts trans-zeatin (Z, 4)[7], the new cytokinin 1'-methylzeatin (1'MeZ, 5)[8] and their corresponding 9-β-ribosides, namely the trans-zeatin riboside (ZR, 6)[7] and the 1"-methylzeatin riboside (1"MeZR, 7)[9] (Table 1) were isolated. The presence of Z, ZR and 1"MeZR in culture filtrates of P.s. pv. savastanoi was confirmed by MacDonald et al. (1986)[10].

The total and stereospecific synthesis of the two new cytokinins made it possible to confirm their structure and to assign the absolute R-configuration to the C-1' of 1'MeZ and C-1" of 1"MeZR[11]. A structure-activity relationship study showed that , when assayed on etiolated cucumber cotyledons, 1'MeZ and 1"MeZR were slightly more active in stimulating chlorophyl synthesis than their related cytokinins (Z and ZR).

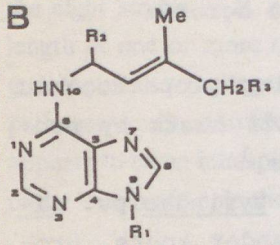
Table 1. Phytohormones (A, auxins; B and C, cytokinins) isolated from Pseudomonas syringae pv. savastanoi* and Pseudomonas amygdali+ culture filtrates

A



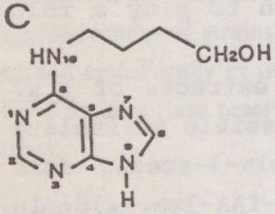
	R
1*	CH_2COOH
2*	$CH_2CONH-(CH_2)_4-CHNHAc$ (COOH)
3*	CHO
8+	CH_2COOCH_3

B



	R_1	R_2	R_3
4*+	H	H	OH
5*	H	CH_3	OH
6*	9-β-D-ribosyl	H	OH
7*	9-β-D-ribosyl	CH_3	OH
9+	9-(β-D-deoxyribosyl)	H	OH
10+	H	H	H

C



11+	Dihydrozeatin

Two other phytopathogenic bacteria, Pseudomonas amygdali and P.s. pv.papulans, were studied in our laboratory for their production of biologically active substances.

P. amygdali, the causal agent of the hyperplastic bacterial canker of almond, also accumulated in culture plant growth substances which seem to be important in the development of the symptoms.[12] From the acid and basic culture filtrate extracts IAA (1)[13], the corresponding methyl ester (8)[14], and trans-zeatin (4)[13], isopentenyl adenine (10)[15], dihydrozeatin (11)[15] were respectively isolated and identified (Table 1).

A further investigation of the basic organic extract made it possible to isolate and to characterize the new cyto-

kinin as 2'-deoxy-zeatin riboside (**9**)[15] (Table 1). This new cytokinin, low levels of which are present in the culture filtrates, is unique in that its sugar moiety consists of a 2'-deoxy-D-ribose residue. Preliminary data indicated that substance **9** is less active, when compared to the related zeatin riboside, in stimulating chlorophyll synthesis in etiolated cucumber cotyledons.[12]

Pseudomonas syringae pv. papulans is the causal agent of blister spot of apple.

Preliminary studies have shown that the acid organic extracts of P.s. pv. papulans culture filtrates had phytotoxic activity when assayed on apple and bean leaves. Three phytotoxic metabolites were isolated from the above extracts. One of the phytotoxic compounds was identified as the methyl ester of the β-phenyllactic acid. The trivial name of papuline has been assigned to this compound (Figure 1). Preliminary spectroscopic data (IR, UV, ^{1}H and ^{13}C-NMR and HR-MS) have indicated that the other two toxic metabolites have a structure related to that of β-phenyllactic acid[16].

Studies are in progress to elucidate the role of the above substances in the disease process.

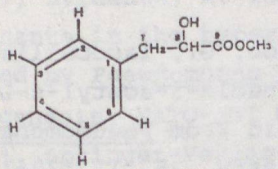


Figure 1. Structure of papuline isolated from Pseudomonas syringae pv. papulans.

Acknowledgements. The technical assistance of Mrs S.L. Lonigro is gratefully aknowledged.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

BIOSYNTHESIS OF SYRINGOMYCIN BY PSEUDOMONAS SYRINGAE VAN HALL

1. SCREENING OF PERSPECTIVE STRAINS

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Many phytopathogenic isolates of Pseudomonas syringae van Hall (PS) produce a broad spectrum of antibiotic syringomycin (SR). Our previous results (Vassilev et al., 1987) suggest that Pseudomonas syringae pv. atrofaciens (McCull) Young et al. (PSA) also produces SR and responses of wheat cultivars Sadovo 1 and Zlatosrui to SR correlated with the reactions to PSA.

In this connection we studied biosynthesis of SR of PS strains which differed in their virulence to SR in in vitro testing of wheat to intensify breeding.

MATERIALS AND METHODS

The strains of P. syringae are isolated from wheat (Table 1). Our previous biotest on PDA (about 100 strains) showed different levels of SR production. Nine of them: V2; V4; V8; V23; V28; I2632; K1392 are PSA, Y7068 is Pseudomonas syringae pv. syringae (PSS) and Y3080- PSS from Syringae vulgaris (L). Inoculation and incubation of cultures on potato-dextrose broth agar (PDB; PDA) used Gross's (1985) method. Virulence of the strains was studied according to Vassilev (1984). The scales of scores are described in Tables 1-3.

RESULTS

Grown in solid medium nearly all observed strains synthesized SR. Strain I2632 was an average producer (Rate II). Strain Y7068 did not produce SR (Table 1).

Table 1 Comparison of strains of P. syringae (vial cell) for production of SR in potato-dextrose agar (PDA)

Strain	Collection	Rate				Average rate
V28	IIPGR	4	4	4	4	4.00
V2	IIPGR	4	4	4	4	4.00
K1392	IMV	4	3	4	4	3.80
Y3080	ICMP	4	4	3	3	3.50
V4	IIPGR	4	3	4	3	3.50
V8	IIPGR	3	3	4	4	3.50
V23	IIPGR	3	3	3	3	3.00
I2632	IMV	2	2	2	2	2.00
Y7068	ICMP	0	0	0	0	0.00

0 - full growth of Rhodotorula sp.
1 - light clearing at the drop place (10 µl)
2 - slight growth at the drop place (10 µl)
3 - fully reduced growth at the drop place (10 µl)
4 - reduced growth around the drop place (10 µl)
IIPGR - Institute of Introduction and Plant Genetic Resources, Sadovo, Plovdiv, Bulgaria,
IMV - Institute of Microbiology and Virology, Kiev, USSR,
ICMP - International Collection of Micro-organisms from Plants, Auckland, New Zealand.

Table 2 Comparison of strains of P. syringae for production of SR in potato-dextrose broth (PDB) media

Strain	Collection	SR Units/cm^3
V28	IIPGR	2700
V8	IIPGR	1200
V23	IIPGR	980
K1392	IMV	780
V2	IIPGR	760
V4	IIPGR	605
Y3080	ICMP	320
I2632	IMV	250
Y7068	ICMP	0

Average of two 50-ml liquid cultures incubated at 25°C for 5 days.
A Unit of SR activity is that amount in an end point dilution which will completely inhibit the growth of Rhodotorula sp. in the area of application of a 10 µl droplet on PDA.

Table 3 Virulence of P. syringae in wheat cv. Rousalka (stage 5-6, large 1954)

Strain	Collection	Colony forming Units/cm^3 rate					
		10^3	10^4	10^5	10^6	10^7	10^8
K1392	IMV	0	2.5Y	2.5Y	2.5Y 1.5N	2.5Y 2N	2Y
I2632	IMV	0	2.5Y	2.5Y	2.5W	3Y	3Y
V8	IIPGR	1.5Y	2Y	2.5Y	2.5Y	2.5Y 1N	2.5 1N
V23	IIPGR	0	2W	2.5Y	2.5Y	2.5Y	2.5Y
V2	IIPGR	0	0	2.5W	2.5Y 1N	2.5Y	3N
Y3080	ICMP	0	0	2.5W	2.5Y 1N	2.5Y 2N	2.5N
Y7068	ICMP	0	0	2.5W	2.5W	2.5Y	2.5W
V4	IIPGR	0	2W	2W	2.5Y	2.5Y 1N	2.5Y 2N
V28	IIPGR	0	0	1W	2.5Y 1N	2.5Y 2.5N	2.5Y 3N

Y - yellow spott
W - watersoaking spot
N - necrotic spot
0 - no symptoms
1 - 1-2 mm spot in place of inoculation
2 - 2-3 mm spot in place of inoculation
2.5 - 3-5 mm spot out place of inoculation; 1/2 from leaf blade spotted
3 - 2/3 from leaf blade spotted
4 - full leaf blade spotted and becomes dry

The strains produced from 0 to 2700 Units/cm^3. Four groups are differentiated (Table 2): I - Strain V28; V8 with production above 1000 Units/cm^3; II - Strain V23, K1392, V2, V4 from 500 to 1000 Units/cm^3; III - Strain Y3080, I2632 - up to 500 Units/cm^3; IV - Strain Y7068 non-producing.

Production levels in in vitro however are not correlated with virulence (Table 3).

DISCUSSION

Syringomycin syntheses from P. syringae play a significant role in pathologic process (Gross, D.C. and Cody, 1985). Pauly et al. (1987) do not find coincidence between SR resistence and viable cultivar PSS. From the

other side, we observed a correlation between cultivar resistance against PSA and SR resistance (Vassilev et al., 1987). Our virulent strains are average producers of SR. The best strain V28 produces 2700 Units/cm^3 which exceeds strain B3A with about 600 Units/cm^3 (Gross, 1985). The original method of producing highly purified SR (Gross and De Vay, 1977) is labour-consuming, and not suitable for production of large quantities. The abridged procedure (Gross, 1985) enables good screening of strain producer and also, toxin with a high efficiency for use in selection in vitro. Disregarding the fact that SR synthesis in vitro does not correlate with bacterium virulence in vivo, toxin is an important factor in the pathologic process. This enables, its use in in vitro screening of the wheat tissue culture resistance studies. These results will be published in our following survey.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

COMPARISON OF OUTER MEMBRANE PROTEINS AND LIPOPOLYSACCHARIDES OF XANTHOMONAS CAMPESTRIS PATHOVARS

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INTRODUCTION

Over 125 pathovars of Xanthomonas campestris are now recognized on the basis of the host from which they were first isolated. Each pathovar has a different set of compatible hosts, however, strains of some pathovars can be pathogenic for host species in different plant families. The molecular basis of host-pathogen specificity is poorly understood. Cell surface components of bacterial pathogens may play a role in the early infection process and in determination of specificity of host-pathogen associations (Minsavage and Schaad 1983, Sequeira 1985). In this study we compared outer membrane protein (OMP) and lipopolysaccharide (LPS) patterns among 55 isolates from 15 different pathovars of X. campestris.

OUTER MEMBRANE PROTEINS

OMP samples were prepared according to Achtman et al. (1983). Bacterial cells grown in Luria broth for 16 h were collected and broken by sonication. Unbroken cells were removed. Membranes were pelleted by centrifugation and suspended in 10 mM Tris-HCl buffer (pH 8.0). Sarcosyl was added to final concentration of 1.5 %. The insoluble outer membranes were pelleted by centrifugation and suspended in electrophoresing sample buffer. Samples were run in sodium dodecyl sulfate polyacryl amide gel electrophoresis (SDS-PAGE) containing urea.

Within one pathovar, the OMP patterns of the strains were closely similar. This is illustrated in Fig. 1 for nine strains of the pathovar X. campestris pv. campestris. The variation mainly involves one of the smaller peptides, which differed significantly in the strain 1648 and less in the strains 528, 1929 and 3290. Similarly, the OMP patterns of five X. campestris pv. translucens strains were almost identical and only one pv. translucens strain (3215) differed clearly from others in the OMP pattern (Fig. 2). The OMP patterns of the different pathovars differed clearly from each other, except those of X. campestris pv. translucens and X. campestris pv. graminis, which differed only in the apparent size of the smallest peptide (Fig. 3).

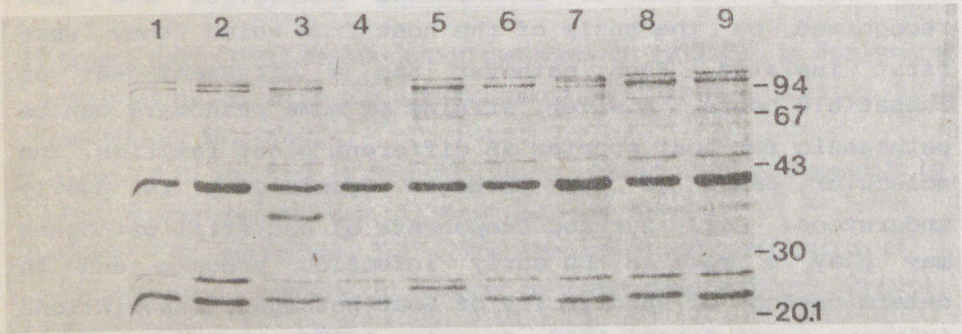


Fig. 1. OMP patterns of isolates of X. campestris pv. campestris. Strains in lanes: 45 (1), 279 (2), 528 (3), 1043 (4), 1648 (5), 1929 (6), 3207 (7), 3290 (8), 3291 (9). The positions of the standard proteins (in kDal) on the right.

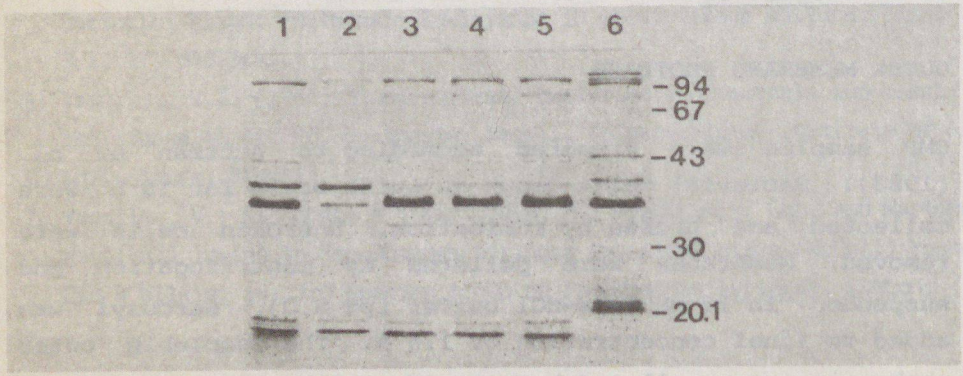


Fig. 2. OMP patterns of isolates of X. campestris pv. translucens. Strains in lanes 973 (1), 2389 (2), 2920 (3), 3170 (4), 3176 (5), 3215 (6). Standard proteins on the right.



Fig. 3. OMP patterns in different X. campestris pathovars. Strains in lanes: X. c. pv. translucens 973 (1), X. c. pv. graminis 2700 (2), X. c. pv. campestris 528 (3), X.c. pv. pelargonii 2985 (4), X. C. pv. begonie 3003 (5), X. c. pv. oryzae 3301 (6), X. c. pv. citri 3135 (7), X. c. pv. cucurbitae 2597 (8). Standard proteins on the right.

LIPOPOLYSACCHARIDES

LPS samples were prepared according to Hitchcock et al. (1983). Bacteria were grown on NYGP agar, collected and suspended in water and sonicated as above. Membrane pellet

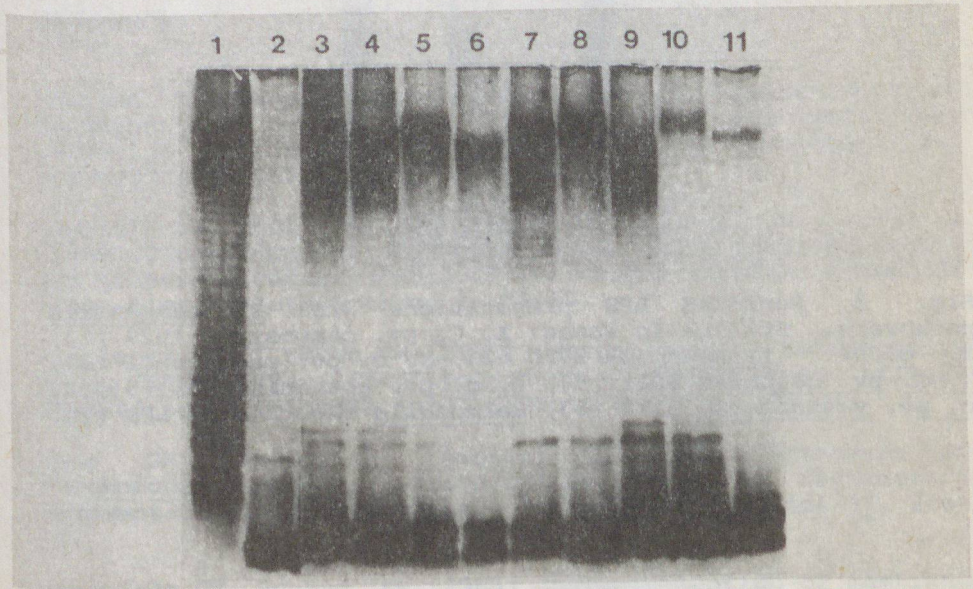


Fig. 4. LPS patterns of isolates of X. campestris pv. campestris. Strains in lanes 3291 (3), 3290 (4), 3207 (5) 1929 (6), 1648 (7), 1043 (8), 528 (9), 279 (10), 45 (11). Two Salmonella LPSs, a smooth one (1) and a rough one (2) are shown for comparison.

was suspended in lysing buffer and heated for 10 min at 100°C. Proteinase K was added and samples were incubated first for 2 h at 58°C and then overnight at 37°C. Samples were heated for 5 min at 100°C and run in SDS-PAGE. From 6 strains LPS was isolated by hot phenol-water procedure (Westphal and Jann 1965).

The LPS patterns of the nine X. campestris pv. campestris strains were closely similar in the rough and the smooth regions of the LPS molecules (Fig. 4). The LPS patterns of the different X. campestris pathovars were clearly different and in most cases of the smooth chemotype (Fig. 5).

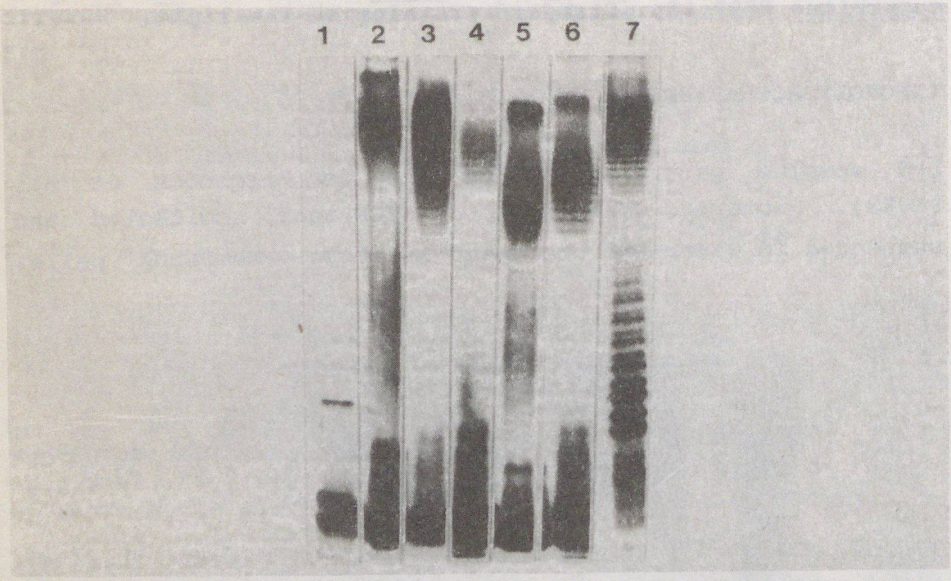


Fig. 5. Purified LPS preparations from X. campestris pathovars. Strains in lanes: X. C. pv. campestris 1929 (1), X. c. pv. translucens 2920 (2), X. c. pv. pelargonii (3), X. c. pv. begoniae 3021 (4), X. c. pv. graminis 3041 (5), X. c. pv. vesicatoria 3178 (6), Salmonella smooth type LPS (7).

DISCUSSION

Lazo et al. (1987) and Gabriel et al. (1988) recently showed that pathovars of X. campestris can be distinguished by restriction fragment-length polymorphism, which indicates a clonal population structure of X. campestris. In E. coli, the clonality of population structure can be constructed also with cell surface structures, including OMP and LPS patterns (Achtman et al. 1983). Our results show that these cell surface structures are clonally distributed in X. campestris strains as well. Within a X. campestris pathovar, the OMP and LPS composition of the strains appear similar. Excepting the OMPs of X. c. pv. translucens and X. c. pv. graminis isolates, individual pathovars have unique OMP and LPS composition. We are currently studying whether surface structures of X. campestris pathovars can be utilized in diagnostics and whether they have a role in initiation of the pathogenetic process.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

EXOPOLYSACCHARIDES OF XANTHOMONAS AND THEIR PROPERTIES

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The members of the various pathovars of Xanthomonas have the ability to produce extracellular polysaccharides (EPS:) on the various media with sugars. Carbohydrate-nitrogen ratio in nutrient medium exerts a considerable influence on this process. The maximal production of polysaccharides is observed on media with carbon excess and some nitrogen deficiency. Most of all the ability for polysaccharide-production was expressed in Xanthomonas campestris pv. campestris (Matyschevskaya et al., 1981).

The reason for such hypersynthesis of this metabolite and its role in bacterial activity has not been cleared. The supposition that polysaccharide is the factor of virulence is not well grounded since virulence of the examined members of genus Xanthomonas does not correlate with the production level of the EPS and their properties. The absence of the toxicity of EPS Xanthomonas for animals is indirect confirmation of it. EPS of Xanthomonas can play, to our mind, the auxiliary role in the development of infection. Due to high viscosity in solution they are able to cork up mechanically plant veins facilitating the development of the pathological process.

Within the first 0.5-1 h of growth on liquid medium the dying off of cells prevails over reproduction, then the culture has slow growth. Lag-phase lasts for 2-6 h followed by exponential phase to 26-30 h from the moment of inoculation. After intense growth the culture changes to stationary phase, the period of which can be rather long (from 9 to 12 h). After 45-48 h of growth cell dying off in culture begins to prevail over reproduction.

Biosynthesis of EPS by cells of Xanthomonas starts in the first hours of growth and reaches a maximum in 2-3 days. The increase of viscosity in the cultural fluid lags behind the accumulation of EPS in cultural fluid

Table 1 EPS-production of Xanthomonas campestris 8162 in dynamics

Period of growth days	EPS	
	quantity, g/l	viscosity, sSt
1	3.5	2.38
2	14.9	11.06
3	14.2	23.25
4	12.2	41.6
5	11.7	23.88
6	11.0	12.1
7	9.5	21.69
8	9.0	17.88

Table 2 The influence of season rotation on EPS-production in X. campestris 8162

Samples of EPS	Months	EPS-level, g/l, M±m
S-1	December	14.5 ± 0.3
S-2	January	13.3 ± 0.46
S-3	February	14.4 ± 0.37
S-4	March	13.0 ± 0.23
S-5	April	17.0 ± 0.3
S-6	May	15.2 ± 0.18
S-7	June	13.5 ± 0.3
S-8	July	11.6 ± 0.69
S-9	August	17.0 ± 1.04
S-10	September	10.5 ± 0.55
S-11	October	11.5 ± 0.18
S-12	October	7.7 ± 0.33
S-13	November	11.0 ± 0.31

and reaches a maximum in 4 days (Table 1). Then EPS-level and its viscosity drops.

This fact, in our opinion, confirms that EPS in Xanthomonas is a reserve material which can be degraded and utilized by cells in case of exhaustion of nutrient medium.

Inhabiliting plants by bacterial population undoubtly leads to its association with the plant vegetation cycle. Thus we can suppose that natural factors influencing plant development affect the main functions of baceterial culture. Two peaks of the activity of EPS-production within a year were establihsed for Xanthomonas campestris pv. campestirs 8162. One of them was observed at the end of March - the beginning of April the second

Table 3 The production of EPS by the variants of X. campestris 8162

Variants of producer	EPS-level		
	g/l	% to "ST"	% to "X"
M - 1	16.8	182.6	90.3
M - 2	16.4	178.2	88.1
M - 3	15.7	170.6	84.4
M - 4	15.5	168.4	83.3
L - 1	17.2	186.9	92.4
L - 2	15.1	164.1	81.1
L - 3	16.2	176.0	87.0
L - 4	15.2	165.2	81.7
"ST"	9.2	100.0	49.4
"X"	18.6	202.1	100.0

"X" - initial culture
"ST" - secondary culture with low activity

one - in August, i.e. prior to plant vegetation and before the end. It is likely that correlative dependence between the life cycle of the pathogen and plant, and also its immunity was predetermined by evolution fixed. Therefore the alterations of the seasons influences one of the forms of pathogenic life activity - the possibility of EPS-production (Table 2).

Season-dependent changes of phytopathogenic activity may be due to other reasons. The alteration of solar activity can be one of the most probable reasons, as effect reported by Chizhevski-Velhover (1973).

When comparing the levels of solar activity and EPS-production in X. c. pv. campestris 8162, there was a high level of correlation between them: the raising of solar activity was accompanied by the increase of EPS-production (Fig. 1). We can suppose that polysaccharide slime has a protective function in solar radiation too /3/.

As some authors reported (Cadmus et al., 1976; Kidby et al., 1977) the culture of X. c. pv. campestris, synthesizing EPS, was able to dissociate into colonies of two types: small ones having low polysaccharide-producing activity and large ones with high activity. As a result, the polysaccharide-producing ability of the whole population decreased. We also observed such a dissociation in variant "ST" of X. c. pv. campestris 8162 which lowered the level of EPS-production 2 times, compared with the initial culture, into colonies of two mentioned types. But these different colonies under the separate growing conditions demonstrated almost such high activity of

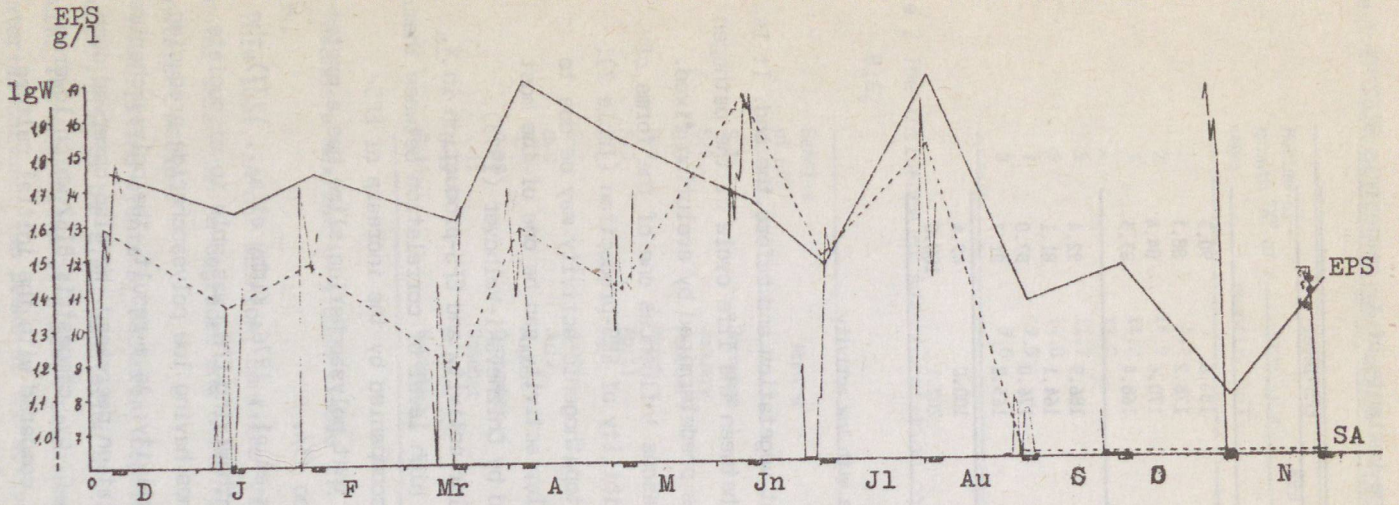


Fig. 1. The influence of solar activity on the EPS-production by X. c. pv. campestris 8162

SA - solar activity

W - Wolph's numbers

- 3-days period of experiment

EPS-production as we observed for the initial culture (Table 3). We can suppose that bacteria of the different types being in the same population compete among themselves and as a result the whole population activity is reduced.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

TRANSMISSION OF PSEUDOMONAS SYRINGAE PV. SYRINGAE BY SEED OF STONE FRUIT TREES

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INTRODUCTION

In South Africa, the causal organisms (*Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *morsprunorum*) of bacterial canker multiply extensively on buds, leaves, and shoots of stone fruit trees (Roos and Hattingh, 1983). Systemically-invaded trees often remain symptomless. However, we found that cherry blossoms inoculated with *P. s.* pv. *morsprunorum* died or gave rise to fruits containing necrotic spots at or near the blossom ends (Roos and Hattingh, 1988). Scanning electron microscopy of developing fruits indicated that the pathogen had invaded the entire pericarp, including the endocarp.

Seeds of stone fruit trees are required for producing rootstocks and are kept for breeding programs and seed banks. Local plant breeders and nurserymen reported that several batches of peach seeds collected in fall and then stratified in cold storage germinated poorly when planted in multipods under greenhouse conditions. Because of these observations and our earlier findings, we investigated the possibility that *P. s.* pv. *syringae* is transmitted in peach seeds.

MATERIALS AND METHODS

***P. s.* pv. *syringae* in germinating seed.** Seeds were randomly sampled from a supply kept for breeding purposes by the South African Plant Improvement Organization and the Fruit and Fruit Technology Research Institute. Seeds were stratified, surface-disinfested, and allowed to germinate according to Endert and Ritchie (1984). The presence of *P. s.* pv. *syringae* was determined in 1,611 individual seedlings. Each seedling was surface-disinfested before the epicotyl, hypocotyl, and cotyledon were separated. Each part of the seedling was sliced into small pieces, placed in 10 ml of sterile distilled water for 4-5 hr, and shaken vigorously. Loopfuls

of suspension were streaked on medium B of King *et al.* (1954). Randomly-selected representative fluorescent colonies were isolated and purified by streaking on this medium.

***P. s.* pv. *syringae* on pollen.** Thirty-three pollen samples were tested for the presence of *P. s.* pv. *syringae*. Each sample (1 g) was suspended in 10 ml of sterile distilled water and processed as described in the previous paragraph.

Identification of *P. s.* pv. *syringae*. The identity of *P. s.* pv. *syringae* was confirmed by tests listed previously (Roos and Hattingh, 1983). Pathogenicity of individual strains was determined by inoculating peach seedlings according to Endert and Ritchie (1984).

RESULTS AND DISCUSSION

In preliminary experiments, more than 80% of 200 peach seeds tested contained *P. s.* pv. *syringae*. Two batches of 20 seeds were vacuum-infiltrated with one of the freshly-isolated strains of *P. s.* pv. *syringae* and the highly aggressive strain NV (originally isolated from nectarine fruit), respectively. Nineteen of the seeds infiltrated with NV failed to germinate; the single seedling that did develop was stunted. High populations of the pathogen were detected in seedlings inoculated with the freshly-isolated strain, irrespective of the appearance of symptoms.

In the present investigation, *P. s.* pv. *syringae* was detected in 73% of peach seedlings showing disease symptoms and from 36% of symptomless seedlings. If detected in a seedling, the pathogen was invariably present in the epicotyl, hypocotyl, as well as the cotyledon. Many saprophytic bacteria also occurred in germinating seedlings. Eight of the 33 pollen samples processed, yielded *P. s.* pv. *syringae*.

Apart from having adverse effects on seedlings, plant pathogenic bacteria overwinter in seed (Schuster and Coyne, 1974). Shipment of infected seed ensures long-distance spread of pathogens. The presence of *P. s.* pv. *syringae* in pollen and seed of stone fruit complicates efforts to manage bacterial canker of this group of host plants.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

METABOLIC CHANGES ASSOCIATED WITH INDUCED SYSTEMIC RESISTANCE: ETHYLENE AND PROTEINS

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INTRODUCTION

Ethylene has long been known as an endogenous regulator of gene expression in higher plants (Abeles 1973). An evolution of ethylene is observed during interactions between the host and pathogens during infection processes (Abeles 1973). Exogenously applied ethylene induced an increase in the amount of lytic enzymes, such as chitinase, β-1,3-glucanase (Abeles et al. 1971; Boller et al. 1983) and peroxidase (Imaseki 1970; Morgan & Fowler 1972; Retig & Rudich 1972) in many different plants. We have indications now that even the amount of hydroxyproline in cell-walls is regulated by ethylene (Ridge & Osborne 1970; Toppan et al. 1982). Hydroxyproline is one of the major compounds of glycoproteins in plant cell walls. Chitinase has no known function in plant metabolism since higher plants do not contain a substrate for this enzyme. It has been proposed therefore, that chitinase participates in the defence against chitin-containing fungi (Abeles et al. 1971). The enzyme β-1,3-glucanes can also be useful in the defense against fungal zoospores because β-1,3-glucanes are major compounds of most cell walls of phytopathogenic fungi (Bartnick-Garcia 1968). The ability of these two enzymes to degrade fungal cell walls has long been established (Wargo 1975; Schlumbaum et al. 1987; Mauch et al. 1988). Our working hypothesis is that induced systemic resistance is regulated by ethylene and that resistance to pathogens is mediated by proteins or enzymes (Fig. 1).

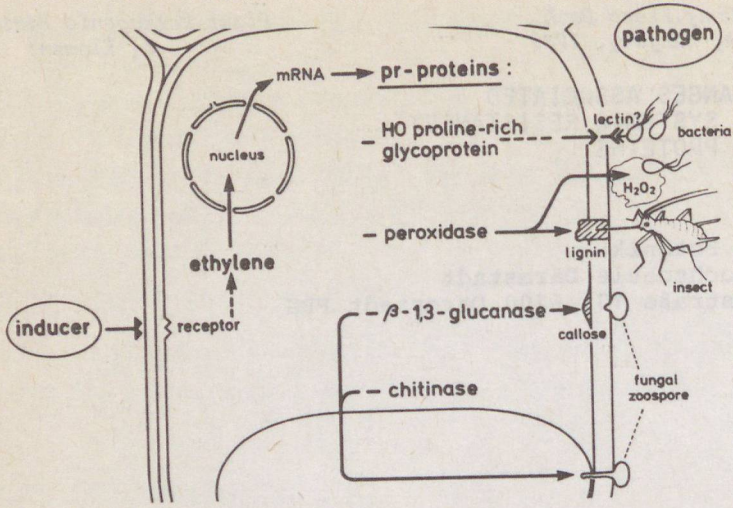


Figure 1 Working hypothesis

MATERIAL AND METHODS

Plant Material: Eight week-old Nicotiana tabacum L. plants ("Samsun NN") with 5-8 leaves were cultivated in porous clay and fertilized with Lewatit HD 5 from Bayer (FRG). Cucumis sativus L. Plants ("Chinese-Snakes") were grown in earth and used when 4-5 weeks old.

Conditions of culture: The plants have been cultivated in a greenhouse with 16 h light and 8 h dark, relative humidity min. 60% during daytime and min. 80% during nighttime. The growth chamber was run by a temperature-program implying rapid changes in temperature every few hours - because constant temperatures prevent induced systemic resistance (Falkhof et al. 1988).

Inoculum:

- o Bacteria: Pseudomonas syringae pv pisi DSM 50291 and Pseudomonas tabaci 5R1
- o Fungi: Erysiphe cichoracearum DC and Spaerotheca fuliginea Poll.

Inducers:

- o Plant extract: the dried leaves of the polygonaceae Reynoutria sachalinensis (F. Schmidt) Nakai were extracted in tap water for 0.5-3 hours (10 g per liter).

- o Bacterial culture filtrate: Pseudomonas fluorescence was incubated for 4 days. After measuring the amount of cells the culture was centrifuged (15 min, 1.000 xg). The supernatant was diluted to a density equal to $5x10^8$ cells per ml.
- o Fungal culture filtrate: the 3 week-old culture of Stachyobotrys chartarum (Ehrenb. ex. Link) Huges obtained from Prof. F. Schoenbeck, Hannover (FRG) was cultivated at 21 °C, then filtrated through cheese-cloth and used as an inducer (Maiss 1987).

Induction and Inoculation:

- o Cucumber: First true leaf of a cucumber plant was sprayed with inducer, 3-4 days later the same and the next (second) leaf were inoculated with $5x10^4$ spores per ml.

  Tobacco: The intercellular space of one half-leaf was infiltrated with the inducer by means of a syringe. 2-4 days later the whole leaf was infiltrated with 10^6, 10^7, or 10^8 cells per ml of the inoculum.

Crude enzyme preparation: Frozen leaf was homogenized in 0.1 M Na-acetate buffer pH 5.2. The homogenates were centrifuged (15 min, 14.000 xg). The supernatants were used as crude enzyme preparations for measuring peroxidase, chitinase and -1,3-glucanase activities. All preparations were performed at 4 °C.

ENZYME ASSAYS

1. Assay for peroxidase: An aliquot of enzyme extract was incubated for 5 min with 0.1 M Na-acetate buffer pH 5.2, guajacol ($1.6x10^{-5}$M/sample) and H_2O_2 (1%). Extinction was measured at 436 nm and 25 °C.
2. Assay for β-1,3-glucanse: An aliquot of crude enzyme preparation was incubated with laminarin (0.5 mg/ml) for 2 h at 44 °C. Another aliquot of the same preparation was incubated after boiling for 5 min. These samples were used as references. Colorimetric determination (Nelson 1944; Somogyi 1945). A modified Nelson-reagent was used containing Na_2HPO_4 instead of $Na_2H\ AsO_4$.
3. Assay for endo-chitinase: The reaction mixture contained crude enzyme extract and colloidal chitin (1 mg/ml). This mixture was incubated for 2 h at 37 °C. Following the incubation the mixture was centrifuged (1,000xg, 5 min). An aliquot of supernatant was incubated with β-glu-

cosidase to hydrolyse chitin oligomers. After 0.5 h at 37 °C in this reaction mixture N-acetylglucosamine (GlcNAc) was determined colorimetrically (Reissig et al. 1955).

4. Assay for l-hydroxyproline: The protein in dried leaf was hydrolysed with 6 N HCl for 24 h at 105 °C and then centrifuged (15 min, 1,000 xg). The supernatant was used for the determination of L-hydroxyproline (Neuman & Logan 1950 modified by Leach 1960).
5. Ethylene determination. Tobacco plants of the same age were used for ethylene determination. Each plant was placed into a plexiglas-chamber, after treatment with inducer. Control plants were treated with water or remained untreated. Ethylene concentration in the atmosphere of the chamber was determined applying a gas sample of 1 ml to a gaschromatograph.
6. Ethylene treatment: Ethylene was applied with a gas-tight syringe through the rubber diaphragm of a plexiglas chamber. The chambers were aerated every 24 h. Ethylene was injected after taking a leaf sample for the enzyme assays (total concentration: 100 ppm ethylene per chamber). Control plants without ethylene were kept in a chamber in the presence of $KMnO_4$ to remove any ethylene naturally formed.

RESULTS

Elicitation of enhanced enzyme activity by various inducers of resistance:

- o Peroxidase: The peroxidase activity reaches a maximum 3-5 days after inducer treatment. The bacterial and fungal culture filtrates cause an increase in activity about 600 and 420% respectively. Other inducers enhance the enzyme activity only about 250-300% relative to 100% activity at the beginning of the treatment. Water treatment does not enhance the activity (Fig. 2).
- o β-1,3-glucanase: Aspirin as the strongest elicitor for this enzyme increases the glucanase activity up to 600% other inducers stimulate only between 200 and 300% (Fig. 3).
- o Chitinase: The culture filtrate of the fungus St. chartarum and the plant extract cause a sharp rise in activity to about 500%. Almost no change of activity is observed after treatment with the bacterial culture filtrate (Fig. 4).
- o L-hydroxyproline: All inducers tested produce almost the same amount

Fig. 2

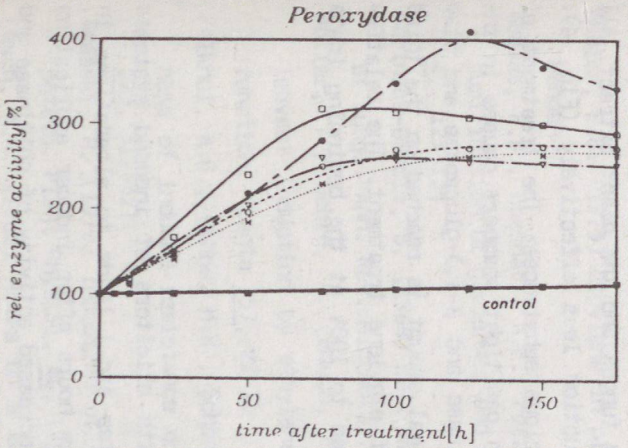


Fig. 3

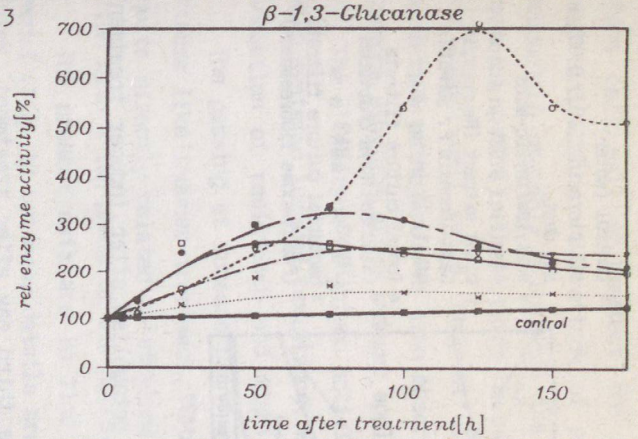


Fig. 4

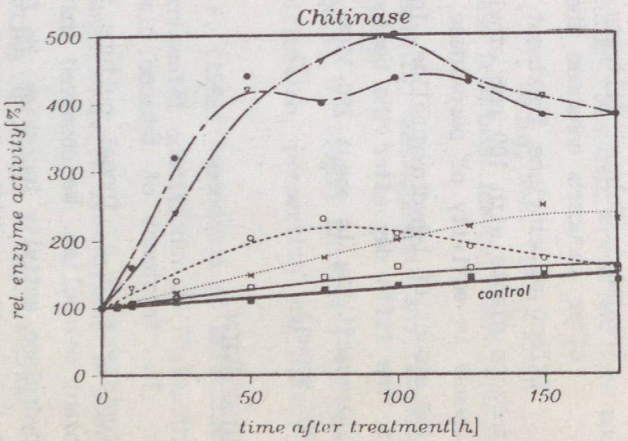


Fig. 5

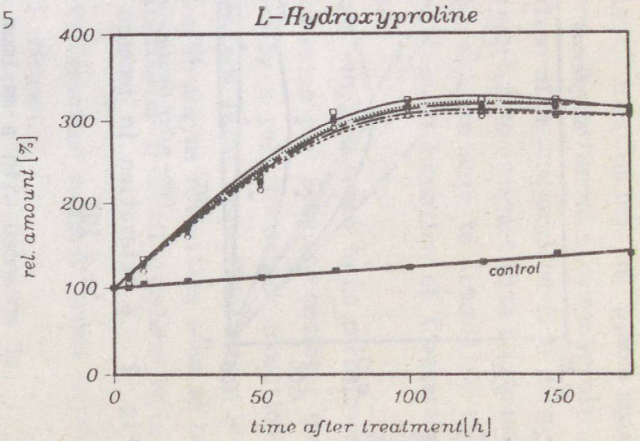


Fig. 6

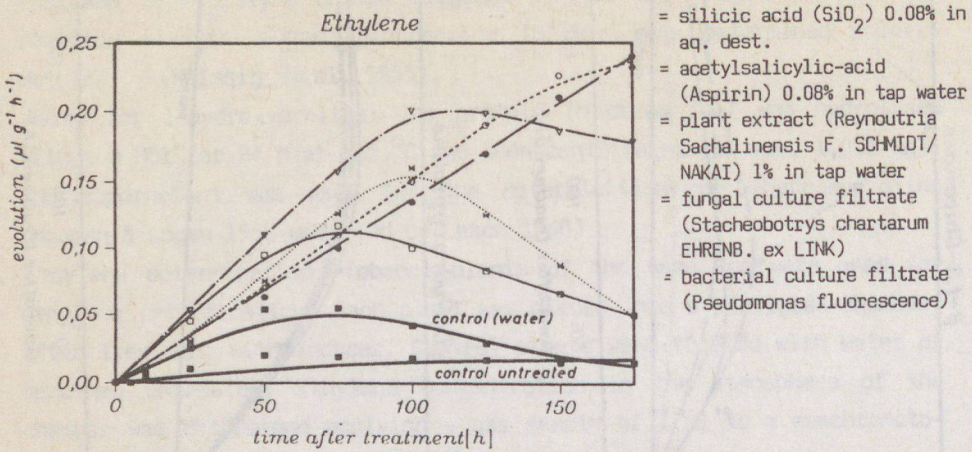


Fig. 2 - 6 Alteration in tobacco leaf metabolism after inducer treatment (n = 10)

of enhanced with a maximum on the third day after treatment. Water treatment (control) does not alter the amoungt(Fig. 5).

o Ethylene: The highest production of ethylene is caused by aspirin treatment. Culture filtrate of the fungus and the plant extract. All other inducers enhanced the production less effectively (Fig. 6).

Effect of ethylene treatment on various substances: The treatment of tobacco plants with 100 ppm ethylene in gas-tight chambers causes in increase in activity of peroxidase, chitinase and β-1,3-glucanase and also the amount of l-hydroxyproline. The maximal amount is reached on the third to the fifth day after the beginning of ethylene treatment. The relative enzymeactivity is about 250-350% compared to 100% at the beginning (data not shown).

DISCUSSION

The treatment of tobacco plants with elicitors of induced systemic resistance (e.g. fungal culture filtrates etc.) leads to alterations in plant metabolism. We observed that a few hours after inducer application hydrolase activity increases. We found increased activity of chitinase and β-1,3-glucanase which are able to degrade fungal cell walls. In addition we measured an increase in peroxidase activity. Peroxidase and β-1,3-glucanase are involved in lignin and callose synthesis. Both substances reinforce the

plant cell wall or protect the cytoplasm, thus making the plant more resistant against the attack of pathogens. The enhanced L-hydroxyproline production probably stimulates the formation of hydroxyproline-rich glycoproteins (HRGPs). Those HRGPs have been reported to interact with bacterial and fungal cell walls (Roby et al. 1985) and are able to immobilize the bacterial pathogens. We also observed an enhanced formation of the stress hormone ethylene after inducer application.

The alteration of enzyme activities and ethylene seems to be correlated with systemic induced resistance. We hypothesize that ethylene mediates the induction of resistance and might also play a role in enzyme induction.

The gassing of tobacco plant with ethylene leads to similar enzyme increase like inducer treatment. Alterations in enzyme activities seem to be quite directly related to ethylene application or internal ethylene evolution with a lag phase of 6-8 hours.

The present data are in line with the reaction sequence shown in the model (Fig. 1) but many details are still unknown.

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Proc. 7th Int. Conf. Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

HISTOLOGICAL STUDIES ON THE EFFECT OF CONTINUOUS DARKNESS ON PRODUCTION OF EXOPOLYSACCHARIDES BY XANTHOMONAS CAMPESTRIS PV. MALVACEARUM IN COTTON COTYLEDONS

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ABSTRACT

When susceptible cotton plants were placed in continuous darkness after inoculation with *X.c.* pv. *malvacearum* the visible plant reaction within 48 h resembled a hypersensitive response. Initially, bacterial multiplication was the same or even faster as in illuminated leaves, but ceased completely after 5 days in darkness. In order to elucidate the reasons for the resistance-like response of darkened leaves, histological studies were carried out by means of both light and electron microscopy of inoculated tissue using ruthenium red for specific staining of acidic polysaccharides. Leaf samples 48 h after inoculation revealed that under permanent darkness considerably less extracellular polysaccharides (EPS) were produced by the bacteria. While the intercellular spaces of illuminated leaves were filled with bacterial EPS, numerous EPS-free spaces were observed in leaf tissues from darkened plants. It was also observed that the bacteria in darkened leaves were more densely packed than in illuminated leaves. It is suggested that the hypersensitive-like response of compatible leaves under continuous darkness is induced by a cell to cell contact, because bacterial EPS-production is suppressed due to deficiency in soluble sugars.

INTRODUCTION

Several studies have shown that typical water-soaked spots do not appear when susceptible plants are placed in continuous darkness after inoculation with phytopathogenic bacteria. Instead, the plant reactions resemble a hypersensitive response [3,4,6,7]. To characterize the resistance-like response of darkened leaves, histological studies were carried out in susceptible cotton cotyledons inoculated with *X.c.* pv. *malvacearum*.

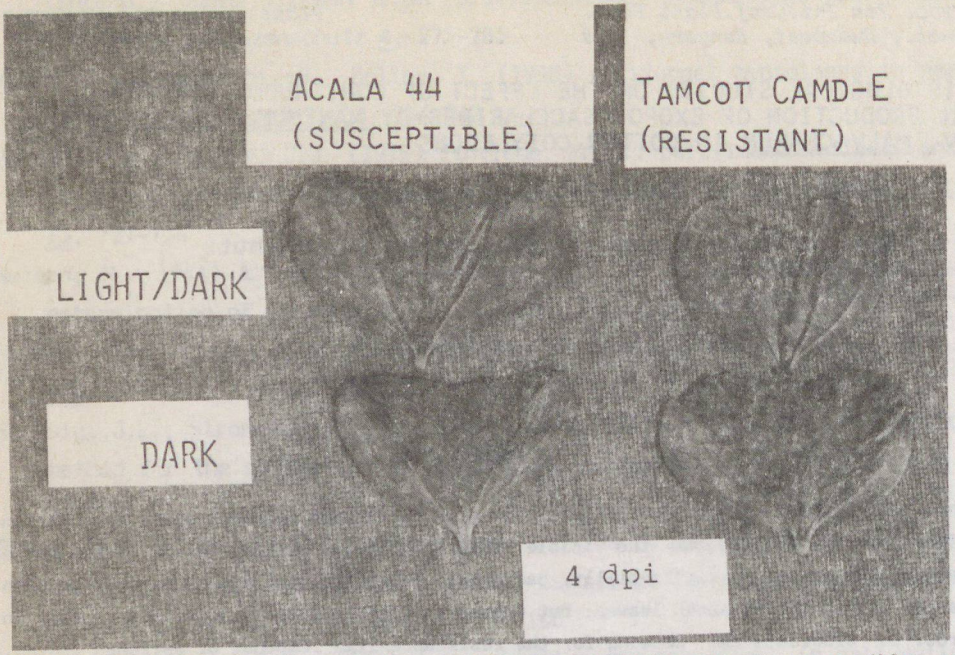


Fig. 1: Effect of continuous darkness on symptom development of susceptible and resistant cotton cotyledons 4 days after inoculation with *X.c.* pv. *malvacearum*.

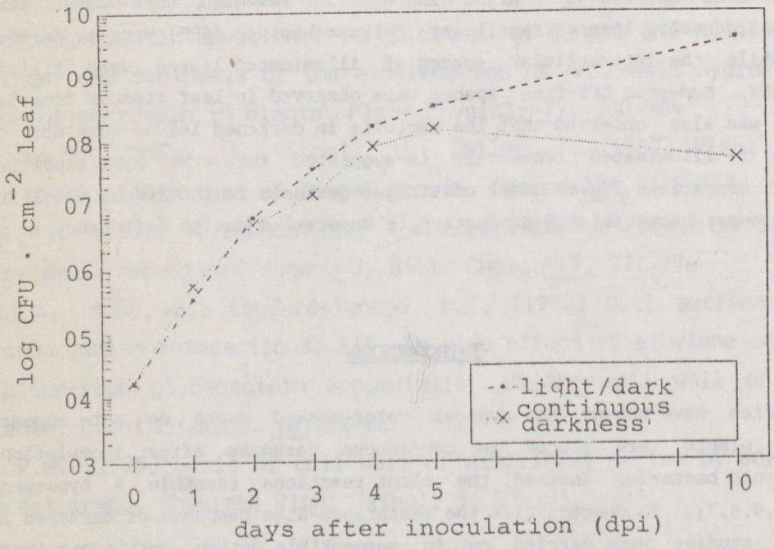


Fig. 2 : Effect of continuous darkness on growth of *X.c.* pv. *malvacearum* in compatible cotton cotyledons (cv. "Acala 44").

MATERIALS AND METHODS

Cotyledons of two week-old susceptible cotton plants (cv. "Acala 44") were infiltrated with race 7 of *X.c.* pv. *malvacearum*, which was isolated from infected cotton leaves collected in Turkey in 1986 [8]. The race identification was carried out on a set of differentials as previously described [9]. Half of the plants were placed in continuous darkness, the other half under light/dark conditions (day 12 h, 30 °C; night: 20 °C). Bacterial growth was monitored by dilution-plating of the homogenate from inoculated cotyledons. Samples of cotyledon tissue (3 mm), approximately 1 mm apart from the wound-infiltrated sites, were excised in 0.1 M cacodylate buffer containing 2 mg ml^{-1} ruthenium red (pH 7.3) 48 h after inoculation [1,5]. The tissues were prefixed in 2.5% glutaraldehyde and 2% paraformaldehyde in the same buffer for 6 h at 4 °C , postfixed for 4 h in 2% osmiumtetroxide in the same buffer at room temperature and dehydrated in an acetone series. The tissues were embedded in Spurrs' medium in flat rubber molds and cured at 70 °C for 24 h. Thin sections were stained with methylene blue [2] and examined and photographed with a Zeiss Axiophot light microscope. The ultrathin sections were stained in 4% uranyl acetate, and in Reynolds' lead citrate, and examined and photographed with an electron microscope.

RESULTS AND DISCUSSION

When the plants were kept in continuous darkness the inoculated areas became flaccid and faint olive green within two days, and after 5-6 days these areas appeared completely dry and changed to beige-coluored. Never did typical water-soaked spots develop (Fig. 1). Initially, bacterial multiplication was the same or even faster as in illuminated plants, but ceased completely 5 days after inoculation when the leaf tissue collapsed and became dry (Fig. 2). Six days after inoculation illuminated leaves showed typical disease symptoms with water-soaked areas along the veins but only slight wilting. The intercellular spaces of leaves kept in the dark contained considerably less fibrillar material (FM) around the bacteria (Figs. 4 and 6) than was observed in illuminated leaves (Figs. 3 and 5). Numerous FM-free spaces were observed in leaf tissues from darkened plants (Fig. 4), indicating an induced resistance under continuous darkness. In normally illuminated leaves an evenly distributed FM surrounded the bacteria, so that large areas of the intercellular space were filled (Fig. 3). Secondly, a very compact electron-dense material (EM) sometimes enveloped the bacteria (Fig. 5).

The results indicate that deficiency of soluble sugars in darkened leaves leads to a drastically reduced rate of EPS-production, so that the bacteria are not surrounded by a thick slime layer and a resistant reaction is induced by a cell to cell contact. The observation of electron-dense material in normally illuminated leaves supported this assumption. Further investigations are required to substantiate this hypothesis.

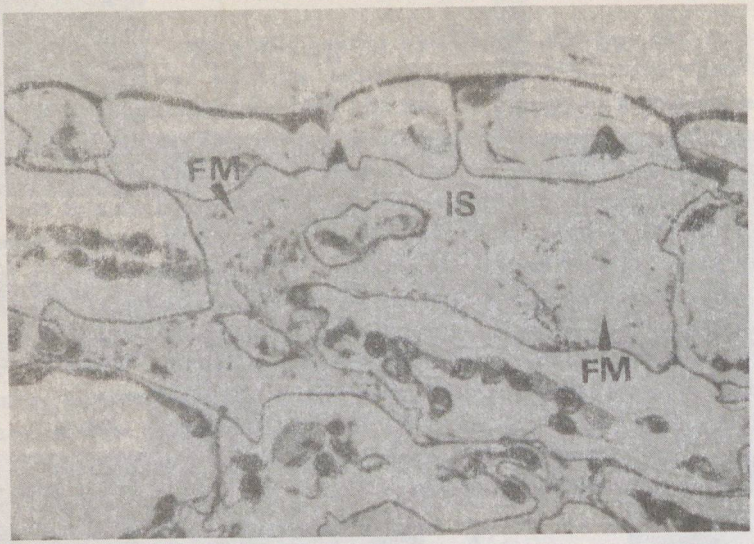


Fig. 3: Light micrograph (bright-field) of the intercellular space of an illuminated "Acala 44" cotyledon, 48 h after inoculation (x 1250). Ruthenium red stained fibrillar material (FM) fills the intercellular space (IS) of the spongy mesophyll. The bacteria occur homogenously distributed in the intercellular space.

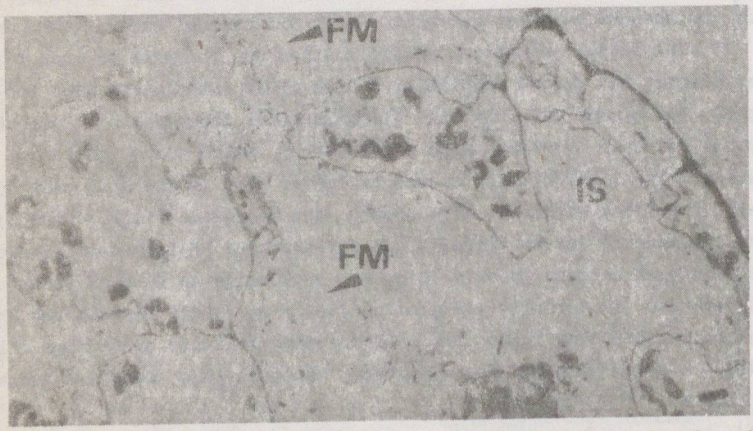


Fig. 4: Light micrograph (bright-field) of the intercellular space of an "Acala 44" cotyledon kept in continuous darkness, 48 h after inoculation (x 1250). The intercellular space (IS) of the spongy mesophyll contains considerably less stained fibrillar material (FM) around the bacteria than observed in illuminated tissue. Numerous FM-free space, occur in the leaf tissue and the bacteria are densely packed.

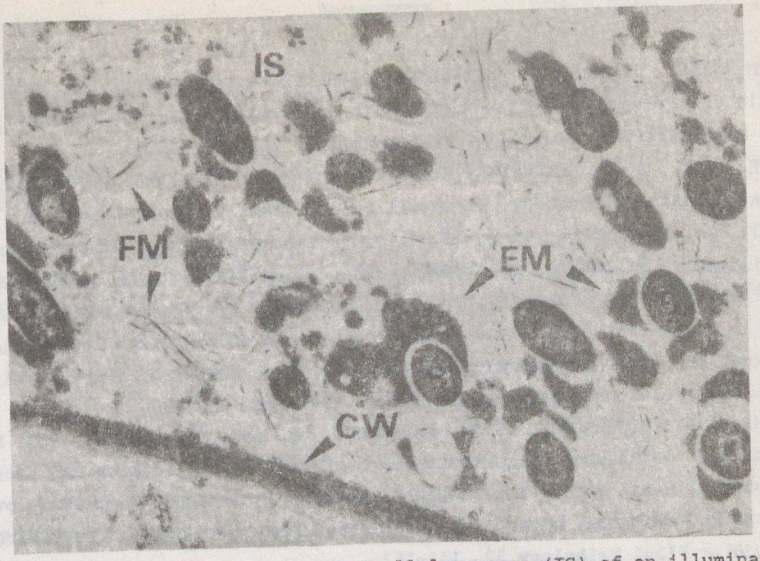


Fig. 5: Electron micrograph of the intercellular space (IS) of an illuminated "Acala 44" cotyledon, 48 h after inoculation (x 35000). Ruthenium red stained fibrillar material (FM) occurs around the bacteria (B). Note the electron-dense material (EM) around some bacteria; CW, cell wall

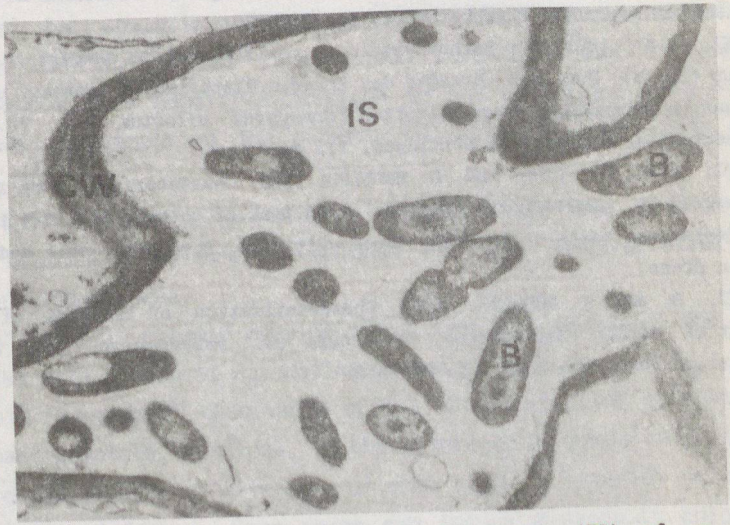


Fig. 6: Electron micrograph of the intercellular space (IS) of an "Acala 44" cotyledon kept in continuous darkness, 48 h after inoculation (x 33000). The bacteria (B) are not surrounded by fibrillar material (FM); CW, cell wall

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogneic Bacteria Z. Klement (ed.)

EFFECT OF CORONATINE AND CORONATINE-FORMING BACTERIA ON PLANT CELL CULTURES

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SUMMARY

The effect of coronatine (I) on the plant cell cultures (PCC) Lycopersicon peruvianum, Chenopodium album and Phaseolus vulgaris is described. The estimation of growth, protein and amino acid content of the cells as well as the "viability" test with triphenyltetrazoliumchloride were used as test parameters. The phytotoxins phaseolotoxine (II) and tentoxine (III) were tested for comparison. Moreover the effect of coronatine-forming bacteria on the culture of Lycopersicon peruvianum was analysed. This culture shows a more sensitive reaction to the bacteria. These reactions were followed by measuring the growth, TTC activity and raster electron microscopy (REM).

Coronatine (I) is a phytotoxin produced by some phytopathogenic bacteria, especially by the Pseudomonas syringae pathovarieties tomato, glycinea or atropurpurea.

The formation is plasmid coupled and a loss or a damage of the plasmids is connected with a loss of coronatine formation.

The main "secondary" observable effect after infection of host plants, e.g. soybean, tomatoes, potatoes, is characterized by small chlorotic halos surrounding the infection sites or in some cases a brownish colouring of parts of infected leaves. Some races can induce a systemic chlorosis of plants together with a stunting of growth.

Effects of coronatine on plants are quite different but the "primary" action is not yet known. With our experiments we were looking for ways to examine the interaction between bacteria and plant cells for a better understanding of the infection processes.

Used Plant cell cultures

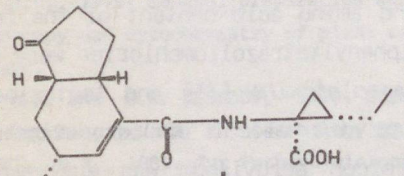
Lycopersicon peruvianum, fine suspension in a modified MS medium (L.p.), Chenopodium album, fine suspension in a modified MS medium (C.a.), Phaseolus vulgaris "Red Kidney", inhomogenous, unstable suspension in B5-medium (Gamborg et al.) (P.v.).

Used strains of bacteria

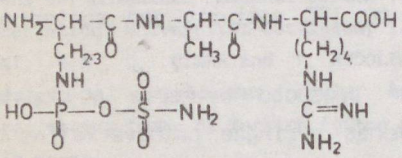
Pseudomonas syringae pv. glycinea, isolated from a soy, with the ability for formation of coronatine in submersed cultures (up to 40 mg/l). Pseudomones syringae pv. tomato, isolated from a tomato. Some isolates are able to form coronatine alone.

Used toxins

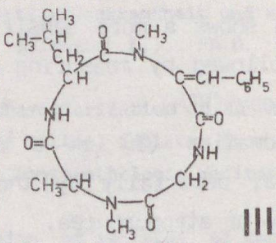
Coronatine (I) and as a comparison phaseolotoxine (II) and tentoxine (III), respectively.



I



II



III

The preliminary experiments with a coronatine-forming bacteria show a stronger reaction of L.p. against bacteria than on coronatine. Throughout the culture of L.p. shows the first distinct reaction earliest 24-36 hours after addition of I, a clear reaction was measurable 2-4 hours after mixing with bacteria. Fig. 1 shows the experiments in an overview and Fig. 2 the biochemical reaction of the cell culture after mixing with the bacteria. The measuring principle was the activity of dehydrogenases measured by a colouring reaction with triphenyltetrazoliumchloride. These dehydrogenases react very sensitively.

The used system cell culture shows some advantages because there are sterile conditions free of other biological, chemical or biochemical interactions as well as without any larger permeation problems. The experiments with the isolated toxins are summarized in Table 1.

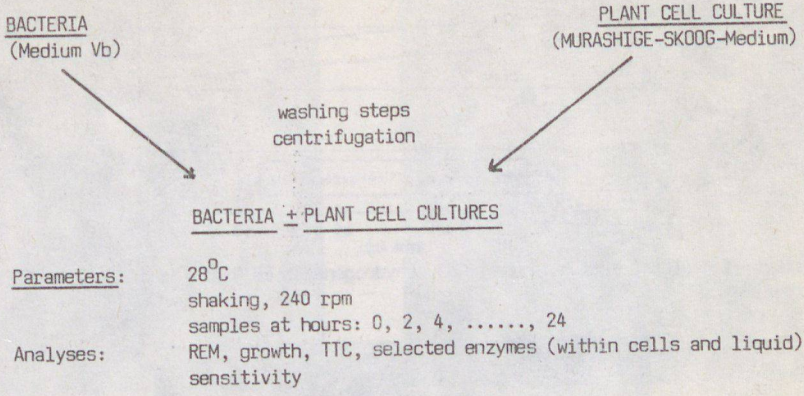


Fig. 1 Scheme for cocultivation of bacteria and PCC. All media of (PCC and mixture PCC/bacteria) are modified media after Murashige and Skoog.

Table 1 Effect of different phytotoxins on PCCs.
The results are given in the following ranges:
a) growth
b) TTC-reaction
c) protein content
d) content of free amino acids within the cells
++++, +++, ++, +: dimension of the effects found after treatment with the toxins.
-: no reaction
First significant reactions can be observed mostly on the 2nd-3rd day of cultivation with the used toxins

Used Toxins (ng/ml culture)		Plant Cell Culture L.p.	C.a.	P.v.
I	15	lethal effects a-d: ++++	a,c,d: ++ later relaxation	a,c: +++ d: ++
	7	lethal effects a-d: ++++	a: + (7th day, 10th day normal reaction) b-d: -	a,c: +++ d: ++
	0.5	a,b: +++ c,d: +		
II	800	not detected	not detected	lethal effects a-d: ++++
III	0.00032	no effects a: -		
	3170	no effects a: -		

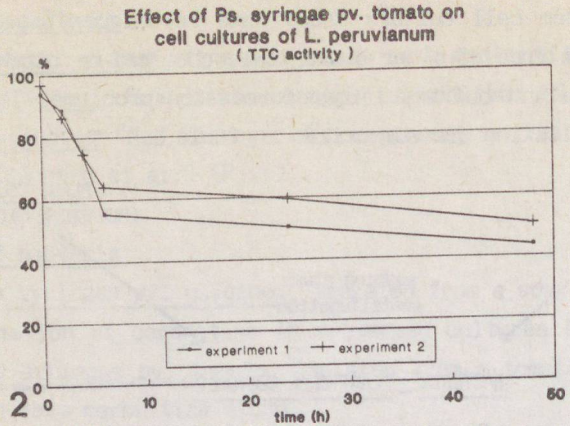


Fig. 2 Effect of bacteria on the activity of dehydrogenases as % of the control (2 different experiments).



Fig. 3-8 REM pictures of bacteria (Fig. 3), PCC alone (Fig. 4) and mixed cultures after mixing (Figs 5 and 6) and after 2 or 4 hours cocultivation (Figs 7 and 8, respectively).

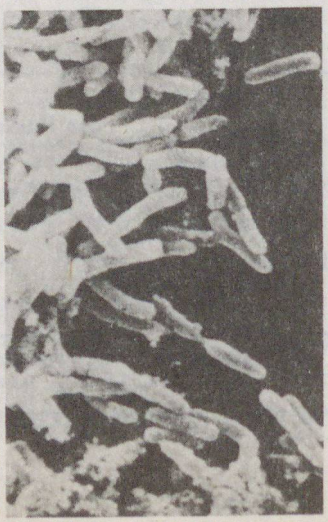


Fig. 4

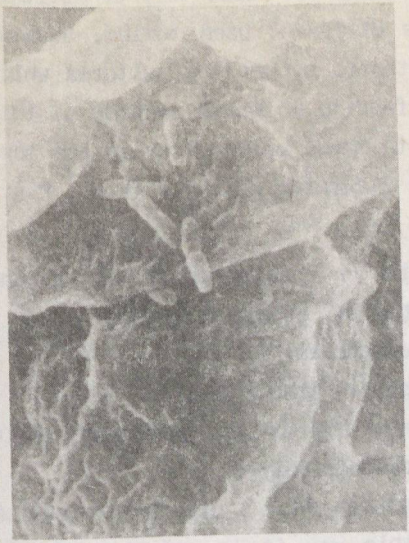


Fig. 5



Fig. 6



Fig. 7



Fig. 8

The conclusions are that the cell cultures can react very sensitively and they are able to select between the different used toxins. Because tentoxine acts primarily on the photosynthetic system the cultures which are not green cannot show any reaction by feeding of larger amounts of this toxin. The toxin I acts mere strongly with these cultures which are more related to the natural host plant of the I-forming bacteria (L.p., P.v.) than with the weed cell culture (C.a.).

Preliminary experiments were done with a cocultivation of PCC and phytopathogenic bacteria, mainly Ps. syringae pv. potato (Fig. 1).

The results show several interesting tendencies:

- The cocultivation is possible in a "short" time 2-3 days.
- The PCC react more quickly on bacteria than on the given phytotoxin coronatine: 2-12 hours or 2-3 days, respectively.
- REM (raster electron microscopy) pictures show a direct interaction between bacteria and PCC. But in this way it is not possible to detect the influence of exopolysaccharides or analogous products formed by the bacteria directly.

In conclusion powerful systems are given for estimation of working mechanisms of phytotoxins by using the methods of plant cell cultivation. The cocultivation of bacteria and plant cell cultures allows better results which can help understand the ways of interaction between bacteria and hosts. More detailed information given by Bagdache (1989), Wichetek and Schmauder (1989) and Schmauder et al. (1989).

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SESSION 2

CONTROL, BIOCONTROL

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

BIOLOGICAL CONTROL WITH BACILLUS SUBTILIS

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The resistance of a plant to disease is not a fixed status, but has to be regarded as a dynamic process, due to a permanent interaction between host, pathogen and environment. Especially factors like soil, climatic and water conditions can modify the predisposition of the plants within the limits of the genetically defined resistance potential. Besides the research and description of the influence of these factors on the resistance of plants, it seems to be useful to investigate the possibilities to shift the predisposition of the plants to a higher degree of resistance. This may offer new ways to protect plants against pathogens.

The enhancement of resistance by suitable methods is well known with terms as 'acquired resistance' or 'induced resistance'. According to our definition, induced resistance means the activation or aquirement of the resistance of plants being otherwise susceptible to the disease without changing their genetic constitution done for example by breeding or genetic engineering.

Inducing agents can be:
- preinoculations with pathogens, apathogens or symbionts
- physical treatments or the application of synthetic substances or natural metabolites

In general, resistance has to be induced before the challenge inoculation with the pathogens follows. In addition, an essential basis for the induced resistance is the lack of any direct effects of the inducing agents on the pathogens. In contrast to the toxic mode of action of fungicides which eliminate the pathogens, the target of induced resistance is the plant, stimulating their resistance mechanisms by metabolic changes. From this point of view the term biological plant protection characterizes this principle more precisely than biological control. Instead of the elimination of the pathogens the plants are stimulated to defend themselves more efficiently.

Resistance can be induced by the application of microbial metabolites. These metabolites are produced by a special *Bacillus subtilis*-isolate in submerged culture. The bacteria are propagated and cultivated in a synthetic nutrient solution. During the fermentation antibiotic or antifungal compounds are not excreted in the culture filtrate. The culture filtrate without bacteria is subsequently applied onto the leaves prior to inoculation with the pathogens. For the protection of the plants an interval of 48 hours between the application of the resistance inducer and the inoculation with the pathogens is necessary (Schönbeck et al. 1981).

The induced resistance is effective only against fungi which depend on a special kind of host-parasite relationships. The plants showed reduced susceptibility only against obligate biotrophic fungi. The development of perthotrophic fungi and bacteria was not affected. On one side the induced resistance is nonspecific being active against members of rusts, powdery and downy mildews and can be induced in several plants independent of their taxonomy.On the other side it is a remarkable attribute of the induced resistance that the effectiveness is restricted to fungi forming haustoria in their host plants.

On induced resistant barley plants the number of colonies of powdery mildew was reduced at about 50% compared with un-

treated plants, but characteristically infection was never completely prevented. This reduction of colony density is correlated with a reduced formation of primary haustoria at about 50%, but neither the germination and the germtube growth nor the appressoria formation of the powdery mildew conidia are impaired.

The mildew colonies were not only fewer, but also remained smaller. The sporulation of the colonies was reduced at about 50% based on a lower number of conidia per conidiophore and haustorium. This indicates, that the parasitic efficiency of the fungal structures was impaired during the overall colony development (Stenzel et al. 1985). Especially under practical culture conditions these aspects of incomplete resistance are of importance determining the epidemiological spread of the pathogens in the field (Dehne et al. 1984).

For practical applications induced resistance will gain significance only if plant productiveness, definitely determined as yield, is not affected. The treatments have to lower disease severity below the physiological threshold to prevent an early impairement of the plant development. The increase of resistance mechanisms should not result in yield reductions due to additional energy consuming processes curtailing the benefits of decreased infection density. Induced resistant plants have to yield as high as fungicide-treated ones.

The plant protecting effect of the induced resistance and the disease - yield loss models of these plants were examined in field trials with the winter barley cultivars 'Igri', 'Mammut' and 'Birgit' differing in disease susceptibility and productiveness.

Especially on the highly susceptible cultivar 'Igri' the powdery mildew quickly reached a very high infection level on the first leaf. In contrast 'Birgit' showed only a low infection

rate due to the adult plant resistance of this cultivar. Independent of the genotype, two fungicide treatments with Bayfidan (0,5l/ha) protected the plants almost completely. The 2 and 4 times repeated applications of the resistance inducer restricted the pathogen development less efficiently, whereby the more frequent applications gave better results. The protection effect depended on the genotype of the plants. Disease reduction was best on the partially resistant cv. 'Birgit'.

In every cultivar reduction of infection density was most efficient between shooting and end of flowering of the barley plants. Thereby induced resistance prevented early epidemics, which would affect the grain yield more substantially than infections at late growth stages.

The impact of the induced resistance on powdery mildew is not restricted to a reduction of diseased leaf area, resulting from an impaired colony growth, but especially the sporulation rate per leaf and colony area was reduced at about 90%. Additionally to the smaller losses of nutrients to the pathogens this results in only little disturbances of translocation processes during grain filling.

The reduced damaging effect of the powdery mildew on induced resistant plants also became obvious in the rate of assimilation and respiration of their leaves. They showed suitable relations between photosynthesis and respiration similar to that of fungicide treated, healthy plants.In spite of higher disease levels the senescence of the induced resistant plants appeared to be delayed.

Although applications of the inducer allowed further development of the pathogens, the grain filling of induced resistant barley was unaffected. Twofold treatments enhanced grain yield similarly as the fungicide applications. After fourfold treatments with the inducer the yields of cvs.

'Mammut' and 'Birgit' exceeded that of fungicide protected plants. This derived from an additional increase of grain weight (Table 1).

Table 1:
Effect of induced resistance with microbial metabolites and fungicide applications on grain yield of three barley cultivars (grains/ear: n=50, kernel weight: n=100).

cultivar	treatment	kernel/ ear	kernel weight	yield dt/ha		mildew EC 69*
'Igri'	untreated	17,3	42,3	63		45,4
	Bayfidan	18,1	45,4	71	112	0,7
	Inducer 2x	18,1	45,0	70	111	12,4
	Inducer 4x	18,7	44,2	71	113	10,6
	GD 0,05	0,7	1,4			
'Birgit'	untreated	31,6	37,3	58		14,2
	Bayfidan	34,2	40,0	67	116	0,9
	Inducer 2x	35,3	39,4	68	118	2,9
	Inducer 4x	35,5	41,7	73	126	0,6
	GD 0,05	1,9	2,3			
'Mammut'	untreated	34,8	38,1	68		28,6
	Bayfidan	37,5	39,5	75	112	0,3
	Inducer 2x	36,6	38,9	72	108	5,4
	Inducer 4x	37,0	43,3	81	121	4,5
	GD 0,05	2,0	1,3			

* mean value of the sporulating colony area on flag- and second leaves at end of flowering.

The yield response of induced resistant plants to disease reduction did not fulfil the yield loss models derived from investigations with fungicides. Grain yields of induced resistant plants indicate an increased tolerance to powdery mildew

(Steiner et al. 1988). Besides effects on resistance and tolerance of plants against powdery mildew the metabolites of *Bacillus subtilis* may improve the yield formation under unfavourable environmental conditions (Oerke et al. 1989).

As shown above, the consequences of biological plant protection with induced resistance are not restricted on a reduction of infection density. Indicated by the high grain yields the productiveness of plants does not depends on complete protection against the pathogens. Inducer treatments do not change only the compatibility of the host-parasite relationships, furthermore, microorganisms have the ability to produce metabolites which modify disease tolerance and physiology of yield formation.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

ANTAGONISTIC BACTERIA AGAINST PSEUDOMONAS SYRINGAE AND ERWINIA AMYLOVORA FROM WILD GROWING PLANTS

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ABSTRACT

Among 245 bacterial isolates obtained from leaves and flowers of 28 species belonging to 18 genera of wild growing plants, 18 isolates showed antagonistic activity against *Erwinia amylovora* and *Pseudomonas syringae* when tested on potato-dextrose (PDA) medium, medium with mannitol and minimal medium for genus *Bacillus*. Ten of those isolates inhibited both *E.amylovora* and *P.syringae*, five only *P.syringae* and three only *E.amylovora*. Eight of all antagonistic isolates were gram-positive, spore-forming bacilli, nine were gram-negative, fluorescent rods and one was gram-negative, non spore-forming and non fluorescent rod. The spore-forming isolate, marked as 28 HTS has been given the largest inhibition zones both to *E.amylovora* and *P.syringae* on all of the media used. When this isolate was grown in liquid minimal medium for genus *Bacillus*, it produced an extracellular substance that inhibited both pathogens to the same degree as the living cells. From the supernatant of the 28 HTS culture two active fractions were obtained; the one dissolvable in n-buthanol, ethanol and methanol, and the second, dissolvable in water and in 0.1% NaOH. The fraction dissolvable in water was dialysable and its molecular weight, resulting from gel filtration, was about 2000 D.

INTRODUCTION

Bacterial canker of stone fruits (*Pseudomonas syringae* van Hall) and fire blight (*Erwinia amylovora* Burr.Winslow et al.) are responsible for great losses in many countries of the world. Controlling them, as well as other bacterial diseases is difficult because of the lack of effective bactericides. In common usage are practically only copper compounds with their well known phytotoxic effect and antibiotics, the use of which is, however, strictly prohibited in many countries including Poland. Thus, new ways of controlling those diseases are need. For many years attempts have been made to find antagonistic bacteria to control bacterial diseases of above-ground parts of plants, especially fire blight, but with no spectacular effects [1,2,7,10,].

This report presents the preliminary results of searching for bacteria antagonistic to *E.amylovora* and *P.syringae* among epiphitic microflora of wild growing plants and for substances responsible for antagonism produced by the most active bacterial isolate.

MATERIALS AND METHODS

Isolation of bacteria

Bacteria were isolated in the spring of 1988 from young leaves or leaves and flowers of 28 plant species from different taxonomical groups by the washing and serial plate method. The samples were placed in a physiological solution enriched by a 0.1% yeast extract and 0,01% Tween 80, and shaken on an orbital incubator for 10 min. every half hour. After 4 hours the washings were seeded on Petri plates with Nutrient Agar (Difco) and Potato Dextrose Agar. To both media 0.004% of Benlate was added. After seven days of incubation in 25°C the selection of the bacterial colonies was made and from each colony of different morphology the bacteria were transferred onto nutrient agar slants.

Activity of isolated bacteria

The activity of bacterial isolates against *P.syringae* and *E.amylovora* was tested with techniques modified by Vidaver et al.[16] using standard microbiological media and a medium with mannitol as a carbon source [8] and a minimal medium for genus *Bacillus* [3]. All of the isolates that inhibited indicatory bacteria were tested several times to leave only those inhibiting *E.amylovora* and/or *P.syringae* persistently.

Identification of antagonistic isolates

Identification of isolates has been carried out to the level of a genus. Gram reaction was checked by the standard staining method [4], as well as by using a 3% KOH solution [15].To distinguish spore-forming from non spore-forming bacteria the heat test according to Bradbury [3] was performed. Fluorescence of isolates was tested on a King B medium.

Isolation of biologically active substances

Active substances were isolated from the culture of 28 HTS isolate, which caused the largest inhibition zones of *E.amylovora* and *P.syringae*. After 3 days of growth in a liquid minimal medium for genus *Bacillus* at 27°C on an orbital incubator bacteria were centrifuged. The supernatant was concentrated and three-fold extracted by n-butanol. The n-butanol extract was then vacuum-evaporated to dryness, and the residue was dissolved in methanol. The active ingredient was precipitated by citric buffer of pH 4,0. The postextracting liquid was concentrated to a small volume,separated by dialysis from high molecular extracellular substances and concentrated once more. From it the other active component was obtained by precipitation with a surplus of methanol.The activity of the two obtained substances was tested by the diffusion zone method developed by van Horn and described by Mayr-Harting [13] on the minimal medium for genus *Bacillus* and on the medium with mannitol.

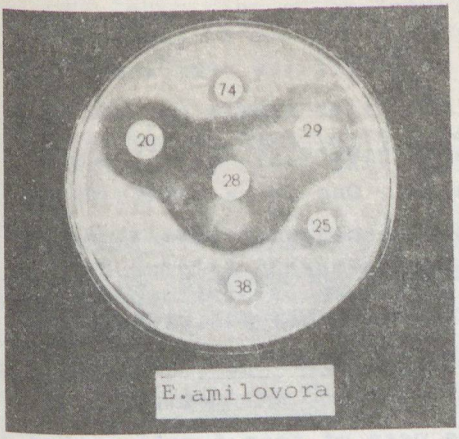
RESULTS AND DISCUSSION

During two months of the spring of 1988 245 bacterial isolates were obtained. The first screening for antagonism selected 45 isolates which more or less inhibited in vitro the growth of *E.amylovora* and/or *P.syringae*.This test was carried out on nutrient agar, and to avoid the possibility that the antagonistic effect might be due to a nonspecific mechanism, i.e.acid production from glucose, all the next tests were carried out on minimal media. Finally 18 active isolates remained, showing unchanged both in vitro activity to pathogens after numerous passages and storage (phot.1,2)

TABLE . The origin and primary characteristic of antagonistic isolates

SOURCE PLANT SPECIES	ISOLATE NUMBER	GRAM REACTION	SPORE FORMING	FLUORESCENCE	ACTIVITY AGAINST: *P.syringae* (A) *E.amylovora* (B)
1.*Berberis vulgaris*	216	-	-	+	A
2.*Betula verrucosa*	321/2	+	+	-	A, B
	14	-	-	+	B
3.*Crataegus oxycantha*	28	+	+	-	A, B
4.*Larix decidua* - - *var.polonica*	99/9	+	+	-	A, B
5.*Ficaria verna*	29	+	+	-	A, B
6.*Malus baccata*	25	-	-	+	A, B
	209	-	-	+	A, B
7.*Ribes aureus*	100	-	-	+	B
8.*Salix caprea*	23	-	-	+	B
9.*Sorbus occuparia*	6	-	-	+	B
	6/1	+	+	-	A
	6/2	+	+	-	B
	20/1	-	-	+	B
	20/2	+	+	-	A, B
	20/3/1	+	+	-	A, B
10.*Taraxaccum officinale*	212	-	-	+	A
11.unidentif. *Umbelliferae*	187	-	-	-	A

The results presented in the table show that practically all of the 18 antagonistic isolates belong to one of the two groups (*Bacillus* or fluorescent pseudomonads), commonly occurring in nature and well known as the antibiotic producers [5,9,11,12, 14]. There is no apparent correlation (except maybe *Sorbus*



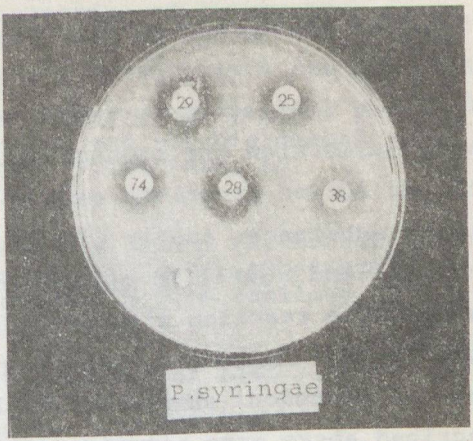
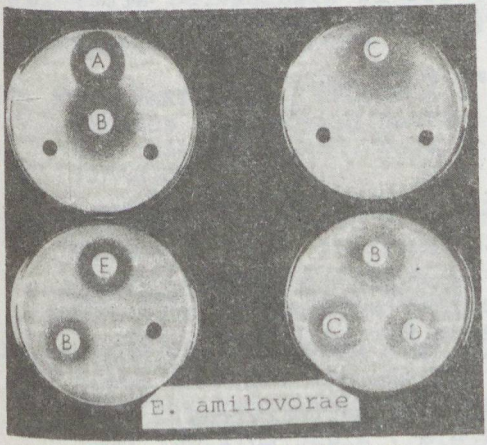


FIGURE 1 AND 2. Inhibition of growth of *Erwinia amylovora* and *Pseudomonas syringae* by 28 HTS isolate on minimal medium for genus *Bacillus*



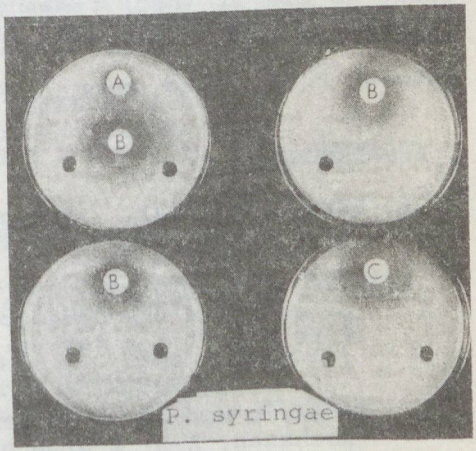


FIGURE 3 AND 4. Activity of crude supernatant of 28 HTS isolate culture (A),n-buthanol fraction (B, B_1) and fraction from postextracting liquid (C) against *Erwinia amylovora* and *Pseudomonas syringae* on minimal medium for genus *Bacillus*

occuparia) between the origin (plant species) and the number of antagonists isolated. It should also be pointed out that the epiphitic microflora on flowers and leaves in the very early stages of their development in many cases contains antagonistic microorganisms in their composition.

In liquid minimal medium for the genus *Bacillus* the 28 HTS isolate, which showed the best in vitro activity to *E.amylovora* and *P.syringae* produced extracellular substances inhibiting indicatory bacteria to a similar degree as the living cells.From the supernatant of the culture of this isolate two active fractions were obtained. One is a brown, amorphous substance, easily dissolvable in methanol,ethanol,n-butanol, and weakly in glycine buffer of pH above 9. It gives a positive reaction with ninhidrine, a weakly positive Molish test and a negative reaction with the Benedict reagent. The second fraction dissolves well in water. All reactions described above were negative in the case of this fraction. Its molecular weight is about 2000-3000 D, because separated on Sephadex G 25 it migrates between insulin and oxitocin.In the boiled crude supernatants activity of those substances (or at least of one of them) remained, but it disappeared after 10 minutes of autoclaving in 121°C at 1 atm. Both fractions inhibit *E.amylovora* more than *P.syringae*.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

BACTERIAL ANTAGONISTS TO PSEUDOMONAS SOLANACEARUM IN POTATOES AND SOME OTHER PLANT PATHOGENS

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SUMMARY

Three different antagonistic bacterial isolates have been obtained from plant tissue and were identified as Erwinia sp., Bacillus sp. 1 and Bacillus sp. 2. On King's B and 523 media the erwinia isolate had bacteriostatic effect on Pseudomonas solanacearum and Corynebacterium fascians, but no effect on two fluorescent pseudomonads, four xanthomonads, three erwinia and Agrobacterium tumefaciens. The two Bacillus isolates eliminate totally in the zone of inhibition all the tested bacteria except Xanthomonas campestris pv phaseoli var fuscans and C. fascians to which they are bacteriostatic, all this in the period of 2 days at 22°C. The two Bacillus isolates produced a fungistatic effect on Phytophthora capsici, P. citricola, P. cinnamomi, Pythium sp. Sclerotinia sclerotiorum, Fusarium oxysporum, F. culmorum, Verticillium dahliae, V. alboatrum, Rhizoctonia solani, Aspergillus niger, Sclerotium rolfsii and Macrophomina phaseolina using PDA medium. The erwinia isolate added to soil in pots, where Alpha variety potatoes and P. solanacearum were placed, prolonged the life of potato plants for 20 days on the average compared to controls. This erwinia can be conveniently preserved in distilled sterile water for more than 20 months. We conclude that these antagonistic isolates seem promising for possible control of some pathogens especially P. solanacearum, and that the two Bacillus isolates have a wide antibiotic effect to bacteria and fungi.

INTRODUCTION

In the past many bacterial isolates have been known to produce antagonistic effect to many microorganisms (1, 2, 3). Selected Agrobacterium radiobacter isolates have been used even commercially against A. tumefaciens (4).

We have obtained bacterial isolates belonging to Erwinia and Bacillus species from plant tissue and air, that are antagonistic to Pseudomonas solanacearum and some other pathogenic bacteria and fungi of agronomic importance. The antagonism was tested on two media and in the case of Erwinia sp. in pots in greenhouse sowed with potatoes. Antagonism is evident and promising.

MATERIAL AND METHODS

Erwinia sp. (E) and one Bacillus (B_1) isolates obtained from plant tissue and one Bacillus (B_2) isolate from air have been employed on King's B (BK) (5) and 523 (3) media to test their antagonism to P. solanacearum, P. syringae pv phaseolicola, P.s. pv syringae, P.s. pv helianthi P.s. pv tagetis, P. marginalis, Xanthomonas campestris pv phaseoli, X. c. pv phaseoli var fuscans, X.c. pv campestris, X.c. pv vesicatoria, Erwinia amylovora pv amylovora, E. carotovora subsp carotovora, E.c. subsp atroseptica, A. tumefaciens and Corynebacterium fascians at 22°C. The inhibition zone was measured after the antagonists growing for a period of five days (E) and two days (B_1 and B_2) respectively and then spraying the plates with bacteria.

The two Bacillus isolates were also tested on PDA medium against Phytophthora capsici, P. citricola, P. cinnamomi, Pythium sp. Sclerotinia sclerotiorum, Fusarium oxysproum, F. culmorum, Verticillium dahliae, V. albo-atrium, Rhizoctonia solani, Aspergillus niger, Sclerotium rolfsii and Macrophomina phaseolina. The Bacillus isolates were grown on PDA medium four days prior to fungal inoculations. Inhibition was observed five days after the fungi had grown on the medium.

For greenhouse work with potatoes, the erwinia isolate previously grown on PDA or BK was suspended in sterile distilled water and 100 ml of 3×10^8 cells/ml was added to a clay pot with 1 kg of soil previously sown with Alpha variety potato tuber. After 24 hrs a suspension of P. solanacearum (100 ml of 3×10^8 cells/ml) per pot was added. In another

TABLE 1. Zone of inhibition (mm) of antagonistic isolates to various bacteria in BK and 523 medium after 2 days of incubation at 22 °C.

	Erwinia		Bacillus 1		Bacillus 2	
	BK	523	BK	523	BK	523
P. solanacearum	4	4	8	8	8	8
P. syringae	0	0	0	0	0	0
P.s. phaseolicola	0	0	2	2	2	2
P.s. helianthi	0	0	0	0	0	0
P.s. tagetis	0	0	0	0	0	0
P. marginalis	0	0	0	0	0	0
X.c. pv. phaseoli	0	0	13	13	14	14
X.c. pv. phaseoli var. fuscans	0	0	15	16	16	17
X.c. campestris	0	0	15	15	15	15
X.c. vesicatoria	0	0	12	12	12	12
E. amylovora var. amylovora	0	0	9	9	9	9
E. carotovora subsp. carotovora	0	0	7	8	6	7
E. car. subsp. atroseptica	0	0	8	8	8	8
A. tumefaciens	0	0	5	5	5	5
C. fascians	5	6	12	13	13	13

TABLE 2. Effect of inhibition of two Bacillus isolates on PDA medium after 5 days of growth at 22 °C.

	Bacillus 1	Bacillus 2
Phytophthora capsici	X	X
P. citricola	X	0
P. cinnamoni	X	X
P. parasitica	X	X
Pythium sp.	X	-
Sclerotinia sclerotiorum	-	0
Fusarium oxysporum	-	X
F. culmorum	-	X
F. tricinctum	-	X
Fusarium sp.	-	0
Macrophomina phaseolina	-	-
Rhizoctonia solani	-	-
Aspergillus niger	-	-
Sclerotium rolfsii	-	-
Verticillium dahlian	-	-
V. albo-atrum	-	0

- = Inhibition total
X = Little growth
0 = Data not taken

trial potato tubers were dipped in the same erwinia suspension for 30 min and again 24 hrs afterwards P. solanacearum was added in quantities and doses as indicated previously. In both cases the pots were well watered and maintained at greenhouse temperature which fluctuated between 15°C at night and 32°C in the daytime. Five replicates of each treatment were performed and the period of observation was extended to 48 days.

The erwinia isolate was preserved in distilled sterile water for more than 20 months and left at 22°C.

RESULTS AND DISCUSSION

After a period of growth of two days the erwinia isolate produced a bacteriostatic effect on P. solanacearum and C. fascians on both media. The zone of inhibition was 4 mm and 5 to 6 mm respectively. No effect was noted on the other bacteria.

The two Bacillus isolates had marked bactericidal effect on all the tested bacteria except on X.c. pv phaseoli var fuscans and C. fascians to which they were bacteriostatic. No effect was observed against P.s. pv tagetis, P.s. pv helianthi, P.s. pv phaseolicola, P.s. pv syringae (Table 1).

The two Bacillus isolates had fungistatic effect on all the fungi tested, although some were more pronounced than others (Table 2).

The erwinia isolate prolonged the life of potato plants for 20 days on the average compared to controls. These control plants to which only P. solanacearum was added lived 27.5 days on the average, dying from wilt. When tubers were externally protected by the erwinia isolate or this was added directly to the soil, the plants lived 47.5 days on the average (Fig. 1). This means that protected plants lived 20 days more than the unprotected ones, and were able to set tubers and produce a small crop.

The erwinia has been conveniently stored in distilled sterile water for more than 20 months at 22°C.

All these results with the antagonistic isolates seem promising especially for P. solanacearum and testing needs to be extended to the field. The two Bacillus isolates have a wide antibiotic effect on bacteria and fungi.

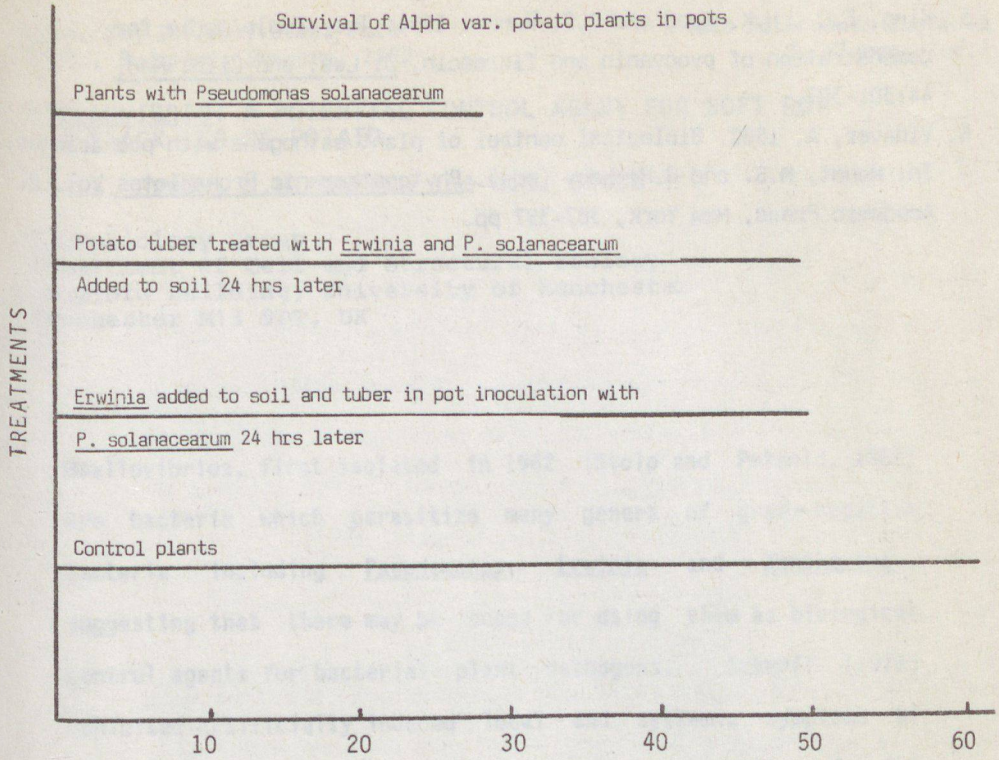


Fig. 1 Survival of plant in days

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Proc. 7th Int. Conf. Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

BDELLOVIBRIO: A POTENTIAL CONTROL AGENT FOR SOFT ROT AND BLACK LEG OF POTATO

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Bdellovibrios, first isolated in 1962 (Stolp and Petzold, 1962) are bacteria which parasitize many genera of gram-negative bacteria including Pseudomonas, Erwinia and Xanthomonas, suggesting that there may be scope for using them as biological control agents for bacterial plant pathogens. Scherff (1973) inhibited artificially induced local and systemic symptoms of bacterial blight of soybean using a ratio of 9:1 Bdellovibrio:Pseudomonas glycinea, but since then no other successful experiments have been reported.

In the present study we are attempting to control soft rot and black leg of potatoes, caused by Erwinia carotovora.

Isolates capable of attacking Erwinia have been obtained from soil in a potato crop, from soil on the bank of a stream and from water in a lake, as well as from culture collections.

Isolation of Bdellovibrio

Isolates of Bdellovibrio were obtained by filtration of the lake water or soil suspensions through a series of filters finishing with a 0.45 um methyl cellulose membrane filter. The filtrate

was mixed with a suspension of the host bacteria and suspended in molten (40°C) DNB agar (Shilo and Bruff, 1965) containing half the normal concentration of agar. This was immediately poured on to DNB agar plates with agar at the usual strength, producing a double layer plate. A lawn of host bacteria grew in the upper layer and this was inspected for plaques after 24h incubation at 25°C. Any plaques developing within 24h were marked and ignored as they were produced by bacteriophage. Plaques which developed in the following six days were examined to eliminate protozoa and those formed by Bdellovibrio were transferred to 24h cultures of host bacteria in NB-10 broth (Stolp and Petzold, 1962).

Growth in liquid culture

The double-layered agar plate technique was used to make viable counts of Bdellovibrio in liquid culture so that optimum yields could be obtained for subsequent experiments. Figure 1 shows the growth curves for Bdellovibrio sp. strain Bd.7 and its host bacterium Erwinia carotovora subsp. atroseptica, which had been growing for 24h in NB-10 in a shaken batch culture prior to inoculation with Bd.7.

Screen for control of black leg

To investigate the ability of Bdellovibrio isolates to control potato black leg, a screen was set up using potato leaflets (method devised by Jane Chard, University of Edinburgh, publication in preparation). For these experiments the Bdellovibrio strains were grown for 24h using Erwinia herbicola as the host bacterium, as this is not a pathogen of potatoes.

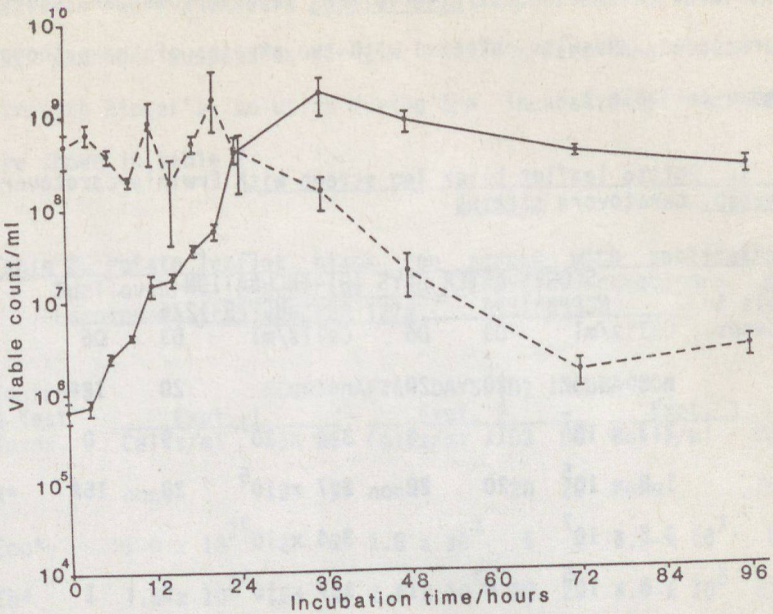


Figure 1. Growth of Bdellovibrio strain Bd.7 on Erwinia carotovora subsp. carotovora in NB-10 broth. Each point is a mean of 9 counts. Vertical bars represent 2 x S.E.

Strains of Erwinia carotovora subsp. carotovora and Erwinia carotovora subsp. atroseptica were screened for their ability to induce black leg-like symptoms in the excised potato leaflets, and two strains of Erwinia carotovora subsp. carotovora were selected as causing the most severe symptoms. Controls consisted of 1/4 strength Ringer's solution, the pathogen, Erwinia herbicola and Erwinia herbicola + pathogen.

To screen each Bdellovibrio strain, ten replicates were set up by placing 0.5 ml of a 10^{-1} dilution of a 24h Bdellovibrio/E.herbicola culture in a sterile Universal bottle, to which 0.5ml of Erwinia carotovora subsp. carotovora was added. Cell numbers were adjusted so that in most experiments a viable cell ratio of 10:1 Bdellovibrio:pathogen was used. After 2 days

leaflets were examined daily for black leg symptoms and scores were recorded. Results obtained with two strains of the pathogen are shown in Table 1.

Table 1. Potato leaflet black leg screen with Erwinia carotovora subsp. carotovora strains

Controls & Test Susps.	SCORE* AFTER DAYS (D) INCUBATION NCPPB*1236 Cells/ml	D3	D6	NCPPB 1276 Cells/ml	D3	D6
R*	none	20	20	none	20	12#
Ecc*	2.3×10^7	11	5	3.4×10^7	9	0
Eh*	1.8×10^5	20	20	3.7×10^5	20	16#
Ecc +	2.3×10^7			3.4×10^7		
Eh	1.8×10^5	8	1	3.7×10^5	9	1
Bd* 2HE	2.7×10^8	14	5	5.6×10^7	11	0
Bd 7	2.4×10^8	15	10	1.6×10^8	11	2
Bd 12	1.5×10^8	10	4	lost	11	1
Bd SP4	1.7×10^8	15	11	2.4×10^8	15	1
Bd. stolpii DSM* 50722	1.8×10^8	17	14	1.9×10^8	14	6

* Scores shown are cumulative scores from 10 leaflets in each treatment: 2 = no black leg symptoms; 1 = decay of petiole and lower leaf tissue, but at least half of leaflet unaffected; 0 = more than half leaflet affected.

R = 1/4 strength Ringer's solution
Ecc = Erwinia carotovora subsp. carotovora
Eh = Erwinia herbicola
Bd = Bdellovibrio strain or species
NCPPB = National Collection of Plant Pathogenic Bacteria UK
DSM = German Culture Collection of Micro-organisms
# Black leg-like symptoms occurred on some Ringer and E. herbicola controls but organisms isolated did not degrade pectate.

It was recognised that pathogen populations may have been reduced by parasitism by Bdellovibrio in the inoculating fluid before leaflet infection occurred, so in a second set of experiments the

leaflets were placed in Bdellovibrio suspensions for 24h, then in the pathogen suspension for 24h. Leaflets were then placed in 1/4 strength Ringer's solution during the incubation period. Results are shown in table 2.

Table 2. Potato leaflet black leg screen with application of Bdellovibrio 24h prior to Erwinia carotovora subsp. carotovora strain NCPPB 1276.

Controls & Test Susps.	SCORE* AFTER DAYS (D) INCUBATION Expt. 1			Expt. 2			Expt. 3	
	Cells/ml	D3	D6	Cells/ml	D3	D6	Cells/ml	D2
R*	none	19	9#	none	20	20	none	20
Ecc*	1.0×10^7	2	0	1.8×10^7	5	0	1.1×10^7	0
Eh*	1.6×10^5	19	8#	2.8×10^5	20	20	1.9×10^5	20
Ecc +	1.0×10^7			1.8×10^7			1.1×10^7	
Eh	1.6×10^5	2	1	2.8×10^5	9	8	1.9×10^5	0
Bd*7	1.1×10^8	18	4	1.4×10^8	20	20	1.1×10^8	0
Bd.stolpii	0.8×10^8	16	0	1.6×10^8	20	20	1.0×10^8	0

Footnotes as Table 1.

Screen for control of soft rot

Considerable variability has been experienced in experiments on the control of soft rot, particularly with reproducibility of results from controls, and there are so far no reliable data to suggest that Bdellovibrio is capable of controlling potato soft rot under experimental conditions, although experiments are continuing with the intention of improving the screening technique.

Discussion

Problems of reproducibilty were encountered in both the screens for control of soft rot and black leg, probably due to physiological variation in the plant material, and particularly variation in leaflet age in the black leg screen. However, the positive results obtained in some experiments suggest that control of black leg using Bdellovibrio may be possible and a new screen based on the use of stem cuttings may yield more consistent results.

Acknowledgements

We are grateful for financial support from the Agricultural Genetics Company, Cambridge, U.K., and the award of a Studentship to N.M.W. by the Science & Engineering Research Council.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

ABILITY OF KASUMIN TO CONTROL SOME PHYTOPATHOGENIC BACTERIA

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Only few chemicals are available for the control of bacterial plant diseases. Bordeaux Mixture and related copper formulations are frequently recommended despite their moderate efficacy. Antibiotics are generally not allowed to be used as pesticides although they have a quite low minimum inhibitory concentration (MIC) to phytopathogenic bacteria. Because most of them are used for therapy in human diseases and because transferable plasmids coding for resistance to these antibiotics have been detected in plant associated bacteria there will never be a general use of them in plant protection.

Kasugamycin is also an antibiotic ; it is produced by Streptomyces kasugaensis ; its use is restricted to agriculture and no plasmid coding for resistance to Kasugamycin has been yet discovered.

Kasugamycin has been shown to be highly effective to control some fungal (rice blast) and bacterial diseases (mainly incited by Pseudomonas spp).

Minimum inhibitory concentrations of Kasugamycin to some Pseudomonas species were demonstrated to vary between 25 and 50 ppm.

In order to know if Kasugamycin could be useful to control bacterial diseases occurring in France experiments were performed with Kasumin liquid which contains 2 % v/v Kasugamycin.

Kasumin was tested for its ability to reduce epiphytic populations of pathovars of P. syringae responsible for plant diseases or involved in frost injury. Moreover Kasumin was applied in field in early spring on grapes to control the leaf stage infection of Xanthomonas ampelina Panagopoulos.

MATERIAL AND METHODS

I - Monitoring epiphytic populations of P. syringae pathovars

Experiments were performed with 2 syringae pathovars :

. pathovar persicae which is the causal agent of a severe dieback of peaches in France.

. pathovar syringae which causes bud necroses, leaf spot and twig canker on kiwis.

. pathovar syringae again involved in frost damages on grape buds in spring.

Young plants (with 10 to 20 leaves) of each host were selected for experiments in climatic chamber in which temperature was kept at 20°C and relative humidity at about 85 %.

Bacterial suspension (10^8 CFU/ml) of each strain was sprayed on homologous host to get the establishment of epiphytic inoculum on young leaves.

One week later Kasumin (1 or 0.5 g/l Kasugamycin) was sprayed on a block of plants. Besides Bordeaux Mixture (1.25 g/l Copper) and Mycoshield (1 g/l Oxytetracylin) were also applied on 2 other blocks of plants as references and water was sprayed on a last block as control.

Just before the chemicals spraying one sample of 30 leaves was collected for each block to get an estimation of the initial epiphytic population.

Bacterial populations were estimated regularly at 1, 4, 7, 10 and 15 days after the chemical spraying for peaches and kiwis and at 1, 3, 6 and 9 days after for grapes.

For each estimation each 30-leaf sample was ground in a blendor (peaches and kiwis) or washed for an hour in a reciprocal shaker (grapes). Serial dilutions of the obtained suspensions were then plated on nutrient agar and plates were incubated for 3 days at 16°C.

II - Control of leaf stage infection of X. ampelina on grapes

In this field experiment Kasumin (0.5 g/l Kasugamycin) was compared to Bordeaux Mixture (0.4 g/l Copper) and to a control.

5 plots of 10 plants were determined for each combination. Experiment started when grapes were at the stage F (3 leaves).

Five combinations were compared : water, Kasumin 0.5 g/l (one and two sprayings), Bordeaux Mixture 0.4 g/l or 0.2 g/l (one and three sprayings).

The second spraying of Kasumin and Bordeaux Mixture was applied 10 days after the first one and the third spraying of Bordeaux Mixture 10 days after the second one.

Bacterial inoculum (10^8 CFU/ml) was sprayed on all the plots one day after the first spraying of chemicals.

Notations of the percentage of infected leaves were done after a 6 to 7 weeks' incubation period.

RESULTS

I - Ability of Kasumin to reduce P. syr. populations

1) on peach leaves (fig. 1)

Compared to the control, epiphytic populations of pv. persicae decreased rapidly (2 log lower at day 2) within the first 2 days for both Kasumin and Bordeaux Mixture. Despite a weak increase between days 4 and 8, the populations on the plants treated with Kasumin at both concentrations remained at a low level until the end of the experiment. Kasumin appeared to be more effective than copper sulfate or Oxytetracyclin to reduce epiphytic populations of P. syr. pv. persicae.

2) on kiwi leaves (fig. 2)

Initial inoculum was particularly high in this experiment (about 10^8 CFU/leaf). Within 4 days, Kasumin (1 g/l) caused a significant reduction of the bacterial populations which remained at about 10^6 CFU per leaf until the 2-week period. Copper sulfate and Oxytetracyclin showed no or only a week effect.

3) on grapes (fig. 3)

A strong reduction of pv. syringae populations was also observed 1 day after the spraying of Kasugamycin copper sulfate and Oxytetracyclin. For the nine next days the populations on Kasugamycin (1 g/l) treated leaves remained at this low level. Reduction was less with the lowest concentration of Kasugamycin and with Oxytetracyclin. Copper sulfate showed only a moderate activity.

Figure 1

EFFECT OF KASUMIN ON THE DYNAMICS OF PATHOGENIC POPULATIONS ON PEACH LEAVES

(Pseudomonas syringae pv.persicae)

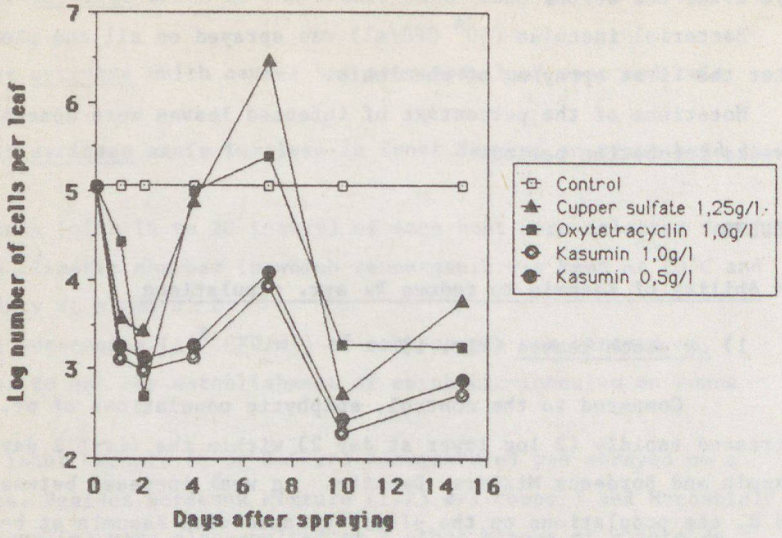


Figure 2

EFFECT OF KASUMIN ON THE DYNAMICS OF PATHOGENIC POPULATIONS ON KIWI LEAVES

(Pseudomonas syringae pv.syringae)

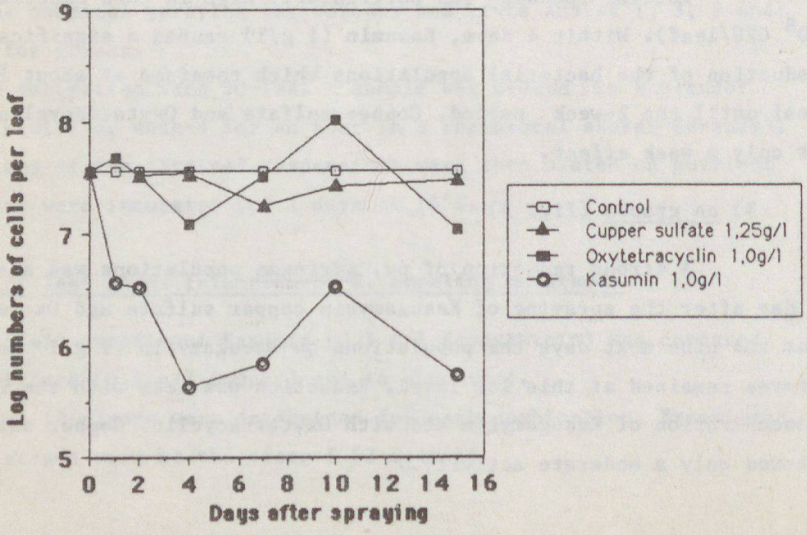


Figure 3

EFFECT OF KASUMIN ON THE DYNAMICS OF INA+ POPULATIONS ON GRAPE LEAVES

(Pseudomonas syringae pv.syringae)

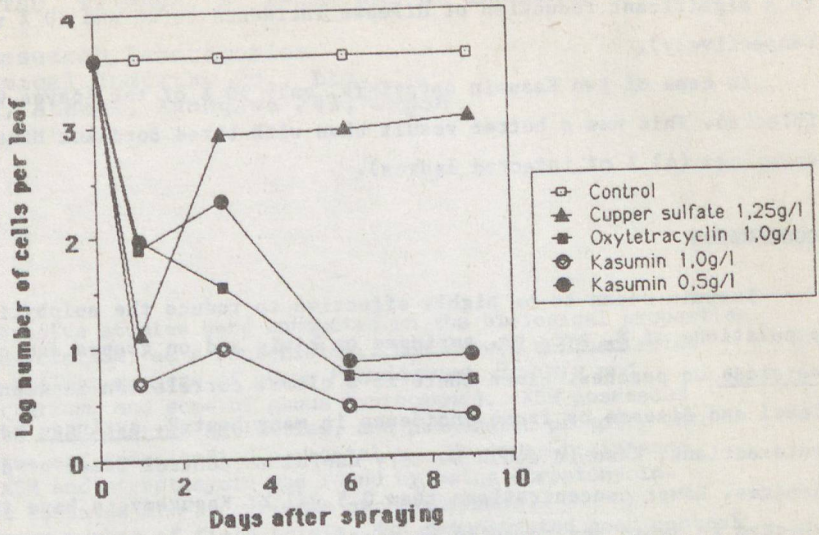
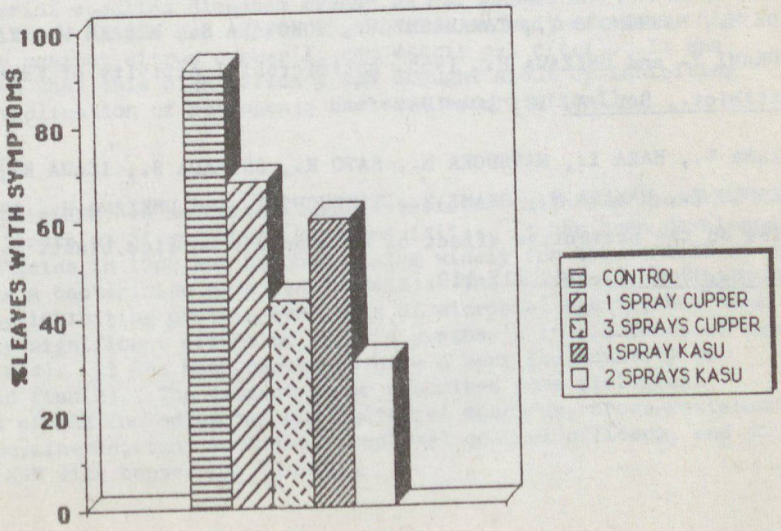


Figure 4

EFFECT OF KASUMIN SPRAYINGS ON GRAPE BACTERIAL "CANKER"

(Xylophilus ampelinus)



II - Control of X. ampelina on grapes (fig. 4)

More than 90 per cent of the leaves of the control showed typical symptoms of the disease.

One spraying of Kasumin (0.5 g/l) or Bordeaux Mixture (0.4 g/l) led to a significant reduction of disease incidence (a 20 and 10 % reduction respectively).

In case of two Kasumin sprayings, only 30 % of the leaves were infected. This was a better result than with three Bordeaux Mixture sprayings (43 % of infected leaves).

CONCLUSION

Kasumin seems to be highly effective to reduce the epiphytic populations of P. syr. pv. syringae on kiwis and on grapes and of pv. persicae on peaches. Since there is a close correlation between inoculum level and disease or frost incidence in many host-P. syringae pathovar interactions, Kasumin could be very useful to control frost or disease damages. Lower concentrations than 0.5 g/l of Kasugamycin have to be checked to avoid any toxicity on plants.

Kasumin is also very active against Xanthomonas ampelina and insures a good protection of grapes when 2 sprayings are applied. Reduced concentrations could be tested for the same reason as above.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

EFFECT OF KASUGAMYCIN ON THE CONTROL OF BACTERIAL DISEASES

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ABSTRACT

Comprehensive studies were conducted on the biological properties of Kasugamycin(KSM) as a bactericide. KSM showed antibacterial activity against members of genus *Pseudomonas*, *Erwinia* and *Corynebacterium*, and some of genus *Xanthomonas*. KSM possessed preventive and curative activities, and penetrated promptly into leaf tissue and translocated acropetally. No cross-resistance between KSM and streptomycin was found by using streptomycin-resistant isolates of *Ps. syringae* pv. *lachrymans*.

In a large number of field trials, KSM demonstrated good control against bacterial canker(*Ps. syringae* pv. *morsprunorum*) and bacterial blossom rot(*Ps.* spp.) of kiwifruit, angular leaf spot (*Ps. syringae* pv. *lachrymans*) of cucumber, bacterial grain rot (*Ps. glumae*) of rice and fire blight(*E. amylovora*) of cotoneaster at low dosages(20-75 g a.i./ha) of foliar application. KSM also was able to be used with soil incorporation. Namely, by incorporating KSM into the nursery soil of rice at a rate of 1.2 g a.i. per 10 liters, it showed outstanding effect for the control of bacterial seedling diseases caused by *Ps. glumae* and *Ps. avenae*.

The mixture of KSM and copper oxychloride showed remarkably high efficacy against citrus canker(*X. campestris* pv. *citri*). It was confirmed that this high efficacy was brought about by inhibiting the multiplication of pathogenic bacteria synergistically.

INTRODUCTION

Kasugamycin(KSM) is an agricultural antibiotic which was isolated from culture filtrate of *Streptomyces kasugaensis*(1). It has been developed as a rice blasticide in 1965 and has been using widely for the control of plant diseases as a bactericide or a fungicide(3). The antibiotic exhibits its activity by inhibiting protein synthesis of microbial agents, but does not exhibit any significant effect on protein synthesis in ribosomes of rat liver cells(4). It has been proved to have a very low toxicity to mammals and fish(2). The present paper describes some biological properties of KSM including the antibacterial spectrum, cross-resistance to streptomycin-resistant isolates, practical control efficacy, and joint action of KSM with copper oxychloride.

MATERIALS AND METHODS

In vitro activity: The antibacterial activity of KSM against phytopathogenic bacteria including streptomycin(SM)-resistant isolates was tested on Noken agar medium impregnated with the antibiotic. After incubation at 28°C for 2 days, bacterial growth was observed and minimum inhibitory concentration (MIC) was determined.

Glasshouse test: The preventive and curative foliar activities were tested by spraying KSM solution on cucumber seedlings (cv. suyo) 1 day before and after inoculation with bacterial cell suspension (10^9 cells/ml) of *Ps. syringae* pv. *lachrymans*. The degree of infection was assessed by counting the number of lesions presented on each leaf, and the results were expressed as percent control.

The systemic activity of KSM against cucumber angular leaf spot was evaluated by a bioassay. For root application, the roots of cucumber seedlings were placed into nutrient solution containing the antibiotic. To assay local systemic activity in leaf tissue, KSM was applied on the proximal or the distal half of 3rd true leaf. One day after treatment, plants were inoculated with cell suspension of *Ps. syringae* pv. *lachrymans* on the abaxial surface of leaves by spraying. The number of lesions on the non-treated parts of each leaf was counted 10 days after inoculation.

Soil incorporation was conducted by applying the granule formulation of KSM (2% a.i.) into the nursery soil of rice. The infected seeds which had been artificially inoculated by steeping into bacterial cell suspension of *Ps. glumae* and *Ps. avenae*, respectively, were sown in nursery box (30x60x3 cm). Ten days later, the percent infected seedlings were determined by sampling about 500 seedlings from each box.

Field trial: A randomized block design was used. Three plants of kiwifruit(cv. Hayward) were applied three times with 2000-3000 l/ha of the antibiotics by a power sprayer. Control efficacy was assessed by counting the number of infected leaves and blossoms after 3rd application.

Joint action of KSM with copper oxychloride(COC):
Each chemical was applied on citrus seedlings (cv. Natsudaidai). One day later, leaves were inoculated by pin-prick method with cell suspension of SM-resistant strain V9-1 (MIC >1000 µg/ml) of *X. campestris* pv. *citri* at a concentration of 10^3 cells /ml. Leaf disks of 7 mm in diameter, including the inoculated points, were punched out from the leaves periodically until 20 days after inoculation. The disks were rinsed with tap water and were homogenized in distilled water. The number of bacterial cells in homogenate was determined by counting the number of colonies formed on potato sucrose agar medium containing SM at a concentration of 500 µg/ml by agar dilution method.

RESULTS AND DISCUSSIONS

In vitro activity

KSM demonstrated a high level of good activity against members of genus *Pseudomonas*, *Erwinia* and *Agrobacterium*, but weak activity against genus *Xanthomonas* and *Corynebacterium* (Table 1). On the other hand, KSM was equally effective against SM-resistant or -sensitive isolates of *Ps. syringae* pv. *lachrymans* which were isolated from diseased plants (Table 2). Sato (1983) reported that no cross-resistance between KSM and other aminoglycoside antibiotics including SM, kanamycin and chloramphenicol was found by using KSM-resistant mutants of several phytopathogenic bacteria which were selected from a successive liquid shake culture containing KSM(5).

Preventive and curative activities

KSM demonstrated good preventive and curative activities against cucumber angular leaf spot at a concentration of 50 µg/ml.

Table 1. Antibacterial activity of KSM against phytopathogenic bacteria

Bacteria		MIC (µg/ml)
Agrobacterium	*tumefaciens*	31.3
Corynebacterium	*michiganense* pv. *michiganense*	> 100
Erwinia	*carotovora* subsp. *carotovora*	50
Pseudomonas	*avenae*	25
Ps.	*marginalis* pv. *marginalis*	25
Ps.	*syringae* pv. *lachrymans*	25
Ps.	*syringae* pv. *mori*	25
Ps.	*syringae* pv. *morsprunorum*	25
Ps.	*syringae* pv. *phaseolicola*	31.3
Ps.	*syringae* pv. *eriobotryae*	31.3
Ps.	*syringae* pv. *tabaci*	50
Ps.	*glumae*	100
Ps.	*syringae* pv. *striafaciens*	> 100
Ps.	*solanacearum*	> 100
Xanthomonas	*campestris* pv. *citri*	> 100
X.	*campestris* pv. *cucurbitae*	> 100
X.	*campestris* pv. *oryzae*	> 100
X.	*campestris* pv. *phaseoli*	> 100
X.	*campestris* pv. *pruni*	

Table 2. Antibacterial activity of KSM against streptomycin sensitive and resistant isolates of *Ps. syringae* pv. *lachrymans*

Isolate	MIC (µg/ml)	
	Streptomycin	KSM
Saitama	< 7.8	25
Nodai - 7432	< 7.8	25
Nodai - 7110	125	25
Nodai - 7313	500	25
Kurume	> 1000	25
Nodai - 7413	> 1000	50

Uptake and movement

KSM when applied as a root dipping on cucumber seedlings was translocated systemically, and provided excellent disease control to foliage. In a test that KSM was applied at the proximal or the distal half parts of young cucumber leaves at 100 mg a.i. per liter, the molecule movement within the leaf provided very excellent protection to the upper distal untreated part and moderate protection to the proximal part (Table 3). In rainfastness studies, 100 mg a.i. per liter application of KSM showed excellent activity when 20 mm of precipitation was made 6 hours after KSM treatment.

Soil incorporation

Recently, a serious occurrence of bacterial seedling rot caused by *Ps. glumae* and bacterial brown stripe caused by *Ps. avenae* of rice were reported in a large area of Japan as a result of introducing the nursery box combined with mechanized seedling transplanting. These diseases appear 4-10 days after sowing with symptoms of yellowing and rotting on seedlings. KSM when applied as a soil incorporation with granule formulation exhibited excellent control against both diseases at a dosage of 1.2 g a.i. per 10 liters of soil without any rice injury (Table 4).

Table 3. Systemic activity of KSM against cucumber angular leaf spot as shown by percent reduction of number of lesions per leaf

Concn. (mg a.i. / liter)	Root application		Foliar application: Leaf treated proximally : infection on distal half	Foliar application: Leaf treated distally : infection on proximal half
	24*	72*	24*	24*
100	-	-	99	73
10	93	100	-	-
1	38	87	-	-
0	(78)**	(78)**	(32)***	(41)***

* Period(hour) from the time of application to inoculation.
** The number of lesions per 3rd leaf on untreated plant.
*** The number of lesions on proximal and/or distal half leaf of untreated.

Table 4. Effect of KSM on the control of bacterial seedling rot(*Ps. glumae*) and bacterial brown stripe(*Ps. avenae*) of rice by soil incorporation

Treatment	g a.i./ 10 lit. of soil	% disease control: Seedling rot	% disease control: Brown stripe*
KSM	1.2	97	99
	0.6	95	-
Untreated	-	(87.4)**	(40.2)**

* Test result at Niigata Agricultural Experiment Station in 1984(6).
** % infected seedlings.

Foliar application

Bacterial canker caused by *Ps. syringae* pv. *morusprunorum* and bacterial blossom rot caused by *Ps.* spp. of kiwifruit have been prevailing in main cultivated areas in Japan. The activity of KSM against these bacterial diseases was evaluated at Shizuoka Citrus Experiment Station and Nagasaki Fruit Tree Research Station in 1986(7,8). KSM exhibited good efficacy against both diseases at 5 g a.i./hl (Table 5). No phytotoxicity was noted on fruits and leaves.

KSM demonstrated good efficacy against many other bacterial diseases in field trials. Some of these are summarized in Table 6.

Table 5. Effect of KSM on the control of bacterial canker (*Ps. syringae* pv. *morsprunorum*) and bacterial blossom rot (*Ps.* spp.) of kiwifruit by foliar application

Treatment	g.a.i/hl	% disease control*		
		Bacterial canker Leaf	Bacterial blossom rot Blossom	Bud
KSM	5	90	82	74
Streptomycin	20	89	-	-
Untreated	-	(60.3)**	(11.7)**	(25.6)**

* Application : 3 times with the following intervals canker 11-12 days, blossom 18-19 days.
** % infected leaves, blossoms and buds.

Table 6. Bacterial diseases effectively controlled by KSM

Crop	Disease	Rate(g a.i.)	Application
Rice	Bacterial grain rot (*Ps. glumae*)	50-75/ha	Foliar
Kidney bean	Halo blight (*Ps. syringae* pv. *phaseolicola*)	30-40/ha	Foliar
Cucumber	Angular leaf spot (*Ps. syringae* pv. *lachrymans*)	30-60/ha	Foliar
Tomato	Bacterial canker (*C. michiganense* pv. *michiganense*)	30-60/ha	Foliar
Carrot, Potato	Bacterial soft rot (*E. carotovora* subsp. *carotovora*)	2/hl	Dipping
Cotoneaster	Fire blight (*E. amylovora*)	15-30/hl	Foliar

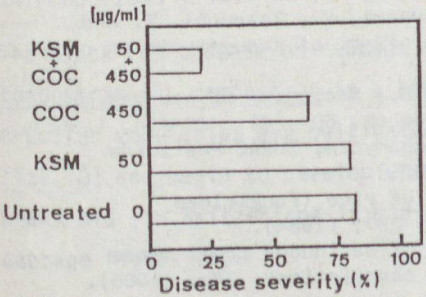


Fig 1. Effect of the mixture of KSM and COC on the control of citrus canker.

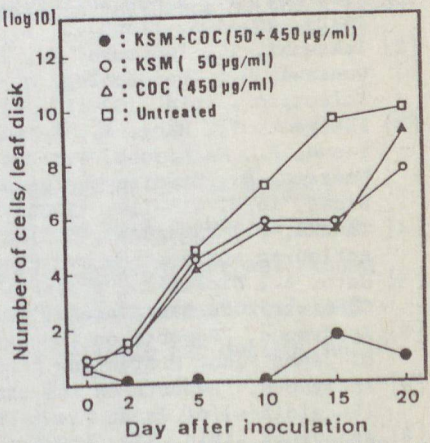


Fig 2. Effect of the mixture of KSM and COC on the multiplication of *X. campestris* pv. *citri* in leaf tissue.

Joint action of KSM with copper oxychloride(COC)

In order to complement the curative and systemic properties of KSM with protectant action and to reduce the risk of KSM resistance development, the mixture of KSM and COC was developed. The levels of disease severity provided by the mixed application of KSM with COC were clearly lower than that of single application of each component when applied to control citrus canker(Fig. 1).

The mechanisms of its high efficacy obtained by mixing KSM with COC were elucidated by assessing the multiplication of *X. campestris* pv. *citri* in citrus leaf after application. The multiplication of bacterial cells in leaf tissue was outstandingly suppressed in comparison with single application of each component(Fig. 2). This result has indicated that the high efficacy obtained by combining KSM with COC was brought about by inhibiting the multiplication of the pathogenic bacteria synergistically.

CONCLUSIONS

1. Kasugamycin has a wide spectrum against plant pathogenic bacteria, and shows good efficacy for the control of economically important diseases caused by members of genus *Pseudomonas*, *Erwinia* and *Corynebacterium*, and some of genus *Xanthomonas*.

2. The mixture of kasugamycin and copper oxychloride is more effective than each component used alone, and will be recommended from the view of reducing the development of kasugamycin-resistance.

3. No cross-resistance between Kasugamycin and streptomycin has been found by using streptomycin-resistant isolates of *Ps. syringae* pv. *lachrymans*.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

BLACK ROT DISEASE ON CABBAGE, IRRIGATION AND SPREADING

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ABSTRACT

Crop losses caused by black rot disease (bacterium Xanthomonas campestris pv. campestris) are increasing. The disease can destroy whole field yields. The pathogen can survive in the soil, on crucifer weeds and can be transmitted by seeds.

Often the infection way is uncertain and after a successful infection, the conditions of the spreading in a field are uncertain.

The influence of the water irrigation systems on the spreading behaviour of black rot disease in a cabbage field was investigated. Wetting of cabbage leaves favored the development of the disease to a considerable degree. The wind direction had only a subordinate influence on the spreading of the disease.

INTRODUCTION

The black rot disease on cabbage caused by the bacterium Xanthomonas campestris pv. campestris is a harmful disease in most countries where crucifer vegetables are cultivated. The disease symptoms are well-known (1,2,10) and begin to develop when the cabbage plants are about 7 to 8 weeks old (in Switzerland in mid July). The disease can destroy entire cabbage fields (4).

There are different ways for the pathogen X.c. pv. campestris to survive: 1) overwinter in the soil, especially where diseased plant material has been left in the field; overwinter on crucifer weeds growing near cabbage fields; 3) the pathogen can be transmitted by seeds (3).

The possibilities to control bacterial diseases are limited. This situation is exacerbated by the fact that the actual way of infection is often insufficiently known (5). According to current knowledge a set of not fully understood conditions must coincide to allow the disease to break out (6).

Therefore, the extension of the black rot disease under different environmental conditions was investigated in cabbage fields. Particular interest was placed in the question whether the irrigation system and the predominant wind direction were important factors affecting the spreading of the black rot disease in the cabbage field. Several experiments were carried out. An example is given below (5,7).

MATERIALS AND METHODS

Seeds of cabbage (Wirsing, Brassica oleracea L. var. sabauda L.) were sown out in sterile compost. After 10 days the seedlings were transferred to sterile compost in plastic pots (diameter 9 cm). At the 6-to 10-leave stage the plants were put outdoor to adapt to outside conditions. After 25 days they were arranged in three groups where they were exposed to different growing conditions.

The first group was set up in a foil greenhouse. The plants were irrigated once a week with a watering can, wetting of the leaves was carefully avoided. The second group was set up in the field thus exposed to ambient weather conditions. In the event of long dry periods the plants were irrigated like those in group 1. The third group was treated like group 2, but sprinkled every second day, whenever there was no natural rainfall. Every plot was divided into four sectors according to wind directions. As a source of infection with X.c. pv. campestris two infected plants were put in the centre of every test plot (Figure 1). The pathogen material consisted of a mixture of 4 strains (529, 541, 590, 595) of our bacteria collection, all isolated from Swiss cabbage cultures.

Plants acting as the source of infection in the field were deliberately infected prior to planting. This was made by injecting 0.1 ml of a bacteria suspension of 10^8 cells/ml directly into the xylem vessels (9). In addition the edges of three leaves of every infection plant were cut at the end of the vessels and dipped 5 minutes into a bacterial suspension of 10^8 cells/ml.

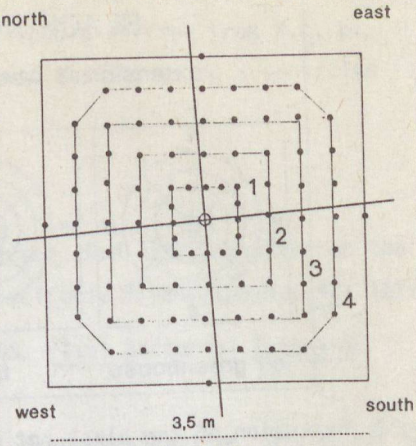


Figure 1.

Layout of a test plot in the experimental field

Legend:

o Infection source

. Test plants

1: Distance class 1 0.35 - 0.5 m distance to the source of infection

2: Distance class 2 0.7 - 1.0 m distance to the source of infection

3: Distance class 3 1.15 - 1.5 m distance to the source of infection

4: Distance class 4 1.4 - 1.8 m distance to the source of infection

In the field experiment samples of cabbage leaves were collected at random at 7 days intervals. The first sample was collected 7 days after the initial deliberately infested exposure of the infected plants. Samples were taken from all sectors of each plot.

The amount of X.c. pv. campestris on the surfaces of the cabbage plants was monitored by isolating the bacteria (2,8) during the whole experiment.

RESULTS

In group 1 (foil greenhouse) no symptoms were observed at all, despite the presence of a source of infection. Towards the end of the experiment all samples showed high numbers of bacteria. In the foil greenhouse latent bacteria were found frequently.

In group 2 (outdoors, no additional irrigation) first symptoms were observed at day 52 in the distance class 1. At day 62, the disease was observed in the distance classes 2 and 3, both in the east sector.

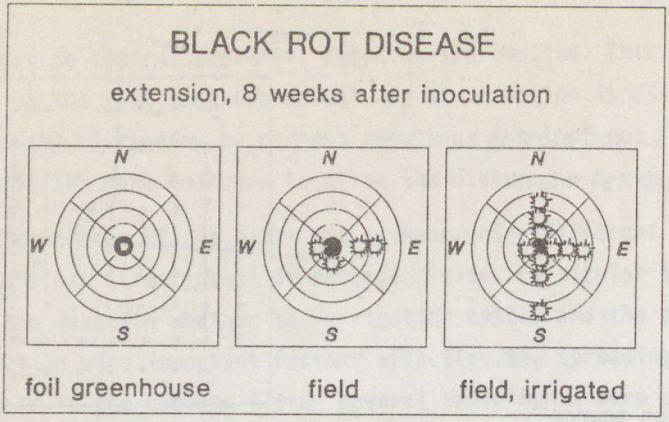


Figure 2: Extension of the black rot on cabbage under different conditions

In group 3 (outdoors, additional irrigation) the first symptoms were already found 26 days after planting (north sector, distance class 2). The percentage of plants showing clear disease symptoms increased to 20% after day 62 in all distance classes in the sectors north, east and south. The increase of newly diseased plants occurred in a rather unsteady way. Wind direction seemed to have only a limited significance in the spreading of the disease (no extention to the west) (Figure 2).

DISCUSSION

Like in the cases of many other bacterial plant diseases the development of black rot in cabbage seems to be strongly influenced by wet leaf periods. Nevertheless the magnitude of their effect was surprising. Contrary to our expectations the wind direction was not significant for the spreading of the disease.

In recent years, the international seed trade has grown steadily, a fact which is of increasing significance for the transmission of bacterial diseases. A certain pathogen can be an endemic organism of little significance in one place. After transfer to other regions or countries, where the pathogen encounters more favorable living conditions and susceptible host varieties it can cause a significant disease. As a result of such an unintentional introduction of a pathogen heavy losses

may occur. Cabbage seeds for example should be free from X.c. pv. campestris according to laboratory test results using a sensitive internationally agreed method.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

DISEASE RISK ASSESSMENT: CHANGES TO BILLING'S SYSTEM FOR FIRE BLIGHT

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ABSTRACT

Experience showed that Billing's original fire blight risk assessment system (BOS) could be improved. The revised system (BRS), described in outline, is simpler, more logical and more precise. A mnemonic, SIRDOw-TIPSuOf is included as a reminder of factors that must be considered when assessing risks (spread of inoculum, infection risks, disease development rates, overwintering risks, target numbers, inoculum potential, host susceptibility and other factors). BRS gives useful guidance on fire blight risks and goes further than other systems. A similar approach to risk assessment should be applicable to other bacterial plant diseases.

INTRODUCTION

Billing's original system (BOS) was concerned with fire blight (Erwinia amylovora) risk assessment over the whole growing season. It considered separately: risks of spread by rain and by insects during bloom, risks of infection (disease establishment) and rates of disease development following infection. It was emphasised that weather analyses should be used in conjunction with, not as a substitute for, other risk factors (including host susceptibility) which must be assessed at the local level (1,2,3). Some limitations of the system were discussed.

Unfortunately, some workers used the term "Billing's system" when they used the system in part, not as a whole, or in ways not intended by the author. The underlying principles were sometimes misunderstood and some criticisms of the system were not soundly based. Nevertheless, though BOS gave useful guidance on disease risks, it had important limitations.

As with other systems, rules for action could not easily be framed and could prove unreliable because the quality and quantity of the data input was inadequate in relation to the desired output. Risk assessment users sometimes overlook this problem.

Again, as with other systems, tests on the validity and precision of BOS could not easily be made because of the difficulty of obtaining field data on disease incidence and severity in a numerical form suitable for statistical analyses.

Some of the misunderstandings and misuse were undoubtedly due to the complexity of BOS. Re-examination indicated that, as well as simplification, improved precision and a more logical approach were possible. The outcome of further work is Billing's revised system (BRS) supplemented with a mnemonic, SIRDOw-TIPSuOf which acts as a reminder of all risk factors that require consideration when making judgements on risks. This mnemonic and some other of the underlying principles used in fire blight risk assessment should be applicable to other bacterial plant diseases.

BILLING'S REVISED SYSTEM (BRS)

Weather analyses

Changes introduced in BRS are described only in outline here; they will be presented more fully in the Proceedings of the Fifth International Workshop on Fire Blight (1989) and discussed in detail elsewhere. A report of the Agrimed Fire

Blight Group of the European Economic Community (in preparation) also contains an account of the revisions.

Schouten's [4] corrected table of PD values (potential daily doublings of the pathogen) is now used for temperatures up to 30°C; with these values, spring modifications to the system are no longer necessary and imprecision of the system in dry weather is reduced. The rain scoring method is slightly changed making it more suitable for use in warm, dry climates but this scoring method may still reflect rather poorly the water relationships of host plant tissue. Reliance on trace rainfall records is a disadvantage but the system loses precision without this data. Local records of heavy dews, of morning mists and of irrigation are the best substitute for trace rainfall records. Humidity data is not included at present because it is not available in a suitable form from most weather stations.

The scoring method (still tentative) for risks of spread (S) of inoculum by insects has been revised. Major changes have been made to the infection risk (IR) scoring method; it is now more firmly based on experience. The method for calculating disease development rates (D) remains the same except that allowance is made for PD value changes and the adjustments to the rain scoring method. However, the time between infection and symptom expression is now called the D-period instead of the I-(incubation) period. The criterion for a potential infection day is changed; cool wet days are no longer considered important for infection unless they follow warm days so fewer D-periods are calculated than in BOS. No attempt is made to score disease overwintering (Ow) risks or factors which depend largely on field observations; these remain a matter for judgement at the local level by the person assessing the risks.

The SIRDOw-TIPSuOf approach

This mnemonic provides a reminder of epidemiological factors that need to be considered when judging disease risks. First, there are factors which are largely weather dependent (SIRDOw) and give guidance on relative risks of disease under a given set of field conditions. They are: spread of inoculum by insects or rain (S), infection risks, especially via flowers or young leaves (IR), disease development rates (D) and risks of inoculum production or disease progression from overwintering sources in spring (Ow). In the case of fire blight, Ow risks depend largely on environmental conditions at the end of the growing season; if they favour late infections and late disease progression, cankers are not sealed. With annual crops, risks of seed infection and or survival of the pathogen in plant debris in the soil would be important.

Other risk factors depend largely on field conditions and include: target numbers, notably open flowers and/or expanding sepals and leaves from bud-break onwards (T); inoculum potential, judged mostly by current disease levels (IP); host susceptibility, (Su); other risk factors (Of). Su risks depend on the genetic character of the host, its age and phenological state and on cultural and environmental factors. Other field risks include: the number of host plants and of nearby infected alternative hosts, beehives in orchards, irrigation and insect or storm damage.

RESULTS AND DISCUSSION

BRS has been tested against more than 200 case histories involving different hosts and different climatic areas. Lack of good field data on disease incidence and severity precluded a statistical approach to validation (a common problem with risk assessment systems) but trends indicated that BRS provides useful guidance for fire blight risk assessment. Details of tests will be published elsewhere.

BRS is easier to use and to interpret than BOS and precision is greater. As with all systems, limitations remain which are not easy to remedy.

BRS uses temperature and rainfall records from official weather stations because of their reliability and the inclusion of trace rainfall amounts; thus, the system may not be directly applicable for use with automated weather recording instruments within a plantation where siting and instrument precision may mean that temperatures and rain scores are not comparable to those at weather stations. Also, BRS is intended to be used as a whole, not in part, and as an aid to an experienced worker, not as a rule generator for action without full consideration of all risk factors. Use of infection risk scores alone without reference to spread risks and other factors which affect overall risks is not recommended when judging timing of spray applications during bloom. The method of graphical presentation of BRS means that all risks can easily be assessed at a glance and appropriate action judged.

The blossom period is the time when maximum spread of fire blight usually occurs, largely through the agency of flower visiting insects. BRS takes account of all risks during this period and goes further than most other systems at that time; also, it considers all hosts and all climates and covers the whole growing season. An added advantage of the graphical presentation is evident when actual events do not seem to match the estimated risks; unusual weather or field factors in the current or previous season may reveal a possible explanation and warn of additional factors that might need to be considered in future assessments.

* I acknowledge the contribution made by the Leverhulme Trust towards expenses incurred in the preparation of this report.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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AN ANTI-BACTERIAL ANTIBIOTIC PREPARATION FOR THE DECONTAMINATION OF THE SEEDS OF TOMATOES FROM THE AGENTS OF BACTERIOSES

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The bacterial diseases of tomatoes - bacterial canker (Clavibacter michiganensis subsp. michiganensis-Cmm), bacterial speck (Pseudomonas syringae pv. tomato-Pst) and bacterial spot (Xanthomonas campestris pv. vesicatoria-Xcv) are some of the most economically important diseases of this crop in Bulgaria (5).

The main source of infection are the seeds of tomatoes (7,8,10,11,12) .That is why their decontamination is necessary which is performed by different means and methods.The hydrothermic method is recommended by several research workers (9, 12, 17).The high quality of the decontamination of the tomato seeds from the agents of bacterioses is achieved by the thermochemical method.(2).Together with the application of chemical preparations (2, 5, 8, 9, 16, 17) the possibility is also investigated of treating the tomato seeds with antibiotics because of the systematic and complex character of their action (5,6,10,13,15) .Their application, however, is rather limited for they are not on one and the same basis with the preparations used in medicine.

The purpose of the present work is the testing of the method of application and action of the new Bulgarian antibiotic preparation for plant protection - bactericin for the decontamination of the seeds of tomatoes from the agents of bacterial diseases.

Material and methods

The relative activity of the separate components of the preparation is determined in percentages by the diffusion method (1), and for its calculation the table of Dmitrieva is used (1) with test-microorganisms: **Bacillis subtilis**(BS) **Cmm** , **Pst** and **Xcv**.

The laboratory tests in vivo are carried out with naturally and artificially infected seeds (vacuum infiltration - 1 atm. for 60 min. in a bacterial suspension of a 36-48 h culture of the respective bacteria in concentration 10^8 cfu/ml).The seeds are inlaid with alcoholic polymer with an antibiotic dissolved in it by the method of **Vitanov** and **Bogatzevska** (3) in dose 3, 10, 30 and 60 gr per 1000 gr of seeds .The testing is performed in diagnostic media for **Cmm**-TTC(18), **Pst** -KBM(18), **Xcv** -NJD (18).The germination of the seeds with the different doses is also determined by the standard method.Its bactericide action is compared to the decontaminating effect in the following cases of treatment: 1.Hydrothermic - t^o 53 C for 30 min.;2.Thermochemical - 0,25% solution of phalisan nasbaize heated to t^o 52 C for 25 min.;3.Wet treatment (a) in 3% perhydrol for 30 min. and (b) in 0,25% solution of phalisan for 60 min.Control - non treated seeds naturally and artificially contaminated with pathogenic bacteria.

Three-year field tests have been carried out under production conditions in which we used naturally infected seeds of the variety Druzhba with **Pst** in the following variants: 1.Bactericin - seeds inlaid with polymer and an antibiotic dissolved in it in dose 10 and 30 gr per 1000 gr of seed;2.Hydrothermic-t^o 53 C for 60 min.;3.Thermochemical - 0,25% solution of phalisan nasbaize heated to t^o 52 C for 25 min.;4.Wet treatment - a)with 3% perhydrol -30 min and b) 0,25% solution of phalisan for 60 min.5.Control - naturally contaminated seeds.

In the phenophase of full fruit-bearing the degree of the attack is recorded on each seventh plant in the central part of the plot (each variant 15 decares),altogether 100 plants for variant.The evaluation is performed according to

the 5-rating scale (0 - healthy;1 - single spots .....4 - over 50% of the surface of the fruit covered by spots.) (2).The percentage of the diseased fruit and the index of the disease are calculated with the formula of **Mc Kinney** (14).The results are processed by the standard statistical methods (dispersion analysis) (4).The differences are proved at static authenticity P=0,01.

Results and discussion

The new antibiotic preparation for plant protection - bactericin is obtained by the cultivation of Streptomyces hydroscopicus 155 and is a complex compound of active (macro-cyclic lactones,nonpolyene and polyene antibiotics) and non-active components.It is soluble in aqueous alcoholic compounds.The preparation is stable in dry state and in solution.Its activity does not change with heating to t° 60 C for 24 h, ultra-violet irradiation.

The relative activity of the separate antibiotic components in the composition of bactericin is presented in table 1.

Table 1

The content /%/ and activity /%/ of the antibiotic components of bactericin

anti-biotic	content	test-microorganisms			
		Bs	Pst	Cmm	Xcv
155-A	17±4	75	81	98	93
155-M	34±8	25	19	2	7
155-H	3±2	0	0	0	0

The activity of antibiotic 155-A (type elaiophilin) is from 81 to 98%, of 155-M (type macrolyde antibiotics) from 2 to 19% depending on the test-microorganism.The antibiotic 155-H (polyene group) with the lowest percentage composition (3±2) has no bactericide activity but only a weak fungicide effect.

The results of the carried out in vivo laboratory tests (table 2) prove that the complete decontamination of

the seeds of tomatoes from bacterial pathogens is obtained with their treatment by bactericin dissolved in alcoholic polymer in doses 10, 30 and 60 gr per 1000 gr of seed and through the thermochemical method.The germination of the seeds inlaid with polymer and bactericin in dose 3, 10 and 30 gr is higher than the control (respectively 98.5,98,98.5% against control 95%), while in dose 60gr/1000 gr of seed a sharp reduction to 72% is recorded.Since with the lowest dose the complete decontamination of the seeds is not obtained and with the highest dose the decrease of germination is observed they are not appropriate for the decontamination of the seeds of tomatoes from the agents of bacterioses.

Table 2

The relative number of the diseased tomato seeds (in %) with the different methods of decontamination

Variants	Pst		Xcv		Cmm	
	natur.	artif.	natur.	artif.	natur.	artif.
1.Control	78	100	82	100	94	100
2.Bactericin						
3gr/1000gr seed	10	12	15	12	10	12
10gr/1000gr seed	0	0	0	0	0	0
30gr/1000gr seed	0	0	0	0	0	0
60gr/1000gr seed	0	0	0	0	0	0
3.Hydrothermic	0	0	0	0	1	2
4.Thermochemical	0	0	0	0	0	0
5.Wet						
a. 3% perhydrol	12	18	28	15	36	18
b. 0,25% phalisan	5	7	7	5	10	3

The field tests confirm the advantages of the decontamination of the seeds of tomatoes with the antibiotic bactericin in dose 10 and 30 gr per 1000 gr of seed (table 3).The statistical difference compared to control at P=0,01 is well provided.The decontaminating effect of the antibiotic is equal to that of the processing of the seeds by the thermochemical method.

Table 3

The effect of the method of decontamination of tomato seeds under field conditions

	1986				1987				1988			
	1	2	3	4	1	2	3	4	1	2	3	4
1.Control	51.75	20.20	-	-	38.0	18.40	-	-	41.18	20.98	-	-
2.Bactericin 10g/1000g seed	20.02	6.35	13.60	+++	4.18	1.13	12.48	+++	9.23	2.79	18.19	+++
3.Bactericin 30g/1000g seed	18.06	5.76	15.03	+++	3.76	0.89	15.65	+++	5.03	1.35	19.63	+++
4.Thermic	25.78	6.15	12.45	+++	7.50	2.40	10.25	+++	5.45	1.50	19.56	+++
5.Thermo-chemical	19.11	8.74	14.33	+++	0.40	0.10	18.28	+++	1.83	0.45	20.53	+++
6.3% perhydrol	64.76	34.61	14.13	000	31.20	14.20	4.18	0	26.52	17.24	3.74	0
7.Phalisan 0.25%	41.31	14.10	6.41	000	26.0	10.20	7.88	+	40.50	13.47	7.51	0
		GD 0.1%-11.61				GD 0.1%-4.61				GD 0.1%-9.87		

1-Diseased fruit (%); 2-E% (index of the disease); 3-*D (difference); 4-Proved difference P=0.01.

The obtained results in the laboratory and field tests prove the high effectiveness of the antibiotic preparation bactericin in the decontamination of the seeds from the agents of bacterioses on tomatoes.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

KASUMIN, A BACTERICIDE AGAINST FIRE BLIGHT IN ORNAMENTAL NURSERY CROPS

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Kasumin is an agricultural bactericide and fungicide with a wide spectrum of activity. The active ingredient is the antibiotic kasugamycin, developed by Hokko Chemical Industry Co., Ltd.. In December 1988 Kasumin was registered in Holland by Duphar Nederland B.V. for fire blight control *(Erwinia amylovora)* in ornamental nurseries.

MODE OF ACTION OF KASUGAMYCIN

Kasugamycin, an aminoglycoside antibiotic, inhibits the incorporation of amino acids in ribosome systems of bacteria and fungi in the process of proteïn synthesis. Contrary to other aminoglycoside antibiotics such as streptomycin or kanamycin, kasugamycin does not cause miscoding in the process of protein synthesis.

NO CROSS RESISTANCE WITH OTHER ANTIBIOTICS

Bacteria, both sensitive and resistant to clinically used antibiotics, were equally sensitive to kasugamycin. And in vitro selected bacteria, insensitive to kasugamycin, were sensitive to other antibiotics. So it can be said that there is no cross resistance between kasugamycin and clinically used antibiotics. In Holland there are no legal restrictions for the use of Kasumin.

RESULTS OF EXPERIMENTS

In 1983 and 1984 efficacy experiments were carried out by the Dutch Plant Protection Service. Also phytotoxicity tests were done on nursery crops. One experiment was done against blossom infection in Cotoneaster. For artificial infection a suspension of bacteria was used (10^7 cells/ml). Treatments

were carried out 5 hours before infection (preventative) or 18 hours after infection (curative). There were about 600 flowering clusters per object. Assessments were done 2 weeks later. 4 Replicates. Results are shown in Table 1.

Table 1. Efficacy of one Kasumin-treatment against fire blight (preventive or curative).

object	dosage	ppm a.i.	% infected	%control
Kasumin 25 WP (1)	0.06%	150	4 b (3)	87
Kasumin 25 WP (1)	0.12%	300	1 b	97
streptomycin (1)	0.06%	106	4 b	87
copper oxychloride (1)	0.30%	1500	5 b	84
Kasumin 25 WP (2)	0.06%	150	9 b	71
Kasumin 25 WP (2)	0.12%	300	4 b	87
streptomycin (2)	0.06%	106	16 ab	48
untreated	-	-	31 a	-

(1) preventive spraying (2) curative spraying (18 hours) (3) t-test ($P<0.01$)

Table 2. Efficacy of Kasumin against fire blight. 6 treatments during blossom period.

object	dosage	ppm a.i.	no. of infected clusters per object	% control
Kasumin 25 WP	0.06%	150	8 c (1)	92
streptomycin	0.06%	106	6 c	93
copper oxychloride	0.20%	1000	31 b	61
untreated	-	-	91 a	-

(1) t-test ($P<0.01$)

Another experiment was done with natural infection in Cotoneaster watereri 'Pendulus'. During blossom period 6 treatments were carried out. Weekly assessments were done on infections. 8 Replicates. Results are shown in Table 2.

Copper oxychloride is causing phytotoxicity in a great number of ornamental nurseries. For this reason in many nursery crops the product cannot be used frequently for fire blight control. Phytotoxicity tests showed that Kasumin 25 WP, sprayed in a dosage of 0.12% (300 ppm a.i.) was safe on most commercially grown nursery crops.

Conclusion of these experiments is that Kasumin, in the tested dosages, has given a good control of fire blight. Efficacy (preventative, curative) was equal to, respectively better than streptomycin and copper oxychloride. On most ornamental nursery crops Kasumin did not cause phytotoxicity.

WIDE SPECTRUM

Kasumin is registered worldwide in many countries for control of a great number of diseases, caused by both bacteria and fungi.

Examples:

bacteria

Erwinia amylovora
Erwinia carotovora
Pseudomonas spp.
Xanthomonas spp.
Corynebacterium michiganense
etc.

fungi

Pyricularia oryzae
Cladosporium fulvum
Collectotrichum lagenarium
Venturia inaequalis
etc.

FUTURE PROSPECTS

Because of its high efficacy, very low toxicity and its environmental safety, the product is very promising, particularly as an agricultural bactericide. Development work is going on.

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

USE OF ANTIBIOTICS, KASUGAMYCIN TO CONTROL BACTERIOSES OF VEGETABLES IN HUNGARY

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For most vegetables (beans, tomato, pepper, cucumber) grown in Hungary, one of the greatest plant protection problems is caused by bacterioses. For their control purposes, copper products have been used up to nowadays. Similarly to other countries of the world, registration has not been issued for antibiotics (e.g. streptomycin) under Hungarian conditions. They are otherwise widely used in human medicine.

Due to the unproper efficiency and long pre-harvest interval (21 days in Hungary) of the copper products, since the beginning of the 198os new, efficient formulations have been searched for and, as a result, laboratory screenings and field trials have been carried out with formulations containing kasugamycin to control bacterioses of the above vegetables.

Materials and methods

In vitro investigations of the effect of the formulations on bacteria were made with liquid dilution method in concentrations of looo ppm, 5oo ppm, 25o ppm, 125 ppm, 63 ppm, 32 ppm, 16 ppm, 8 ppm, 4 ppm and 2 ppm calculated for active ingredient.
The bacteriostatic effect was determined after incubation of 24, 48, 72 and 96 hours, the bactericidal concentration within 96 hours.

Based on results obtained with the in vitro investigations between 1981 and 1985, small plot and large plot field trials were carried out in beans, tomato, pepper and cucumber by broadcast spraying, in different dis tricts of Hungary. The small plot trials were made on areas of 3o-5o sq. meters, in 4 replicates, in a randomised block design. Treatments were

made with Wan Der Weij sprayer (Tee-Jet 11006 nozzles) using 600 l/ha spray volume. The large plot trials were made on areas of 0,1-0,2 ha, in 2 replicates in row desing. Sprayer Novor 1005 (Tee-Jet 11006 nozzles) was used with 600 l/ha spray volume. Treatments were initiated at the first appearance of the disease symptoms and were repeated four to five times every 8 to 10 days.

Three evaluations were made: immediately before the first and third treatments, and on days 8-10 after the last treatment. By evaluating 10 plants per treatment and replicate, respectively, the infection percentage and the infection index (with a scale 0 to 6) were determined.

Results

In investigations in vitro, the bacteriostatic and bactericidal concentrations of active ingredient kasugamycin ranged between wide limits depending on the genus and species of bacteria.
Bacteria belonging to the Pseudomonas sp. under study were more sensible to the kasugamycin than those of Xanthomonas sp., while the Xanthomonas sp. were more sensible to copper oxychloride (Table 1).

Results of 6 small plot trials and 9 large plot trials carried out in beans with Kasumin 2L (kasugamycin) and with Kasuran 50 WP (kasugamycin + copper oxychloride) showed control action similar to that of copper oxychloride 50 WP and Bordeaux powder against Xanthomonas campestris pv. phaseoli and Pseudomonas syringae pv. phaseolicola. Tables 2 and 3 show trial results obatained with beans.

Result of 5 small plot trials and 11 large plot trials carried out in tomato with Kasumin 2L showed a control action similar to that of copper oxychloride 50 WP against Xanthomonas campestris pv. vesicatoria and Pseudomonas syringae pv. tomato. Result of control trials in tomato are shown in table 4.

Results of 6 small plot trials and 6 large plot trials carried out in pepper with Kasumin 2L showed a control action slightly better than that of copper oxychloride 50 WP and almost similar to that of Bordeaux powder against Xanthomonas campestris pv. vesicatoria. The Kasuran 50 WP showed a biological efficiency better than both standard products and slightly better than Kasumin 2L. Table 5 shows results of control trials carried out against bacterial diseases of red pepper.

Table 1 - Bacteriostatic and bactericidal effects of Kasumin 2L on bacteria of vegetables

Pathogens	Bacteriostatic concentration in ppm active ing. 96 after 96 hours		Bactericidal	
	Kasumin 2L	copper oxychloride 5o WP	Kasumin 2L	copper oxychloride 5o WP
Xanthomonas campestris pv. phaseoli	125	63	25o	125
Xanthomonas campestris pv. vesicatoria	25o	125	5oo	125
Pseudomonas syringae pv. phaseolicola	16	25o	63	25o
Pseudomonas syringae pv. tomato	16	25o	63	125
Pseudomonas syringae pv. lachrymans	32	25o	63	25o

Table 2 - Study on the control of bacterial diseases of beans, Szekszárd, 1983

Treatments	Doses kg; l/ha	Bacterial blights[1] leaf infection index	pod infection %
Untreated	-	2,3	14,3
copper oxychloride 5o WP (standard)	3,2	1,3	5,5
Bordeaux powder (standard)	3,o	1,1	6,8
Kasumin 2L	1,5	o,8	3,3

Note: 1) at the last evaluation, average of 2 replicates

Table 3 - Study on the control of bacterial diseases of beans, Pécs, 1984

Treatments	Doses kg; l/ha	Bacterial blinghts[1] leaf infection index	pod infection %
Untreated	-	3,2	91,o
copper oxychloride 5o WP (standard)	3,2	1,9	67,o
Kasuran 5o WP	1,5	1,6	67,o
SD_5		o,56	8,7

Note: 1) at the last evaluation
average of 2 replicates

Table 4 - Study on the control of bacterial diseases of tomato, Eger, 1981

Treatments	Doses kg; l/ha	Bacterial blights[1] fruit infection %
Untreated	-	13,2
copper oxychloride 5o WP (standard)	2,4	6,4
Kasumin 2L	1,5	4,o

Note: 1) at the last evaluation
average of 2 replicates

Table 5 - Study on the control of bacterial diseases of red pepper, Kecskemét, 1982

Treatments	Doses kg; l/ha	Bacterial blights[1] leaf infection index
Untreated	-	6.o
copper oxychloride 5o WP (standard)	2.4	1.7
Bordeaux powder (standard)	7.5	1.5
Kasumin 2L	1.5	1.3
Kasuran 5o WP	1.o	1.o
SD_5		o.35

Note: 1) at the last evaluation
average of 2 replicates

Table 6 - Study on the control of bacterial diseases of cucumber, Balassagyarmat, 1985

Treatments	Doses kg; l/ha	Bacterial blights[1] leaf infection index
Untreated	-	5.5
copper oxychloride 5o WP (standard)	2.5	2.4
Kasumin 2L	2.o	3.5
Kasuran 5o WP	2.o	2.4
SD_5		o.8

Note: 1) at the last evaluation
average of 2 replicates

Results of 4 small plot trials carried out in cucumber with Kasumin 2L showed a control action weaker than that of copper oxychloride 5o WP, while Kasuran 5o WP was similar to copper oxychloride 5o WP against Pseudomonas syringae pv. lachrymans. Table 6 shows the trial results.

Consequences

The biological efficiency of Kasumin 2L (active ingredient: kasugamycin) is almost similar to that of the formulations containing cupper. The advantage over the copper products is the extremely short pre-harvest interval (2 days in Hungary). On the basis of the experimental results, the antibiotics kasugamycin obtained a registration in Hungary for use against bacterial diseases of vegetables, because it is applied only in plant health. Since the issuance of registration document for Kasumin 2L, the formulation has been incorporated in the plant protection technology of vegetables. It is therefore used on the growth stages of 2-4 leaves and flowering of the crops, and, if necessary, before harvest. Treatments made before harvest are of particular importance in beans, tomato and pepper, because the yield safety increases in years with occurrence of bacteria, due to the pre-harvest interval of two days.

In other periods of the year formulations containing copper are used to control bacterial diseases.

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

PROTECTION OF THE CUCUMER AGAINST PSEUDOMONAS SYRINGEA PV. LACHRYMANS BY SAPROPHITIC MICROORGANISMS

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INTRODUCTION

Studies on biological control of pathogens that affect aerial parts of plants developed more slowly than in the case of pathogens affecting roots. The ecology of microorganisms present on aerial surfaces, an understanding of which facilitates decisions affecting successful biocontrol, only recently became, a popular field of investigation. In addition, in the case of bacterial diseases we have a limited number of pesticides on the market. *Pseudomonas syringae pv. lachrymans* causes diseases of leaves and fruits of cucumber. Under cool, wet conditions it can be one of the limiting factors in the production and marketing of field grown cucumbers. The purpose of this work was to investigate the potential of saprophytic microorganisms as biological control agents for *P. syringae pv. lachrymans*.

MATERIALS AND METHODS

Isolations of microorganisms were made from 10 grams of roots (adhering soil was removed) or leaves or young small fruits of cucumber (*Cucumis sativus* L., cv. Wisconsin SMR 18). The materials were suspended in sterile water to appropriate dilutions. The isolations were made on potato-dextrose agar (PDA), and 150 isolates were maintained on PDA slants at +4°C. We evaluated 35 isolates, which were representative of the tested populations.

To evaluate the antagonistic potential of each the microbes were grown on yeast extract agar (YE) or on PDA, the *in vitro* agar disc method was used on the YE. Eight isolates, which showed antagonistic activity *in vitro* were used for *in vivo* laboratory tests. The antagonistic microorganisms were cultivated on mineral liquid medium (6) by 8-days at 28°C on

shaker. Seeds of cucumber (cv. Wisconsin SMR 18) were soaked in tested cultures for 24hrs at 18°C. They were placed on wet filter paper and after germination the cotyledons were covered with tested cultures, again. One day later, they were covered with the *P.s.pv. lachrymans* PCM 1410 (48hrs-old cells - 10^8 c.f.u.). The pathogen was cultivated on YE plates. The cells were removed from the agar with water. Tested plants were kept in high humidity atmosphere in plastic boxes at 21°C in darkness for 48hrs. After that time they were kept in a growth chamber. Two weeks later plants were estimated visually and a percentage of the infection was determined on each plate.

The four most promising antagonists were further studied for their ability to control pathogens in sterile soil. They were characterized by Dr. R. Galinska-Stypa, Silesian University, Department of Microbiology, Katowice. The white yeast was characterized by Dr. Z. Krężel from our Department. The antagonistic microbes were cultivated as described above. The colony forming units (c.f.u.) were determined by plate counts on PDA. Soil (clay brown soil mixed with sand 1:1, pH 6.3) was autoclaved. Ten seeds were sown *per* pot (150 grams of soil) after being disinfected with 6 % H_2O_2. Investigated saprophytic microbes were applied as follows: A - seeds were soaked and in the stage of first true leaves they were covered by tested cultures (10^8 c.f.u./1 mL); B - water-washed cells of the antagonistic microbes were added to the pots in the range of 10^8 c.f.u./1 g, one day before sowing. Culture of the pathogen was handled as described above. Infection was done at the stage of first true leaves. Laboratory experiments were repeated three times.

The selected isolates were studied in natural soil in laboratory experiments as well as in field conditions. Micropot experiments were done as described above with sterile soil. Seeds were dusted with lyophilized microbial cells mixed with sterile dust of brown coal (10^{12} c.f.u./1 g). These preparations were applied in the range of 2 g/100 grams of seeds. In the field experiments we used nondisinfected seeds of the cv. Wisconsin SMR 18 at Piastów and cv. Wiktoria F1 at Zgorzelisko. Additionally, plants were sprayed with tested microbes (10^{10} c.f.u./1 mL) on the beginning of the fructification in the dose of 2.5 dcm^3/10 m^2. We included two control series of plots. One had not been protected by any treatment. The second one had been protected by chemicals; seeds were dressed with Nexion L and plants were sprayed four times with Cynkomiedzian (zineb plus copper oxychloride). Fungi were controlled with Topsin M (four times). The fruits were collected several times during the season depending on their maturity. All experiments were carried out in 5 replications.

RESULTS

Biological Control Potential of Phylloplane and Rhizosphere Microorganisms

The potential of 35 isolated microorganisms to control *P.s.pv. lachrymans* was evaluated in a series of *in vitro* and *in*

Table 1 *In vivo* antagonistic activity of isolated strains against *Pseudomonas syringae pv. lachrymans*

TEST STRAINS[x]	12b	8a	12a	8b	3K	40T	22T	27T
INHIBITION ZONE (mm)	17a	15a	11b	10bc	8c	5d	4de	2e

[x] - the rest of 27 tested microorganisms did not show any antagonistic activity,
a-e date, in the same line followed by the same letter are not significantly different P(0.05) by Duncan's test.

vivo tests. All 35 isolates included in all tests were ranked in the same order each time indicating consistency among tests. The eight isolates showed stable antagonistic activity (Tab. 1), which varied from 2 mm to 17 mm inhibition zone around the discs. The significantly best antagonistic activity was observed in the case of isolates 12b and 8a. These eight isolates were compared with each other *in vivo* to choose the best ones for further research. In this final comparison the positive controls (covered with antagonistic microbes) did not show any influence on the growth of tested seedlings up to two weeks. In the negative control, (covered with water and

Table 2 Protection of the cucumber seedling in laboratory experiment

STRAINS	H_2O	12a	8b	8a	12b	3K	27T	40T	22T	C[x]
% OF INFECTED PLANTS	0a	6ab	10b	22c	42d	48d	61e	62e	82f	84f

[x] - infected control,
a-f date, in the same line followed by the same letter are not significantly different (P(0.05) by Duncan's test.

Pathogen) 84% of the plants were infected (Tab. 2). The four most promising isolates were a *Bacillus sp.* 12a, an *Enterobacter sp.* 8b, a *Brevibacterium sp.* 8a, amd a *Saccharomyces sp.* 12b, which reduced the infection to the level of 6, 10, 21, and 42%, respectively.

Biocontrol of *P. syringae pv. lachrymans* in Sterile Soil

None of the tested antagonistic microbes significantly influenced the germination or growth of the plants. The

Table 3 Protection of the cucumber plants in soil

Tested microorganisms	Percentage of infected plants		
	sterile soil		natural soil
	A[x]	B	C
Control unifected	0a	0a	0a
Bacillus sp. 12a	8ab	48c	54c
Brevibacterium sp. 8a	34c	28b	43b
Enterobacter sp. 8b	14b	5a	39b
Saccharomyces sp. 12b	42c	50c	68d
Control infected	73d	93d	97e

[x]A - seeds and leaves treated with antagonistic microorganisms,
B - antagonistic microorganisms added to the soil,
C - seeds dusted with lyophilized cells of the anatagonistic microorganisms.
a-c data, in the same row followed by the same letter are not significantly different P(0.05) by Duncan's test.

percentage of infection in negative controls varied from 73% to 93%. *Enterobacter sp.* 8b gave the best protection for the seedlings (5 - 14% infection) after two weeks of growth in both experiments (Tab.3, A & B). Treatment of the seeds and later on of the first true leaves with *Bacillus sp.* 12a gave a level of protection similar to *Enterobacter sp.* 8b (Tab.3, B). In all other cases tested microorganisms showed significantly less control than the *Enterobacter sp.* 8b and *Bacillus sp.* 12a, when applied as described above. Additionally, enumeration of the c.f.u. during the experiment B showed , that the *Enterobacter sp.* 8b, *Brevibacterium sp.* 8a, *Bacillus sp.* 12a, and *Saccharomyces sp.* 12b multipled 5-fold, 40-fold, 100-fold, and 100-fold, respectively.

Biocontrol of *P. syringae pv. lachrymans* in Natural Soil and in Field Experiments

The percentage of infection averaged about 97% in negative controls in micropot experiments (Tab.3, C). Similarly, as in sterile soil *Enterobacter sp.* 8b and additionally *Brevibacterium sp.* 8a gave the best control, 39% and 43% of infection, respectively. The other two isolates showed significantly less control. As we can see the level of control was

Table 4 Yield of the cucumber fruits in field experiments (kg/plot)

OBJECTS	Piastów		Zgorzelisko	
	1sth week	Sum	1sth week	Sum
Chemical control	0.96 b	28.12 a	1.30 a	32.30 a
Bacillus sp. 12a	1.00 b	19.63 b	0.72 d	23.12 b
Brevibacterium sp. 8a	1.09 ab	21.54 b	0.97 c	23.14 b
Enterobacter sp. 8b	1.30 a	26.78 a	1.14 b	21.70 b
Saccharomyces sp. 12b	0.78 c	18.40 b	0.72 d	24.00 b
Control untreated	1.03 b	20.09 b	0.90 c	21.97 b

[a-c] data, in the same row followed by the same letter are not significantly different P(0.05) by Duncan's test.

significantly lower than in sterile soil. The results of the field experiments showed that *Enterobacter sp.* 8b protected cucumber plants cv. Wisconsin at Piastów, significantly (Tab. 4). The yield was not significantly lower than that given on the plots chemically protected (Tab. 4). The total yield of the fruits was not affected by *Enterobacter sp.* 8b in comparison with unprotected control plots on a field at Zgorzelisko (Tab. 4). However, during the first week of the fructification we collected significantly higher yields (27%) on plots biologically protected than on control plots.

CONCLUSIONS

In the rhizosphere and phylloplane of cucumber plants exist microorganisms (*Bacillus*, *Brevibacterium*, *Enterobacter*, *Saccharomyces*), which have a potential as biocontrol agents for *P.s.pv. lachrymans*. This bacterial disease was controlled as effectively by *Enterobacter sp.* as by the chemical treatment Mor cultivar Wisconsin SMR 18. Greenhouse and field trials are needed for further evaluation of the *Enterobacter sp.* to establish optimum application concentrations, timing, and effectiveness on different cultivars.

The reduction of the disease observed in these experiments was consistent with that found by other researchers when populations of phylloplane and rhizosphere residents were augmented before applications of a pathogen. Several examples of biocontrol of leaf pathogens, mainly fungi, by bacteria were

reviewed in several papers (3,5). However, only a few examples of biocontrol of bacterial pathogens were noted. For example, the *Bdellovibrio bacteriovorus* controlled *P.s.pv. glicinea* on soybean (4). Populations of *Erwinia herbicola*, the common leaf bacteria, were used by Riggle & Klos (2) to protect against infections with *E. amylvora*. The disease was controlled up to 50% in orchard trials. Similar effects using *E. herbicola* and *P. fluorescens* in protection of apple and pear blossoms was described by Beer *et al* (1). Visser *et al* (7) showed an isolate of *L. plantarum* to be effectively antagonistic to the bean pathogen *P. syringae* in pot trials. The pathogen of sugar beet seedlings *Bacillus mycoides* has also been controlled by *Streptomyces roseofulvus* (6).

The capacity of saprophytic microbes to reduce the disease level caused by *P.s.pv. lachrymans* could be attributed to the ability to reduce the level of exogenous nutrients as well as the production of a bacteriocins. Such competition has been observed between epiphytic microorganisms and *P.syringae* (1,2). Examples of bacteriocin production have been described by several authors, however examples of natural production of such compounds on aerial plant surfaces have not been demonstrated. Further research is needed to determine the mechanism of action in the cucumber pathosystem.

ACKNOWLEDGEMENT
This research was partly supported by Grant no. 3.19.06.02K awarded by Institute of Plant Protection in Poznań.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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ALKYLBENZYLDIMETHYLAMMONIUM SALTS AS REAGENTS INHIBITING THE ICE NUCLEATION ACTIVITY OF ERWINIA ANANAS

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The frost in early spring often damages buds and shoots of plants and their immature fruits. It has been reported that some strains of ice nucleation-active (INA) bacteria cause frost injury as they act as heterogeneous ice nuclei in dewdrops (Maki et al., 1974; Hirano et al., 1978; Lindow, 1983; and Makino, 1985). The ice crystals thus formed grow and propagate into the plant tissues where they injure the cells.

Several methods for preventing frost injury are available, such as covering of the crops with plastic film, sprinkling with water, air blowing with frost-protecting fans, and smoking with burning heavy oil. These methods, however, have limitations as to effectiveness, material costs and expensive labor. Antinucleating agents against the bacterial action have been reported by some research groups (Fischer, 1976; Lindow et al., 1978; Yankofsky et al., 1981; and Makino, 1985); the agents include bacteriocides, hydroxamic acid derivatives, heavy metals, and cationic detergents. Two biotechnological approaches for reducing INA bacteria have been developed; the case is the use of bacteriophages to reduce the living cell number of INA bacteria (Kozloff et al., 1983) and the other is the use of a bioantagonist which can reduce their population density (Lindow, 1983).

The present paper describes a systematic approach to developing new types of antinucleating chemicals for practical use in regulating the INA bacterium, Erwinia ananas.

MATERIALS AND METHODS

INA bacterium.

E. ananas IN-10 (Goto et al., 1988) was used. The bacterial cells were cultured in the medium reported by Watanabe et al. (1988) and then washed twice with physiological saline.

Outer membrane.

The cells were disrupted with a French press. The outer membrane fraction was obtained from the lysate by sucrose density gradient ultracentrifugation (Miura and Mizushima, 1969) at 4°C.

Antinucleating agents.

n-Hexyl-n-octylamine and n-alkylbenzylamines (carbon number of the alkyl moiety: 8-18) were synthesized from the respective n-alkylamines through reaction with a 1.1 molar equivalent of n-hexyl iodide or benzyl bromide in chloroform under refluxing conditions for 3 days in the presence of a 1.0 molar equivalent of K_2CO_3 as a catalyst. Each of the synthesized secondary amines was purified by silica gel column chromatography with chloroform-methanol (19:1) as a solvent system. The second peak (main peak) was concentrated in vacuo and the resulting product, oil or crystal, showing a single spot on TLC (silica; ethyl acetate-pyridine, 9:1), was evaluated for antinucleating activity. The amines were also used to derive quarternary ammonium salts. The ammonium salts other than tetramethyl ammonium were derived by methylating the respective secondary amines in chloroform with a large excess of methyl iodide in the presence of a 1.2 molar equivalent of K_2CO_3 at room temperature overnight. The product was purified by silica gel column chromatography with n-butanol-acetic acid-water (4:1:1) as a solvent system. The second peak (main peak) was collected and evaporated. n-Alkylphenyldimethyl-ammonium salts (carbon number of the alkyl moiety: 6-11) were synthesized by reacting an appropriate alkyl bromide with dimethylaniline in acetonitrile. Each of the reaction products was purified by silica gel column chromatography with n-butanol-acetic acid-water (4:1:1).

Antinucleating activity test.

1.8 ml of 0.1 M phosphate buffer (pH 6.5) was put in a test tube (10 mm inside diameter) and 10^8 cells were suspended. The outer membrane fraction was similarly suspended at a concentration corresponding to an optical density of 10^{-2} at 280 nm. Each suspension was mixed with water (0.2 ml) containing a given amount of one of the test agents at 0°C and then kept at 0°C overnight. The suspension was then cooled in an ethylene glycol bath kept at -5°C in order to examine the degree of supercooling (DS) by the method described in the previous paper (Watanabe and Arai, 1987). The DS value was expressed as T_m-T_f, where T_m is the melting point and T_f the temperature at which freezing of the bulk water is initiated. The final concentration of each agent in suspension was expressed by a ppm unit.

Freezing prevention test.

Young tea plants (Thea sinensis L) grown in pots were subjected to the test. Shortly before tea picking, the plants were sprayed with aqueous dispersions of n-octylbenzyldimethylammonium iodide at concentrations of 100, 250, 500 and 1000 ppm. Immediately after, each pot was coated with cotton to keep the soil and roots warm. Part of the ground surface was covered with polyethylene film to prevent evaporation of soil water. The test plants were stored at -3°C overnight. The film was removed from the cold-treated plants, which were then warmed at 20°C under mild flow of air. After 3 hr, the test agent was evaluated for effectiveness.

RESULTS AND DISCUSSION

The screening of antinucleating agents was first performed by determining the degree of supercooling of bulk water in the presence of the INA bacterial cells or their outer membrane fraction. Organic acids, amino acids and amino acid esters were ineffective for the cells, whereas some compounds with amphiphilic structures were effective for the membrane fraction. Assuming that an amphiphilic molecular structure may contribute to antinucleation, we tested several kinds of surfactants and

obtained the following results: (1) the nucleating activity of the outer membrane is inhibited by many kinds of surfactants, and (2) that of INA bacterial cells is inhibited only by a cationic surfactant. These results indicate that the ice-nucleation active site of the cell is comprised of an anionic environment, the conformation of which easily changes during the membrane preparation process.

These findings prompted us to examine systematically a series of amines as to their antinucleating ability. Table 1 shows that primary, secondary and tertiary amines whose hydrophobic groups are substituted with alkyl chains having carbon numbers of more than 8 were effective in increasing DS values in the presence of cells. This suggests the importance of a structure having both a hydrophobic group and a cationic group. In the case of amines, an increase in the carbon number of the alkyl group caused a decrease in water solubility.

Table 1. Antinucleating abilities of amines.

Amine	Conc. (ppm)	DS (°C)	Amine	Conc. (ppm)	DS (°C)
		Av. ± s.e.			Av. ± s.e.
Methyl	1000	0.5 ± 0.5	Di-n-hexyl	1000	0.5 ± 0.1
Ethyl	1000	0.3 ± 0.3	Dibenzyl	1000	2.4 ± 0.8
n-Butyl	1000	0.2 ± 0.1	Di-n-octyl	1000	>5*
n-Hexyl	1000	0.9 ± 0.7		100	>5*
n-Octyl	1000	4.4 ± 0.2	n-Hexyl-n-octyl	1000	>5*
n-Decyl	1000	4.9 ± 0.1		100	>5*
n-Dodecyl	1000	4.9 ± 0.1	n-Octylbenzyl	1000	>5*
n-Tetradecyl	1000	0.7 ± 0.4		100	>5*
n-Hexadecyl	1000	0.5 ± 0.2	Tri-n-butyl	1000	0.1 ± 0.1
Benzyl	1000	0.3 ± 0.2	Tri-n-hexyl	1000	0.7 ± 0.2
Cyclohexyl	1000	0.3 ± 0.2			

*Effective.

In order to improve the water solubility we synthesized ammonium salts and tested their abilities as to the inhibition of the nucleating activity of the cells. As shown in Table 2, quarternary ammonium salts showed greater inhibitory abilities

than the corresponding amines (Table 1). As to an effective structure, it was thus suggested that one substituent should be a hydrophobic alkyl group having a carbon number of more than 8. It was also found that a benzyl group acted as another favorable substituent. Alkylbenzyldimethylammonium salts (carbon number of the alkyl moiety: 8-18), even when dissolved at 100 ppm, showed a DS value of more than 5°C.

Table 2. Antinucleating abilities of ammonium salts.

Ammonium salt	Conc. (ppm)	DS (°C) Av. ± s.e.	Ammonium salt	Conc. (ppm)	DS (°C) Av. ± s.e.
Tetramethyl	1000	0.3 ± 0.1	n-Dodecylbenzyl-dimethyl	1000	>5*
n-Butyltrimethyl	1000	0.4 ± 0.1		100	>5*
n-Hexyltrimethyl	1000	0.8 ± 0.2	n-Tetradecylbenzyl-dimethyl	1000	>5*
n-Octyltrimethyl	1000	4.6 ± 0.3		100	>5*
n-Decyltrimethyl	1000	4.9 ± 0.1	n-Hexadecylbenzyl-dimethyl	1000	>5*
n-Dodecyltrimethyl	1000	4.6 ± 0.4		100	>5*
n-Tetradecyltrimethyl	1000	3.6 ± 0.3	n-Octadecylbenzyl-dimethyl	1000	>5*
Benzyltrimethyl	1000	0.2 ± 0.1		100	>5*
Di-n-hexyldimethyl	1000	1.5 ± 0.5	n-Hexylphenyldimethyl	1000	0.2 ± 0.1
n-Hexyl-n-octyl-dimethyl	1000	>5*	n-Heptylphenyldimethyl	1000	3.3 ± 0.3
	100	>5*	n-Octylphenyldimethyl	1000	4.0 ± 0.4
n-Octylbenzyl-dimethyl	1000	>5*	n-Nonylphenyldimethyl	1000	>5*
	100	>5*	n-Decylphenyldimethyl	1000	>5*
n-Decylbenzyldimethyl	1000	>5*	n-Undecylphenyl-dimethyl	1000	>5*
	100	>5*			

*Effective.

In actuality, the freezing of water never occurs at a subzero temperature of higher than -3°C unless an effective heterogeneous nucleus is present. E. ananas is one of the most potent nuclei and causes frost injury to tea plants. Therefore, a required attribute of an antifrost agent for tea plants should be that a DS value of more than 3°C is attained on spraying of the agent. It is also necessary that its concentration at the time of spraying should not be harmful to the plants. When tea plants were sprayed with the octylbenzyldimethylammonium salt at

a concentration over 250 ppm and then stored at -3°C, cryoinjury hardly occurred as far as a pot test was concerned.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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MECHANISM BY WHICH THE ANTIBIOTIC OF ERWINIA HERBICOLA Eh318 INHIBITS ERWINIA AMYLOVORA Ea273

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Biological control of fire blight, caused by *Erwinia amylovora*, has shown great promise in studies done in a research orchard. Spraying apple blossoms with suspensions of *E. herbicola* has given control of fire blight equivalent to that achieved with streptomycin, the most commonly used and effective chemical control agent.

The ability of 12 strains of *E. herbicola* to produce antibiotic *in vitro* correlated well with the effectiveness of the strains in preventing infections of apple blossoms induced by *E. amylovora* strain Ea273 in field tests. Although antibiotic production clearly is not the only mechanism of inhibition, the ability to produce antibiotic may distinguish excellent and good antagonists of *E. amylovora*.

Erwinia herbicola strain Eh318 was rated as an excellent antagonist of Ea273 in field tests. It produces an antibiotic that inhibits Ea273 *in vitro*. However, the Eh318 antibiotic is not inhibitory to Ea273 in the presence of mixtures of amino acids, such as casein amino acids. No single amino acid fully inhibits the toxicity of the Eh318 antibiotic. The purpose of this investigation was to determine the mechanism by which the Eh318 antibiotic inhibits Ea273.

A crude preparation of the Eh318 antibiotic, which inhibits Ea273, was not inhibitory to Ea273 in the presence of a combination of arginine and histidine. Arginine had a much greater effect on reducing the toxicity of the antibiotic to Ea273 than did histidine. It was hypothesized that the antibiotic inhibits arginine biosynthesis by Ea273.

Intermediates in the putative pathway of arginine biosynthesis of Ea273 were tested for their ability to mimic the ability of arginine to inhibit the toxicity of the Eh318 antibiotic. Glutamic acid and

N-acetylglutamic acid, which occur at the start of the pathway, did not inhibit the toxicity of the Eh318 antibiotic. However, N-acetylornithine, ornithine and citrulline, which occur late in the pathway, did inhibit the toxicity of the Eh318 antibiotic. Thus, the Eh318 antibiotic probably inhibits the biosynthesis of arginine after the formation of N-acetylglutamic acid and before the formation of N-acetylornithine.

A crude preparation of N-acetylornithine transaminase, which converts N-acetylglutamyl semialdehyde and glutamic acid to N-acetylornithine and α-ketoglutarate in arginine biosynthesis, was prepared from Ea273. The transaminase was purified and assayed by the procedure of Albrecht and Vogel (J. Biol. Chem., 239:1872-1876, 1964). Since the transaminase reaction has an equilibrium constant near one, and N-acetylglutamyl semialdehyde was not commercially available, the reaction was studied in the reverse direction. Pure antibiotic, obtained from the laboratory of Professor Jon Clardy (Department of Chemistry, Cornell University) inhibited the transaminase activity. Crude preparations of the transaminase from Eh318 and Ea273R318 (a spontaneous mutant of Ea273 that is resistant to the Eh318 antibiotic) also were inhibited by the pure antibiotic to about the same extent as the transaminase of Ea273. Thus, the resistance of Ea273R318 and Eh318 to the Eh318 antibiotic is not the result of an altered (resistant) transaminase.

Kinetic studies were done with pure Eh318 antibiotic and transaminase that was purified 13-fold by ammonium sulfate precipitation and isoelectric point precipitation. When kinetics were done in the presence and absence of pure Eh318 antibiotic, with N-acetylornithine as the varied substrate, the Eh318 antibiotic was a competitive inhibitor of the enzyme, indicating that it competes with N-acetylornithine for the active site of the enzyme. If this is true, the Eh318 antibiotic should be an uncompetitive inhibitor of the transaminase when α-ketoglutarate is the varied substrate. When the kinetic studies were repeated with α-ketoglutarate as the varied substrate, the Eh318 antibiotic indeed was an uncompetitive inhibitor of the enzyme.

This work was supported (in part) by a grant from the Cornell Biotechnology Program which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, the U.S. Army Research Office and the National Science Foundation.

SESSION 3

EPIDEMIOLOGY

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

OCCURRENCE OF PSEUDOMONAS SYRINGAE PV. PAULANS IN BUDWOOD OF 'MUTSU' APPLE

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ABSTRACT

Blister spot is a serious fruit spot disease of 'Mutsu' apple in Ontario, Canada. To investigate the possibility that budwood for propagation may be the source of inoculum, it was collected from 11 sites including six major nurseries. Buds were aseptically excised, comminuted in buffer, and aliquots plated onto King's medium B agar plates. Representative bacterial colonies were passed through a series of physiological, biochemical, and pathological tests. Pseudomonas syringae pv. papulans was detected at four sites in 1987 and six in 1988, suggesting that P. syringae pv. papulans in budwood could be a significant source of inoculum in young orchards of 'Mutsu' apple.

INTRODUCTION

Blister spot is a fruit spotting disease of Malus domestica (Borkh.) cv. Mutsu in Ontario (Dhanvantari, 1969) and under favorable environmental conditions, a necrosis of the leaf blade midvein (Bonn and Bedford, 1986). The fruit lesions are superficial, however, they seriously reduce the quality of apples for the fresh market.

Good evidence exists for overwintering of Pseudomonas syringae pv. papulans (Rose 1917) Dhanvantari 1977, in the bud tissue of 'Mutsu' apple (Bazzi and Calzolari, 1987; Bedford et al., 1984; Burr and Katz, 1982). Later, it becomes an epiphyte on new green tissue (buds, leaves, flowers, fruit) in the spring (Bedford et al., 1988). These epiphytic populations are presumed to form the inoculum for the infection of fruit stomates

during a 2-4 wk period beginning about 2 wk after petal fall (Bedford et al., 1988; Burr and Hurwitz, 1981). Lenticular lesions on the fruit appear in late July and early August; by harvest, the lesions are raised circular areas 1-5 mm in diam., 1-2 mm deep, with a blistered brown center surrounded by a dark purple border.

Bedford et al. (1988) have shown that epiphytic populations of P. syringae pv. papulans are present in the orchard from bud break in spring until the end of September overlapping the period when budwood is selected for propagating. The possibility of budwood contamination from epiphytic bacteria or bacteria washing out of infections exists. This investigation was undertaken to determine whether budwood for propagation of 'Mutsu' apple was contaminated with the blister spot pathogen.

MATERIALS AND METHODS

Orchard and nurseries sampled

Studies incorporated budwood samples collected from three commercial apple orchards, one of which was cv. Wellington, two institutional orchards, and six commercial nurseries in southern Ontario. The six nurseries supply the bulk of the sales of 'Mutsu' nurserystock to commercial apple growers in the province. The samples collected from nurseries were subsamples of budwood used for propagation. The budwood was shipped to the Harrow Research Station and placed in cold storage at 4°C until processed. Each sample contained 10 budsticks consisting of a minimum of 10 buds per stick.

Budwood assay

Budsticks were surface disinfested by dipping them in a 10% water solution of household bleach (6.0% NaOCl) for one minute, rinsing them in distilled water, and air drying on paper towels. Each of 10 buds was aseptically removed, observed for tissue discoloration, and placed into a 7 ml glass tissue grinder along with 1 mL of a 0.1 M sodium phosphate buffer (pH 7.0). The bud was macerated and an additional 1 mL of phosphate buffer was added to the grinder prior to vigorous agitation. Aliquots of 0.1 mL each from the original and subsequent 10-fold dilution series were plated in triplicate onto King's medium B (King et al., 1954). The plates were incubated at 24°C and observed after 3 days for fluorescent colonies typical of P. syringae. Counts of total bacterial colonies and those

typical of P. syringae were made from plates of the appropriate dilution. Representative colonies exhibiting morphological characteristics, color, and fluorescence typical of the P. syringae pathovars, were selected, streaked onto King's B medium for purity, and placed in phosphate buffered (0.01M) saline (0.85%).

Characterization and pathogenicity of selected isolates

Bacterial isolates were transferred from storage in phosphate buffered saline to King's medium B for further identification. Following verification of colony morphology and color they were subjected to a series of tests to identify Pseudomonas spp. (Schaad, 1988) and to

Table 1 Tests for characterizing Pseudomonas syringae pv. syringae and P. syringae pv. papulans

Test*	Reaction	
	pv. syringae	pv. papulans
1. Gram stain	-rod, non-spore forming	-rod, non-spore forming
2. Presence of catalase	+	+
3. Presence of oxidase	-	-
4. Fluorescence on King's B medium	+	+
5. Oxygen requirements	aerobic	aerobic
6. Presence of arginine dihydrolase	-	-
7. Hypersensitive reaction to tobacco	+	+
8. Proteolysis of gelatin	+	-
9. Proteolysis of casein	+	-
10. Growth and acid prod'n from arbutin	-	+
11. Growth and acid prod'n from salicin	-	+
12. Growth on leucine	+	-
13. Levan production	+	-
14. Longevity on sucrose nutrient agar	-	+
15. Pathogenicity on 'Mutsu' apple fruit	-	+

* Tests 1 through 7 characterize P. syringae, tests 8 through 14 distinguish pv. papulans from pv. syringae (Dhanvantari, 1977).

differentiate between the pathovars syringae and papulans (Bedford, 1982; Dhanvantari, 1977). The tests for the differentiation of P. syringae pv. syringae and P. syringae pv. papulans are presented in Table 1. Pathogenicity of a selected number of P. syringae pv. syringae and P. syringae pv. papulans isolates was confirmed by inoculation of 'Mutsu' apple in the orchard according to Bedford (1982).

RESULTS

Commercial

P. syringae pv. papulans (PSP) was detected in the buds from two of three commercial orchards in 1987 and three of four orchards in 1988 (Table 2). One orchard remained free of detectable PSP in both years. The orchard

Table 2. Percentage of buds from which Pseudomonas syringae pv. papulans (PSP) and P. syringae pv. syringae (PSS) were isolated in 1987 and 1988

Orchard Site *		Buds (%)			
		PSP		PSS	
		1987	1988	1987	1988
Commercial	1	3	2	20	5
	2	36	9	13	1
	3	0	0	17	8
	4	-	16	-	0
Institutional	1	0	0	79	8
	2	0	0	37	3
Nursery	1	1	4	38	1
	2	0	1	33	15
	3	0	0	26	4
	4	19	1	8	6
	5	0	0	38	2
	6	0	1	3	12

* 'Mutsu' orchards were chosen from sites in southern Ontario. Commercial 1 consisted of the cultivar Wellington.

described as commercial 1 had a low percentage of buds containing PSP even though containing 'Wellington' which is not a host of the pathogen. Pathogen numbers varied between sites and years (Table 3) with a low of 4.4×10^2 cfu/bud and a high of 9.1×10^4 cfu/bud. Total bacterial counts varied over the same time and site by 26-fold with a low of 4.9×10^3 cfu/bud and a high of 1.3×10^5 cfu/bud.

Table 3. Mean population of Pseudomonas syringae pv. papulans detected in infested buds of 'Mutsu' apple in 1987 and 1988

Site		1987 Infested buds (%)	1987 Mean population (cfu/bud)	1988 Infested buds (%)	1988 Mean population (cfu/bud)
Commercial	1	3	9.6×10^3	2	1.1×10^4
	2	36	4.7×10^3	9	3.2×10^4
	3	0	NA	0	NA
	4	-	-	16	6.3×10^3
Institutional	1	0	NA	0	NA
	2	0	NA	0	NA
Nursery	1	1	2.7×10^3	4	5.0×10^4
	2	0	NA	1	4.4×10^2
	3	0	NA	0	NA
	4	19	5.9×10^3	1	9.1×10^4
	5	0	NA	0	NA
	6	0	NA	1	1.1×10^4

Institutional

Orchards of 'Mutsu' at the Harrow Research Station and the Horticultural Research Institute of Ontario at Vineland Station had a high percentage of buds containing P. syringae pv. syringae (PSS), however, no PSP were detected in the buds in either year (Table 2). Populations of total bacteria and fluorescent bacteria P. syringae plated onto King's medium B

ranged from 4.4×10^4 to 1.5×10^5 cfu/bud and 4.3×10^2 to 1.2×10^5 cfu/bud, respectively.

Nursery

PSP was detected in buds excised from budsticks collected from two of the six nurseries sampled in 1987 and an additional two nurseries sampled in 1988 (Table 2). Populations of PSP ranged from 2.7×10^3 cfu/bud in site 1 where only one bud was infested to 5.9×10^3 cfu/bud at site 4 where 19 buds were infested (Table 3) in 1987. In 1988 these sites were again infested where site 1 had 4% and site 4 had 1% of the buds containing PSP (Table 2). Populations of PSP were somewhat higher in 1988 with 9.1×10^4 cfu/bud detected at site 4 and 5.0×10^4 cfu/bud at site 1 (Table 3). Two additional sites yielded PSP when plated onto King's medium B in 1988. In each case, only 1% of the buds were infested (Table 2). Total populations ranged from 1.2×10^3 to 6.2×10^5 cfu/bud and fluorescent bacteria from 4.2×10^2 to 2.6×10^5 cfu/bud.

DISCUSSION

Buds of 'Mutsu' contained substantial numbers of bacteria, especially fluorescent *P. syringae*, in late summer when budsticks were collected from trees for use in propagating nurserystock. Some of the isolated *P. syringae* were in fact PSP, the causal agent of blister spot. PSP-contaminated buds used in the propagation of 'Mutsu' nurserystock could provide an important inoculum reservoir in newly planted orchards.

PSP was positively identified in symptomless buds obtained from the cvs. Wellington and Mutsu adjacent to each other in a commercial orchard. The source of inoculum for budwood contamination was most likely the epiphytic population of PSP maintained on 'Mutsu' which had a long history of blister spot disease. In a previous study (Bedford, 1982), 'Wellington' did not support an epiphytic population of PSP nor were blister spot symptoms recorded on this cultivar. The current work suggests that budwood obtained from commercial 'Mutsu' orchards can have substantial numbers of buds carrying the pathogen in late summer when budwood is collected for nurserystock propagation.

Institutional orchards maintained free of disease and under close observation appear to offer good sources of PSP-free budwood. Even though the buds carry substantial numbers of bacteria including fluorescent P. syringae, PSP was not detected in the buds in either 1987 or 1988. The institutional site 2 contains two blocks of about 20 trees each and is used to provide about 7,000 buds yearly to nurserymen. Under close scrutiny, this orchard could provide disease-free buds to the nursery industry for the propagation of 'Mutsu'. Currently, it is underutilized but as the awareness of the need to use disease-free budwood increases, there should be greater use of this resource.

PSP was detected in two of six nurseries in 1987 and in four of the same six nurseries in 1988. The percentage of buds contaminated was low, however, the numbers of PSP per bud were fairly consistent at 10^2-10^4 cfu/bud (Table 3). The detection of PSP in budwood used by the nurseries in 1987 and 1988 suggests that their sources of budwood did not come from either disease - or pathogen-free orchards. Either low levels of disease were present unobserved in the budwood orchards or that PSP was present as an epiphyte in these orchards. There is a good chance that nurserystock originating from sites with contaminated buds may bring about blister spot problems when newly planted orchards begin to fruit, e.g. nursery site 4 in 1987 (Table 2).

The results show that PSP occurs in symptomless buds from both commercial orchards with disease and in buds used for propagation by nurseries in producing nurserystock. In the former situation, the buds are overwintering sites for PSP (Bazzi and Calzolari, 1987; Bedford, 1982; Burr and Katz, 1984); in the latter, the buds are reservoirs of inoculum for contamination of new orchards, initially as an epiphyte of growing parts (Bedford, 1982) and later as a pathogen of the 'Mutsu' fruit. Under careful scrutiny, institutional orchards could act as important sources for pathogen-free budwood for the propagation of 'Mutsu' trees by both nurseries and the individual grower.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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DETERMINATION OF EARLIEST BLOSSOM BLIGHT SYMPTOMS ON MALUS AND PYRUS FOLLOWING IN SITU INOCULATION WITH VARIOUS INOCULUM LEVELS OF ERWINIA AMYLOVORA

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The blossom blight phase of fire blight usually occurs prior to the appearance of blight symptoms on leaves or vegetative shoots (10). The absence of blossom blight may be explained by a lack of sufficient inoculum (3), unfavorable environmental conditions before bloom (5), or during and after bloom (4), or the innate resistance of blossoms to the disease (8).

Rosen (6) made the first detailed observations of the penetration and invasion of E. amylovora in apple and pear blossom tissues. He showed conclusively how the bacteria can be traced through several layers of cells below the nectarthode chamber soon after artificial inoculation of blossoms. In New York, Hildebrand (3) demonstrated that 60% of apple blossoms became infected when single cells of E. amylovora were introduced into blossom nectaries of excised apple flowers. However, similar tests with intact flowers on pear trees in the greenhouse failed to produce infection.

More recently, Aldwinckle and Beer (1) reported that they obtained blossom infection in 'Golden Delicious' apples using as few as 10 cells per blossom. In England, Billing (2) determined that 10^9 cells of E. amylovora were required to obtain blossom infection and that about 2-3 days were required to achieve completion of an incubation period.

The objectives of this research were: 1) to study the degree of artificial blossom blight infection with low numbers of bacterial cells and 2) to obtain some supplementary, fundamental information to improve the effectiveness of the recently developed MARYBLYT predictive model (7). Blossoms were inoculated with various dosages of E. amylovora and were analyzed under existing weather conditions during the spring seasons of 1987, 1988, and 1989. Preliminary results were reported earlier (9).

MATERIALS AND METHODS

The inoculation experiments were carried out in a 2 acre (1 ha) block of fruit trees and in growth chambers at the Appalachian Fruit Research Station. In the field, trees of 'Jonathan' apple and 'Bartlett' pear in alternate rows were used. Weather records were collected in the block with a computerized recorder as well as with a manual hygrothermograph to serve as back-up. The lighted growth chambers were maintained at specified temperatures as indicated in the tables and a relative humidity of 95%. In two experiments, inoculated excised shoots were rotated to chambers with different temperatures.

Cultures of virulent isolates of E. amylovora were maintained on nutrient yeast-dextrose agar (NYDA) slants and specific concentrations (10^2-10^8 CFU/ml) of bacterial cells were prepared in phosphate buffer suspension and densities determined on a Klett-Summerson photoelectric colorimeter. Inoculations were performed in replicate experiments by placing desired quantities of inoculum (10, 25, or 50 ul) in individual blossoms with a micropipette dispenser. To verify numbers of bacterial cells in specific concentrations of inoculum, replicate platings were performed on NYDA plates. Counts were made after 72 hours.

RESULTS AND OBSERVATIONS

In 1987, significantly more pear and apple blossoms blighted following inoculation with 10^8 than with 10^5 or 10^2 CFU/ml (Table 1). This was true with each isolate of E. amylovora as well as with the combination of all 3 isolates. With one exception, this was also reflected in the number of blighted blossom clusters, counted about 10 days after initial symptoms were observed. Blossom blight was considerably more severe in 'Bartlett' pear than in 'Jonathan' apple.

In 1988, similar significant results were obtained on 'Bartlett' pear and 'Yellow Delicious' apple, inoculated with 10^8, 10^5, and 10^2 CFU/ml of a mixed suspension of 3 isolates of E. amylovora. When blossoms were sprayed with streptomycin (60 ppm), using a one-liter spray bottle, at intervals of 24, 48, or 72 hours, only minor significant differences in blight infection were observed. This was due to the placement of the inoculum in the flower cup at least 24 hours before the antibiotic was applied. In 1988, significantly fewer blossoms became infected with the two lower concentrations (10^5 and 10^2) than those inoculated with 10^8 CFU/ml.

Table 1. Severity of blossom blight on 'Bartlett' pear and 'Jonathan' apple following artificial inoculation with three isolates of Erwinia amylovora. (Spring 1987)

E. amylovora isolate dosage	Percent blighted blossoms[X]		Number blighted blossom clusters[Y]	
	Bartlett (May 4)	Jonathan (May 12)	Bartlett (May 15)	Jonathan (May 20)
Isolate 259				
10^8 CFU/ml	74.0 bc[Z]	61.5 ab	36 a	33 ab
10^5 CFU/ml	49.5 de	20.0 de	31 ab	21 cd
10^2 CFU/ml	0.0 g	12.5 de	3 f (14)	3 f
Isolate 260				
10^8 CFU/ml	79.0 abc	46.5 bc	39 a	6 f
10^5 CFU/ml	51.5 d	18.0 de	25 bc	9 ef
10^2 CFU/ml	26.0 f	2.5 e	17 e	0 f (6)
Isolate 1112				
10^8 CFU/ml	87.5 ab	79.5 a	40 a	40 a
10^5 CFU/ml	59.5 cd	30.0 cd	33 a	29 bc
10^2 CFU/ml	16.5 fg	5.0 e	6 ef	7 f
All 3 isolates				
10^8 CFU/ml	95.5 a	55.0 b	39 a	31 abc
10^5 CFU/ml	31.0 ef	18.5 de	19 cd	18 de
10^2 CFU/ml	14.5 fg	1.5 e	13 de	8 ef
(25)				
Controls				
Buffer only	3.0 g	0.0 e	2 f	0 f
Natural infection	1.0 g	2.0 e	1 f	3 f

X Based on inoculation (pear, April 24 and apple, May 1) of 200 blossoms (5 blooms in each of 10 blossom clusters and 4 replications) with 50 ul of bacterial suspension in a micropipette.

Y Based on examination of 40 blossom clusters with infections advanced into the woody tissue, showing necrosis in nearest leaves; figures in parenthesis indicate number of blighted blossoms.

Z Values with different letters within columns are significantly different based on t-test of regression coefficients (P=0.05).

In 1989, when individual 'Jonathan' blossoms were inoculated with 10^6-10^3 CFU/ml, and 50, 25, or 10 ul of each concentration was applied, significantly fewer blossoms became infected with 10^3 CFU/ml than the higher concentrations (Table 2). Except for this lowest concentration, the different inoculum dosages did not result in significant differences in infection.

In the earliest (April 24 and May 1) experiments in 1987, the incubation period was 10 days for both 'Bartlett' pear and 'Jonathan' apple. When blossoms were inoculated one week later (May 7), these periods were reduced to 5 and 8 days, respectively. In 1988, the

Table 2. Degree of blossom blight on 'Jonathan' apple following inoculation with various inoculum dosages and bacterial cell concentration. (Spring 1989)

Bacterial concentration CFU/ml	Inoculum dosage	Percent blighted blossoms[X]	Number blighted blossom clusters[Y]
10^6	50	32 a[Z]	14 ab
	25	27 ab	15 ab
	10	27 ab	16 a
10^5	50	24 abc	12 abc
	25	24 abc	12 abc
	10	20 abcd	9 abcd
10^4	50	25 abc	8 abcd
	25	16 abcd	6 bcd
	10	23 abcd	8 abcd
10^3	50	10 bcd	4 cd
	25	7 cd	2 d
	10	4 d	2 d
Check (buffer)	50	11 bcd	7 abcd
	25	9 bcd	3 cd
	10	15 abcd	6 bcd
Check (natural)		6 cd	1 d

X Based on inoculation of 100 blossoms (5 blooms in each of 5 blossom clusters and 4 replications).

Y Based on examination of 20 blossom clusters with infections advanced into woody tissue, showing necrosis in nearest leaves.

Z Values with different letters within columns are significantly different based on t-test of regression coefficients (P=0.05).

incubation period ranged 10-13 days for 'Bartlett' and 9-11 days for 'Jonathan'. During the prolonged cool, wet weather of 1989, this period remained the same for 'Bartlett, but was extended to 16 days for 'Jonathan'. For all three years, the average incubation period was 8.8 days for 'Bartlett' and 10.8 days for 'Jonathan'. Earliest symptoms detected before necrosis usually were a tiny ooze droplet on the stem just below the floral cup on 'Bartlett' pear and a distinct bright orange coloration of the base of the stamen on 'Jonathan' apple.

When open 'Bartlett' blossoms on excised branches, maintained in a growth chamber at 75F (24C) and 95% RH, were inoculated with 10^6-10^3 CFU/ml, earliest symptoms were detected about 72 hours after inoculation (Table 3). Major differences (some significantly so) were recorded between inoculum concentrations; and even with the lowest application (10 ul) of the lowest concentration (10^3 CFU/ml), 8% of the blossoms became infected. Replicate platings of this concentration on NYDA confirmed accuracy of this concentration. When excised 'Bartlett' blossoms were

Table 3. Percent blighted 'Bartlett' pear blossoms, following artificial inoculation with various concentrations of Erwinia amylovora and incubation in a growth chamber at 24C (75F) and 90% RH.

Bacterial concentration CFU/ml	Inoculum dosage	Percent blighted blossoms	Platings CFU/ml
10^6	50 ul	92 a	25300 a
	25 ul	75 ab	16200 b
	10 ul	67 abc	4800 c
10^5	50 ul	67 abc	2550 d
	25 ul	33 cdef	1390 de
	10 ul	58 abcd	590 e
10^4	50 ul	67 abc	296 e
	25 ul	58 abcd	140 e
	10 ul	17 ef	64 e
10^3	50 ul	50 bcde	35 e
	25 ul	25 def	19 e
	10 ul	8 f	10 e
Check	50 ul	0 f	0 f
	25 ul	0 f	0 f
	10 ul	0 f	0 f

At each concentration, first symptoms appeared 72 hours after inoculation.

inoculated with 10^8 or 10^4 CFU/ml for 1hr (24C) and some of the flowers were moved to chambers maintained at 50F (10C) and then 33F (1C), still 40% of the blossoms became infected after exposure for 4 hours at 33F.

DISCUSSION

Blossom blight symptoms were obtained in artificially inoculated flowers in the orchard, with various concentrations of E. amylovora, even in two (1988 and 1989) years, when natural blight did not occur. Additionally, symptoms appeared in blossoms inoculated under adverse weather conditions, with only 10 ul of 10^3 CFU/ml, or approximately 10 bacterial cells, as shown in plating on NYGA medium in the laboratory. This confirmed previous results by Aldwinckle and Beer (1). Also, in 1989, we noticed blossom infections on uninoculated trees, presumably due to unusually high bee population activity. These observations proved also that extremely low numbers of E. amylovora bacteria were disseminated by bees and eventually were able to cause infection. In 1987, the occurrences of natural infection were due to epiphytic bacteria.

Close examinations of inoculated blossoms resulted in the observation of the earliest detectable blossom blight symptoms. Depending upon climatic conditions, the incubation period varied from 5 to 13 days for 'Bartlett' pear and from 8 to 16 days for 'Jonathan' apple. Calculations of degree days over 40F (5C) for 'Jonathan' confirmed those reported for the MARYBLYT model (220 DD) to predict fire blight in the eastern United States (7). The figure 140 DD (range 130-150) for 'Bartlett' pear is a new recording. Detecting infected pear blossoms in the growth chamber, after excised flowers were inoculated with 50 ul of 10^4 CFU/ml and maintained at 33F for 4 hours, was unexpected. Bacteria apparently survived the low temperature and continued multiplication when blossoms were replaced at favorable temperatures. Results reported here will help to improve the ultimate blossom blight predictive system for the eastern United States.

ACKNOWLEDGMENT

The author wishes to thank John Walter, Research Technician, for invaluable assistance in orchard and growth chamber experiments.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

CLIMATIC DATA AND FIRE BLIGHT OCCURRENCE IN GREECE

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SUMMARY

A severe epidemic of fire blight on pears and other pomaceous trees, appeared in Greece in the spring of 1987. The assessement of fire blight activity risks according to Billing's "revised" system gave satisfactory explanation for the distinct differences in disease appearance and severity in two places of Greece. No satisfactory explanation can be given for the severity of the epidemic and its wide spread. The appearance of an epiphytic population of Erwinia amylovora before an epidemic occurrence is obvious.

INTRODUCTION

Fire blight caused by the bacterium Erwinia amylovora was found, for the first time in Greece in 1984 (Psallidas, 1987) and up to 1987 it had not caused any serious problem for pear and apple trees, except probably for the cases of the islands of Crete and Lesbos. A serious outbreak of the disease suddenly occurred in the spring of 1987 when the disease was found almost in every pome fruit growing area.

The influence of climatic factors, especially temperature and rain, on the epidemiology of the disease has been recognized and risk assessement systems based on these factors have been developed in order to predict disease occurrence (Billing, 1980 a,b; Thomson et al., 1982; Zoller, 1985).

These risk assessement systems have also been used to explain past disease epidemics and to evaluate the potential risks for fire blight establishment in a given area. Applying Billing's

system in four places of Greece, Jacquart and Paulin (1986) distinguished them in high risk (Korinth and Larissa) and low risk (Tripolis and Aghialos) areas. After the fire blight epidemic in 1987, Lecomte and Tsiantos (1988) compared the climatic risks of fire blight in Aghia, Larissa from 1984 to 1987.

In the present work a comparison between the theoretical risks for fire blight activity, based on the climatic data and the actual disease appearance in the areas of Tripolis (low risk) and Korinth (high risk) is attempted.

METHODS

Billing's "revised" system (Billing unpublished report) was used for fire blight risk assessement. D-periods start when the infection risk score is ≥ 2. For the graphical presentation the old Billing's system was followed (Billing 1980, a,b.). The climatic data were obtained from the local stations of the Meteorological Office of Greece.

For the detection of epiphytic populations of Erwinia amylovora, samples of 25 flowers were collected randomly at about 50% bloom and after full bloom.

The leaf samples consisted of 20 leaves, collected from different parts of the tree canopy at different dates. Both leaf and flower samples were processed according to the method of Ishimaru and Kloss (1984). Nutrient Agar + 5% sucrose + cyclohexamide was in addition to CCT medium used for Pseudomonas syringae growth. For the detection of Erwinia amylovora on branch surfaces pieces of adhesive paper tape (5x5 cm) were stuck on the surface and then laid on the surfaces of CCT medium in petri dishes and incubated at 26°C.

RESULTS AND DISCUSSION

The theoretical potential risks for fire blight activity during the spring of the years 1985 to 1989 are shown in figure 1.

From the analysis of these graphs it can be concluded that: 1985 and 1986. Both years were unfavourable for fire blight in Tripolis for the main pear varieties since there is no D-period ending before or during bloom. 1985 seems to be more dangerous than 1986 especially for Passa-crassana because of the 5 warm

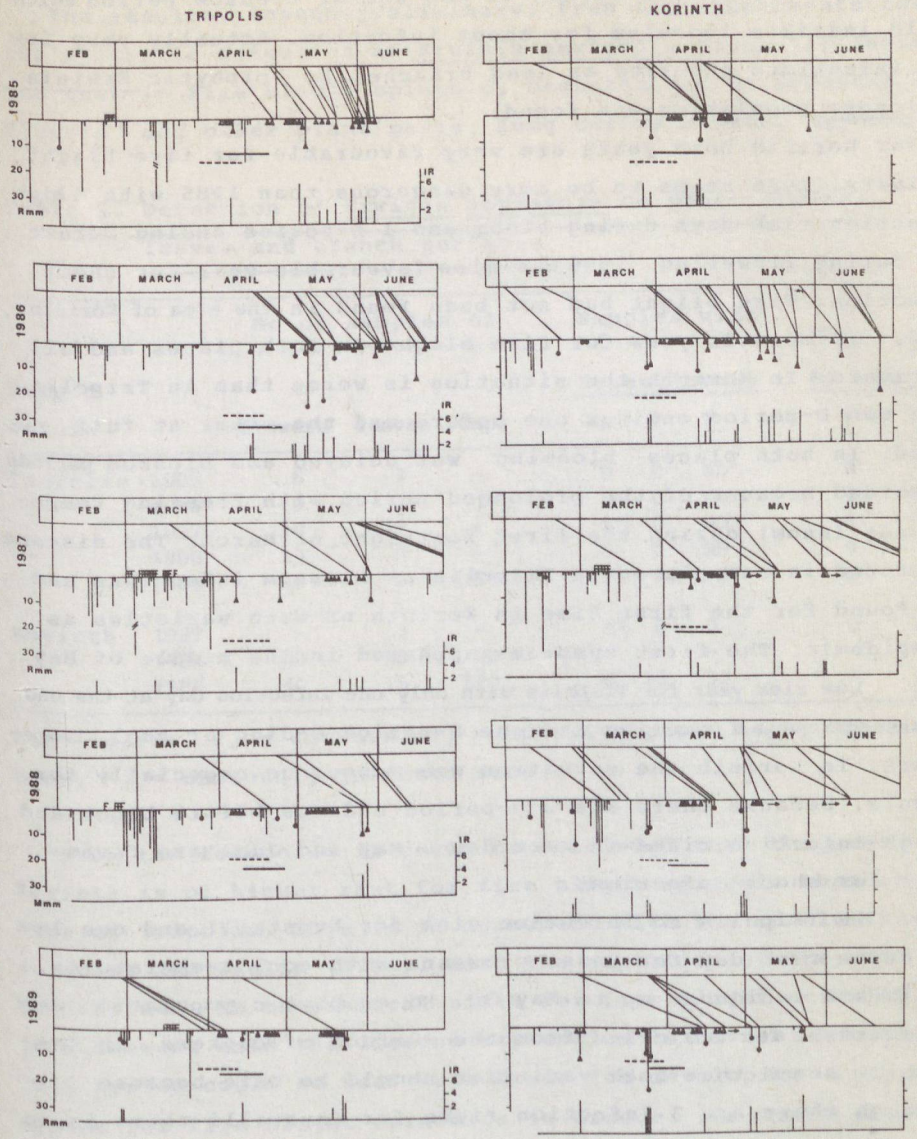


Fig. 1. Theoretical potential risks for fire blight activity in Korinth and Tripolis for the period from 1985-1989. Sloping lines : calculated D-periods. Downward vertical lines : rain. Upward vertical lines : Infection Risks (IR) score. ●,=5 < PD < 9 ▲ =PD ⩾ 9., F,=Frost Blossom period---Krystalli, ——— Kondula

days and the D-period ending at the end of blossom period which could initiate inoculum for shoot infection. Actually very few new infections occurred as dead branches. No epiphytic Erwinia amylovora population was found.

For Korinth both years are very favourable for fire blight activity. 1986 seems to be more dangerous than 1985 with 4 high infection risk days during bloom and 1 D-period ending before and during flowering. 1986 was also favourable year for shoot infection. Fire blight had not been found in the area of Korinth. 1987. Favourable year for fire blight in both places and all cultivars. In Korinth the situation is worse than in Tripolis with two D-period endings one before and the other at full bloom. In both places blooming was delayed and blossom periods coincided because of the prolonged period with freezing temperatures (snow) during the first fortnight of March. The disease was found in new places in Tripolis as blossom infections and was found for the first time in Korinth on both varieties as an epidemic. The first symptoms appeared in the middle of May. 1988. Low risk year for Tripolis with only one infection day at the end of Passa-crassana flowering and one D-period ending at cool temperature. In Korinth the situation was dangerous especially for Kondula. Because there are 3 D-period endings before bloom and enough infection risks in warm days. May and June are favourable for shoot infections.
1989. In Tripolis no infection risk for Krystalli and one low infection risk day for Passa-crassana with cool temperatures. No disease incidence up to May 30. No epiphytic population was found except for two cases from the surface of cankers. In Korinth it seems that both varieties should be safe because although there are 3 infection risks for Krystalli there is no D-period ending before or during bloom. The real situation of the disease does not fit the theoretical one. Inoculum was detected on the 7th of March on the surface of branches with cankers and on flowers collected on March 21 and 29. The first symptoms appeared on Krystalli on the 3rd April. Krystalli was badly affected by the disease through blossoms while Kondula was rarely infected. It seems that the D-periods were overestimated because of the dry and warm winter.

The results, though preliminary, from the experiments for the epiphytic detection of Erwinia amylovora (Table 1) indicated that in fire blight epidemic, bacteria can be detected on flowers and other plant parts, long before symptom appearance.

Table 1. Detection of Erwinia amylovora on pear, flowers, leaves and branch surfaces

Sample origin		No of samples of: flowers	leaves	tape	Samples with: Er.amylovora L	F	T	Ps.syringae L	T
Tripolis	1985	6	3	-	0	0	ND	0	3
	1986	9	1	-	0	0	ND	0	6
	1987	10	2	-	1*	0	ND	1	7
	1988	13	0	-	0	0	ND	0	4
	1989	30	0	50	0	0	2*	0	0
Korinth	1987	-	3	-	1*	0	0	0	0
	1988	ND	ND	ND	-	-	-	-	-
	1989	40	15	120	0	10	53	-	-

L, = leaves, F, = flowers, T, = adhesive paper tape, ND, = Not Done, *, = obviously infected tree.

From the foregoing it could be concluded that the area of Korinth is of higher risk for fire blight epidemic than the area of Tripolis. This is in agreement with the conclusions of Jacquart and Paulin (1986). With the exception of the year 1989 for Korinth the theoretical risks for fire blight activity are in agreement with the disease expression in nature. Consequently this system could be used as a basis for developing a prediction system for fire blight epidemics in Greece. Our results are also in agreement with those of Lecomte and Tsiantos for the area of Aghia. The years 1985 to 1987 are considered favourable for fire blight activity at bloom and post bloom periods.

The sudden epidemic in 1987 although is justified by the weather data analysis is still unexplained because the risks for fire blight activity during the spring of 1987 were not higher than the risks of the previous years 1986 and 1985. The only exception was the prolonged period with temperatures below zero during March;which occurred

all over Greece (unusual). The first detection of the disease in an area with the lower risk, according to weather analysis is another mystery about fire blight occurrence in Greece.

ACKNOWLEGEMENTS

The authors are very grateful to Dr E. Billing for her kindness to help us to initiate this work and to provide us all the details about her "revised" system.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

CHARACTERISTICS OF THE TRANSMISSION OF PSEUDOMONAS SYZYGII, THE CAUSE OF SUMATRA DISEASE OF CLOVES, BY HINDOLA SPP. (HOMOPTERA: MACHAERODIDAE) IN INDONESIA

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Repeated transmission tests showed that the tube-building cercopoids *Hindola fulva* (in Sumatra) and *H. striata* (in Java) were natural vectors of Sumatra disease of cloves, caused by *Pseudomonas syzygii*. In tests on experimental acquisition and transmission of *P. syzygii*, *H. striata* collected from a disease-free area acquired bacteria within 4h of access to infected plants and transmitted them within 4h of access to healthy plants. Transmission was persistent and occurred within 24h of acquisition feeding but there was some evidence for a short latent period between acquisition and transmission of bacteria. After acquisition feeding, *P. syzygii* was isolated from 67% of insects that transmitted the disease and also from 3% of those that did not. Times to death of test plants following first exposure to both naturally- and experimentally-infective insects showed log-normal distributions with similar slopes. Younger (1 yr) seedlings died slightly sooner (RT_{50} = 200 days) than older (2-3 yr) test plants (RT_{50} = 280 days) and the bacterial content of test plants was positively correlated with time to death. These features were similar to those observed in mechanically-inoculated test plants.

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

SURVEY OF PHYTOPATHOGENIC BACTERIA RECOVERED FROM COMMERCIAL TOMATO SEED AND THEIR SECONDARY SPREAD WITHIN TOMATO TRANSPLANT FIELDS

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ABSTRACT

Nine semiselective media were compared for seedborne recovery of the major bacterial pathogens of tomato. Clavibacter michiganensis subsp. michiganensis (CMM) was the most prevalent bacterium and was detected in 1.7% of lots tested at an estimated level of one infested seed in 30,000. Pseudomonas syringae pv. syringae and pectolytic xanthomonads were recovered on modified KMB and a carboxymethylcellulose medium, respectively. Except for CNS, P. aeruginosa was a major problem as a background contaminant and occurred with high frequency in tomato seed lots. Passage of seed washes through a 5.0 um filter reduced some bacterial and most fungal contamination but also resulted in a 10 fold reduction of the target organism. Modified CNS medium was the most consistent medium for the recovery of CMM and was capable of detecting 10^2 cfu/ml in controlled studies. CMM was detected in 0% and 8.3% of transplant fields in 1987 and 1988, respectively. Although latent periods ranged from 3-5 wks, under optimum conditions plants were infectious within 2 days. Plant clippers, used in the routine culture of southern tomato transplants, were capable of disseminating the bacterium at least 50 m in seedling beds and could result in 5-7 secondary cycles in transplant beds.

INTRODUCTION

Tomato (Lycopersicon esculentum Mill.) transplants produced in southern Georgia for use in the eastern United States and Canada are subject to inspection by the Georgia Department of Agriculture for plant diseases. The certification of transplants begins with the testing of seed and includes inspections of plants in the field. Clavibacter

michiganensis subsp. michiganensis (Smith) Davis, Gillaspie, Vidaver, and Harris (CMM), which causes bacterial canker, Xanthomonas campestris pv. vesicatoria (Doidge) Dye (XCV), which causes bacterial spot, and Pseudomonas syringae pv. tomato (Okabe) Young, Dye, and Wilkie (PST), which causes bacterial speck, have resulted in significant losses in past seasons. All three bacteria can be seedborne, and once present are disseminated by various means, including the clipping of transplants. The current seed tests used by the State of Georgia are nonspecific and one cannot draw definitive conclusions from them about the potential infestation of tomato seed with bacterial pathogens.

Recently, there have been reports of semiselective media for CMM (and related organisms) [3,4,6], XCV [1,8], and PST [2] (and related fluorescent pseudomonads [9]). This report describes the evaluation of direct plating assays with these media as part of a comprehensive program for detecting the three major bacterial seedborne pathogens of tomato. The relationship of seedborne infestation to incidence and dissemination in the field is discussed.

MATERIALS AND METHODS

Bacteria were extracted from seed using the procedures of Fatmi and Schaad [4]. Briefly, a total of 400 seedlots each containing 72 g of tomato seed (approximately 30,000 seeds) were tested. Each lot was divided into three subsamples of 24 g and was processed in 150 ml of buffer (0.05 M K_2HPO_4-KH_2PO_4, pH 7.4) in a Stomacher homogenizer. Rinsates were concentrated by centrifugation at 12,000 g for 15 min. Samples were suspended in 5 ml of buffer and serially diluted (1:9). Aliquots of 0.1 ml taken from the source out to the 10^{-3} dilution were spread onto various media. Portions of select samples were passed through a 5.0 um filter prior to plating and were compared to nonfiltered counterparts. To test recovery, 0.1 ml of suspensions containing 10^9 cfu/ml and 10^5 cfu/ml of the three pathogens was added to different seed samples prior to homogenization. The following semiselective or diagnostic media were evaluated: KBC [9], KMB [7], and GAL [2] for PST; CNS (modified by the omission of chlorothalonil and lithium chloride) [6], SCM [4], and KBST [3] for CMM; and for XCV tween medium B [8], ND [1], and CMC which contained 1 g/L of NaCl, 0.2 g/L of $MgSO_4 * 7H_2O$, 1 g/L of $(NH_4)_2HPO_4$, 0.5 g/L of KH_2PO_4, 0.025 g/L of phenol red, 3.33 ml/L of 60% DL-lactate syrup, 1.5 ml of 1% methyl green, 25 g/L of sodium-

carboxymethylcellulose, 10g/L of agar, 0.05 g/L of cephalexin, and 0.05 g/L of cycloheximide. To deter the formation of clumps, the CMC and agar were added while slowly blending, and to avoid boiling over, the highly viscous medium was sterilized in quantities of 500 ml in a 2 l flask. Antimicrobial agents were filter-sterilized and added aseptically after autoclaving.

CNS was used with a stem-printing technique [5] to assess the incidence of bacterial canker, which generally is in the incubation phase and symptomless in transplant beds. The dissemination of CMM in the field also was examined with the stem-printing technique.

RESULTS

Compared to standard microbiological media, efficiency of recovery of CMM on CNS, SCM, and KBST was 64, 24, and less than 1%, respectively. Recovery of XCV on tween B, CMC, and ND was 92, 92, and 0%, respectively. Recovery of PST on KBC and GAL was 83 and 90%, respectively. Colonies of the various bacteria appeared on their respective media as described in the literature. Colonies of XCV were recognized on the CMC medium by the deep pits that they formed. CMM was detected on CNS, SCM, and KBST when added at levels of 10^6 cfu/ml and 10^2 cfu/ml of buffer prior to centrifugation. XCV was detected at both concentrations on Tween B and CMC when added in the same manner, and PST was detected on KMB and KBC only at the higher concentration. Passage of the concentrated seed washes through a 5.0 um filter decreased fungal contamination and alleviated the problem of plugged pipets, but resulted in an approximate 10 fold reduction in the recovery of the pathogens in question.

A survey of 400 tomato seedlots resulted in the detection of CMM in 1.7% of the lots tested, whereas PST and XCV were not detected. However, P. syringae pv. syringae and an avirulent, pectolytic Xanthomonas were recovered in 0.5% of the seedlots, and once within the same seedlot. In those lots positive for CMM, the bacterium was recovered 50% of the time from the nondiluted wash concentrate in only one of three subsamples of 10,000 seed tested. When present, CMM was detected in two or all three subsamples 37.5% and 12.5% of the time, respectively. CNS was the only medium on which all seedlots positive for CMM were detected. Only 25% and 63% of the seedlots determined positive with CNS were considered positive with KBST and SCM, respectively.

Based on the detection of CMM in all subsamples and by the number of colonies on the 10^{-3} dilution plate, a seed source of tomato cv. H-722 was used for estimating infestation of individual seeds. Log_{10} mean populations of CMM were 2.57 and 3.12 for individual seeds and bulk sampling, respectively, where n=100. The Log_{10} mean of CMM populations from individual seeds when there was no saprophytic background was 1.95, however when there were numerous saprophytic competitors the Log_{10} mean population from single seeds was 3.43. The range of population in single seeds was from 1 x 10^1 to a maximum of 2 x 10^6 cfu/seed/ml, with 38% of the seed infested.

CMM was detected in 0% and 8.5% of the commercial transplant fields sampled in 1987 and 1988, respectively. However, only 0.01% of the total plants surveyed (n=30,000) were positive and none displayed symptoms. Presence of CMM in the field was correlated to infestation of seedlots.

Sampling of transplants down the plant bed at different distances from a point source of inoculum revealed that the bacterium was transmitted by clipping. The bacterium was detected at a distance of 50 m from the source of inoculum, and a gradient occurred within the first 10 m. Out of 3200 plants tested, only one plant showed signs of bacterial canker at harvest, which was 5 wks after inoculation. However, the bacterium could be detected 1-2 cm below the clipped terminal end only 2 days after clipping.

DISCUSSION

Of the current possible techniques for detection of plant pathogenic bacteria in tomato seed, the use of an agar plating system seemed best suited for the detection of several different pathogens and for use in a diversity of laboratories. Furthermore, most of the techniques [4] and different media required [1,2,3,4,6,7,8,9] have been reported. The CMC medium proved useful as it was unlikely to confuse XCV with any other bacterium as most bacteria do not form pits on this medium. This was beneficial when a saprophytic, yellow, bacterium capable of forming white crystals was found on the tween medium. However, the largest problem encountered was the routine presence of Pseudomonas aeruginosa. It was particularly troublesome for the detection of PST. However, the results of this survey generally indicate that an agar plating method could be used on a large-scale basis for screening of the three major seedborne bacteria likely to be in tomato seed. Because of the diverse background

microflora encountered, it is recommended that 2-3 different semiselective medium be employed for each pathogen being tested. Thus a failure of one medium due to background contamination would not result in an ineffective test.

Based on the results of the detection of CMM in individual seeds, one could speculate that the low levels and distribution of CMM detected in most of the samples were due to one seed in 30,000 being infested. However, even with only one seed in 30,000 being infested, the total number of infected plants per hectare in a transplant operation would range from 32-48 plants.

In 1988, the detection of CMM in transplant beds was correlated to positive seedlots as determined by the agar plating methods outlined. Furthermore, plants remaining symptomless for up to 5 wks were infectious after only 2 days. Because transplants are routinely clipped, it is possible for bacterial canker to produce multiple secondary cycles long before symptom development. This potential for spread demonstrates the need for starting with clean, healthy seed.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

RELEASE OF SOIL-BORNE XANTHOMONAS CAMPESTRIS PV. CAMPESTRIS IN THE PHYLLOSPHERE OF CABBAGE PLANTS

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INTRODUCTION

Xanthomonas campestris pv. *campestris* (Xcc), the causal agent of the black rot disease, is a worldwide threat for cruciferous crops. A number of agricultural practices are recommended to prevent or to control this disease (Williams, 1980). These recommendations are mainly based upon the knowledge of the various aspects of the disease cycle. Many, but not all, aspects are understood in detail. One of those less understood aspects is the role of soil-borne inoculum in the disease cycle. From the work of Schaad and White (1974) it is known that Xcc can survive in the soil for at least one year. Our question is how important soil survival is for the initiation of a black rot epidemic as compared to seed-borne inoculum. Quantifying the different phases in the disease process in relation to environmental factors will be necessary. Before this can be done we must know what phases in the disease cycle can be distinguished. In this paper experiments are described in which soil-borne Xcc was able to reach the phyllosphere and first estimations are made of important epidemiological parameters like latency period and incubation time in relation to relative humidity. The release of Xcc in the phyllosphere was recorded by its presence in the guttation fluid.

MATERIAL AND METHODS

Host plant. Cabbage plants (*Brassica oleracea var. gemmifera* cv. Titurel) were used in the 3 to 5 leaf-stage. Each treatment consisted of 4 to 6 plants.

Pathogen. *Xanthomonas campestris* pv. *campestris* (Pammel)Dowson 1939 isolate LUF107 (Xcc) was used. Suspensions for inoculation were made from two-day-old cultures on Yeast Peptone Glucose Agar (0.5% w/v yeast extract, 1.0% w/v peptone, 0.5% w/v glucose, 1.5% w/v agar Oxoid nr.3).

Root inoculation. This was done by damaging the roots of the plants by cutting through the soil with a knife. A bacterial suspension (20 ml with about 10^8 cfu/ml) was carefully poured into the soil through a funnel in order to avoid contamination of the soil surface with bacteria. The roots were damaged in order to obtain a higher frequency of infected plants.

Conditions for plant growth. After inoculation the plants were incubated in a hand-in or walk-in growth chamber with a temperature of 20°C during the night (8 h) and 25°C during the day (16 h). Relative humidity (RH) was the subject of study and was 100 or 80 % continuous or varied from 100% during the night period and 75% during the day period.

Collection of the guttation fluid. At the beginning of each day period guttation droplets were collected with a sterile pipette. In the first experiments the fluid from all the plants were pooled and in later experiments it was collected from each leaf separately. Small aliquots of these droplets were streaked onto Tween medium A (McGuire et al. 1986) and YPGA and incubated at 27°C. For confirmation of the isolations pathogenicity tests were carried out by injecting the bacteria into the leaves of cabbage plants.

RESULTS

Xcc in guttation fluid

It appeared to be relatively easy to find Xcc in guttation fluid of root-inoculated cabbage plants. Even when the droplets from the same leaves harboured large populations of other bacteria in the previous samplings, Xcc became dominant soon after its first appearance in the guttation fluid. This was determined by comparing colony counts on YPGA and on Tween medium A. Xcc was found in densities up to 10^6 cfu/ml. The guttation fluid could only be collected from turgid leaves. Wilting leaves do not guttate. The

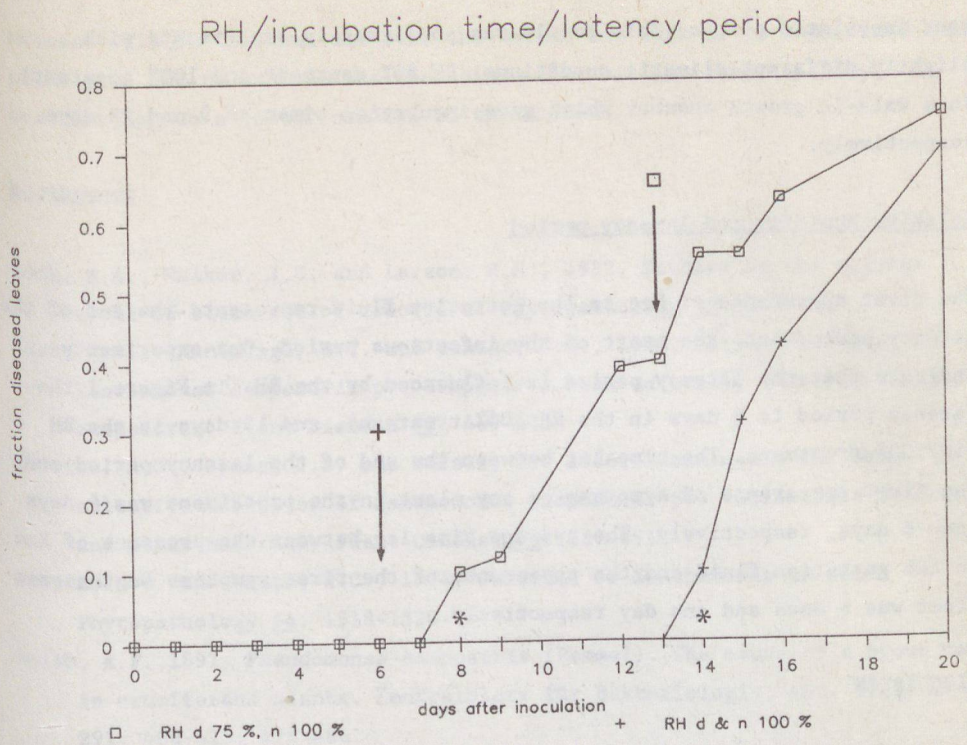


Figure 1. The effect of relative humidity (RH) on the fraction of total number of leaves with black rot symptoms on root-inoculated cabbage plants. The arrows indicate the first appearance of Xcc in the guttation fluid. The asterisks (*) indicate the first visible symptoms.

leaves with guttation droplets did not show any symptoms of the black rot disease at the time of sampling the fluid. Xcc was not observed in the guttation fluid from all plants that eventually showed black rot symptoms.

Relative humidity and incubation time

Figure 1 shows that the relative humidity (RH) influenced the incubation time. With root-inoculated plants incubated in a constant RH of 100% the first symptoms appeared after 13 days. With a RH of 75% during the day period and 100% during the night period the first symptoms were visible after 7 days. Thus, drier conditions accelerated the symptom development in

root-inoculated plants. This result was confirmed in experiments with slightly different climatic conditions (RH 80% constant and 100% constant) in a walk-in growth chamber which gave incubation times of 9 and 23 days, respectively.

Relative humidity and latency period

The first appearance of Xcc in the guttation fluid represents the end of the latency period and the start of the infectious period. Our experiments indicate that the latency period is influenced by the RH. In Figure 1 the latency period is 6 days in the RH 100% treatment, and 13 days in the RH 75%/100% treatment. The time lag between the end of the latency period and the first appearance of symptoms on any plant in the experiment was 6 days and -8 days, respectively. The average time lag between the presence of Xcc in the guttation fluid and the appearance of the first symptoms on the same plant was 6 days and one day respectively.

DISCUSSION

The role of guttation fluid and hydathodes as the entrance of Xcc into the host plant has been emphasized many times in literature and is already known from older literature (cf. Smith, 1897, Cook et al. 1952, and Stapp,1958). In contrast, the guttation fluid as source of inoculum received little or no attention. Xcc has been found in aerosols and in the phyllosphere of native cruciferous and non-cruciferous weeds (Kuan et al. 1986). However, it is not clear from where these populations originate. Do they originate from diseased plants or are they the result of growth of Xcc populations in the phyllosphere? For a more profound understanding of the epidemiology of this plant disease knowledge of inoculum sources, qualitatively as well as quantitatively is of major importance. In combination with data concerning latency period, infectious period and to a lesser extent the incubation time it belongs to the major parameters in plant disease epidemiology. Quantifying these parameters will help to understand what is important in the black rot disease.
The disease cycle of the black rot disease is known. Although the relative importance of contaminated seed, survival of the pathogen in plant residues and non-host plants are difficult to estimate and will certainly be different in each situation. Nevertheless, the principles of the disease

process will be the same all over the world. Our task is to understand these principles and to analyse the mechanisms that are involved, thereby contributing to a better control of the disease.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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CLAVIBACTER XYLI SUBSP. CYNODONTIS: AN ENDOPHYTIC BACTERIUM IN CORN

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Clavibacter xyli subsp. *cynodontis* (Cxc) was originally isolated from bermudagrass (*Cynodon dactylon*) imported from Taïwan to the United States (2, 7). Subsequently, Cxc was found to be distributed widely in the United States (1, 5, 8). The bacterium endophytically colonizes the xylem of the plants without external effect in normal growth conditions.

Cxc has been demonstrated recently to serve as a systemic endophyte in corn (*Zea mays*) and other plant species (3, 4, 9). In the United States Cxc is being developed as an artificial symbiont for the delivery of biopesticidal gene products inside plants. Specifically, a single gene from *Bacillus thuringiensis* subsp. *kurstaki* (Bt) encoding for delta endotoxin production has been introduced into Cxc to obtain a recombinant strain. The target of this product is the European Corn Borer (8).

A research program has been conducted in France with an indigeneous isolate of Cxc (5). The two main objectives of this study were :

- to study the effects of Cxc colonization on corn under the European conditions of cultivation.
- to analyze various environmental fate parameters of Cxc.

MATERIALS AND METHODS

Corn cultivation

The experiments were conducted in Avignon (southeastern France) under mediterranean climatic conditions and using cultural practices common to this area. All plots were irrigated.

Inoculation technique

Plants were inoculated at the 4-6 leaf stage (3-4 cm above the soil level) by using a modified N° 20 sewing needle inoculating device, calibrated to deliver approximately 4 ul of inoculum (1×10^{10} to 1×10^{11} CFU/ml of indigeneous isolate CDA 15 or CDA 15 S+, a spontaneous streptomycin-resistant mutant). Control plants where inoculated with the sterile buffer.

Detection of Cxc in plants

Colonization by Cxc was assessed 6-8 weeks after inoculation in both treated and control plants. Sap was extracted from surface disinfested (30 sec. in 95% ethanol) of the oldest healthy leaf midribs and placed onto SCM or GCAB agar, and by radioimmunoassay (10).

Spread from colonized plants

Uninoculated corn growing in plots adjacent to Cxc-colonized corn were assayed in each of the varieties in yield tests in 1987. The oldest viable leaf was removed from each plant and isolations and radioimmunoassay made from surface-disinfested leaf midribs.

In 1988, 20 bermudagrass plants were interplanted between corn plants colonized by Cxc and placed direct contact to optimize conditions for transmission. To test the reciprocal, 42 grass plants colonized by Cxc were interplanted within untreated corn. In autumn, all plants were sacrificed and analyzed for colonization by Cxc as described above.

Bermudagrass and other plant species were collected at various distances from a bermudagrass plot at Frejus (Var) where Cxc had been previously isolated (5). Plants were collected immediately adjacent to the plot, up to 16 m. Samples were collected in July and October along an axis running perpendicular to the plot. Isolations were made as previously described.

Persistence of Cxc on plant surfaces

Four different species, two known hosts of Cxc, corn (Zea mays) and marigold (Tagetes patula), and two non hosts, madagascar periwinkle (Catharanthus roseus) and radish (Raphanus sativus) were sprayed with a Cxc suspension (CDA 15 S+ : 1×10^8 cells/ml). Plants were grown in a greenhouse in 1987 and in an insect proof tunnel in 1988. Enumeration of the epiphytic population has been regularly analyzed (detection limit 1×10^{-3} to 1×10^4/g fresh weight).

Persistence of Cxc in corn plant debris

Persistence studies were conducted in mature Cxc (CDA 15 S+)-colonized plants (left standing) or debris (cut into fragments left on the soil or buried).

Samples were collected at three-week intervals, disinfested and presence of Cxc analysed by dilution plating on selective medium.

Transmission of Cxc by aphids

The ability of 3 aphid species (Rhopalosiphum padi, Sitobion avenae, Myzus persicae) to transmit Cxc from colonized to uncolonized corn plants was tested within growth chambers.

Aphids populations were transferred onto Cxc-colonized corn plants (30 days post-inoculation) for an acquisition feeding period of 1, 2 or 3 days. Subsequently, aphid population were transferred to uninoculated corn (trap plants). Isolations were made from trap plants after at least 8 weeks.

In a second laboratory study each of there aphid species were fed through a parafilm membrane, on sucrose solution (2%) containing Cxc in suspension ($\sim 1 \times 10^8$ CFU/ml), then transferred and allowed to feed through a membrane into sterile solution of sucrose. After transfer time, Cxc has been detected in this second solution by plating on appropriate medium (level detection : 2.5 bacteria/ml).

TABLE 1 - EFFECT OF Cxc COLONIZATION ON CORN GROWTH (average of final heights of plants)

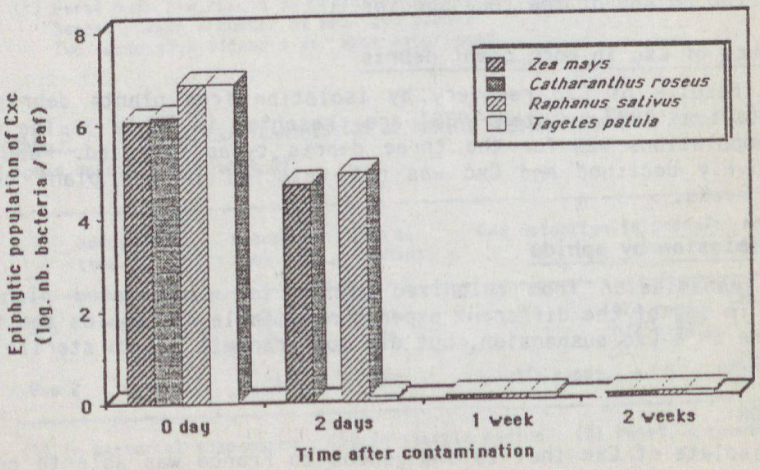
	1987 (6 varieties)						1988
	LORENA	CAROLA	SABRINA	SIRENA	LG 22 Auto	Adda R 90	CAROLA
Colonized plants (+ Cxc)	203.7	190.1	199.0	179.4	185.7	204,0	150.4
Control plants (- Cxc)	198,6	180.0	194.2	178,5	183.0	204.3	152.4
Difference	NS	*	NS	NS	NS	NS	NS

TABLE 2 - EFFECT OF Cxc COLONIZATION ON CORN YIELD (average 5/6 blocks at harvest)

	1987 (6 varieties)						1988
	LORENA	CAROLA	SABRINA	SIRENA	LG 22 Auto	Adda R 90	CAROLA
Colonized plants (+ Cxc)	39,7	41,0	31,2	35,5	28.8	40.4	24.9
Control plants (- Cxc)	40,9	40,0	30,4	36.6	30.2	39.2	26.5
Difference	NS	NS	NS	NS	NS	NS	- 6%**

NS : not significant * : significant (0,05) ** : significant (0,01)

FIGURE 1 : PERSISTENCE OF Cxc ON LEAF SURFACES (EPIPHYTIC STAGE)



RESULTS

Corn colonization

Cxc colonized all corn varieties but there are some genetic differences, for example in 1987 we observed an excellent colonization with 2 varieties (Lorena 100%, Carola 85%) moderate with 3 varieties (Sabrina 75%, LG 22 Auto 65%, Sirena 55%) and limited in one variety (Adda Rx 90 28%).

Colonization had no apparent effect on plant appearance. Growth was not altered by the presence of bacterium (Table 1). On the contrary, in 1987, with the variety Carola those plants inoculated with Cxc were significantly taller than the control plants. This difference is not observed in 1988 with the same variety.

Colonization had no effect on the yield and total biomass for the 6 varieties compared in 1987 (Table 2). However, a reduction (-6%) was observed in 1988 with the variety Carola.

Spread from colonized plants

Of 1134 corn plants, grown adjacent to colonized corn and analyzed by culture on SCM agar or radioimmunoassay, none were found to be colonized by Cxc.

No plant to plant transfer of Cxc by direct contact was observed between Cxc-colonized corn and 20 bermudagrass plants between Cxc colonized bermudagrass and 42 corn plants.

Cxc was isolated form bermudagrass growing up to 2 meters from the edge of a variety test plot containing Cxc-colonized bermudagrass planted 4 years before. Individual analysis (isolation) of 291 wild plants belonging to 11 different species growing at various distances from the plot did not yield Cxc.

Persistence of Cxc on plant surfaces

In 1987, the bacterium was not detected on any of the species after eight days (Figure 1). In 1988 the results were comparable. After 2 days Cxc was detected only on corn, redish and marigold. After 4 days Cxc was not detected on any of the four species.

Persistence of Cxc in corn plant debris

The results of Cxc recovery by isolation from plants debris after harvest (autumn 1987, winter 1988) are presented in Table 3. The decline of Cxc populations was for the three debris types compared. Populations progressively declined and Cxc was not detected in the plant sections after 12 weeks.

Cxc transmission by aphids

No transmission from colonized plants to uninoculated plants was observed in any of the different experiments (Table 4). Aphids fed through a membrane in a Cxc suspension, but did not transmit Cxc to sterile medium (Table 5).

DISCUSSION

An isolate of Cxc that is indigenous to France was able to colonize corn under normal conditions of cultivation in the south of France. As observed in the United States, colonization generally had little effect on

TABLE 3 : PERSISTENCE OF Cxc IN CORN DEBRIS

Analysis time (1)	Placement of samples					
	Standing plants		on soil surface		burried	
Harvest	29/35[2]	83%	29/35	83%	29/35	83%
3 weeks	22/24	91%	11/24	45%	6/22	27%
6 weeks	10/23	43%	6/17	35%	7/17	41%
9 weeks	2/23	8%	0/10	0%	3/20	15%
12 weeks	0/23	0%	0/17	0%	0/18	0%

(1) after harvest
(2) first number are isolations with Cxc and second number is the total number of samples analysed (maximum number of samples after harvest is 24. In a number of instances, final results were obtained from less than 24 samples due to excessive contamination by soil-borne organisms.

TABLE 4 : STUDY OF Cxc TRANSMISSION BY 3 SPECIES OF APHID

3 aphid species :
Rp = Rhopalosiphum padi ; Sa = Sitobion anevae ; Mp = Myzus persicae

	Contact time on colonized plant	No of aphids	Rp		Sa		Mp	
Exp 1	2 days	20	0/18[1]	0/6	0/16	0/10	0/4	
Exp 2	3-1 days	100	0/8	0/2	0/3	0/2	0/3	0/2

(1) First number = result of detection of Cxc in transfer plants (trap-plants)
Second number = number of analyzed plants
Two successive attempts for each experiment

TABLE 5 : STUDY OF Cxc TRANSMISSION BY APHIDS FEEDING THROUGH A PARAFILM MEMBRANE

3 aphid species (see Table 4)

	acquisition time (1)	transfer time (2)	No of aphids	Cxc detection in transfer medium Rp	Sa	Mp
Exp 1	30mn	30mn	20		0/4 repet. (3)	
	2h	2h	20		0/2 repet.	
Exp 2	2h	2h	100	0/3 repet.		0/3 repet.

(1) in bacterial suspension (2) in sterile medium (3) repet. = repetitions

the growth and the yield. Yield studies are being continued.

The results reported in these studies corroborate results of experiments conducted in the United States with wild-type isolates. In addition, the lack of transmission of Cxc by aphid species has been demonstrated. In 1988, field releases, designed to analyze the environmental behaviour of a genetically engineered strain of Cxc (Cxc/Bt) were conducted at two sites in the United States (Beltsville, MD and Ingleside, MD). This strain behaved similarly to the wild-type strain (6). Field releases comparing Cxc and Cxc/Bt were expanded in 1989 to eight sites in four states (Maryland, Nebraska, Minnesota and Illinois).

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

BACTERIAL DISEASES OF GRAMINEAE IN BULGARIA

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The bacterial diseases of Gramineae have been the object of significantly smaller number of investigations than viral and fungal diseases. The state of this problem in Bulgaria is discussed in the present review.

ECONOMIC IMPORTANCE

The losses caused by bacterioses in Gramineae are both quantitative and qualitative. The basal glume rot of barley induces reduction in the total productive tillering and the grain weight per plant [15]; the water balance is also disturbed [2]. In wheat bacterioses a decrease of yield by 5 to 60% is established [9]. In wheat plants artificially inoculated with Pseudomonas syringae pv. atrofaciens (PSA) a reduction is observed in: the weight of 1000 grains, the weight of grains per ear, the germination ability and energy. The changes in the physical, technological and biochemical characters of the infected grains are rather unfavourable [24].

SPECIFIC DIVERSITY AND FEATURES OF PATHOGENS

Of the Gramineae bacterioses in Bulgaria, the basal glume rot is of major importance. The PSA agent is isolated from wheat [5,8], barley [13], triticale and cereal grasses [6]. In addition to this pathogen the following have also

been reported: Pseudomonas syringae (PS), Xanthomonas campestris pv. translucens (XCT), Erwinia carotovora, and Erwinia herbicola [9]. The latter are represented by less virulent strains as compared to bacteria from the group of P.syringae. Four PSA races have been described on the basis of quantitative evaluation of the resistance of cvs Sadovo 1 and Thracia [18]. The serotyping of PSA has shown a great diversity in the surface structures [4], which is related to the greater variety in the virulent characters, too.

SYMPTOMOLOGY

The basal glume rot symptoms vary in dependence of the cultivar, pathogen and environment conditions [23]. In general, the symptoms in wheat, barley, rye, and triticale can be referred to three groups: root and basal rot, spotting of ear, leaves and stem, ear deformation with sterility, porous grain formation or speltoidity. Necrotic root damages of the cv. Kenya Leopard have been established at embryo-culture inoculation with PSA [1]. PSA is also isolated from damages in glume tips, a symptom described as typical for XCT.

INFECTION SOURCES AND FACTORS FAVOURING THE DEVELOPMENT OF BACTERIOSES

Primary infection sources of bacterial diseases in Gramineae are seeds, vegetable wastes, and some perennial cereal grasses [6,9,16,19]. With PS the existence of epidemiological relation between fruit and cereal plants is very probable [6]. A common infection of PSA and Fusarium culmorum has been established on winter soft wheat [22]. Both pathogens develop in almost the same climatic settings and are not inhibited in vitro.

Moisture is a main factor for the distribution of patho-

gens causing bacterioses in cereals, especially when combined with suitable sensitive stages of development and a sensitive cultivar [25]. Early sowing is favourable for the development of bacterioses [9].

Epiphytoties from basal glume rot of wheat were registered in Bulgaria in 1971, 1973, and 1975 [7,12]. In separate regions of the country, the disease reached a strong development in 1979, 1981, and 1986, too.

IMMUNITY EXAMINATIONS

During the epiphytoty of wheat basal glume rot in 1971, the cultivars Burgas 2, Russalka, and Caucasus proved to be most sensitive [11]. A comparatively high degree of resistance to PSA was established in the cvs Sadovo 1, Sadovo S, Momchil, Katja, and Pobeda (Victory), distributed in wheat production, triticale No 7291, barley MB-35, all rye cultivars, Aegilops, and a number of cereal grasses [14,19,27,28].

The methods of artificial inoculation of plants are of great importance for the correct evaluation of varietal resistance [25,26]. The quantitative character of Gramineae resistance the application of the method makes useful for infective bacterial titration [20,21]. By the use of a single concentration, which is high in most cases, the evaluation of cultivars is not precise [3,17]. A possibility for accelerating the process through <u>in vitro</u> screening and breeding is found [29]. The confusion of the notions resistance and tolerance [10] is a common mistake [24]. Tolerant wheat cvs to PSA are Skitija, Russalka, K224, Sadovo S, and Pliska.

In Bulgaria, the investigations on cereal bacterioses are mainly directed to the examination and timely discovery of highly sensitive cultivars as early as in the initial ma-

terial and the first links of the process of breeding, the conducting of a strict control of the newly-developed cultivars, and the improvement of screening and breeding for resistance.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

A TWO DECADE EPIDEMIC OF PSEUDOMONAS SOLANACEARUM ON TOTATO IN PERU

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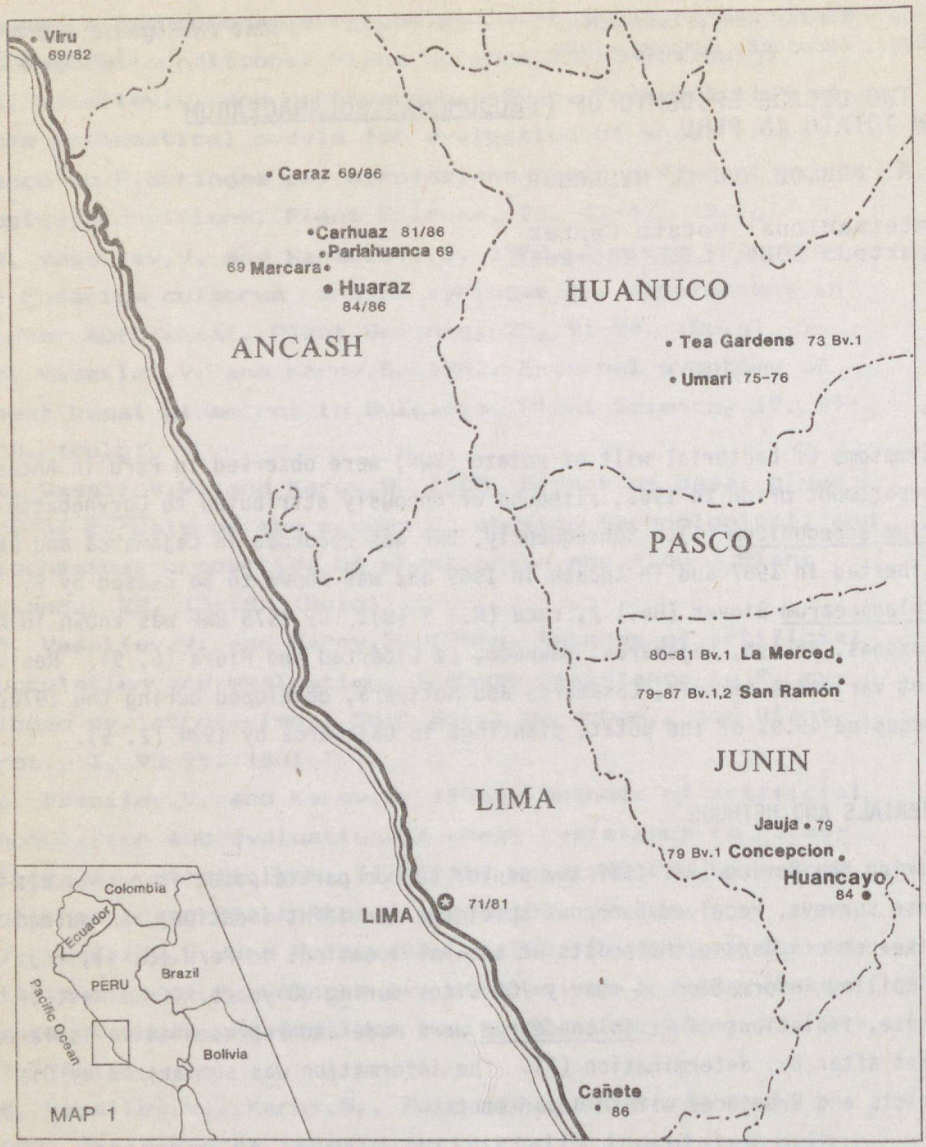
Symptoms of bacterial wilt of potato (BWP) were observed in Peru in Ancash Department prior to 1965, although erroneously attributed to Corynebacterium sepedonicum (1). Subsequently, BWP was recorded in Cajamarca and La Libertad in 1967 and in Ancash in 1969 and was shown to be caused by P. solanacearum Biovar (Bv.) 2, race (R.) 3 (8). By 1975 BWP was known in Amazonas, Ancash, Cajamarca, Huanuco, La Libertad and Piura (6, 9). Resistant varieties Amapola, Caxamarca and Molinera, developed during the 1970s, occupied 49.9% of the potato plantings in Cajamarca by 1984 (2, 5).

MATERIALS AND METHODS

During the period 1967-1987 the senior author participated in potato disease surveys, received numerous specimens for identification, and guided research of plant pathologists at several locations in Peru (8, 9), thus compiling information on nearly 700 sites during 20 years. From most of these, isolations of P. solanacearum were made and representative isolates kept after Bv. determination (7). The information was summarized by Districts and Provinces within Departments.

RESULTS

Ancash Department (Map 1): The first determination of BWP (1) was confirmed by isolations from Caraz, Marcara and Pariahuanca in the Santa River Valley of Ancash in 1969. In 1975 it occurred in all provinces of the valley, in 1981 only in Carhuaz Prov., in 1984 in Huaraz and in 1986 again throughout the valley. Altitudes of these locations ranged from 2,300 to 3,000 m. All isolates were Bv. 2. Spread was clearly linked to seed distribution patterns.



Map 1. Part of Central Peru showing incidence of bacterial wilt of potato. Numbers are the last two digits of the years of record. Years spanned are hyphenated, interruptions are indicated by a slash. Instances of Bv.1 or both Bv. 1 and 2 are shown, others were Bv. 2.

Amazonas Department (Map 2): Symptoms of BWP were seen at Pipos (2,300 m) in 1965, but isolations were not made. Samples received for isolation and identification from Chachapoyas (2,500 m) in 1987 were Bv. 2.

Cajamarca Department (Map 2): Potato cultivation is at altitudes of 2,300-3,100 m, with more than 20,000 ha of potatoes planted annually in



Map 2. Part of Northern Peru showing incidence of bacterial wilt of potato (see legend for Map 1).

two seasons (2). BWP was first recorded in 1967 in Cajamarca Province (2,700 m). Rapid spread occurred in subsequent years, and was linked to latent infection in a new highly productive potato variety, Renacimiento. In Chota, Huambos and Cutervo Provinces (valleys) fields were heavily infec-

ted by 1974 and farmers ceased to grow potatoes. From 1977, varieties resistant to BWP became very popular, especially because they were also resistant to late blight, and potato production soon surpassed its previous area. Pastures and woodlands in warmer lands (as low as 2,100 m) were plowed and planted as the potato became less a subsistence crop and markets developed in large coastal cities (2, 5). However, under warmer growing conditions they were susceptible, leading to moderate BWP incidence in the 1980s. BWP spread through the use of latently infected tubers to more distant parts of Cajamarca and to the Departments of La Libertad, Lima and possibly Piura.

Piura Department (Map 2): The Cajamarca epidemic appeared to spread into Huancabamba Province in 1970, although there are no direct roads linking these highland sites. From there it spread to Chalaco. A case of BWP in 1974 at Morropon (which at 2,000 m is too low for potato production), was caused by Bv. 1.

La Libertad Department (Map 2): BWP occurred throughout Huamachuco and Santiago de Chuco Provinces in 1970 and Tayabamba in 1979. In 1981 it was detected in Otuzco where 37% of fields were diseased by 1985. The coastal irrigated desert valley of Viru had more than half its winter potato crops infected in 1969. It was sown with seed from a Santiago de Chuco highland seed farmer. However, it did not persist in soil as was demonstrated when potatoes were replanted after one month and remained disease-free (4). A similar outbreak occurred in 1982.

Loreto Department (Map 2): At Yurimaguas (170 m), the International Potato Center tests adaptation of potatoes to hot climates. Almost every time potatoes were planted in newly cleared tropical rainforest from 1975 to 1987, BWP occurred. Isolates were either Bv. 1 or 2, or both.

Junin Department (Map 1): San Ramon and La Merced in the Chanchamayo Valley, where potatoes have also been grown experimentally in a tropical rainforest climate at altitudes of 800-1,200 m, gave similar results to Loreto. The three sites shown to the South are in the Mantaro valley at altitudes of 3,300 to 3,500 m. Only one isolation was made per site, from plants suffering from other diseases and in which BWP was latent because of low temperatures. The Huancayo and Jauja (Bv. 2, R. 3) cases were probably due to latently infected seed brought, as is known in practice when seed is scarce, from the Lima market. The Concepcion planting was with seed origi-

nating at a mid-elevation site close to San Ramon, which may explain this unusual case of Bv. 1 (R. 1) in a highland site.

Huanuco Department (Map 1): At Umari (2,400 m) in Pachitea Province, in 1975 47.5 ha of land had BWP. Numerous small farms were planted with seed from one local farm. Planting potatoes was prohibited for four years on these farms. Annual surveys of the entire province did not result in further findings. The soils of Umari were suppressive to P. solanacearum so that replanting potatoes experimentally resulted in no BWP after one year (3). BWP was caused by Bv. 1 at Tea Gardens (1,300 m) in 1973.

Lima Department (Map 1): BWP was introduced to fields in Lima in 1971 and 1981 for experimental purposes. At Cañete in 1986 varieties Molinera and Amapola were planted with seed from low, warm areas of Cutervo, Cajamarca Dept. (Map 2), and 5-10% BWP resulted. Potatoes planted 6-12 months later did not develop the disease.

DISCUSSION

BWP became a serious disease of epidemic proportions in Peru from 1967 until 1977. The unknown initial focus of infection must have been a warm climate site. Spread was principally by transport of latently infected seed tubers. Circumstances suggest that the epidemic began in Cajamarca Province, spread North and South within the Department and then to La Libertad, Lima and Piura Departments. It spread repeatedly from the highlands to the coast, with the traditional seed flow.

The periodic flare-ups in Ancash which preceded records in Cajamarca, may have had indigenous origins, or the two may be interrelated. The short lived Huanuco epidemic is probably an introduction from Ancash to which it is linked by road. The Junin highland incidences of R. 3 are of no local economic significance, however, latently infected seed (Bv. 1, R. 1) from Concepcion that was planted in San Ramon had 100% BWP (H. Torres, unpublished).

The coastal irrigated desert valley cases in La Libertad and Lima provided circumstantial evidence of suppressive factors occurring in those soils, as was also the case of Huanuco.

The epidemic situations described here involved strains of P. solanacearum, Bv. 2 (R. 3). This upland wilt can be controlled through integrated con-

trol measures. Isolated cases of lowland wilt were caused by Bv. 1 (R. 1) in Huanuco, Junin, Loreto and Piura. The lowland strains of Bv. 2 in Junin and Loreto cannot be considered as R. 3 which by definition is a cool climate "potato strain" (3). These indigenous strains differ from the highland strains in host range (9) and nutritional characteristics (Hayward et al., these proceedings).

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

EPIDEMIOLOGY OF BACTERIAL BLIGHT OF COWPEA

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Bacterial blight of cowpea (Vigna unguiculata (L.) Walp.-a legume), caused by Xanthomonas campestris pv. vignicola (Burk.) Dye, is a widespread disease which causes extensive damage in Rajasthan (1). Disease severity ranged between 7 to 16% in Udaipur region (6). Paper briefly reports the epidemiology of disease and survival of the bacterium.

MATERIALS AND METHODS

Bacterial suspension of 8.6×10^8 CFU/ml conc. was used in all inoculations. For foliar infection, on one-month-old plants were sprayed twice at 24 h interval.

For seed-borne inoculum, five seed samples were tested for XCV by 'agar plate method' (5). Seeded plates were incubated at 28 ^{o}C for 10 days and observed daily for bacterial growth. Surface sterilization of seed was done with 2% sodium hypochlorite for 5 min. Transmission of XCV was determined by growing seedlings in quartz sand.

Determining survival of bacterium, three-seed samples (Table 1) were stored in plastic containers at low (5-10 ^{o}C) and room (10-40 ^{o}C) temperatures; 10 seeds per lot were crushed in 10 ml sterile water, stirred and supernatent was streaked on Kado and Heskett's agar medium (KHAM-cellobiose 10g, K_2HPO_4, 3g, NaH_2PO_4 1g, NH_4Cl 1g, $MgSO_4.4H_2O$ 0.3g, agar 15g, dist. water 1l, pH7) at 10-day intervals for bacterial growth.

Cowpea seeds were swelled for 12h in sterile water and wax microtomy was done with the method of Dowson (2).

Symptoms

Cotyledonary infection and blight and defoliation of leaves were most common. Frequency of seedling mortality and stem canker formation was low. Pods developed patches. Wilting of seedlings was not observed in natural field conditions.

A minimum inoculum conc. of $8.6x10^3$CFU/ml was essential for initiation of blight; conc. $8.6x10^8$ was best. Bacterial population in leaf tissue, one day after inoculation, was $7.2x10^3$CFU/leaf disc which incrased to $3.7x10^8$ on 4th day at water-soaking and reached a maximum of $4.3x10^9$ on 11th day in blighted tissues. Population dropped to $7.5x10^9$ with initiation of necrosis on 17th day.

Survival

The cultures of XCV in distilled water survived for 1020 days in refrigerator and 410 days in room.

Direct sun heat adversely affected the bacterium in host tissues which, though survived for 10 months in laboratory, could survive only for 40 days on roof top and 70 days on soil surface in field. It survived for 20 days in unsterilized and for 200 days in sterilized soil, when added as cell suspension.

Seed-borne nature of XCV has been suggested (3,4) but no satisfactory method of detection of bacterium in seed was demonstrated. In present case, bacterium was successfully isolated from 0.5 to 8% seeds, in five samples, by plating them on KHAM as bright yellow growth (Fig. 1). This medium supported very good growth of the bacterium.

Cotyledonary infection developed in seedlings from infected seed in sterilized quartz sand. Some seedlings developed stem canker and suffered mortality whereas others developed blight on primary leaves and served as a source of infection. Seedling wilt occurred only in inoculated seeds; more inoculum caused more seedling wilt (Table 1).

The bacterium is systemic and was isolated from various parts of infected seedlings.

Infected seeds could be visually recognised in being small, dark, wrinkled with few discoloured patches on the surface. Sections of such seeds revealed the presence of bacterial pockets in mesocarp and cotyledonary tissues (Fig. 2).

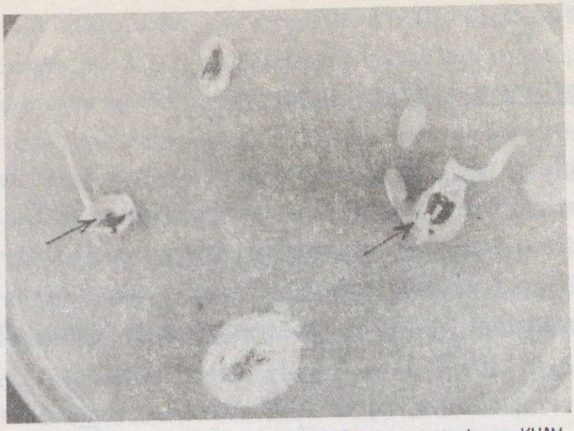


Fig. 1 Isolation of blight bacterium from sterilized cowpea seeds on KHAM

Table 1. Transmission of XCV in cowpea seeds by growing in quartz sand

Seed sample	Germination (%)	Seedling infection	
		Cotyledon	Wilt
Cultivator's field	75.0	13.33	0
Inoculated filed	75.0	24.0	-
Inoculated seed 1 h	75.0	20.0	5.0
3 h	74.0	27.0	11.0
6 h	57.0	45.0	40.0
9 h	50.0	100.0	100.0
Check	90.0	-	-



Fig. 2 Bacterial pockets in cotyledonary tissue

Bacterium survived in cowpea seed for 390 days at low and 250 days at room temperature.

Environmental factors and disease progress

Field experiments were carried out for two years to determine the influence of weather factors on intensity development of bacterial blight in cowpea. The disease occurs only during monsoon and not summer crop.

In year 1986, initial development of disease was slow as the climatic conditions - temperature 31-33 ^{o}C, maximum humidity above 70% which greatly

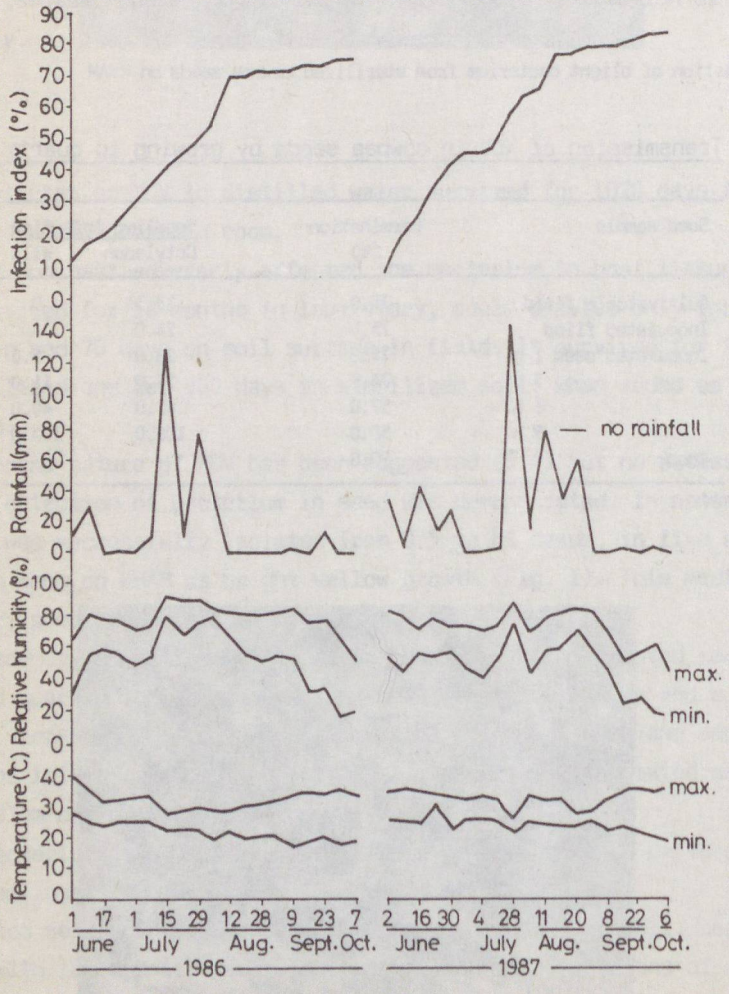


Fig. 3 Bacterial blight development in field during year 1986 & 1987

fluctuated, and rain during first week of June followed by a dry period till second week of July, were not very favourable. Disease evolution was fast from second week of July and reached the highest point of disease progress in second week of August. Disease severity made a steep rise in first two weeks of August when ideal climatic conditions prevailed i.e. temperature 38-30 ^{o}C, maximum humidity 80-91%, minimum humidity high (above 67%) and good rain (Fig. 3).

Initial development of blight was better during 1987. The temperature and humidity conditions were almost identical to the corresponding period of 1986 but moderate rain that year favoured better establishment of disease. The most favourable period (Temp. below 30 ^{o}C, high humidity and good rain) of disease evolution was from third week of July to third week of August when the disease reached the highest point of progress. Prior to favourable period, first two weeks of July were dry with temperature above 33 ^{o}C and humidity below 70% and the evolution of blight remained practically static (Fig. 3).

The results of weekly inoculations for two years (Nov., 1985 to Nov., 87) were similar to field experiment. Two monsoon months - July and August, were best for disease evolution. The disease was negligible during cool December and low during dry hot summer months. Temperature below 25 and above 33 ^{o}C adversely affected cowpea blight development.

CONCLUSION

The same isolate of XCV produced blight, stem canker and seedling wilt symptoms. Wilting in seedlings from inoculated seeds signifies the necessity of higher inoculum load in seed for the production of wilt. Such a condition was rarely possible in naturally infected seed lot.

Low survivability in unsterilized soil and at higher temperatures suggests that XCV was unlikely to survive in plant debris for long under Rajasthan conditions. The seed-borne nature of cowpea blight was suggested, not demonstrated, on the basis of obtaining seedling infection from diseased and inoculated seeds (4,7). In the absence of any specified medium, the isolation of XCV from seeds on agar plates was not successful (3). The bacterium was isolated successfully on KHAM in present case. The medium contained cellobiose which supported very good growth of XCV. The role of such nutrients in isolating seed-borne bacterium may be of interest. The presence of bacterium in seed tissue, its easy isolation, production of

disease and presence of bacterium in diseased seedlings and its long survival in seed strongly supports that seed is the primary source of infection in this area.

The analysis of two-year data has shown that temperature between 28-30 ^{o}C and humidity above 80% coupled with rain favoured rapid progress of cowpea blight; humidity to a minimum of 70% and temperature upto 33 ^{o}C on higher and 25 ^{o}C on the lower side, were within favourable limits of disease development. Temperature above 33 and below 25 ^{o}C and humidity below 70% were detrimental. The production of disease in summer months, during weekly inoculations, can be attributed to systemic nature of bacterium which can reach xylem even during dry months and can induce disease but rapid progress will always depend on favourable climatic conditions.

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Proc. 7th Int. Conf. Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

LONGEVITY OF XANTHOMONAS CAMPESTRIS PV. TRANSLUCENS IN WHEAT SEED UNDER TWO STORAGE CONDITIONS

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INTRODUCTION

Black chaff, caused by Xanthomonas campestris pv. translucens, is a seedborne disease which has become a major concern in the last decade to sprinkler-irrigated wheat growers in parts of southern Idaho. Disease losses are estimated to be up to 40% in individual fields. Published reports on the longevity of the pathogen in seed vary from 1 to 3 years (1,3). The objective of this study was to determine the longevity of the pathogen associated with seed of Triticum durum under two storage conditions in Idaho.

MATERIALS AND METHODS

Seed of cv. WAID was harvested from a severely diseased field in south central Idaho in August, 1982, and stored in an outdoor building at ambient temperature and relative humidity (annual range of -20 to 40°C and 10-100% RH) and in the laboratory (18-23°C, 30-40% RH) at Kimberly, Idaho. Seed assays (2) to determine the longevity of the pathogen were conducted semi-annually on three 120 g subsamples (ca. 3000 seeds) per treatment for 5 yrs.

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* This study was supported in part by the Idaho Wheat Commission and regional Idaho Hatch Projects H-840 and H-850 contributing to NC-135 regional research.

In 1987, a subsample of seeds which had been stored in outdoor conditions and which had a population of 1.1×10^3 cfu's of X. c. pv. translucens per gram of seed was planted in the field in 10 m^2 plots to observe disease development. Plants were sprinkler-irrigated at weekly intervals and grown using recommended cultural practices.

RESULTS

The population of X. c. pv. translucens on the seed after harvest was 3.6×10^5 cfu's/g of seed (Fig. 1). It decreased ca. 100 times in the first 2.5 years of storage in the laboratory, while the population on seed stored outside remained unchanged. After an additional year of storage, it had decreased an additional 100 times in the laboratory, but was still above 1×10^5 cfu's/g on seed stored outside. Pathogenicity tests of random colonies from the assays were always positive.

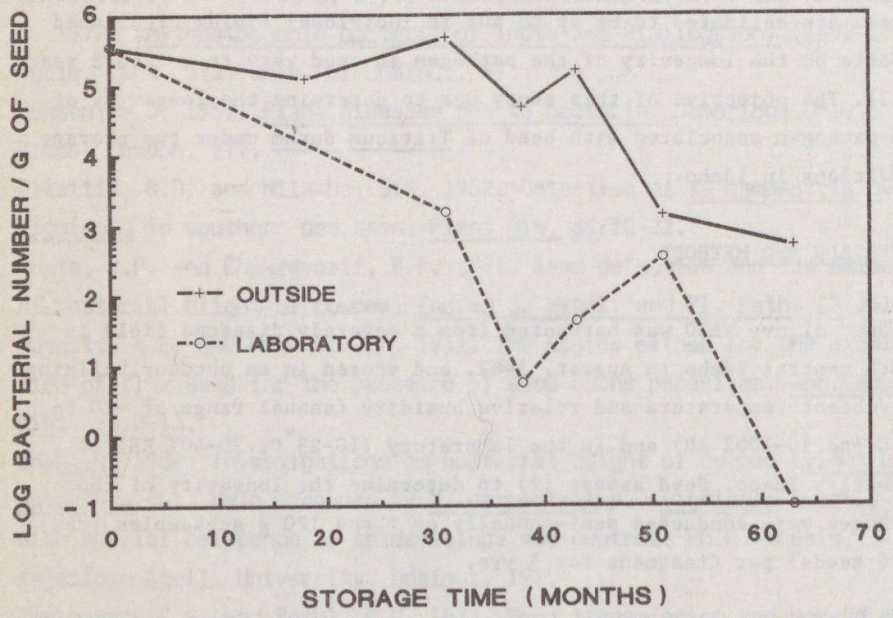


Fig. 1. Longevity of Xanthomonas campestris pv. translucens on wheat seed (Triticum durum) stored in an outdoor building and in the laboratory at Kimberly, Idaho.

Regression analysis of the data (outside: $Y = 12.49 + 0.074X - 0.0028X^2$; $R^2 = .73$; laboratory: $Y = 12.29 - 0.187X$; $R^2 = .72$; where Y = the log of the pathogen population per g of seed and X = storage time in months) predicts an extinction point (when the pathogen is not detectable in the seed) of 81 and 66 months, respectively.

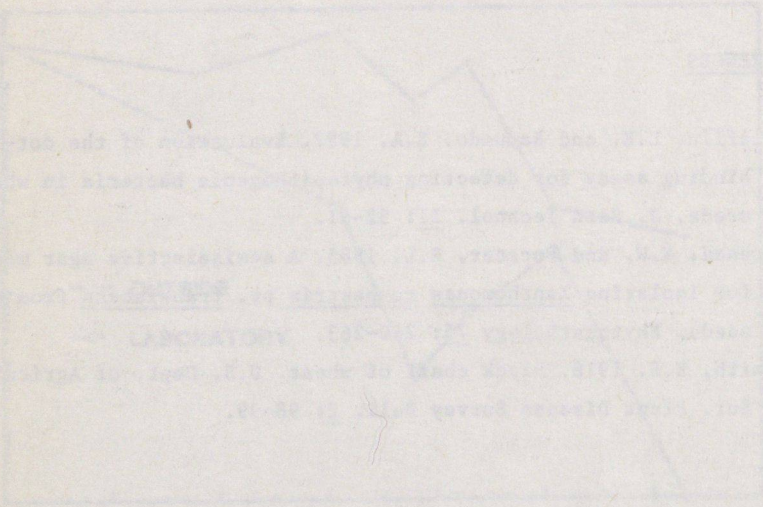
When the seed stored outside for 4.5 years was planted in the field in 1987, disease developed normally in those plants.

DISCUSSION

X. c. pv. translucens can survive on wheat seed stored outside in Idaho for more than 5 years and may cause disease under favorable environmental conditions. The population on seed stored in the laboratory at relatively constant temperature and humidity decreased rapidly and was not detectable after 5 yrs. The cooler, usually drier, desert climate of southern Idaho may account for the longer survival under uncontrolled conditions.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

EPIPHYTIC SURVIVAL OF PSEUDOMONAS FUSCOVAGINAE ON RICE PLANTS IN RELATION TO INCIDENCE OF BACTERIAL SHEATH BROWN ROT

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Bacterial sheath brown rot caused by Pseudomonas fuscovaginae was first observed at northern hemisphere (6). At present time, however, it has been reported from the tropics as Asia, Central Africa, Latin America and Madagascar (1, 2, 5, 7). The cool, humid conditions prevailing during booting stage result in the intensive and widespread occurrence of the disease in those highland areas as well as the northern regions. Consequently, the disease results in the yield losses and the quality deterioration of rice grains.

This paper describes the epidemiological behavior of P. fuscovaginae that the appearance of disease is apparently associated with a gradual build up of resident population of P. fuscovaginae prior to infection on rice plants at susceptible growth stage.

HOST SUSCEPTIBILITY

Bacterial sheath brown rot usually became noticeable on flag leaf sheath at booting stage of rice plants in the paddy fields. Susceptibility of plants at different growth stage and leaf sheaths at the booting stage were studied by different inoculation methods.

Inoculation with a bacterial suspension to rice plants at different growth stages using both spray and injection methods in growth chamber revealed that all the rice plants inoculated using injection at seedling, tillering, flag leaf and booting stages showed a high percentage of affected plants, on the other hand, spray inoculation only resulted in disease on the plant at the booting stage but not at any one at the other stages. The results show that most rice plants at any developmental stage may be infected through wounds whereas only booting plant may be infected through natural entry points.

Typical water-soaked dark green lesions initially appeared on flag leaf sheath but not on leaves or lower sheaths at the booting stage of rice plants under field conditions. Both spray inoculation and injection into inner leaf sheath without injuries with a bacterial suspension to the individual sheaths at different position showed the severe disease development on the flag leaf sheath whereas slight or absent disease developed on lower sheaths.

Microscopic examination showed that most stomata on the adaxial epidermis of all sheaths were open whereas those of abaxial side of the sheath were closed, and the aperture of stomata on adaxial epidermis of

flag leaf sheath were 1.0-1.1μm, and these of lower sheaths were 0.6-0.8 μm during the daytime.

Histological examination showed that masses of bacterial cells could be seen on the epidermis cells of adaxial side of flag leaf sheath after inoculation at booting stage. They entered the stomata, then multiplied in intercellular spaces of the parenchyma and in the lysigenous aerenchyma. However, no bacterial cells were observed in the stomata on the abaxial side or vascular bundles.

EPIPHYTIC SURVIVAL OF PSEUDOMONAS FUSCOVAGINAE

Monitoring of Pseudomonas fuscovaginae population on rice plants using the bacteriophage method (4) showed that the causal bacteria survive on the aerial parts of healthy seedling grown from artificially inoculated seed and the population of P. fuscovaginae was significantly higher at 12C° than at 20 or 25C°. Time from planting to seedling emergence varied depending on temperature. The mechanism of movement from the seed to the seedling is unknown, but it has been suggested that the bacteria swim up the seedling or are carried up by the growing points.

In the greenhouse experiment, the population of causal bacteria increased on healthy rice plants at booting stage 12, 34 and 72 days after a bacterial suspension was sprayed at the seedling, tillering and flag leaf sheath stages, respectively. The disease subsequently occurred during the booting to flowering periods (Table 1).

TABLE 1. Recovery of Pseudomonas fuscovaginae on healthy rice plants inoculated with bacterial suspensions at different stages(Phage method)

Inoculation stage[a)]	Recovery from:	Propagation of phage[b)]			
		3	7	18	72* day
5th leaf	leaves	-	-	+	-
	sheaths	-	-	+	+++
		2	6	21	34*
7th leaf	leaves	+++	+++	++	-
	sheaths	+++	+++	-	+
		2	7	12*	16
flag leaf	leaves	+++	+++	++	++
	sheaths	+++	+++	+++	+

a; 10^{6-7}cfu/ml bacterial suspension was sprayed.

b; Degree of phage propagation, -: no propagation, +: 10, ++: 10^2, +++: 10^3 times over.

c; *: Booting stage of rice plants.

Known lysotype A of P. fuscovaginae (4) naturally contaminated seed was recovered from the aerial parts of healthy rice plants grown from these seeds eight days before it developed bacterial sheath brown rot symptoms in the paddy field, and then from all healthy plants two days after the diseased plants were visible (Table 2). It is evident from the data as present here that the causal bacteria survive on healthy rice plants and reach high population at the booting stage following establishment of the disease.

TABLE 2. Seasonal variation in epiphytic population of Pseudomonas fuscovaginae on rice plants (Phage method)[a)]

Applied seed[b)]	Bacteriophage propagation[c)]						
	6.20	25	7. 4	14	22	28	8. 1
A	-	-	-	-	+++	NT[d)]	+++
B	-	-	-	-	+	-	+++
C	-	-	-	-	-	-	+++

a: Initial disease occurrence was on 30 July.
b: A and B; Diseased seeds were transplanted on 19 May and 16 June, respectively. C; Healthy seeds on 16 June.
c: Degree of phage propagation, -; no propagation, +; 10, ++; 10^2, +++; 10^3 times over.
d: Not tested.

An epiphytic phase of P. fuscovaginae on rice plants has important implication in the epidemiology of bacterial sheath brown rot. Widespread outbreaks have appeared suddenly while the rice plant is in the booting stage. Natural inoculum, whether from seed or other sources, may result in the build up of epiphytic populations of P. fuscovaginae which could be spread with each successive rainfall. These epiphytic populations also can be a source of disease inciting inoculum. Under the predisposing conditions these populations may be become pathogenic, resulting in the observed sudden, widespread outbreaks of bacterial sheath brown rot.

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SESSION 4

GENETICS AND
MOLECULAR BIOLOGY

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

ORIGIN OF IAA GENES AMONG TUMORIGENIC BACTERIA*

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Some bacteria are responsible for diseases in plants; however, only a few of many species encountered are capable of establishing a disease symptom. Certain specialized functions therefore, establish the pathogenic relationship between the plant pathogenic bacteria and the host plant (Kosuge et al. 1985)

The bacterium Pseudomonas syringae pv. savastanoi incites the formation of tumor at the site of infection on olive and oleander plants. Tumor formation by these plants is a response to high concentrations of indoleacetic acid (IAA) introduced into infected tissues by the bacterium (Smidt and Kosuge 1978). IAA is a natural plant hormone required for the normal plant tissue development. Under normal circumstances only a small quantity of the compound is needed and regulatory mechanisms in the plant assure that their production and delivery to the sites of action occur in keeping with the developmental needs of the plant (Kosuge and Yamada 1987). In the diseased state, production and secretion of phytohormone in the normal regulatory systems would be disturbed by the pathogen. The resulting phytohormone imbalances may cause the host tissues to respond by undifferentiated proliferation (Kosuge et al. 1985; Kosuge and Yamada 1987; Nester and Kosuge 1981)

Genes for IAA production

The bacterium produces IAA from tryptophan with indoleacetamide (IAM) as the intermediate (Kosuge et al. 1985; Kosuge and Yamada 1987). The enzymes involved are tryptophan 2- monooxygenase, which catalyzes the conversion of L-tryptophan to IAM, and IAM hydrolase, which catalyzes the conversion of IAM to IAA. The genetic determinants for the two enzymes,

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*This paper is dedicated to the memory of Dr. Tsune Kosuge (deceased 13 March 1988).

iaaM and iaaH, respectively, are part of an operon that is borne on a plasmid, pIAA, in oleander strains of pathogen; in olive strains the determinants are on the chromosome (Comai and Kosuge 1980). A 2.8kb EcoRI restriction fragment (fragment-M) bearing iaaM was cloned from pIAA into the plasmid RSF1010 to construct a recombinant plasmid, pLUC1 (Comai and Kosuge 1982). Transformation of Iaa$^-$ mutant of P. savastanoi with pLUC1 restores high levels of tryptophan 2-monooxygenase activity and partial virulence(Comai and Kosuge 1982).

Structure of iaa operon and the nucleotide sequence of iaaM and iaaH

The nucleotide sequences reveal that the iaaM locus contains an open reading frame encoding 557 amino acids that would comprise a protein with a molecular weight of 61,783; the iaaH locus contains an open reading frame of 455 amino acids that would comprise a protein with a molecular weight of 48,515 (Yamada et al. 1985). Significant amino acid sequence homology was found between the predicted sequence of the tryptophan monooxygenase of P. savastanoi and the deduced product of tms-1 gene on T-DNA of the octopine-type plasmid pTiA6NC from Agrobacterium tumefaciens, which is responsible for the auxin synthesis(Schroeder et al. 1984). The tms-1 together with tms-2 on T-DNA are the genes encoding the enzymes converting tryptophan into IAA (Van Onckelen et al. 1984). The nucleotides sequence of tms-1 reveals that it encodes 755 amino acids (Klee et al. 1984), and the linear homologous relationship between iaaM and tms-1 resides on the C-terminal of tms-1 protein of approximately 550 amino acids. Homology was also found in the amino acid sequences representing the central region of the putative product of iaaH and tms-2 T-DNA. While iaaH and tms-2 show lesser homology (27% for the perfect homology), but yet significant (Yamada et al. 1985).

Transposable DNA elements occurring in iaaM

Two types of transposable elements, IS51 and IS52, were found in iaaM that spontaneously insert into and inactivate tryptophan monooxygenase; the insertion results in the loss of IAA production and attenuation of virulence (Comai and Kosuge 1983). The nucleotide sequences of both IS elements have sizes and structural features common to other prokaryotic IS elements; IS51 is 1311 base pairs(bp) long and has terminal inverted repeats of 26 bp; IS52 is 1209 bp long and has the terminal inverted repeat of 10 bp with a 1 bp mismatch (Yamada et al. 1986). In the

insertion involving IS51, the trinucleotide sequence CAG is duplicated within iaaM sequences at the recombination junction; in those involving IS52, the tetranucleotide sequence TTAG or CTAG are duplicated within iaaM sequences at the recombination junction (Yamada et al. 1986). A copy of IS51 occurs 2.5 kb downstream from the 3'-end of iaaH in a wild type strain. In contrast to the high copy number of IS51 in the genome of the bacterium, only a few copies of IS52 are present (Yamada et al. 1986).

A striking nucleotide sequence homology was found between a 531bp of IS51 and the corresponding region of a portion of the cerntral region of T-DNA in the octopine type plasmid from A. tumefaciens,in which Tc (T-DNA center) region of T-DNA is not integrated into the plant genome. .

The origin of IAA gene

These observations on the structural homology between IAA producing genes of P. savastanoi and A. tumefaciens in addition to the IS51 sequence suggest that genes for IAA production in the two systems have a common origin. The homology observed between IS51 and Tc region of A. tumefaciens T-DNA may indicate a remnant of an earlier transpositional event by which iaaM and iaaH were introduced into A. tumefaciens T-DNA from P. avastanoi (Yamada et al. 1986). The copy of IS51 that occurs 2.5kb downstream from iaaH may represent one border of a transposon originally introduced into A. tumefaciens T-DNA from P. savastanoi; the hypothetical P. savastanoi transposon that contained iaaM and iaaH subsequently underwent DNA rearrangement in A. tumefa-

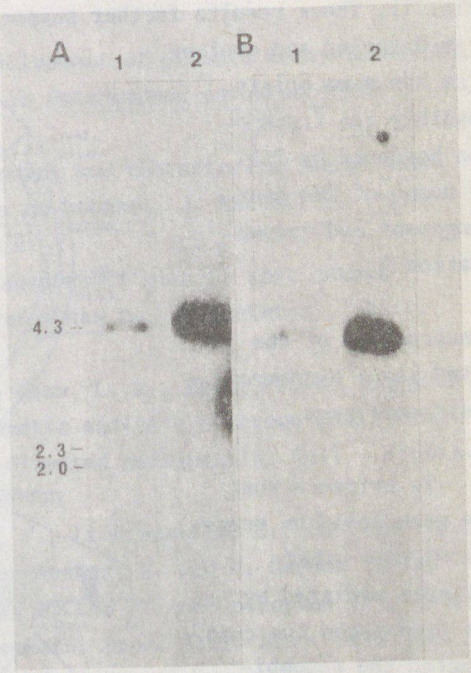


Fig. 1. Southern blot hybridization analysis of the iaa and IS51 in plasmid pRi1000 and pTiC58. EcoRI-digested pRi1000 from A. rhizogenes 1000 (A) and pTiC58 from A. tumefaciens C58 (B) were fractionated on 0.7 % agarose gel and blotted onto a nylon membrane filter. The filters were hybridized with iaa gene probe, pLUC 2 (Fragment H::pBR328) (Lane 1), or iaa + IS51 probe, pLUC12 (FragmentQ1M::pBR328)(Lane 2). The numbers on the left are the position of the size marker.

ciens , leaving only functionally active iaaM and iaaH conserved in the T-DNA.

Sequencing homology to other pathogenic or symbiotic bacteria

The presence of sequence homology of P. savastanoi- iaa genes and -IS51 in other plant pathogenic bacteria, P. phaseolicola, P. solanacealum, P. pruni, Xanthomonas campestris pv. pisi and X. pruni were investigated by Southern blot hybridization analysis. At least 4-5 fragments were hybridized to iaa + IS51 probe with EcoRI-digested total DNA fragments from these bacteria. Hybridization analyses with the purified Ti plasmid (pTiC58) from A. tumefaciens C-58 and pRi1000 plasmid from A. rhizogenes 1000 against iaa or iaa + IS51 probe shows that at least a single copy of the homologous sequence of iaa and IS51 is present on these plasmids (Fig. 1). These results further support the hypothesis that iaa genes have been diverged and evolved from the same origin, possibly iaa transposon bordered by IS51, by means of DNA rearrangement and recombination.

Construction of the fused iaa + hygromycin resistance gene

To evidence that iaa gene could be transferred into other bacteria mediated by iaa transposon, we constructed the recombinant plasmid pTOM1 in which hygromycin resistance gene (hpt) was fused in iaa operon in a wide host range vector pRK415 (Fig. 2). E. coli HB101 (pTOM1)

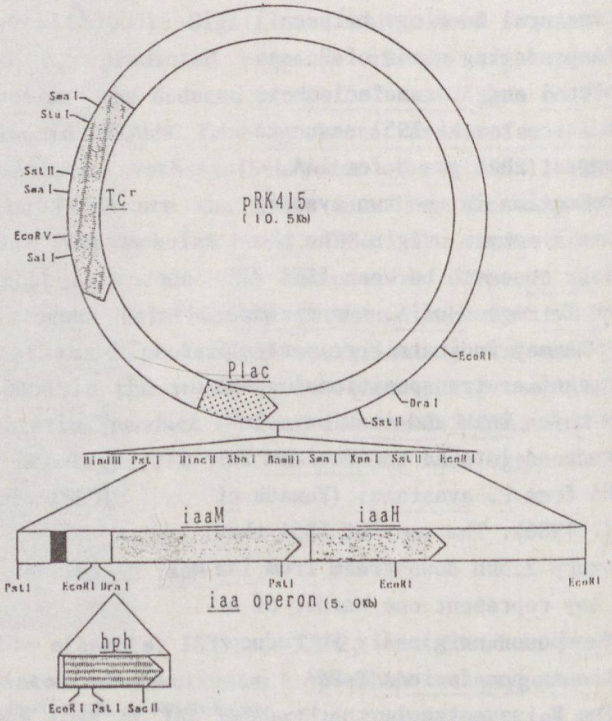


Fig. 2. Restriction map of the recombinant plasmid pTOM1. pTET40 in which iaa operon was cloned in a wide host range vector pRK415 was partialy digested with DraI restriction endonuclease. hpt gene was inserted in DraI site in front of iaaM.

expresses both iaa and hpt. pTOM1 was transconjugated into a wild type P. savastanoi by tri-mating with E. coli (pRK2013) harboring the helper plasmid pRK2013. A mutant in which iaa operon was replaced by hpt-iaa on pIAA1 was selected by a marker exchange eviction method as described by Garfinkel et al. (1981).

ACKNOWLEDGEMENT

We deeply thank Dr. G. Ditta, Dr. F. White and Dr. C. I. Kado for providing us E. coli HB101 (pRK415), A. rhizogenes and A. tumefaciens, respectively. This research was supported by the grant from the Ministry of Education, Science and Culture of Japan No. 63560046 and 62480045.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

GENETIC AND MOLECULAR CHARACTERIZATION OF THE AVIRULENCE GENE avrBs3 FROM XANTHOMONAS CAMPESTRIS PV. VESICATORIA

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INTRODUCTION

Xanthomonas campestris pathovar (pv.) vesicatoria (Xcv) is the causal agent of bacterial leaf spot disease on pepper and tomato. Resistance against Xcv depends on the presence of two different loci: a resistance locus in a particular cultivar of pepper and an avirulence locus in a particular race of Xcv. The phenotype of this race-specific reaction is a hypersensitive response (HR), which is a defense reaction accompanied by a rapid necrosis of the infected plant tissue (Maclan et al. 1974; Sequeira 1984). Induction of an HR prevents the accumulation of bacteria in the intercellular spaces of leaves. Several dominant, non-allelic resistance genes (e.g. Bs1, Bs2, Bs3) mediating an HR toward particular races of Xcv have been identified in pepper (Cook and Stall 1963; Kim and Hartmann 1985; Hibberd et al. 1987) and were crossed into the susceptible cultivar Early Cal Wonder (ECW; R.E. Stall, unpublished results). Use of these nearly isogenic cultivars of pepper and genetically engineered pathogens allows us to approach an understanding of the molecular mechanisms of specific resistance.

Xcv race 1 isolates induce an HR only on pepper cultivar ECW-30R carrying the resistance gene Bs3. This race-specific interaction is due to the presence of the avirulence gene avrBs3 in Xcv race 1 strains. This paper describes the isolation and molecular characterization of the avrBs3 locus from Xcv race 1.

RESULTS AND DISCUSSION

Isolation of avrBs3

AvrBs3 was cloned from Xcv race 1 strain 71-21 using the broad host range vector pLAFR3 (Staskawicz et al. 1987) and identified by complementation analysis of Xcv race 2 transconjugants (Bonas et al. 1989). Normally Xcv race 2 causes a compatible reaction on pepper cultivars ECW and ECW-30R. However, transconjugants carrying an active avrBs3 gene induce an HR on ECW-30R.

The avrBs3-clone pL3XV1-6, with an insert of about 5 kb, was chosen for detailed analyses (Fig. 1). Southern blot hybridizations showed that the avrBs3 gene is located on an endogenous plasmid of approximately 45 kb. Genetic studies revealed that this plasmid is self-transmissable. To ascertain that the cloned fragment contained a gene required for HR induction in ECW-30R we inactivated the gene by random insertion mutagenesis of pL3XV1-6 and introduced it by subsequent marker gene replacement into wild type race 1 strains. The positions of independent insertions by a transposon (the so-called Gus-poson; B. Staskawicz, unpublished results) and a site-specific insertion by the Ω-fragment (Prentki and Krisch 1984) respectively are illustrated in Fig. 1. Except for Gus-poson-insertion #1 all insertion mutants showed a compatible reaction on pepper cultivars ECW and ECW-30R suggesting that the function of avrBs3 in induction of HR on ECW-30R had been destroyed by the insertions.

To determine the minimal size of the DNA fragment required for avrBs3 activity, a deletion analysis was performed. The avrBs3 activity of several deletion clones was analyzed for their reactions on ECW-30R. The reaction was either incompatible or compatible. In no case an intermediate phenotype between watersoaking and HR or a slower HR was observed. These results indicated that a DNA region spanning 3.6-3.7 kb is necessary to confer avrBs3 activity.

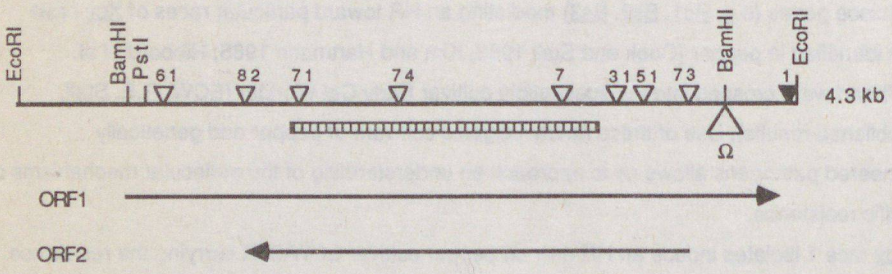


Fig. 1: Physical map of the 4.3.kb EcoRI fragment of the avrBs3 clone pL3XV1-6. The positions of independent insertions into avrBs3 are indicated by triangles. The triangles and numbers above the line refer to the insertion of the Gus-poson. Only # 1 leaves the avrBs3 gene intact. The striped bar indicates the region of direct repeats. The lines with single-headed arrows mark the position and direction of the two longest ORFs in this fragment.

DNA homology of avrBs3 to other Xanthomonas campestris pathovars

Total genomic DNA of different races of Xcv and of different X. campestris pathovars was hybridized in a Southern blot experiment to the 3.3 kb BamHI fragment which is an internal fragment of avrBs3 (see Fig. 1). DNA from strains of other Xcv races or from strains of the X. c. pathovars begoniae, fragariae, holcicola, pruni and vignicola did not hybridize to avrBs3. However we did find homologous DNA sequences in strains of the X. c. pathovars alfalfae, campestris, carotae, glycines, malvacearum and phaseoli. When inoculated into different pepper cultivars the pathovars containing avrBs3 homologous sequences induced an atypical HR suggesting that the hybridizing fragment either contains a non-functional or incomplete avrBs3 gene. The observed light-brown HR may be due to the expression of non-host resistance which in some cases has been shown to be induced by avirulence genes (Whalen et al. 1988).

DNA sequence analysis

The DNA sequence of the 4.3 kb EcoRI fragment was determined (Bonas et al. 1989) and reveals interesting structural features: a region of direct repeats is located in the middle of the fragment. These repeats, each 102 bp long, are present 17 times and are 91-100% homologous to each other. Striking is the presence of several long ORFs (Fig. 1). ORF1 is 3.4 kb, ORF2 2.4 kb in length. The calculated molecular weights of the corresponding proteins Pep1 and Pep2 are 125.3 kDa and 82 kDa respectively. Since the size of ORF1 and the DNA fragment needed for avrBs3 activity coincide it seems very likely that Pep1 is AvrBs3.

A sequence comparison of avrBs3 to sequences of other avirulence genes, i.e. avrBs1 from X.c. pv. vesicatoria race 2 (Ronald and Staskawicz 1988) and avrA, avrB and avrC from Pseudomonas. syringae pv. glycinea (Napoli and Staskawicz 1987; Tamaki et al. 1988) and a homology search in the sequence data bank did not reveal significant sequence homology on the nucleic acid and amino acid level to known proteins.

Expression of avrBs3

To determine promoter activity of avrBs3 we fused DNA fragments of different lengths containing putative promoter regions of ORF1 and ORF2 respectively to a reporter gene. For this purpose pLAFR6 (pLAFR1-derivative with *trp* terminators flanking the insert; B. Staskawicz, unpublished) containing a promoterless β-glucuronidase gene (Jefferson et al. 1986) was used. The constructs were transferred into Xcv race 1 and race 2 by triparental mating and GUS-activity of the cells was measured under different conditions. Only for ORF1

the promoter could be defined: a 125 bp sequence upstream of the putative translational start site was sufficient for full activity. If ORF2 is expressed is presently not clear.

ORF1 seems to be constitutively expressed. Cells grown in rich medium contained significant GUS-activity which was unaltered after inoculation of the bacteria into pepper plants. The observation that avrBs3 seems to be expressed before the bacteria enter the host plant is confirmed by protein data. A polyclonal antiserum which was generated against a β-galactosidase/Pep1-fusion protein specifically detects a 125 kDa protein in protein extracts of Xcv race 1 cells. This protein is only present in race 1 and in race 2 transconjugants harboring pL3XV1-6 and not in other Xcv races. In avrBs3 insertion mutants the size of the protein is altered dependent on the integration site.

The subcellular location of AvrBs3 is important for an understanding of the mechanism of induction of hypersensitivity. Having a specific antibody we tried to biochemically fractionate Xcv race 1 cells and follow the avrBs3 protein by Western-blot analysis. Preliminary results suggest that the majority of AvrBs3 is localized in the cytoplasm. Due to rapid degradation of the soluble avrBs3 protein it can only be estimated to be more than 60% of the total amount. The inner and outer membranes were separated by sucrose gradient centrifugation and tested for marker enzyme activity and distribution of AvrBs3. The intensity of the AvrBs3 Western-blot signals correlates with NADH-oxidase activity of the respective fractions indicating that the membrane-bound AvrBs3 is in the cytoplasmic membrane.

The biochemical function of AvrBs3 in induction of HR in ECW-30R is unknown.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
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PLASMID-ENCODED COPPER RESISTANCE IN XANTHOMONAS CAMPESTRIS PV. VESICATORIA

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ABSTRACT

Copper resistance in *Xanthomonas campestris* pv. *vesicatoria* (Xcv) has been associated with a large self-transmissible plasmid (pXvCu). Over 500 strains of Xcv isolated from pepper and tomato were examined for growth on media supplemented with 0.8 mM of copper sulfate and for the presence of the megaplasmid. At least one large plasmid ranging in size from 170 to 300 kbp was detected in eighty percent of the strains. Eighty-four percent of the strains carrying the putative pXvCu were able to grow on copper-supplemented media. Copper resistance was always correlated with the presence of at least one large plasmid. Large plasmids were also present in copper-sensitive strains. Several plasmids were self-transmissible and three plasmids exhibited unique fragment patterns. A DNA fragment conferring copper resistance hybridized to the large plasmid band in copper-resistant strains, and to different restriction fragments in three strains.

INTRODUCTION

Xanthomonas campestris pv. *vesicatoria* (Doidge) Dye (Xcv) causes the bacterial spot disease in pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.). The disease is endemic in several regions of the world where these plants are cultivated. Copper-containing bactericides are commonly used for control. Stall et al. (1986) reported that the copper-resistance (Cu^R) phenotype was encoded by a self-transmissible plasmid in Xcv strains isolated in Florida (USA). Copper resistance is also encoded by plasmids in the plant pathogenic bacterium *Pseudomonas syringae* pv. *tomato* (Bender and Cooksey, 1986) and in other bacteria (Silver, 1988).

This study was undertaken to determine the frequency of copper resistant strains

in Xcv from several locations in the world and if this phenotype is encoded by homologous plasmids and/or homologous genes.

MATERIALS AND METHODS

Five-hundred and twenty-four strains of Xcv isolated from pepper and tomato in several regions of the world were included in this study. Bacteria were maintained in sterile tap water for long term storage or on Lima Bean Agar (Difco), pH 7.0, for short term storage. Copper resistance was determined by growth on nutrient agar supplemented with 0.8 mM of $CuSO_4.5H_20$. A nalidixic acid-resistant mutant of *Xanthomonas* sp. strain T-55 (Stall, 1987), used as recipient in conjugation experiments, was obtained by selection on nutrient agar plates supplemented with 25 ppm of nalidixic acid (nal).

For conjugation experiments bacteria were grown with vigorous shaking at 30°C for 16 to 24 hrs in nutrient broth tubes, mixed and incubated with gentle shaking for 8 hrs, then plated on appropriate selective media.

Plasmid extraction was by a modification of the alkaline lysis method for large plasmids according to the procedure of Kado and Liu (1981). Preparations were maintained for several months at 4°C after adding 25 mM of EDTA. Large scale plasmid preparations were done by the total DNA extraction method of Marmur(1961) and followed by cesium chloride centrifugation (Maniatis et al., 1982).

Plasmid sizing was done by comparison of relative mobility in 0.5% agarose gel under an electrical current of 5V/cm for two hours. The plasmids of the SW2 strain of *Erwinia stewartii* (Coplin et al., 1981) were used as molecular weight markers.

Southern blots were prepared according to Maniatis et al. (1982) and hybridization was done with the GENIUS nonradioactive DNA labeling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) following the manufacturer's instructions. Restriction endonuclease analysis was performed in accordance with the manufacturer's recommendations.

RESULTS AND DISCUSSION

Large plasmids (170-300 kbp) were detected in eighty-four percent of the strains. They were separated into three size classes (Table 1). Seventy percent of all strains

grew on a copper–supplemented medium (Table 2). Only strains carrying large plasmids were copper–resistant but large plasmids were also detected in copper–sensitive strains (14% of the strains) (Table 2). Those strains with the largest plasmids (class A) were almost always Cu^R (99.3%), whereas the percentage of Cu^R strains were less in those in class B (82.3%) and only half (51.8%) of those in class C were Cu^R.

Table 1. Frequencies of strains of *Xanthomonas campestris* pv. *vesicatoria* having large plasmids in various size classes

Population	Size class[(a)]		
	A	B	C
UF collection	47/218(b)	98/218	36/218
Taiwan collection	0/28	8/28	7/28
BA collection (Arg.)	0/30	22/30	0/30
BV tomato field (Arg.)	0/52	26/52	0/52
FL tomato field (USA)	5/84	79/84	0/84
FL pepper field (USA)	83/97	6/97	13/97
Ohio tomato field (USA)	0/15	15/15	0/15
Total	135/524	254/524	56/524
Percentage	25/8	48/5	10/7

(a) A= >260 kbp; B=201-259.9; C=170-200.9
(b) No. of positive strains/total number in collection

Table 2. Frequencies of strains of *Xanthomonas campestris* pv. *vesicatoria* with copper resistance among size classes of large plasmids.

Population	Size class			
	A	B	C	Total Cu^R
UF collection	47/47(a)	76/98	21/36	144/218(b)
TW collection	0/0	5/8	4/7	9/28
BA collection	0/0	5/22	0/0	5/30
BV tomato field	0/0	26/26	0/0	26/52
FL tomato field	5/5	79/79	0/0	84/84
FL pepper field	82/83	4/6	4/13	86/97
Ohio tomato field	0/0	14/15	0/0	14/15
Total	134/135	209/254	29/56	368/524
Percentage	99.3	82.3	51.8	70.2

(a) Number of Cu^R strains/number of strains in class
(b) Number of Cu^R strains/number of strains in population

Selected strains were used in conjugation experiments. They were mated to the plasmidless strain T−55 nal^R and transconjugants were selected on a copper and nal supplemented medium. Only large plasmids were detected in transconjugants. Some plasmids were transmissible at high frequencies, whereas others were at low frequencies, or not transmissible (data not shown).

The pXvCu plasmids in three strains were conjugated into T−55 nal^R, and the plasmid DNA was recovered from the transconjugants after CsCl centrifugation. The plasmid DNA was subjected to restriction with the endonucleases *Xba*I and *Sma*I and concentrated by ethanol precipitation. Restriction patterns for the three plasmids in agarose gel were not the same with either enzyme. The patterns for *Sma*I are in Fig. 1.

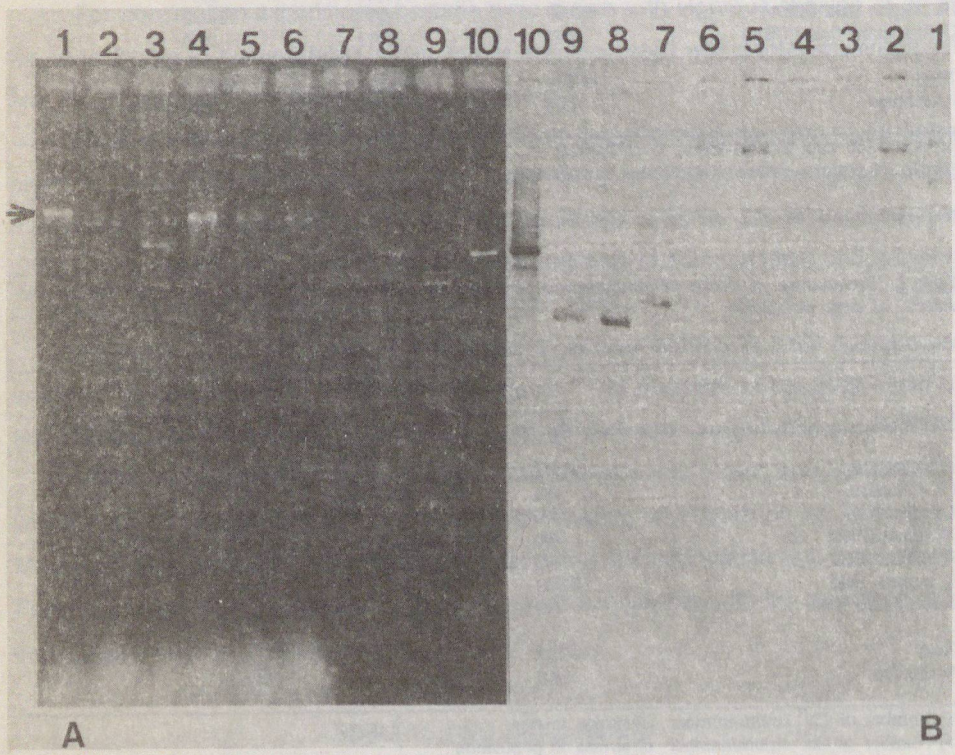


Fig. 1 Hybridization of the copper resistance clone pXvCu1-13 to the plasmid DNA from three strains of *Xanthomonas campestris* pv. *vesicatoria*. (A) Gel stained with ethidium bromide. (B) Southern blot of the gel in panel A probed with pXvCu1-13 clone. Lanes in both panels are: 1,2,3 = total plasmid DNA; 4,5,6= transconjugants from the matings to the strain T-55; 6,7,8=*Sma*I digested plasmid DNA of the transconjugants. Lines 1,4, and 7=BV5-4a; lines 2,5, and 8=TB-1; lines 3,6, and 9= 71-31. Line 10= pXvCu1-13. Arrow indicates chromosomal and linear plasmid DNA

A DNA fragment coding for Cu^R in Xcv strain 81–23, provided by Dr. B.J. Staskawicz, Univ. of California, Berkeley, was used in hybridization experiments. Several Cu^R strains and one copper–sensitive strain containing a large plasmid were probed. The probe hybridized to the large plasmid band in all Cu^R strains and also to a 55–75 kbp plasmid in the BV group of strains from Argentina (Fig. 1). No hybridization occurred with the copper–sensitive strain.

To further determine the degree of homology of the Cu^R plasmids, the *Sma*I digest of the CsCl extracted plasmids were probed with the fragment encoding Cu^R. The probe hybridized to fragments of different sizes within the digested plasmid DNA from the strains from Florida and Argentina (Fig. 1).

Apparently, polymorphism exists among these large plasmids in Xcv in spite of their conserved size and their conjugative properties. In hybridization experiments polymorphism also occurred in the DNA fragments encoding Cu^R in two strains from Florida (TB–1 and 71–31) and one strain from Argentina (BV5–4a). Some of the polymorphism may result from an insertion sequence (IS element) present in at least some of the pXvCu plasmids (Kearney et al., 1988). A smaller plasmid present only in strains from Argentina also hybridized to the fragment encoding Cu^R but is not known if it contains an active gene for copper resistance.

Whether the Cu^R sequences were distributed from a single source or arose independently in every location cannot be determined from this study. What is clear, however is that resistance to copper is a widespread phenomenon in Xcv strains.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

REGULATION OF GENE EXPRESSION AND PATHOGENICITY OF XANTHOMONAS CAMPESTRIS PV. CAMPESTRIS

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INTRODUCTION

The development of molecular genetic procedures and their application to plant pathogenic bacteria has led to some rapid advances in our understanding of pathogenicity mechanisms and specificity determination. By cloning genes encoding putative pathogenicity factors such as plant cell wall degrading enzymes it is possible, for example, to construct strains carrying single mutations in precisely known positions in the genome, and such mutants can be used to evaluate the role of particular genes and their products in pathogenicity.

The expression of most bacterial genes is tightly regulated so as to exploit the environment in the most economical way. It is to be expected therefore that expression of pathogenicity genes is regulated. Bacterial pathogens occupy ecological niches with sharply differing and changing physicochemical properties, for example soil, leaf surfaces, roots, intercellular spaces, xylem fluids, rotting tissues, etc. Little is known of the adaptive phenomena and mechanisms which operate in transitions between these niches, and which are probably very important in determining the nature and course of host-pathogen interaction.

In this paper we describe three approaches to studying genes which do or may regulate pathogenicity gene expression in *Xanthomonas campestris* pathovar *campestris*, a pathogen of crucifers.

COORDINATE POSITIVE REGULATION OF SYNTHESIS OF EXTRACELLULAR ENZYMES AND POLYSACCHARIDE

Mutants of *X. c. campestris* defective in pathogenicity were isolated by direct screening of mutagenised bacteria in *Brassica* seedlings (Daniels *et al*, 1984a). One of the mutants (8237) was found to be defective in

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[2]The Sainsbury Laboratory is supported by the Gatsby Charitable Foundation.

production of extracellular protease, polygalacturonate lyase, amylase, endoglucanase and extracellular polysaccharide (EPS, xanthan gum) (Daniels *et al*, 1984b; Tang, 1989). Pathogenicity and the production of all the enzymes, and EPS, were restored by a cosmid pLAFR1 clone, pIJ3020, isolated from a library of wild-type DNA. The defect in 8237 was shown to be in the synthesis of the factors, rather than export from the cells. pIJ3020 is not related to another cosmid clone pIJ3000, which contains a cluster of genes necessary for export of the enzymes, but not for their synthesis (Turner *et al*, 1985; Dow *et al*, 1987).

The DNA in pIJ3020 (*ca* 27kb plus the vector moiety) has been studied by subcloning, deletion analysis, promoter probing, mutagenesis with Tn*5* and Tn*5lac*, and by DNA sequencing. There is evidence for at least seven genes (A-G) in this region of the genome which are needed for enzyme synthesis. It should be emphasised that all genetic manipulations performed on this DNA have strictly coordinate effects on production of all the enzymes and EPS. Complementation experiments suggest that the genes act as a regulatory "cascade", although much remains to be learned about the functioning of the system.

A 3.2kb *Bam*H1 fragment containing the presumptive gene C was sequenced to reveal an open reading frame capable of encoding a 74kd protein which showed strong homology of amino acid sequence to both the "sensor" and "regulator" components of the family of two-component prokaryotic regulatory genes (Ronson *et al*, 1987), and the N-terminal region has hydrophobic regions which could span membranes, like other sensors. In all other cases the sensors and regulators are encoded by separate genes, except for one recently described example (Stibitz *et al*, 1989). From amino acid residues 138 to 376 there is homology with the C-terminal sensor domains of the EnvZ and PhoR proteins, and residues 408 to 563 show homology to the regulator regions of NtrC, OmpR, SpoOF, CheY, PhoB and Dye (Fig 1). Although *X. c. campestris* appears to have a two-component regulatory system encoded by one gene, we cannot yet rule out the possibility that the gene product is cleaved post-translationally in the "linker" region to yield independent functional molecules. The sequencing experiments also revealed the presence of a gene called F, in opposite orientation to C, showing homology to the PhoM protein of *Escherichia coli*, which is involved in regulation of the phosphate assimilation proteins. Transposon mutagenesis shows that gene F is required for enzyme and EPS synthesis. A 5.1kb *Bam*H1 fragment adjacent to genes C and F, believed to contain genes D and G has also been sequenced and appears to contain 4 ORF's, one of which shows homology to regulators.

Analysis of more than 60 Tn*5lac* inserts in pIJ3020 has enabled minimal transcription units to be assigned (Fig 1).

Two-component regulatory systems usually regulate gene expression in response to changes in the environment. It is not known what triggers the system in this case, but since the gene products ultimately regulated have no obvious common features apart from a role in causing damage to plants, some plant-derived effect or substance is an obvious possibility. Two of the "target" structural genes, protease and endoglucanase, have been sequenced (Tang *et al*, in preparation; Gough, 1989) so that it should be possible to search for conserved sequences which could be binding sites for regulatory proteins which in turn could be products of one or more genes in the pIJ3020 region.

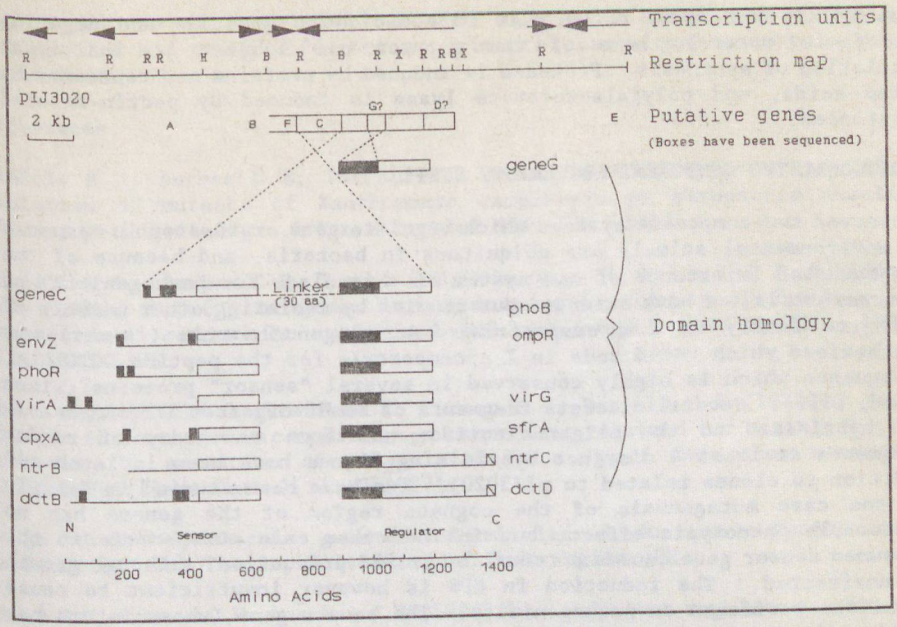


Figure 1. Organisation of the region of the genome of *X.c. campestris* cloned in pIJ3020. The arrows at the top indicate minimal transcription units deduced from Tn*5lac* mutagenesis. The second line is a restriction map showing positions of sites for *Bam*H1 (B), *Eco*R1 (E) *Hind*III (H) and *Xho*1 (X). The third line shows the positions of putative genes A to G, and the regions in boxes have been sequenced. From the derived amino sequences of ORFs presumed to be encoded by genes C and G there is clear homology with conserved domains of two component regulatory systems shown in the lower part of the figure. In the "sensor" proteins the small black boxes are transmembrane domains, and the open boxes at the C-terminal ends are the sensor domains which interact with the conserved black N-terminal boxes of the corresponding "regulator" proteins.

NEGATIVE REGULATION OF EXTRACELLULAR ENZYME SYNTHESIS

The pIJ3020 genes activate the synthesis of extracellular enzymes and EPS, and it might be supposed that a "balancing" negative regulation system should exist. A candidate gene (or group of genes) has been identified in the pLAFR3 recombinant cosmid pIJ3079. When this plasmid is introduced into the wild type *X. c.campestris* (thereby increasing the copy number of functional alleles), the production of all the enzymes and EPS is severely depressed, and the strain shows reduced virulence. However, the growth rate is not reduced, indicating that there is no reduction of general protein synthesis. Disruption of the chromosomal copy of the gene in the wild type strain causes increased enzyme and EPS production. The regulatory systems of pIJ3079 and pIJ3020 appear to be independent.

Finally, it should be noted that in addition to positive and negative coordinate control, some of the enzymes are subject to individual regulation of synthesis. Protease is induced by proteins and repressed by amino acids, and polygalacturonate lyase is induced by pectin-related substances.

ADDITIONAL TWO-COMPONENT REGULATORY SYSTEMS

Conserved two-component systems which regulate gene expression in response to environmental stimuli are ubiquitous in bacteria, and because of the demonstrated importance of one system of this class for pathogenicity of *X. c. campestris*, we have extended our studies by isolating other members of the gene family in *X. c. campestris*. An oligonucleotide (24-mer) was synthesised which would code in *X. c. campestris* for the peptide GGTGLGLA, a sequence which is highly conserved in several "sensor" proteins (Nixon *et al*, 1986). Several discrete fragments of *Bam*H1-digested *X c campestris* DNA hybridised to the oligonucleotide, and from a library of random fragments at least 8 distinct hybridising clones have been isolated (in addition to clones related to pIJ3020). Two have been studied in detail. In one case mutagenesis of the cognate region of the genome has no detectable phenotypic effect, but in the other case mutagenesis in the presumed sensor gene causes a reduction in EPS production, although growth is unaffected. The reduction in EPS is however insufficient to cause significant effects on pathogenicity. The sensor gene (whose nature has been confirmed by sequencing) appears to be linked to a regulator gene because an oligonucleotide which would code for a conserved internal peptide of a regulator gene hybridises to adjacent DNA.

Although this work has not yet led to the discovery of additional genes required for pathogenicity, it illustrates a novel approach to the study of regulatory genes, some examples of which have been shown to be important for pathogenicity not only of *X. c. campestris* but also of *Agrobacterium* (*vir*A and *vir*G).

DISCUSSION

The main theme of our research is the identification and characterisation of genes which are necessary for pathogenicity of *X c campestris*, and the study of regulation of gene expression has proved to be an important part of the story. The regulation of extracellular enzyme synthesis is surprisingly complex, with both individual and global positive and negative regulatory systems. EPS synthesis also involves an additional two-component system. Several strategies have been used for cloning bacterial pathogenicity genes, three being illustrated by the work described in this paper. (1) Genes, including the pIJ3020 cluster, have been cloned by virtue of their ability to complement a pathogenicity mutant. Initially no assumptions are made about the possible function of the genes, and this is discovered by subsequent analysis. (2) Genes to which a specific function may be ascribed are cloned using a screening test related directly to the function of the gene product (eg pIJ3079, or the protease structural gene (Tang *et al*, 1987)) and (3) A simple but "noisy" preliminary screen is used to isolate a sample of genes belonging to a certain class (eg conserved regulatory genes), and then more detailed work can be carried out to determine which genes are of interest in the context of pathogenicity.

The importance of using complementary strategies based upon different assumptions and using different experimental techniques cannot be over emphasised.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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GENETIC AND PATHOLOGICAL CHARACTERIZATION OF PATH$^-$ AND HR MUTANTS OF PSEUDOMONAS SYRINGAE PV. PHASEOLICOLA COMPLEMENTED BY CLONES FROM A WILD-TYPE GENOMIC LIBRARY

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INTRODUCTION

Pseudomonas syringae pv. phaseolicola, the causal agent of halo blight of bean, Phaseolus vulgaris, causes so-called watersoaked lesions on leaves and pods of susceptible bean cultivars. On the other hand, resistant bean cultivars and non-host plants like tobacco react to its attack by a rapid local necrosis called hypersensitive reaction (HR) (Klement, 1982). Mutants of P. s. pv. phaseolicola and other P. syringae pathovars have been obtained in several laboratories which due to the insertion of the transposon Tn5 have lost their ability to induce disease symptoms (Path$^-$) and, simultaneously, to elicit a hypersensitive reaction (HR$^-$).

We also previously isolated prototrophic Tn5 induced mutants of P. syringae pv. phaseolicola which were Path$^-$ and HR$^-$ and had a reduced multiplication capacity in susceptible host plants (Somlyai et al., 1986). Two of these mutants (533 and 989), each of which contained a single independent Tn5 insertion, were chosen for further studies. Our objective was to isolate clones from a wild-type gene library which complement these mutants to the wild-type phenotype and to study those clones genetically and as to their behavior in planta.

MATERIALS AND METHODS

A genomic library of the race 2 wild-type strain S21 was constructed in the wide host-range cosmid vector pVK102 according to standard procedures (Maniatis et al., 1982). The clones of this gene bank were conjugated in groups of 10 into the mutants via triparental matings. The resulting transconjugants were tested on tobacco leaves for HR induction and on bean leaves for symptom development as described below. In the case of a positive reaction, all ten clones of the respective library group were conjugated individually into the mutants to obtain transconjugants from single clones with which the plant test was repeated. Those clones complementing the mutants were then taken from the library for further analysis.

Restriction mapping of the clones was performed using single and double digests with the endonucleases HindIII, EcoRI, BamHI, and BglII. Hybridization of ^{32}P-labelled cloned DNA to Southern blots of digested DNA of all clones was carried out according to Maniatis et al. (1982).

The pathogenicity of the transconjugants was tested by high pressure infiltration of $5X10^6$ cells.ml^{-1} into the susceptible bean cultivar "Red Kidney". On one hand, the number and size of water-soaked lesions were measured. On the other hand, the population rate of mutants in the bean leaf tissue was followed over a six-day period, comparing them to the Path$^-$ mutants and the wild-type strain as controls. HR was observed in leaves of the tobacco cultivar "White Burley" infiltrated by 10^6 cells.ml^{-1} suspensions of bacteria (Klement, 1982).

RESULTS

Three clones (12-7, 33-1, 33-15) were isolated from the wild-type gene library, which restored the Path$^+$ HR$^+$ phenotype to mutant 533. Clone 12-7 could also complement

mutant 989, which contains its Tn5 insertion at a different site presumably coding for different proteins (El-Kady et al., 1986).

The typical water-soaked symptoms on bean leaves inoculated both by the transconjugants and the wild-type occurred on the 3rd to 4th day. The number of lesions related to 1 cm^2 leaf area varied greatly, from 150 to 1120 lesions with the transconjugants as compared to 1200 lesions with the wild-type (Table 1).

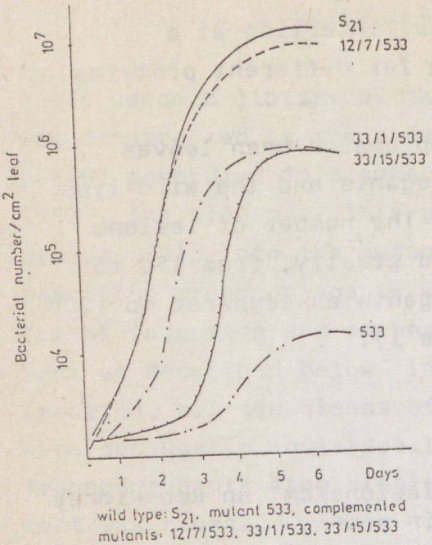
Table 1 Number of water-soaked lesions/cm^2 on Red Kidney bean leaves

Trans-conjugants	12/7/533	33/1/533	33/15/533	12/7/989	S 21
Number of lesions	1080	150	402	1120	1200

This difference between the transconjugants was also evident from their multiplication rate in planta. While clone 12-7 restored bacterial cell number of both Path$^-$ mutants to that of the wild-type (Fig. 1) mutant 533 could be complemented only partially by clones 33-1 and 33-15 (Fig. 2).

When the HR-causing ability of the transconjugants was examined in tobacco leaves it was found that all of them induced a hypersensitive reaction.

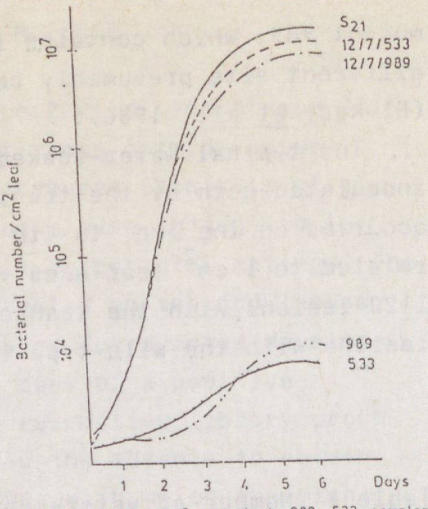
After restriction mapping, cosmid 12-7 turned out to harbor an insert almost identical with the so-called hrp cluster isolated by Panopoulos and co-workers from a race



wild type: S_{21}, mutant 533, complemented mutants: 12/7/533, 33/1/533, 33/15/533

Fig. 1

Multiplication of P.s.pv. phaseolicola wild type, mutant and complemented mutants in bean leaves



wild type: S_{21}, mutants: 989, 533, complemented mutants: 12/7/533, 12/7/989

Fig. 2

Multiplication of P.s.pv. phaseolicola wild type, mutants and complemented mutants in bean leaves

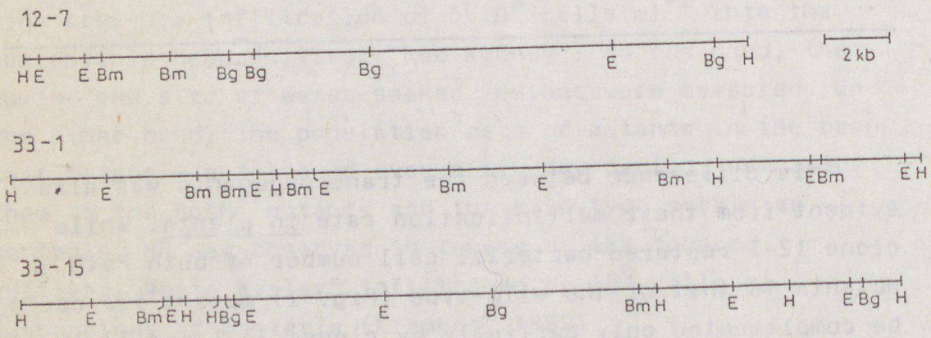


Fig. 3 Restriction site map of 3 clones complementing Path⁻ HR⁻ mutants of P. s. pv. phaseolicola to the wild-type phenotype

1 isolate of P. s. pv. phaseolicola (Lindgren et al. 1986). The other two recombinant plasmids (33-1, 33-15) neither share homology with the above clone 12-7 (data not shown), nor are they identical to each other on the basis of their restriction maps (Fig. 3) and hybridization experiments.

DISCUSSION

These results confirm that there is a correlation between the ability of bacteria to cause disease and to multiply in the intercellular spaces of susceptible leaves.

Furthermore, it could be shown that there is more than the one gene already described (Lindgren et al. 1986) which is involved in HR and pathogenicity. The two additional clones isolated (33-1, 33-15) lead only to a partial restoration of the wild-type phenotype in one mutant, 533, however. On the other hand, both mutants which as was shown are not identical to each other could be complemented by clone 12-7 which resembles the DNA region carrying the above hrp genes. The simplest explanation for the latter observation might be that the two Tn5 insertion sites are adjacent to each other so that they can be complemented by one large fragment spanning the whole region. Neverheless, as the three clones do not harbor overlapping sequences this possibility cannot explain satisfactorily the first finding that all three clones can complement one mutant. It seems quite possible, however, that the additional clones do not carry those sequences switched off in the Tn5 mutant but genes with similar functions which being present in the cell in several copies can partially restore the wild-type phenotype. Further speculations as to the meaning of these unusual findings should be postponed until more experimental support is available.

Currently we are repeating conjugation of clones 33-1 and 33-15 into mutant 989 which so far has not yielded

$Path^+HR^+$ transconjugants. Also, hybridization of labelled DNA of all clones to immobilized total DNA of the mutants and their complemented derivatives is being carried out to clarify the relationship between the $Path^-$ mutants and the complementing clones.

Yet the question remains whether the genes isolated have a direct influence on pathogenicity and HR induction, or whether they affect other general functions such as bacterial multiplication.

ACKNOWLEDGEMENT

This work was supported by grants of the Hungarian Academy of Sciences and the German Academic Exchange Service (DAAD) to M.N.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

THE USE OF *hrp* GENES TO IDENTIFY OPPORTUNISTIC XANTHOMONADS

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ABSTRACT

Genomic DNAs from 20 strains of opportunistic xanthomonads and from 33 strains of 17 pathovars of *Xanthomonas campestris* were restricted with *Eco*R1 and probed with a segment of DNA from *X. campestris* pv. *vesicatoria* that complimented nonpathogenic mutants of that bacterium to pathogenicity (*hrp* genes). The probe did not hybridize with DNA from 11 of the 20 opportunistic xanthomonads and hybridized weakly to the others. The probe hybridized with restricted DNA from all 33 plant pathogenic strains. Polymorphism of hybridization profiles existed within the plant pathogenic strains.

INTRODUCTION

Many genera of bacteria that have yellow pigments can be isolated from plant material. Occasionally colonies of *Xanthomonas*, which are not pathogenic to the plant from which they were isolated, have been obtained. These xanthomonads have been identified by the presence of xanthomonadins and by their unique fatty acid profiles. Physiologically, the group belongs to the species *X. campestris* and members have been called opportunistic xanthomonads (Gitaitis *et al.*, 1987).

Whether the opportunistic xanthomonads are pathogenic to some unknown plant, pathogenic only under conditions of plant stress, or not pathogenic is undetermined. Hypersensitivity in tobacco, pepper, or tomato, which is sometimes used as an indicator of pathogenicity, does not occur with these xanthomonads, but hypersensitivity to those plants is variable among known pathogenic xanthomonads (Irey and Stall, 1982). Many of the opportunistic xanthomonads produce pectolytic enzyme(s) and can cause a soft-rot of mature tomato and pepper fruit (Gitaitis *et al.*, 1987).

If the opportunistic xanthomonads are indeed plant pathogens, they should have genes that determine growth in plant tissue. A gene cluster that appears to be involved in the growth of *X. campestris* pv. *vesicatoria* (Xcv) in host tissue was isolated by recombinant DNA techniques in our laboratory (Minsavage and Stall, Unpublished). This cluster compliments nonpathogenic mutants of Xcv, which do not grow in host tissue, to pathogenicity. Complimented mutants grow in host tissue equally as well as the wild strains. The gene cluster also restores to nonpathogenic mutants the characteristic of hypersensitivity in tobacco and other nonhost plants. Random insertion of the transposon, Tn*5*, into a region of at least 17 kbp of the gene cluster prevents complementation of nonpathogenic mutants. These characteristics are comparable to the *hrp* genes isolated from other plant pathogenic bacteria (Lindgren *et al.*, 1986, Boucher *et al.*, 1987).

The purpose of this paper is to report hybridization experiments involving DNA of the opportunistic and selected plant pathogenic xanthomonads with the *hrp* DNA from Xcv. The results may help to decide if the opportunistic xanthomonads are indeed plant pathogenic.

MATERIALS AND METHODS

Twenty strains of opportunistic xanthomonads and 33 strains of 17 pathovars of *X. campestris* were used in this study. Among the 20 opportunistic xanthomonads 10 strains were included in a previous study (Gitaitis *et al.*, 1987), four strains were isolated from citrus, one strain from *Bidens pinnata* L., a weed in a citrus grove, and the rest were from various plants. The pathovars of *X. campestris* were obtained from many sources. All strains were maintained in sterile tap water in our laboratory.

Pathogenicity of bacteria to their hosts of origin and hypersensitivity to tobacco (*Nicotiana tabacum* L., cultivar Bottom Special) was determined by inoculation of leaves by infiltration with a hypodermic needle and syringe. Inocula were produced in nutrient broth, centrifuged to a pellet, and suspended in sterile tap water. Suspensions were standardized to 0.3A at 600 nm wavelength with a Spectronic 20 spectrophotometer. The concentrations ranged from 3–5 X 10^8 cell/ml.

The gram reaction was determined by the KOH method (Suslow, *et al.*, 1982) and the presence of xanthomonadins was determined by thin–layer chromatography (Irey and Stall, 1982). Fatty acid profiles were determined with the Microbial Identification

System (Microbial ID, Inc., 115 Barksdale Prof. Center, Newark, DE 19711) according to the instructions accompanying the system.

Genomic DNA was extracted from cultures of the xanthomonads by the method of Boucher et al. (1987). For hybridization, genomic DNA was digested with *Eco*R1 for 2 hrs at 37°C and with RNAse A for 0.5 hr at the same temperature. Samples were electrophoresed in a gel containing 0.5% agarose in TAE buffer overnight at 2V/cm of gel. The DNA was then transferred from the gel to Gene Screen Plus hybridization membranes (DuPont Company, NEN Products, Boston, MA 02118) by manufacturer's instructions. Hybridization was accomplished according to the protocol for the Genius nonradioactive DNA labeling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, IN 46250). Random primed DNA of the *hrp* gene cluster inserted into the cosmid, pLAFR–3 was labelled with digoxigenin–dUTP. Hybridization occurred at 68°C for 18–24 hrs after which the membranes were washed either (No. 1) two times for 15 minutes with 0.5X SSC and 0.1% SDS at 65°C, or (No. 2) two times for 15 min with 0.1X SSC and 0.1% SDS at 68°C. Detection of hybrids was by enzyme immunoassay.

RESULTS AND DISCUSSION

All of the bacteria used in this work were gram negative and contained xanthomonadins. They were identified as *Xanthomonas campestris* by computer comparison of their fatty acid profiles with the profiles from pathovars of *X. campestris*. The pathovars were pathogenic to their known hosts and those identified as opportunistic were not pathogenic to the host from which they were isolated. None of the opportunistic xanthomonads produced hypersensitivity in tobacco.

Fragments of different sizes were obtained by digestion of the genomic DNA from the strains of pathovars and opportunistic xanthomonads with *Eco*R1. Hybridization bands occurred with DNA from all 33 strains of the pathovars and the *hrp* DNA. Eight fragments of DNA from *X. campestris* pv. *vesicatoria* hybridized with the *hrp* gene cluster isolated from that bacterium. The numbers of fragments of DNA from the 33 strains of pathovars of *X. campestris* that hybridized with the *hrp* DNA were variable. Different strains of a pathovar had similar hybridization profiles. The number of hybridization bands for each pathovar remained the same with different post–hybridization washes except for pv. *dieffenbachiae* and pv. *translucens*. Results with one strain of each pathovar are in Table 1 and Fig. 1.

Table 1. The number of bands of hybridization between DNA of *hrp* genes of *Xanthomonas campestris* pv. *vesicatoria* and *Eco*RI restricted total DNA of opportunistic xanthomonads and pathovars of *X. campestris*.

Pathovar	Strain	Stringency[a] 1	2	Opportunistic xanthomonad	Strain	Stringency 1	2
vesicatoria	E-3	8	8	tomato	pk	2	1
phaseoli	G-24	5	5	tomato	peto	2	0
glycines	8701	2	2	tomato	H6653	1	1
vignicola	82-38	6	6	tomato	wal	2	0
alfalfae	82-1	9	9	tomato	663	3	1
dieffenbachiae	79-2	3	2	pepper	jal	2	0
malvacearum	R-4	6	6	pepper	bb	3	0
campestris	2669	5	5	nightshade	T-55	1	1
raphani	86-10	2	2	orange	S52	4	2
carotae	13	5	5	orange	ina69	1	0
poinsettiicola	424	5	5	bidens	2-8	1	0
zinniae	1984	4	4	toadflax	tdf	2	2
maculifolium-gardeniae	2410	8	8				
vitians	164	11	11				
pelargonii	1370	10	10				
begoniae	G-13	3	3				
translucens	80-1	10	2				

[a]Stringency refers to post-hybridization washes, which were (1) two times for 15 minutes with 0.5X SSC and 0.1% SDS at 65°C and (2) two times for 15 minutes with 0.1X SSC and 0.1% SDS at 68°C.

Hybridization of *hrp* DNA to restricted DNA of 11 of 20 strains of opportunistic xanthomonads was not detected after the most stringent post–hybridization wash. Only weak hybridization of DNA from the remaining 9 strains of opportunistic xanthomonads occurred with the *hrp* DNA based on the weak signal and the variation in numbers of bands with washes of different stringencies. Results of 10 strains of the opportunistic xanthomonads are in Table 1 and Fig. 1.

The polymorphism in *hrp* genes among pathovars complicates the use of them to formulate conclusions about the pathogenicity of the opportunistic xanthomonads. Whereas all of the sequences of the *hrp* DNA appear to be necessary for growth of *X. campestris* pv. *vesicatoria* in its hosts, the extent of polymorphic variation that can occur in a *hrp* gene cluster with no change in function of gene products is not known.

At least some of the *hrp* gene sequences seem to be present in some of the opportunistic xanthomonads. If an intact *hrp* gene cluster is necessary for growth in plant tissue, possibly an internal fragment of the *hrp* gene cluster could be more useful in distinguishing the pathogenic xanthomonads from the opportunistic types.

The opportunistic xanthomonads appear to be a group distinct from pathovars of *X. campestris* based on hybridization of genomic DNA with the *hrp* genes from *X. campestris* pv. *vesicatoria*. The percentages of total DNA hybridization, similarity

coefficients of restriction fragment length polymorphism with several probes, and more complete phenotypic comparisons of members of the group with members of *X. campestris* should be completed to determine the taxonomic position of the group.

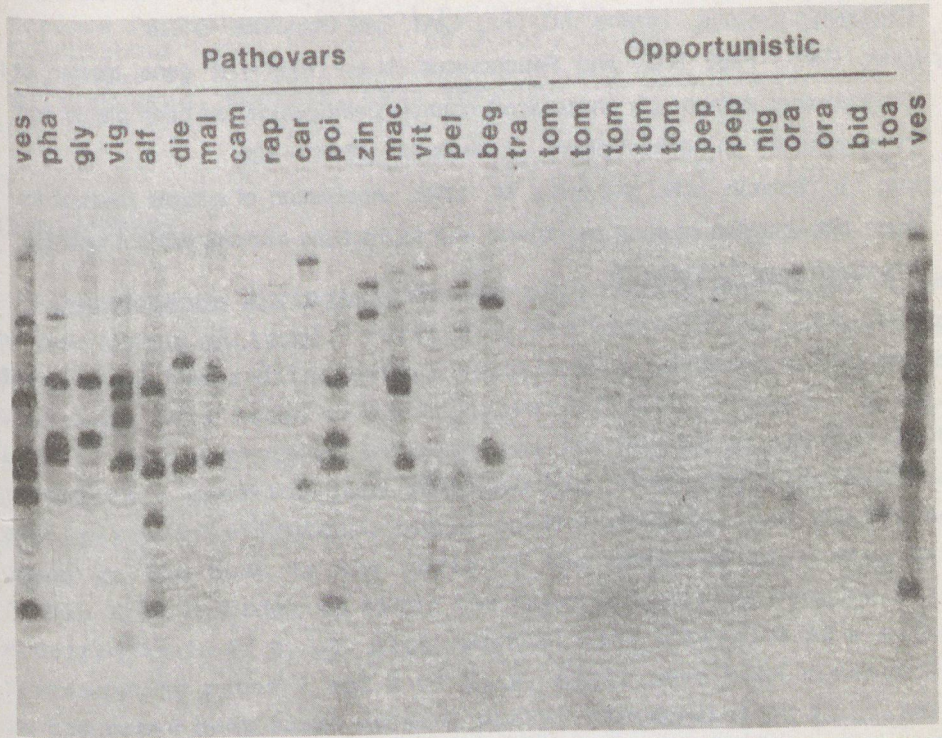


Fig. 1. Southern blot analysis of *Eco*R1 digested total DNA of pathovars of *Xanthomonas campestris* and opportunistic xanthomonads probed with *hrp* gene cluster inserted into the cosmid, pLAFR

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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CLONING OF SPECIFIC AVIRULENCE GENES FROM PSEUDOMONAS SYRINGAE PATHOVAR PISI

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INTRODUCTION

Pseudomonas syringae pathovar pisi causes bacterial blight of pea (Pisum sativum). Currently, six races of the pathogen are defined by their differential reactions in stem-inoculations of seedlings of a range of pea cultivars (Table 1; Taylor et al., 1989). This pattern of interactions led Taylor et al. (1989) to postulate a gene-for-gene relationship involving five matching gene pairs of avirulence genes in the pathogen and resistance genes in the host as the basis of the observed race structure (Table 1). In order to study the genetic basis of host cultivar specificity in P. s. pv. pisi, partial gene libraries of strains, representing races 2 and 3 of P. s. pisi, have been constructed in the broad host range cosmid, pLAFR3 (Staskawicz et al., 1987), in Escherichia coli strain 803 (Vivian et al., 1989). The resulting clones were mobilized into rifampicin-resistant derivatives of wild-type strains of P. s. pisi using the helper plasmid pRK2013 (Figurski & Helinski, 1979).

CLONING OF A RACE 2-SPECIFIC AVIRULENCE GENE

The race 2 strain 203 (Taylor, 1972) was used to construct a gene library (Atherton, 1987), which was mobilized as individual recombinant cosmids into a rifampicin-resistant derivative of the race 1 strain, 299A (Taylor, 1972). Both races 1 and 2 were compatible on the universally-susceptible cultivar Kelvedon Wonder, but on cv. Early Onward, race 2 was incompatible

Table 1. Postulated gene-for-gene relationship

		Races of P. s. pisi					
		1	2	3	4	5	6
	A genes	1,3	2	3	4	2,5	none
Pea cultivars	R genes						
Kelvedon Wonder	none	+	+	+	+	+	+
Early Onward	2,5	+	-	+	+	-	+
Shasta/Puget	1,3	-	+	-	+	+	+
Partridge	1,3,4,5	-	+	-	-	-	+
Hursts Greenshaft	1,4,5	-	+	+	-	-	+
Vinco	1,2,3,5	-	-	-	+	-	+
Fortune	1,2,3,4,5	-	-	-	-	-	+

Dominant avirulence (A) genes and resistance (R) genes are shown in pathogen and hosts respectively + = compatible, - = incompatible.

Table 2. Reactions of pea cultivars to races harbouring pAV200

	Pea cultivar *				
	KW	EO	Sh	V	F
Pathogen race					
1	+	+	-	-	-
1 + 2 **	+	-	-	-	-
2	+	-	+	-	-
3	+	+	-	-	-
3 + 2 **	+	-	+	-	-
4	+	+	+	+	-
4 + 2 **	+	-	+	-	-
6	+	+	+	+	+
6 + 2 **	+	-	+	-	-

* KW, Kelvedon Wonder; EO, Early Onward; Sh, Shasta; V, Vinco; F, Fortune.
** Transconjugant harbouring pAV200.
+ = compatible, - = incompatible.

and race 1 compatible. Transconjugants of race 1 harbouring the race 2 clones were tested by stem-inoculation in cv. Early Onward, which resulted in the identification of a single cosmid clone, designated pAV270, which conferred avirulence on race 1

towards cv. Early Onward, but which did not affect the compatible interaction of race 1 with cv. Kelvedon Wonder (Vivian et al., 1989). Sub-cloning of pAV270 has resulted in the localisation of the avirulence gene (provisionally designated avrAspil) to a 4.0kb EcoRI fragment.

Introduction of pAV200 (the 4.0kb fragment cloned in pLAFR3) into races 1, 3, 4 and 6, followed by screening on a range of pea cultivars has confirmed the race-specific nature of the avirulence gene (Table 2). However, in one interaction, an anomalous result was obtained, for which at present, we have no explanation. Introduction of pAV200 into race 3, which is incompatible on cv. Shasta (due to the postulated presence of resistance gene, R3), changed the reaction to compatible.

CO-SEGREGATION OF RESISTANCE TO RACE 2 AND A RACE 1 TRANSCONJUGANT HARBOURING pAV270

A cross between host cultivars Kelvedon Wonder (lacking all race-specific resistance genes) X Early Onward (postulated to possess R2 and R5) showed that the ratio of progeny resistant or susceptible to race 2 segregated in a sample of F_2 progeny in a ratio 3:1, consistent with the presence of a single dominant gene R2 being present in cv. Early Onward. Sequential inoculation of the F_2 progeny with race 1, race 2 and race 1 + pAV270, showed co-segregation of resistance to race 2 and to race 1 harbouring

Table 3. Co-segregation of resistance in F_2
A cross between pea cvs. Kelvedon Wonder (r) X Early Onward (R2), F_2 progeny were tested by sequential inoculation with race 1 (299A), race 2 (203) and race 1 harbouring pAV270.

	Phenotypes	
Expected ratio	3	:1
race 1	+	+
race 2	-	+
race 1 + pAV270	-	+
no. of progeny	154	39

data simplified from Vivian et al. (1989)

pAV270 (Table 3). A sample of F_2 progeny from a further cross, involving cvs. Kelvedon Wonder X Fortune (harbouring all five R genes), also confirmed the specific interaction of avrAspil with R2, consistent with a gene-for-gene interaction (Vivian et al., 1989).

CLONING OF RACE 3-SPECIFICITY

A mutant strain of the race 1 strain 299A, designated PF24 was isolated by Malik et al. (1987). This mutant was one of a class showing reduced pathogenicity towards a particular pea cultivar and was obtained following introduction of pSUP2011 into strain 299A. Subsequently, it has been shown to lack one of two plasmids present in 299A, and also to show a change of interaction with the group of cultivars represented in Table 1 by cv. Shasta; whereas 299A is incompatible with cv. Shasta, PF24 is compatible (A.D. Bavage & A. Vivian, unpublished results). Although postulated to harbour R1 and R3, in crosses involving the group to which cv. Shasta belongs, no segregation of resistance to races 1 and 3 has been observed (J.R. Bevan, pers. comm.), suggesting that this group of cultivars may harbour only R1 or R3 alone.

Therefore a race 3 gene library of strain 870A, was introduced into a rifampicin-resistant derivative of the mutant PF24, and the transconjugants were tested on cv. Puget (of similar resistance genotype to cv. Shasta). A total of eleven clones were isolated that conferred avirulence on PF24 towards cv. Puget. Seven of these appear to share common insert fragments when digested with Eco RI (data not shown). One of these clones, designated pAV271, was radiolabelled and used to probe digests of plasmid DNA from races 1 and 3 and from the cosmid clones. This confirmed the presence of similar-sized and cross-hybridising fragments in the cosmids and strain 870A (race 3), and also the presence of cross-hybridising fragments in strain 299A (race 1) but not PF24 (A.D. Bavage & A. Vivian, unpublished results). This suggests that race 1 may harbour an avirulence gene for race 3-specificity, which can restore PF24

to avirulence towards cvs. Puget and Shasta. Furthermore this gene is located on plasmid DNA in race 3 and possibly also in race 1. It would also indicate the presence of a single resistance gene in cultivars such as Puget and Shasta. The remaining 4 clones which are not related in DNA sequence to pAV271 on the basis of cross-hybridisation have not been further investigated, but clearly might indicate the presence of further avirulence genes in 870A.

Acknowledgements

This work was supported by project grants from the Agricultural & Food Research Council to A.V. and was carried out under MAFF licences numbers PHF 678/46(26), PHF678/47(26) and PHF 428A/42(96) issued under the Plant Pests (Great Britain) Order 1980.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

HOMOLOGOUS STREPTOMYCIN RESISTANCE GENE PRESENT AMONG DIVERSE BACTERIA IN NEW YORK APPLE ORCHARDS

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INTRODUCTION

Resistance to streptomycin in Pseudomonas syringae pv. papulans, the causal agent of blister spot of apple, is associated with a conjugative plasmid. The disease is predominant on, but not restricted to the Mutsu cultivar. Previously growers in New York successfully controlled the disease with streptomycin sprays, first applied 10 to 14 days after petal fall, but in 1985 incidences of control failure were brought to our attention. We subsequently isolated strains of P. syringae pv. papulans that were resistant to streptomycin from several orchards in New York and demonstrated their ability to overwinter in buds. There was a significant correlation between the number of streptomycin sprays applied the previous season and the detection of streptomycin-resistant strains of P. syringae pv. papulans, and we showed that resistance to streptomycin is associated with a conjugative plasmid (Burr et al. 1988).

The streptomycin resistance determinant of P. syringae pv. papulans has been cloned and was used to develop a DNA probe specific for streptomycin-resistant strains of P. syringae pv. papulans. The probe is a 250 bp DNA fragment that is a portion of a 1.1 kb region shared among 3 different DNA clones of the P. syringae pv. papulans streptomycin resistance gene. When the probe was hybridized to a Southern blot of digested plasmid and organismal DNA of 11 field strains of P. syringae pv. papulans from eight different orchards in New York, it did not hybridize with any DNA from 4 streptomycin-sensitive strains but hybridized with DNA from all seven of the resistant strains.

We report here that many strains of Pseudomonas and apparently unrelated bacteria that were isolated from apple orchards in New York were resistant to streptomycin and carry genes for resistance that are homologous to the P. syringae pv. papulans resistance gene.

METHODS

Bacteria were isolated from apple leaves and soil collected in 12 New York orchards three weeks after petal fall (7 June 88). Three orchards were selected from each of the following 4 categories: Mutsu orchards previously treated or not treated with streptomycin and non-Mutsu orchards previously treated or not treated with streptomycin.

500 to 1000 leaves were collected at random from each orchard and 6 replicate 5 gm sub-samples of leaves were sonicated in 50 ml of 0.05M phosphate buffer, pH 6.5, for 10 min. In each orchard, 40 soil samples consisting of 2.5 cm soil cores of the top 5 to 10 cm of soil were collected at the tree drip line. Soil samples from a single orchard were combined, mixed, and 3 replicate 10 gm sub-samples were shaken in 100 ml of phosphate buffer for 30 min. Serial 100-fold dilutions of leaf and soil washes were plated on nutrient agar amended with 200 ug cycloheximide and 50 ug streptomycin per ml and on the P. syringae selective KBC media (Mohan and Schaad 1987) amended with 50 ug of streptomycin per ml.

After 48 hr incubation at 28°C, one to two colonies representative of each morphological type present on a isolation plate was selected for further analysis. Isolates were maintained on fifty-grid Luria-Bertani plates amended with 50 ug of streptomycin per ml and used for colony hybridizations with the streptomycin resistance probe of P. syringae pv. papulans. Each plate contained the P. syringae pv. papulans strains Psp 36 (streptomycin-resistant) and Psp 32 (streptomycin-sensitive) as positive and negative controls.

Based upon a Gram test and the ability to fluoresce on King's B medium bacteria were placed in three general groups: gram positive bacteria, non-fluorescent gram negative bacteria and fluorescent Pseudomonads. P. syringae pv. papulans was distinguished from other fluorescent Pseudomonads by a negative reaction for the following characteristics: oxidase, gel liquefaction, levan production from sucrose, and ice-nucleation at -3°C. Six strains characterized as P.

syringae pv. papulans by these tests were inoculated onto immature Mutsu fruit in the orchard and all produced typical blister spot symptoms.

RESULTS

39% of 398 streptomycin resistant bacteria isolated from the orchards were homologous to the P. syringae pv. papulans streptomycin-resistance probe in colony hybridizations. Homology was found in both leaf epiphytes and soil bacteria. Observed homologies were: 100% of 36 Psp strains, 0% of 75 gram positive strains, 44% of 93 fluorescent Pseudomonads (excluding Psp), and 58% of 194 gram negative strains (excluding fluorescent Pseudomonads). Homologous fluorescent Pseudomonads included both oxidase positive and negative strains.

Plasmid DNA was isolated from 103 strains showing homology, digested with AvaI, size fractionated by gel electrophoresis, Southern transferred to hybridization membrane, and hybridized with the streptomycin-resistance probe of P. syringae pv. papulans. Homology was conserved to the same size restriction fragment in 100% of 8 P. syringae pv. papulans strains, 100% of 17 fluorescent Pseudomonads isolated from leaves and 83% of 12 strains isolated from soil, and 79% of 43 non-fluorescent gram negative bacteria isolated from leaves and 52% of 23 strains isolated from soil.

In bacteria other than Psp, homology occurred on plasmids of several different sizes.

These data indicate that the streptomycin resistance gene of P. syringae pv. papulans is shared among the gene pool of a diverse group of gram negative bacteria.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

DEVELOPMENT OF A NONRADIOACTIVE DNA PROBE SPECIFIC FOR CORONATINE-PRODUCING BACTERIA

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ABSTRACT

Pseudomonas syringae pv. tomato, causal agent for bacterial speck of tomato, produces the chlorosis-inducing toxin coronatine. In a previous study, we used Tn5 mutagenesis to identify and characterize a region of the P. syringae pv. tomato genome involved in coronatine production. In the present report, a 5.3-kb XhoI fragment from this region was subcloned into pGB2, labeled with a nonradioactive labeling kit, and tested as a hybridization probe specific for coronatine-producing bacteria. Thirteen P. syringae pv. tomato strains and 18 strains from other P. syringae pathovars including syringae, maculicola, lachrymans, morsprunorum, atropurpurea, coronafaciens, persicae, pisi, phaseolicola, tabaci, and glycinea were screened for homology to the probe using slot blot hybridization. Only those bacteria shown by leaf bioassay to produce coronatine reacted with the probe. The probe did not hybridize to the tomato plant epiphytes P. putida, P. marginalis, and P. viridiflava, nor to the tomato pathogens P. solanacearum and Clavibacter michiganense subsp. michiganense. The leachate from one macerated bacterial speck (tomato) or bacterial blight (soybean) lesion gave a strong response to the probe while healthy tissue or diseased tissue from bacterial wilt- or bacterial canker-infected tomato plants was nonreactive. The detection limit for this stable, nonradioactive probe was approximately 3,000-10,000 P. syringae pv. tomato cells.

INTRODUCTION

Pseudomonas syringae pv. *tomato* causes bacterial speck, one of the major bacterial diseases of tomato in Canada. Traditionally, distinction of this pathogen from other bacterial tomato pathogens, such as *P. syringae* pv. *syringae* and *Xanthomonas campestris* pv. *vesicatoria*, has relied upon a series of time-consuming biochemical and pathogenicity tests. A more rapid means of diagnosis is available through serological methods and phage typing (1, 6). Unfortunately, these procedures are not yet specific enough to serve as the sole means of identification . Another rapid, simple method for disease diagnosis involves the use of nucleic acid hybridization probes (8). Provided the appropriate DNA sequences have been selected, such probes offer high specificity and sensitivity. The one *P. syringae* pv. *tomato* DNA probe currently available exhibits a weak homology to DNA from several of the other *P. syringae* pathovars, including the tomato pathogen *P. syringae* pv. *syringae* (4). The present report describes a nonradioactively-labeled *P. syringae* pv. *tomato* DNA probe that binds specifically to the DNA of bacteria that produce the toxin coronatine. Since *P. syringae* pv. *tomato* is the only coronatine-producing bacterium normally found in tomato fields, this probe should prove useful in the diagnosis of bacterial speck.

MATERIALS AND METHODS

Bacteria, media, and cultural conditions. In addition to the strains listed in Table 1, the following bacteria were used in this study: *Pseudomonas solanacearum* K60, *Clavibacter michiganense* subsp. *michiganense* JD83-1, *Xanthomonas campestris* pv. *vesicatoria* XV21 and XV34, *Pseudomonas fluorescens-putida* group 9A2, 8B2, 10A3, 9B3, and 4A3, *Pseudomonas viridiflava* AP-1, T9B1, and MF-2, and *Pseudomonas marginalis* DC83-1. The media used to grow and maintain these bacteria have already been described (1).

Isolation and manipulation of DNA. Genomic and plasmid DNA isolation, nick translation labeling of DNA, Southern blotting, restriction enzyme mapping, and molecular cloning were performed according to standard procedures (7) or have been described previously (2).

Preparation of slot blots. A BRL hybri-slot manifold apparatus was used to deposit purified DNA, bacterial cells, and tomato leaf leachate onto a nitrocellulose membrane filter. Procedures for cell lysis, DNA denaturation, and hybridization of the blots to labeled probes have been described by Maniatis et al. (7).

Nonradioactive labeling procedure. The P. syringae pv. tomato DNA probe was labeled using Chemiprobe, a nonradioactive DNA labeling kit (FMC Corp., Mandel Scientific Company LTD., Guelph, Ontario.). After the probe was hybridized to the blot, the hybrid complexes were visualized using the combined immunoenzymatic reaction described in the Chemiprobe manual.

RESULTS AND DISCUSSION

In a previous study Tn5 mutagenesis was used to identify and characterize a region of the P. syringae pv. tomato genome involved in coronatine synthesis. A 5.3-kb XhoI fragment from this region was cloned into the SalI site of the cloning vector pGB2 (4 kb)(3). The resulting recombinant plasmid, pTPR1, was labeled with Chemiprobe and tested for homology to the P. syringae pathovars listed in Table 1. Only those strains shown by leaf bioassay to produce coronatine hybridized with the probe. Of the four coronatine- and probe-positive pathovars, only tomato is likely to be found in Canadian tomato fields (1). The P. syringae pv. tomato strains included in this assay came from Ontario, four different regions of the U.S., New Zealand, France, and Switzerland. NCPPB1008 (USA) was the one P. syringae pv. tomato strain tested that did not react with the probe or synthesize coronatine. This strain was first isolated in 1942; whether or not it produced the toxin initially is unknown. Since the value of pTPR1 as a diagnostic probe depends

upon the frequency of the coronatine sequences among field strains, we currently are surveying fresh isolates of the pathogen from Ontario's major tomato-growing regions for toxin production.

The coronatine probe, tested against both pure cultures and infected leaf tissue, had no homology with DNA from the tomato pathogens P. syringae pv. syringae (Table 1), P. solanacearum

Table 1. Response of various P. syringae pathovars to the P. syringae pv. tomato DNA probe pTPR1.

Pathovar	Number of strains	Coronatine positive	Probe positive
tomato DC3000, DC84-1, AV80, G13, JL1035, SM78-1, DCT6D1, NCPPB1008, CNBP1318, 188B, OH314, PDDCC3357, CNBP1323	13	12	12
antirrhini PDDCC2738	1	0	0
atropurpurea 340, 1304	2	2	2
coronafaciens 345	1	0	0
glycinea F111, B3	2	2	2
lachrymans 419	1	0	0
maculicola 437,438	2	1	1
morsprunorum 436	1	0	0
persicae PDDCC5846	1	0	0
phaseolicola HB10Y	1	0	0
pisi 150	1	0	0
syringae B78*, NCPPB2747*, NCPPB281, 84-15	4	0	0
tabaci GB1	1	0	0

* tomato isolates.

(one strain), X. campestris pv. vesicatoria (two strains), and C. michiganense subsp. michiganense (one strain). P. marginalis (one strain), P. viridiflava (three strains), and members of the P. fluorescens-putida group (five strains), all common to tomato plant surfaces (1), also did not react with the probe.

The sensitivity of pTPR1 was tested using purified P. syringae pv. tomato genomic DNA, bacterial cells, and infected leaf tissue. The limit of detection for both Chemiprobe-labeled and ^{32}P-labeled (overnight exposure) probe was approximately 2 ng of purified genomic DNA. Chemiprobe-labeled probes have a strong advantage over ^{32}P-labeled probes in that they are more stable (up to one year at -20 C), cheaper, and less of a biohazard. The smallest number of bacterial cells that could be detected with Chemiprobe-labeled pTPR1 was between 3×10^3 and 10^4 cfu. These numbers are in agreement with those obtained using diluted suspensions of bacterial speck lesions (Fig. 1). Since a typical bacterial speck lesion contains $3\text{-}6 \times 10^6$ P. syringae pv. tomato cells (1) and since plant tissue may clog the filter, hybridization assays are best performed with dilutions of the infected material. Conceivably, this probe,

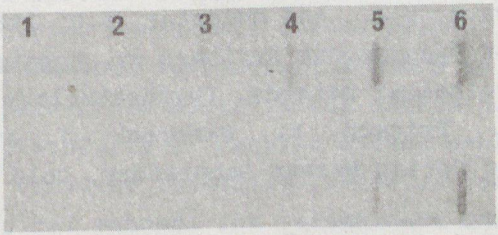


Figure 1. Slot blot hybridization of DNA from diluted suspensions of bacterial speck lesions to the P. syringae pv. tomato DNA probe pTPR1. Top row (P. syringae pv. tomato DCT6D1): lane 6, 1.5×10^6 cfu; lane 5, 3×10^5 cfu; lane 4, 3×10^4 cfu; lane 3, 3×10^3 cfu; lane 2, 3×10^2 cfu; lane 1, uninoculated leaf tissue. Bottom row (P. syringae pv. tomato DC3000): lane 6, 1.6×10^5 cfu; lane 5, 3×10^4 cfu; lane 4, 3×10^3 cfu; lane 3, 3×10^2 cfu; lane 2, 3×10^1 cfu; lane 1, uninoculated leaf tissue.

because of its high sensitivity, could be used in tomato transplant certification programs to screen for the presence of the bacterial speck pathogen on symptomless tissue. It may even be applicable to a disease forecasting system such as the one recently proposed by Jardine and Stephens (5).

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

PATHOGENICITY IS ASSOCIATED WITH SURFACE ANTIGENS OF PSEUDOMONAS SYRINGAE PV. SYRINGAE

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Introduction

Bacterial brown spot disease on bush beans (Phaseolus vulgaris) is caused by Pseudomonas syringae pathovar syringae (P. syringae) strain R32. A non-pathogenic mutant, PS9021, also unable to cause the hypersensitive reaction (HR) on tobacco (Anderson et al. 1984, Niepold et al. 1985) was generated by transposon (Tn5) mutagenesis from P. syringae strain R32. Subsequent application of recombinant DNA techniques led to the detection of an 8.5 kilobase pair (kb) wild type DNA fragment that could complement the non-pathogenic mutant PS9021 and restore both pathogenicity on bush bean and the capacity to induce the HR in tobacco. Since PS9021 also exhibited fluidal colony morphology, a change in the cell surface components seemed obvious.
Application of DNA-DNA hybridization could be used for identifying phytopathogenic bacteria and determining their relationships, particularly if genetic marker genes related to pathogenicity could be identified. Here we report on the use of serological methods and recombinant DNA techniques to identify antibodies to pathogenicity-related, cell surface specific antigens.

Materials and Methods

All bacterial strains used were routinely cultured in Rhodes medium (Rhodes 1959), and the antibiotic selection media were supplemented, as appropriate, with rifampicin (Serva,

50µg ml^{-1}), kanamycin (Sigma, 30µg ml^{-1}) or tetracycline (Sigma, 12.5µg ml^{-1}). Recombinant cosmids were transferred into P. syringae (rifampicin resistant) by using the triparental mating system (Ditta et al. 1980). Small-scale plasmid extractions were performed using the alkaline-lysis procedure (Mills et al. 1987), and genomic DNA from pseudomonads was isolated using the alkaline-lysis procedure (Mills et al. 1987) but the denaturation step with NaOH was omitted. Labelling of DNA probes with ^{32}P-dCTP was achieved using a nick-translation procedure (Maniatis et al. 1982). Dot blots were made using 1µg and 0.1µg of chromosomal DNA from each Pseudomonas strain and blotting it onto a nitrocellulose filter (Schleicher & Schuell), using the Southern blot procedure (Maniatis et al. 1982). Antibodies were obtained and purified as described by Hurn et al. (1980).

Exponentially growing cells of P. syringae strain PS9021 were pelleted and washed twice with 0.9% NaCl solution and divided into several equal portions. Each portion was separately suspended in 10ml purified and resuspended R32 AB solution, incubated for 5 min at room temperature, and then centrifuged for 15 min at 2500g. This procedure, later referred to as purging, was repeated with the same AB solution four more times, until no further agglutination was visible with P. syringae strain PS9021.

Pathogenicity tests of bacteria were done as described earlier by Niepold et al. (1985). Temperature-dependent pathogenicity tests were performed on bush bean leaves (Phaseolus vulgaris cv. "Red Kidney") using cultures of P. syringae that were grown at one degree temperature intervals from 20°C to 37°C. Prior to inoculating the plants, the bacteria were diluted in fresh medium (1:20 dilution) of the same temperature. The bacteria were inoculated into bean plants using a hypodermic syringe. The inoculated plants were kept at 15°C and the development of symptoms evaluated after 3 days.

Results

Antibodies were raised against the wild type to identify any differences between the mutant and the wild type in antigenic determinants of their cell surfaces. Non-specific agglutination was excluded using corresponding pre-immune antiserum "zero AB", which produced no agglutination. To verify that during purging a release of agglutinins from mutant strain PS9021 did not cause non-specific agglutination, "zero AB" was purged with the mutant strain PS9021. The supernatant obtained did not agglutinate strain R32, confirming the specificity of the purged AB. This result indicates that a successful immunization had occurred against Pseudomonas syringae R32. However, the AB was obtained after purging only agglutinated cells of the pathogenic strains R32 and not cells of the mutant PS9021. The purged AB agglutinated the R32 strain with an end-point dilution of 1:10 of the original blood volume. It was concluded, therefore, that this AB recognized pathogenicity factors of P. syringae strain R32 that were located on the cell surface.

When P. syringae pv. syringae strain R32 was cultivated at a temperature of 30°C or higher, no agglutination was detectable with purged AB. However, when cultivated at temperatures between 20°C and 30°C the cells were agglutinated by purged AB. Cells of strain R32 that were grown at or above 30°C produced much less severe symptoms in bean leaves compared with those produced by bacteria grown at 20°C.

Recently it was demonstrated that the pathogenicity-related DNA sequences from strain R32 which are cloned in pOSU3105 have homology with DNA from other pseudomonads pathogenic to beans (Mills et al. 1987).

To determine if the DNA sequence is widespread among other, less closely related, phytopathogenic pseudomonads, DNA from several phytopathogenic and non-phytopathogenic pseudomonads was isolated and probed with pOSU3105 (Fig. 1). The intensity of the hybridization signals (amount of homology) was compared visually with the titre of purged AB which just agglutinated pseudomonad cells. To exclude non-specific agglutina-

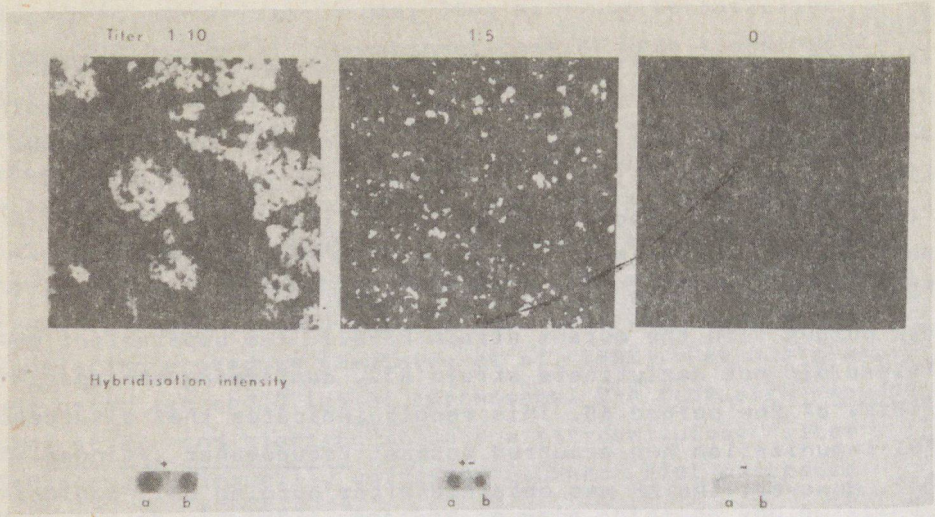


Fig. 1. Visual classification of agglutination and DNA-DNA hybridization intensity. For the agglutination tests the pathogenicity specific antibodies (AB) raised against P. syringae pv. syringae strain R32 were purified and freeze-dried. After resuspending the AB in a 0.9% NaCl solution to the original blood volume, it was purged and described in Materials and Methods. 10µl of the purged AB solution (representing a titre of 1:10) were mixed with 5µl of bacterial solution (about 10^8 cells ml^{-1}) on a clean microscope slide and agitated for 2 min at room temperature. The intensity of agglutination was checked visually against a dark background.

tion, all bacteria were initially screened with purified "zero AB" but none showed non-specific agglutination.
The results show a positive correlation between the amount of homology and the titre of the purged AB of each individual Pseudomonas strain.

Discussion

Antibodies were detected for specific cell surface components of wild type R32 but not for the mutant itself (PS9021). This result strongly suggests that the gene identified by the Tn5 insertion in PS9021 either encodes a product essential for

pathogenicity to bean, or regulates the expression of another gene product that is present at the cell surface of strain R32. That strain R32 cells do not agglutinate when incubated with purged AB grown at or above 30°C was a striking result. The reason for this observation is unclear but it could be due to: (1) a temperature-dependent synthesis of the cell surface component, (2) the failure of the components to be properly incorporated into the cell surface at the elevated temperatures, or (3) the occurrence of a heat-dependent modification of the antigenic determinant. Experiments are currently in progress to determine whether the two proteins react with the purged AB.

DNA homology studies involving several different P. syringae isolates that were probed with the insert of pOSU3105 revealed that there was a complete correlation between the titre of purged AB and the DNA homology. Different intensities of hybridization signals and titres among the tested Pseudomonas strains imply that the pathogenicity-related genes had diverged. This divergence could involve the amino acid composition of the proteins they encode including the antigenic site, thereby resulting in the reduced agglutination ability of the purged AB.

A rapid and inexpensive procedure for proof of the occurrence of homologous cell surface antigens for P. syringae is provided by serological screening procedures. Since hybridization studies showed a direct correlation between DNA homology and the titre of the purged AB, AB agglutination represents an easy and inexpensive procedure for detecting strain-specific bacterial infections of plant material.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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SCREENING FOR ALGINATE AND LEVAN DEFECTIVE STRAINS OF PSEUDOMONAS SYRINGAE PV. PHASEOLICOLA MUTAGENIZED BY Tn5 TRANSPOSON

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ABSTRACT

A screening method for the detection of alginate and levan defective Tn5 mutants of *Pseudomonas syringae* pv. *phaseolicola* is described. It comprises the cultivation of the mutants in microtiter plates (96 wells), the recording of growth behaviour (optical density), and an additional dot-blot assay for acidic polysaccharides. The use of different carbon sources helps to eliminate auxotrophic mutants and most of the mutants affected in other sites than polysaccharide synthesis and excretion.

Six out of 20 probably alg- strains obtained from the screening program exhibited reduced virulence (development of water-soaked spots) on bean leaves. Extracellular polysaccharides (EPS) were prepared from 500 ml batches of these 6 strains and analysed for alginate and levan contents. Although the total amount of EPS did not differ considerably from the wild type, in all but one case the alginate content was drastically reduced. These data confirm that the alginate production is an important virulence factor of *P. phaseolicola*.

INTRODUCTION

Pseudomonas syringae pv. *phaseolicola* synthesizes extracellular polysaccharides (EPS) which consist of the two main components levan (EL-BANOBY and RUDOLPH 1980) and alginate (GROSS and RUDOLPH 1987a,b). The appearance of water-soaked spots on bean leaves during the compatible interaction correlated with the production of alginate *in planta* (GROSS and RUDOLPH 1987d). In order to prove this correlation we employed Tn5 mutagenesis. Since a full EPS defective phenotype was not expected, a

quantitative and differentiating screening program for both polysaccharides had to be developed.

MATERIAL AND METHODS

Bacterial strain and media. Strain K1-S_2 of *Pseudomonas syringae* pv. *phaseolicola* race 2 termed *P. phaseolicola* in the following text originated from the institute's collection. Tn5 transposon mutagenesis was performed with the suicide plasmid 1011. All 2500 mutants were cultivated in microtiter plates for two days at 25° C. In order to provide a standardized inoculum a preculture in 250 µl KW-medium (KEEN and WILLIAMS 1971) was set up in microtiter plates using toothpicks. From the preculture the main cultures (mineral medium, GROSS and RUDOLPH 1987a) were inoculated with a simple transfer using a 96-pins stamp. All media were completed with 100 ppm kanamycin, 0.03% sodium desoxycholate (DOC), and glucose, glycerol or gluconate, respectively, as the carbon source. For selecting the auxotrophic mutants an amino acid cocktail (50 ppm peptone/50 ppm yeast extract) was added to the MM-medium.

Dot-blot assay. Microtiter plates containing cultures of 96 mutants were centrifuged after the optical density had been recorded in a microplate reader (660 nm). Aliquots of the supernatants were vacuum blotted on a nytran membrane and the acidic polysaccharides visualized with toluidine blue.

Immuno dot-blot. Aliquots of the culture supernatants were vacuum blotted on nitrocellulose membrane which was blocked with skim milk and incubated in antiserum against bacterial alginate. The anti-alginate-antibodies were then detected with peroxidase-conjugated anti-immuno globulins and developed with diaminobenzidine.

Preparation and analysis of the EPS. To confirm the screening results obtained with the different microtiter plate assays EPS was prepared from 500 ml batches of selected strains and analysed for alginate and levan in the usual manner (GROSS and RUDOLPH 1987a).

Infection of plants. Bean plants (*Phaseolus vulgaris* cultivar "Red Kidney") were inoculated using an atomizer with a carefully adjusted suspension (2.5×10^5 cfu/ml) of selected strains as described by RUDOLPH (1984). Symptom expression was recorded after 4, 7, 10, and 14 d.

RESULTS AND DISCUSSION

1. Screening for alg⁻ and lev⁻ mutants

Since EPS produced by strains of *Pseudomonas phaseolicola* on agar usually is highly contaminated with protein, LPS, and a vast of autolysis products of bacterial cells making it difficult to detect low amounts of alginate (GROSS and RUDOLPH 1987a), we developed a liquid culture screening program to select alginate and levan defective (alg⁻, lev⁻) strains (fig. 1). The program was based on the optical density of the cultures after growth in different media, and on an additional dot-blot assay for alginate.

Lev⁻ mutants were detected in an indirect manner, assuming that the levan production *via* levan sucrase (GROSS and RUDOLPH 1987c) were strictly correlated. Levan sucrase splits sucrose into its sugar monomers, polymerizing the fructose residues to form levan and liberating the glucose units which serve as substrate for the bacteria.

Starting from a sucrose containing preculture, three main cultures were inoculated (fig. 1). Lev⁻ mutants were expected not to grow in mineral medium with sucrose as the only carbon source, but grow well in the same medium with glucose, gluconate or glycerol instead of sucrose. Thus, the general carbohydrate metabolism was proved to be intact, and at the same time the defect was localized at the initial enzyme for sucrose catabolism (levan sucrase) which simultaneously synthesizes the levan. Auxotrophic mutants also grew hardly in sucrose mineral medium, but in contrast to lev⁻ mutants they developed a high OD in the presence of the amino acid cocktail.

For the detection of alg⁻ mutants a dot-blot assay for acidic polysaccharides was developed. The procedure was optimized in order to eliminate positive responses of compounds (protein, LPS, levan) other than strongly acidic polysaccharides like alginate. The specificity of the dot-blot assay was confirmed with an immuno dot-blot assay using antisera directed against bacterial alginate. The correlation of these two procedures reached nearly 100%. However, the immuno blot discriminated slightly better between alg⁻ and alg⁺. The alg⁻ phenotype was characterized by the growth behaviour of the mutants under different conditions as described in figure 1, and by the dot patterns on the membranes taken from these cultures. Only those mutants which showed normal growth and no visible spot on the membranes were designated to be probably alg⁻.

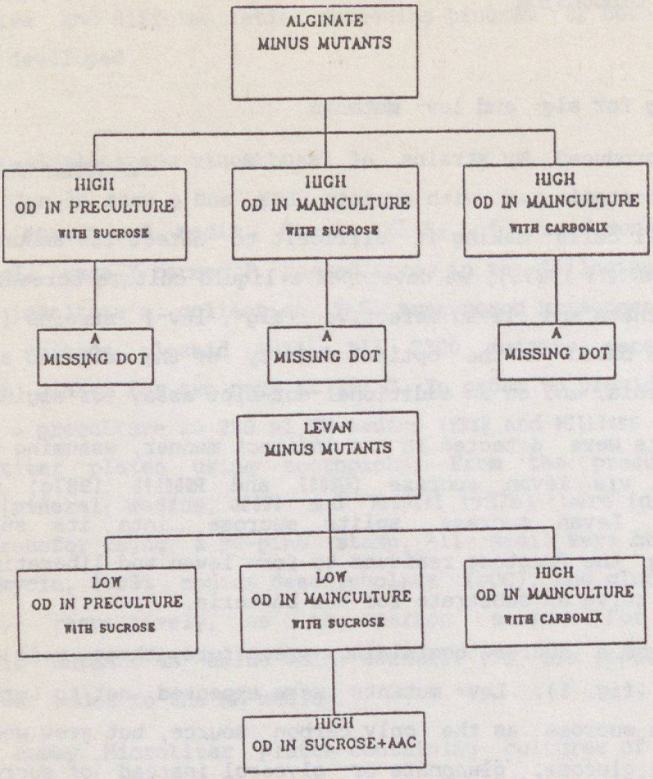


Figure 1. Screening scheme for lev- and alg- Tn5 mutants of *P. phaseolicola*.
OD = optical density
PRECULTURE = KW-medium
MAINCULTURE = MM-medium
CARBOMIX = glucose, gluconate and glycerol
AAC = amino acid cocktail.

Furthermore, DOC was added to the media since it had a stimulatory effect for the alginate synthesis. This was necessary because the alginate production of a given strain *in vitro* is not a constitutive feature, but seems to depend on various unknown conditional parameters. Therefore, the detergent DOC was employed as a "synthetic stress factor" in order to stimulate high alginate levels and thus facilitating the detection of alg- strains.

2. Symptom expression

With the aid of this screening program 31 probably lev- and 20 probably alg- mutants were selected. The 20 probably alg- mutants were further tested on bean and tobacco leaves. Few of them showed markedly reduced virulence in comparison with the wild type, and finally six were taken for EPS analysis.

3. EPS production *in vitro*.

The EPS from the six probably alg- mutants exhibiting reduced virulence on bean leaves was prepared and analysed (fig. 2). Although the absolute amount of EPS produced by each mutant did not differ considerably from the wild type EPS, the contents of alginate exhibited big differences. This confirms the correlation between alginate production and the appearance of water-soaked spots during pathogenesis. However, the presence of a high alginate level in one of the mutants with reduced virulence suggests, that also features other than EPS production are involved in pathogenesis.

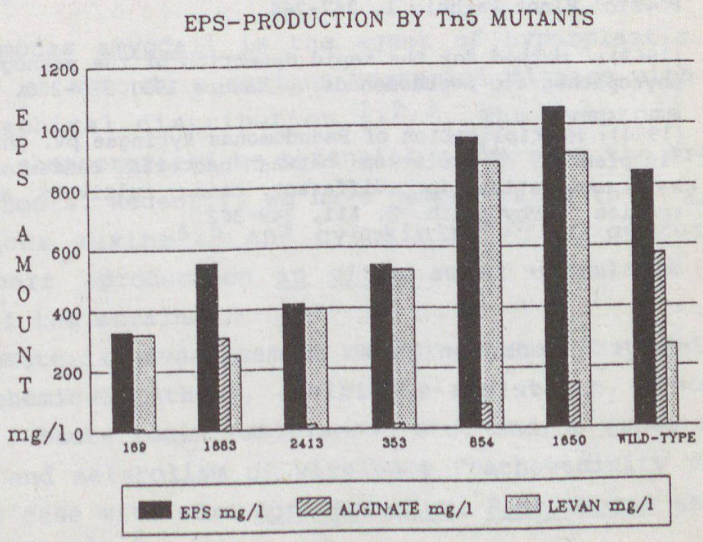


Figure 2. Amount of EPS, alginate and levan produced by six mutants of *P.phaseolicola* in comparison with the wild type strain.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

INVOLVEMENT OF PLASMID DNA IN THE PRODUCTION OF CYTOKININS BY PSEUDOMONAS AMYGDALI

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ABSTRACT

Pseudomonas amygdali strain NCPPB 2610, a producer of high levels of cytokinins, contains four plasmids with molecular masses of about 79, 70, 69 and 36 Kb. A spontaneous mutant (NCPPB 2610-1), no longer capable of secreting detectable cytokinins in culture, has lost the 69 Kb plasmid (pPAC1). Hybridization studies showed that pPAC1 DNA sequences have a high homology with ipt gene(s) from P. s. pv. savastanoi.

INTRODUCTION

Pseudomonas amygdali is the agent of hyperplastic bacterial canker of almond, a serious bacterial disease with a limited geographical distribution [1,2,3]. The symptoms of the disease are characterized by perennial cankers on the branches, twigs and shoots. Recently, we have demonstrated that P. amygdali produces auxins[4,5] and cytokinins[4,6] in culture and, moreover their production in vitro seems correlated to the virulence of the strains[7].

Many bacteria have plasmids carrying genes that codify for unique biochemical pathway, antibiotic resistance, bacteriocin production , bacteriocin resistance, etc. and/or genes for the production and metabolism of virulence /pathogenicity factors. This is the case with some Agrobacterium, Pseudomonas and Corynebacterium species[8].

Here we report evidence that cytokinin production in P. amygdali is mainly specified by plasmid-borne gene(s).

MATERIAL AND METHODS

Strain NCPPB 2610 and its mutant NCPPB 2610-1 were grown in liquid culture, as previously reported[4,6]. The cells were removed by centrifugation and then by membrane filtration; the resulting broth culture was extracted with ethyl acetate at pH 8.5.

The cytokinin activity of the alkaline extracts was determined by the cucumber cotyledon bioassay[9].

The alkaline extracts were analysed by TLC and by HPLC as previously reported[7].

Plasmid DNA was isolated by a modified alkaline lysis procedure[10]. The agarose electrophoresis of plasmid DNA was carried out on horizontal slab gel apparatus in a 0.5% agarose gel in Tris borate buffer. The plasmid DNA was transferred to a nitrocellulose membrane[11] and then hybridized with the isopentenyl transferase (ipt) gene probe. The ipt gene, isolated from P.s. pv. savastanoi is contained in a 3 Kilobase (Kb) Eco RI/Bam HI fragment (pFR1A3) cloned in E. coli pRK415 (F. Roberto, personal comunication). The 3 Kb fragment was electrophoretically purified and radioactively labeled by random primer method[12].

RESULTS AND DISCUSSION

P. amygdali strain NCPPB 2610 accumulates in culture high amounts of cytokinins also when compared to other phytopathogenic bacteria inducers of hyperplasia and/or plant growth malformation. In particular, we detected a high level of t-zeatin (Z) (4.621 mg/l) and a moderate quantity of isopentenyl adenine (iP) and 2'-deoxyzeatin riboside, a novel cytokinin[6]. Estimation of the cytokinins was performed by HPLC.

Alkaline extracts of the mutant NCPPB 2610-1 were inactive in the cucumber cotyledon bioassay.

The comparative TLC analysis of the alkaline extracts of both strains showed the absence of Z and iP in extracts of the mutant strain NCPPB 2610-1. The HPLC analysis confirmed the absence of Z in the mutant extracts also when aliquots ten-fold concentrated were injected.

When analysed for its plasmid content, strain NCPPB 2610 showed the presence of four plasmids with molecular masses of about 79, 70, 69 and 36 Kb. On the other hand, the spontaneous mutant NCPPB 2610-1 has lost the 69 Kb plasmid suggesting that the biosynthetic gene(s) for cytokinins are located on the above-mentioned plasmid (Fig. 1).

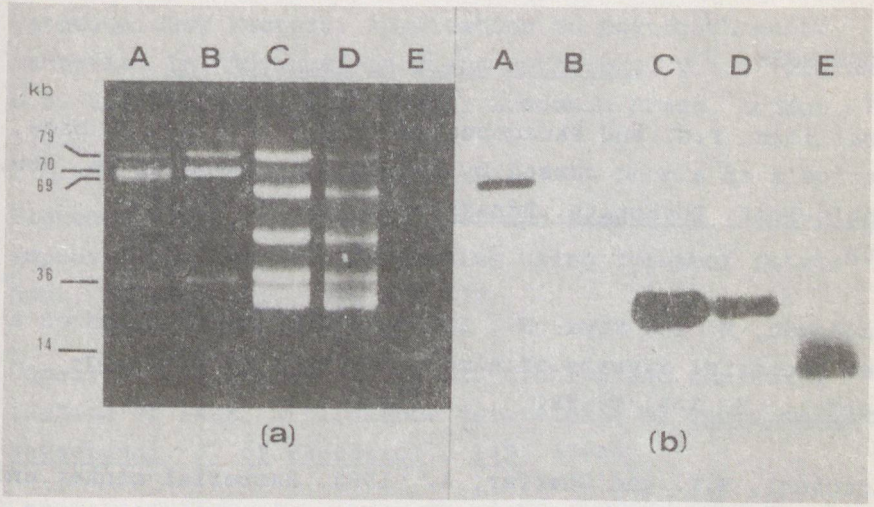


Fig.1.(a) Agarose gel electrophoresis of plasmid DNA purified from: P. amygdali NCPPB 2610 (lane A) and NCPPB 2610-1 (lane B), P.s. pv. savastanoi ITM 519 (lane C and D) and plasmid pFR1A3 (lane E) used as control. The estimated sizes (Kb) of the plasmids from P. amygdali NCPPB 2610 are shown on the left. (b) Plasmid-DNA from gel in Fig. 1 (a) was hybridized with ^{32}P-labelled 3 Kb Eco RI/Bam HI fragment containing the ipt gene from P.s. pv. savastanoi

To determine whether or not the cytokinin gene(s) are located on the 69 Kb plasmid , plasmid DNA preparations of both strains were hybridized with an available ipt gene probe from P.s.pv,savastanoi. Under high-stringency wash conditions, the P.s. pv. savastanoi ipt-gene-containing probe strongly hybridise to the 69 Kb plasmid. (fig.1) This finding confirmed the presence of the biosynthetic gene(s) on the 69 Kb plasmid and, furthermore, showed a high homology between the ipt gene(s) of P.s. pv. savastanoi and the cytokinin biosynthetic gene(s) of P. amygdali.

Our present efforts are directed at cloning the cytokinin gene(s) in E. coli in order to characterize them.

Acknowledgements. We wish to thank Dr. F. Roberto, University of California, Davis, California for generously providing pFR1A3 plasmid.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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CONJUGAL TRANSFER OF COSMID PVK102 INTO PSEUDOMONAS SYRINGAE PV. SAVASTANOI

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A study was undertaken to develop an efficient conjugation system in Pseudomonas syringae pv. savastanoi, the causal agent of olive and oleander knot disease, in order to study some characters of the pathogen. The ultimate goal of this research project is the construction of a genomic library from wild-type strains of the pathogen in a plasmid vector which can be mobilized into and maintained in P.s. pv. savastanoi. Here we report evidence that vector pVK102 (4) can be conjugally introduced into certain P.s. pv. savastanoi strains, using pRK2013 as a helper plasmid (2).

Conjugation experiments were performed by using the triparental mating system of Ditta et al. (1980) with some modifications indispensable for overcoming the refractority of the bacterium to conjugation. Bacterial matings were made using E.coli HB101 (pVK102) and HB101 (pRK2013) as donors and P.s.pv.savastanoi as a recipient. An appreciable conjugation frequency was observed when NCPPB640 and ITM601, both isolated from oleander plants, were utilized as recipients. They acquired cosmid pVK102 at a frequency ranging from 0.83×10^{-6} to 6.3×10^{-6} transconjugants per recipient cell (Table 1). NCPPB640 and ITM601 transconjugants, with the same colony morphology as the recipient strains, expressed the same levels of antibiotic resistance as the donor strains, were able to accumulate indoleacetic acid (IAA) in the liquid growth medium, and did not grow at 37°C.

The plasmid profile analyses of donors, recipients and transconjugants showed that plasmid transfer to recipients

Table 1. Conjugal transfer frequencies of pRK2013/pVK102 binary plasmid system for various strains of Pseudomonas syringae pv. savastanoi

Donor	Recipient	Host origin	Number of Tc^r and Km^r transconjugants/recipients
E.c.HB101(pRK2013)+E.c.HB101(pVK102)	P.s.s.NCPPB640 Rif^r	Oleander	8.3×10^{-7}
" " " " " "	P.s.s.ITM601 Rif^r	"	6.3×10^{-6}
" " " " " "	P.s.s.PB230 Rif^r	Olive	0
" " " " " "	P.s.s.ITM519 Rif^r	Oleander	0
" " " " " "	P.s.s.PB219	"	0
" " " " " "	P.s.s.PB229 Rif^r	Olive	0
" " " " " "	P.s.s.PB215 Rif^r	Privet	0

Tc^r: tetracycline resistant; Km^r: kanamycin resistant; Rif^r: rifampicin resistant. pRK2013 codes for resistance to kanamycin; pVK102 codes for resistance to kanamycin and tetracycline. E.c.: Escherichia coli; P.s.s.: Pseudomonas syringae pv. savastanoi

occurred in all cases examined with a plasmid of the correct mass being present only in donors and transconjugants (Figure 1).

NCPPB640 transconjugants after seven days of growth in absence of drug selection, maintained the vector pVK102 in 42% of the colonies. Moreover, the transconjugants of P.s. pv. savastanoi NCPPB640 and ITM601 strains, inoculated into olive tissues, induced the formation of typical knots and from them, transconjugants with kanamycin and tetracycline resistance were isolated.

Repeated attempts to introduce cosmid pVK102 into other oleander, olive and privet strains of pathovar savastanoi were unsuccessful, indicating a genetic diversity in the P.s. pv. savastanoi population (Table 1). These differences could occur at the plasmid transfer stage, e.g. some strains may not be compatible with the pRK2013 transfer apparatus, or they could occur at subsequent stages, including DNA restriction systems and plasmid maintenance in the recipients. Recently, Glass and Kosuge (1988), transferred the cloned gene for IAA-lysine synthetase from E.coli to P.s. pv.

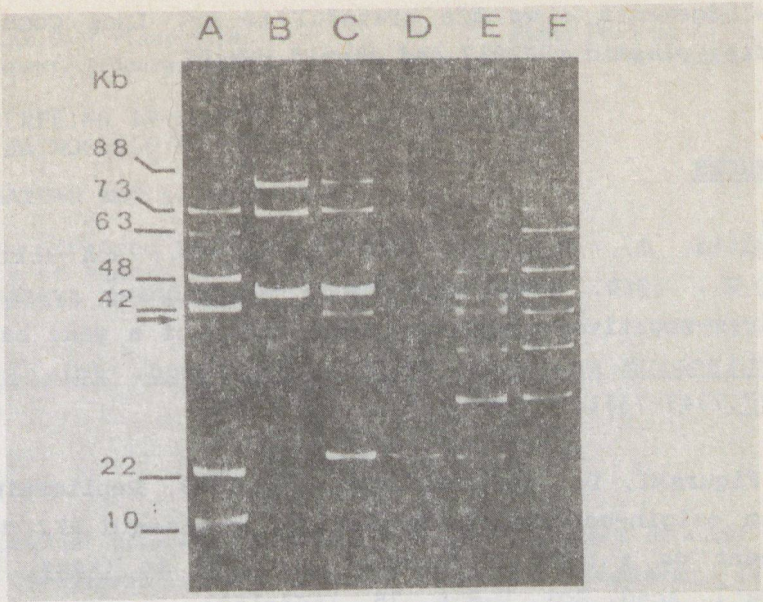


Figure 1. Agarose gel electrophoresis of plasmid DNA purified from transconjugants of Pseudomonas syringae pv. savastanoi wild-type strains NCPPB640(B) and ITM601 (F). C, transconjugant of NCPPB640; E, transconjugant of ITM601; D, E. coli HB101(pVK102). pV102 is also present in transconjugant plasmid preparations as nicked circular or linear DNA (see arrow). On the left, the estimated sizes (Kb) of the plasmids from P.s. pv. savastanoi ITM317 used as size markers (5) are reported. Analysis was performed in 0.5% agarose gel on a horizontal slab electrophoresis apparatus.

savastanoi, by triparental mating using the cloning vector pLAFR3. However, in this case, the resulting pLAFR3 constructs were not stable in planta.

The good stability of pVK102 in P.s. pv. savastanoi under non-selective conditions indicates that this cloning vector can be used to generate a clone bank of genomic DNA from P.s. pv. savastanoi strains.

Acknowledgements : we are grateful to Dr. Luca Comai for providing plasmid pRK2013 and cosmid pVK102.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

PLASMID SCREEING IN SOME OF THE INDIAN STRAINS OF PLANT PATHOGENIC BACTERIA

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INTRODUCTION

Bacterial diseases substantially reduce the yield of many plants in India (Rangaswami, 1988; Gnanamanickam and Mahadevan, 1988). Though much research has been made on the distribution of diseases, the mechanism of infection remains to be understood except in a few plant pathogenic bacteria (Gnanamanickam and Mahadevan, 1988). Furthermore, lack of genetic knowledge has resulted in the absence of efficient and effective control measures.

Plasmids are widespread among plant pathogenic bacteria. They code for several important characters including virulence in Agrobacterium tumefaciens (Waston et al, 1975), A.rhizogenes (Chilton et al, 1982), Pseudomonas syringae pv.savastanoi (Comai and Kosuge, 1980), P.s. pv. atropurpurea (Sato et al, 1984) and P. solanacearum (Xu et al, 1988); production of auxin and cytokinin in A. tumefaciens (Liu and Kado, 1979, Binns 1983) and IAA production in P.s.pv.savastanoi (Comai and Kosuge, 1980); toxin production in P.s.pv.atropurpurea (Sato et al, 1984); host range in A.tumefaciens (Whatley et al, 1976); cell wall proteins in P.s.pv.syringae (Farraza and Levy, 1980), resistance to copper in X.c.pv.vesicatoria (Stall et al, 1986); catechin dissimilation in P.solanacearum (Boominathan and Mahadevan, 1987) and gene transfer to higher plants by A.tumefaciens (Caplan et al, 1983). Plasmids are also used as means of identification and dispersal of pathogens (Lazo and Gabriel, 1987).

As part of our study on the mechanism of disease development by bacterial plant pathogens and to develop a method to monitor the dispersal and spread of pathogens, we screened 30 bacterial strains of Indian origin for plasmids and the results are presented.

MATERIALS AND METHODS

Xanthomonads were maintained in PSA medium (Wakimoto, 1967). Pseudomonads were cultured in MaNy medium, and Erwinia strains were maintained in nutrient agar. For plasmid isolation, cells were grown in Luria broth until the late log phase and pelleted by centrifugation at 8,000 x g for 10 min. Cells were washed in NaCl (0.5 M) and Tris buffer (30mM) to remove excess polysaccharide. Washed cells were suspended in 25 mL of lytic buffer (0.05 M Tris, 0.020 M EDTA, 1% w/v SDS, pH 12.3) and incubated at room temperature for 30 minutes. The pH of the lysate was adjusted to 8.5 by the addition of 1.5 mL of 2 M Tris (pH 7.0). The SDS content of the lysate was increased to 4% by adding 3.5 mL of a 25% SDS solution. Immediately 7 mL of 5 M NaCl was added and the lysate was kept at 0°C. After 1 h, the precipitate was pelleted by centrifugation at 20,000 x g in a Beckman JA 20 rotor for 40 minutes and discarded. To the supernatant, 8 mL of a 50% PEG 8000 solution was added (final concentration ca. 10%) and stored overnight at 4°C. Preparation of buffers and reagents was according to Maniatis et al (1982).

The precipitate was collected by low speed centrifugation (5,000 x g, 5 min) and resuspended in 0.2 to 0.5 mL of TES buffer (500 mM Tris, 5 mM NaCl, 5 mM EDTA, pH 8.0). A 50 µL sample was subjected to agarose gel electrophoresis; 1% agarose was used in a horizontal gel at a constant 40 v for 4 h. The gel was stained for 20 min in ethidium bromide (2 µg/mL) and photographed under U.V.

RESULTS AND DISCUSSION

Xanthomonas campestris pv.oryzae strains contained 1-2 plasmids per strain. Strains 1 and 2 isolated from the same locality in Tamil Nadu displayed difference in their plasmid content. Strain 1 had a moderately mobile plasmid band while strain 2 had two fast moving bands, away from the wells of the gel (Fig. 1). Strains 3 and 4 isolated from Andhra Pradesh and West Bengal contained a single

plasmid of identical mobility (Fig. 1). Plasmid banding pattern within the same pathovar differed markedly. But the exact relationship between the different plasmids is yet to be ascertained.

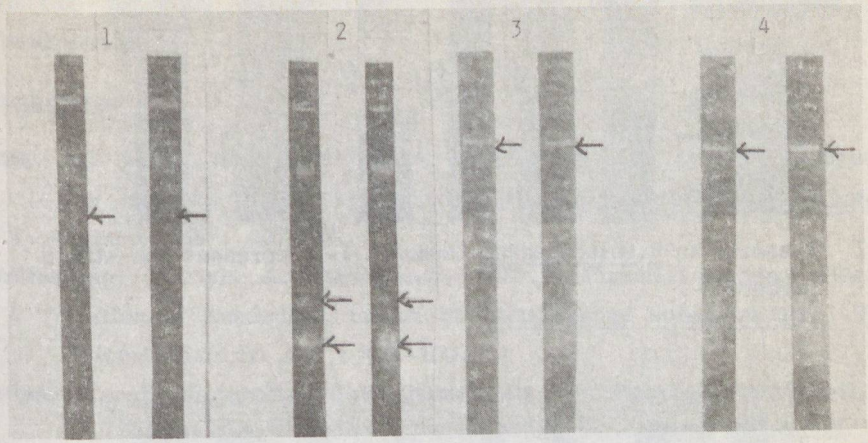


Fig. 1 Plasmids in X.c.pv.oryzae (Numbers 1-4 represent the strain numbers)

A single plasmid of moderate mobility was observed in strains of X.c.pv.vesicatoria isolated from the same locality. We did not detect any plasmid from strains of X.c.pv.cannas, X.c.pv.citri, X.c.pv.malvacearum, X.c.pv.mangifera and X.c.pv.translucens. The absence of plasmids in these pathovars indicates that plasmids might not influence pathogenesis. A similar observation was made by Lazo and Gabriel (1987) on 13 pathovars of X.campestris.

Pseudomonas solanacearum strains isolated from potato and cabbage harboured plasmids. Strain 1 had 4 plasmid bands of fast mobility. A single plasmid band of low mobility was observed in strain 6. Strain 3 harboured 7 plasmid bands of varying mobility while strain 7 had 5 plasmid bands coinciding with the mobility of 5 plasmid bands in strain 3 (Fig. 2). Strains 1, 2, 3, 4 and 5 were isolated from Himachal Pradesh and Assam. Strain 6 was from Himachal Pradesh and strain 7 was from Ooty in Tamil Nadu. Earlier Boominathan and Mahadevan (1987) recorded the presence of a single plasmid from P.solanacearum isolated from banana. Rosenburg et al (1982) and Morales and Sequeira (1985) reported the occurrence of 1-2 plasmids in P.solanacearum strains.

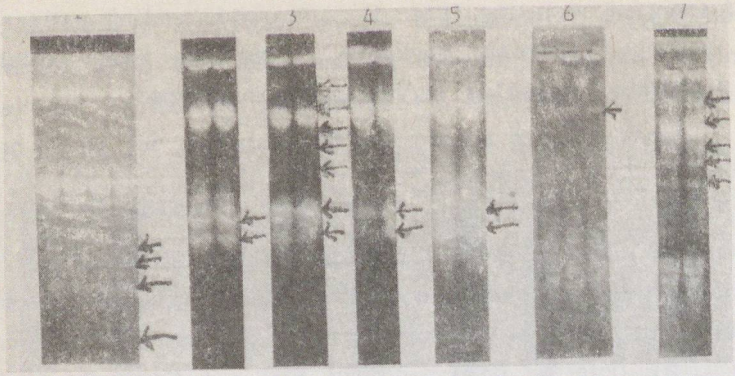


Fig. 2 Plasmids in P.solanacearum (Numbers 1-7 represent the strain numbers)

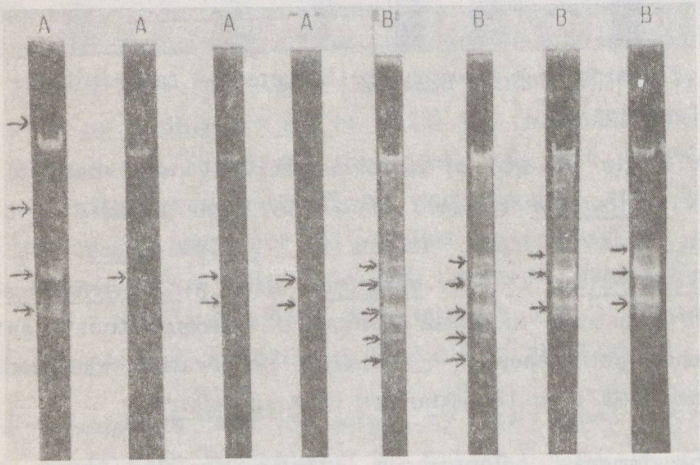


Fig. 3 Plasmids in P.syringae (A = P.s.pv.syringe; B = P.s.pv. tomato)

P.s.pv.syringae strain 1 had a single plasmid while in strains 2 and 3, 2 plasmid bands of identical mobility were present. The upper band was identical in all the 3 strains while the lower band was absent in strain 1 (Fig. 3). Two strains of P.s.pv.tomato contained 3 fast moving identical bands while two additional bands of fast mobility were recorded in two other strains (Fig. 3).

A nonpathogenic strain of Erwinia herbicola harboured two plasmids while E.carotovora contained a single plasmid.

Our observations indicate that plasmid banding pattern as such cannot be used as a means of identification of different pathovars. Effort is underway to find out the relationship between different plasmids by restriction enzyme cutting pattern and DNA hybridization.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

ORGANIZATION AND EXPRESSION OF hrp GENES IN PSEUDOMONAS SOLANACEARUM

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Pseudomonas solanacearum is the causative agent of a wilt disease which affects a large variety of host plants. In an attempt to identify pathogenicity determinants of this organism we had previously isolated transposon insertion mutants of strain GMI1000. Certain of these mutants had simultaneously lost the ability to produce disease of tomato and the ability to induce the hypersensitive response (HR) on the heterologous host tobacco (Boucher *et al.*1985). Genes governing both properties are now currently referred to as *hrp* genes (LINDGREN *et al* 1986). Previous studies have shown that a majority of the *hrp* genes map in a single cluster of which the right border had previously been identified (BOUCHER *et al.* 1987). In this paper we present further characterization of this cluster and information concerning the regulation of the transcription of these genes.

1. DELIMITATION OF THE BORDERS OF THE *hrp* GENE CLUSTER

Two overlapping cosmids (namely pVir2 and pAFE8) have been isolated from a genomic bank of the wild-type strain made in the wide host range cosmid pLAFR3 (LINDGREN *et al.* 1986). These plasmids have been selected as showing homology with regions in which insertion of a transposon had previously led to identification of *hrp* genes.

These two plasmids were mutagenized with transposon Tn5-B20 which can promote transcriptional gene fusions between the promotor of the target gene and the coding sequence of *Escherichia coli* ß-galactosidase (Keller *et al.* 1988). Each insertion was mapped on the plasmids and all the insertions which had occurred within the plasmid inserts were marker exchanged into the wild-type strain. For each recombinant strain thus obtained, pathogenicity on tomato and ability to induce the HR on tobacco were tested. In all cases mutants which were affected in one property were also affected in the other. All the *hrp* deficient mutants mapped in a continuous stretch of DNA demonstrating the presence of an *hrp* gene cluster which extends over 22 kb (figure 1). Insertions in the 3kb rightward end of this cluster led to a leaky phenotype which resulted in decreased aggressivity towards tomato and ability to

induce a partial and/or delayed HR on tobacco. Therefore it appears that the right end of this cluster corresponds to *hrp* genes rather than to *dsp* (disease specific) genes as we had previously reported (Boucher *et al.*1987). Several insertions located within the *hrp* cluster did not affect interactions of the bacteria with plants. It is assumed that these insertions occurred in non coding sequences located at the end of transcriptional units or between adjacent transcriptional units.

2. TRANSCRIPTIONAL ORGANIZATION OF *hrp* GENES

Because strain GMI1000 does not produce ß-galactosidase activity and because Tn5-B20 promotes transcriptional gene fusions with the coding sequence of ß-galactosidase, measurement of this enzyme activity could be used to monitor *hrp* gene expression. For each individual *P. solanacearum* strain carrying a Tn5-B20 insertion within the *hrp* cluster or in the flanking regions, ß-galactosidase activity was measured following growth till late log phase in minimum medium. In these conditions, about fifty percent of the strains did not show a significant level of activity (<5 Miller's units) (Miller 1972) although this level was above the background level mesured on the wild-type strain. These strains probably correspond to strains carrying an insertion in a region which is not transcribed or an insertion in which the orientation of the transposon is such that the ß-galactosidase coding sequence is not placed under the control of the promoter of the target gene.This is in agreement with the fact that within the *hrp* gene cluster regions are found in which all the insertions oriented in one direction expressed ß-galactosidase activity whereas all the insertions oriented in the reverse direction were silent. Such regions probably correspond to individual transcriptional units or to adjacent transcriptional units transcribed in the same direction. It is also interesting to note that ß-galactosidase activity measured for mutants with a significant activity may vary within the range of 10 to more than 300 Millers units but that adjacent insertions usually led to the similar level of activity suggesting that they reflected the activity of a common transcriptional unit.

Both orientation of the transcription and level of ß-galactosidase activity have been used to tentatively define transcriptional units within the *hrp* cluster. Altogether these data suggest the existence of a minimum of nine transcriptional units within the *hrp* cluster. These putative transcriptional units are presented in figure 1. It has to be noted that insertions within the *hrp* gene cluster which do not affect properties on plants all map between individual transcriptional units as previously defined.

3. REGULATION OF THE EXPRESSION OF *hrp* GENES

For all insertions which occurred within the *hrp* gene cluster ß-galactosidase activity was also measured following growth of the mutant strain in rich medium. In all cases no significant activity was found (<5 units), demonstrating that expression of *hrp* genes is repressed in these conditions. A similar repression was also observed when minimum medium was complemented by addition of casamino acids ($10g.l^{-1}$).

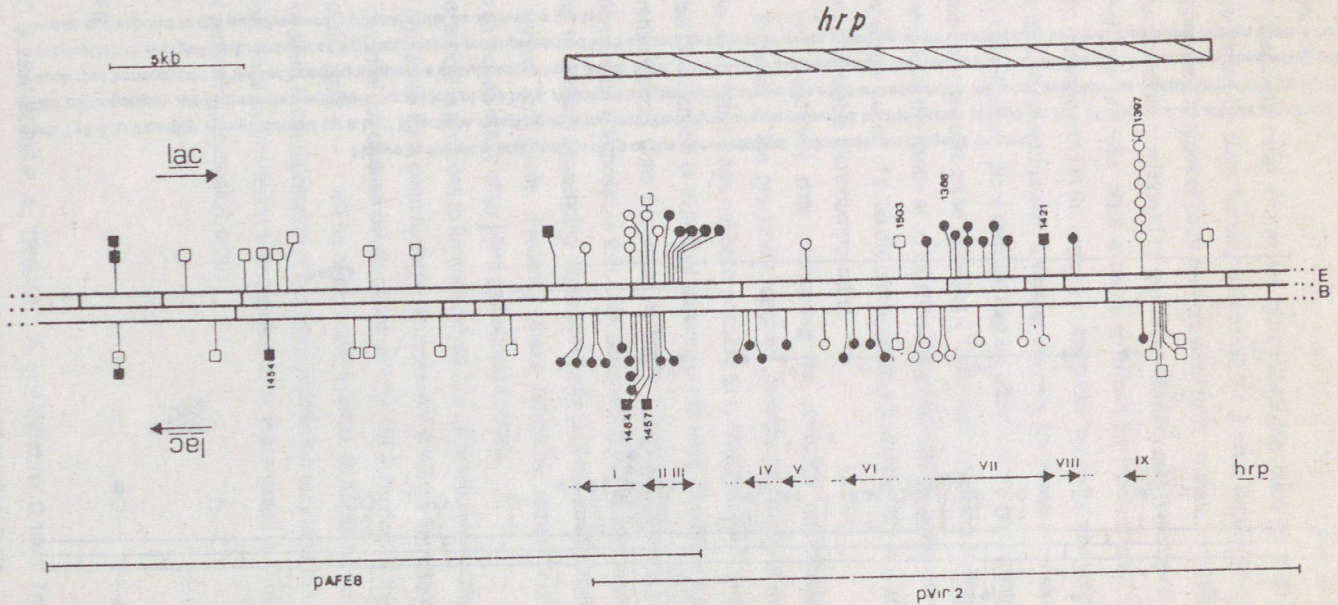


Figure 1: Physical and genetic map of the *Pseudomonas solanacearum hrp*gene cluster.

Each Tn5-B20 insertion is represented by a bar. Insertions resulting in a mutant phenotype are represented by the circles placed on top of the bars; insertions which do not affect pathogenicity are represented with squares on top of the bars. All insertions presented above the map correspond to the ones in which the orientation of the transposon is such that transcription of the *lacZ* coding sequence should occur from left to right, insertions presented below the map are in the reverse orientation. Insertions which result in production of a significative amount of ß-galactosidase are represented with closed symbols whereas open symbols correspond to silent insertions. Arrows placed below the map correspond to the putative transcriptional units as defined in the text.

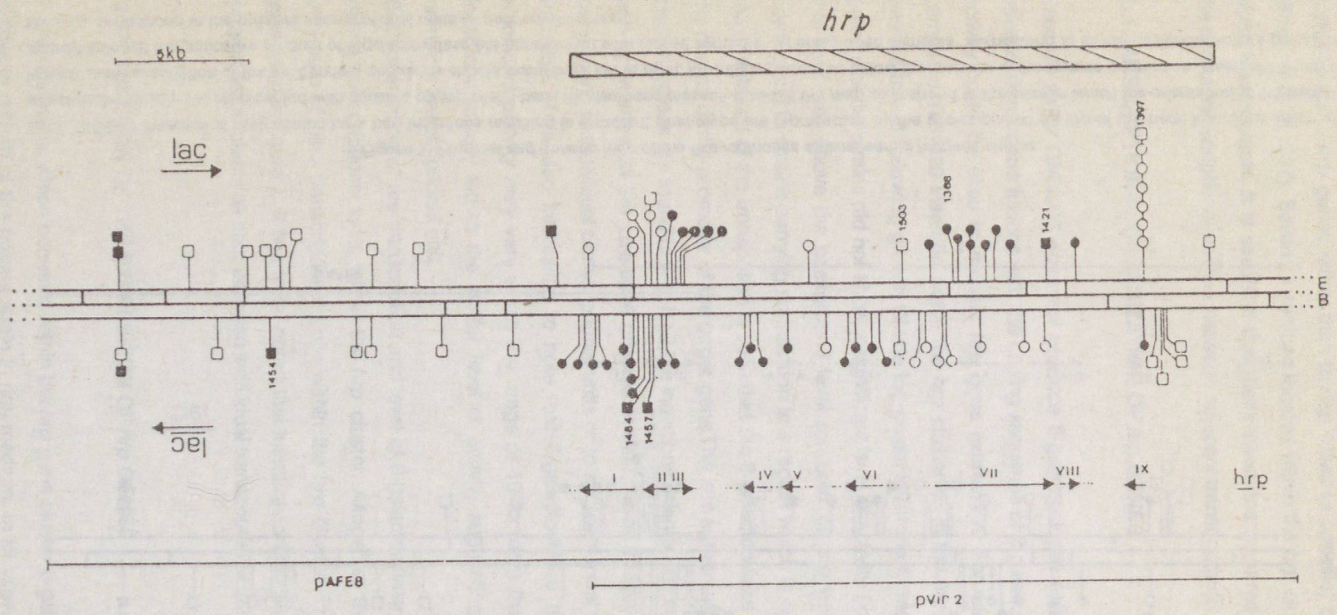


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On the other hand expression in minimal medium was independent of the nature of the sugar provided as sole carbon source.

Because it has previously been shown for plant-interacting bacteria that genes involved in this interaction are regulated by plant factors, we looked for such a regulation for *P. solanacearum hrp* genes. For this purpose two types of plant extracts were used : tomato root exudates (compatible host) or plant cell culture medium conditioned by growth of tobacco cells (incompatible host).

Addition of either one of these plant extracts to minimum medium stimulated up to 3 folds the expression of *hrp* genes located in the regions identified as transcriptional units IV, VI and VII (figure 1). Stimulation was also observed but with a weaker intensity for transcriptional units I, II and VIII. When added to rich culture medium, plant extracts had no effect on *hrp* genes expression. The stimulating effect of plant extracts was conserved following a 10 mn incubation in a boiling water broth and activity could be recovered from the filtrate of an ultrafiltration performed on anAmicon ultrafiltration membrane with a molecular weight cut off of 2000 dalton suggesting that this effect was at least partly due to a low molecular weight heat stable plant compound(s).

These results are preliminary data concerning the structural organization of *hrp* genes in *P. solanacearum*. Because of the previously reported inability to construct meridiploid strains for this cluster (Boucher *et al.* 1987) it has not been possible to conduct complementation assays which could allow the precise identification of individual transcriptional units. Therefore it is possible that certain of the putative transcriptional units presented in figure 1 may correspond to more than one simple complementation group. It is possible that DNA sequencing in this region will help to solve certain of these questions. In addition sequencing might eventually lead to identification of biochemical functions encoded by certain *hrp* genes. Presently these functions remain completely unknown in *P. solanacearum* as well as in any other plant pathogenic bacteria.

Additional work is now in progress to further analyse *hrp* gene regulation. One important concern is the stimulation of *hrp* genes by a plant factor(s). In particular it will be important to know whether such a stimulation is essential for disease development and/or HR induction. If this is the case biochemical identification of the plant will be of particular interest. In the same way the understanding of the mechanisms involved in the repression of *hrp* gene expression may lead to interesting developments in plant protection; since by interfering with expression of these genes it should be possible to develop new means of protecting susceptible crops.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

CULTIVAR SPECIFIC AVIRULENCE GENE FROM PSEUDOMONAS SYRINGAE PV. PISI AFFECTS THE VIRULENCE OF P.S. PV. PHASEOLICOLA

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Pseudomonas syringae pv. *pisi* (*Pspi*) is the causal agent of bacterial blight of pea. Six races are currently recognised on the basis of their differential virulence towards cultivars of the host *Pisum sativum* (Taylor *et al.*, 1984). Three races of *Pseudomonas syringae* pv. *phaseolicola* (*Psph*) the causal agent of halo-blight of bean are clearly distinguished by their differential virulence towards cultivars of the host *Phaseolus vulgaris* L. (Harper *et al.*, 1987). Genetic studies of both pathogens and their respective hosts have indicated race-cultivar specificity to be controlled by a 'gene-for-gene' relationship in which an incompatible (or hypersensitive) response requires the presence of a single dominant allele for resistance in the host and a complementary gene for avirulence in the pathogen. Incompatibility (or hypersensitivity) is manifested as rapid, localised necrosis of plant tissue in the vicinity of the invading pathogen, accompanied by the accumulation of phytoalexins and other resistance-related products. Susceptibility of the host occurs where either allele is absent from the host or pathogen, and is characterised by extensive water-soaking or delayed collapse of the infected plant tissue.

Several cultivar-specific avirulence genes from *Pseudomonas syringae* pathovars have been cloned and in some cases sequenced (Hitchin *et al.*, 1989; Napoli & Staskawicz, 1987; Staskawicz *et al.*, 1984; Vivian *et al.*, 1989). The genetic determinants controlling the specialisation of pathovars of *P. syringae* to one or two particular plant species are, however, less well defined but it is thought that such 'species-specificity' may also be determined by pathogen avirulence genes and corresponding resistance genes in the host. We have characterised a gene cloned from a genomic library of *Pspi* race 2 which controls *Pspi* race-cultivar specificity on pea and which, when expressed in *Psph* races 1 and 2

elicits a hypersensitive response on bean in a cultivar-specific manner. A single pLAFR3-based cosmid clone designated pAV270, altered the wild type virulent phenotype of *Pspi* race 1 to avirulence towards the pea cv. Early Onward. The clone pAV270 was mobilised into *Psph* and transconjugants were screened for alterations in virulence by inoculation of a range of bean cultivars. Unlike wild type *Pspi* race 2 which elicits the hypersensitive response on all bean cultivars, the phenotypes of two races of *Psph* containing pAV270 were altered to avirulence towards some originally susceptible cultivars but remained virulent on others (Table 1). It appears therefore that an avirulence gene located on pAV270 is interacting with a previously unrecognised, complementary resistance gene present in some bean cultivars. Subcloning pAV270 indicated that the avirulence gene is located on a 4.1 kb *Eco*R1 DNA fragment. Transposons located within this 4.1 kb fragment abolish the avirulence phenotypes conferred on both *Pspi* and *Psph* indicating that the same gene controls race-cultivar specificity in *Pspi* and 'non-host' cultivar specificity in *Psph*.

Table 1 The effect of a cultivar-specific avirulence determinant from *Pspi* on the virulence of *Psph* race 1 towards leaves of *Phaseolus vulgaris*

Psph race/Transconjugant	Bean cultivar				
	Canadian Wonder	Red Mexican	Tendergreen	Horsehead	Seafarer
Wild type race 1	S	HR	S	S	S
race 1 (pAV270)	HR	HR	HR	HR	S
race 1 (pAV200)	HR	HR	HR	HR	S
race 1 (pAV270p203)	S	HR	S	S	S

HR - hypersensitive response displayed as rapid, localised collapse, necrosis and desiccation of leaf tissue at the inoculation site

S - susceptible response displayed as delayed collapse of leaf tissue around the inoculation site

pAV270 - cosmid clone from a genomic library of *Pspi* race 2

pAV200 - 4.1 kb *Eco*R1 subclone of pAV270

pAV270p203 - pAV270 harbouring a Tn5 insertion in the 4.1 kb *Eco*R1 fragment of DNA

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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CHARACTERIZATION OF A GENE FOR CULTIVAR-SPECIFIC AVIRULENCE FROM PSEUDOMONAS SYRINGAE PV. PHASEOLICOLA RACES 3A AND B

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ABSTRACT

Cosmid clones of genomic libraries of P.s. pv. phaseolicola (sph) races 3A and 3B were found to contain similar determinants of avirulence towards the gene for resistance in Phaseolus vulgaris cv. Tendergreen. No genes for cultivar-specific virulence were identified. Sub-cloning and transposon mutagenesis with Tn1000 identified a DNA fragment of <0.9kb with avirulence, recognised by induction of the hypersensitive reaction and associated phytoalexin accumulation. Co-segregation studies of the reaction of the F_2 progeny of crosses between cvs. Tendergreen and Canadian Wonder, respectively resistant and susceptible to race 3, indicated that a gene-for-gene relationship between single genes for avirulence and resistance controls the interaction between cv. Tendergreen and isolates of race 3.

A 2.2kb Pst1 fragment containing the avirulence gene from race 3B was sub-cloned into pBR322 and sequenced by a dideoxy method using Tn1000 primers. Three open reading frames (ORFs) were identified. Sub-cloning and site directed mutagenesis demonstrated that ORF1 of only 384-bp functioned as the gene for avirulence named as avr, sphA.

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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SECRETION OF PECTATELYASE IN ERWINIA CHRYSANTHEMI: THE OUTJ GENE

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The phytopathogenic enterobacterium *Erwinia chrysanthemi* secretes a number of enzymes involved in plant tissue degradation, notably the five isoenzymes of pectate lyase. We have cloned a region involved in pectate lyase, pectin methylesterase and cellulase secretion by complementation of non secretory *outJ* mutants of the *E. chrysanthemi* strain 3937. The cloned region maps near the *ade-377* marker on the *E. chrysanthemi* 3937 chromosome. A R-prime containing a chromosomal DNA insert of about 30 kb was first obtained; subcloning permitted the isolation of a 4 Kb ClaI/SspI fragment able to complement *outJ* mutations in *E. chrysanthemi*. The isolation of *phoA* fusions in this fragment allowed us to determine the direction of transcription of the encoding region, which extends over about 2.5 kb, and demonstrate that this region encodes exported protein(s). Identification of the products encoded by the ClaI/SspI fragment demonstrated that *outJ* encodes an 83 polypeptide which is processed to an 81 kD polypeptide by cleavage of a signal sequence.

INTRODUCTION

Erwinia chrysanthemi is responsible for soft rot of many plant species. The pathogenicity of this enterobacterium is related to its secretion of plant cell wall-degrading enzymes such as pectinases, cellulases and proteases. We studied the secretion of pectinases in *E. chrysanthemi* 3937, in order to better understand the mechanism of secretion in Gram negative bacteria, to determine the implication of the secretory mechanisms in phytopathogenicity, and to know whether the translocation of pectinases is linked with their synthesis.

RESULTS

Isolation of *outJ* non-secretory mutants, expression of the *outJ-lacZ* fusions

E. chrysanthemi 3937 was mutagenized with phage Mu-*lac* or with nitrosoguanidine. Polygalacturonate-non-fermenting (PGA^-) mutants were analysed to determine the site of their mutation. Some were affected for the pectinolytic pathway (Hugouvieux-Cotte-Pattat &

Robert-Baudouy, 1985; Condemine et al., 1986). Other mutants, exhibiting either a reduction or an absence of pectinase production, were retained as potentially affected in the secretion of these enzymes. Seven Mu-*lac* mutants (A265, A341, A655, A692, A769, A770 and A839) and one NTG mutant (A420) showing normal total pectate lyase activity were analysed for the localization of the three types of enzyme secreted by the wild-type strain (Table 1). In each *outJ* mutant the pectate lyase and pectin methylesterase activity were found bound to the cell and essentially in the periplasm. Cellulase activity was also mainly located in the periplasmic space, whereas the protease activity remained extracellular (Table 1).

Table 1. Distribution of enzyme activities in subcellular fractions. Enzyme activities are expressed as a percentage of the total activity. Total activities are expressed in units/mg cell dry weight. Cells were fractionated by a lysozyme-EDTA method. The location of the periplasmic enzyme ß-lactamase and of the cytoplasmic enzyme ß-galactosidase was determined as control. (ND = not detectable)

	A622 (parental strain)				A341 (*outJ* mutant)			
	Supernatant	Periplasm	Cytoplasm	Total	Supernatant	Periplasm	Cytoplasm	Total
Pectate lyase	97	1	2	1.95	2	95	3	2.46
Cellulase	85	6	9	$2x10^{-3}$	9	86	5	$2.2x10^{-3}$
Protease	100	ND	ND	$3x10^{-3}$	100	ND	ND	$2x10^{-3}$
ß-galactosidase	2	10	88	25	1	2	97	160
ß-lactamase	2	77	21	12.8	2	92	6	1.62

Four of the Mu-*lac* insertion mutants, A341, A655, A769 and A770, expressed ß-galactosidase and then probably contained *outJ* - *lac* fusions. The expression of these *outJ-lacZ* fusions was tested in various growth conditions (Ji et al, 1987). While synthesis of pectate lyases increased in presence of PGA or galacturonate, under semi-aerobic conditions, at low temperatures or in late growth phases, that of ß-galactosidase from the *outJ-lacZ* fusions was not changed. The various cultural conditions tested did not alter ß-galactosidase expression (presence of good carbon sources, variation of phosphate concentration, iron concentration, the pH of the medium, addition of ethanol, various growth rates), suggesting that the *outJ* gene is expressed constitutively.

Composition of membrane and periplasmic proteins in the *outJ* mutants

The pectate lyases present in the periplasmic fraction of each mutant were compared to that of culture supernatant of the wild-type strain by electrofocusing and specific staining of pectate lyase activity. The apparent isoelectric points of the five pectate lyases (PLa to PLe)

from the periplasm of *outJ* mutants or from supernatant of the 3937 strain were identical, and the respective concentrations of each pectate lyase were similar. Therefore, the pectate lyases of *E. chrysanthemi* are not modified during export through the outer membrane.

We also compared the total protein composition of the outer membrane and the periplasmic space of *outJ* mutants with that of the wild-type strain by SDS-PAGE. The outer-membrane protein patterns in the mutants were identical to that of the wild-type. In contrast, the protein profiles of the periplasmic space were different; the *outJ* mutants contained several more intense bands (78, 50, 35 kDa) than did the wild-type. These proteins are probably retained in the periplasm of the *outJ* mutants, in the same way as pectinases or cellulases. Moreover, one band, corresponding to a protein of 65 kDa was absent in the periplasmic fraction of these mutants. This protein could correspond to the *outJ* product.

Isolation of R-prime plasmids carrying the *outJ* region , subcloning of *outJ*

To obtain a positive selection for the *outJ* gene, we isolated an insertion of the transposon Tn*5* near the *outJ* region. In strain A1417, the Tn5 insertion gave a cotransduction of 30% with *outJ* mutations. We used this strain to isolate R-prime plasmids bearing both the Tn*5* insertion and the wild-type *outJ* gene. The presence of *outJ* on plasmids was tested by complementation of the *outJ* mutation (PGA^+ phenotype). Assay of enzyme activity in the different cellular compartments of *outJ* mutants containing RJ551 showed that this R-prime was able to complement the *outJ* mutation of the 8 mutants isolated suggesting that only one genetic locus was affected in these non-secreting mutants.

Digestion of the RJ551 plasmid by *Pst*I, *Hin*dIII or *Eco*RI showed that this plasmid carried an insert of about 30 kb of chromosomal *E. chrysanthemi* DNA (Fig. 1) (Ji et al, 1989). Transposon Tn*5* is inserted at one extremity of the chromosomal DNA fragment and next to one copy of Mu3A.

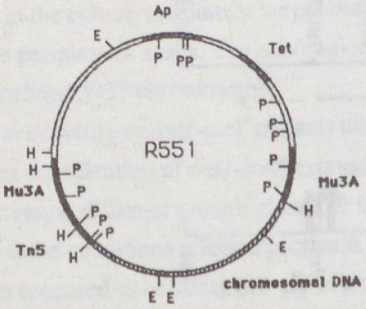


Fig. 1. Map of RJ551 plasmid harboring the *outJ* region and the Tn5 insertion. (H, *Hin*dIII; E, *Eco*RI;P, *Pst*I) *Pst*I sites inside the chromosomal DNA insert are not indicated.

We first subcloned a 29 kb *Hin*dIII fragment of plasmid RJ551. Since there was apparently no *Hin*dIII site in the insert of chromosomal DNA, this fragment probably spread from a Tn*5* *Hin*dIII site to a Mu3A *Hin*dIII site (Fig. 1). The resulting plasmid, pSH10, complemented the *outJ* mutation. Further subcloning showed that the smallest completely functional fragment was the 4 kb *Cla*I/*Ssp*I insert of plasmid pSCSs7 (Fig. 2) (Ji et al, 1989).

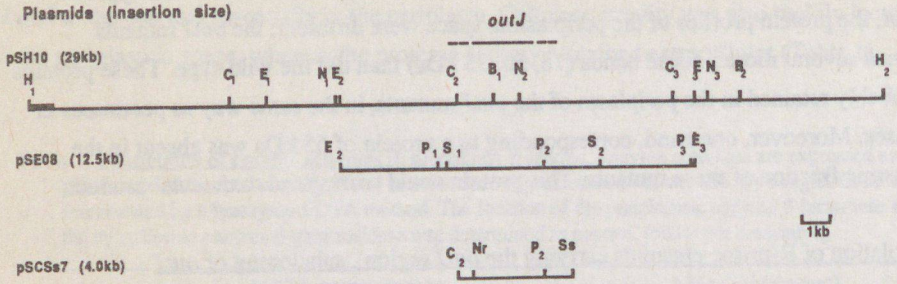


Fig. 2. Restriction map of the pSH10 plasmid and its subclonings. (heavy line = transposon Tn*5* DNA)
H: *Hin*dIII, E: *Eco*RI, P: *Pst*I, C: *Cla*I, B: *Bam*HI, N: *Nco*I, S: *Sal*I, Ss: *Ssp*I, Nr: *Nru*I.

Construction of *phoA* protein fusions

Transposon Tn*5*::*phoA* was inserted at random on the *Cla*I/*Ssp*I fragment. If Tn*5*::*phoA* is inserted in the proper orientation and translational reading frame, into a gene encoding an exported protein, a hybrid protein with alkaline phosphatase activity is produced (Manoil and Beckwith, 1985). Many alkaline phosphatase-positive insertions were found in the *Cla*I/*Ssp*I fragment (Fig. 3), indicating that this region encodes for exported protein(s).

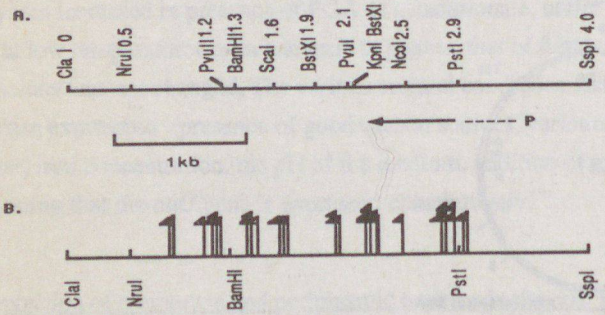


Fig. 3. Restriction map of the *Cla*I/*Ssp*I fragment (A) and location of *phoA* protein fusions (B).
The letter P and an arrow indicate the presumed *outJ* promoter and its transcriptional direction.

These Tn*5*::*phoA* fusions spread on a 2.3 kb region and the direction of transcription of the *phoA* gene was from the *Ssp*I site towards the *Cla*I site (Fig. 3). Plasmids containing these insertions showed no complementation of the *outJ* mutations.Some of these insertions were introduced in the chromosomal DNA of wild-type strain 3937 by homologous recombination. The recombined clones containing these Tn*5*::*phoA* insertions acquired the phenotype of *outJ* mutants; i.e., no secretion pectinases or cellulases.

Identification of the *outJ* products

The system that employs phage T7 RNA polymerase to direct synthesis of genes located downstream from a phage T7 promoter (Tabor and Richardson, 1985) was applied to the *outJ* region. Electrophoretograms of the radioactive polypeptides produced, showed two polypeptides of 81 and 83 kD synthesised in addition to ß-lactamase and its precursor. Since the *outJ* region has been shown to encode exported proteins, we postulate that the 83 kD protein is the primary product of the *outJ* gene and is the precursor of the 81 kD protein. After addition of ethanol (which inhibit peptidase signal activity) in the medium, only the 83 kD protein was produced, in addition to the precursor form of ß-lactamase. The simultaneous disappearance of the mature ß-lactamase and the 81 kD protein suggests that the 81 kD polypeptide results from cleavage of the 83 kD polypeptide by the action of signal peptidase.

CONCLUSION

We isolated mutants altered in secretion of pectate lyases by using Mu-*lac* insertion in *Erwinia chrysanthemi* 3937. In the *outJ* mutants the total amounts of pectate lyases, pectin methylesterase or cellulases were similar to that of the wild-type, but the localization of these enzymes markedly differed. The wild-type produced approximately 80-90% of these enzymes in the culture medium, whereas *outJ* mutants retained 80-90% of these enzymes within the periplasmic space. The *outJ* region maps near the *ade*-22 marker on the *E.chrysanthemi* 3937 chromosome.

The availability of *outJ-lacZ* mutants allowed us to assay *outJ* expression. There was no significant modification of *outJ-lacZ* expression under semi-aerobic conditions, at low temperatures, at different growth phases or in the presence of galacturonate or PGA, although these conditions affected pectinase production. In contrast to pectinolytic genes, the *outJ* gene appeared to be constitutively expressed.

We cloned a 4 kb *Cla*I/*Ssp*I fragment which complements the *outJ* mutations and is then required for secretion of pectate lyases and cellulases by *E.chrysanthemi* . Subcloning and Tn*phoA* insertions suggested that the *outJ* region encodes exported protein(s) and

extends over a distance of about 2.5 kb. Use of the T7 polymerase promoter system showed that the OutJ product is an 83 kD polypeptide which is the precursor form and an 81 kD mature polypeptide. In the *outJ* mutants, we noted the disappearance of a 65 kD periplasmic protein; this 65 kD protein could be totally distinct from the OutJ product but, another possibility is that the 81 kD protein is submitted to subsequent cleavage(s), giving rise to the 65 kD protein. In conclusion, we identified an exported protein, with a precursor form of 83 kD, involved in secretion of pectinases and cellulases. The *outJ* product may act at the level of transfer across the outer membrane of *E. chrysanthemi* but is probably not the sole protein involved in this secretory mechanism.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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FUNCTIONAL CONSERVATION OF A LOCUS REQUIRED FOR LESION FORMATION WITHIN MOLECULARLY DISTINCT ISOLATES OF PSEUDOMONAS SYRINGAE PV. SYRINGAE

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INTRODUCTION

Pseudomonas syringae pv. *syringae* (*P. s. syringae*) strain B728a is a causal agent of bacterial brown spot disease of bean (*Phaseolus vulgaris*). We are interested in the molecular characterization and evolution of genes in phytopathogenic bacteria that are involved in the ability to cause disease. By transposon mutagenesis, we have identified a locus in B728a, designated *lemA*, that is required for the formation of disease lesions on bean leaves and pods (Willis *et al.*, 1989). The *lemA1*::Tn*5* mutant strain NPS3136 does not form lesions but does retain other bacterial-plant interactions, such as the abilities to grow epiphytically and to induce the hypersensitive reaction on the non-host tobacco. Cosmid clones containing the genomic DNA fragment affected in NPS3136 and flanking sequences have been isolated from a genomic library of the wild-type B728a. These clones restore the pathogenic phenotype to the *lemA1* mutant strain NPS3136.

The *lemA1*::Tn*5* mutation is located within a 6.1 kb genomic *Eco*RI DNA fragment. A fragment of similar size that shares homology with the *lemA* locus of B728a has been found in various *Pseudomonas syringae* strains (Willis *et al.*, 1989). In the work presented here, we extended this analysis to include bean pathogenic *P. s. syringae* field strains and have found that these strains can be grouped into seven distinct classes based on restriction fragment length polymorphism (RFLP) patterns. Recombinational exchange mutagenesis was used to demonstrate that the *lemA* locus is required for lesion formation by these field isolates. In addition, we have begun to investigate the possibility that the *lemA* locus may share functional homology between different *P. syringae* pathovars. Towards this end, we have isolated a clone from *P.*

syringae pv. *phaseolicola* (*P. s. phaseolicola*) that restores lesion formation to the *P. s. syringae lemA1*::Tn*5* mutant.

RESULTS AND DISCUSSION

Molecular diversity of *P. s. syringae* field isolates

P. s. syringae isolates were recovered from experimental bean plots in Wisconsin by dilution plating of homogenized bean leaflets and identified by standard methodology (positive for fluorescence under long wave ultraviolet radiation; negative for cytochrome oxidase; appropriate colony morphology on King's B and glycerol agar media) (Hildebrand *et al.*, 1988). Each strain was tested for its ability to cause brown spot disease on bean pods by a laboratory assay which has been successfully used to screen bean lines for genetic resistance to brown spot (Daub and Hagedorn, 1979). Eighty isolates were selected at random from bacterial collections spanning several years. Chromosomal DNA from each isolate was digested separately with the restriction endonucleases *Eco*RI and *Hin*dIII and analyzed for RFLP using the 6.1 kb *lemA Eco*RI fragment as a DNA hybridization probe (Willis *et al.*, 1989). With the exception of one isolate, three *Eco*RI patterns and two *Hin*dIII patterns were observed in all permutations, resulting in six distinct classes of RFLP. A single isolate (5B1458) had a unique pattern for each enzyme and formed a seventh RFLP class. Three of the four *Eco*RI patterns (from strains B728a, 4B10, and S1B1129) are shown in Figure 1 (lanes A, D, and G). Preliminary results from hybridizations with another *P. syringae* probe containing 32 kb of chromosomal DNA indicate some additional RFLP groupings. We have also investigated whether nonpathogenic variants of *P. s. syringae* can be distinguished from the pathogens by their RFLP patterns. Evidence to date suggests that nonpathogenic strains do not bear patterns distinct from those identified in pathogenic strains.

Correlations have been drawn between RFLP groupings and pathogenicity in *Xanthomonas campestris* (Lazo *et al.*, 1987; Gilbertson *et al.*, 1987), in *P. solanacearum* (Boucher *et al.*, 1988; Cook *et al.*, 1989), and in *P. syringae* (Denny *et al.*, 1988). These studies report the potential of RFLP typing as an alternative to identification methods based on pathogenicity or physiological tests. Most of the RFLP studies cited above compared strains isolated from a wide variety of host plants. In contrast, we examined a large number of isolates recovered from a single host species. Although we have

not attempted an exhaustive analysis of RFLP types, the finding that a single probe, shorter in length than many probes used in other studies, revealed at least seven RFLP groups suggests that there is a great deal of variability at the DNA level between strains in a field situation. Ultimately, it will be necessary to gain an understanding of the diversity exhibited within indigenous bacterial populations in order to confidently utilize any classification system based on DNA sequence polymorphism. The results described here for *P. s. syringae* suggest that RFLP analysis may be an effective means of strain identification in such population studies.

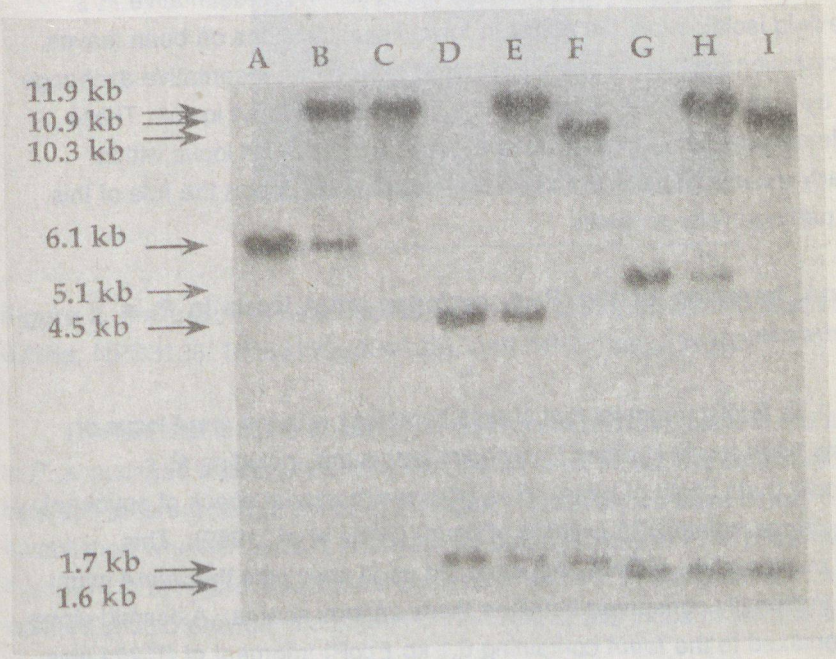


Figure 1. Southern blot of *Eco*RI digested DNA from wild-type *P. s. syringae*, exchange intermediate (merodiploid), and exchange mutant strains probed with the 6.1 kb *lemA* containing *Eco*RI fragment from *P. s. syringae* B728a. Lanes **A**, **B**, and **C**: B728a, B728a(pKW25), and B728a(*lemA1*::Tn*5*), respectively; lanes **D**, **E**, and **F**: 4B10, 4B10(pKW25), and 4B10(*lemA1*::Tn*5*), respectively; lanes **G**, **H**, and **I**: S1B1129, S1B1129(pKW25), and S1B1129(*lemA1*::Tn*5*), respectively. (Taken from Willis *et al.*, 1989).

Conserved function of *lemA* within *P. s. syringae* field isolates

The RFLP shown by the *lemA* probe within *P. s. syringae* field strains led us to question whether the function of this locus is conserved. The *lemA1*::Tn*5* mutation, contained in the cosmid clone pKW25, was exchanged by homologous recombination into representatives from each of the seven RFLP classes of field isolates. As seen in Figure 1, the 6.1 kb *Eco*RI *lemA* containing fragment of the wild-type B728a (lane A) has increased by 5.8 kb, the length of Tn*5*, to yield a fragment of 11.9 kb in the exchange mutant B728a(*lemA1*::Tn*5*) (lane C). Both the 6.1 kb and the 11.9 kb fragments are present in the merodiploid exchange intermediate, B728a(pKW25) (lane B). Figure 1 also shows the introduction of the *lemA1*::Tn*5* mutation into *P. s. syringae* field isolates 4B10 and S1B1129 (lanes D - I).

The resulting *lemA1*::Tn*5* mutants of these seven representative *P. s. syringae* field isolates lost the ability to form disease lesions on bean leaves. Lesion formation was subsequently restored to three representative exchange mutants by a cosmid clone containing the intact B728a *lemA* locus. These results demonstrate the functional conservation of the *lemA* locus within molecularly diverse populations of *P. s. syringae* and support the role of this locus in pathogenicity on bean.

Functional homolog of the *P. s. syringae lemA* locus in *P. s. phaseolicola*

A 6.1 kb *Eco*RI fragment that shares homology with the *lemA* locus of B728a has been found in other *P. syringae* pathovars, including *P. s. phaseolicola* (halo blight of bean), *P. s. glycinea* (bacterial speck of soybean), and *P. syringae* isolate BR2 (wildfire of bean) (Willis *et al.*, 1989). This apparent conservation of DNA sequence led us to speculate that *lemA* might also be functionally conserved between these various strains. A cosmid clone which hybridized to the *lemA* containing 6.1 kb *Eco*RI fragment of B728a was isolated from a genomic library of *P. s. phaseolicola* NPS3121, a causal agent of halo blight of bean. This clone, designated pKW12b, restored lesion formation to the *lemA1*::Tn*5* mutant (NPS3136) to an equivalent extent as did pKW352, a clone containing the wild-type *lemA* locus from *P. s. syringae* B728a (Figure 2). Neither pKW12b nor pKW352 conferred pathogenicity to the nonpathogenic *P. syringae* isolate Cit7 or to *E. coli* strain HB101.

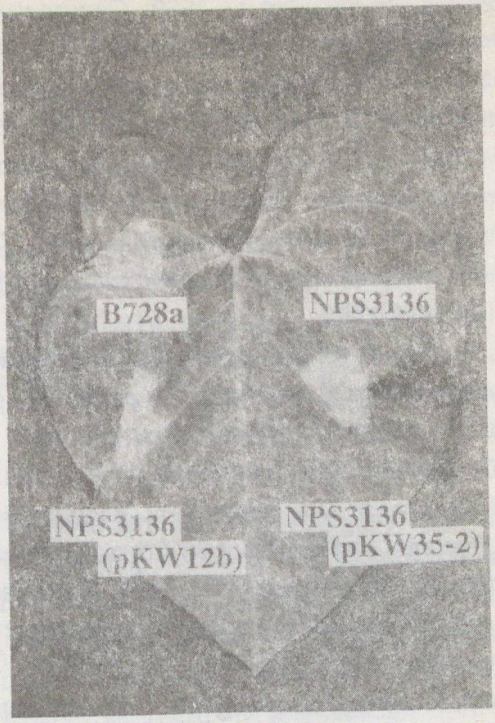


Figure 2. Infiltration of bean leaves with ~10^6 cfu/ml suspensions of strains B728a, NPS3136, NPS3136(pKW12b), and NPS3136(pKW352).

The fact that a clone from *P. s. phaseolicola* can complement the mutation in *P. s. syringae* supports the idea that the function of *lemA* is conserved between different pathovars of *P. syringae*. However, we have not as yet established whether *lemA* is essential for pathogenicity in *P. s. phaseolicola* as it is for *P. s. syringae*. Mutational analysis of *lemA* in this and other *P. syringae* isolates should provide information on the role of this locus in several bacterial-plant interactions.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

ROLE OF ANTIBIOTIC PRODUCTION BY ERWINIA HERBICOLA EH252 IN REDUCING FIRE BLIGHT INCIDENCE

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INTRODUCTION

Certain strains of *Erwinia herbicola*, a non-pathogenic epiphytic bacterium, were shown to be effective in controlling fire blight. Fire blight is a devastating disease of apple, pear and other rosaceous plants caused by the related bacterium *Erwinia amylovora*. Infection usually begins in blossoms, but when blossoms are treated first with *E. herbicola* and then inoculated with *E. amylovora*, infection is often reduced to a similar extent as when blossoms are treated with streptomycin.

Strains of *E. herbicola* that are particularly effective in control of fire blight have been found to inhibit *in vitro*, the growth of *E. amylovora*, by an antibiotic-based mechanism (Wodzinski *et al.* 1987). To determine the role of antibiotic production by *E. herbicola* strain Eh252 in the control of fire blight, two approaches were taken. One approach was based on the use of a derivative of *E. amylovora* that is resistant to the antibiotic produced by strain Eh252, the other approach is based on the use of mutants of Eh252 that are deficient in antibiotic production.

I. INHIBITION IN IMMATURE PEAR FRUIT OF *E. amylovora* STRAINS Ea273 AND Ea273R BY *E. herbicola* STRAIN Eh252.

A derivative of Ea273, designated Ea273R, was selected for its resistance to the antibiotic produced by Eh252. We then compared the percentage of infection in immature pear fruits that had been treated first with *E. herbicola* Eh252, and then inoculated with Ea273 or Ea273R.

Pear fruits harvested when 1.5-3 cm in diameter are susceptible to infection by *E. amylovora* and can be manipulated easily in the laboratory. Suspensions of Eh252 (10^6 CFU/fruit or 10^7 CFU/fruit) were introduced into a well bored into the cheek of an immature pear fruit. Suspensions of *E. amylovora* Ea273 or Ea273R (2 x 10^6 CFU/fruit) were then introduced into the same well. The fruits were incubated at 27°C in a moist chamber and scored daily for evidence of infection. A fruit was considered infected when drops of bacterial exudate were detected in and around the well. When buffer was used instead of the suspension of *E. herbicola*, 100% of the fruits were infected after 3 days of incubation. The presence of Eh 252 reduced infection of the pear fruits by both strains of *E. amylovora*, and the inhibition was greater when higher populations of *E. herbicola* were used. However, disease induced by the strain Ea 273R was always much greater than that induced by the wild-type strain. These data suggest that antibiotic production is involved in the inhibition of *E. amylovora* in pear tissue, but that other mechanisms also are involved.

II. INHIBITION OF *E. amylovora* IN IMMATURE PEAR FRUIT BY Eh252 AND MUTANTS OF *E. herbicola* THAT LACK ANTIBIOTIC PRODUCTION.

Mutants of Eh252 deficient in antibiotic production were selected after Tn*5* mutagenesis. A derivative of the bacteriophage lambda (*b*221, *O*am29, *P*am80, rex::Tn*5* 132, *c*I857) was used as a vector for Tn*5* (De Bruijn and Lupski 1984). Because Eh252 is not sensitive to lambda, the plasmid pTROY9 (De Vries *et al.* 1984), which contains and expresses constitutively the *lam*B gene of *Escherichia coli*, was introduced into Eh252. Strain Eh252(pTROY9) was transfected with the lambda derivative containing Tn*5*. Of 1,500 kanamycin-resistant colonies screened, three prototrophic mutants were identified that did not show any antibiotic activity. Southern blots were done using total DNA isolated from these mutants cut with either *Eco* RI or *Bam* HI. Hybridization of these blots using the Tn*5*-containing plasmid pRZ102 (Jorgensen *et al.* 1979) as probe, revealed that two mutants, 10:12 and 17:22, had single Tn*5* insertions. The strain Eh252 contains three indigenous plasmids ranging from 100 kb to 180 kb in size. The plasmids isolated from 10:12 and 17:22 did not hybridize with the probe pRZ102 indicating that for these two mutants, the Tn*5* insertions were in the chromosome.

The ability of the non-antibiotic-producing mutants, 10:12 and 17:22, to inhibit the development of fire blight in immature pear fruits was compared to that of Eh252. The fruits were first treated with suspensions of *E. herbiciola* (Eh252, 10:12, or 17:22) ranging from 4 x 10^3 to 4 x 10^6 CFU/fruit and then inoculated with a suspension of *E.*

amylovora (Ea273) of 5 x 10^5 CFU/fruit. In all cases, pear fruits treated with the wild-type strain Eh252, exhibited less infection than those treated with the non-antibiotic-producing mutants. At low concentrations of *E. herbicola* (4 x 10^3 or 4 x 10^4 CFU/fruit), most fruit were infected after 6 days, and suppression of infection was proportional to the concentration of *E. herbicola* used. The highest amount of *E. herbicola* tested (4 x 10^6 cells/fruit) resulted in the greatest suppressive effect, even when the non-antibiotic-producing strains 10:12 or 17:22 were used. This suggests again that other, as yet undefined, mechanisms may also be involved in the protective effect against fire blight by *E. herbicola.*

CONCLUSION

Two approaches were used to evaluate the role of antibiotic produced by *E. herbicola* strain Eh252 in reducing fire blight infection. Both approaches, using a derivative of *E. amylovora* resistant to the antibiotic produced by Eh252 and mutants of Eh252 deficient in antibiotic production, indicated that antibiotic is involved in controlling fire blight infection, though other mechanisms may be involved.

Further evidence for the role of antibiotic production by Eh252 will be provided by genetic complementation. Antibiotic production will be restored to the mutants 10:12 and 17:22 by complementation with a genomic library of Eh252. The protective ability of the mutants, complemented mutants and Eh252 will be compared in immature pear fruit.

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This work was supported (in part) by a grant from the Cornell Biotechnology Program which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, the U.S. Army Research Office and the National Science Foundation.

SESSION 5

IDENTIFICATION

A) AND B)

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

CHEMOTAXONOMIC AND PHENOTYPIC CHARACTERIZATION OF PHYTOPATHOGENIC PSEUDOMONADS

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ABSTRACT

Chemotaxonomic studies based on the analyses of quinones and fatty acids distinguished 4 groups among the phytopathogenic pseudomonad species. This grouping coincided well with that by genetic studies based on the DNA-rRNA hybridization. Some newly described phytophatogenic pseudomonad species of uncertain affiliation were also assigned to any one of these 4 groups. None of single phenotypic characteristics investigated could distinguish these groupings exclusively, however, some may give useful information for primary identification.

INTRODUCTION

The genus Pseudomonas is now considered as a heterogeneous taxon, and it is divided into several groups, based on some phenotypic characteristics and DNA-rRNA hybridization (Palleroni 1984, DeVos et al. 1985). There, however, exist many plant pathogenic species whose relationships to these groups or to each other are uncertain.

Recently, two chemotaxonomic methods have been examined extensively by many workers. Those are the analyses of quinones and fatty acids. The former exist in the electron transport system and the latter exist in membranes, LPS and so on. Both of them are very important molecules, and their differences are considered to reflect the genus level differentiation (Oyaizu and Komagata 1983).

In this study, we tried to clarify the grouping of phytopathogenic pseudomonads through analyses of these two kinds of compounds, and to evaluate conventional identification methods based on some phenotypic characterization.

MATERIALS AND METHODS

Pseudomonad species used in this study were listed in Table 1. Several strains of each species were investigated of which the origins are heterogeneous. Some xanthomonad species were also included for comparisons.

Quinones were extracted by ether-ethanol, purified with TLC and chromatographed on filter papers to reveal the number of isoprenoid units (Yamada et al. 1969, Collins and Jones 1981). Fatty acids were methylated by hydrogen chloride and methanol, extracted with hexane, and gas-chromatographed. Obtained peaks were compared with those of standards, and some peaks were analyzed by mass-spectrometry (Suzuki and Komagata 1983, Komagata and Suzuki 1987).

Phenotypic characteristics were investigated through conventional methods (Sands et al. 1980). Accumulation of poly-β-hydroxybutylate (PHB) and degradation patterns of aromatic compounds were determined with the method described by Stanier et al. (1966).

RESULTS AND DISCUSSION

From the analysis of quinones, the pseudomonad species were divided into two large groups (Table 1). The one possesses ubiquinone with 9 isoprenoid units (Q-9) and the other with 8 units (Q-8). No other kind of quinones was detected such as menaquinone.

As far as we investigated, pseudomonads with Q-9 have same pattern of fatty acid profile, now referred to as group A. Pseudomonads with Q-8 were divided into 3 groups based on the fatty acid profiles, referred to as groups B, C and D. Representative chromatograms of these groups were shown in Fig. 1. These 4 groups could be divided on the basis of presence or absence and the ratio of some fatty acids shown in Table 2. As a matter of fact, these groups could be easily distinguished from each other at a glance of their chromatograms.

Group A: This group includes fluorescent pseudomonad species such as P. syringae pathovars and P. cichorii. P. corrugata, P. ficuserectae and P. meliae were revealed to be related to fluorescent pseudomonads.

Group B: Group B includes P. cepacia, P. caryophylli and P. gladioli. P. glumae, P. plantarii and P. andropogonis were also included in this group. P. andropogonis has been considered to relate to P. avenae on the basis of phenotypic properties (Goto and Starr 1971) and biosynthetic path-

Table 1. Grouping of Phytopathogenic Pseudomonas Species

	Quinone	Fatty acids	1	2	3
P. syringae					
pv. mori	Q-9	A	I	I	I
pv. photiniae	Q-9	A			
pv."magnoliae"	Q-9	A			
pv. dendropanacis	Q-9				
pv. tremae	Q-9				
pv. actinidiae	Q-9				
P. ficuserectae	Q-9	A			
P. meliae	Q-9	A			
P. cichorii	Q-9	A	I	I	
P. corrugata	Q-9	A'	I		
P. alcaligenes	Q-9		I	I	
P. pseudoalcaligenes	Q-9		I		
P. caryophylli	Q-8	B	II	II	II
P. andropogonis	Q-8	B	II	III	
P. cepacia	Q-8	B	II	II	
P. gladioli	Q-8	B	II	II	
P. glumae	Q-8	B	II		
P. plantarii	Q-8	B			
P.(C.) acidovorans	Q-8	C	III	III	III
Comamonas terrigena	Q-8	C	III		III
P. avenae(=cattleyae)	Q-8	C	III	III	IX
P. pseudoalcaligenes ssp. konjaci	Q-8	C			
ssp. citrulli	Q-8				
P.(X.)maltophilia	Q-8	D	V	V	V
P. cissicola	Q-8	D	?		
X. campestris pv.campestris	Q-8	D	V	V	
pv.mangiferaeindicae	Q-8	D	V		
pv.oryzae	Q-8	D'			

1: Grouping based on rRNA-DNA hybridization study. (DeVos et al. 1985)
2: Grouping based on biosynthetic pathway. (Byng et al. 1980)
3: Grouping based on the analysis of quinone and fatty acid. (Oyaizu and Komagata 1983)
Fatty acid profiles were divided into 4 groups (A, B, C & D).
Some minor differences were observed in A' and D'.

Table 2. Characteristics of four types of fatty acid profile

	A	B	C	D
12:0	+	-	+	-
16:1/16:0	ca.1	0.4	ca.1	ca.1
18:0	1%	1%	1%	1%
3-OH 10:0	+	-	+	-
3-OH 16:0	-	+	-	-
2-OH 12:0	+	-	-	-
2-OH 16:0	-	+	-	-
iso & anteiso branched fatty acids	-	-	-	+

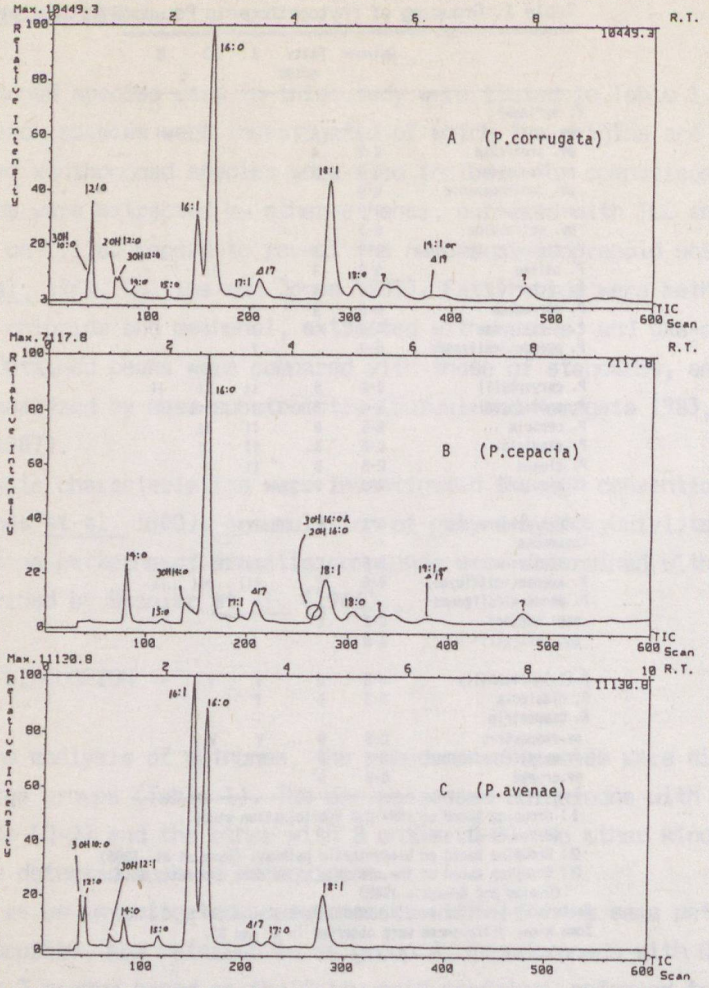


Fig. 1. Chromatogram of the methylated fatty acids from the cells of A: P. corrugata PDDCC5819), B: P. cepacia (ALO8281) and C: P. avenae (PAVEL8101). Analysis were made on a OV-1 column

way (Byng et al. 1980), however, the result of fatty acid analysis revealed that it is related more closely to P. cepacia or P. caryophylli rather than to P. avenae.

Group C: This group includes P. acidovorans (=Comamonas acidovorans, Tamaoka et al. 1987) and Comamonas terrigena. P. avenae, P. pseudoalcaligenes ssp. konjaci and ssp. citrulli were included in this group. These two subspecies of P. pseudoalcaligenes were considered to be inadequately classified in P. pseudoalcaligenes.

Group D: Group D consists of xanthomonad species. P. cissicola was also included in this group.

Table 3. Phenotypic Properties of Phytopathogenic Pseudomonas Species

	PHB	Ring cleavage	Growth at 40C	OF test	LOPAT test	NO_3 reduc.	Utilization[a] Glc	Suc	Val	βAla	pigment[b] FL	Y
P. syringae												
pv. mori	−	−	−	0	+−−−+	−	+	+	−	−	+	−
pv. photiniae	−	ortho	−	0	−−−−+	−	+	+	−	−	−	−
pv."magnoliae"	−	−	−	0	+−−−+	−	+	+	−	−	−	−
pv. dendropanacis	−	ortho	−	0	−−−−+	−	+	+	−	−	+	−
pv. tremae	−	−	−	0	−−−−+	−	+	+	−	−	−	−
pv. actinidiae	−	−	−	0	+−−−+	−	+	+	−	−	−	−
P. ficuserectae	−	−	−	0	+−−−+	−	+	+	−	−	−	−
P. meliae	−	−	−	0	−−−−−	−	−‡	−‡	−‡	−‡	−	−
P. cichorii	−	ortho	−	0	−+−−+	−	+	−	−	−	+	−
P. corrugata	+	ortho	−	0	−+±++	+	+	+	+	+	−	−
P. alcaligenes	−	−	+	0/-	−+−+−	+	−	−	−	+	−	−
P. pseudoalcaligenes	−	−	+	0/-	−+−−−	+	−	−	−	+	−	−
P. caryophylli	+	ortho	+	0	++−−−	+	+	+	−	+	−	−
P. andropogonis	+	ortho	−	0	−−−−−	−	+	−	−	+	−	−
P. cepacia	+	ortho	+	0	−+−−−	+	+	+	+	+	−	−
P. gladioli	+	ortho	V	0	−±V−−	V	+	V	+	+	−	−
P. glumae	+	ortho	+	0	−−+−−	+	+	−	+	+	−	−
P. plantarii	+	ortho	−	0	−±−−−	+	+	−	+	+	−	−
P. acidovorans	+	meta	−	0/-	−+−−−	+	+	−	+	+	−	−
Comamonas terrigena	+	−	−	0/-	−+−−−	+	−‡	−‡	−‡	−‡	−	−
P. avenae	+	−	+	0/-	−+−−+	+	+	−	−	+	−	−
P. pseudoalcaligenes ssp. konjaci	+	−	−	0/-	−+−−+	+	+	−	−	+	−	−
ssp. citrulli	+	−	−	0/-	−+−−+	+	+	−	−	+	−	−
P.(X.)maltophilia	−	−	−	0	−−−−−	+	−‡	−‡	−‡	−‡	−	±
P. cissicola	−	−	−	0	+−−−−	−	+	+	−	−	−	−
X. campestris pv.campestris	−	−	−	0	+−+−−	−	+	+	−	−	−	+
pv.mangiferaeindicae	−	−	−	0	+−−−−	−	+	+	−	−	−	−
pv.oryzae	−	−	−	0	+−−−−	−	−‡	−‡	−‡	−‡	−	+

a): Glc:glucose, Suc:sucrose, Val:L-valine, βAla:β-alanine, ‡require growth factor.
b): Fl:fluorescent pigment production, Y:yellow xanthomonadin like pigment production.
V: variable results among the strains.

Our chemotaxonomic results coincided well with the classifications based on DNA-rRNA hybridization (DeVos et al. 1985) and on biosynthetic pathway (Byng et al. 1980), with few exceptions such as P. cissicola and P. andropogonis.

Results of phenotypic characterization were shown in Table 3. Almost all the strains of group A could not accumulate PHB, with the exception of P. corrugata, which accumulate PHB on the medium containing β-hydroxy butylate as a sole source of carbon, but not on the Yeast Peptone Agar or Nutrient Agar media. P. pseudoalcaligenes and P. ficuserectae were also reported to accumulate PHB in their original descriptions. Therefore, it may be inadequate to divide primarily the pseudomonad species solely on the basis of PHB accumulation. All the strains of group B and C were able to accumulate PHB. We also determined the degradation pattern of aromatic

compounds using protocathecuate as a substrate. Although, many species of group A could not degrade these compounds, ortho type was observed if present. All the group B bacteria degrade protocathecuate through ortho pathway. In group C, only P. acidovorans showed meta type and others showed no degradation. All the fluorescent strains were included in group A. Yellow pigmented strains are grouped in D group that is Xanthomonas group. However, some strains of this group did not produce yellow pigment. Some other properties were also investigated such as LOPAT test, nitrate reduction, growth factor requirements, and utilization of some organic compounds. But none of them could distinguish these groups. Despite this fact, there exist some tendencies in the reaction of the species belonging to the same group. Investigation of many more characters and their numerical analysis will be needed to be compared with chemotaxonomy.

As mentioned above, it is revealed that the analyses of quinones and fatty acids may be useful tools in classification and identification of phytopathogenic pseudomonads. Furthermore, we clarified the relationships of some phytopathogenic pseudomonads of uncertain affiliation. The groups in the genus Pseudomonas should become independent genus, and it will be needed to change their generic name. Chemotaxonomic study may support this work.

An important thing is that the analyses of quinone and fatty acids are easy to perform. It is much easier to handle these compounds than to handle isotopes as used in DNA and RNA studies. Thus, it may be useful to use chemotaxonomic methods in a preliminary identification. For example, if we found a new disease and the pathogen were thought to be a new species, we would be able to choose appropriate reference strains for fine identification through the analyses of quinones and fatty acids.

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Proc. 7th Int. Conf. Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

PHENETIC ANALYSIS AND PRELIMINARY GENETIC CHARACTERIZATION OF NON-FLUORESCENT PLANT PATHOGENIC PSEUDOMONAS SPP.

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ABSTRACT

Numerical analysis of results of biochemical and nutritional tests with strains of hitherto poorly characterized or recently named non-fluorescent plant pathogenic *Pseudomonas* spp. produced clusters of strains which allowed *P. andropogonis*, *P. caryophylli*, *P. cissicola*, *P. corrugata*, *P. glumae*, *P. plantarii*, *P. pseudoalcaligenes* subsp. *konjaci*, and *P. rubrisubalbicans* to be discriminated by determinative tests. Strains of *P. ficuserectae* and *P. meliae* together formed a single cluster. Strains of *P. avenae*, *P. cattleyae*, *P. pseudoalcaligenes* subsp. *citrulli*, and *P. rubrilineans* also formed a single homogeneous cluster ('avenae cluster'). The close relationship between members of the 'avenae cluster' was confirmed using colony hybridization with total genomic DNA probes of *P. avenae* and *P. rubrilineans*. Although biochemical and nutritional tests differentiated *P. pseudoalcaligenes* subsp. *konjaci* as a single separate cluster, strains of this sub-species showed partial colony hybridization with DNA probes, which suggests some affinities with the 'avenae cluster'. Preliminary results of pathogenicity tests support the close relationship of *P. avenae*, *P. pseudoalcaligenes* subsp. *citrulli* and *P. rubrilineans*. These results indicate that *P. cattleyae*, *P. pseudoalcaligenes* subsp. *citrulli*, and *P. rubrilineans* are synonymous with *P. avenae*, and that *P. pseudoalcaligenes* subsp. *konjaci* shares a close, but distinct, relationship with *P. avenae*.

INTRODUCTION

Plant pathogenic *Pseudomonas* spp. are usefully divided according to their ability to produce fluorescent pigments on iron-deficient media. The non-fluorescent pathogenic species *P. cepacia* (ex Burkholder 1950) Palleroni & Holmes 1981, *P. gladioli* pv. *alliicola* (Burkholder 1942) Young et al. 1978, *P. gladioli* pv. *gladioli* Severini 1913, and *P. solanacearum* (Smith 1896) Smith 1914 have been well described (Palleroni 1984). However, other non-fluorescent plant pathogenic pseudomonads are either poorly characterized (Palleroni 1984), or are recently described and have not been included in comparative studies. This study reports a comparison of strains of such poorly characterized or recently described taxa using biochemical, nutritional and nucleic acid hybridization tests.

METHODS

Strains Used

All strains used in this study were from the International Collection of Micro-organisms from Plants (ICMP), Plant Diseases Division, DSIR, Auckland, New Zealand (Table 1).

Initially, their identities as non-fluorescent pseudomonads was confirmed by tests for Gram reaction, fluorescent pigment production, and oxygen requirement. Gram reaction of air-dried bacterial suspensions from yeast-phosphate agar (YPA; Young 1987) were tested using the staining modification of Hucker (Manual of Microbiological Methods 1957), and the elution procedures of Bartholomew (1962). Oxygen

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requirement was tested by the method of Young (1977).Fluorescent pigment production was investigated using modified King's medium B (mKB; Dansk Standard - Merck 1982 Handbook) in which 1.8g K_3PO_4 replaced K_2HPO_4. Only Gram negative, aerobic, non-fluorescent strains were included in the study (Table 1). Cultures were maintained by serial transfer at two-month intervals on YPA and stored at 8C°.

Table 1. *Pseudomonas* spp. and ICMP strains used in the comparative study

P. andropogonis (Smith 1911) Stapp 1928 2806, 2807*, 2808, 2809, 3377, 3994, 3995, 3996, 3997, 3998, 4001, 5980, 6779, 6780, 6781, 7076, 7854, 7855, 7856, 8039, 8042, 8665, 9214

P. avenae Manns 1909 3178, 3179, 3180, 3181, 3182, 3183*, 3184, 3185, 3186, 3254, 3960, 7083, 7084

P. caryophylli (Burkholder 1942) Starr & Burkholder 1942 512*, 2824, 5848, 8691, 8692

P. cattleyae (Pavarino 1911) Savulescu 1947 2826*, 8654

P. cissicola (Takimoto 1939) Burkholder 1948 4289*, 4290, 4291, 8561

P. corrugata (ex Scarlett et al. 1978) Roberts & Scarlett 1981 5819*, 8231, 8270, 8271, 8890, 8891, 8898

P. ficuserectae Goto 1983 7848*, 7849, 7850

P. glumae Kurita & Tabei 1967 3655*, 3727, 3728, 3729, 8657

P. meliae Ogimi 1981 6289*

P. plantarii Azegami et al. 1987 9424*, 9425, 9426, 9427, 9428, 9429

P. pseudoalcaligenes subsp. *citrulli* Schaad et al. 1983 6521, 6522, 7500*, 7713, 7714, 7715, 7716, 8663

P. pseudoalcaligenes subsp. *konjaci* Goto 1983 7733*, 7734, 7851

P. rubrilineans (Lee et al. 1925) Stapp 1928 251, 252, 254*, 255, 1656, 3106, 3107, 3139, 3166, 3167, 3168, 5811, 7460

P. rubrisubalbicans (Christopher & Edgerton 1930) Krasilnikov 1949 792, 793, 2850, 3108, 3109, 3110, 3112, 5714, 5777*, 6268

* type strain

Test Methods

For nutritional and biochemical tests, media were inoculated with a faintly turbid suspension (~10^8 colony forming units (cfu)/ml) prepared by dispersing cells from 24-48h YPA cultures in sterile de-ionized water. Solid media in tubes were inoculated by streaking suspensions with a 2 mm dia. nichrome wire loop or by stabbing with a wire dipped in the suspension. Agar plates were inoculated using a 10-point inoculator. Liquid media were inoculated with 1-2 drops of the suspension using a Pasteur pipette.

For pathogenicity tests, inoculum was made from cultures grown for 48h on YPA and suspended in sterile de-ionized water to give 10^8 cfu/ml.

Biochemical and Nutritional Tests

Strains were compared using the methods described by Dhanvantari et al. (1978) for their ability to grow on: adonitol, β-alanine, anthranilate, L-arabinose, benzoate, 1,3-butylene glycol, cellobiose, dextrin, dulcitol, erythritol, glutarate, *m*-inositol, inulin, lactate, laevulinate, malonate, maltose, melezitose, melibiose, DL-norvaline, oxalate, propionate, raffinose, rhamnose, salicin, sorbitol, succinate, sucrose, D(-)tartrate, L(+)tartrate, *m*-tartrate, trehalose, tryptamine, and D-xylose. Results were scored after 7, 14, 21, and 28 days. Studies showed that some strains made limited growth on the basal medium. Comparison of test plates with controls showed that best discrimination between such limited growth and sustained growth was achieved using data recorded at 14 days.

Isolates were examined using the methods of Lelliott et al. (1966) for levan production, oxidase activity, arginine dihydrolase activity, and production of a hypersensitive reaction in tobacco. Pectolytic activity was tested on pectate gels at pH 5 and 8 using the method of Hildebrand (1971). Pitting of gels at pH 5 is caused by the action of polygalacturonase, and at pH8, by pectate lyase.

Strains were tested for the following attributes, using the appropriate methods as referred to: ability to reduce nitrate and nitrite (Dye 1968); production of urease (2 methods: Dye 1968; Cowan & Steel 1965); reactions on purple milk, amylase activity with potato starch and soluble starch, utilization of Tween 80, and growth at 4°C and 41°C, (Dye 1968); ability to liquefy gelatine (Crosse & Garrett 1963); accumulation of poly-β-hydroxybutyrate granules in cultures grown on mKB (Fahy & Hayward 1983). Production of nitrate reductase was tested using the medium of Young (1977), but strains were cultured in the presence and in the absence of nitrate in anaerobic conditions. Growth in the presence, but not in the absence, of nitrate was evidence of nitrate reductase activity.
Results were recorded as '+' or '−' and analysed using centroid linkage to form similarity clusters. Identified clusters were analysed to discover which combination of associated tests uniquely identified each cluster.

DNA Colony Hybridization
Type strains of all species, and all strains of *P. avenae*, *P. cattleyae*, *P. pseudoalcaligenes* subsp. *citrulli*, *P. pseudoalcaligenes* subsp. *konjaci*, and *P. rubrilineans* were examined using a 'dot-blot' colony hybridization method to determine the homology of their DNA to ^{32}P-labelled probes prepared from the type strains of *P. avenae* and *P. rubrilineans*. The methods for nick translation, colony hybridization, and DNA extraction of Maniatis et al. (1982) were used, except that during cell lysis by sodium dodecyl sulphate, the concentration of EDTA was increased to 0.1M to inhibit nuclease activity. A hybridization temperature of 65C° was used.

GC Ratios
The GC ratios of the type strains of *P. avenae* and *P. rubrilineans* were determined by the method of Ulitzer (1972) using DNA prepared from *Escherichia coli* (Migula 1895) Castellani & Chalmers 1919 ICMP 6107 as the standard.

Pathogenicity Tests
Citrullus lanatus (Thunb.) Masum. & Nakai (watermelon) cv. Sundance Mixed, *Cucumis sativus* L. (cucumber) cv. Moneymaker, *Zea mays* L. (maize) cv. 'XP74', and *Z. mays* var. *rugosa* Bonaf. (sweetcorn) cv. Golden Treasure, were grown from seed in sterile potting mix in a glass-house until seedlings had produced 1-2 fully expanded true leaves.
Inoculations of strains of *P. avenae*, *P. cattleyae*, *P. pseudoalcaligenes* subsp. *citrulli*, *P. pseudoalcaligenes* subsp. *konjaci*, and *P. rubrilineans* were made. A hypodermic syringe containing bacterial suspension was used to prick through the leaf-sheath for maize and sweetcorn, and through the lamina of the expanding leaf for cucumber and watermelon.
Maize and sweetcorn were then sprayed to run-off with an atomizer using inoculum diluted to $2x10^7$ cfu/ml in 100ppm Tween 80 which was infiltrated into host tissue by subjecting the plants to a partial vacuum of 35kPa. The expanding leaves of cucumber and watermelon were spray-infiltrated to water-soaking with inoculum using an atomizer.
After inoculation, plants were allowed to dry until leaves had lost all appearance of water-soaking, then transferred to an electronically-controlled mist cabinet in which free surface water was maintained continuously, and held for 24h at 22-24°C. Plants were then returned to a glass-house in which the temperature range was 16-30°C. Pathogenic reactions were recorded 4, 6, and 14 days after inoculation.
All strains of *P. avenae* (13) and *P. rubrilineans* (13) were inoculated into cucumber, maize, and sweetcorn. Subsequently, representative strains of the 2 species were inoculated into sweetcorn, cucumber and watermelon.
All strains of *P. cattleyae* (2), *P. pseudoalcaligenes* subsp. *citrulli* (8), and *P. pseudoalcaligenes* subsp. *konjaci* (3) were inoculated into cucumber, watermelon, and sweetcorn in duplicated experiments.
Isolations from inoculated plants were made from small sectors of tissue from the margins of lesions, comminuted in sterile water. The resultant suspensions were spread on surface-dried agar plates of mKB.

RESULTS

Numerical Analysis of Biochemical and Nutritional Tests

Ten clusters were identified from the dendrogram formed by centroid linkage of data.

Separate clusters containing almost all strains of the respective taxa were formed by *P. andropogonis* (23/23), *P. caryophylli* (5/5), *P. cissicola* (3/4), *P. corrugata* (7/7), *P. glumae* (5/5), *P. plantarii* (6/6), *P. pseudoalcaligenes* subsp. *konjaci* (3/3), and *P. rubrisubalbicans* (9/10). *P. ficuserectae* (3/3) and *P. meliae* (1/1) formed a single cluster. *P. avenae* (12/13), *P. cattleyae* (2/2), *P. pseudoalcaligenes* subsp. *citrulli* (7/8), and *P. rubrilineans* (12/13) formed a single cluster. In all, 5 strains were unclustered. Tests which distinguish the clusters are listed in Table 2. Only tests which identified >90% of strains per cluster are included; the tests listed allow, where possible, differentiation between any pair of clusters using a minimum of 3 tests. Only 2 tests distinguished *P. pseudoalcaligenes* subsp. *konjaci* from the *'P. avenae'* cluster, and *P. andropogonis* from *P. ficuserectae* - *P. meliae*.

DNA Colony Hybridization

All strains of *P. avenae*, *P. cattleyae*, *P. pseudoalcaligenes* subsp. *citrulli*, and *P. rubrilineans* gave a positive reaction to probes prepared from the type strains of *P. avenae* and *P. rubrilineans*, equivalent to the reaction of the homologous strains. Strains of *P. pseudoalcaligenes* subsp. *konjaci* showed partial homology to the probes. No strain of other species hybridized with these probes.

GC Ratios

The GC ratio of a reference sample of DNA from *E. coli* was set at 51%. The GC ratios of the type strains of *P.* avenae and *P. rubrilineans* were calculated to be 65.26% and 64.4%, respectively.

Table 2. Tests which distinguish clusters of non-fluorescent *Pseudomonas* spp.

Cluster*	Tests																				
	glutarate	lactate	L(+)tartrate	benzoate	oxalate	propionate	xylose	dulcitol	adonitol	m-inositol	DL-norvaline	laevulinate	arg. dihyd.	growth 4°C	pept. milk	alk. milk	nit. red.	tween-80	polygalacturonase	pectate-lyase	PHB-acc.
1	+	+	v	−	v	v	+	−	−	−	+	+	−	−	v	−	−	+	−	v	+
2	+	+	+	−	−	v	−	−	−	−	−	v	v	−	+	−	−	+	−	+	+
3	v	+	+	v	v	+	+	−	+	−	v	v	−	−	−	+	−	−	−	−	+
4	−	v	−	v	−	−	−	−	+	v	−	v	−	v	−	+	−	−	v	−	+
5	−	−	−	−	−	−	−	−	−	−	−	−	−	v	−	+	−	v	+	−	−
6	v	+	−	−	−	+	+	+	+	+	−	v	−	−	−	v	−	v	v	+	+
7	−	v	+	−	−	−	+	−	v	+	−	−	+	−	−	+	−	−	−	−	v
8	+	+	v	−	−	+	+	−	−	+	v	v	+	+	v	v	+	v	−	−	+
9	v	+	−	+	v	+	+	−	+	+	−	−	−	−	−	v	+	+	+	−	+
10	−	v	+	−	−	+	+	+	−	+	−	−	−	−	+	−	+	+	+	−	+

*Cluster Name

1 *avenae* – *cattleyae* – *pseudoalcaligenes* subsp. *citrulli* – *rubrilineans*; 2 *pseudoalcaligenes* subsp. *konjaci*; 3 *rubrisubalbicans*; 4 *andropogonis*; 5 *meliae* – *ficuserectae*; 6 *glumae*; 7 *cissicola*; 8 *corrugata*; 9 *caryophylli*; 10 *plantarii*.

\+ positive reaction in strains of a cluster within 14 days

− negative reaction in strains of a cluster within 14 days

v differing reactions between strains of a cluster

Pathogenicity

a. Cucumber and Watermelon

Strains of *P. pseudoalcaligenes* subsp. *citrulli*, *P. avenae*, and *P. rubrilineans* produced similar symptoms on cucumber and watermelon. Pale brown progressive necrosis of the lamina, mostly dry, but occasionally accompanied by water-soaking, was seen after 3 days. Only strains of *P. pseudoalcaligenes* subsp. *citrulli* gave reproducible disease reactions in all experiments. Strains of *P. avenae* and *P. rubrilineans* caused necrosis which was less severe than that caused by *P. pseudoalcaligenes* subsp. *citrulli*. Strains of *P. pseudoalcaligenes* subsp. *konjaci* produced distinctive necrotic lesions in cucumber and watermelon.
Isolations from representative lesions gave rise to unmixed populations of organisms producing characteristic non-fluorescent colonies on mKB.
No symptoms were induced in cucumber or watermelon by strains of *P. cattleyae*.

b. Maize and Sweetcorn

Inoculations of maize and sweetcorn with *P. avenae* or *P. rubrilineans* produced similar symptoms in both hosts. Symptom expression was more severe in sweetcorn than in maize. First symptoms were water-soaked streaks which appeared after 3 days. These increased in length and became brown, desiccated, and necrotic. Length of fully developed lesions varied from a few mm to the full length of the leaf blade. Lesion production was accompanied by extensive chlorosis of the leaf tissue. Strains expressed a range of severity, from those which produced no symptoms, to those which, particularly in sweetcorn, caused the lamina to become entirely necrotic and collapsed. Two (of 8) strains of *P. pseudoalcaligenes* subsp. *citrulli* produced symptoms in sweetcorn similar to those described above, but expression was not as severe as that caused by *P. avenae* or *P. rubrilineans*.
Isolations from representative lesions gave rise to unmixed cultures of organisms producing characteristic non-fluorescent colonies on mKB.
No symptoms were produced in either maize or sweetcorn inoculated with strains of *P. cattleyae* or *P. pseudoalcaligenes* subsp. *konjaci*.

Results are summarized in Table 3.

Table 3. Summary of symptoms produced in cucumber, watermelon, maize, and sweetcorn, inoculated with selected *Pseudomonas* spp.

	Maize	Sweetcorn	Cucumber	Watermelon
P. avenae	+	+	v	v
P. rubrilineans	+	+	v	v
P. p. citrulli	*	v	+	+
P. p. konjaci	*	v	v	v
P. cattleyae	*	−	−	−

\+ reproducible progressive severe necrotic reaction
− no reaction
v variable reaction for limited numbers of strains
* not tested

DISCUSSION

Increasingly, classification of bacterial species is considered to be on the basis of DNA-DNA homologies (Wayne et al. 1987). So far, relatively few species within *Pseudomonas* Migula 1894, have been established using this criterion. Even when all species are so established, the requirement for laboratory determination of phenotypic characters for the identification of species will remain.

Although the non-fluorescent plant pathogenic *Pseudomonas* spp. have been commonly considered as a single group, they are not homogeneous. Analysis of rRNA-DNA homologies has shown that they fall into several distinct genetic groups (Palleroni 1984) which could be considered to be separate genera. So far, the inability to characterize these groups except by genetic means has inhibited their formal classification.

Numerical analysis of biochemical and nutritional reactions of the non-fluorescent plant pathogenic *Pseudomonas* spp. in this study indicated that strains of most species form clusters. Thus, strains of *P. andropogonis*, *P. caryophylli*, *P. cissicola*, *P. corrugata*, *P. glumae*, *P. plantarii*, *P. pseudoalcaligenes* subsp. *konjaci*, and *P. rubrisubalbicans* formed separate clusters comprising almost all strains of each species.

Strains of *P. ficuserectae* and *P. meliae* collectively formed a single cluster. However, only three strains of *P. ficuserectae* and one strain of *P. meliae* were available for study and their status as a single taxon must be regarded as provisional.

Most strains of *P. avenae*, *P. cattleyae*, *P. pseudoalcaligenes* subsp. *citrulli*, and *P. rubrilineans* formed a single cluster comprising 33 of 36 strains. DNA hybridization using probes to the type strains of *P. avenae* and *P. rubrilineans* indicated that, irrespective of whether they had clustered or not, all strains of these species also showed a higher level of DNA homology than did the strains of other species. An unpublished study using genomic probes to pathovars of *P. syringae* van Hall 1902 indicates that positive reactions occur if DNA homologies are greater than 40-50%. *P. pseudoalcaligenes* subsp. *konjaci* also showed partial homology with the types of *P. avenae* and *P. rubrilineans*.

The value calculated for the GC ratio of *P. avenae*, 65%, is close to that calculated here for *P. rubrilineans*, 64%, and close to the values of 66% for *P. pseudoalcaligenes* subsp. *citrulli* (Schaad et al. 1978; Goto 1983), and of 66-68% for *P. pseudoalcaligenes* subsp. *konjaci* (Goto 1983).

The indistinguishable pathogenic reactions to strains of *P. avenae* or *P. rubrilineans* in maize and sweetcorn confirms the synonymy of these pathogens (Claflin & Ramundo 1987).

The progressive disease caused by some strains of *P. avenae* and *P. rubrilineans* in cucumber and watermelon, and of *P. pseudoalcaligenes* subsp. *citrulli* in sweetcorn suggests that these are homologous reactions. In the absence of inoculum dose response data it is not possible to determine if the differences in pathogenicity are attributable to the natural variation in virulence between strains of a single pathogen, or to the existence of host specific pathogenic variants of *P. avenae*.

P. avenae, *P. cattleyae*, *P. pseudoalcaligenes* subsp. *citrulli*, and *P. rubrilineans* form a cluster and have a relatively high level of DNA homology when examined using genomic DNA probes. The evidence presented indicates that these species are synonymous; *P. cattleyae*, *P. pseudoalcaligenes* subsp. *citrulli*, and *P. rubrilineans* being synonyms of *P. avenae*.

P. pseudoalcaligenes subsp. *konjaci* showed some DNA homology with *P. avenae* and is similar in its biochemical and nutritional reactions. It is unclear whether this taxon should be retained as a separate species.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

BACTERIAL LEAF SPOT OF CHERRY LAUREL: VARIABILITY AND TAXONOMY OF P.S. PV. SYRINGAE ISOLATED FROM LESIONS AND LEAF SURFACE

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Bacterial leaf spot of cherry laurel was described initially in Canada (De Boer, 1980). In France, this disease was observed first in 1976 (Gardan et al., 1989). It occurs mainly on young plants during propagation in containers and in the field.

The most important symptoms are : leaf spot, leaf hole, necrosis and cankers on herbaceous shoots. Damage is sometimes very severe, since up to 40% of diseased plants are not marketable. Cultivar Caucasica is more susceptible than are cultivars Otto Luyken and Rotundifolia which are used extensively in France.

From 1976 to 1988, 241 strains were isolated from lesions and leaf surface of cherry laurel and identified as P.s. pv. syringae. We present in this paper the variability and taxonomy of strains of P.s. pv. syringae isolated from cherry laurel in comparison with strains of P.s. pv. syringae isolated from other host-plants, pathovars of P. syringae and some other plant pathogenic fluorescent Pseudomonas.

MATERIAL AND METHODS

Strains :

- P.s. pv. syringae : 50 representative strains isolated from cherry laurel (lesions and leaf surface) selected from a collection of 241 strains - 58 strains isolated from 1950 to 1986 from various host-plants classified in 31 genera.

- 53 strains of type strains of pathovars of P. syringae and species of plant pathogenic Pseudomonas.

Biochemical tests : we used 20 classic biochemical tests performed in tubes or in Petri dishes and three sets of 49 microtests each, corresponding to carbohydrates (50 CH), organic acids (50 AO) and amino-acids (50 AA) prepared by Api System SA, France. Growth was observed up to 6 days.

Numerical taxonomy : the number of characters studied was 167. The results of biochemical and physiological tests were coded 1 (positive) or 0 (negative). Similarity matrices were computed using the Jaccard and Sneath coefficient (Veron, 1982). Strains were clustered using unweighted pair group method with averages (UPGMA) (Veron, 1982). Computation was made on a 3090 IMB computer and a program written in Cobol.

RESULTS

- variability of 50 strains of P.s. pv. syringae isolated from cherry laurel in comparison with type strain of P.s. pv. syringae.

At the distance 0.0819 there were 6 groups plus ten isolated phenotypes which were differentiated by assimilation of malonate, glucosamine, DL-5 aminovalerate, L-cysteine, O-OH-benzoate, sarcosine, D-lyxose, propionate and N-caproate.

One strain (U21-1) and the type strain of P.s. pv. syringae (C.F.B.P.* n° 1392) were very far from all the other strains.

- variability of strains of P.s. pv. syringae (50 strains isolated from cherry laurel and 58 strains isolated from various host-plants mixed together).

On the basis of Hildebrand characters the 108 strains are distributed among 7 biotypes. Biotype 3 is predominant since it grouped 45 strains from cherry laurel and 37 strains from various hosts. The other biotypes were insignificant since they had less than 6 strains each. The type strain of P.s. pv. syringae was assigned in biotype 2 (Table 1).

Fig. 1 showed the hierarchical classification of the 161 strains.

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*C.F.B.P. = Collection Française des Bactéries Phytopathogènes - Angers - France

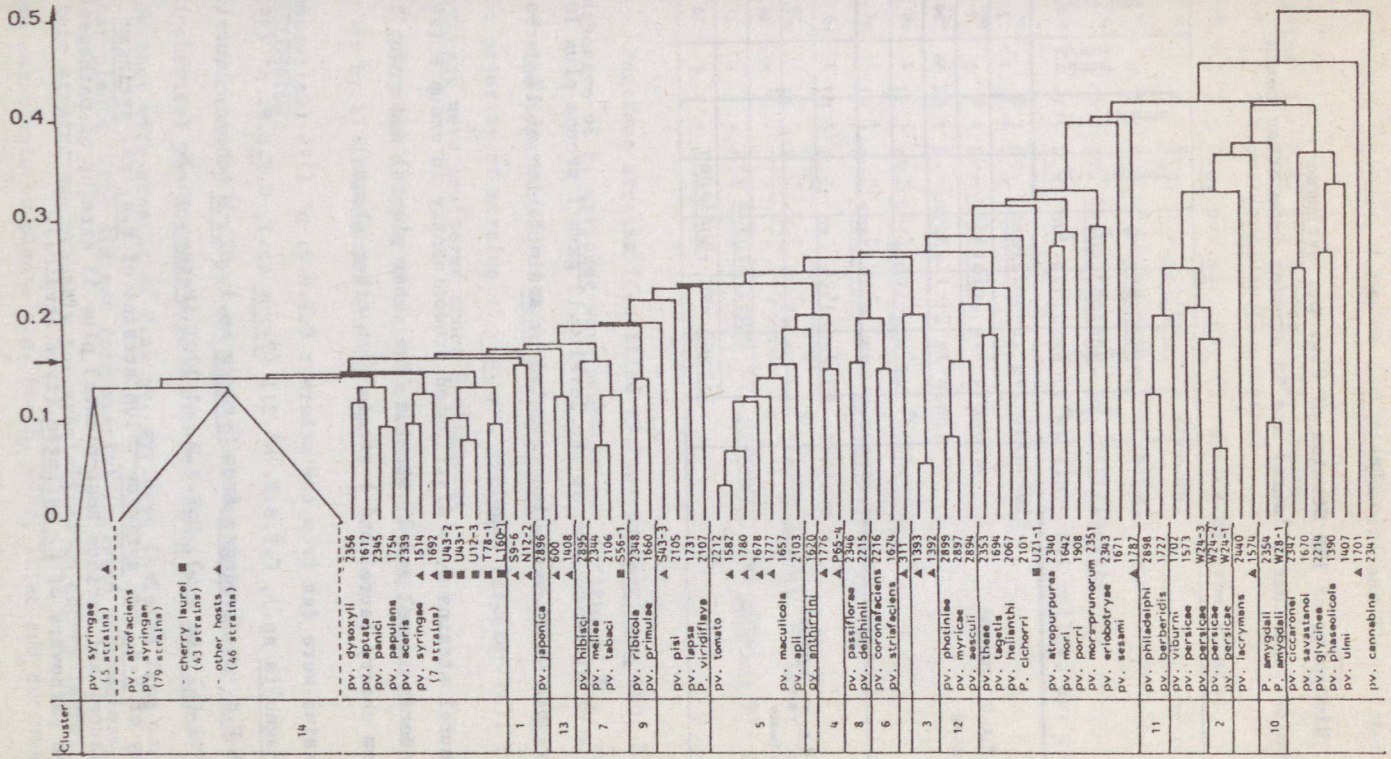


Figure 1

DENDROGRAM OF SIMILARITY BASED ON THE DISTANCE OF THE 161 STRAINS OF P.S. PV. SYRINGAE PATHOVARS OF P. SYRINGAE AND PATHOGENIC FLUORESCENT PSEUDOMONAS

(= P.s. pv. syringae from cherry laurel, = P.s. pv. syringae from other hosts)

Table 1

Biotyping of 108 Strains of P.s. pv. syringae : 50 strains from cherry laurel and 58 strains from other hosts

	Biotype	Sorbitol	Erythritol	DL Lactate	L(+) Tartrate	D(-) Tartrate	Number of strains	Percentage
50 strains of P.s. pv. syringae from Cherry laurel	1	+	+	-	-	+	1	2
	2	+	+	+	-	-	1	2
	3	+	+	+	-	+	45	90
	4	+	+	+	+	+	2	4
	5	-	-	+	-	+	1	2
58 strains of P.s. pv. syringae from different hosts	1	+	+	-	-	+	3	5
	2	+	+	+	-	-	11	19
	3	+	+	+	-	+	37	64
	6	+	-	+	-	+	1	2
	7	+	-	-	-	+	6	10

Dendrogram of distances cut at the level 0.13 gave 7 groups plus 10 isolated phenotypes which were discriminated by assimilation of 17 carbon substrates.

Cherry laurel strains were distributed predominantly in group 6 (44 strains from cherry laurel and 25 strains from other plants) and group 2 (5 strains from cherry laurel and 1 strain from other plants).

Seven strains were far from the others : C.F.B.P. n° 1776 (Calistemon sp.), P65-4 (Magnolia sp.), C.F.B.P. n° 311 (Pyrus sp.), C.F.B.P. n° 1392 (type strain of P.s. pv. syringae from Syringa sp.), U21-1 (cherry laurel), C.F.B.P. n° 1787 (Allium sp.), C.F.B.P. n° 1701 (Persea sp.).

- taxonomy of P.s. pv. syringae : 108 strains of P.s. pv. syringae (from cherry laurel and various host-plants) plus 53 strains of pathogenic Pseudomonas and pathovars of P. syringae (type strains).

At the distance 0.158 we had 14 clusters and 31 isolated phenotypes :

cluster 1 : P.s. pv. syringae (2 strains)
" 2 : pv. persicae (2 strains)
" 3 : P.s. pv. syringae (2 replica of type strain)
" 4 : P.s. pv. syringae (2 strains)
" 5 : P.s. pv. syringae (4 strains)
" 6 : pv. coronofaciens, pv. striafaciens
" 7 : P.s. pv. syringae (1 strain), pv. mellea, pv. tabaci
" 8 : pv. passiflorae, pv. delphinii
" 9 : pv. ribicola, pv. primulae
" 10 : P. amygdali (2 strains)
" 11 : pv. philadelphi, pv. berberidis
" 12 : pv. photiniae, pv. myricae, pv. aesculi
" 13 : P.s. pv. syringae (2 strains)
" 14 : P.s. pv. syringae (48 cherry laurel, 43 other host- plants), pv. atrofaciens, pv. dysoxyli, pv. aptata, pv. panici , pv. papulans, pv. aceris

Fourteen strains identified as P.s. pv. syringae that lie among pathovars of P. syringae were probably not strains of P.s. pv. syringae.

The type strain of P.s. pv. syringae was not in the same cluster as the majority of strains of P.s. pv. syringae (cluster 14). Pathovars of P. syringae were scattered among 9 clusters.

Strains of each of the 14 clusters were differentiated from each other by 22 biochemical characters.

DISCUSSION

Strains of P.s. pv. syringae isolated from cherry laurel had variable biochemical characters, were scattered along the dendrogram and mixed together with strains of P.s. pv. syringae isolated from various host-plants. They did not form special isolated subclusters. Moreover, these strains are probably not specialized to cherry laurel since, some are pathogenic on plants e.g. peach, pear, lilac and cucumber.

Cluster 14 regrouped the majority of strains of P.s. pv. syringae isolated from cherry laurel and other host-plants and 6 pathovars of P. syringae (pv. atrofaciens, pv. dysoxyli, pv. aptata, pv. panici, pv. papulans, pv. aceris). The member of this cluster could be considered as P.s. pv. syringae. However the type strain of P.s. pv. syringae isolated from lilac is distinct from this cluster and our results suggest that a new type strain should be chosen and perhaps the pathovar should be renamed. The boundary of the cluster might be estimated more precisely by DNA/DNA hybridization.

In earlier work (Gardan et al., 1987) we demonstrated clearly that P.s. pv. savastanoi is a species and not a pathovar of P. syringae since for the former level of hybridization with type strain of P.s. pv. syringae is only about 43% and the reciprocal level of hybridization of type strain of P.s. pv. syringae with P.s. pv. savastanoi is about 60 % (Gardan et al., 1987). Misaghi and Grogan (1969), Sands et al. (1970) and Pecknold and Grogan (1973) provided the evident basis for the taxonomy of fluorescent pathogenic Pseudomonads. However many new pathovars of P. syringae or new species have been described since their work and new technics of DNA/DNA hybridization have been published. Perhaps, the taxonomy of this group should be revised completely.

When DNA/DNA hybridization groups are clearly defined, we might revise the notion of pathovar (Young et al., 1978) to preserve it for strains pathogenic on a specific host-plant. As a matter of fact it is possible to verify cross pathogenicity on several plants (less than 10) corresponding to each group of DNA/DNA hybridization, but not on a great number of plant-hosts.

ACKNOWLEDGMENTS

We thank S.V. Beer and J.P. Paulin for advice and reviewing the manuscript.

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Proc. 7th Int. Conf. Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

OCCURRENCE AND IMPORTANCE OF BACTERIAL STEM PITH NECROSIS OF TOMATO IN THE GDR

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INTRODUCTION

In 1978 Scarlett et al. described a new bacterial species, which is able to induce stem pith necrosis in mature tomato plants. Such symptoms have been observed in United Kingdom since 1955. For this pathogen belonging to the genus Pseudomonas the name Pseudomonas corrugata Roberts & Scarlett was validated. In the same year, 1978 we found wilting tomato plants showing the characteristic symptoms of this bacteriosis in a glasshouse holding nearby Halle/GDR.

Following this the disease had also been discovered in at least 8 further European countries. However, in some cases other bacterial species were identified as causal agents of this syndrome. Already Wilkie & Dye (1974) had reported on a similar disease caused by Ps. cichorii in New Zealand. Later on, in different countries Erwinia chrysanthemi /1,6,7/, Ps. viridiflava /2,3,6/, Ps. fluorescens and an unidentified Pseudomonas species /5/ could also be proved as stem pith necrosis-inducing organisms.

During the last years the stem pith necrosis spread in the GDR and was notified from an increasing number of districts. Simultaneously, the losses caused by this bacteriosis grew. For these reasons we have tried to clarify

- which pathogens are responsible for this disease in the GDR

- and how the causal agent is transmitted and can attack glasshouse crops especially under hydroponic conditions.

RESULTS AND DISCUSSION

Thus, since 1978 tomato plants with suspect symptoms from different parts of the country were analysed with regard to bacterial pathogens. In this way we were able to isolate more than 100 stem pith necrosis-inducing bacterial strains from samples out of 8 districts. That means, that this bacteriosis occurs in more than half of the districts of the GDR.

From these pathogenic isolates 25 were selected for identification. Nearly 30 properties were determined. For comparison

- the type strain Pseudomonas corrugata NCPPB 2445 and
- reference strains of Ps. cepacia, Ps. gladioli pv. alliicola and Ps. cichorii

have been tested.

In their morphological and physiological characters 20 of the 25 isolates under test extensively agreed with the type strain of Ps. corrugata, had additional properties described for this pathogen or showed divergences only in one or two characteristics.

Corresponding to this result, 11 of the isolates under test gave positive reactions using ELISA with 3 specific antisera that have been produced against Ps. corrugata.[x)] From the tests it can also be concluded that 3 or more serogroups of Ps. corrugata may exist.

A comparison of the protein pattern using polyacryl amide gel electrophoresis technique also showed a good agreement between the type strain and the majority of the isolates tested.[xx)]

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x) For the serological analysis we have to thank Dr. R. Zielke, Aschersleben,

xx) For the protein profile analysis we thank Mr. L. Plobner, Aschersleben.

The 5 isolates up to now not completely identified also belong to the genus Pseudomonas. Three of them even reacted positively with antisera against Ps. corrugata. However, two of them also showed similarities with Ps. fluorescens.

These results suggest that this disease in the GDR is caused first of all by Ps. corrugata. However, it could also be confirmed, that some phenotypical properties of the isolates under test vary considerably, for example pigmentation and the morphology of the colony surface. This also proved right especially for their pathogenicity, as the following table demonstrates (Table 1). So, the type strain NCPPB 2445 caused an average necrosis length in tomato stems of 4.1 cm ranging from 6.5 to 1.0 cm dependent on each single test plant inoculated.

Table 1: Virulence of some stem pith necrosis-inducing isolates selected on different test plants
(Length of stem pith necrosis (cm))

	Test plants				
	Tomato			Egg plant	Dat. stram.
Isolates	$\bar{x}$	max.	min.	$\bar{x}$	$\bar{x}$
Type strain NCPPB 2445	4.1	6.5	1.0	0.8	1.5
I V 40	3.4	7.4	0.5	0.2	2.6
I V 51	2.4	10.0	0	0.2	4.3
I V 70	1.6	3.0	0	0.3	1.1
I V 82	3.1	6.5	0	0.6	3.9
Da-do 3	3.7	8.0	0.5	0	4.1
Pc 1	2.6	5.5	0.5	0.5	3.9
Pc 32	5.1	14.0	0.5	0.5	3.8

Taking the average values as a basis, most of our isolates have not been so virulent, however isolate Pc 32 induced a necrosis length of more than 5 cm on an average varying from 14.0 to 0.5 cm. These findings are in keeping with the valuation of Scarlett et al. (1978), about which Ps. corrugata belongs to the under-grade pathogens.

Further on, the conditions necessary for causing this disease

were studied. At first the survival of a Ps. corrugata strain labelled by streptomycin resistance (400 ppm) in fluid substrates to be used in modern hydrophonic systems was tested. From these experiments it can be concluded, that Ps. corrugata is able to survive more than 6 months in such nutrient solutions without essential decrease of the cell concentration added before. Thus this substrate can continuously act as an infection source for the whole cultivation period.

By contrast, transmission of the disease by seeds is very improbable. All efforts to isolate the pathogen from fruits or seeds produced by infected plants failed.

Further on, different possibilities given for infection of tomato stems by Ps. corrugata should be checked. For this end the following plant organs and places were inoculated:

- intact and shortened roots
- wounds originated from pruning sprouts
- wounds produced by detaching leaves and
- inflorescences.

The results obtained demonstrate, that the pathogen as a rule cannot attack its host via roots or flowers. The entrance into the stem above all succeeded via pruning wounds (Table 2).

Table 2: Symptoms produced by a stem pith necrosis-inducing strain after inoculation of different plant organs

place	Inoculation method	Portion of plants with symptoms (%)
roots	submerging	0
stem wounds caused by pruning	spreading	53
stem wounds produced by detaching leaves	spreading	33
inflorescences	submerging	6

As a further question the influence of different environmental circumstances on the occurrence and intensity of stem pith necrosis was studied. Surprisingly, in model experiments with tomato seedlings different temperatures ranging fom 18

to 24 °C and air humidity conditions regulated by over-head irrigation or cultivation in moist chambers did not influence the symptom development and strength.

However, under conditions of practical production always a higher incidence of this bacteriosis was observed during the spring cultivation period than in autumn. It can be assumed that this fact may be a consequence of the lower temperature level existing during the early stages of plant growth in springtime.

Under these circumstances higher nitrogen amendments (2.5 g N per plant) remarkably increased the development of stem pith necrosis symptoms.

SUMMARY

Summarizing the results the following can be stated:

1. Tomato stem pith necrosis is a frequent and in several districts occurring disease in the GDR.
2. Up to now only Pseudomonas corrugata could be surely identified as causal agent.
3. The various isolates of this pathogen partly show remarkable differences in their phenotypical and pathological properties.
4. Under glasshouse conditions Ps. corrugata can survive more than 6 months in nutrient solutions used in hydroponic systems.
5. An inoculation of the stem succeeded above all via pruning wounds.
6. Under conditions of practical tomato production the disease especially develops during the spring cultivation period, which is characterized by a lower temperature level than the autumn period. Fertilizing with high nitrogen doses favours the symptom formation.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

HETEROGENEITY OF VIRULENCE IN NATURAL POPULATIONS OF ERWINIA AMYLOVORA

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Erwinia amylovora is known to be a rather stable bacterial species: strains of different origins (host plant or geographic) are usually very similar when compared on the basis of physiological or serological tests in the laboratory. Nevertheless, few differences between strains can be noticed. These refer to pathogenicity, resistance to antibiotics, plasmids profile or capsulation.

Differences in pathogenicity, between strains, are described, with "differential virulence" of some strains v.s. certain resistant apple cultivars (Norelli, 1986). Another kind of distinction between strains is the amount of disease produced by inoculation on host plants: avirulent strains, weakly virulent and strongly virulent strains can be described. Such strains can be found in type culture collections. We have tried to study the origin of such naturally occurring avirulent strains. It is probably useful to precisely define two words used during the course of this work.

We call STRAIN a pure culture of a bacterial species (here: Erwinia amylovora). It originates usually from a purification after isolation from a lesion caused by the bacteria: one colony described as "typical" has been selected and subcultured. Its identity to E. amylovora has been checked and this culture is then considered to be a representative of the species E. amylovora.

We call POPULATION the collection of bacterial cells which is isolated from a lesion (here: fire blight) and which belongs to the pathogenic species (here: E. amylovora). A picture of this population is given by a sample of several colonies issued from the same lesion. We call ISOLATE the subculture of such a colony. A group of a sufficient number of isolates is a representative of the population of the lesion.

We have analysed the virulence of isolates from populations of E. amylovora of different origins.

MATERIAL - METHODS

The only original technique which was used was the sampling method of colonies isolated from lesions, and the associated pathogenicity test.

From each lesion, the following successive stages had been followed.

- First step

Isolation by grounding in sterile distilled water, dilution and plating (KB, 26°) of a known volume of the suspension. After incubation, 96 separate colonies were suspended in sterile distilled water (1 ml), and one drop of each of these suspensions was inoculated by deposit on pieces of apple fruits in suitable plastic plates. The suspension was then deep-frozen for the second step.

After incubation (4 days 17°C in the dark) the production of exudate on apple slices was noted. The corresponding bacterial suspension was discarded as "virulent". The "negative" suspensions were kept for further analysis.

- Second step

The suspensions unable to produce exudates were collected from the deep freezer and inoculated on actively growing pear or apple seedlings in the greenhouse, as well as infiltrated on tobacco leaves.

The isolates whose pathogenicity was negative on seedlings, after repeated inoculation (3 times) were kept as avirulent isolates.

RESULTS

- Avirulent colonies, after the first step of analysis

Most of the colonies produced exudate on apple pieces. Nevertheless, for each type of symptom, a certain proportion of the population (up to 20% in canker) did not produce exudate. The total number of negative isolates at this stage was 171, originating from 18 cankers and 30 necroses on old parts.

- Avirulent isolates, after the second step

When checked on seedlings a certain proportion of previously "avirulent" colonies gave rise to typical symptoms. The total number of avirulent isolates was finally 77.

- Repartition of the avirulent isolates

Avirulent isolates could be found in a proportion of the total population of the lesion which ranging between 0 and 2.5%. This proportion was usually higher in cankers (sampled in winter).

DISCUSSION

Our technique of analysis of bacterial population of lesions allowed pointing out a certain level of heterogeneity among cells of E. amylovora present in these lesions.

The first step is to analysis (relying only on a single inoculation on an apple piece) the number of so-called avirulent isolates, because the test may not allow the production of exudate by a virulent isolate for diverse reasons (dry surface of the piece of fruit inoculated, unsuitable deposit of the drop of bacteria ... etc.). Nevertheless, it revealed as well a certain level of heterogeneity. It is likely that, among the isolates discarded at the second step (inoculation on seedlings) some were actually of moderate pathogenicity. Our technique of screening did not allow the selection of such isolates (except very weakly pathogenic strains).

The avirulent components of bacterial population were more frequent in symptoms sampled in winter that is during the dormant period (cankers and necrosis on woody parts). This could mean that the exudate produced when cankers and other dormant lesions are reactivated in spring could contain a mixture of avirulent and virulent cells. We have checked that avirulent cells may multiply in plant tissues, even when virulent cells are present (data not shown).

These avirulent components of the population of a lesion could then play a role in epidemiology. For example, their presence in a mixed inoculum with virulent cells could decrease the severity of infections, as it can be shown experimentally.

The origin of naturally occurring avirulent strains which can be collected in type culture collection is probably linked with the heterogeneity of virulence as seen here. When selecting one colony for pure culture it is possible — at least occasionally — to choose by chance one of the avirulent colonies of the population of the pathogen.

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

EVALUATION OF CELLULAR FATTY ACID PROFILES FOR RAPID IDENTIFICATION OF PLANT PATHOGENIC BACTERIA

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ABSTRACT

Cellular fatty acid profiles are presented for many plant pathogenic bacteria representing *Agrobacterium*, *Clavibacter*, *Curtobacterium*, *Erwinia*, *Pseudomonas* (rRNA groups, 1,2,3), *Rhodococcus*, *Xanthomonas* and *Xylophilus*. Most of these groups have unique profiles usually characterised by specific types of fatty acid, eg. branched or hydroxy acids. At generic level differences were usually qualitative but differences between pathovars, biovars, subspecies and closely related species were usually quantitative. Some but not all pathovars of *Pseudomonas syringae* and *Xanthomonas campestris* have unique profiles. Results of a computer-assisted comparison of profiles of a wide range of strains from the National Collection of Plant Pathogenic Bacteria (NCPPB) with a commercially available library further demonstrate the value of this technique in rapid, accurate diagnosis of bacterial diseases of plants.

INTRODUCTION

Whole cell fatty acid (FA) profiles have been used to identify bacteria for many years [1,2,3] but good reproducibility of results especially for profiles of Gram negative bacteria which contain polar hydroxy FAs was greatly improved by the fairly recent development of capillary columns. There are very few papers in the literature which use FA profiles to identify plant pathogenic bacteria. [4,5,6,7,8,9,10] This paper investigates the FA profiles of a wide range of plant pathogenic bacteria from the NCPPB (610 strains) and compares the profiles with a commercially available FA library containing approximately 300 species of aerobic bacteria. Only those taxa included in the library are compared.

MATERIALS AND METHODS

The major taxa included are listed in Tables 1, 2 and 3. A total of 610 strains representing 43 taxa were profiled. Cultures were grown on trypticase soy agar for 24 hours at 28°C except for *Xylophilus ampelinus* (6 days). Approximately 50 mg fresh weight of cells was harvested and the fatty acid methyl esters (FAMEs) derived and profiled as previously described [9,10,11]. The system used an automated GC system with autosampler, integrator, printer and computer. FAMEs were quantified and identified and profiles compared with a commercially available library using software obtained from Microbial ID, Inc. Newark, Delaware, USA.

TABLE 1. PRINCIPLE FATTY ACIDS IN PROFILES OF A SELECTION OF GRAM NEGATIVE PLANT PATHOGENIC BACTERIA

Range of % fatty acid in profile

	Agrobacterium biovars	Erwinia amylovora	carotovora	chrysanthemi	Pseudomonas syringae pv. syringae	corrugata	rubrisubalbicans	andropogonis	gladioli	solanacearum	avenae	Xylophilus ampelinus
8:0 3 OH												0- 2
10:0 3 OH					1- 4	2- 8	1- 3				2- 5	
12:0 2 OH					1- 5	1- 7	2- 5					
12:0 3 OH					1- 6	1- 8						
14:0 2 OH							2- 5					
14:0 3 OH	3-10	5-10	6-10	5-10		1- 4		3- 5	3- 6	5-10		
16:1 2 OH								1- 3	1- 3	0- 5		
16:0 2 OH								3- 6	1- 3	0- 2		
16:0 3 OH	2- 4							2- 5	4- 6			
18:1 2 OH								1- 3	1- 3	2- 6		
12:0		4- 6	4- 8		2- 8	2- 7	1- 4				2- 5	
14:0		4- 6	1- 3	5-12				4- 5	3- 5	3- 6	0- 3	2- 5
16:1 CIS 9	2- 6	15-35	30-40	20-35	30-40	20-35	30-40	15-20	10-25	25-35	35-45	40-50
16:0	5-10	30-40	20-35	25-35	20-30	22-32	20-30	15-20	15-35	20-35	30-40	20-35
17:0 CYCLO		2-15			0- 7	0-12	0- 2	1- 7	2-25	1-10		
18:1 CIS 11	60-80	5-12	5-25	10-25	20-25	10-30	20-30	25-40	15-35	10-25	15-20	15-30
19:0 CYCLO 11-12	1- 6							5-15	0-20			

TABLE 2. PRINCIPLE FATTY ACIDS IN PROFILES OF A SELECTION OF GRAM NEGATIVE AND GRAM POSITIVE PLANT PATHOGENIC BACTERIA

Range of % fatty acid in profile

	Rhodococcus fascians	Clavibacter michiganensis subsp. michiganensis	subsp. sepedonicus	Curtobacterium flaccumfaciens pv. flaccumfaciens	Xanthomonas albilineans	Xanthomonas maltophilia	Xanthomonas populi	Xanthomonas campestris pv. campestris	pv. hyacinthi	pv. manihotis	pv. oryzae	pv. pelargonii
12:0 3 OH					1- 3	1- 5	1- 3	2- 6	1- 3	1- 3	2- 5	2- 5
11:0 ISO 3 OH					1- 5	1- 5	1- 3	3- 4	1- 3	1- 3	1- 3	2- 3
13:0 ISO 3 OH					2- 4	1- 6	2- 4	2- 6	1- 3	3- 6	2- 5	2- 4
14:0	4-10					1- 6	3- 6	1- 5		1- 2		1- 5
15:0	5-20						4- 6					
16:1 B						1- 5	10-20	1- 4		1- 2	2- 4	1- 3
16:1 CIS 9					5-20	8-15		12-25	10-15	15-20	15-30	20-30
16:0	15-30	3- 8	1- 9	0- 2	7-20	3-10	5-10	2-10	3-6	2- 8	10-30	2- 8
17:1 B	1-10						2- 5					
18:1 CIS 9	5-35				0- 5	1- 4	6-10				1- 5	
18:1 CIS 11				0-10	1- 5	1- 4					1- 4	
11:0 ISO					3- 8	1- 5	3- 5	3- 6	2- 5	3- 5	2- 4	3- 5
14:0 ISO								1- 7				
15:1 ANTEISO A		1-5	1-15									
15:0 ISO		0-5	0- 2	1-12	0- 3	30-45	28-40	20-35	5-25	20-30	1- 7	20-35
15:0 ANTEISO		45-55	35-50	45-55		5-20	1- 3	5-20	20-40	10-15	0- 2	10-20
16:0 ISO		5-15	5-17	4-13	1-10	1- 5		1- 3	5-15	1- 5		1- 2
17:1 ISO F					25-35	2- 8		1-12	1- 5	5-10	5-10	2- 5
17:0 ISO			0- 2	0- 3	15-25	1-10	3- 6	3-10	1- 8	5-10	10-20	3- 5
17:0 ANTEISO		27-35	27-37	20-40	1- 5				1- 2	0- 2		
10 Me 18:0	1-13											

TABLE 3. IDENTIFICATION OF PLANT PATHOGENIC BACTERIA BY COMPARISON OF FATTY ACID WITH LIBRARY OF PROFILES

Group/species	% strains correctly identified (first choice) at specific or infraspecific rank
Agrobacterium	biovar 1 50 (100) [1] biovar 2 100 biovar 3 100
Clavibacter	*michiganensis* 100
C. michiganensis	subsp. *michiganensis* 19 (100) subsp. *sepedonicus* 80 (100)
Curtobacterium	*flaccumfaciens* 94 (100)
C. flaccumfaciens	pv. *flaccumfaciens* 75 (100) pv. *poinsettiae* 77 (92)
Erwinia	*amylovora* 85 (100) *herbicola* 57 (100)
	carotovora 100 *chrysanthemi* 100
E. carotovora	subsp. *carotovora* 83 (100) subsp. *atroseptica* 90 (100) subsp. *betavasculorum* 100
Pseudomonas	*agarici* 44 (78) *cichorii* 44 (78) *corrugata* 100
	rubrisubalbicans 100 *syringae* 98 (100) *viridiflava* 67 (100)
	andropogonis 91 *cepacia* 56 (78) *caryophylli* 100 *gladioli* 71(81) *solanacearum* 96 (100) *rubrilineans* 83
P. syringae	pv. *syringae* 0 (8) pv. *lachrymans* 8 (23) pv. *maculicola* 33 (79)
	pv. *morsprunorum* 10(30) pv. *phaseolicola* 67 (100) pv. *pisi* 5 (45)
	pv. *savastanoi* (ash) 18 (46) pv. *tomato* 14
Rhodococcus	*fascians* 100
Xanthomonas	*albilineans* 100 *campestris* 99 (100) *maltophilia* 100
X. campestris	pv. *campestris* 10 (38) pv. *hyacinthi* 100 pv. *manihotis* 88 pv. *pelargonii* 88 (94) pv. *phaseoli* 17 (42) pv. *prunt* 29 (100)

RESULTS & DISCUSSION

Investigation of FA profiles

Summary profiles are presented in Table 1 (*Agrobacterium*, *Erwinia*, *Pseudomonas* and *Xylophilus*) and Table 2 (*Xanthomonas*, *Rhodococcus*, *Clavibacter* and *Curtobacterium*). Only FAs of major diagnostic value are included.

Comparison of profiles with library

The numbers of correct first choice identifications for different taxa are given in Table 3. Figures in brackets indicate that the correct identification was given among the first three choices in a list of reference taxa.

Agrobacterium. The three biovars had fairly discrete profiles which allowed excellent identification especially of biovars 2 and 3. Some biovar 1 strains were mis-identified as biovar 3. At present specific nomenclature is based on plasmid governed virulence eg tumefaciens, rhizogenes. Since plasmids do not influence profiles, identification at specific rank is not possible. Agrobacteria have large amounts of 18:1 cis 11. All contain 14:0 3 OH and 16:0 3 OH.

Erwinia. All strains contain 14:0 3 OH. Most species could be differentiated. This technique provided excellent differentiation between *E. carotovora* subsp. *atroseptica*, E.c. subsp. *carotovora* and *E. chrysanthemi*.

Pseudomonas. On the basis of hydroxyacids, 3 discrete groups were found. These coincided with the rRNA groups of Palleroni [12] and De Vos *et al.* [13]. Group 1 contained the fluorescent species *P. agarici*, *P. cichorii*, *P. marginalis*, *P. viridiflava* and *P. syringae*. It also contained *P. corrugata* and *P. rubrisubalbicans*. All contained 10:0 3 OH and 12:0 2 OH. Some of the species had discrete profiles. With others including *P. viridiflava* and many pathovars of *P. syringae*, there was overlap. However some pathovars were identified quite accurately by the Microbial ID library eg pv. *phaseolicola* and pv. *maculicola*.
The second group all contained 14:0 3 OH. This group included *P. andropogonis*, *P. cepacia*, *P. caryophylli*, *P. gladioli* and *P. solanacearum*. All had discrete profiles from which accurate

identification could be made although there was some overlap between *P. cepacia* and *P. gladioli* strains.
The third group contained *P. avenae* and *P. rubrilineans*. Their profiles were very similar to each other and to those of *Commamonas (Pseudomonas) acidovorans*. The only hydroxy acid occurring in all three was 10:0 3 OH.

Xanthomonas. These profiles were very different from any of the other Gram negative plant pathogens. Table 2 shows that all species tested had discrete profiles containing many branched acids. All strains also contained 1 .:0 3 OH, 11:0 ISO 3 OH and 13:0 ISO 3 OH. Within X. *campestris* many pathovars eg. pv. *hyacinthi*, pv. *pelargonii*, pv. *manihotis* and pv. *oryzae* had unique profiles but pv. *campestris* profiles overlapped with profiles of other pathovars eg pv. *armoraciae*, pv. *vesicatoria* and pv. *dieffenbachiae*. Other pathovars or groups of pathovars not covered in this study but known to have unique profiles are the "translucens" group and the "graminis" group [10]. Many of these differences were qualitative as well as quantitative (Table 2).

Clavibacter and *Curtobacterium*. These had simple profiles containing mainly branched acids. No hydroxy acids were found. Profiles were similar but *Curtobacterium* strains did not contain 15:1 anteiso A whereas *Clavibacter* strains did. There were some differences between *Curtobacterium flaccumfaciens* pathovars and *Clavibacter michiganensis* subspecies but the variation within profiles did not always allow accurate infraspecific identification. Comparison of ratios of some of these acids may facilitate differentiation.

Rhodococcus. *Rhodococcus fascians* had a unique profile and contained tuberculostearic acid (10 methyl 18:0) a unique feature among plant pathogenic bacteria.

Xylophilus. *Xylophilus ampelinus* had a very simple but unique profile in contrast to those of *Xanthomonas* with which it was classified until recently. It was the only plant pathogen to contain 8:0 3OH.

The overall percentage accurancy of identification at different taxonomic ranks was: Genus 99 (99), Species 93 (96), Subspecies 70 (96), Biovar 75 (100), Pathovar 33 (57). Figures in brackets refer to correct identifications being made among the first 3 choices.

CONCLUSIONS. Fatty acid profiling provided an excellent rapid and accurate method for identifying most plant pathogenic bacteria. Within the genus *Pseudomonas*, groupings based on FA profiling agreed with those based on nucleic acid homology. For several of the strains misidentified at generic or specific level, re-examination by other diagnostic techniques showed that they had been wrongly classified when acceded to the NCPPB. Some taxa had very similar profiles which did not allow ready differentiation. This was much more common at pathovar level than at higher taxonomic ranks. Although there was much more overlap in *Pseudomonas syringae* pathovar profiles than for *Xanthomonas campestris* profiles, some pathovars had profiles of diagnostic value. Within *Xanthomonas campestris* the variation indicated that some pathovars should have specific rank. Thus for many plant pathogenic bacteria the fatty profile is of such diagnostic value that presumptive diagnosis can be made with greater confidence and very often confirmation of diagnosis even at pathovar level can be made without recourse to time consuming host tests. All the results presented were obtained within 28-48 hours of streaking an isolated colony onto the test medium except for the slow growing *Xylophilus ampelinus*.

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Proc. 7th Int. Conf. Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

USE OF FATTY ACID PROFILES AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM TO TRACE A QUARANTINE OUTBREAK OF PSEUDOMONAS AVENAE ON FRENCH AND ITALIAN MILLET

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INTRODUCTION

In February 1988 a severe bacterial foliage blight was observed on 9 of 11 cultivars of Panicum mileaceum (French millet) at three experimental sites planted in December 1987; Gatton and Dalby in Queensland and Moree in New South Wales. The disease was minor on Setaria italica (Italian millet). The sites were remote from each other, being 130 to 260 km apart and separated by mountain ranges. The sites were part of a national breeding programme, and cultivars had been selected from 213 seed-lines imported from 7 countries. Plants were quarantined in glasshouses in winter 1986, with seed increase and field selection in spring 1986 at Gatton.

The causal organism was presumptively identified as Pseudomonas avenae, a pathogen not previously recorded in Australia (Moffett, 1983). Trial sites were quarantined and all plants destroyed. Surrounding areas were surveyed, and isolations were made from germinated French millet seed (harvested in 1987) to determine if the disease was present prior to the outbreak.

This paper reports on the use of fatty acid methyl ester (FAME) profiles and restriction fragment length polymorphism (RFLP) to trace the origin of the disease and to identify the pathogen groups involved.

METHODS

Bacteria were isolated from lesions by diffusion into sterile distilled water and streak plating on King's B agar. Isolation from seed was by a grow out method through a 50 mm layer of wet vermiculite held at 26-28C in controlled temperature and humidity chambers. Cultures were identified using the determinative tests described by Hayward (1983). Cultures found to be virulent in glasshouse pathogenicity tests on French millet were freeze dried.

Extracted cellular fatty acids were methylated and esterified for gas chromatographic analysis essentially as described by Gitaitis et al (1987) using the Hewlett-Packard (Avondale, PA, USA) Microbial Identification System (MIS) with profile library version 2.0 (HP5898A). Cultures from trypticase soy agar (40h, 28C) were prepared as described by the May 1987 revision of the MIS operating manual. MIS identifies fatty acids between C9 and C20 chain length and compares the profiles to a library of known strains.

Nucleic acids were isolated using standard phenol/chloroform procedures (Marmur, 1961) and digested with Hae III and RNAse A according to the manufacturers instructions. Electrophoresis was performed using 5% polyacrylamide and TAE buffer (Maniatis et al, 1982). Fragments were visualised using a silver stain (Igloi, 1983). Marker tracks of Hae III and digested lambda DNA were included in each gel run.

RESULTS

Fifty eight bacterial isolates from quarantine outbreak sites, field surveys and seed isolations were identified as Pseudomonas avenae on standard biochemical tests. FAME and RFLP analysis of these isolates, and two reference cultures, divided P. avenae into 10 groups; A1 to A9 and C, labelled in order of collection and determination. RFLP patterns confirmed separations based on FAME patterns and established groups A6 to A9, which were not readily separated by FAME analysis until fatty acid concentrations were closely standardised.

All A strains isolated from French millet had similar FAME profiles, but were distinguishable by the titre of minor fatty acid peaks at standardised concentrations (Figure 1). A4 (ICMP 3183, type strain from Zea mays, USA 1958) and A5 (ICMP 3960, from Oryza sativa, Japan 1955) differed markedly from millet isolates (Figure 2). Both exhibited a high similarity index to P. acidivorans and P. rubrilineans on numerical analysis of FAME profiles. Similarly all A strains from millet had related RFLP patterns, whilst A4 and A5 had patterns more distantly related to the millet group.

Group A1 was isolated from all quarantine outbreak sites and directly from the original imported seed from Hungary, East Germany and USA. A1 was not detected in local seed or from field surveys. Group A2 was isolated from several local seed samples and from imported seed from China and Japan. Group A3 was isolated from a quarantine site, 20 km from a quarantine site and from seed imported from India. Group A6 was isolated from several local seed samples, a quarantine site and from seed of Italian millet from USA

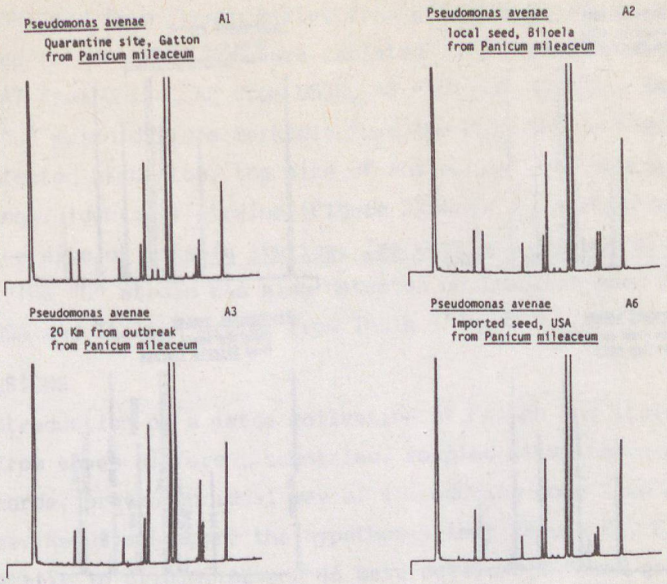


Figure 1: FAME profiles representing the range of A groupings of P. avenae isolated from Panicum mileaceum.

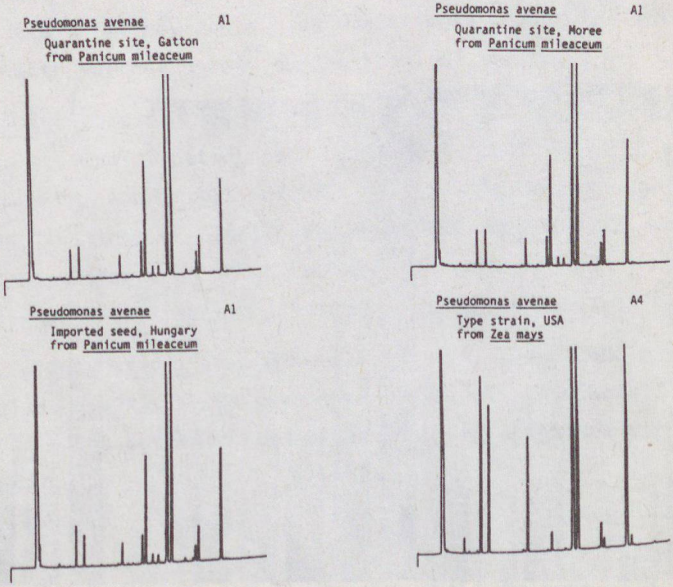


Figure 2: FAME profiles of P. avenae from three quarantine sites, compared with type strain A4.

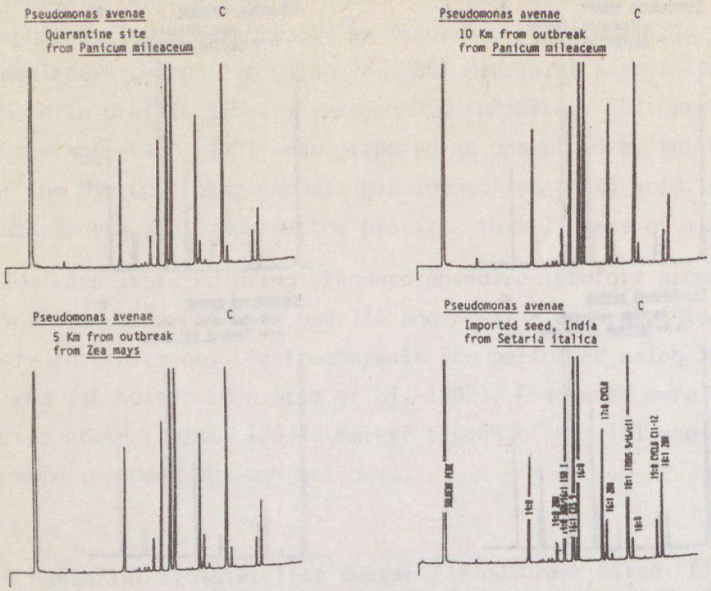


Figure 3: FAME profiles of P. avenae C from a quarantine site and two possible escapes, compared with a group C isolate from Indian seed.

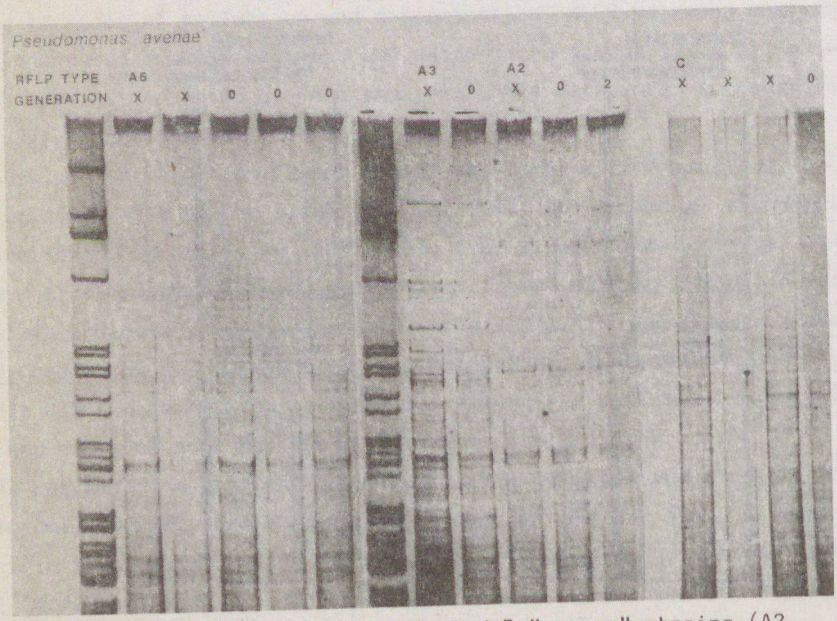


Figure 4: Hae III digests of selected P."avenae" strains (A2, A3, A6 and C) generations; 0 = from imported seed, 2 = glasshouse, X = from commercial, non-quarantine crops.

and USSR, and from French millet from USA. Groups A7, A8 and A9 were not detected in the field but were isolated only from imported French millet seed; A7 from China, A8 from USSR, A9 from USA and East Germany.
Group "C" which differs markedly from the A groups in FAME and RFLP patterns, was detected at Gatton, the site of the spring 1986 and summer 1988 plantings. Identical strains (Figure 3) were also detected 1, 5 and 10 km from the site on Setaria italica, Zea mays and Panicum mileaceum respectively. The "C" strain was also detected on imported seed of French millet from USA and Italian millet from India (Figure 4).

CONCLUSIONS

The introduction of a large collection of French and Italian millet cultivars from seven different countries, coupled with inadequate quarantine safeguards, proved an ideal way of introducing more than one new bacterial disease. Results support the hypothesis that Groups A1, C and probably A3 were exotic in origin. A2 and A6 were detected in seed grown in areas remote from the outbreak and may represent earlier introductions or previously undetected indigenous pathogens. However all groups of millet pathogens could be isolated directly from imported seed. The seed increase plots were visited by several millet farmers at crop maturity and there is the possibility they could have unwittingly spread the infection to remote areas, with further spread during the 1987 season.

The combined use of FAME and RFLP patterns proved a powerful tool in tracing strains of pathogens from the introduction. RFLP patterns confirmed the sensitivity of FAME analysis to detect significant subspecies groupings of plant pathogens. RFLP patterns provide a definitive taxonomic standard on which to base FAME profiles and indeed to check the validity of identifications by conventional culture tests.

The P. avenae group, including all the non fluorescent pseudomonads on Graminiae, clearly needs taxonomic revision. This work illustrates the dangers in the current trend of lumping bacterial species on inadequate grounds.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

DEVELOPMENT OF A NATIONAL COMPUTERIZED NETWORK FOR CULTURE COLLECTIONS OF MICROBIAL AND SUBCELLULAR GERMPLASM ASSOCIATED WITH PLANTS

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ABSTRACT

A databank and computer-supported network are being developed to expedite exchange of cultures and data about microbial and subcellular germplasm resources of scientific, biological, agricultural, and industrial importance. The network will serve scientists with "working" collections and will facilitate (i) rescue and reassessment of germplasm in collections, (ii) evaluations of regulatory, patent, and environmental impact issues, and (iii) interconnect with national and international networks in related fields. Approximately 200,000 accessions have been identified to date, consisting of bacteria, fungi, nematodes, protozoa, algae, fastidious prokaryotes, and subcellular elements (viruses, plasmids, cloning vectors, etc.). The database for the network is being developed using Informix 4GL. User-friendly software for individual collections is also being written to aid entry of data in a standardized format. Quarterly newsletters are being published, and a directory of scientists with a brief summary of their accessions will be published later in the year.

INTRODUCTION

Collections of microorganisms and subcellular germplasm maintained and used by scientists within State Agricultural Experiment Stations, USDA/ARS Research Labs, and private industry represent a diverse an extremely valuable national, and even international, resource. These specialized research collections ("working" collections) maintained by individual scientists and laboratories are often limited in the number of species but rich in the number of strains maintained. [1]

Unfortunately, this resource has been under-appreciated [2] and under-funded in the past. Furthermore, working collections constantly face extinction as funding for research becomes sparse, principal investigators retire, and institutional research interests shift. The value of these resources, both commercially and for basic research, lies in: (i) the rich pool of genetic information present in the research collections, and (ii) the scientist's expert knowledge of the germplasm and its biology. Advances in biotechnology and genetic engineering have helped bring the importance of microbial and subcellular culture collections more sharply into focus.

DEVELOPMENT OF A NATIONAL COMPUTERIZED NETWORK

An accurate assessment is unavailable of the extent and variety of germplasm maintained by scientists in their research collections, and our present knowledge of "who has what" germplasm is at best fragmentary (Table 1). For this reason a computerized, distributive network is being developed to link together scientists who, as part of their research or teaching programs, maintain working collections. The network will focus on collections of microorganisms or subcellular entities which have some relationship to plants and which comprise "working" collections.

The long range goal is to develop a decentralized database, accessible by all researchers, which contains minimum data sets describing these individual collections (see DaSilva and Taguchi for discussion of the advantages and disadvantages of decentralization [3]). Inquiries can then be made via a distributive, computerized network to the individual laboratory in which the culture of interest resides. A catalog will be published which identifies individual collections in the United States and their general contents. A similar catalog will be made available on line so that it can be searched at will and updated constantly. Not only will sources of germplasm be listed but the scientists associated with these collections provide a potential pool of expertise which coulld address regulatory, patent and environmental-impact issues.

Publication of a periodic newsletter has been instituted to inform scientists, administrators, and the public of (i) the importance of microbial germplasm and (ii) progress in the development of the database and the network. It also provides a forum for discussion of problems and issues that affect working culture collections, and it regularly contains articles on; innovative laboratory techniques, notification of upcoming events, pertinent literature citations, software criticisms, questions or suggestions and news items that bear upon culture collections.

NEED FOR STANDARDIZED SOFTWARE

Communications between laboratories would be facilitated greatly if records could be stored in personal computers in a standardized format. The availability of public domain software that is intuitive to operate and designed specifically for management of "working" culture collections could be instrumental in achieving this goal.

Table 1. Characterization of working culture collections based on type of germplasm, size, usage and documentation

A. Number and size of collections containing various types of germplasm.

TYPE OF GERMPLASM	NUMBER OF COLLECTIONS	NUMBER OF ACCESSIONS TOTAL	NUMBER OF ACCESSIONS RANGE
Bacteria	92	38,456	1-5,000
Fastidious Procaryotes	5	797	12-400
Fungi	115	60,239	2-15,000
Mycorrhizae	18	940	2-400
Nematodes	28	8,078	1-3,000
Viruses	29	1,201	1-600
Plasmids	13	816	3-250
DNA Clones	8	2,723	11-2,000
Sequenced DNA cultures	8	1320	1-1,225

B. Usage and Documentation of culture collections

Usage	
research	80%
teaching	31%
other	17%
Support and Service:	
Receive support funds for collection	10%
Provide cultures to other scientists	99%
Do not charge for cultures	95%
Documentation	
Maintain Documentation	90%
Documentation on Computer	6%
Interested in developing such a system	60%

[1]Data taken from ESCOP Subcommittee survey completed 20 July, 1984. Data based on information from 224 respondents, 195 of which maintained collections.

SCIENTIST'S MAJOR CONCERNS

There have been three recurring concerns raised by scientists regarding the Microbial Germplasm Database: (i) How will the proprietary nature of personal collections be maintained? (ii) How will the database and network insulate scientists from numerous and often frivolous requests? (iii) What impact will these first two concerns have on the free exchange of information and materials? No easy answers have been found to these questions, but the concerns are real and must be addressed if the effort is to succeed.

INTERNATIONAL DATABASES/NETWORKS

The above concerns may have been examined in part by one or more of several international organizations concerned with microbial culture collections. Based on the literature available, it appears that many of these organizations have given careful, analytical thought to the needs of scientists and culture collections. We are starting to contact these groups and are working carefully to avoid unnecessary duplication of effort.

The following groups are illustrative of programs already in force: ECCO, European Culture Collections Organisation; (BDT), Tropical Database; JFCC, Japan Federation for Culture Collections; MIRCENS, Microbial Resource Centers; MiCIS, Micobial Culture Information Service; MINE, Microbial Information Network Europe; NORCC, Nordic Register of Microbiological Culture Collections; CODATA, Committee on Data for Science and Technology; WFCC, World Federation of Culture Collections; MSDN, Microbial Strain Data Network; WDC, World Data Center on Microoganisms [taken from 4,5]

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ACKNOWLEDGMENTS

The progress made to date in developing the Microbial Database Network was made possible by the encouragement and support of the Biotechnology Committee of the National Association of State Universities and Land Grant Colleges (NASULGC) and the Experiment Station Committee on Policy (ESCOP), by Dr. Orville Bentley's (former Assistant Secretary of Agriculture) appointment of a Special USDA Committee on Microbial Collections , and a financial grant from the Cooperative State Research Office of the USDA.

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

RESTRICTION FRAGMENT LENGTH POLYMORPHISMS DISTINGUISH XANTHOMONAS CAMPESTRIS STRAINS ISOLATED FROM FLORIDA CITRUS NURSERIES FROM XANTHOMONAS CAMPESTRIS PV. CITRI

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ABSTRACT

Hartung, J.S., and E.L. Civerolo. 1989. Restriction fragment length polymorphisms distinguish Xanthomonas campestris strains isolated from Florida citrus nurseries from Xanthomonas campestris pv. citri.

Genomic DNA prepared from 35 strains of X. campestris pv. citri and 14 strains of X. campestris isolated from Florida citrus nurseries was used for RFLP analysis. Seven cosmid clones selected from a library of strain XC62 of X. c. pv. citri (group A) were used to screen for EcoRI and PvuII polymorphisms, which were quantified by calculating coefficients of similarity (F). Cluster analysis of the RFLP dintinguished the group A strains from the remaining strains of X. c. pv. citri, which formed a second distinct B/C/D group. A relatively heterogeneous, but related, group of X. campestris strains has been isolated from Florida citrus nurseries. Cluster analysis of the RFLP data revealed a significant separation between the 14 strains of X. campestris isolated from Florida citrus nurseries and all strains of X. c. pv. citri. This collection of strains is not closely related to previously recognized strains of X. c. pv. citri. We conclude that the disease in Florida citrus nurseries is not a form of citrus bacterial canker disease and that the disease is not a strain of X. c. pv. citri.

Several forms of citrus bacterial canker disease, CBCD, caused by Xanthomonas campestris pv. citri, have been described (8): A for Asiatic CBCD, which occurs worldwide and is the most virulent [synonym X. citri (ex Hasse) Gabriel et. al (2)] and groups B, C, and D

[synonym X. campestris pv. aurantifolia Gabriel et. al (2)], which occur in Argentina, Brazil, and Mexico, respectively.

In 1984, the discovery of leaf and stem lesions in Florida citrus nurseries caused by X. campestris [synonym X. campestris pv. citrumelo Gabriel et. al (2)] prompted an extensive and unsuccessful eradication effort (6). Strains of X. campestris isolated from citrus nurseries cause flat water-soaked lesions instead of raised lesions on diseased plants, as do all strains belonging to groups A-D of X. campestris pv. citri, and are generally found only on nursery stock.

MATERIALS AND METHODS

The 35 bacterial strains studies included 14 of group A (Argentina, Japan, Pakistan, S. Yemen, Guam, and Florida, USA); 5 of group B (Argentina); 1 each of groups C and D (Brazil and Mexico); and 14 strains of X. campestris. Individual clones from a complete genomic library of strain XC62 (CBCD group A, Japan) were labeled with biotin-11-dUTP by nick translation. The seven probes used had an average insert size of 25 kb and did not cross hybridize. Seven ug of digest DNA (EcoRI or PvuII) were separated in 14 cm 0.8% agarose gels using TPE buffer at 5 V/cm. Southern blots were done to nitrocellulose membranes which were hybridized under standard stringent conditions. Coefficients of similarity, (F), (5) were calculated for strains appearing in the same gels and were averaged for the seven probes and two endonucleases. Cluster analyses were performed on the data using the unweighted pair group method with numerical averages (UPMGA), single link and complete link algorithms.

RESULTS

The 14 A strains tested formed a very homogeneous group (Figure 1A). The B/C/D group of strains (XC64 - XC90) was also closely related (F greater than 0.82). However, the A and the B/C/D groups of strains were clearly distinct (e.g. XC62 vs. XC64-XC90, F = 0.6). The group of Florida citrus nursery strains showed only limited relationship to any strains of X. c. pv. citri (F1-F97 vs. XC62-XC90, F = 0.3)

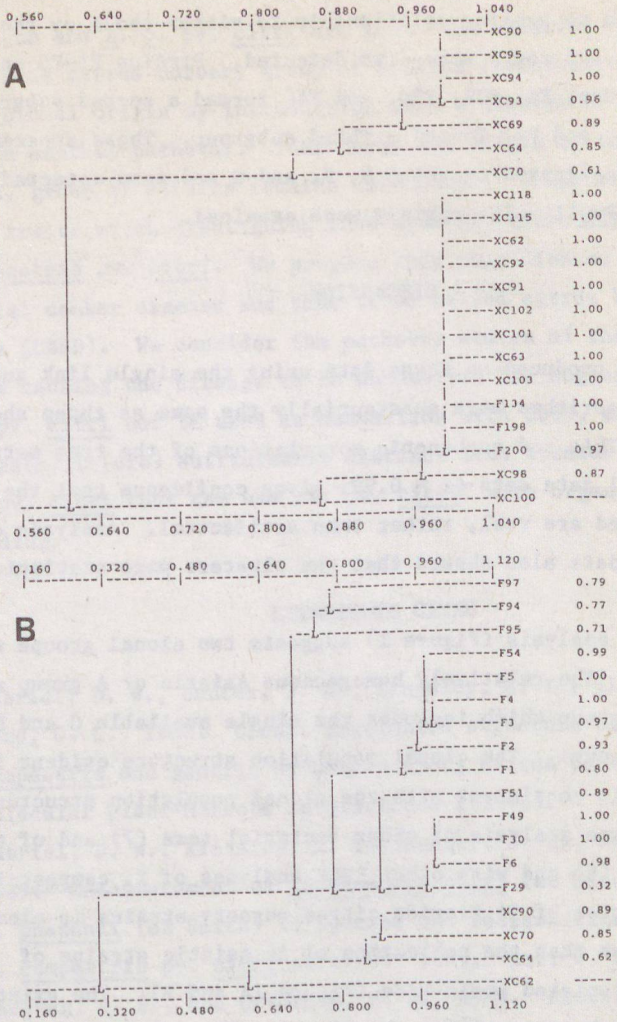


Figure 1. A) Dendrogram based on RFLP similarity coefficients for strains of X. c. pv. citri calculated by the UPGMA algorithm. The upper cluster is the B/C/D complex; The lower cluster is the A group of strains. The cophenetic correlation coefficient (r) was 0.99. B) Dendrogram based on RFLP similarity coefficients for strains of X. campestris isolated from Florida citrus nurseries and strains of X. c. pv. citri representing groups A-D calculated by the UPGMA algorithm. The top group of related clusters are the Florida citrus nursery strains; The bottom cluster are the strains of X. c. pv. citri. The cophenetic correlation coefficient (r) was 0.99.

(Figure 1B), with no greater relationship to either the A or the B/C/D group. Apparent subgroups were also detected. Strains F1-F5 and F54 formed one subgroup; F6, F29, F30, and F49 formed a second subgroup; strains F94, F95, and F97 formed a third subgroup. These apparent subgroups were designated as group E, F, and G and were detected when either EcoRI or PvuII polymorphisms were examined.

DISCUSSION

Dendrograms produced on these data using the single link and complete link algorithms were substantially the same as those shown in Fig. 1A and B. This and cophenetic correlations of the tree matrices with the original data sets ($r = 0.99$) gives confidence that the clusters displayed are real, rather than artifactual. Analysis of variance of the data also showed that the clusters were statistically significant (3).

The cluster analysis (Figure 1) suggests two clonal groups within X. c. pv. citri: The relatively homogeneous Asiatic or A group and the cancrosis B group which includes the single available C and D strains (B/C/D group). The clonal population structure evident in X. c. pv. citri is consistent with the clonal population structure revealed by isozyme analysis of other bacterial taxa (7) and of this group of strains (4) and with other RFLP analyses of X. campestris (1). The collection of 14 Florida citrus nursery strains is clearly more heterogeneous than the collection of 14 Asiatic strains of X. c. pv. citri isolated world-wide (Figure 1A and B). The existence of (RFLP based) subgroups within the Florida citrus nursery group of strains should not obscure the fact that they are in fact a group of related strains (Figure 1B) but may complicate any taxonomy of the group if supported by other characteristics. The diversity of RFLP observed with the Florida citrus nursery group of strains is consistent with previously observed wide variation in electromorphs detected during multilocus isozyme analysis (4).

The RFLP analyses conducted in this study indicate that the Florida citrus nursery strains are not derived from any recognized group of X. c. pv. citri as the coefficients of similarity between

this group and X. c. pv. citri are low. The observed heterogeneity of the Florida citrus nursery group of strains is inconsistent with a single clonal origin by introduction from elsewhere or by a mutational event in another pathovar. Therefore, the origin of this related, yet diverse, group of strains remains unresolved. These strains share common traits which distinguish them from all known strains of X. campestris pv. citri. We propose that this disease is not citrus bacterial canker disease and that it be called citrus bacterial spot disease (CBSD). We consider the pathovar status of the group of strains causing the disease to be unresolved but suggest that X. c. pv. citri not be used in connection with CBSD, since the pathogen(s) is(are) sufficiently distinct from strains A-D of X. c. pv. citri that the use of "pv. citri" with organism(s) is misleading.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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X-RAY MICROANALYTICAL DETERMINATION OF THE IONIC COMPOSITION OF PLANT PATHOGENIC BACTERIA

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GENERAL TECHNIQUE

Transmission electron-probe X-ray microanalysis has been widely used in the electron microscope investigation of both eukaryote and prokaryote cells, where it provides information on elemental composition - including the occurrence of major anions and cations.

In general, the technique is able to detect elements with an atomic number of 11 or over (ie. Na and above) at detection limits of 10[-17]g (absolute units) or 0.1% on a mass fraction basis. The area analysed has a minimum diameter of about 0.3μm in the case of a microprobe, and considerably less with a nanoprobe.

ANALYSIS OF BACTERIAL CELLS

(a) Range of bacterial cells examined

X-ray microanalysis has been used to determine the elemental composition of a variety of bacterial cells, including *Escherichia coli*, halophilic bacteria, marine fouling bacteria, freshwater bacteria and plant pathogenic bacteria (reviewed in Sigee,1988).

(b) Plant pathogenic bacteria

Studies at Manchester have been carried out on pathovars of *Pseudomonas syringae* and on *Erwinia amylovora* These

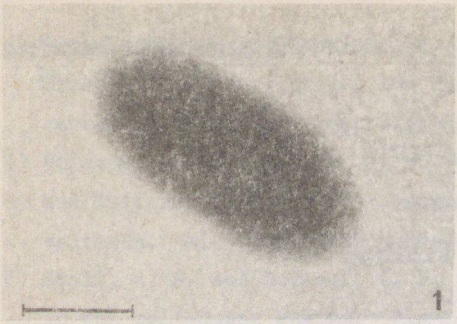


Fig.1 Transmission electron micrograph of air-dried cell of Erwinia amylovora . No contrast enhancement. Bar scale = 0.5µm

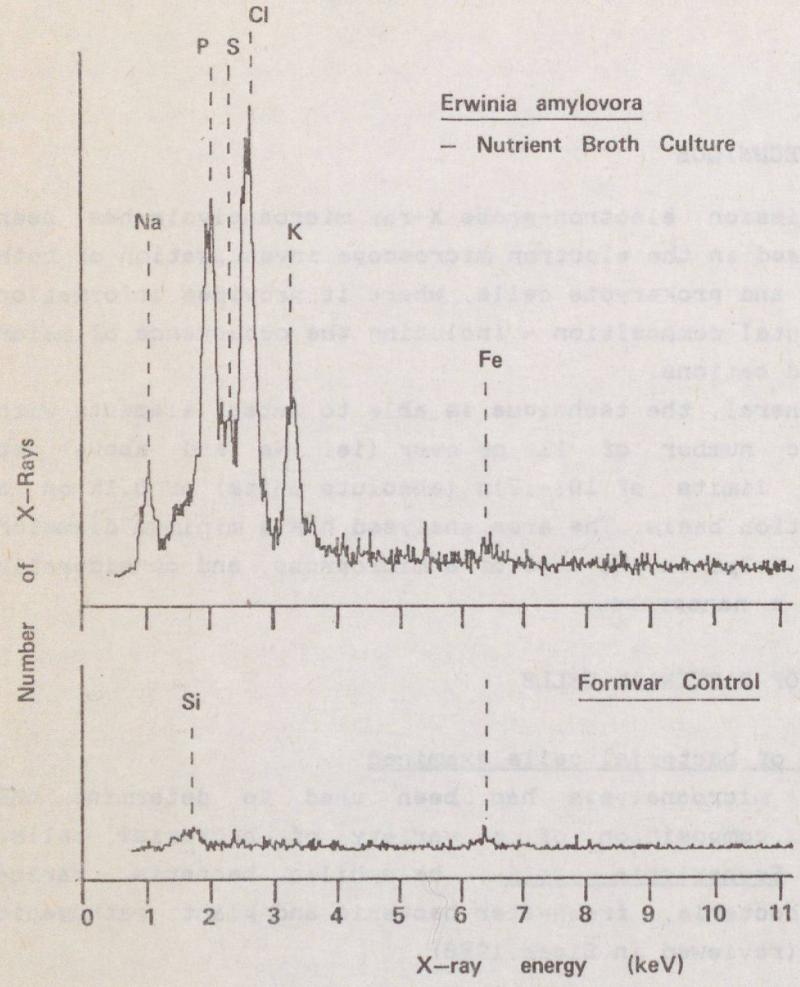


Fig.2 X-ray emission spectra from an air-dried cell of Erwinia amylovora and Formvar support film (control).

studies initially examined the effect of different preparation techniques for X-ray microanalysis, including cryosections, resin sections of fixed, dehydrated cells, lysed cells and whole-cell preparations (Sigee et al.,1985).

Whole-cell preparations are particularly useful in investigating the overall levels of elements in bacteria, since the relatively high mass in the probe area generates a correspondingly high level of X-rays, with optimal statistical definition of elemental peaks. Whole bacterial cells can be clearly distinguished (Fig.1) and analysed in the transmission mode, without the need for contrast enhancement.

A typical X-ray emission spectrum from an air-dried cell of Erwinia amylovora, isolated from a washed culture of cells grown in nutrient broth, is shown in Fig.2. This spectrum is closely similar to spectra obtained from an equivalent preparation of Pseudomonas syringae which show consistent peaks of Na, P, S, Cl and K, plus occasional peaks of transition metals (particularly Fe and Zn). Chemical treatment of these cells (glutaraldehyde fixation and ethanol dehydration) prior to air-drying leads to a loss of soluble constituents (eg.K) and an enhancement of insoluble divalent cation peaks - particularly Ca and transition metals (Sigee al., 1985).

Typical values for the level of detectable elements vary considerably, and range from about 6% (P) to about 0.1% in the case of transition metals. With a detection limit of 0.1%, this technique is able to determine and quantify major elements in the cells, but will not detect minor elements or small elemental fluctuations.

X-ray microanalysis of phytopathogenic bacteria has so far been used to determine -

The elemental composition of bacterial DNA

Nucleic acids are a major site of cation binding in bacterial cells. This was investigated in P.s.pv tabaci

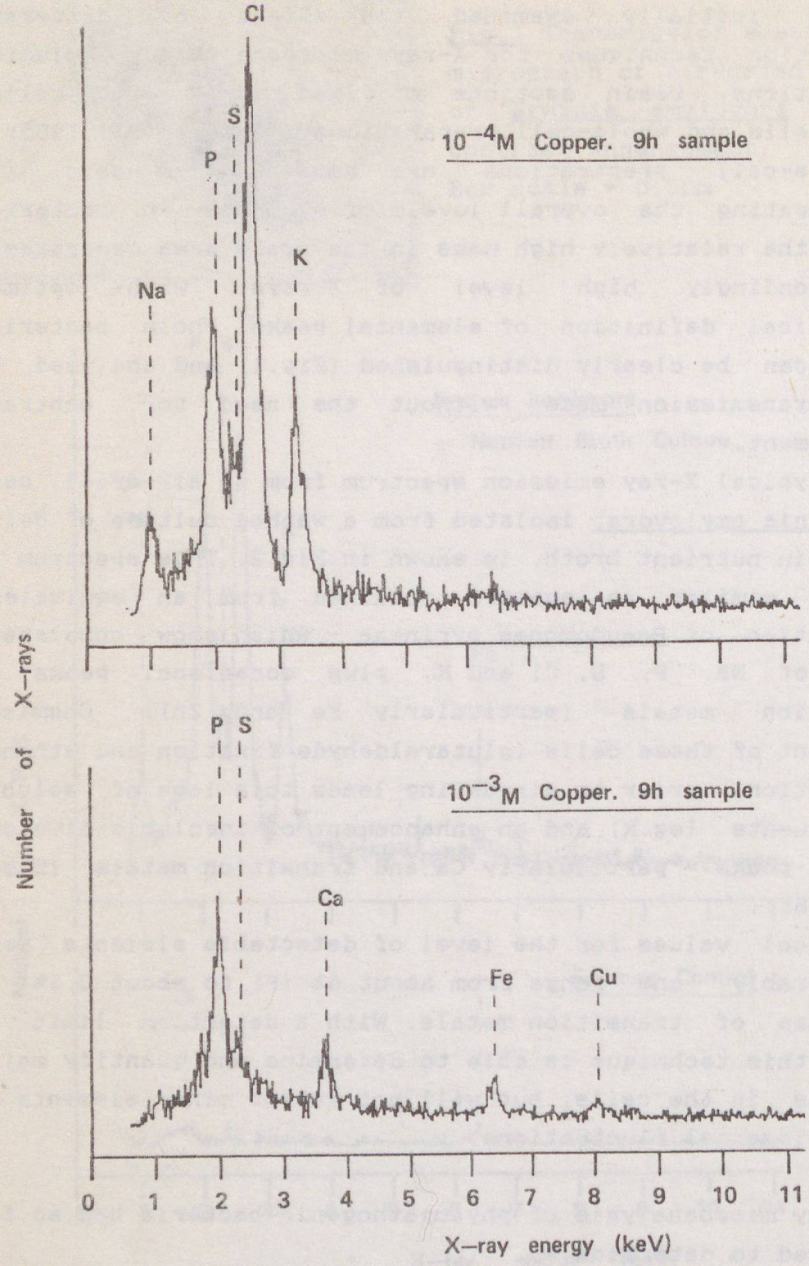


Fig.3 X-ray emission spectra from air-dried cells of Erwinia amylovora grown in non-toxic (top spectrum) and toxic (bottom) levels of copper. The 10[-3]M spectrum shows loss of Na,Cl and K and enhanced peaks of Ca,Fe and Cu.

(Sigee & El-Masry,1988) by extraction and purification of DNA by conventional procedures, deposition of intact molecules on an EM grid, then determination of the cation content by X-ray microanalysis. Spectra from the highly condensed molecules show clear peaks of P, K, Ca and transition metals Ni, Cu and Zn, but no S (absence of protein).

Both monovalent and divalent cations show a clear correlation in occurrence with P, but not with each other. This suggests that both types of cation are associated with electronegative phosphate groups - but in different ways. Approximately 50 detectable cations were present per 100 phosphorus atoms, which is equivalent to one cation per two phosphate groups or per two nucleotides.

Heavy metal toxicity

Transition metals are highly toxic to bacterial cells, and copper ions are widely used in the chemical control of epiphytic populations of phytopathogenic bacteria. The toxic effect of heavy metals on bacterial growth and elemental composition has been studied for nickel (Sigee & Al-Rabaee, 1986), and is currently under investigation for copper. In both cases, *in vitro* growth of bacteria is limited at a cation level of 10[-3]M and above.

Cells of *P.s. pv tabaci* grown in nickel at this concentration show clear peaks of Ni (plus other transition metals) with decreased levels of K, suggesting a general effect on cation exchange at the cell surface.

Current studies on the toxic effect of copper ions on *Erwinia amylovora* (Fig.3) show similar effects, with a sharp decrease in soluble cations (Na,K) and enhanced peaks of Cu and Fe in cells grown at 10[-3]M compared to 10[-4]M Cu.

Bacterial changes in planta

Changes in the elemental composition and dry mass of *P.s. pv tabaci* have been monitored during the development of wildfire disease in tobacco (El-Masry & Sigee,1989). Cells of the phytopathogen were extracted from infected leaf tissue by

maceration / filtration, followed by deposition of cells on an E.M. grid, then X-ray microanalysis of air-dried and chemically-processed cells.

The results show that the elemental composition of bacteria *in planta* is closely similar to that *in vitro* - with the exception of Ca, which is particularly high in the former case. Little change occurs in the cation levels of these bacteria until about 96h after infiltration, when a there is a sharp fall in K and other elements - consistent with bacterial cell death and cation leakage.

Comparable studies are currently being undertaken to determine changes in the elemental composition of bacterial cells inoculated into incompatible tissue.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

FURTHER INVESTIGATIONS ON PSEUDOMONAS SYRINGAE PATHOVAR AS PATHOGEN OF SOUR CHERRY FRUITS IN YUGOSLAVIA

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ABSTRACT

Biochemical and physiological characteristics of bacterial isolates obtained from sour cherry fruits and leaves were studied. The results indicated that the isolates investigated belonged to an intermediate form between Pseudomonas syringae pv. syringae and Pseudomonas syringae pv. morsprunorum.

In recent past years bacterial necrosis of sour cherry (Prunus cerasus) fruits has become an economically important disease in Yugoslavia.

The results of our previous investigations (Arsenijević and Balaž, 1986; Balaž et al., 1988) showed that the necrotic changes were of bacterial origin and the causal agent of disease belonged to the pathovar of Pseudomonas syringae group.

Regarding to the unusually severe occurrence of this new bacterial disease for Yugoslavia, as well as considering the doubts appearing most frequently in the determination of Pseudomonas syringae pathovars, we wanted to get an answer to the following: Which bacterium causes necrotic changes of sour cherry fruits? Does this bacterium belong to the pathovar P.s.pv. syringae or P.s. pv. morsprunorum?

MATERIAL AND METHODS

The strains V-79, V-85, V-88 and V-93 isolated from the diseased sour cherry fruits (Fig. 1) and leaves were used. They were also used in our previous study (Balaž et al., 1988).

Further investigations for the identification of these isolates were conducted by differential tests for distinguishing P.s.pv. syringae from

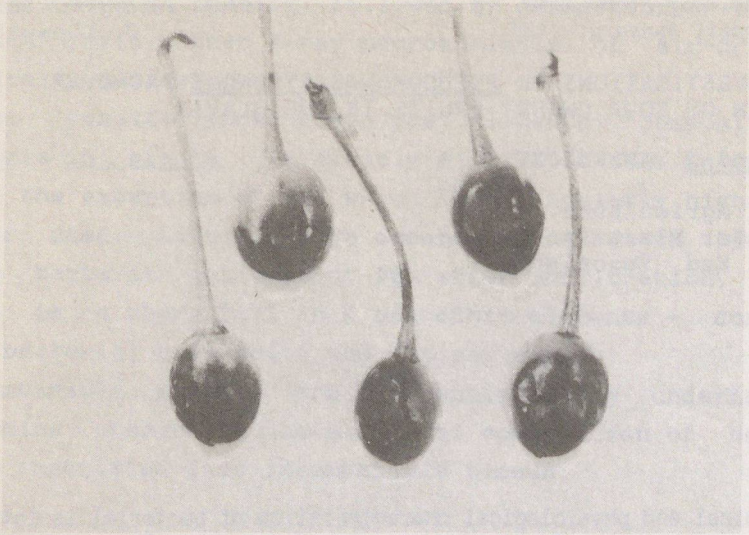


Fig. 1. Pseudomonas syringae pathovar. Necrotic lesions on immature sour cherry fruits. Natural infection.

P.s.pv. morsprunorum. (Crosse, 1966; Garrett et al., 1966; Latorre and Jones, 1979; Sletten, 1979; Simon et al., 1980; Sobiczewski, 1984). For the comparative investigations we included the referent isolates P.s.pv. syringae H-1 (isolated from leaf of sour cherry by S. Süle) and P.s.pv. morsprunorum C-46 (isolated from branches of cherry by J.E. Crosse, supplied to us by S. Süle).

The following tests were used: gelatin liquefaction (Smith et al., 1952); hydrolysis of aesculine (Sneath, 1956); tyrosinase activity (Lelliott et al., 1966); utilization of tartaric acid (Simon et al., 1980); utilization of lactic acid (Fahy and Hayward, 1983); hydrolysis of casein (Panagopoulos, 19719; growth characteristics in nutrient sucrose broth (Jones, 1971); syringomycin production (Seemüller and Arnold, 1978); ice nucleation activity (Paulin and Luisetti, 1978); sensitivity to bacteriophage A-7 (isolated by Crosse and Garrett, supplied to us by S. Süle) investigated after Latorre and Jones (1979).

RESULTS

The results of investigations of some biochemical and physiological properties of isolates, being characteristic for distinguishing P.s.pv. syringae from P.s. pv. morsprunorum are given in Table 1.

According to the results presented the isolates obtained from sour cherry liquiefied gelatine, did not hydrolize aesculin, the activity of tyrosinase was negative and they did not utilize tartaric acid. These reactions indicated that investigated isolates wer GATTa$^{+}_{-}$, after Latorre and Jones (1979). They utilized lactic acid, did not hydrolize casein and gave white growth in sucrose broth. Syringomycin production, INA test and sensitivity to A-7 bacteriophage were negative.

Table 1. Physiological and biochemical characteristics of bacterial isolates

	Isolates					
	V-79	V-85	V-88	V-93	H-1	C-46
Gelatine liquefaction	+	+	+	+	+	-
Hydrolysis of aesculine	-	-	-	-	+	-
Tyrosinase activity	-	-	-	-	-	+
Use of tartaric acid	-	-	-	-	-	+
Use of lactic acid	+	+	+	+	+	-
Hydrolysis of casein	-	-	-	-	+	-
Growth in nutrient sucrose broth	white	white	white	white	yellow	white
Syringomycin production	-	-	-	-	+	-
Ice nucleation activity	-	-	-	-	+	-
Sensitivity to bacteriophage A-7	-	-	-	-	-	+

*) Reaction: + positive; - negative.

Comparing biochemical and physiological characteristics of the isolates obtained from sour cherry with the referent isolates H-1 and C-46, it could be seen that there were some similarities between them but differences too.

The results of pathogenicity tests (Balaž et al., 1988) on immature sour cherry fruits inoculated by needle showed that the isolates investigated caused small, brown spots, while the strain P.s. pv. syringae V-109 (isolated from branch of sweet cherry) caused large, sunken, darkbrown spots (Fig. 2). After Sletten (1979) brown, small, superficial spots on inoculated fruits of cherry were characeristic for P.s.pv. morsprunorum, and black, sunken spots for P.s.pv. syringae.



Fig. 2. Pseudomonas syringae pathovar. Circular, brown spots on artificially inoculated green sour cherry fruits (left). P.s.pv. syringae. Large, sunken, darkbrown spots on artificially inoculated green sour cherry fruits (right).

On the basis of the biochemical and physiological characteristics, as well as pathogenicity of the isolates investigated, it could be concluded that the bacterial isolates obtained from sour cherry belonged to an intermediate form between P.s.pv.syringae and P.s.pv. morsprunorum.

DISCUSSION

Our investigations showed that the causal agent of necrotic changes of sour cherry fruits and leaves was a bacterium which belonged to an intermediate form between P.s.pv. syringae and P.s.pv. morsprunorum.

The fact that P.s.pv. syringae and P.s.pv. morsprunorum isolates have not been found in the investigated bacterial population but only an intermediate form, points out the occurrence of bacterial varieties adapted to our ecological conditions and to the particular sour cherry varieties. The specifity of our isolates was also expressed in the pathogenic ability, in that they were first of all the parasites of sour cherry fruits, while the majority of authors emphasized mainly their importance as the causal agent of bacterial canker on the branches of sour and sweet cherries (Crosse, 1966; Garrett et al., 1966; Slleten, 1979; Lyskanowska, 1979; Sobiczewski, 1984; Kûdela and Trynerová, 1986).

The ability of these bacteria to adapt to different ecological conditions was pointed out by Crosse (1966). A great variability among isolates from sour cherry was proved by Sobiczewski (1984) and Zeller (1984). Kůdela and Trynerová (1986) showed also the occurrence of an intermediate type between P.s.pv.syringae and P.s.pv. morsprunorum.

ACKNOWLEDGEMENTS

We should like to express our thanks to Dr. M. Hevesi and Dr. S. Süle (Research Institute for Plant Protection, Budapest, Hungary), for their kind help.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

PATHOGENIC AND PHYSIOLOGICAL PROPERTIES OF PSEUDOMONAS SYRINGAE PV. HELIANTHI

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ABSTRACT

Bacterial spot of sunflower leaves becomes more and more frequent in Yugoslavia. Several bacterial cultures were isolated from infected tissues and three of them were studied in detail. Judging by morphological, cultural, physiological and pathogenic characteristics, the isolates were determined as Pseudomonas syringae pv. helianthi.

INTRODUCTION

In the course of 1986 and 1987, a new form of leaf spot disease was observed on sunflowers in Yugoslavia (Aresenijević and Maširević, 1987, 1988 a, b, 1989). The disease was manifested on the lamina in the form of brown necrotic spots which tended to expand and coalesce. The necrotic process of the infected tissues caused drying and dieback of entire leaves. The symptoms of the disease were also observed on sunflower petioles and stems. Since the causal agent has not been studied sufficiently either in Yugoslavia or elsewhere, we decided to investigate the pathogen and try to identify the causal agent of the disease.

MATERIAL AND METHODS

Samples and isolation

Several bacterial strains were isolated from sunflower leaves and stems in August 1986 and June 1987 on a nutrient medium (NA) following a conventional procedure. Three of them (5415, 5419 and 5421) were studied in detail. Isolates Ps-4 (P.s. pv. syringae) and B-4 (P.s. pv. phaseolicola) were used

as positive controls. The first was isolated from wheat spike and the second originated from string bean pod.

Pathogenicity

Twenty-four hrs-old cultures grown on the nutrien medium NA were used. Leaves of the tobacco variety White Burley were used for infiltration (Klement, 1963). Isolates possessing positive hypersensitivity were used for inoculating young sunflower plants (2-4 pairs of well-developed leaves), string bean pods, and peach shoots. The shoots were inoculated by priking, using the bacterial suspension 10^8 cells/ml. A suspension with the concentration of 10^7 cells/ml was injected in string bean pods.

Bacteriological properties

Fluorescence was examined on King's B medium. Lopat tests and other physiological properties (Table 1) were tested by conventional procedures (Schaad, 1980; Fahy and Persely, 1983; Lelliott and Stead, 1987; Arsenijević, 1988).

RESULTS

Symptoms

The symptoms of the disease may be observed in the field at the beginning of June. First small brown spots, on the lamina occur about 2 mm in diameter, with a chlorotic halo. The spots extend and become irregular. After the spots coalesce, the major part of the infected leaf turns necrotic. At that point, the disease symptoms resemble those caused by leaf blight. The infected leaves become deformed and dry. Bacterial exudate is observed seldom.

At the beginning, the spots on the stem are oily and moist on the surface and later on they turn brown or darker. They also expand and coalesce causing necrosis of the major part of the infected tissue.

Necrotic changes may also be observed on the petiole. The pathogen usually infects the basal part of the petiole which becomes necrotic and carcks, resembling canker-like symptoms. The infected tissue is brown, besprinkled with amber coloured exudate drops.

Inoculation of sunflower plants

First changes on sunflower leaves inoculated by infiltration technique became evident after 2-3 days. Those were dark-green spots which kept expanding and changing colour. After 5 days, the spots were light-brown and dry in the centre and dark-brown and moist on the periphery. Later on, the periphery too turned dry while the spots themselves became irregularly shaped or elongated, over 1 cm long. Continuing to expand, the spots coalesced, causing intensive necrosis and the drying of entire leaves. The symptoms resemble those caused by bacterial blight. The reisolations of the bacterium from the necrotic tissue were successful and HR on the tobacco leaves were positive. However, there were no changes on the control plants.

The inoculation of main leaf nerves caused necrosis of the entire nervure and the surrounding tissue while the entire leaf became deformed.

The inoculation techniques using a portable sprayer or a highpressure sprayer were also found to be efficient, irrespective of whether the leaf tissue was preliminarily injured or not.

The inoculations with the sprayers brought about almost identical changes. First small moist spots occurred with cholorotic halos and then large brown spots which expanded and merged causing necrosis and blight-type changes resulting in the drying of the infected leaves.

Inoculation of string bean pods and peach shoots

Light-brown spots occurred already after 24 hours on the tissue of string bean pods treated with isolates from sunflower. There were no further changes throughout the 6-day observation period.

The control isolates of Ps. s. pv. phaseolicola did not cause any changes within 24 hours after the inoculation. Only after 48 and 72 hours could round, oily-looking, moist sots be seen. The spots tended to expand and whitish exudate drops appeared on their surface. The control pods inoculated with water did not express any change while those inoculated with Ps. s. pv. syringae formed dark-reddish, sunken spots, a characteristic symptom for this bacterium.

It was obvious that the Ps.s. pv. helianthi isolates from sunflower did not cause typical changes on the inoculated pods. This may relate to the development of HR resulting from the incompatibility between the pathogen and the host (Klement, 1968).

The isolates from sunflower failed to induce any change in the inoculated peach shoots. However, the isolate PS-4 (Ps. s. pv. syringae), used as a positive control, caused considerable changes, the wilting of leaves at the beginning and then the drying up of entire shoots.

Bacteriological characteristics

On nutrient medium, colonies were circular, pale cream to white. The bacterial cells were rod shaped and motile, fluorescent pigment produce on King B and levan on NAS medium. Oxydase, potato rot and arginine dihydrolase tests were negative and HR on tobacco leaves positive. Gelatine, esculin, starch, tween hydrolysis and milk proteolysis were negative; Simmens citrate test was positive and B glucosidase negative.

Table 1. Biochemical characteristics of Ps. pv. helianthi

Isolates	5415	5419	5421	Published
Gram reaction	-	-	-	-
Shape	rod	rod	rod	
Motility	+	+	+	+
Fluorescence (King B)	+	+	+	
Levan	+	+	+	
Oxydase	-	-	-	-
Potato rot	-	-	-	-
Arginine dihydrol.	-	-	-	-
Tobacco hypersen.	+	+	+	+
Gelatine hydrol.	-	-	-	-
Nitrate red.	-	-	-	-
Simmons' citrate	+	+	+	
Esculin hydrol.	-	-	-	
Starch hydrol.	-	-	-	(+) -
Tween hydrol.	-	-	-	
Milk prot.	-	-	-	-
O/F test	O	O	O	
Acid product.				
Glucose	+	+	+	(-) +
Manose	+	+	+	
Arabinose	-	-	-	+
Xylose	+	+	+	+
Rhamnose	-	-	-	-
Lactose	-	-	-	-
Inositol	-	+/-	+/-	-
Mannitol	-	-	-	-
Adonitol	-	-	-	

Acid production from carbohydrates was more or less in agreement with authors cited (Table 1), except arabinose, mannitol and inositol.

DISCUSSION

The bacterial spot of sunflower leaves is gaining importance in Yugoslavia. This fairly obscure disease had been reported to attack sunflowers in Romani, Yugoslavia, Spain and Czechoslovakia (Stancescu and Severin, 1983; Arsenijevi and Maširević, 1987, 1988a; Duran et Sobrino, 1987; Kudela, 1988).

The fluorescent isolates investigated induced HR in tobacco and strong necrosis of sunflower leaves following incolation by pricking, spraying and infiltration techniques. Their pathogenicity was not proved on peach shoots and string bean pods.

The morphological and physiological properties of the investigated strains (Table 1) were similar to those found by other authors (Piening, 1976; Stancescu and Severin, 1983).

On the basis of the pathogenicity, Lopat tests and other biochemical characteristics it was shown that these isolates belong to Pseudomonas syringae pv. helianthi (Kawamura, 1934; Young, Dye et Wilkie, 1978).

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
Z. Klement (ed.)

OCCURRENCE OF PSEUDOMONAS GLUMAE IN NEPAL

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ABSTRACT

A pathogenic non-fluorescent Pseudomonas sp different from P. avenae was isolated from seeds and seedlings of rice in Nepal. This bacterium is oxidase negative and positive in gelatine liquifaction. Acid is produced from mannitol, inositol, sorbitol, and arabinose but not from rhamnose, and sucrose. This pathogen agglutionated with antiserum of P. glumae but not with P. avenae. On the basis of biochemical tests Pathogenicity, and serology, this pathogen was identified as Pseudomonas glumae which causes grain and seedling rot of rice. This disease was first reported from Japan and now known to occur in other countries also. This is the first report on occurrence of P. glumae in Nepal.

INTRODUCTION

Pseudomonas glumae [Kurita and Tabei, 1967] which causes grain rot and seedling rot of rice is previously known to occur only in Japan [Kurita and Tabei, 1967]. However, now the occurrence of this pathogen has been reported from a few other rice growing countries. It is a serious seedborne pathogen of rice in Japan [Tsushima et al 1986 Uematsu et al., 1976]. So far P. avenae is the only reported pathogen among Pseudomonads causing rice diseases in Nepal [Shakya et al. 1985, Shakya and Ranjitkar 1988]. P. glumae and P. avenae are two non-fluorescent Pseudomonas Spp that cause rice disease . A pathogenic non-fluorescent Pseudomonas Sp different from P. avenae has been isolated from seeds and seedlings of rice in Nepal. This paper reports the identification of this pathogen.

MATERIALS AND METHODS

Isolation: Rice seed samples of different cultivars collected from different parts of the country were grown on a germination stand in a growth chamber at 24°-28°C. Bacterial isolates were made from infected seedlings with brown water

soaked diffused stripes on the sheath and leaves. Isolates were also made from bacterial ooze on rice seeds plated on a moist blotter incubated at 21 to 25°C in a growth chamber. The media used for isolation of bacteria were nutrient agar [NA], nutrient sucrose agar with 1% [NSA], and a selective medium [S-PG] as described by Tsushima et al. [1986], but without Pheneticillin Potassium.

Identification: The preliminary sorting of the isolates were undertaken by Gram reaction [Suslow et al. 1982], tobacco hypersensitivity [Klement et al. 1964], a oxidase [Kavacs, 1956], and the production of fluorescin on king's medium B [King et al. 1954]. A pathogenicity test was performed on 3 to 4 week-old rice seedlings by hypodermic injection, leaf clip, and spray methods, with bacterial suspension of approximately 10^8 cells/ml.

Biochemical tests were performed in API 20 E bacteriological profile kits [API system S.A., 38390 Montalieu Vercin, France]. The comparative study of the rice isolates was made with named cultures of P. glumae [NCPPB No. 2391] and P. avenae [NCPPB No. 3356] obtained form the National Collection of Plant Pathogenic Bacteria, Harpenden, England.

Serological: It was conducted by agglutination reaction with antisera of P. glumae and P. avenae obtained from the Danish Government Institute of Seed Pathology, Denmark. The specificity of antisera had been evaluated by this method with the following bacteria: P. avenae, P. glumae, P. phaseolicola, P. viridiflava, xanthomonas phaseoli, X. campestris pv. oryzae, P. campestris pv. oryzicola, Erwinia chrysanthemi, E. caratovora, and two non-pathogenic isolates obtained from rice.

RESULTS

Rice seed samples belonging to the cultivars Apajhute, Kanchan, Pokharali Mashino, and Sataraj from the districts of Dhanding, Bhairahawa, Kathmandu, Baradia respectively, were found infected, producing brown water-soaked diffuse strips or lesions on the sheaths, and sometimes on the leaves of the seedlings. The affected seedlings often turned brown.

Bacterial isolates were Gram negative, non-fluorescent, tobacco hypersensitive positive, and oxidase negative. They were pathogenic to rice seedlings, producing brown water-soaked diffuse stipes. Besides hypodermic injection, leaf clip, and spray methods were also very effective in the pathogenicity test producing distinct brown water-soaked symptoms. But the symptoms were only produced by the spraying inoculation with P. avenae when tween 20 was added.

The colonies were circular, smooth, and pale dirty-white on the NA and NSA, while on the S-PG medium colonies were pink or purple at the centre with white circles. Rice isolates agreed in biochemical characters and serology with the named culture of P. glunae, but differed distinctly with the named culture of P. avenae. [Table 1]. These test isolates agglutinated strongly [complete clearing with cottony flucose clumps] only with the P. glumae antiserum but not with the P. avenae antiserum. The antisera used were specific to the respective antigens only.

DISCUSSION

On the basis of pathogenicity, serological, and biochemical tests, the present bacterium isolated from seeds and seedlings of rice is identified as Pseudomonas glumae [Kunta and Tabei 1967]. This pathogen is oxidase negative, gelatine

Table 1. Comparative tests of pathogenic rice isolates with named cultures of Pseudomonas glumae and Pseudomonas avenae

Characters	Rice Isolates	P. glumae	P. avenae
Gram reaction	-	-	-
Tobacco hypersensitivity	+	+	+
Fluorescent pigment	-	-	-
Oxidase	-	-	-
Beta-galoctosidase	+	+	-
Arginine dihydrolase	-	-	-
Lysine decarboxylase	-	-	+
Ornithine decarhoxylase	-	-	+
Utilization of citrate	-	-	+
H_2S Production	-	-	-
Urease	-	-	+
Tryptophane deaminase	-	-	+
Formation of indole	-	-	-
Voges-Proskauer reaction	-	-	-
Gelatin hydrolysis	+	+	-
Acid production from:			
Glucose	+	+	-
Mannitol	+	+	-
Inositol	+	+	-
Sorbitol	+	+	-
Rhamnose	-	-	-
Saccharose	-	-	-
Melibinose	+	+	-
Amygdalin	-	-	-
Arabinose	+	+	-
Serology:			
P. glumae	+	+	-
P. avenae	-	-	+

\+ Positive reaction
\- Negative reaction.

liquefaction and Beta-galactosidase positive, and produced acid from glucose, mannitol, inisitol, sorbitol, and arabinose. It is clear from the results that the identified P. glumae is different from P. avenae, the other non-fluorescent Pseudomonas sp that causes similar rice seedling disease. Furthermore, the identification of the pathogen has been confirmed by serology, which is a useful method to distinguish it from P. avenae [Tsushima et at. 1986; Uematsu et al. 1976.].

Recently P. glumae has been considered a serious rice disease, not only in Japan, but also in other countries, threatening rice productin both in quantity and quality. From the detection of P. glumae on the cultivars of rice from different districts of Nepal, it is clearly indicated that this pathogen is probably widespread in the country there being a possible incidence of disease in the fields. This is the first report on the occurrence of P. glumae in Nepal. More studies are needed to investigate further.

ACKNOWLEDGEMENTS

I express my sincere gratitude to the Royal Nepal Academy of Science and Technology [RONAST] for providing financial support to conduct this investigation. I am also thankful to the Danish Government Institute of Seed Pathology for Developing countries, Denmark for the cooperation and providing antisera. I am grateful to the International Foundation for Science Sweden [IFS] for equipment grant under No. C/0955-1. I wish to thank Mrs. H. D. Ranjitkar and Miss R. Malla for their practical help.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

ISOLATION OF *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS* FROM CRUCIFER SEEDS USING A HIGHLY SELECTIVE MEDIUM

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ABSTRACT

Ampicillin, penicillin G, and tetracycline completely inhibited the growth of *Xanthomonas campestris* pv. *campestris* (*X.c.c.*) B-24 at 6.25 µg/ml or less whereas carbenicillin, cephalothin, gentamicin, and kanamycin inhibited *X.c.c.* at 25 to 80 µg/ml. Bacitracin, neomycin, and streptomycin were least inhibitory with the minimal inhibitory concentrations at >400, 160, and 640 µg/ml respectively. CS20ABN medium was developed for isolating *X.c.c.* from crucifer seeds. Samples of 50,000 seeds per lot were washed for 2 hr at room temperature in 250 ml saline. The cheesecloth-filtered washings were centrifuged, and the pellets, resuspended in 8 ml saline, were diluted to 10^{-2}. A 0.1 ml sample was pipetted onto CS20A and CS20ABN. Saprophytic bacteria were too numerous to count and overgrew *X.c.c.* on all media except CS20ABN where 59-100% of recovered colonies were *X.c.c.*

INTRODUCTION

Among the various methods for the detection of seedborne pathogens, plating washings of seeds onto a semiselective medium to isolate pathogens has played a significant role for obtaining healthy and clean seeds (4). Media including NSCA (3), NSCAA (3) BSCA (3), and FS (5) have been widely used in seed laboratories for the detection of *Xanthomonas campestris*

pv. campestris on and in crucifer seeds. In July of 1988 when the seed wash time was changed from 3 min to 2 hr at room temperature, we observed the overgrowth of the saprohytic bacteria over the X.c.c. on NSCA, NSCAA, and BSCA plates which resulted in no recovery of X.c.c. A redilution of the washings to 10^{-3} and 10^{-4} became necessary in order to isolate X.c.c. from readable plates. An alternative approach to handle seed lots heavily infested with saprophytes was to develop a medium that inhibited the growth of saprophytes without significantly suppressing X.c.c. This work described how a new semiselective medium was developed and how it helped in the seed assay program.

MATERIALS AND METHODS

Antibiotic spectrum of X. c. pv. campestris grown in CS20 medium. X.c.c. strain B-24 was used for the study of its antibiotic spectrum to the following ten antibiotics (Sigma, St. Louis, Missouri): ampicillin sulfate, bacitracin (59,400 U/g), carbenicillin, cephalothin, gentamicin sulfate, kanamycin sulfate, neomycin sulfate, penicillin G potassium salt, streptomycin sulfate, and tetracycline hydrochloride. Stock solutions were made with sterile distilled water. Eight two-fold dilutions starting with the following concentrations were used: ampicillin, carbenicillin, cephalothin, penicillin G, and tetracycline, 100 µg/ml; gentamicin, kanamycin, neomycin, and streptomycin, 640 µg/ml; and bacitracin, 400 µg/ml. One ml of each antibiotic stock solution was added into 499 ml of CS20 agar medium (1,2) when cooled to 45 °C. Control medium was of CS20 agar without antibiotics. The molten agar with antibiotic was poured to a depth of 2.5-3.0 mm, in 100 x 15 mm plastic Petri plates (Fisher). All plates were stored at 4 °C and allowed to come to room temperature prior to usage.

A 0.1 ml sample of the log-phase culture of X.c.c. B-24 was pipetted onto duplicate agar plates and spread using an alcohol flamed L-shaped glass-rod and turn table. After 5 days at 30 °C, the plates were examined under a binocular

microscope at 50x. The minimal inhibitory concentration (MIC) was defined as that concentration resulting in no bacterial growth.

Formulation of a new semiselective CS20ABN medium. The CS20ABN agar medium was prepared by adding stock solutions of bacitracin, neomycin, and cycloheximide into CS20A medium before pouring the plates to obtain a final concentration of bacitracin, neomycin, and cycloheximide at 100 µg/ml, 40 µg/ml, and 100 µg/ml respectively. The CS20A agar medium contained all the ingredients found in CS20 agar with the following two modifications: (1) the omission of hemin chloride and phenol red and (2) the change of the potato starch concentration to 10 g. CS20A broth medium was prepared as the above without agar.

Evaluation of CS20ABN medium. Five pathogenic strains of X.c.c., originally isolated from commercial crucifer seed lots, were grown in CS20A broth overnight. Each strain was then adjusted to a Klett reading of 50 and from which a series of 10-fold dilution was made to 10^{-8} with CS20A broth. A 0.1 ml sample from each of three dilutions, 10^{-6}, 10^{-7}, and 10^{-8}, was pipetted onto triplicate plates of CS20A and CS20ABN. After 3 days at 30°C, the colonies per plate at 10^{-6} dilution for each medium and strain were recorded and the average diameter (mm) of 10 colonies from each plate at 10^{-6} dilution were measured.

Both CS20A and CS20ABN media were used for the detection of X.c.c. in commercial seed lots following the procedures reported by Randhawa and Schaad (3) except samples of 50,000 seeds per lot were washed for 2 hr at room temperature in 250 ml saline.

RESULTS

The antibiotic spectrum of X. c. pv. campestris strain B-24. The minimal inhibitory concentration of ampicillin, bacitracin, carbenicillin, cephalothin, gentamicin, kanamycin,

neomycin, penicillin G, streptomycin, and tetracycline were 6.25, >400, 25, 50, 80, 80, 160, 3.13, 640, and 1.56 μg/ml respectively. Bacitracin, neomycin, and streptomycin were least inhibitory to X.c.c. among the ten antibiotics.

Evaluation of a new semiselective medium CS20ABN. Based on the mean sizes of colonies and average numbers of colonies recovered per plate of 5 strains of X.c.c., there was no significant difference in growth when grown on CS20A and CS20ABN (Table 1) indicating that the antibiotics incorporated in CS20A medium were not inhibitory to X.c.c.

Of the twelve commercial seed lots assayed, saprophytes overgrew X.c.c. in all cases on CS20A medium (Fig.1) whereas 59-100% colonies recovered on CS20ABN were X.c.c. (Fig. 1 and Table 2), indicating the high specificity of the new medium.

Table 1. Comparison of the size of colonies and the mean colonies recovered per plate of 5 strains of Xanthomonas campestris pv. campestris grown on CS20A and CS20ABN media

	Colony diameter[a] (mm)		Mean colonies[b] per plate	
Strain	CS20A	CS20ABN	CS20A	CS20ABN
1	4.6	4.2	15.3	14.3
2	2.9	2.7	10.0	13.3
3	3.0	3.5	23.3	15.7
4	3.3	3.6	20.7	18.7
5	2.6	3.8	19.7	17.7
Average	3.3	3.6	17.8	15.9

[a]Average diameter of 30 colonies for each strain and medium.
[b]Average of colonies of 3 plates for each strain and medium.

DISCUSSION

A new medium CS20ABN was developed for detecting X.c.c. on or in crucifer seeds that were heavily contaminated with saprophytes which usually overgrew the pathogens on NSCA and NSCAA media. The CS20A medium supports better growth for X.c.c. than NSCA and NSCAA (C. J. Chang, unpublished). This

made CS20A an ideal basal medium for the development of a semiselective medium for the detection of X.c.c. from crucifer seeds. Through the investigation of the minimal inhibitory concentrations of antibiotics to X.c.c., not only could the kinds of antibiotics be selected for the incorporation into basal medium but also the concentrations of the antibiotics could be determined. For example, we were able to select bacitracin and neomycin at 100 and 40 µg/ml respectively for the semiselective medium CS20ABN. The concentration of bacitracin (100 µg/ml) used in CS20ABN happened to be the same as the maximum uninhibitory concentration determined by Randhawa and Schaad (3). They, however, did not use bacitracin in NSCA, NSCAA, and BSCA (3) because bacitracin offered no advantage over nitrofurantoin and vancomycin. A combination of bacitracin and neomycin in CS20A was able to reduce the background saprophytes to less than 20% and in many cases to no saprophytes at all (Table 2).

Table 2. Recovery of Xanthomonas campestris pv. campestris from commercial seed lots on CS20ABN medium[a]

	Mean colonies per plate[b]		
Seed lot	Seed bacteria(S)[c]	X.c.pv.campestris(X)	X/S(%)
1	103	103	100
2	17.5	17	97
3	52	52	100
4	66	66	100
5	97	57	59
6	16.5	16.5	100
7	178.5	153	86
8	60	60	100
9	72.5	72.5	100
10	132	106.5	81
11	85.5	85.5	100
12	96	96	100

[a]Assayed by agar plating of washings from 50,000 seeds (3).
[b]Two replications.
[c]Seed bacteria included colonies of saprophytes and X.c.c.

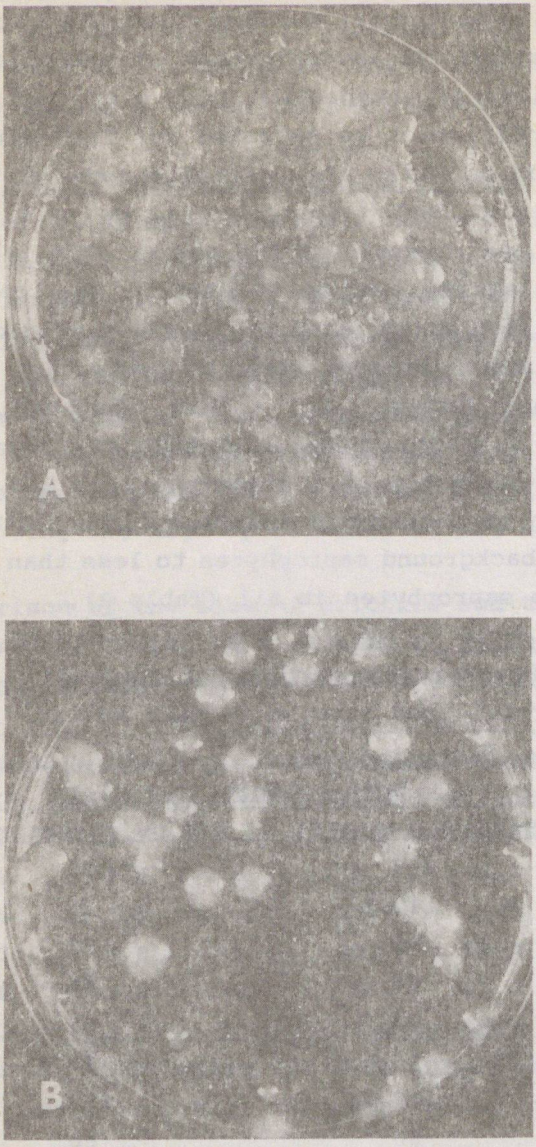


Fig. 1. The overgrowth of saprophytes on CS20A (A) and pure culture of X. c. pv. campestris on CS20ABN (B).

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

VARIATION IN PATHOGENICITY AMONG NIGERIAN ISOLATES OF XANTHOMONAS CAMPESTRIS PV. MALVACEARUM (SMITH) DYE

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ABSTRACT

In determining the range of virulence within the Nigerian population of Xanthomonas campestris pv. malvacearum, 68 isolates were each inoculated on 11 cotton differential lines. Fifty percent of the isolates (34) were the most prevalent and pathogenic to resistance genes B_2, B_7, B_{In} and B_N, while less than two percent of the isolates (2) were the least prevalent and attacked either a polygenic background and, or, genes B_2, B_7 and B_{In}. No isolate was pathogenic to the B_2 B_3 gene combination, B_4 gene, and the recently designated B_{12} gene. The isolates were categorized into nine race groups, using the U.S. and Indian systems. The implications of these findings in breeding for stable resistance to bacterial blight of cotton is Nigeria are discussed.

INTRODUCTION

Variation in levels of virulence and aggressiveness within populations of Xanthomonas campestris pv. malvacearum (Smith) Dye (XCM), the casual organism of bacterial blight of cotton, has been established in almost all cotton producing regions of the world (5, 6, 7). Resistance, conferred by major genes and polygenic complexes, has almost always been subsequently overcome by new, virulent forms of the pathogen (1, 2, 4, 6).

Two methods of categorizing isolates of XCM varying in levels of virulence have been adopted by most researchers in different parts of the world. On the basis of reactions of a set of ten host differential lines, possessing different major gene(s) for resistance, nineteen races have been identified using the U.S. Verma and Singh (9) in India proposed a new system of designating races of the bacterium. Thirty two race groups were proposed and biotypes identified on the basis of virulence to the differential line, Gregg. Although the two systems have been shown to be valuable; they are un-

likely to define the whole range of virulence in the population of XCM. In Africa, for example, the recent evolution in Burkina Faso, Chad and Sudan of highly virulent isolates (HV_1, HV_3, HV_7, CH_1, CH_2, CH_3, SU_1, SU_2, SU_3) with associated virulence to all known major genes for resistance have rendered all the ten host differential lines susceptible (4, 6, 7). These virulent isolates are yet to be assigned race numbers since no suitable differentials have been identified.

The importance of defining the range of virulence in pathogen populations is to enable breeders and pathologists breed for durable resistance in the host (1, 2, 4, 6). Researchers, through recurrent selection, in Chad identified a cotton line (S295) which is highly resistant/immune to the most highly virulent isolate (HV_1). The gene conferring the resistance in S295 was shown to be monogenic and dominant (4, 8). It was designated gene B_{12} (8).

Differences in virulence among isolates of the pathogen from the three cotton zones of Nigeria have been demonstrated (3). Poswal (7) identified races 6, 7 and 10 and showed race 10 to be the most virulent and widely distributed. This paper provides additional information on the pathogenicity and virulence levels within the Nigerian population of XCM.

MATERIALS AND METHODS

The 68 isolates of XCM used in this study were obtained from diseased (angular leaf spot and/or vein-blight) cotton leaf samples collected from different locations within the three cotton zones of Nigeria. For inoculation purposes, bacterial suspensions, from pure cultures, were prepared and standardized in vials according to Poswal (7).

The 11 cotton host differential lines used are presented in Table 1. Acid-delinted seed of each of the lines were planted in plastic pots, in a glasshouse. Five plants of each line, at the 8 to 10 true leaf stage were inoculated on the underside of the leaves, with each of the 68 isolates of the pathogen, using the tooth-pick scratch technique (1).

Plants were graded twice, for disease reactions, from 15 to 21 days after inoculation using the method of Hunter et al. (5). The isolates were categorized into virulence groups and races designated according to the U.S. and Indian systems (1, 5, 6, 7, 9).

Table 1. Cotton host differential lines and their bacterial blight resistance genes

Line	Resistance gene(s)
Acala 44	None
Stoneville 2B-S9	Polygenes
Stoneville 20	B_7 + polygenes
Mebane B-1	B_2 + polygenes
1-10B	B_{In} + polygenes
20-3	B_N + polygenes
101-102B	B_2B_3 + polygenes
Gregg	?
Empire B_4	B_4
DP x P_4	?
S295	B_{12}

RESULTS

The reaction of the isolates of XCM on the 11 host differential lines are presented in Table 2. The 68 isolates are categorized into nine isolate-virulence groups, namely A, B, C, D, E, F, G, H, and I. Group A was the most prevalent (50.0%) and pathogenic, attacking the polygenic background in Stoneville 2B-S9 and major genes B_7, B_2, B_{In} and B_N. The least virulent

Table 2. Pathogenic (+) and non-pathogenic (-) reactions of sixty eight Nigerian isolates of X.c. pv. malvacearum on eleven host differential cotton lines

Host Differential line	Resistance gene(s)	Isolate virulence group[a]								
		A (50.0%)	B (26.5%)	C (5.9%)	D (4.4%)	E (4.4%)	F (4.4%)	G (1.5%)	H (1.5%)	I (1.5%)
Acala 44	None	+	+	+	+	+	+	+	+	+
Stoneville 2B-S9	Polygenes	+	+	+	+	+	+	+	-	+
Stonevilla 20	B_7+polygenes	+	+	+	-	-	+	+	+	-
Mebane B-1	B_2+ "	+	+	-	+	+	-	+	+	-
1-10B	B_{In}+ "	+	-	+	+	+	-	+	+	-
20-3	B_N+polygenes	+	+	+	+	-	+	-	-	-
Gregg	?	+	+	-	+	+	+	+	+	+
DPx P_4	?	+	+	-	+	-	+	+	+	+
Empire B_4	B_4	-	-	-	-	-	-	-	-	-
101-102B	B_2B_3+polygenes	-	-	-	-	-	-	-	-	-
S295	B_{12}	-	-	-	-	-	-	-	-	-

[a]In parenthesis below each virulence group is the percentage of the isolates in that group.

Table 3. Race categorization of the nine isolate-virulence groups, representing the sixty eight isolates of X. c. pv. malvacearum

Virulence group[a]	Resistance gene(s) matched	Race categorization	
		Hunter et al. (1968)	Verma and Singh (1974)
A	P,B_2,B_7,B_{In},B_N	10	32A
B	P,B_2,B_7,B_N	16	28A
C	P,B_7,B_{In},B_N	14	29
D	P,B_2,B_{In}	7	30A
E	P,B_2,B_{In},B_N	6	20A
F	P,B_7,B_N	17	19A
G	$P,B_7\ B_2,B_{In}$	8	27A
H	B_7,B_2,B_{In}	-	23A
I	P	11	2A

[a]The polygenic background represented by P is that of the differential line, Stoneville 2B-S9.

group (I) was only pathogenic on polygenes (Stoneville 2B-S9). The least prevalent groups were G, H and I. All the isolates were pathogenic on Acala 44 (no resistance gene) and non-pathogenic on Empire B_4 (B_4), 101-102B ($B_2\ B_3$), and S295 (B_{12}). With the exception of isolates in group C, the rest was pathogenic on Gregg, while only isolates in group C and E were pathogenic on $DPxP_4$.

The isolate-virulence groups A, B, C, D, E, F, G and I are similar to races 10, 16, 14, 7, 6, 17, 8, and 11 of the U.S. system respectively (Table 3), the corresponding races under the Indian system being races 32A, 28A, 29, 30A, 20A, 19A, 27A and 2A, respectively.

DISCUSSION

Evidence for the variation in pathogenicity among Nigerian isolates of XCM was first provided by Dransfield (3). However, it was Poswal (7) who established the presence of races 6, 7 and 10 in the three cotton zones of Nigeria. It was concluded that these races evolved within the plant populations of the current commercial varieties, Samaru 71 (no genes for resistance) and Samaru 77 (possessing the B_2 gene). The results of the present

study have further established the presence of additional virulence groups in the Nigerian population of the pathogen. The determination of the full range of virulence would depend on the further testing of large numbers of widely distributed isolates of XCM.

The wide distribution of the most virulent isolate (race 10) has some implications for the breeding for bacterial blight resistance in Nigeria. Potential replacements for Samaru 71 and Samaru 77, and indeed all breeding materials, need to be screened against the array of virulence genes of XCM (7). Bird (1) and Brinkerhoff et al. (2) in the U.S. have successfully bred highly resistant bacterial blight cotton cultivars, by using mixtures of races of the pathogen in inoculations of segregating breeding populations.

None of the Nigerian isolates of SCM were pathogenic to the B_4, B_2 B_3 and B_{12} genes for resistance. The differential line 101-102B with the B_2 B_3 gene combination has been immune for the past 20 years (2,6,7). It was rendered susceptible, however, by the highly virulent isolates (HV_1, HV_3, HV_7, CH_1, CH_2, CH_3, SU_1, SU_2, SU_3) that evolved in Burkina Faso (Upper Volta), Chad and Sudan (4, 6, 7). While the results of the present study point to the fact that these highly virulent isolates from these neighbouring countries may not be present, at this time, in Nigeria, it would be necessary to continuously monitor for their appearance. Meanwhile, in preparation for the eventual occurrence of these isolates in Nigeria, steps have been taken at Samaru to incorporate the B_{12} gene into the breeding materials.

ACKNOWLEDGEMENT

I am grateful to Professors I.D. Erinle and A.M. Emechebe, and Dr. A.D. Akpa for reviewing the manuscript.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

A NEW XANTHOMONAD: CAUSAL AGENT OF BACTERIAL BLIGHT OF MILKWEED (ASCLEPIAS SPP.)

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ABSTRACT

Milkweed (Asclepias spp.), often a serious perennial weed in agricultural crops, is now being grown for its commercial value. A bacterium was isolated on NBY from blighted areas of leaves from field grown milkweed. The bacterium could also be isolated from seed and rhizomes. The causal agent was identified by comparative analyses with other bacteria using polypeptide profiles and fatty acid analyses, substrate utilization and xanthomonadin pigment extraction, and growth on semi-selective medium. Host range studies with members of the milkweed family and various commercial crops grown in Nebraska showed that the bacterium was specific for certain species of Asclepias. The name proposed for this bacterium is Xanthomonas campestris pv. asclepiadis.

INTRODUCTION

Milkweed is currently being grown for properties of its floss to be used in clothing, blankets, and other items. It has previously been grown for other purposes as well (Berkman 1949; Gaertner 1979; Schwartz 1987). Plants with blighted areas of leaves were observed in two Nebraska fields and collected for diagnosis (Figure 1).



Fig.1. (A) Healthy milkweed; (B)(C) Diseased milkweed in field; (D) Greenhouse reproduction of symptoms

MATERIALS AND METHODS

Isolation of the pathogen. Leaves were washed under running water for 15 min. Tissue adjacent to chlorotic areas was macerated using a mortar and pestle. Dilutions were plated onto a general purpose medium, NBY (Vidaver 1967).

Inoculations. Resultant colonies were obtained and inoculated into healthy milkweed tissue. Plants were inoculated in the lower stem with 10^6 CFU/ml of cells using a 1cc syringe and 26 1/2 G needle. Isolates that reproduced symptoms upon reinoculation were saved for further study.

Identification of the pathogen. The pathogen was identified using several techniques. Growth was evaluated on the semi-selective medium MXP (Claflin et al. 1987). The yellow pigmented bacterium was tested for the presence of xanthomonadin pigment using a thin layer chromatography procedure modified from the method of Irey and Stall (1981). Fatty acid content was analyzed separately by Dr. M. Sasser. Substrate utilization profiles were obtained using Biolog Microplates (Biolog, Inc., Hayward, California). Polypeptide profiles were obtained and examined using a modified procedure of Carlson and Vidaver (1982).

Host Range. The host range of the organism was tested by inoculating the pathogen into five species of Asclepias, as well as into six major crops of Nebraska, including maize, sorghum, wheat, soybean, alfalfa, and dry bean. Tomato, a common home-garden plant was also tested.

RESULTS

The bacterium was readily isolated from diseased plant tissue. The organism was isolated from seed as well as leaf, root, stem, and flower parts. Symptoms were reproduced upon inoculation into healthy plants. Growth occurred on MXP with starch hydrolysis. Pigment analysis

indicated the presence of xanthomonadin pigment. Polypeptide profiles showed a similarity to the genus Xanthomonas, especially pathovars of Xanthomonas campestris. Fatty acid analysis also indicated a similarity to pathovars of Xanthomonas campestris. Substrate utilization profiles were as indicated in Table 1. Four of the five Asclepias spp. tested showed susceptibility to the pathogen. None of the major crops nor tomato were susceptible to the bacterium.

TABLE 1. CARBON UTILIZATION ASSAY[a]

BACTERIUM	CARBON SOURCE												
	glycogen	N-acetyl glucosamine	m-inositol	lactose	maltose	D-mannitol	D-sorbitol	acetic acid	citric acid	malonic acid	glycerol	dextrin	D-melibiose
Milkweed strain 0006	+	+	-	-	+	-	-	-	+	+	+	+	-
X. campestris pv. campestris 528 NCPPB	+	+	-	-	+	-	-	-	+	+	+	-	+
X. campestris pv. glycines 11766 ATTCC	+	+	-	+	+	-	-	-	+	+	+	+	-
X. campestris pv. holcicola 3103 PDDCC	-	+	-	-	-	-	-	-	-	-	-	-	-
X. campestris pv. phaseoli LB-1	+	+	-	-	+	-	-	+	+	+	+	+	+

[a]Biolog GN microplates were used for carbon utilization assays. Of 95 carbon sources, 13 are shown here.
+ = carbon utilization - = no utilization

DISCUSSION

Bacterial blight of milkweed presents a formidable deterrent to the cultivation of common milkweed. Isolation of the pathogen from seed and other plant parts indicates a systemic nature of the disease. At the present time methods for control of the organism are not established. The organism, however, may be an effective biological control agent for milkweed in situations where it is undesirable.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
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A NEW HOST PLANT OF BACTERIAL BLACK SPOT OF MANGOES: PEPPER TREE (SCHINUS TEREBENTHIFOLIUS RADDI)

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Bacterial black spot of mangoes (*Mangifera indica* L.) caused by *Xanthomonas campestris* pv. *mangiferaeindicae* is the most serious disease of Mango tree in many countries (1, 2, 4, 5). Patel *et al.* (6, 7) described the disease in India in 1948. They mentioned Cashew (*Anacardium occidentale* L.) and Imara (*Spondias mangifera* L.) as other hosts for the disease. Robbs *et al.* (9) showed pathogenicity of pv. *mangiferaeindicae* to Mombin (*Spondias mombin* L.) by artificial inoculation. All these plant species belong to *Anacardiaceae*. Recently, Rott and Frossard (10) isolated an apigmented *Xanthomonas* causing a dieback on Ambarella (*Spondias cytherea* Sonn. = *Spondias dulcis* Forst.) in Martinique (French West Indies). These strains were pathogenic to Mango by artificial inoculation (8).

In this study, the authors put forward a new host plant that is a member of the *Anacardiaceae*. This species is very common in Reunion Island and probably all over tropical and subtropical areas.

On Pepper tree under Reunion Island conditions, symptoms of the disease occur only on leaves. The bacterium causes black raised angular spots (Fig. 1), as on mango leaves, but their size is smaller on Pepper than on Mango.

Bacterial colonies that have cultural characteristics similar to pv. *mangiferaeindicae* were isolated on LPGA medium. The five strains isolated from Pepper tree were identified as *Xanthomonas campestris* , using classical biochemical tests (3).

Serological studies, using immunofluorescence, were conducted with an antiserum raised against *Xanthomonas campestris* pv. *mangiferaeindicae* NCPPB 490 (neopathotype strain). A strong affinity of Pepper isolates (similar to the homologous reaction) with this antiserum occurred.

Three Pepper strains (CFBP 2928, 2935 and 2940) and one Mango strain (CFBP 2933), used as a control, were inoculated on Mango leaves cv. José (10^8 and 10^6 c.f.u./ml).

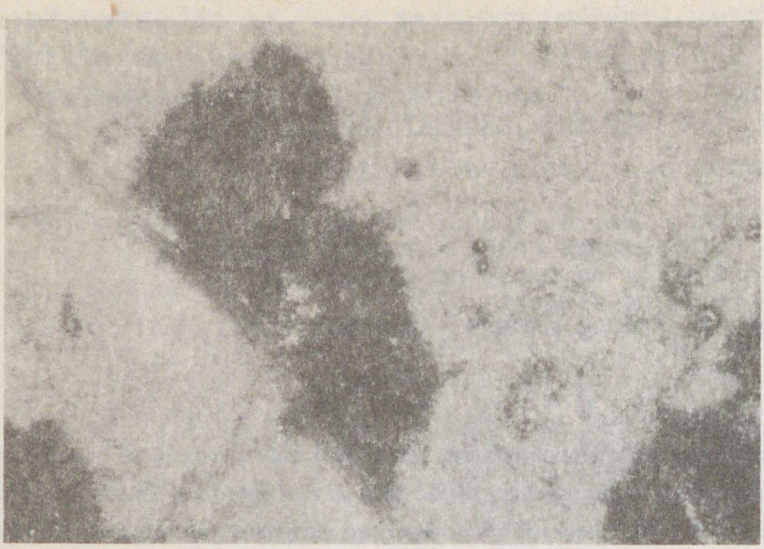


Fig. 1 : Lesion located on a Pepper leaf.

The two bacterial concentrations assayed induced typical symptoms of the disease. After 42 days, percentages of induced lesions were respectively about 90% for 10^8 c.f.u./ml inocula and 60% for 10^6 c.f.u./ml inocula (Fig. 2 and 3). Virulence of Pepper strains compared to the mango isolate was statistically equivalent or higher, depending on dates and bacterial concentrations.

The aggressiveness of 94 pv. *mangiferaeindicae* strains from several hosts (Mango, Pepper, and Ambarella) and with variable cultural characteristics (apigmented and pigmented isolates) was studied on Pepper tree, by wound-inoculating leaves. All the isolates, except strain NCPPB 3078 were shown to be pathogenic on Pepper tree after five weeks. Percentages of induced lesions fluctuated respectively from 0 to 92.5% for a 10^8 c.f.u./ml inoculum and from 0 to 31.3% for a 10^6 c.f.u./ml inoculum.

According to the Duncan's multiple range and Kramer's tests, strains have been classified in 3 classes :

A) high aggressiveness

B) intermediate aggressiveness

C) low aggressiveness

Ranging the studied strains in the 3 described groups, showed that only homologous isolates (from Pepper tree) belong to the A class. Most of the apigmented isolates from Mango, as well as strains isolated from Ambarella were classified into the B class. A few Mango apigmented isolates and all the pigmented strains from Brazil and Reunion Island were classified into the C class.

Fig. 2 : Percentages of induced lesions after inoculation of Pepper and Mango isolates on Mango tree (10^8 c.f.u./ml). Asin-transformed data were compared for each date.

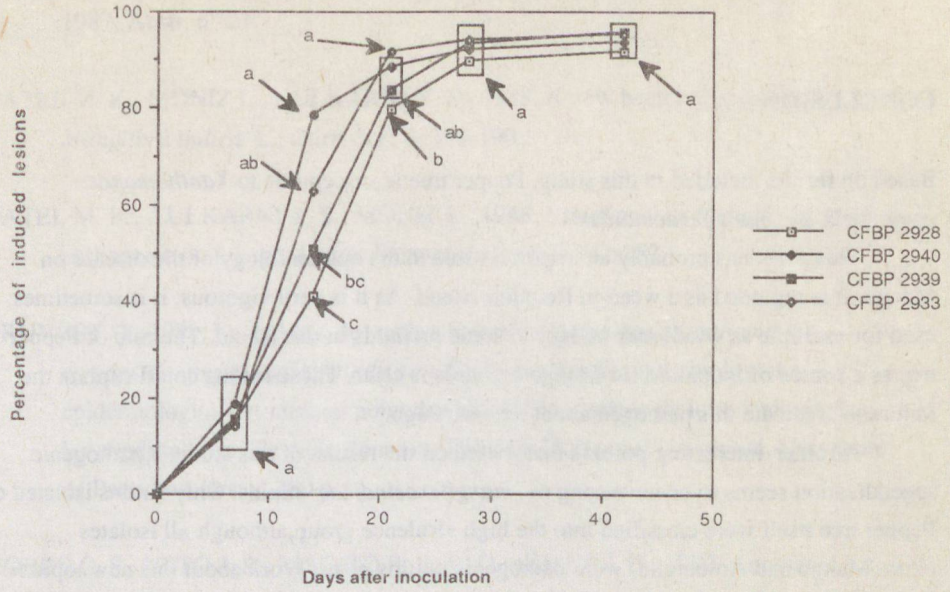
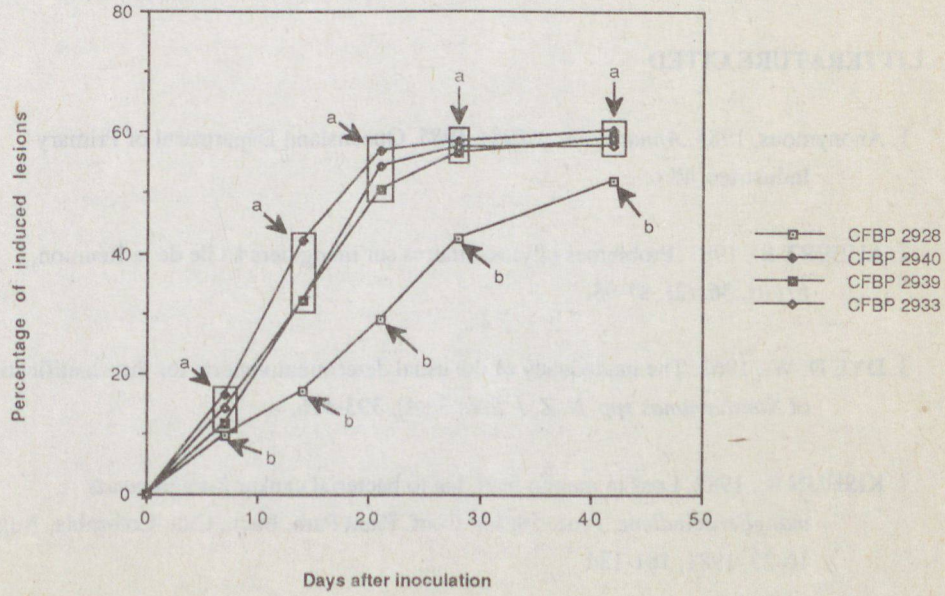


Fig. 3 : Percentages of induced lesions after inoculation of Pepper and Mango isolates on Mango tree (10^6 c.f.u./ml). Asin-transformed data were compared for each date.



In the case where only apigmented strains from Mango (81 isolates) are considered, there is no correlation between virulence, geographical distribution, and year of isolation.

CONCLUSION

Based on results included in this study, Pepper tree is susceptible to *Xanthomonas campestris* pv. *mangiferaeindicae*.

This plant has probably an important role in the epidemiology of the disease on Mango. It is regarded as a weed in Reunion Island. As it is very vigorous, it is sometimes used for example as windbreak hedges in some orchards in this island. The role of Pepper tree as a source of inoculum for Mango orchards is clear. These results could explain the failure to eradicate this pathogen about ten years ago.

Another interesting point is that, based on the results of this study, a pathogenic specialization seems to occur among pv. *mangiferaeindicae* strains. Only strains isolated on Pepper tree itself were classified into the high virulence group,although all isolates (from Mango and Ambarella) were pathogenic on this plant. Work about this new topic is being done further.

Finally, these results confirm the low virulence of pigmented strains of pv. *mangiferaeindicae* , that has been previously demonstrated on Mango (8).

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

POLYAMINES OF PHYTOPATHOGENIC BACTERIA

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Tests which can be used for rapid identification and differentiation of phytopathogenic bacteria aimed to work out the effective measures for their control, and also the estimation of their affinity with other bacteria are needed. The preliminary experiments showed that bacterial polyamines are long-term (1). Interest in polamines greatly increased after it was proved these substances were specific for the related microorganisms, and their grouping by polyamine composition correlated with the characteristic of their 16S rRNA (2). Such experiments appeared to be successful (3).

The present work shows the distribution of classic amines (putrescine, spermine, spermidine) among phytopathogenic bacteria of the various systematic groups. The objects of study were as follows: phytopathogenic bacteria, the members of *Enterobacteriaceae* (genus *Erwinia*), *Pseudomonadaceae* (genera *Pseudomonas*, *Xanthomonas*), *Rhizobiaceae* (genus *Agrobacterium*) and coryneform bacteria (genera *Corynebacterium*, *Curtobacterium* and *Rhodococcus*). The polyamines were isolated from intact or ultrasound-destroyed bacterial cells, extracted with perchloric acids, separated by electrophoresis, identified with standards (4).

The obtained results demonstrated that the members of the various systematic groups differed from each other in the properties of polyamines extracted from cells of these bacteria with perchloric acid. Putrescine and spermidine were extracted from cells of *A. tumefaciens* isolated from the various host-plants in the various geographic regions of the world. The

Table 1. Bacterial strains used in this study

Family, genus, species, strains numbers	Obtained from:
1. *ENTEROBACTERIACEAE:*	
E. amylovora ATCC 15580, *E. cypripedii* NCPPB 3004, *E. herbicola* NCPPB 2771, *E. mallotivora*NCPPB 2851, *E. nigrifluens* NCPPB 564, *E. rhapontici* NCPPB 1578, *E. rubrifaciens* NCPPB 2021, *E. tracheiphila* PDDCC 5845, E. lathyri	Dr. De Ley, Belgium, Rijksuniversiteit
E. carotovora subsp. *atroseptica* 21A, 27A, 30A, 36, 36A, 37A, 39A, 8349, 8912, 8913, 7200 NCPPB 3386, NCPPB 3390, NCPPB 3404, 3406, NCPPB 549	CPPIMV Inst. of Microb. and Dr. de Boer S.H., Canada, Vancuver
E. carotovora subsp. *carotovora* 67, 246, 73, 144a, 173, 718, 741, 566, 48, 53, 56, 71, 92, 133, 216, 258, 921, 743, 808, 890, 7869, 8634, 8941, ATCC 16713, NCPPB 438, NCPPB 392, NCPPB 468, NCPPB 547, NCPPB 550	Dr. De Ley, CPPIMV Ukr. Acad. of Sci., USSR
E. chrysanthemi NCPPB 402, 73, B-73, 141, 248, B-100, G18-3, NCPPB 516, NCPPB 1955, EP3, 910, B-27, NCPPB 898, EA-15	Dr. M. Kocur, Czechoslovakia; Dr. De Ley, Dr. Dickey R.S. USA, Cornell Univ.
Escherichia coli ATCC 11775	Dr. M. Kocur
2. *RHIZOBIACEAE:*	
Agrobacterium tumefaciens B_6, 2x, 5, 7x, 57, 58, 59, 60, 61, 62, 1948, 8592, 8628, 8459, 8460, 8465, 8466, 8468, 8469, 8471, 8891 8892, 8996, 8998, 8999, 9000, 9001	CPPBIMV Ukr. SSR Acad. of Sci., USSR
A. rubi ATCC 13335, *A. radiobacter* 8994,	
Rhizobium leguminosarum 250a, 254, *R. trifolii* 311, 348, *R. melilotti* 425a, 435a,	
R. japonicum 629	
3. *PSEUDOMONADACEAE:*	
P. syringae and other species, *X. campestris* (pathovars and numbers indicated in text)	CPPBIMV of Ukr. Acad. of Sci., USSR

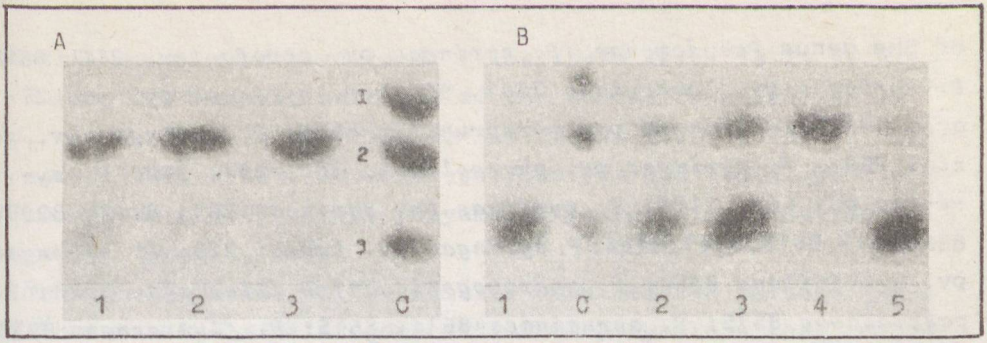


Fig. 1. The composition of polyamines: A-1-*A. tumefaciens* CCM I000; 2-*A. radiobacter* CCM 2054; 3-*A. rubi* ATCC 13335. B-1-*E. carotovora subsp. carotovora* ATCC 15713; 2-*Escherichia coli* ATCC 11774, 3-*P. syringae* pv. *syringae* 281; 4-*X. campestris* pv. *campestris* 8162; 5-*X. beticola* 7325; C-control: 1-spermine, 2-spermidine, 3-putrescine. Paper electrophoresis, sodium-citrate buffer, pH 3.6? the period separation 2,5 h.

prevalence of spermidine over putrescine which remained within the period of culture growth was observed (Fig. 1A).

The composition of polyamines from members of other species of genus *Agrobacterium* (*A. radiobacter* CCM 2054, *A. rubi* ATCC 13335) and genus *Rhizobium* (*E. leguminosarum* 250a, 254; *R. trifolii* 311, 348; *R. melilotti* 425, 435a; *R. japonicum* 629) was similar to that of strains of *A. tumefaciens*.

In contrast to the members of the family *Rhizobiaceae* for which polyamines appeared to be specific on the family level, polyamine composition of the members of the family *Pseudomonadaceae* was specific on genus level (for some exclusions). In bacteria of genus *Xanthomonas* (*X. campestris* pv. *campestris* 8162, 8003, 8659; *X. campestris* pv. *begoniae* 1, 8660; *X. campestris* pv. *cucurbitae* 8684; *X. campestris* pv. *glycines* 3,8393; *X. campestris* pv. *holcicola* 18a, 8662; *X. campestris* pv. *juglandis* 8865; *X. campestris* pv. *malvacearum* 8838; *X. campestris* pv. *papavericola* 8661; *X. campestris* pv. *phaseoli* 262, 7992; *X. campestris* pv. *pruni* 8590) irrespective of agent specialization and their virulence and differences by other properties, the extracted amines are presented by spermidine except for *X. beticola*, the polyamines of which are presented by putrescine and spermidine (Fig. 1B 4,5). In members of the majority of species

of the genus *Pseudomonas (P. syringae* pv. *atrofaciens* 2442, 8611; *P. syringae* pv. *lachrymans* 7591, 9003; *P. syringae* pv. *maculicola* 181; *P. syringae* pv. *morsprunorum* 8655; *P. syringae* pv. *panici* 7693; *P. syringae* pv. *phaseolicola* 300, 301, 303; *P. syringae* pv. *pisi* 7156; *P. syringae* pv. *syringae* 281, 8414, 8297, 8300,8511,8512,8547,8566; *P. syringae* pv. *tabaci* 229; *P. syringae* pv. *viridiflava* 8868; *P. andropogonis* 71; *P. wieringae* 7923; *P. pastinaceae* 8575; *P. aeruginosa* 8614, 8615; *P. fluorescens* 8938; *P. putida* 4126, 8617; *P. aurantiaca* 8613; *P. mendocina* 8676; *P. xanthochlora* 8536, 8538) the extracted amines are presented by putrescine and spermidine (Fig. 1B, 3), in strains of *P. marginalis (syn. P. alliicola)* - by spermidine, in P. cepacia - by putrescine. No correlation was found between bacteria pathogeneity and the composition of their polyamines. Phytopathogens of the genus *Pseudomonas*, conditionally-pathogenic and saprophyties, highly specialized agents and polyaphages had similar composition of extracted amines.

Bacteria of genus *Erwinia* differ by polyaminecomposition. In typical pectobacteria, *E. carotovora (E. carotovora subsp. carotovora, E. carotovora subsp. atroseptica)* the extracted amines are presented by putrescine; spermidine is absent or is presented in trace amounts. The identical polyamine composition was observed in *E. carnegieana* 671, two strains of *E. rhapontici* 572, 1578, one strains of *E. quercina* Acorn 2. By polyamine characteristic these agents differ considerably from Escherichia coli, type members of the family *Enterobacteriacea*, from *E. amylovora*, type member for the genus *Erwinia* and also from the members of other species of the genus *Erwinia* and the members of the family *Pseudomonadaceae* and *Rhizobiaceae*.

Escherichia coli, *E. amylovora*, the members of the various pathovars of *E. chrysanthemi* and members of the other species of the genus *Erwinia (E. tracheiphila, E. mallotivora, E. rubrifaciens, E. quercina, E. cypripedii, E. nigrifluens, E. herbicola)* are presented in our work as single but in most cases type culture. In perchloric extracts of these bacteria it was observed that putrescine content was higher than spermidine. Polyamines are similar in the wide range of species of the genus *Erwinia* to other enterobacteria and members of most species

of the genus *Pseudomonas* and *X. beticola* (Fig. 1B 2, 3, 5).

In phytopathogenic bacteria of the genera *Corynebacterium* (*C. sepedonicum* 7719, 7752; *C. insidiosum* 7770, 7775; *C. michiganense* 7738, 7744), *Rhodococcus fasciens* 8331; *Curtobacterium flacumfaciens* 6567, 6566, polyamines in perchloric extracts were not found.

Thus on the basis of the results obtained the related group of bacteria had similar composition of polyamines that can be used for their identification and differentiation. The level of the relationship among microorganisms having adequate polyamine composition at present is difficult to estimate due to the lack of study of polyamines from bacteria of the other systematic groups. The obtained data also confirm the uncertainty of the systematic position of some species of the genus *Erwinia*, and also of the genera *Pseudomonas* and *Xanthomonas*.

We know that the method we used for separation and identification of polyamines is not sensitive and does not help to find amines and their derivatives having close or similar electrophoretic mobilities. Use of more precise methods (liquid chromatography with high permitting ability, etc.) for polyamine separation can help to make some corrections in polyamine identification that would facilitate the deeper study of these agents. Besides, in the present work we studied the distribution among phytopathogenic bacteria of so-called classical amines, and in addition having weak links with cell organelles. The more complete information of these agents will be given undoubtely after analysis of polyamines from bacterial cells including so-called "unusual" amines observed in numerous microorganisms (5, 6).

The members of family *Enterobacteriaceae*, *Pseudomonadaceae*, *Rhizobiaceae* presented in the given work can be divided into three groups by characteristic of easily extractable amines. The first group consists of bacteria in which putrescine prevails in the form of amine. The second large group includes the members of most of the species of the genera *Erwinia* and *Pseudomonas* containing putrescine and spermidine. The members of genus *Xanthomonas* and family *Rhizobiaceae* form the third group. They possess spermidine as the main amine.

ACKNOWLEDGEMENTS

The authors thank Drs. De Ley, de Boer S.H., M. Kocur and R.S. Dickey for cultures kindly presented for comparative study.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

CHARACTERISTICS OF PHYTOPATHOGENIC PROPERTIES OF KLEBSIELLA GENUS BACTERIA

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Bacteria of Klebsiella genus are widely distributed in nature. They occur in samples of soil, of sea and fresh water, and are found as epiphytic microflora on grains, seeds, vegetables and green parts of plants, in sewage discharged by different industrial enterprises like cotton mills and breweries, etc. Moreover, bacteria of Klebsiella genus are a special object of medical microbiology [1]. Klebsiella are known, in particular, to be agents of hospital infections, diseases of newborns, etc. These bacteria are isolated from different things taken from hospital departments, including water from vases where flowers were kept.

K. ozaenae and K. rhinoscleromatis are agents of chronic klebsiellosis in man. Endemic character of their distribution determines the expediency to disclose the infection foci of these diseases in nature.

In connection with this the study of the property of Klebsiella to infect plants is of both theoretical and practical interest.

MATERIALS AND METHODS

Phytopathogenic properties were tested in strains of bacteria of Klebsiella genus recently isolated from patients and laboratory cultures which were continuously grown on nutrition media. In the present study 75 cultures of K. pneumoniae, 69 strains of K. rhinoscleromatis and 49 strains of K. ozaenae were used.

The property of Klebsiella to infect plants was tested by means of artificial infection. The infected objects were leaves of growing potatoes, beans cabbage, as well as cotyledons of cucumbers and squashes. Supersensitivity test of the studied cultures was performed on the leaves of young tobacco plants. Suspension of one-day-old bacterial culture in sterilized tap water

with concentration of 1 billion cells per 1 ml was used for inoculation. The bacterial suspension was injected under the epidermis of the cotyledons or leaf blades. Each strain infected every 5 plant , 4 - 5 leaves being infected in every plant. Content of pectolytic enzymes in Klebsiella was determined by biological method on potato slices.

Isolation of plasmids from seven strains of K. rhinoscleromatis and five strains of K. ozaenae possessing phytopathogenic properties was performed by Clewel's method [2]. Plasmid DNA molecular mass was defined by means of electron photographs. Preparations for electron microscopy were made by the technique of Kleinschmidt [3]. Electron micrography was made by electron microscope EM-100B under the accelerating voltage of 60 kv. Images of molecules were projected onto paper, the lengths were measured by a curvimeter. The final magnification was 100,000. In calculations the results of 20 or more measurements were used. E.coR1 DNA plasmids were used as the internal standard.

RESULTS AND DISCUSSION

The studies have shown that as a result of artificial infection by K. rhinoscleromatis strains used in the experiments 30 - 36% of the cultures caused lesion of the leaves of horse beans and potatoes, 40 - 45% caused lesion of the cotyledons of cucumbers and squashes, and 55% - of the cabbage leaves. The occurrence of pathogenic properties in the studied strains of K. ozaenae was similar.

20 to 65% of the studied K. pneumoniae cultures affected the leaves of growing potatoes, beans, cabbage, and the cotyledons of cucumbers and squashes as well. Pathogenic changes on the leaf blades of the plants were like necrotic lesions. Most often they appeared on the 5th - 7th day after the injection of the suspension of the studied cultures under the leaf epithelium. A considerable number of K. ozaenae strains, up to 65%, caused lesion of the cabbage leaves. Necrotic lesions appeared on the cabbage leaf blade in 6 - 8 days after the infection. In the case of epicutical injection of the bacterial suspension the affected leaf tissue acquired at first a darker shade than the surrounding unaffected tissue. It looked like greasy dark brownish-green spots. Then the affected places became thin and dry. Necrotic spots were surrounded by the chlorosis zone. The manifestation of the infection was not confined to the place of the injection of the culture, but it extended behind the veins, further affecting a considerable portion of the leaf.

In the case of injecting the Klebsiella culture suspension into the central vein of the cabbage leaf the spread of the infection was more intensive, and it caused the leaf blade to become first segmentally and then completely yellow and dry.

In the affected leaf we usually found the bacteria on the border of the normal and pathologically changed tissues. Bacteria of Klebsiella genus were not isolated from outwardly normal areas.

As the studies have shown, there is marked age dependence of susceptibility of plants on the klebsiellous infection. The plants possessed the highest sensibility to the infection agent in spring, when they were in the stage of shoots or had 3 to 5 leaves. At that time the manifestation of lesion on the cabbage leaves was observed after application of the infectious dose which was 2 or 3 orders lower (10^5 microbic bodies per 1 ml), than usual (10^7 microbic bodies per 1 ml). In the summer time, particularly at the stage of head setting, the resistance of plants to the bacterial infection caused by Klebsiella, increased greatly.

It should be noted that the cultures recently isolated from patients possessed, as a rule, greater virulence than those continuously cultivated on artificial nutrition media. Above all, bacteria, recently isolated from patients, affected a greater number of plants. Cabbage leaves were infected by 83% of recently isolated strains, potatoe leaves - by 72%, cucumber cotyledons - by 56%. Four years later phytopathogenic activity of these bacteria declined. We observed the loss of phytopathogenic properties in some strains of Klebsiella just after single re-inoculations of media. It is of interest that the loss of the phytopathogenic properties by the strains recently isolated from the infected plants was often observed by a number of researchers.

Hypersensitivity reaction of plants to the injection of the agent is an important and independent test of phytopathogenic properties of the bacteria.

To study the possibility of inducing the hypersensitivity reaction on tobacco leaves, affected by bacteria of Klebsiella genus, we used 192 strains in the experiment. The reaction was observed in a considerable number of the studied cultures. The percentage of the positive reactions was 87 for K. pneumoniae, 84 for K. ozaenae, 74 for K. rhinoscleromatis.

The conditions of the reaction corresponded to the optimum temperature regimes of growing plants, 23 to 25°C. Symptoms of affections developed rapidly. Initially a yellow portion of the leaf blade turned into necrotic area

in 24 to 36 hours, the leaf tissue became thin, dark, glistening, and looking like oil paper. At the same time the affected places were distinctly confined to the limits of culture suspension injection into intercellular space.

Phytopathogenic properties of the bacteria are connected in separate cases with production of pectolytic enzymes. Therefore, having stated the property of individual strains of Klebsiella to affect some plants, we determined the presence of pectinases in them. The studies have shown, that the pectolytic activity is only observed in 21% of K. pneumoniae, 14% of K. rhinoscleromatis, 24% of K. ozaenae. Hence, the phytopathogenic properties in Klebsiella do not correlate with the presence of the pectinase enzyme in them.

It should be pointed out that along with the phytopathogenic properties, entomopathogenic properties were also found in the bacteria cultures. Mass death of Lymantria dispar L. caterpillars in early and middle stages was observed caused by all the species of bacteria of Klebsiella genus, being 100% by the 15th day after the infection. The death of the insects due to the klebsiellous infection was accompanied by the symptoms of the bacterial infection and was confirmed by the bacteriological analysis of the bowels contents, emesis and hemolymph.

Taking into account the consideration of pathogenicity of bacteria as the function of their extrachromosomal hereditory factors, we studied the plasmid composition of 7 cultures of K. rhinoscleromatis and 7 strains of K. ozaenae which in the conditions of artificial infection were pathogenic for plants. As the studies have shown, 3 cultures of K. rhinoscleromatis and 4 cultures of K. ozaenae contained cryptic plasmid genomes in autonomic state. In these strains plasmids were present in a small number of copies - from 1 to 5. Their molecular mass was defined within the range $20{:}10^6$-110.10^6 dalton.

The studies do not give reasons for considering the presence of plasmids to be the necessary factor, ensuring the phytopathogenicity of bacteria of Klebsiella genus.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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THE REACTION OF SOME VARIETIES OF WEEDS TO VACUUM INFILTRATION WITH PSEUDOMONAS SYRINGAE PV. TOMATO

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During the last 20 years it has been widely recognized that a number of bacteria are capable of multiplying on healthy plants (host and non-host) (1, 2, 6, 11, 12). Research has been carried out mainly upon leaves thought to be the main habitats.The best known of the epiphytic bacteria belong to the **Pseudomonas syringae** group. (6, 11, 12).

Pseudomonas syringae pv. tomato (Pst) - the pathogen of bacterial speck on tomatoes develops symptomlessly on the leaves of tomatoes (5, 13) and epiphytically on weeds in areas with diseased tomato plants (1, 2, 8, 13).The weed plants in the tomato sowing can be differentiated into three groups according to whether **Pst** develops on them as an epiphyte:**typical weed hosts** on which the bacterium develops in the risosphere and the foliage always in a diseased tomato sowing;**non typical** - the bacterium develops epiphytically mainly on the foliage not always on diseased plants; **non-hosts** - on which the bacterium never develops. (2).Notwithstanding the intensive research in this field very little is known on how the bacteria develop epiphytically, what the type of reaction is , how long they survive on the weeds and if they can develop to the epiphytic phase on all of them.

The purpose of the following work is to establish the reaction of some varieties of weeds common to the tomato sowing to artificial contamination through vacuum infiltration and the duration of the survival of **Pst** on them.

Material and methods

From free areas, where tomatoes had never been cultivated, the seeds of 14 varieties of weeds have been taken: typical - **Amaranthus retroflexus, Solanium nigrum, Datura stramonium, Portulaca oleracea**; non typical - **Andropogon halepensis, Echinochloa crus galli, Abutilon avicennae, Chenopodium album, Setaria sp.**; non-hosts - **Polygonum sp., Sinapis arvensis, Setaria viridis, Hibiscus trionum, Sanguisorba minor.**

We contaminated the plants obtained from them in the phase first leaf through vacuum infiltration (2) with a bacterial suspension in concentration $10, 10^4, 10^8$ cfu/ml with a rifampicin -resistant strain of **Pst** (R_R) obtained by the method of Jardine et al (7). We cultivated the infiltrated plants in the nutritive solution of Knop in room temperature. For controls vacuumed plants of the same varieties were used with sterile water and tomatoes of the variety Chico III with **Pst** (R_R) 10^4 cfu/ml. We recorded their reaction 24 hours after their contamination as well as the appearance and type of symptoms on them. From the single spots on the separate varieties of weeds we made isolations by the method of **Jardine et al** (7).

In order to establish the duration of the survival of **Pst** on groups of weeds on the 5th, 12th, 18th, 25th, 35th and 40th day (after the inoculation) from the leaf part of each weed variety we obtained water extracts (filtrates) according to the method described by Bogatzevska (1) separately for each concentration. The presence of **Pst** in the filtrates was established by the vacuum-infiltration method with a test-plant tomato of the Chico variety. The infiltrated test-plants were cultivated in greenhouses under 20-25C . The presence of **Pst** in the water extracts was determined by the appearance of symptoms typical of bacterial speck on the leaves of tomatoes on the 4-5th day. From the single spots on the test plants isolations were made accor-

ding to the described method.We recorded the average number of spots on one test plant.The pathogen properties of the isolates from the weeds and tomatoes were determined by HR of tobacco (10) and through the inoculation of tomatoes (1, 8).For determining the phenotypic characteristic and the type of the isolated bacteria we used differentiating tests for **Pst** (3, 4, 9, 14).

Results and discussion

HR is induced in the leaves of **D. stramonium** 18 hours after the infiltration with **Pst** Rs (10^4,10^m cfu/ml) and just 36 hours in the concentration of the bacterium 10cfu/ml.On the leaves of **S. nigrum** HR is provoked 24 hours after vacuum infiltration with concentration of the bacterium 10^4 and 10^m cfu/ml and three days later between the necrotic areas (HR) single small necrotic spots with a cholorotic halo appear.These spots do not differ from spots characteristic of bacterial speck on the leaves of tomatoes.This picture is not observed after the contamination of **S. nigrum** with **Pst** Rs-10 cfu/ml.On the 4-5th day after the inoculation on the leaves of **A. halepensis** brownish-reddish spots are formed along the nervation (long from 1 to 5 cm) and on the leaves and stems of **A. avicinnae** and **H. Trionum** -dark brown necrotic spots without a chlorotic halo with d 2-5 mm.On the leaves of **S. arvensis** at first watery chlorotic areas appear (d 1-2 mm) on the 4-5th day.The centre necrotizes and around it a yellowish chlorotic halo forms .These spots are typical of bacterial speck.

In all the remaining weeds: **A. retroflexus, P. aleracea, E. crus galli, Ch. album, Setaria sp., S. viridis, Polygonum sp., S. minor** visible changes on the leaves and stems were not observed and in their outer appearance they did not differ from the control with distilled water.

From the single spots on the leaves of **S. nigrum, A. halepensis, A. avicennae, H. trionum** was isolated only strains resistant to rifampicin which in pathogeny and phenotype characteristic do not differ from the **Pst** and the initial **Pst** Rs.Other bacteria were not isolated.

Pst develops epiphytically (symptomlessly) during the whole test period on the leaves of **A. retroflexus, P. oleracea, Ch. album, Polygonum sp.** (in the three concentrations) and on the leaves of **S. nigrum** infiltrated with 10 cfu/ml. The proof for this is the different number of spots (from 15 to 124) which appear on the test-plants after vacuum infiltration with the water extracts of the respective weeds (according to variety, concentration and days). The **Pst** does not develop epiphytically on the leaves of **E. crus galli, S. viridis** and **S. minor** and the test-plants (tomatoes-Chico), processed with their filtrates not differ from the controls infiltrated with distilled water.

In all the remaining 11 varieties of weeds the maximum development of the bacterium is recorded on the 18th day after the contamination in all three concentrations. The indirect proof of the reproduction of **Pst** on these weeds is the average number of spots on the test-plants artificially contaminated with the filtrates from them. The highest degree of development of the bacterium is recorded on the leaves of **D. stramonium, A. retroflexus, P. oleracea** and **S. nigrum** (respectively 124 , 80 , 47 , 112 average number of spots on 1 test-plant) - on the 18th day after contamination - 10^4 cfu/ml). They all belong to the group of the typical weed hosts for the epiphytic development of the **Pst.**

After the 25th day from the beginning of the contamination the decrease of the density of the bacterium is observed expressed in the very small number of spots on the test plants (from 1 to 10). The single spots on the tomato test-plants are recorded on the 40th day only on those processed with filtrates of **A. retroflexus** (10^4 and 10^8 cfu/ml) and **D. stramonium** (10^8 cfu/ml).

The rifampicin-resistant strains are isolated from single spots on the test plants - tomatoes processed with the filtrates of the respective weeds which do not differ from the **Pst** as to pathogen and phenotype characteristic.

The weeds **S. viridis** and **S. minor** on which **Pst** was not

observed as an epiphyte are probably antagonistic to it or a special epiphytic microflora develops on them which represses its development. They belong to the group of the non-hosts for the epiphytic development of the **Pst.**

New hosts for the pathogenic phase of **Pst** with artificial contamination through vacuum infiltration are the weeds: **S. nigrum, A halepensis, H. trionum** and **A. avicennae.**

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

BACTERIAL BLACK SPOT OF MANGOES IN KENYA

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ABSTRACT

During a crop plant disease survey carried out in upper midland (UM) agro-ecological zones I (humid), 2 (sub-humid), 3 (semi-humid), and 4 (transitional) of Embu District, Kenya, a disease was observed on mangoes grown under small scale farming and inter-cropping systems. Predominant symptoms were black spots, usually covered with exudates on leaves. Spots also appeared on fruits. Disease severity was relatively high in upper midland zones I and 3 than in 2 and 4. In an on-going varietal trial plots located in UM3 zone, there were marked differences in host susceptibility under natural infection. Following isolation, the organism was subjected to a number of diagnostic tests. The latter showed that the bacterium associated with the disease had properties, characteristic of Xanthomonas campestris pv mangiferaeindicae (synon. Pseudomonas mangiferaeindicae). These results differ from early reports made in Kenya regarding the identity of a pathogen causing black spot of mangoes which was then attributed to Erwinia mangiferae.

INTRODUCTION

Occurrence of black spot of mangoes has been reported in South Africa (Doidge, 1915; Steyn et al, 1974), India (Patel, et al 1948), Kenya (Robinson, 1960), Pakistan (Khan and Kamal, 1966) and Australia (Moffett et al, 1979). Doidge (1915) described the causal agent as Bacillus mangiferae. This bacterium which was yellow pigmented, gram negative and peritrichously flagellated was later reclassified as Erwinia mangiferae by Breed et al (1957). Khan and Kamal (1966) also isolated a bacterium which they described as Erwinia mangiferae.

On the other hand Patel et al (1948) isolated a polar flagellated organism which they described as Pseudomonas mangifeareindicae. In 1974, Steyn et. al observed that Erwinia mangiferae organisms could be isolated from old fruit lesions, but were never the cause of black spot of mangoes. They found that Pseudomonas mangiferaeindicae Patel, Moniz and Kulkarni (1948) was the causal agent of the disease in South Africa and concluded that the bacteria was probably the cause of all earlier reported cases of the disease elsewhere. This bacterium has been recently reclassified as Xanthomonas campestris pv. mangiferaeindicae (Patel, Moniz and Kulkarni, 1948) Robbs, Ribeiro and Kimura, 1974.

The first report on the occurrence of black spot of mangoes in Kenya was made by Robinson (1960). The disease was then serious but confined to Nyanza (Western Kenya). Robinson (1960) attributed Bacterium mangiferae as the causal agent but was later reclassified as Erwinia mangiferae (Nattrass, 1961).

During a crop plant disease survey carried out in 1987 to assess diseases of economical importance under small-scale farming in upper midland (UM) agro-ecological zones I (humid), 2 (sub-humid), 3 (semi-arid) and 4 (transitional) of Embu district (Eastern Kenya), a disease was observed on mangoes with symptoms similar to those described for black spot of mangoes. Considering previous inconsistent reports on the identity of the causal agent of the disease both in Kenya and elsewhere, a study was carried out to determine occurrence, distribution, and the identity of the causal agent.

MATERIALS AND METHODS

Disease Survey

A disease survey was conducted in Embu district in Eastern Province. Farms were selected at random along the main and minor roads in the agro-ecological zones UM 1, 2, 3, and 4 in such a manner as to cover a representative area. Crop plants in individual small holdings and a varietal trial farm were inspected for occurrence of the disease. Intensity of the disease was also noted.

Identification of isolates

Initial bacterial isolation was done on nutrient agar (NA). Bacterial colonies thereof were purified by a series of colony transfers and

maintained on NA slants at 4°C. Pathogenicity of isolates was proved by using an inoculum from 48 hr-old cultures on yeast dextrose calcium-carbonate agar (YDCA), spectrophotometrically adjusted to a concentration of 5×10^7 colony forming units per millilitre. Young mango plants of the variety 'Ngowe' were spray - inoculated on the under surface of leaves using a devilbliss atomizer attached to an airline at 1.05 kg of force per square centimetre. Inoculated plants were then covered in a transparent polythene bag for 48hr in the greenhouse.

In identification of bacterial isolates a series of cultural, physiological and biochemical tests were carried out. Unless specified the tests were carried out according to methods of the Society of American Bacteriologists (1957). The gram stain was determined according to a method described by Schaad (1980). Colony characteristics were determined on NA, YDCA and King's medium B. LOPAT properties of the isolates were determined using the methods described by Lelliot et al (1966). Gelatine liquefaction was determined as described by Cowan (1974). Production of hydrogen sulphide was determined according to Dye (1968). Urease activity, reduction of nitrate, tolerance to Nacl and TTC salts were determined as described by Lelliot and Stead (1987). Electron microscopy was done according to Gaard as described by Schaad (1980). All cultures were incubated at temperatures between 25 - 27°C.

RESULTS AND DISCUSSION

Disease symptoms and distribution

Symptoms on leaves consisted of black angular necrotic areas delimited by veins. The necrotic spots were surrounded by yellow margins and in some cases gave exudates. On fruits there were dark spots in some cases showing cracks. Generally the symptoms were severe on young succulent shoots or leaves. The symptoms resembled those that have been described by other workers (Styne et al 1974; Weber, 1973; Moffett and Croft, 1983) for black spot of mangoes. Occurrence of the disease in Embu means that the disease is no longer confined to Western Kenya as reported (Robinson, 1960) earlier. Although the disease occurred in all the zones surveyed, it was relatively more severe in UM1 and UM3 than in UM2 and UM4 zones. The differences could not

be attributed to any specific factor but was considered to be possibly due to variation in micro-environments and varieties grown. In the varietal trial plots, there were differences in severity of the disease under natural conditions. 'Ngowe' and 'Apple' were more susceptible than 'Kiet', 'Alphonse' and Tommy Atkins varieites. This implies possible existence of disease resistance among some cultivars which may be used in the control of the disease.

Colony and Physiological characteristics

Results of the tests carried out are shown on Table I. The bacterial cells were gram-negative short rods with long single polar flagellum. On NA, colonies were creamy-white, shiny, flat and round. On YCDA colonies were creamy white, mucoid, round and bigger than on NA.

Table I Properties of bacterial isolates from mango plants grown in Embu, Kenya.

Properties	Reaction[a]
Gram	-
Levan	+
Arginine dihydrolase	-
Oxidase	-
Urease activity	-
Motility	+
Flourescent pigment	-
H_2S	+
NO_3 reduction	-
Starch hydrolysis	+
Tobacco hypersensitivity	+
Gelatine liquefaction	+
Potato soft rot	-
Protein digestion	+
Growth in media on	
D-arabinose	+
Dextrose	+
Lactose	+
Sucrose	+
Glucose	+
Maltose	-
Sorbitol	-
Asparagine	-

a + = Positive reaction
\- = Negative reaction.

Colonies on 0.02% TTC were small, white and shiny with tinges of purple centres. There was little or no growth at 1% Nacl, slightly restricted, and restricted at 3% and 5% respectively.

The bacterium associated with black spot of mangoes by Doidge (1915) and Robinson (1960) and which was reclassified as Erwinia mangiferae was later found by Dye (1968) to be E. herbicola which is a common saprophyte. This means that although Robinson (1960) described the disease symptoms on mangoes in Kenya, he attributed a wrong agent as the cause of the disease. Based on the keys by Bradbury (1970) and in Bergey's Manual (Buchanan and Gibbons, 1974; Dye and Lelliot, 1974) and descriptions by Lelliot et al (1966) and (1979), the cellular, physiological and biochemical, and pathogenic characteristics of the bacteria in the present study is identified as Xanthomonas campestris pv mangiferaeindicae (Patel, Oniz and Kulkarni, 1948) Robbs, Ribeiro and Kimura 1974. Its lack of a yellow water-insoluble pigment was an unusual feature for Xanthomonad, but which has however, been observed in some nomenspecies of Xanthomonas campestris (Hayward 1979).

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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STATUS OF BACTERIAL LEAF SPOT AND BLIGHT OF CASTOR IN RAJASTHAN, INDIA

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Bacterial leaf spot and blight of castor caused by Xanthomonas campestris pv. ricini (Yoshi and Takimoto) Dye is a serious disease in India. The disease also occurs in other countries including Sudan,U.S.A., U.S.S.R.,Japan, Brazil, Korea, South Africa, Uganda, Rhodesia.

Arachibald (1927) first described the disease in Sudan as 'black rot' caused by Phytomonas ricini. Yoshi and Takimoto (1928) reported the disease from Japan and identified the bacterium as Bacterium ricini.Sabet(1959) concluded that the disease in Sudan was caused by a typical strain of Xanthomonas ricini.

PREVALENCE OF DISEASE: In India the disease was first recorded from Gujarat state by Patel et al.(1951).It now occurs in many parts of the country. In Rajasthan, castor fields in 14 districts were surveyed. The disease occurrence was highest in Sriganganagar(23%) followed by Banswara(21%) and Sirohi(18%) respectively. The disease was not recorded from Jodhpur and Bikaner districts while its incidence ranged from traces to 13% in remaining district(Table 1). PATHOGEN :Two different pathogenic strains of Xanthomonas campestris pv.ricini, the yellow pigmented and the albino, were isolated from diseased castor leaves.Bacterial cells examined under JEOL TEM SX 100 electron microscope after negative staining (Cole and Popkin,1981) were found to be rod-shaped with a single polar flagellum(Fig.1). The

Table-1: Prevalence and severity of castor leaf spot and blight in Rajasthan

Place	Prevalence	Infection Index
Alwar	8	Trace
Banswara	21	10
Barmer	Trace	Negligible
Bikaner	Nil	Nil
Chittorgarh	Trace	Negligible
Bhilwara	Trace	Negligible
Jaipur	9	5.5
Kota	8	5.00
Pali	10	6.00
Calore	5	Negligible
Sriganganagar	23	17.00
Sirohi	18	11.00
Jodhpur	Nil	Nil
Udaipur	Trace	Negligible

cells possess a thick slime layer(Fig.2).During the cell division, a depression is initially formed on the transverse axis of the cell and then apparently a line is drawn transversaly across the width of the rod-shaped cell,later, there is a transverse split and the rod-shaped bacterium divides into two almost equal parts(Fig.3 & 4). In case of certain animal cells, multiplication may be by binary fission with vertical splitting (Lamanna and Mallette,1965). With the present bacterium, the binary fission was always found to take place by transverse splitting.

DISEASE DEVELOPMENT : The minimum concentration of inoculum required for disease development was found to be c.4.00×10^4 and c. 4.00×10^3 cfu/ml by spray inoculation and carborandum abrasion method, respectively. Appreciable amount of disease was produced in field, when the suspension contained c.4.00×10^6 cfu/ml by spray inoculation method (Table 2).



Fig. 1. Bacterial cells with single polar flagellum



Fig. 2. Bacterial cell with thick slime layer

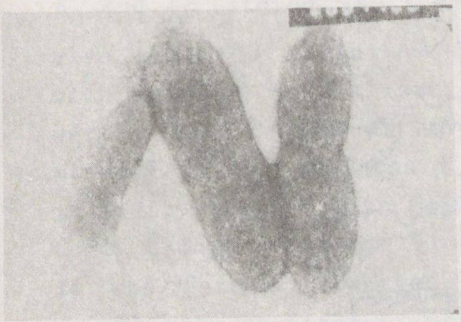


Fig. 3. Initiation of binary fission in bacterial cell

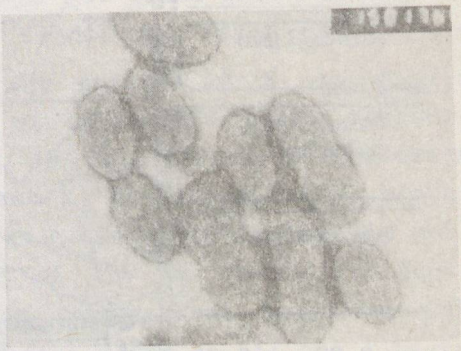


Fig. 4. Different stages of transverse splitting during binary fission

Table 2 : Inoculum concentration X.campestris pv.ricini in relation to disease development

S.No.	Inoculum concentration (cfu/ml)	Spray inoculation*		Carborandum abrasion*	
		Incubation period (days)	Infection index	Incubation period (days)	Infection index
1.	4.00×10^8	6 - 7	55	4 - 5	63
2.	4.00×10^7	6 - 7	43	4 - 5	62
3.	4.00×10^6	6 - 7	42	4 - 5	58
4.	4.00×10^5	8 - 9	31	4 - 5	57
5.	4.00×10^4	10 -12	05	5 - 6	58
6.	4.00×10^3	-	-	7 - 8	17
7.	4.00×10^2	-	-	-	-

* Average of two experiments

During field survey the disease was invariably observed in rainy season crop. The disease development was studied throughout the year at Udaipur under artificial inoculation conditions. Castor plants of variety 'Aruna' were inoculated when the plants attained an age of one month and infection index was recorded 21 days after inoculation. It was found that high humidity(70-75%)and temperature around 30-35% were

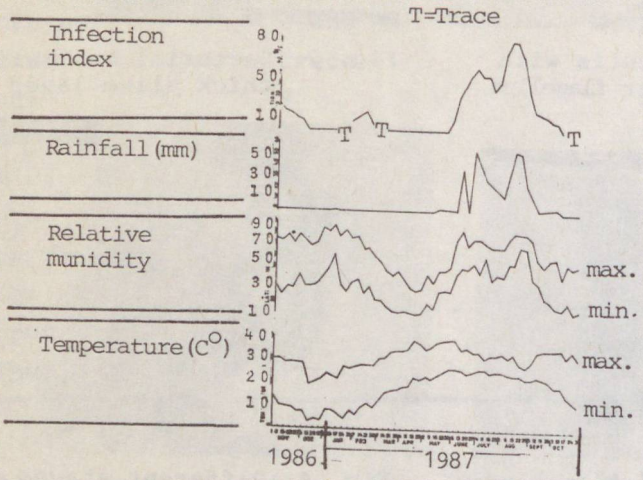


Fig. 5. Bacterial leaf spot and blight development on castor variety Aruna, in cage house after weekly inoculation

conducive to disease development (Fig.5). The progress and spread of disease were further increased with rainfall.

NUTRITIONAL REQUIREMENTS OF THE PATHOGEN: Studies on nutritional requirements with respect to inorganic nitrogen sources, amino acids, carbon sources and mineral sources of 4 isolates of X. campestris pv.ricini revealed that all the isolates grew well when the inorganic nitrogen sources were KNO_3, $NaNo_3$, NH_4Cl, $(NH_4)_2\ SO_4$ and $NH_4H_2PO_4$, while $NaNO_2$ did not serve as a good source of inorganic nitrogen. Similar results were also obtained by Patel and Kulkarni (1949) in case of X.campestris pv.malvacearum. In respect

of utilization of amino acids all the 4 isolates were found similar and the differences were not significant. However, out of 11 amino acids, L-Hydroxyproline and L-Histidine were found to be adequate carbon and nitrogen sources for all the 4 isolates of the bacterium.

The bacterial growth was not similar on all the 15 carbon sources used and different isolates showed variation in the utilization of sugars. However, in general,cellobiose and maltose proved very good sources for all the 4 isolates, but the former was good source for isolates x_1 and x_2(both yellow pigmented),whereas latter was found good for isolates X_3 and X_4 (albino isolates). Jindal and Patel (1972) have also reported variation in the strains of X. ricinicola with respect to utilization of sugar. All the 4 isolates were significantly different in utilization of different mineral salts. Isolates X_1 and X_2 displayed better growth in media supplied with $FeSO_4$ as compared to isolate X_3 and X_4 $CuSO_4$ did not support growth of any of the 4 isolates. It was further observed that the present bacterium had no obligate requirement of mineral salts as all the 4 isolates grew best in salt-free medium.

It appears that the isolates differ with respect to utilization of several nutrients and possibly the 4 isolates of X. campestris pv.ricini are different and there appears to be physiologic specialization within the pathovar. However, the differences cannot be specifically differentiated as the number of isolates used were not large enough.

CHEMICAL CONTROL: Plantomycin, Streptocycline and Paushamycin in combination with Fytolan (50% copper as copper oxychloride) were effective in controlling the disease and these also increased the crop yield . Plantomycin 250 ppm + Fytolan 2000 ppm was most effective followed by Streptocycline 250 ppm + Fytolan 2000 ppm. The yield increase rendered by these treatments may possibly be on account of control of both bacterial and fungal pathogens.

Three sprays of plantomycin 250 ppm + Fytolan 2500 ppm applying first spray with the onset of monsoon followed by 2 more sprays at an interval of 14 days can be suggested for the disease control under field conditions.

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Proc. 7th Int. Conf. Plant Path. Bact., Budapest, Hungary, 1989

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DIAGNOSIS OF THE POTATO RING ROT AGENT BY DNA DOT-BLOT HYBRIDIZATION USING NON-RADIOACTIVE PROBES

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The diagnosis of the bacterial ring rot of potato, a disease of quarantine relevance caused by *Clavibacter michiganensis* subsp. *sepedonicus* is difficult because of the slow growing rate of the pathogen and the occurrence of latent infections in tubers, which appear symptomless. A reliable method for the detection of latent infections was proposed by an expert group of the European Comunity (EC), and consists essentially in an indirect immunofluorescence antibody stain (IFAS) and a pathogenicity test on eggplants (EP) (Janse & Van Vaerenbergh, 1987). However cross reactions had been observed by several authors (Calzolari *et al.*, 1982; De Boer, 1982) while the EP test, despite its high sensitivity, is very time consuming, difficult to read and must be carried out under critical conditions with many replicate plants. Furthermore, a diagnosis based on the EP test can be confirmed only after a successful reisolation of the pathogen from eggplants, so increasing the work involved.

The use of cloned diagnostic DNA probes for the detection of diseases by plant protection services, had not been evaluated until recently, since diagnostic laboratories usually lack facilities for working with radioisotopes. The advent of non-radioactive labeling methods allows DNA to be linked to an apten which is readily detected by enzyme-linked immunoessay.

Recombinant DNA technology will offer the specificity requisites which are often difficult to attain with other techniques: in the case of *Clavibacter michiganensis* subsp. *sepedonicus*, several authors have shown the presence of a highly conserved plasmid of about 30MD, named pCS1 or pLC50 (Gross *et al.*, 1979; Clark & Lawrence, 1986; Mogen *et al.*, 1987) whose restriction fragments have been cloned recently (Firrao, submitted for publication). The suitability of these cloned DNA fragments for specifically detecting the potato ring rot agent with non-radioactive dot-blot hybridization is discussed.

The pCS1 plasmid was extracted following the method described by Clark & Lawrence (1986), based on an alkaline SDS treatment at 56°C and purified by CsCl - EtBr gradient. The purified CCC-DNA was digested by SalI endonuclease, the

single fragments ligated to the plasmid vector pUC19 and inserted into *E. coli* DH5 Alpha competent cells. Recombinant plasmids from transformant clones were extracted according to the method of Birnboim & Doly (1979) followed by a PEG selective precipitation of CCC-DNA and used as probes in hybridization experiments. Five recombinant plasmids with insertions of fragments ranging from 1.7 kb to 3.5 kb were selected for further diagnostic use. Probes were prepared by incubating lug DNA in a standard random priming mixture (BMB) at 37°C for 3 hrs in presence of digoxigenin-UTP. This reaction produces about 250 ng digoxigenin labelled DNA, which was hybridized to target DNAs from various bacteria, prepared as follows: mid-log phase liquid cultures were centrifuged for 5' at 10,000 g at 4°C, and resuspended in 250mM sucrose - 50 mM TrisCl pH 8. After shaking with phenol:chloroform:isoamyl alcohol (25:24:1), the emulsion was spun at 6,000 rpm for 5' and the DNA ethanol precipitated.

The EC method recommended preparing potato tubers by homogenation in maceration buffer (Mazucchi *et al.*, 1981) and differential centrifugation. The same method was followed with healthy potatoes; the resulting final pellet was suspended in 250mM sucrose - 50mM TrisCl pH 8, to which known bacterial suspensions were added. The resulting mixture was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and the aqueous phase ethanol precipitated.

Washed pellets were suspended in 20mM Tris - 1mM EDTA pH 7.4 and heated for 10' at 100°C to partially depurinate DNA. After cooling in ice and spinning down insoluble residues for 2' at 10,000g, 1ul was spotted on nylon membranes (S&S) which were fixed by UV irradiation for 3' and/or baking in an oven for 2 hrs at 80°C. For sensitivity determination a 3 days' growth of strain NCPPB 299 was collected, counted by standard plating methods and submitted to the same treatment described for bacterial DNAs.

Filters were rehydrated in 5x SSC, prehybridized in an Hybrid Ease box (Hoefer) at 42°C for at least 6 hrs with shaking and occasional mixing the 25mL prehybridization buffer (45% formamide, 0.1% Sarkosyl, 0.02% SDS, 5% BMB Blocking reagent). Hybridization was carried out in a similar buffer containing 50 ng/ml digoxigenin labelled DNA for 10-16 hrs at 42°C. Stringency washings were 2 x 5' at room temperature in 2x SSC, 0.1% SDS and 2 x 15' in 0.1% SSC, 0.1% SDS. Filters were then left 30' in blocking buffer and incubated 30' in the presence of an anti digoxigenin - alkaline phosphatase conjugated. The excess of antibody was washed off (2 x 15' in 100 mM TrisCl, 150 mM NaCl, pH 7.5), filters were left overnight in the developing buffer (100 mM TrisCl, 100 mM NaCl, 50 mM $MgCl_2$ pH 9.5) in the presence of NBT and X-phosphate as colour substrates and finally photographed with an AGFAORTHO 25 professional film.

A similar processing of filters was used for Southern blot experiments carried out according to Maniatis *et al.*(1982) using DNA samples purified following the procedure of Lewington *et al.* (1987); in these experiments strong hybridization

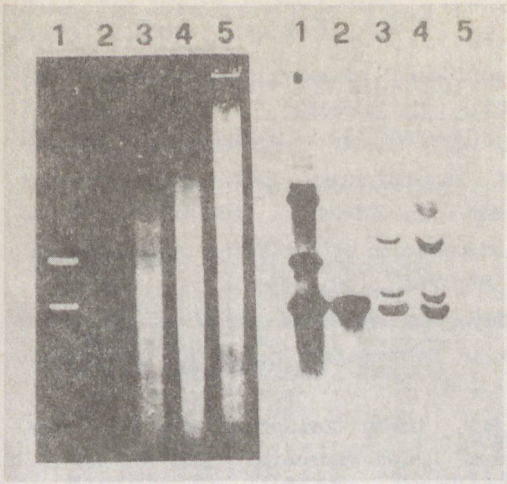


Fig.1: A recombinant plasmid (1) containing a pLC50 fragment used as a non-radioactive digoxigenin labelled probe hybridized with itself and DNA purified from different strains of *C. michiganensis* subsp. *sepedonicus* (3 and 4) but not with another related organism (5) in a Southern blot analysis.

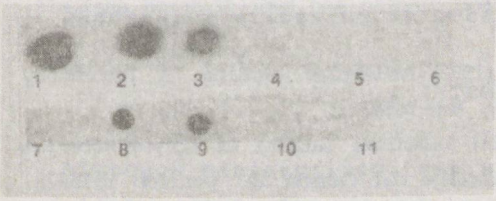


Fig.2: Dot-blot hybridization to a mixture of five recombinant plasmid DNAs labelled with digoxigenin: serial ten-fold dilution of DNA from *C. michiganensis* subsp. *sepedonicus* corresponding to 10^8 to 10^3 cells (spot 1-6), maceration buffer from healthy potatoes (7), *C. michiganensis* subsp. *sepedonicus* strains NCPPB 299 and DPDU 904 (8-9), *Arthrobacter simplex* strain NCIB 8929 (10) and maceration buffer from healthy potatoes with addition of about 10^5 cells of strain NCPPB 299, which represent the minimum signal detected in this work (11).

signals with absence of background were obtained with short, conventional prehybridizations (fig.1). For dot-blot hybridizations with low purity samples we found the prehybridization step very critical, to be performed with large volumes, continuous shaking and occasional complete mixing or substitution of the prehybridization solution. Moreover potato extract cannot be spotted directly on filters; fig. 2 shows that background can be eliminated by phenol: chlorophorm:isoamyl alcohol extraction and ethanol precipitation. The probe described has revealed to be highly specific (Firrao, submitted for publication) with a sensitivity of 10^6 c.f.u. (fig.2); the sample preparation and the hybridization conditions described here are reliable for the setting up of a workable method for the rapid and specific diagnosis of potato ring rot latent infections: the non-radioactive dot-blot DNA-DNA hybridization analysis of naturally infected tissues is actually under study in our laboratory.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

THE USE OF MICROTITER PLATES IN THE PHENOTYPIC CHARACTERIZATION OF PHYTOPATHOGENIC PSEUDOMONADS

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ABSTRACT

Microtiter plates (96-well) were used for a range of characterization tests including carbon source utilization and acid production from carbohydrates, primarily for differentiation of biovars of Pseudomonas solanacearum. One hundred and thirty-eight isolates of P. solanacearum biovar 2, from 28 countries, of which all but seven were from potato, were clearly differentiated into two phenotypes differing in metabolic activity.

INTRODUCTION

Although miniaturized test systems have been developed, and widely used for bacteria of medical interest, systems specifically designed for phytopathogenic, soil or other environmental bacteria are generally lacking. Generally the commercial systems provide insufficient information to discriminate between strains of specialised groups of microorganisms isolated from a specific ecological niche (O'Connor et al, 1988). We set out to evaluate the use of 96-well microtiter plates with a range of solid, semisolid and liquid media. The following describes our experience of the method and the results of its application to a collection of isolates of Pseudomonas solanacearum, in particular to biovar 2 (Hayward, 1964). Although the equation of race 3 (Buddenhagen and Kelman, 1964) with biovar 2 has been widely supported, recent work (Swanepoel

and Young, 1988) suggests that there are marked differences between certain isolates classified as biovar 2.

MATERIALS AND METHODS

The bacterial cultures examined comprised 312 isolates of Pseudomonas solanacearum held in the collection of the International Potato Center and the reference cultures P. syringae pv. syringae UQM 213 and P. cepacia UQM 3153. The solid media used were medium B of King et al. (1954) for production of fluorescent pigment and the aesculin medium of Lelliott and Stead (1987). Acid production from carbohydrates was determined using the semi-solid medium of Hayward (1964), and carbon source utilization in the defined mineral liquid medium of Ayers et al. (1919). Both basal media without added carbohydrate or other carbon source were included in the plates to facilitate recognition of acid pH changes and growth on carbon sources. Linbro/titertek microtitration multi-well plates (ca 0.35 ml capacity) were loaded with a variety of solid, semi-solid or liquid media, either with ca. 150 µl using a Pasteur pipette or 200 µl using a multi-channel pipette. Suspensions of each isolate in sterile distilled water (OD $\leqslant$0.05 at 600 nm) were delivered as one drop (30-40 µl) to each well using a Pasteur pipette. Plates were well sealed with parafilm, enclosed in plastic bags, sealed, and incubated at 30 $\pm$ 1 ^{o}C for 21 days. Observations of growth, acid production and fluorescent pigment production were made over a light box and UV lamp. A range of other tests which were not amenable to the microtiter plate were made using standard procedures.

RESULTS

Preliminary experiments showed that it was essential to prevent dehydration of the medium by sealing the microtiter plates in parafilm and enclosing them in sealed plastic bags, when prolonged periods of incubation were required. The order in which media were loaded into rows of microtiter wells was of critical importance to avoid artefacts caused by the release of alkaline or acid metabolites into the well head space. Where media high in

peptone such as medium B of King et al., or the aesculin medium, were adjacent to the medium used for the production of acid from sugars, the pH of the latter medium became alkaline even where acid was produced from the sugar. Similarly where several rows of sugar-containing media, uniformly positive for acid production, were adjacent to uninoculated medium for acid production, change to acid pH had occurred, suggesting adsorption of a volatile acid metabolite. This problem was overcome by separating high peptone media from the rest, and reduction to one or at most two of the number of adjacent rows of sugar-containing media. The carbon source utilization media were used to separate the rows of sugar containing media. It was also necessary to have no carbon source controls for both acid production and utilization. A third artefact arose where growth occurred to give a visible turbidity in the defined medium without added carbon source. However, this turbidity was usually easily differentiated from the denser turbidity occurring where carbon sources had been utilized. Since P. solanacearum contains the endogenous energy reserve poly-β-hydroxybutyrate, utilization of this substrate may be the reason for the appearance of turbidity. It is possible that this artefact could be eliminated by standardisation of the inoculum to a lower level than that used in this work.

Microtiter plates were used successfully for biovar classification of 312 isolates of P. solanacearum. It was shown that 138 isolates of biovar 2 could be differentiated into two phenotypes A and B one of which was metabolically more active than the other (Table 1).

All strains positive for Kovacs' oxidase, catalase, solubility in 3% KOH; acid production from glucose, sucrose, lactose, maltose, cellobiose, glycerol, L-arabinose, mannose, fructose and galactose; utilization of gluconate, ornithine, L-alanine, L-asparagine, L-aspartate, histidine, tyrosine, malate and citrate; esterase on Tween 80. All strains negative for acid from mannitol, sorbitol,

Table 1. Differentiation of 138 isolates of Pseudomonas solanacearum Biovar 2 into two phenotypes on the basis of differences in metabolic versatility.

Test	Phenotype A (19 isolates)* % positive	Phenotype B (119 isolates)* % positive
Acid from ribose	100	0
Acid from trehalose	100	4
Utilization of L-tryptophan	100	0
Utilization of L(+)tartrate	95	3

*Phenotype A comprises 7 isolates from Yurimaguas, Peru (170 m.asl), 3 from La Chincana, San Ramon, Peru (1200 m.asl) and 9 (of 20) isolates from Brasil; phenotype B comprises isolates from 28 countries (Argentina, Australia, Brasil, Burundi, Chile, China, Colombia, Costa Rica, Egypt, Greece, India, Indonesia, Iran, Israel, Kenya, Malaysia, Mexico, Nepal, Nigeria, Panama, Peru, Philippines, Puerto Rico, Rwanda, Sri Lanka, Sweden, Uruguay and Venezuela).

dulcitol, L-rhamnose, D-arabinose, xylose and adonitol; arginine dihydrolase; fluorescent pigment production, and profuse gas production from nitrate. Variable reactions which follow given as number and percent positive (in parenthesis); results for phenotype A given first and for phenotype B second: utilization of hippurate; 18/19 (95), 115/119 (97); propionate 8/19 (42), 54/119 (45); Beta-alanine 18/19 (95), 108/119 (91); meso-tartrate 2/19(11), 3/119 (3); D-tartrate 2/19 (11), 15/119 (13); citrulline 19/19 (100), 117/119 (98); para-hydroxybenzoate 18/19 (95), 118/119 (99); L-proline 18/19 (95), 119/119 (100); D-quinate 19/19 (100), 113/119 (95); L-serine 18/19 (95), 118/119 (99); succinate 19/19 (100), 116/119 (98); pimelate 6/19 (32), 32/119 (27); succinate 19/19 (100), 116/119 (98); lactate 5/19 (26), 8/119 (7); glutamate 18/19 (95),

119/119 (100); nitrite from nitrate 19/19 (100), 115/119 (97); acid production from meso-Inositol 19/19 (100), 113/119 (95).

DISCUSSION

Microtiter plates can be used in a very flexible way, either to test many isolates for one property such as β-glucosidase activity (aesculin hydrolysis), fluorescent pigment production, or tolerance to an inhibitor; or one isolate for many different properties on a single plate, as in most commercial identification kits. The main advantages of the microtiter system over conventional systems are: ease of filling, handling and reading with manual, automatic or robotic microtiter equipment, if desired; economy in the use of medium constituents and substrates; where several isolates are tested on the same plate, ease of pattern recognition; the increased rate at which some reactions occur, and elimination of cross-feeding and other diffusion artefacts in carbon source utilization tests, which may occur in Petri dishes.

Many tests are not readily adapted to microtiter plates, for example gas production from nitrate and pitting of pectate gel. O'Connor et al. (1988) found that the tests for arginine dihydrolase, Simmons'citrate and urease could not be performed in multidishes because the diffusion of an alkaline product, presumably ammonia, through the head space above wells resulted in false positive reactions. Similarly in this study artefacts due to release of alkaline products from high peptone media, and acid metabolites from sugar media were observed. However, these problems could be overcome by separation of certain tests and adequate spacing of test media, including the use of uninoculated rows of test media.

The method was used to define two phenotypes A and B within a collection of 138 isolates of P. solanacearum biovar 2. Only phenotype B appears to conform with equivalence of race 3 and biovar 2 (Buddenhagen and Kelman, 1964).

Phenotype A is also similar to strain 3 of Swanepoel and Young (1988), but the latter organism differs in that it is a vigorous gas producer from nitrate and fails to hydrolyse Tween 80. Heterogeneity in biovar 2 is substantially confirmed.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

A SENSITIVE AND QUICK ASSAY FOR DETECTION OF PSEUDOMONAS SYRINGAE PV. PHASEOLICOLA IN BEAN SEED

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ABSTRACT

Pseudomonas syringae pv. phaseolicola (Burkholder 1926) Young, Dye and Wilkie 1978, the causal agent of halo blight of beans (Phaseolus vulgaris), could be detected in very low concentrations. The procedure combines dilution plating on a semi-selective medium and determining phaseolotoxin production of suspected colonies by a E. coli bioassay, which was improved by several modifications.

The whole procedure for detection of bacteria requires 6 days to perform. Less than 18 CFU of P. phaseolicola per ml soakate could be detected. Furthermore, P. phaseolicola originating from one diseased bean seed added to 50,000 healthy seeds was identified without problems.

High sensitivity, reliability and ease of operation make the semi-selective medium / E. coli bioassay well adapted for assaying large batches of bean seeds for infestation by P. phaseolicola.

INTRODUCTION

Several methods have been described to detect P. phaseolicola in seeds (Wharton 1967, Schaad 1982, Venette et al. 1987). Mostly serological techniques are applied (Bazzi and Calzolari 1982, Barzic and Trigalet 1982, Guthrie et al. 1965, Van Vuurde et al. 1983). By using specific antibodies these methods are rapid and more secure than physiological tests. However, viability and pathogenicity of the bacteria cannot be determined. Since tests for pathogenicity on the natural host require considerable time, it was the aim of these studies to combine classical phytopathological methods with a few specific tests for a quick detection of P. phaseolicola in bean seeds (Jansing and Rudolph 1989).

MATERIAL AND METHODS

Bacterial strains

All bacterial strains used in these experiments originated from the institute's collection (Jansing and Rudolph 1989).
21 strains of Pseudomonas syringae pv. phaseolicola, 35 strains of Pseudomonas syringae pv. syringae, 3 strains of Pseudomonas fluorescens, 1 strain of Pseudomonas syringae pv. pisi, 1 strain of Pseudomonas syringae pv. tabaci and 1 strain of Escherichia coli (N 100) were used.

Media

The semi-selective medium (MSP) of Mohan and Schaad (1987) was prepared as follows: dissolve in 1000 ml of distilled water: 15 g sucrose, 15 g Bacto peptone, 0.3 g $MgSO_4$ x 7 H_2O, 3.0 g K_2HPO_4, 1.0 g Na H_2PO_4, 17 g Bacto agar, adjust to pH 7.0 and autoclave, add sterile filtrated solutions of 0.15 mg bromthymol blue, 200 mg cycloheximide, 80 mg cephalexine and 10 mg vancomycin.
For routine cultivation of P. phaseolicola the medium B of King et al. (1954) was used. The nutrient medium for Escherichia coli (Staskawicz and Panopoulos 1979) was composed of 1 g KH_2PO_4, 1 g K_2HPO_4, 1 g NaCl, 0.5 g sodium citrate, 4 g $(NH_4)_2SO_4$, 2 g glucose, 0.7 g $MgSO_4$ x 7 H_2O, 18 g Bacto agar, 1000 ml H_2O and adjusted to pH 7.0.
The synthetic medium for production of phaseolotoxin (Watanabe 1963, Rudolph et al. 1978) was prepared as follows: dissolve in 1000 ml water: 14 ml glycerol, 1 g glutamic acid, 0.5 g L-cysteine, 3.0 g $(NH_4)_2HPO_4$, 2.0 g KH_2PO_4, 0.01 g $MnSO_4$ x 4 H_2O, adjust pH to 8.0, dissolve 0.5 g $MgCl_2$ x 6 H_2O and pour immediately into culture vessels because a precipitate will appear.

Isolation of P. phaseolicola from bean seed

1 Artificial inoculation of bean seed:

The detection of P. phaseolicola in bean seeds was carried out according to Mohan and Schaad (1987). Aliquots of the bacterial suspension were added to vessels which contained healthy bean seeds (cv. "Pascal 1533", Van Waveren 1985) soaked in 0.85% NaCl containing 0.01% Tween 20 (Merck No. 822 184) (Jansing and Rudolph 1989).
After soaking 1000 bean seeds in 1000 ml soaking solution at 4-6°C and vigorously stirring the bean seeds with a disinfected glass rod, samples of 10 ml of solution were taken. These were centrifuged at 6000 g for 20 min and the pellet suspended in 0.01 M $MgSO_4$ to give a 10-100 fold concentration of extracted bacteria. One tenth ml of each sample was streaked on a plate of semi-selective medium. After incubation the plates for 2-3 d at 24°C, bacterial growth was evaluated.

2 Naturally infested bean seeds:

Diseased seeds were wrinkled with longitudinal grooves (Jansing 1987) or had intense yellow spots and were clearly separated from the white, smooth healthy seeds. Preceding studies had shown that all the seeds with these symptoms were heavily infected by P. phaseolicola. Such diseased bean seeds were added in known quantities to healthy bean seeds of cv. "Pascal 1533" which were then soaked to detect bean seed infestation of different degrees.

Detection of phaseolotoxin by E. coli

Preparing of test plates: plastic Petri dishes are filled with 10 ml E. coli nutrient agar and overlayed with 4 ml of a mixture of 2% Bacto Agar : sterile distilled water : E. coli-suspension = 50 : 45 : 5. After solidifying holes (58 mm diameter) are punched into the agar and sucked out by a glass tube connected to an aspirator. The plates can be stored for 2 weeks at 4 °C.

Toxin production: Glass tubes (70 x 10 mm) are filled with 0.4 ml nutrient solution (Watanabe 1963), pH 8.0. The tubes are inoculated with a loop full of bacteria taken from agar grown colonies and incubated at 18 °C on a rotating incubator (slope of the tubes 16°) for 1 - 2 d (in most cases 24 h are sufficient).

Toxin test: After autoclaving, 20 µl of each bacterial culture is filled into a hole in the test plates. A second hole obtains additionally 10 µl 0.1% citrulline. The plates are incubated for 5 h at 4 °C, followed for 12 h at 37 °C. Only clear inhibition zones, which do not appear when citrulline was added, are evaluated as a positive reaction.

RESULTS

Growth and appearance of P. phaseolicola on the semi-selective medium

20 strains of P. phaseolicola from different geographical origin belonging to different races were precultured on nutrient agar (Rhodes 1959) in Petri dishes and scrapped from the agar surface in 0.01 M $MgSO_4$. After adjusting the suspension to OD_{660nm}=0.06, 1:10 dilution plating was made on semi-selective medium, and the nutrient medium for comparison.
In no case inhibition of growth was observed on the semi-selective medium. In fact, about 10% more colonies were counted on the selective medium in comparison to Rhodes' medium. After 2 days of growth on MSP, P. phaseolicola formed light yellow, weakly turbid, domed, smooth, circular colonies of ca. 2 mm diameter. After 4 days of growth the margin of the raised colonies became broader and transparent, the yellow colour more intense, whereas the center of the colonies appeared less dense in translucent light. Yellow haloes were formed around the colonies due to acid later on.

Several strains of P. syringae pv. syringae were tested on the MSP-medium. All strains grew well and were undifferentiable from P. phaseolicola colonies during the early stage of growth (up to 1 week). Because P. syringae causes bacterial brown spot of bean and occurs in West Germany (Rudolph 1979), it is necessary to differentiate the two pathogens when bean seeds are assayed.

Detection of phaseolotoxin production by the E. coli-bioassay

An alternative test for differentiation between pathovars syringae and phaseolicola is the production of phaseolotoxin (negative for pv. syringae, positive for pv. phaseolicola).

After autoclaving the toxin-containing suspensions, E. coli-inhibition zones were caused mainly by samples obtained from P. phaseolicola, and the haloes were never turbid. All the tested strains of P. phaseolicola produced clear inhibition zones under these conditions. The results were reversed by the addition of citrulline (Fig. 1). When bean seeds naturally infested by P. phaseolicola were tested by this method, sometimes bacteria were obtained which produced inhibition zones with E. coli that could not be nullified by citrullin (Fig. 1).

In addition, these strains formed atypical colonies on the semi-selective medium. Such strains were therefore not considered to be P. phaseolicola.

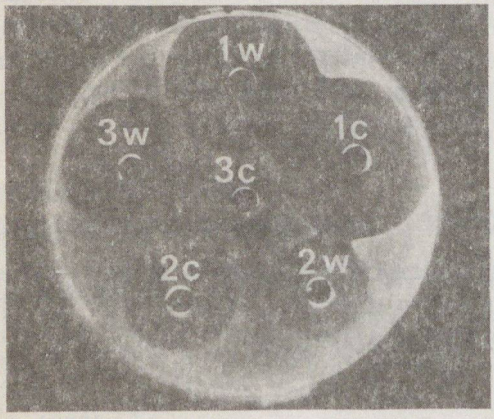


Fig. 1: Bioassay for phaseolotoxin by inhibition of Escherichia coli. Culture fluids of bacterial isolates 1, 2 and 3 were applied together with water (w) or citrulline (c). The inhibition by isolate 3 was reversed by citrulline, identifying this isolate as P. syringae pv. phaseolicola. (From Jansing and Rudolph, 1989)

Sensitivity for detecting P. phaseolicola in infested bean seed

Addition of a suspension of P. phaseolicola to bean seed soaking solutions resulted in a calculated final concentration of 18, 36 or 90 CFU/ml. After 2, 20 or 30 h of soaking, increasing numbers of P. phaseolicola cells could be detected in the soaking solution. Increases ranged from 17 to 124% of the original concentration.

Further experiments were done to mimic natural infestations. Heavily infected bean seeds also resulted in a higher number of detected CFU/ml than did lightly infected bean seeds. Soaking of seeds for 20 h resulted in greater numbers of bacterial cells than did soaking for 2 h. Incubating P. phaseolicola with bean seed increased the number of detectable cells when compared with water alone.

To confirm identification of colonies, from each sample representative colonies were selected and tested by the E. coli bioassay. All the presumptively identified bacteria caused citrulline reversed inhibition-zones for E. coli.

To determine the sensitivity of this method one naturally infested been seed was added to 30 l soaking solution containing 50,000 healthy bean seeds. Also in this case P. phaseolicola could be isolated from the soaking solution without doubt.

Pathogenicity tests

Pathogenicity tests with strains which were isolated from soaked bean seeds and confirmed as P. phaseolicola by the bioassay, always resulted in typical water-soaked lesions and early chlorosis within 5 - 7 days. Generally, leaf inoculations allowed an unequivocal diagnosis within two weeks. However, all strains which had been presumptively identified as P. phaseolicola produced typical halo blight disease symptoms. Therefore, a time-consuming confirmation of the results obtained by using the semi-selective medium combined with the E. coli bioassay for phaseolotoxin production did not appear necessary. Only when determination of bacterial race is needed inoculation tests are required.

DISCUSSION

The semi-selective medium of Mohan and Schaad (1987) allowed good growth of all 20 strains of P. phaseolicola tested. However, within a few days these colonies could not be differentiated from P. syringae.

Acid formation from mannitol and erythritol has been reported to differentiate between these two pathovars (Sands et al. 1980). Although this test was generally satisfactory it did not lead to clear results in every instance and sometimes needed more than 4 days.

Instead it appeared more reliable to specifically identify P. phaseolicola by the E. coli-bioassay for phaseolotoxin production. Staskawicz and Panopoulos (1979) had already reported a 100% correlation between the ability of P. phaseolicola isolates to induce chlorotic haloes in bean leaves and to inhibit E. coli. By addition of sterile filtrated citrulline the phaseolotoxin-caused inhibition zones were nullified and thus specifically identified.

Several experiments showed that P. phaseolicola multiplies in the bean soaking solution at temperatures of 4-6°C. Therefore, a rather long soaking time (20 h) increased the sensitivity of this method. Most of the saprophytes are inhibited by the antibiotics contained in the semi-selective medium of Mohan and Schaad (1987).

Also centrifugation of the soakate enhanced the sensitivity of the test approximately 10-fold. Addition of bacterial suspension to a bean soakate leading to 18 CFU/ml as well as the addition of one infected seed to 50,000 healthy seeds could be detected without problems. Therefore, it can be assumed that even a lower degree of bean seed infestation by P. phaseolicola could be detected by this method.

The required time for the here described assay procedure can be estimated as follows:

extraction of bacteria from bean seed by soaking	1 d
growth on semi-selective medium	3 d
production of phaseolotoxin	1 d
bio-assay for phaseolotoxin	1 d
total	6 d

The procedure reported here to detect P. phaseolicola thus offers several advantages: It is easy to perform, very sensitive and reliable and requires only 6 days. Compared with the numerous physiological tests for identification of pathovars, the E. coli bioassay for phaseolotoxin production is a simple, dependable and specific test (Jansing and Rudolph 1989).

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

HYPERSENSITIVE REACTION INDUCED BY ACETIC-ACID BACTERIA IN TOBACCO LEAF

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The acetic-acid bacteria belong to the genera Acetobacter and Gluconobacter of the new family Acetobacteraceae [2]. For the great similarity of their morphological, physiological, and biochemical characters with the Pseudomonas bacteria the former were until recently, referred to the fam. Pseudomonadaceae [1,7]. Irrespective of the taxonomic differences between these bacteria, the presence of phylogenic relationship of acetic-acid bacteria with representatives of the genus Pseudomonas is emphasized in more recent studies, too [4,9]. In addition to that, there are announcements about the isolation of acetic-acid bacteria as plant pathogens like many species of Pseudomonas [10]. Strains of Acetomonas oxydans (now Gluconobacter oxydans), Acetobacter xylinum and Acetobacter sp. have been established on leaves, flowers, and fruits of oil palm tree (Elaeis guineensis) [3]. The species Acetobacter melanogenus and A. oxydans are reported as agents causing pineapple pink disease [8]. Strains of Acetobacter and Gluconobacter are isolated from rotten apples and pears during their postharvest storage. After an artificial inoculation of ripe apple and pear fruits with these strains, browning and necrosis with no softening of tissue have been observed [11].

Having in view the announcements related to the phytopathogenic manifestation of some acetic-acid bacteria and their phylogenetic relationship with Pseudomonas, we carried out the present study. Its object was to test our different collection strains of Acetobacter and Gluconobacter for their pathogenicity by inducing a hypersensitive reaction on tobacco leaves and by inoculation of orchard fruits.

MATERIAL AND METHODS

Twenty eight acetic-acid bacterial strains from the collection of the Department of Microbiology and Biotechnology at the Higher Institute of Food and Flavour Industries in Plovdiv were included in the experiments. Five of them were provided from other collections as is shown in Table 1. The strains isolated by us were identified in accordance with Bergey's Manual [2] and refer to the species Acetobacter aceti, A. hansenii, A. pasteurianus, and Gluconobacter oxydans. For strains synthesizing cellulose and having different specific biological characters, the specific name Acetobacter xylinum used in the past has been retained [5].

As an indicator for a rapid manifestation of pathogenic properties was used the tobacco cv. Samsun infiltrated by the method of Klement [6] with a suspension of living or killed cells prepared from a 48-hour culture containing from $4.5.10^8$ to 10^9 cells/ml. The occurrence of hypersensitive reaction (HR) was recorded in 6, 12, 24, and 48 h after the infiltration and that of delayed chlorosis (C) or necrosis (N) - on the 3rd, 7th and 14th day.

The inoculation of fruits was made by dropping the same suspension on the fruit surface and pricking with a sterile needle. Green and ripe fruits of apples (cv. Golden Delicious) and cherries (cv. Bing), green fruits of pears

(cv. Passe Crassane), plums (cv. Stanley) and peaches (cv. Red Haven) have been used. The infected fruits were incubated for 48 h in a wet chamber at room temperature. The appearance of necrotic spots on them was registered by the 7th day after inoculation.

Sterile distilled water was used as control instead of bacterial suspension. For comparison, tobacco was infiltrated with a suspension of Pseudomonas syringae that induced a typical hypersensitive reaction (HR).

RESULTS

Acetic-acid bacteria from different species of the genera Acetobacter and Gluconobacter develop phytopathogenic properties. When infiltrated in tobacco leaves, strains of Acetobacter unduce a hypersensitive reaction in 6 to 24 h. With other strains a delayed chlorosis (C) is observed. The tobacco leaf responses to different strains and species of acetic-acid bacteria are shown in Table 1.

It is seen that only strains from the genus Acetobacter induce a hypersensitive reaction and chlorosis in tobacco leaves. With strains from the genus Gluconobacter no such reactions occur (Table 1). Pathogenic properties have been established in strains from our and foreign collections referring to the species A. aceti, A. hansenii, A. xylinum, and A. pasteurianus. With the species A. aceti four of the seven strains induce HR and C. One of the three strains used, A. hansenii, provokes a highly expressed chlorosis, and the other two - a slight HR. The bacteria of the species A. xylinum induce more often HR (6 of the 9 strains used) and only 3 strains cause chlorosis. The four tested strains from A. pasteurianus induce HR, and two of them - C. No difference is established in the HR caused by acetic-acid bacteria and P. syringae.

Table 1. Responses of Tobacco Cv. Samsun Induced by Acetic-Acid Bacteria

Species, Strain		Type and Rate of Response HR	C
Acetobacter aceti	023	1	2
	055	4	2
	100	1	3
AUC	897	3	1
Acetobacter hansenii	014	0	3
	016	1	0
	086	1	0
Acetobacter xylinum	001	1	0
	002	1	1
	059	2	2
	070	2	0
	071	4	0
ATCC	10245	2	2
Acetobacter pasteurianus	061	1	0
	062	1	2
	063	1	0
ATCC	9433	2	1
Gluconobacter oxydans	069	0	0
	099	0	0
	106	0	0
NCIB	4339	0	0
CCM	1792	0	0
Pseudomonas syringae pv. syringae	440b$_2$	3	0
Control		0	0

HR - hypersensitive reaction C - chlorosis

Rates - affected area
0 - no symptoms
1 - 1/3
2 - 1/2
3 - 2/3
4 - whole inter neural area

AUC - All-Union Collection, USSR
ATCC - American Type Culture Collection, Rockville, USA
CCM - Czechoslovak Collection of Microorganisms, Brno
NCIB - National Collection of Industrial Bacteria, UK

The acetic-acid bacteria manifest pathogenicity in orchard fruits, too. All the tested strains from Acetobacter and Gluconobacter cause rotting in ripe apples and cherries. With respect to green apple fruits, necrotic spots are produced by of strains. The acetic-acid bacteria develop pathogenic properties mostly on green pear fruits, more rarely on plums and not a single strain forms necrotic spots on peach fruits.

CONCLUSIONS

Acetic-acid bacteria from genera Acetobacter and Gluconobacter develop phytopathogenic properties. In green fruits of pear and plums, green and ripe fruits of apples and cherries, pathogenicity is displayed by representatives both of Acetobacter and Gluconobacter. When infiltrated in tobacco leaves by the method of Klement a hypersensitive reaction is induced only by species of the genus Acetobacter (A. aceti, A. hansenii, A. pasteurianus, and A. xylinum). Some of these species induce chlorosis.

The tested Gluconobacter strains induce neither hypersensitive reaction nor chlorosis on tobacco leaves. That test could be used for the differentiation of the two genera.

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Proc. 7th Int.Conf. Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria
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DESCRIPTION OF A CORYNEFORM PLANT GROWTH-PROMOTING RHIZOBACTERIUM (PGPR) STRAIN AND ITS EFFECTS ON PLANT DEVELOPMENT

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INTRODUCTION

Recent efforts to increase plant growth via inoculation of seeds with beneficial bacteria have focussed on the use of PGPR - plant growth-promoting rhizobacteria (Kloepper et al. 1989a). Rhizobacteria are a subgroup of rhizosphere bacteria which exhibit root colonization. Root colonization is a process whereby the bacteria survive inoculation onto seeds or into soil, multiply in the spermosphere in response to seed exudates rich in carbohydrates and amino acids, attach to the root surface, and colonize the developing root system (Kloepper et al. 1989b). Most PGPR strains reported to date are members of the fluorescent pseudomonads (Kloepper et al. 1988a); however, some Serratia spp. (Kloepper et al. 1988b), Bacillus spp. (Turner & Backman 1989), and a coryneform strain (Kloepper et al. 1988b) have also been reported.

The coryneform PGPR strain 44-9 was first reported on canola (rapeseed - Brassica campestris) (Kloepper et al. 1988b) where it increased leaf area in greenhouse trials and yield in field trials. We now report a summary of results from more extensive tests with strain 44-9 and its potential use as a microbial inoculant to enhance plant growth.

SOURCE AND IDENTIFICATION OF STRAIN 44-9

Strain 44-9 was isolated as follows. Soil samples were collected from 27 sites in eastern Canada. Five-gram samples were mixed with 100mL of 30% canola seed exudate broth. After incubation for 10 days at 4°C and 50 rpm, the samples were diluted and plated onto nutrient agar and Pseudomonas agar F (PAF), and plates were incubated for 7 to 10 days at 4°C. The fastest growing strains were purified and stored at -80°C.

Strain 44-9 was selected from nutrient agar (NA) from a soil sample in the subarctic. The strain was Gram-positive with aging cultures sometimes retaining crystal violet unevenly. Cells were asporogenous, predominantly rod-shaped with tapered ends; branching and swelling of cells were periodically observed. Strain 44-9 was identified by the American Type Culture Collection as either Curtobacterium or Aureobacterium sp.

INITIAL TESTS FOR GROWTH PROMOTION

Strain 44-9 was first tested for growth promotion on canola in a greenhouse assay. Bacteria were grown for 3 days at 10°C on PAF, scraped off plates, and mixed with an aqueous solution of 0.1 M $MgSO_4$. Canola seeds were agitated in the bacterial suspension for 10 min. Control seeds were treated in the same manner with noninoculated PAF plates substituted for plates with bacteria. Two seeds were planted per pot in a 1:1 mix of field soil and perlite. Pots were placed at 10°C for 1 week, then transferred to a greenhouse with day and night temperatures of 22 and 15°C, respectively. Seedlings were thinned to one per pot after emergence.

The leaf areas of canola plants from treatments with 44-9 were compared to controls 3-4 wks after emergence. A visual scale of 0-3 was used where 0 = leaf area equivalent to the control, 1 = slightly greater leaf area, 2 = moderately greater leaf area, and 3 = much greater leaf area than the control. Strain 44-9 induced a leaf area rating of 2 or 3 in 8 of 11 repeat tests.

EFFECTS OF STRAIN 44-9 ON OTHER PLANTS AND ON FUNGI

Strain 44-9 was tested for growth promotion on other crops including maize, tomato, wheat, bean (Phaseolus), and conifers. In all cases, seeds were treated with suspensions of washed cells grown in tryptic soy broth. Experiments were conducted over a three-year period as part of a screening program to select additional PGPR strains for each crop. Assays consisted of greenhouse tests with field soil. Total dry weight of plants was recorded for treatments and controls. Root colonization capacity of strain 44-9 was measured using a spontaneous mutant resistant to 100 ug/mL rifampicin.

Strain 44-9 induced significant increases in dry weight of all tested crops in several repeat experiments (Table 1). Roots of all crops were colonized by strain 44-9 at population densities from Log 3 to Log 7 cfu/g root.

Strain 44-9 was tested for *in vitro* antibiosis toward several pathogenic fungi (Table 1) on PAF, NA, tryptic soy agar, and potato dextrose agar. No antibiosis was observed against any of the fungi on any of the media.

TABLE 1 EFFECTS OF PGPR STRAIN 44-9

Colonizes Roots of and Promotes Dry Weight of:	No Antibiosis Toward:
Maize	*Pythium ultimum*
Canola (*Brassica napus*)	*Rhizoctonia solani*
Tomato	*Fusarium solani*
Wheat	*Thielaviopsis basicola*
Bean (*Phaseolus*)	
Conifers	

PROMOTION OF CANOLA YIELD

Numerous field trials were conducted over a four-year period with strain 44-9 on canola. All trials used a randomized block design with 6 to 8 replicate 2 or 4 row plots. Five to six other PGPR strains were tested in each experiment. Formulations tested changed each year. Two controls were used, a nontreated control and a formulation control.

The first year of trials (Table 2) used mainly a formulation of non-sterile peat. Strain 44-9 induced an all-site average yield increase of 10% compared to the nontreated control and 30% compared to the formulation control, which indicates that the nonsterile peat was deleterious to canola.

TABLE 2 EFFECT OF INOCULATION WITH 44-9 ON YIELD OF CANOLA RELATIVE TO CONTROLS IN 1985 (NONSTERILE PEAT CONTROLS)

TRIAL*	YIELD, % OF CONTROL	
	NONTREATED	FORMULATION
1	+10	+113
2	-10	-8
3	-17	-6
4	+39	+22
5	+10	+30
6*	+30	+30
YEAR AVERAGE	+10	+30

*Trials 1-5 used a formulation of non-sterile peat; trial 6 used an in-furrow suspension of bacteria

In 1988, a sterile peat formulation of strain 44-9 induced a similar all-site average yield increase (10%) without deleterious effects. The best experimental formulation of strain 44-9 was a sterile liquid polymer which induced an all-site average yield increase of 20% compared to the nontreated control in 1988. The four-year average yield increase using strain 44-9 was 8% compared to nontreated controls and 14% compared to formulation controls (Table 3).

TABLE 3 SUMMARY-EFFECT OF INOCULATION WITH 44-9 ON YIELD OF CANOLA RELATIVE TO CONTROLS IN FOUR YEARS OF FIELD TRIALS

YEAR	FORMULATION USED	% AVERAGE INCREASE	COMPARISON
1985	Nonsterile peat	+10	Nontreated
		+30	Formulation
1986	Oil-based	+1	Nontreated
			Formulation
1987	In-furrow spray	+4	Nontreated
1988	All tested	+15	Nontreated
	Sterile peat	+10	Nontreated
	Sterile polymer	+21	Nontreated
Four Year Average			vs. Nontreated = 8%
			vs. Formulation = 14%

ENHANCEMENT OF SEEDLING EMERGENCE

In some of the initial field trials, an enhanced seedling emergence rate was noted with treatment of strain 44-9. Follow-up experiments were conducted to assess emergence promotion by strain 44-9 on canola and maize in field trials. The bacterial treatment induced increases in emergence rate and final stand in canola and maize. Emergence promotion was also assessed in phytotrons at a constant temperature of 10 - 14°C in field soil for tomato, wheat, conifers, and beans. All crops except conifers demonstrated enhanced seedling emergence after seed treatment with strain 44-9.

SURVIVAL IN FORMULATIONS

Strain 44-9 was formulated in four different ways over the years of field testing (Table 3). The strain consistently demonstrated survival 1 to 3 Log units greater than most pseudomonad strains. The long-term shelf life of strain 44-9 was assessed in a sterile peat formulation at 4 and 30°C (Figure 1). At 4°C, populations dropped from Log 11 cfu/g peat to Log 10 after storage for 250 days, while the corresponding decline at 30°C was from Log 10 to Log 5. When sterile peat was used to treat seeds, and the seeds were stored for 250 days, populations were relatively stable at Log 7 cfu/seed at 4°C and Log 6 cfu/seed at 30°C.

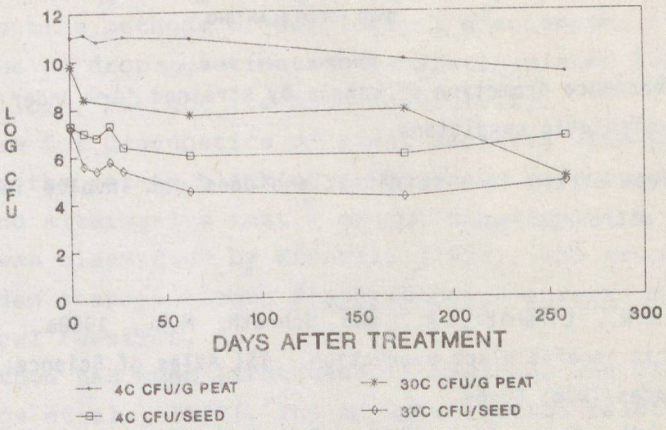


Fig. 1. Long-term shelf life of PGPR strain 44-9 in sterile peat stored at 4 and 30°C.

MECHANISMS OF GROWTH PROMOTION

The mechanism for growth promotion appears to be direct stimulation of plant growth rather than inhibition of pathogens. No in vitro antibiosis was observed for pathogens listed in Table 1. Strain 44-9 did not elongate roots in the gnotobiotic assay used by Lifshitz et al. (1987) to detect direct growth promotion by PGPR strains.

The potential for strain 44-9 to promote plant growth directly was further assessed using a gnotobiotic emergence assay with canola. Seeds were treated with bacterial suspensions of washed cells and planted in tubes containing a 1:1 mix of sterile sand and vermiculite with nutrient solution. Tubes were incubated at a day/night temperature of 18/10 C. Strain 44-9 enhanced the emergence rate and final stand under these gnotobiotic conditions (Figure 2). These results support the theory that growth

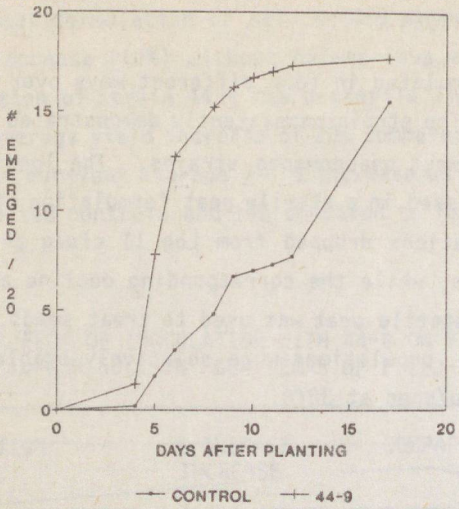


Fig. 2. Emergence promotion of canola by strained 44-9 under gnotobiotic conditions.

promotion from strain 44-9 is direct and does not involve inhibition of native soil microorganisms.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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POTATO BLACKLEG DIAGNOSTICS BY CO-AGGLUTINATION REACTION WITH STAPHYLOCOCCUS

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Among routine methods of serological diagnostics of bacteria the technique of drop agglutination is distinguished for its relative simplicity but not its high sensitivity.

Therefore for diagnostics of plant bacteria with particular reference to the potato blackleg pathogen we have proposed more sensitive and alternative test - co-agglutination with staphylococcus. It was classified by Kronvall (1973), who proposed to use the golden staphylococcus Staphylococcus aurens as a mediator in serological reaction.

This method has been first used in medicine and plant virology (Chirkov et al., 1981). The co-agglutination reaction is based on a unique ability of the golden staphylococcus to absorb on its surface blood gamma-globulin fraction where antibodies are concentrated. Specific antibodies of diagnostic sera are also easily absorbed by immunosorbent forming staphylococcus which is able to react with homologous antigen. The principal pattern of such reaction is shown in Figure 1.

In tests specially treated staphylococcus preparation (non-pathogenic strain Cowan 1) and immune serum are prepared for the potato blackleg pathogen. Afterwards 0.1 ml serum was added to 1.0 ml 20% staphylococcus suspension and mixed in a magnetic mixer for 1 h. The mixture was diluted with 9 volumes of tris-buffer 0.1 M pH 8.0, washing off in such a way excessive antibodies from staphylococcus. The centrifugation was performed at 5000 uv./min for 15 min and precipitate was carefully suspended in the same buffer, adjusting staphylococcus concentration to 2.0%. In the same way staphylococcus with control rabbit serum

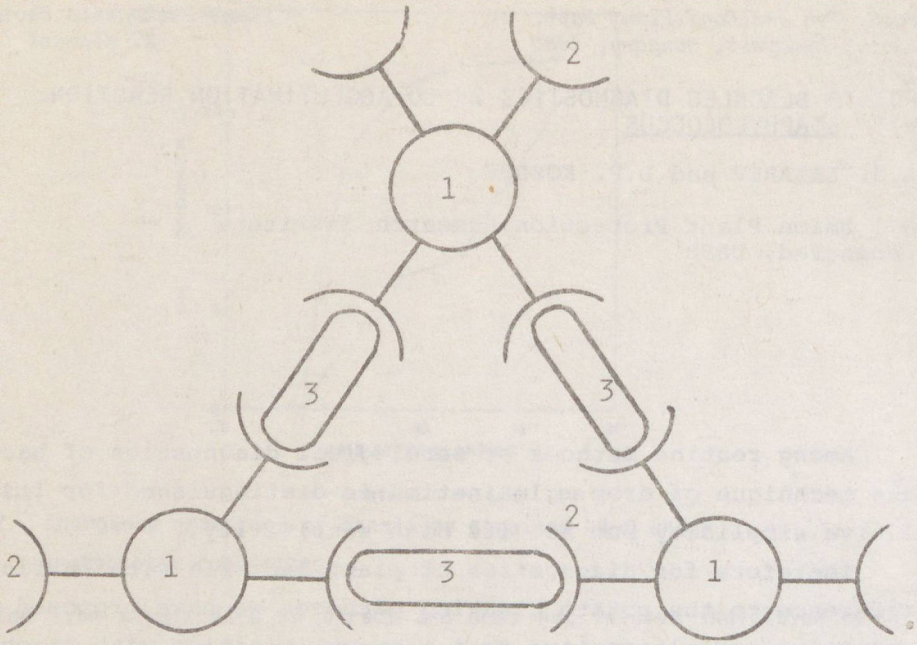


Fig. 1. Co-agglutination reaction pattern with staphylococcus.
1 - staphylococcus
2 - antibody
3 - bacterium, causal agent of blakleg

was prepared. The produced reagent was a homogenous suspension of milk diluted colour.

In order to carry out the reaction 24 h culture of the potato blackleg pathogen (pathogenic strain Ervinia carotovora subsp. atroseptica G784) was used that was grown in potato agar. The bacterial suspension was prepared by phosphate buffer (pH 7.0) washing and serial dilutions were made from 10^9 to 10^6 cells/ml. The reaction was carried out as follows: a drop of suspension from each dilution was mixed with the staphylococcus reagent on a slide. The co-agglutination proceeded immediately and enchanced for 1-2 min. It reasulted in forming large flaky aggregates. The mixture of bacterial suspension with control staphylococcus reagent remained homogeneously turbid. The sensitivity of co-aglutination appeared to be 10 times higher than the generally accepted drop aglutination test (Table 1).

Table 1. Comparison of aglutination and co-agglutination reactions on blackleg pathogen culture

Reaction	Number of bacteria (million/ml)									
	1000	500	250	125	100	50	25	10	5	1
Agglutination	++	++	+	+-	-	-	-	-	-	-
Co-agglutination	++	++	++	++	++	++	+	+-	-	-

(++) - strong and (+) - weal reaction; (+-) - doubtful;
(-) - negative

To determine the phytopathogen in tubers and stems diseased tissue by volume 0.5 cm^3 was macerated with 3-4 ml saline or buffer solution in porcelain pestle to get a homogenous mass which was passed several times through double filter. The reaction was performed as with pure bacteria culture. It was determined in this case that the sensitivity of co-aglutination reaction was higher than in the drop aglutination. During mass analyses of infected potato material non-specific reactions are possible. The test proposed is successfully used for differentiating isolates of various pathogen serogroups.

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Proc. 7th Int. Conf. Plant Path. Bact., Budapest, Hungary, 1989

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NUMERICAL TAXONOMY AND PROBABILISTIC IDENTIFICATION OF GRAM POSITIVE PLANT PATHOGENS

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A numerical taxonomy study was undertaken on one hundred strains, including fresh isolates and reference organisms, in order to establish possible affinities between phytopathogenic corynebacteria and to provide a scheme of probabilistic identification of the microorganisms by using the most diagnostic characters to construct a matrix for the identification of unknowns.

A description of the methods followed to assess the 132 morphological, biochemical and physiological features of the 90 bacterial strains (including 70 phytopathogenic coryneform bacteria), is given in Locci *et al.* (1989) and Firrao and Locci (1989a).

Multivariate analysis was carried out with CLUSTAN 1C (Wishard, 1978) using Simple Matching (S_{SM}) as similarity coefficient and the UPGMA method for clustering. The matrix for the probabilistic identification was constructed by a program named CREMAPI (Firrao, unpublished) which integrated algorithms developed by Sneath (1979b, 1980a,b). Probabilistic identification was carried out using the MATIDEN program (Sneath, 1979a), which calculates Willcox probability, taxonomic distance and its standard error score, and the AFFIN program (Goodall *et al.*,1987), which calculates dissimilarity indices.

Average probability of erroneous results was calculated from the pooled variance ($s^2 = 0.0435$), and was equal to $p = 4.56$.

At 81.6% S_{SM} level six major clusters can be recognized (Fig.1). Plant pathogenic corynebacteria are mainly (62 cultures out of 71) recovered in Clusters A (*Curtobacterium betae* and *Curt.flaccumfaciens*), D (*Clavibacter iranicus, Clav.michiganensis,* including its subspecies *insidiosus, michiganensis, nebraskensis* and *sepedonicus, Clav.rathayi* and *Clav.tritici*) and F (*Rhodococcus fascians*). *Arthrobacter ilicis* does not associate with these phene and similarly *Corynebacterium beticola* shows no relationship whatsoever with other phytopatogenic corynebacteria (Firrao and Locci,1989b).

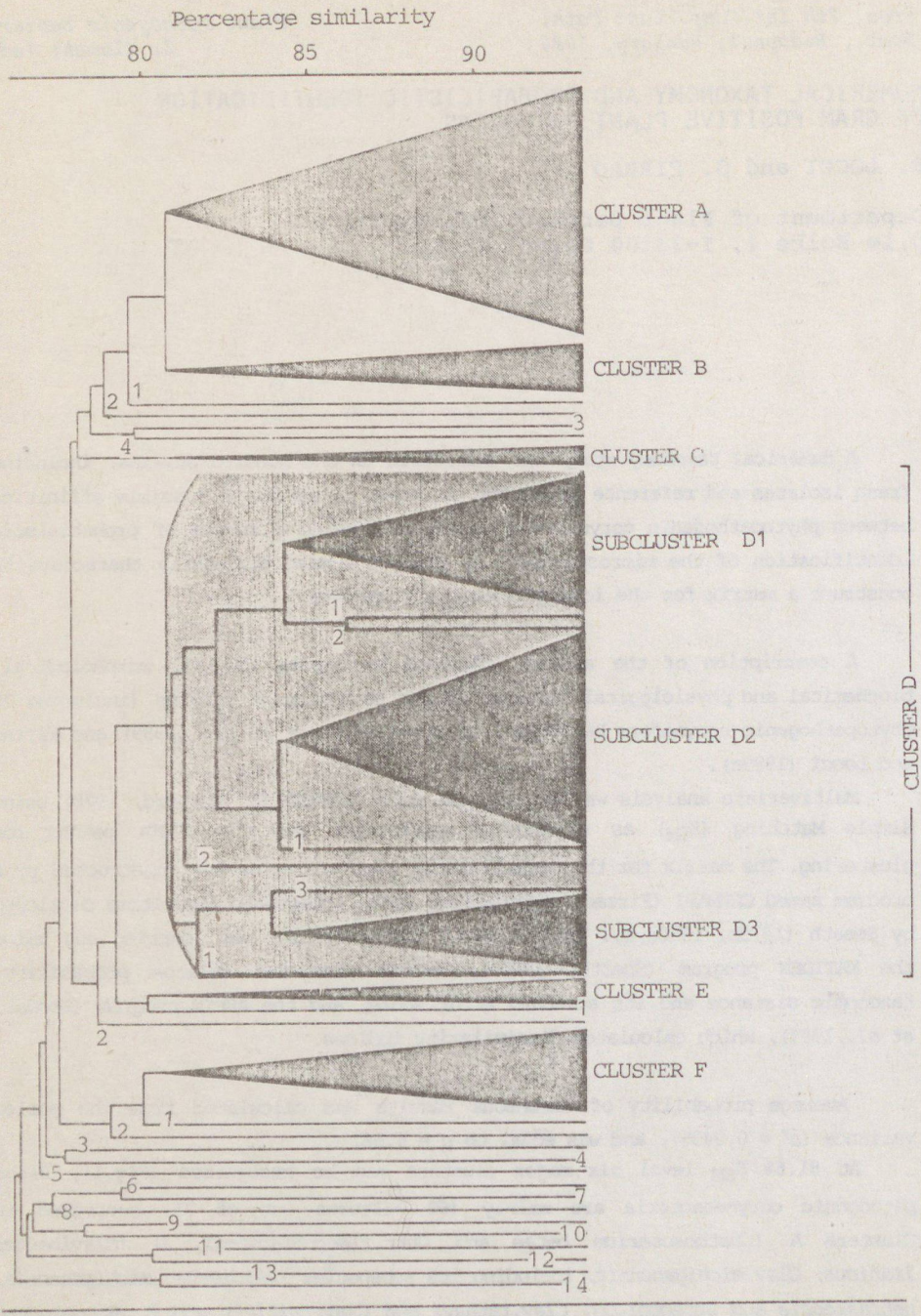


Fig. 1: Dendogram derived from the UPGMA Analysis. Clusters and subclusters were formed at 81.6% and 84.6% S_{SM} respectively

Inside Cluster A, which harbours organisms now attributed to the genus *Curtobacterium*, some subclustering is possible, but there appears to be no association between possible groupings and recognized species (*Curt.betae* and *Curt.flaccumfaciens*) or subspecies (*oortii* and *poinsettiae*) names.

Cluster D comprises all the species at present classified in the genus *Clavibacter*. In particular eight out of nine cultures of *Clav.rathayi* and *Clav.tritici* are recovered in Subcluster D1, together with corynebacteria not identified at the specific level but isolated, as were the cultures of *Clav.rathayi*, from *Dactylis glomerata* in the USA.

Clav.michiganensis subsp.*sepedonicus* and a series of unidentified potato tuber isolates, make up Subcluster D2, together with the subspecies *insidiosus*. The latter clusters separately at 89.4% S_{SM}. Subcluster D3 consists of the two subspecies *michiganensis* and *nebraskensis*, while the only culture of *Clav.iranicus* examined appears among unclustered organisms.

Five out of six named cultures of *Rhodococcus fascians* are recovered in Cluster F. Clusters B and E are composed by five cultures, four of which are of phytopathological interest. Two marker organisms are included in Cluster C.

←

Cluster B

1. Arch. haemolyticum NCTC 9697
2. Curt. flaccumfaciens NCPPB 558
3. Brev. linens NCIB 9910
4. Cory pyogenes HIK A205

Subcluster D_2

1. Clav. mich subsp. insidiosus DSM 20157
2. Clav. iranicus NCPPB 2253
3. Clav. mich subsp. michiganensis DSM 20134

Subcluster D_1

1. Clav. rathayi NCPPB 2392
2. Clav. mich subsp. sepedonicus NCPPB 2138

Subcluster D_3

1. Clav. mich susp. nebraskensis NCPPB 2579

Cluster E

1. Curt. albidium NCIB 11030
2. Corynebacterium sp. NCPPB 1624 (b)

Cluster F

1. Rhod. fascians NCPPB 764
2. Clav. mich nebraskensis NCPPB 2582
3. Xanth. campestris NCPPB 1420
4. Pseud. syringae ATCC 19304
5. Noca. vaccini ATCC 11092
6. Arth. polychromogenes NCIB 10267
7. Cory. 'manihot' NCIB 9097
8. Arth. globiformia NCIB 9220
9. Arth. ilicis NCPPB 1228
10. Micr. imperiale NCIB 9888
11. Arth. simplex NCIB 8929
12. Phaseolus sp. isolate n.6
13. Euph. pulcherrima isolate
14. Erwinia herbicola NCPPB 3128

In order to create the probabilistic identification matrix for phytopathogenic coryneform bacteria, the 132 tests used in the numerical study were evaluated by the CREMAPI program, which selected 19 characters for the recognition of the 3 main clusters, namely clusters A, D and F and 27 characters for identification at subcluster level (table 1). The sum of scores of the most diagnostic character-states, the identification scores for the Hypothetical Median Organism of each cluster and the estimated overlaps between clusters (calculated as in Sneath, 1980c) are very satisfactory and insure against the possibility that no members of a cluster remain unidentified or misidentified.

At present there is general agreement that the phytopathogenic corynebacteria do not belong to the genus *Corynebacterium*, though it is a matter of controversy where they should be placed at the generic level (Davis, 1986).

Numerical taxonomy has been widely employed for clarifying relationships among microorganisms, however few studies have been carried out exclusively on plant pathogenic corynebacteria.

Results obtained in the present study confirm the validity of separating phytopathogenic corynebacteria into different generic taxa. In addition they show some clear-cut relationships among the organisms investigated.

Three main clusters accommodate plant pathogenic corynebacteria at present classified in the genera *Curtobacterium* (Cluster A), *Clavibacter* (Cluster D) and *Rhodococcus* (Cluster F) respectively. The species *Cory.ilicis*, attributed to the genus *Arthrobacter*, does in fact not cluster with any of the previously mentioned groupings. At the same time the illegitimate taxonomic attribution of

Tab.1: List of test to be performed in order to achieve probabilistic identification with the programs AFFIN (Goodall *et al.*, 1987) or MATIDEN (Sneath, 1979a)

Tests required for identification at cluster level:

Rods > 3.5µ in length	Motility
Colony opaque	Colony colour orange
Sediment flaky	Sediment viscid
KOH reaction	Tolerance of NaCl
Inhibition by K tellurite	Inhibition by K thiocyanate
Inhibition by Na taurocholate	Degradation of casein
Utilization of citrate	Acid production from glucose
Acid production from arabinose	Acid production from glycerol
Acid production from sorbitol	Acid production from fructose
Litmus alkaline reaction	

Additional tests required for identification at subcluster level :

Inhibition by Na selenite	Degradation of aesculin
Degradation of arbutin	Degradation of gelatine
Degradation of hippurate	Colony punctiform
Colony elevation convex	Colony colour yellow

Cory.beticola to corynebacteria (Firrao and Locci, 1989b) is confirmed by its recovery among unclustered organisms.

In general terms data obtained confirm the results of Dye and Kemp (1977) and of Seiler (1983), that is the existence of at least four major supraspecific taxa possibly corresponding to the proposed generic denominations of *Arthrobacter, Clavibacter, Curtobacterium* and *Rhodococcus.*

In regard to identification criteria there are no general reference values for probabilistic identification (Williams *et al.*, 1983). Many workers applying the Willcox coefficient, require different minimal values for positive identification ranging from 0.85 to 0.999. In the case of coryneform phytopathogenic bacteria identification should not be regarded as positive when taxonomic distance is greater than 0.35-0.40 and its standard error score higher than +3.0 - +3.2 even if the Willcox probability is very high. Furthermore the Willcox probability must not fall below 0.95, a value that can be considered minimal for positive identification using the scheme proposed.

Many taxonomic approaches have been used for the classification and identification of phytopathogenic gram positive bacteria and consequently many different taxonomic arrangements have been postulated on the basis of results obtained by testing different microbial features. The probabilistic scheme presented here has been objectively tested and should provide a workable system for the identification of these organisms.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

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DIAGNOSTICS OF XANTHOMONAS CAMPESTRIS PV. PELARGONII

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PATHOGEN

Bacterial leaf spot and stem rot of Pelargonium (called also bacterial blight) is caused by Xanthomonas campestris pv. pelargonii. The pathogen is a Gram negative rod, 0.4 x 1.0 µm, with single polar flagellum. On YDCa agar (Griesbach, Tegtmeier, Schmatz 1985) the bacteria create mucoid, convex, shining colonies with a yellow slime. For the detection of the pathogen from systemically infected plants, living slides were prepared. Bacteria were streaming from the edges of the cut stem tissue.

INOCULATION METHODS

Materials

Isolate no 9, obtained from naturally infected Pinto variety plant was used for inoculation. Five-day-old bacteria in the form of suspension were applied in concentration of 10^6 cells/ml. Experiments were conducted on 3 varieties belonging to Pelargonium x hortorum group: Purpurball, Bruni, Lachball.

The inoculation was carried out by five methods:

A: spraying of the entire plant

B: rubbing of leaves powdered with carborundum

C: injecting the main stem with the syringe

D: introducing inoculum to the cut petioles of the two lowest leaves using Durham's tubes (Goldenia, Pajewska,1988)

E: dipping of cut roots into inoculum for 1 h.

In the cases of A and B plants were kept for 24 h in a moist chamber.

Results

All investigated varieties reached in a similar way. There was no evident difference in the degree of infection among them. The symptoms were labelled as follows:

1 - round, necrotic leaf spots, surrounded by chlorotic edge /'halo'/, yellowing of leaves

2 - large, angular necrosis of leaf blades, yellowing of leaves

3 - drying of lower leaves apart from turgid petioles

4 - drying of entire individual leaves

5 - wilting of leaves

6 - 'water soaking' spots on petioles

7 - 'water soaking' spots on main stem

8 - 'water soaking' spots on flowering stems

9 - decreasing of development and breaking of flowering stems

10 - death of flower buds

Table 1. Main symptoms of infection according to inoculation method

Inoculation method	Time of first appearance of symptoms	Symptoms (according to the importance)
A-spraying	14 days	1, 2, 3, 4, 6, 8
B-carborundum	12 days	1, 2, 3, 4, 6
C-injecting	4.5 weeks	2, 5, 6, 7, 3, 4, 8, 9, 1
D-Durham s tubes	5 weeks	2, 5, 6, 1, 7, 8, 9, 3, 4
E-root dipping	4 weeks	2, 6, 3, 7, 1, 9, 10

SEROLOGICAL TESTS

A: Double diffusion in agar
(Ouchterlony 1948)

Materials

In this test 5 isolates of Xanthomonas campestris pv. pelargonii were used: isolate 8/2 from var. Minipel Orange
isolate 9 " " Pinto
isolate 18 " " Rubin
isolate 19 " " Alex
isolate 20 " " Ina.

Bacteria were suspended in saline with the addition of phenol-/1 drop/1ml/ at concentrations of: 10^6, 10^4, 10^2 cells/ml. The antiserum applied in all serological tests was received from the Institute of Plant Protection Aschersleben GDR.

The test also included the detection of bacteria present in sap from plants infected by spraying. The sap was obtained from leaves with visual symptoms of bacteriose, leaves without symptoms, petioles of both kinds of leaves, main stems.

Results

Isolates 9, 18, 19, 20 produced precipitation bands when using concentration of 10^6 cells/ml. Concentrations of 10^4 and 10^2 cells/ml were too small to obtain any positive result.

Isolate 8/2 produced definite negative results in 35% of the trials, the remaining 65% were less readable. The sap from plants infected by spraying showed positive results only when obtained from leaves with distinct symptoms.

B: ELISA
(Clark and Adams 1977)

Materials

Isolate 9 was used in 3 concentrations: 10^6, 10^4, 10^2 cells/ml. Bacteria and stem sap from plants suspected of disease were suspended in 3 kinds of agents: sterile water, saline solution

0.8% and PBS. As a control of antiserum sensitivity, Xanthomonas campestris pv. phaseoli from naturally infected Phaseolus vulgaris plant, was used. Gamaglobuline was diluted in coating buffer in 3 concentrations: 1:1000, 1:500, 1:250. Bacterial suspensions and plant samples were incubated at temperature of 25° and 4°C.

Results

The most readable concentration of gamaglobulin was 1:250. The temperature of 4°C was not effective - results were positive only in the case of 10^6 cells/ml.

Table 2. Results of ELISA test with an incubation temperature of 25°C

Suspension agent	Conc. of X. pelargonii 10^2	10^4	10^6	X. phaseoli 10^6	Stem sap
sterile water	-	+	+	-	-
saline	-	+	+	-	-
PBS	+	+	+	-	+

CONCLUSIONS

According to the inoculation method Xanthomonas pelargonii can appear as parenchymal tissue invader (in the case of spraying and rubbing leaves with carboroundum) or a systemic pathogen (in the case of injection, Durham's tubes, root dipping). This experiment showed that Xanthomonas pelargonii must be in contact with parenchyma to develop external symptoms or they can appear later as second-rate symptoms.

Test of double diffusion showed that concentrations of 10^4 and 10^2 cells/ml were not sufficient to obtain positive results. Isolate 8/2 was not reliable enough to be used in any further experiments. The plants inoculated by spraying showed the presence of bacteria in the double diffusion test only in leaves with the distinct symptoms.

In ELISA test, PBS was the only dissolving agent in which stem samples with systemic infection showed positive results. Since the occurrence of the disease in Poland is evident, the selection of propagation material on the basis of symptoms and serological tests should ensure that the cuttings are free from the disease.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

BACTERIAL UMBEL LIGHT OF CORIANDER IN WEST GERMANY

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SUMMARY

For several years a serious bacterial disease has occurred on coriander in West Germany. The pathogen infects the inflorescences and causes seed decay. A Pseudomonas species was isolated which caused identical disease symptoms after artificial inoculation. According to phenotypical characteristics and biochemical tests the bacterium falls into the group Ia of Pseudomonas.

INTRODUCTION

Coriander (Coriandrum sativum L.), an annual herb of the family Umbelliferae, unknown until recently in West-German agriculture, may gain importance as a field crop for industrial purposes. During 1987 and 1988 an unknown disease affected coriander in nurseries and fields of a plant breeding company in Northern Germany as well as at the university of Göttingen.

The disease, apparently bacterial in nature, was rarely observed on the leaves, petioles and stems but occurred frequently on floral parts and seeds of coriander. This umbel blight and seed decay of coriander caused widespread damage and reduced yield considerably. Reports on a similar disease of coriander appeared in Poland (Godlewska-Lipowa, 1966), Hungary (Németh et al., 1969), Britain (Taylor and Dudley, 1980) and the German Democratic Republic (Plescher, 1983).

The goal of this study was to identify the causal agent of the disease and test contemporary breeding lines of coriander for resistance against the disease agent.

SYMPTOMS

In the early stage of infection, young plants show dark-green water-soaked lesions on leaves, petioles and shoots, which later become brown. The symptoms appear predominantly on the inflorescences. Attacked flowers are discoloured to dark-brown and may decay. Water-soaked lesions may occur on the green, unripened fruits, later turning dark-brown and causing many of the seeds to become shrivelled (Fig. 1). Severely infected plants are ususally stunted and may wilt completely.

ISOLATION AND IDENTIFICATION OF THE PATHOGEN

Isolation: Microscopic examination of water-soaked and dark lesions on different parts of coriander plants revealed the presence of large numbers of bacteria. In most cases isolations resulted in either grey or pale-yellow colonies on King's medium B agar (King et al., 1954). The majority of the obtained isolates (38) showed grey colonies on King's medium B and produced a diffusible yellow-green pigment without fluorescence under UV light (360 nm), while 5 isolates with pale-yellow colonies produced a diffusible green fluorescent substance on the same medium.

Pathogenicity: All 43 isolates induced a hypersensitive reaction on tobacco (White Burley). Artificial inoculations with 12 representative non-fluorescent and 3 fluorescent isolates were performed by spraying bacterial suspensions (10^7 CFU/ml) onto young flowering coriander plants in the greenhouse.

When the plants were incubated at 80-90% relative humidity and 22 ^{o}C the first water-soaked spots appeared after 7 days, and later on all the described disease symptoms developed (Fig. 2 and Fig. 3). In all instances where symptoms followed inoculation, the inoculated bacterium was re-isolated.



Fig. 1. Disease symptoms on coriander infructescences after natural infection

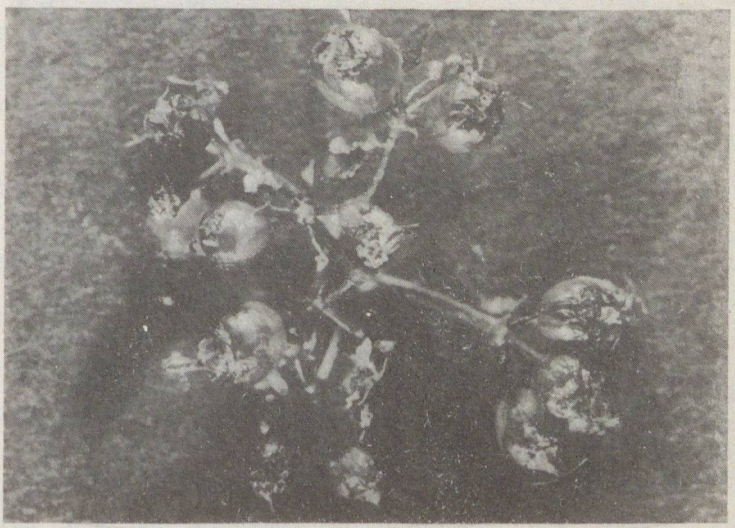


Fig. 2. Water-soaked and brown lesions on unripened fruits of coriander 2 weeks after artificial infection



Fig. 3. Flowerstand blight on artificially infected coriander with bacterial exudates



Fig. 4. Formation of filamentous bacterial exudates out of the water-soaked tissue of coriander 4 weeks after artificial inoculation

If the inoculated coriander plants were held for more than 15 days at 18-20 °C and at high relative humidity (70-90%) infection often resulted in the formation of filamentous bacterial exudates out of the water-soaked tissues (Fig. 4). Only the non-fluorescent strains induced the typical disease symptoms. The rested fluorescent strains solely caused a rot on a few unripened fruits.

Seven breeding lines of coriander tested for resistance agains two highly virulent, non-fluorescent isolates reacted as very susceptible in the greenhouse.

CHARACTERIZATION OF THE PATHOGEN

The pathogenic isolates were Gram-negative, rod-shaped, motile bacteria, mainly with 1-2 flagella. Tests for LOPAT reactions (Lelliott et al., 1966) with 6 representative non-fluorescent isolates showed that all isolates were positive for levan production and tobacco hypersensitivity, but negative for oxidase and arginine dihydrolase and negative or weakly positive for the ability to rot potato slices. A non-fluorescent isolate of Pseudomonas sp. (NCPPB 3115) pathogenic to coriander behaved similarly in comparison with our isolates.

According to these preliminary studies the coriander pathogenic isolates belong to group Ia of Pseudomonas which includes Pseudomonas syringae. However, the bacterium differs from the pathovar syringae.

DISCUSSION

The studies indicated that a Pseudomonas sp. was responsible for the umbel blight and seed decay of coriander in West Germany. Similar results were obtained by Taylor and Dudley (1980) in Britain. The isolates of Taylor (e.g. NCPPB 3115) seemed to be identical with those from Germany. In contrast, Németh et al. (1969), described the same disease symptoms on coriander in Hungary, but the bacteria isolated were fluorescent. Bacterial isolates obtained from Németh and tested in Göttingen did not cause typical disease symptoms, instead only a rot on a few unripened fruits occurred.

According to the determinative scheme of Lelliott et al. (1966) the pathogenic isolates belonged to group Ia of Pseudomonas. An exceptional feature was the non-fluorescence and a weak rot of potato discs in some tests.

The pathogen seems to be seed-borne because infested seeds yielded diseased seedlings, while seedlings of dry hot treated seeds showed no symptoms (Toben, unpublished).

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

DIAGNOSIS OF BACTERIAL SHEATH BROWN ROT OF RICE (PESUDOMONAS FUSCOVAGINAE)

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ABSTRACT

Three identification tests were assessed for rapid and accurate diagnosis of bacterial sheath brown rot of rice caused by *Pseudomonas fuscovaginae* :

- a biochemical profile comprised of eight characteristics : levan and 2 ketogluconate production, presence of cytochrome C oxidase and of arginine dihydrolase, production of acids from inositol, sucrose, sorbitol and trehalose ;
- antigenicity, by tube agglutination, with an antiserum to a strain of *P. fuscovaginae* from Madagascar.
- pathogenicity on rice seedlings.

They were applied to seven known strains of *P. fuscovaginae* and to 136 fluorescent bacterial strains isolated from rice plants originating from Brazil, Cameroon, Madagascar, Réunion and Rwanda. Application of at least two out of three tests was required in order to distinguish *P. fuscovaginae* from other fluorescent pseudomonads. The presence of the pathogen was confirmed in Madagascar and Rwanda.

Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

OCCURRENCE OF BASAL GLUME ROT (PSEUDOMONAS SYRINGAE PV. ATROFACIENS) ON CEREALS IN WEST GERMANY AND TESTING FOR RESISTANCE IN WHEAT

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Abstract

A survey in 1987 and 1988 revealed that basal glume rot, caused by *Pseudomonas syringae* pv. *atrofaciens*, occurred nearly everywhere in West Germany. The symptoms of the disease usually consisted of water-soaked dark green lesions on unripe wheat heads, mainly at the basal end of the glumes, which later became dark brown. Forty-six isolates of *P.s.*pv. *atrofaciens* were obtained from glumes, seeds and leaves of wheat and barley. For a fast identification of the isolated bacteria, a bioassay was used. Four to five day-old wheat seedlings, grown on wet filter paper in Petri dishes, were pricked at 2-3 sites with a dissecting needle contaminated with bacteria. After two days, pathogenic isolates induced brown to black spots. During screening resistance, several cultivars of spring and winter wheat were tested in the greenhouse and/or field tests. The results showed marked differences in the susceptibility of different cutivars.

INTRODUCTION

P. syringae pv. *atrofaciens*, the incitant of basal glume rot of wheat, barley and rye, has been long known in many wheat growing areas of the world (FAHY & LLOYD, 1983). In Europe it was described in USSR (GALATCHIAN, 1941), in Bulgaria (VASSILEV & KAROV, 1982) and only recently in Italy (VARVARO, 1983). In West Germany the disease has been observed for several years by plant-breeders and the bacterium was first isolated in 1985

(RUDOLPH & MAVRIDIS, 1987). In order to gain more knowledge on the occurrence and distribution of the disease in West Germany we tried to isolate the pathogen from different regions. In addition, resistance tests were carried out.

MATERIALS AND METHODS

Isolation of bacteria: Small pieces of plant tissue were comminuted in a small volume of sterile distilled water and this dilution was plated on the semi-selective medium of MOHAN & SCHAAD (1987). Typical colonies which proved to be oxidase negative were transferred onto King-B medium (KING et al., 1954). Only fluorescent colonies were selected for further characterization.

A bioassay on wheat seedlings proved very useful for speeding up identification. Four to five day-old wheat seedlings grown on wet filter paper in Petri-plates were pricked at 2-3 sites by a fine dissecting needle which was contaminated by bacterial slime. After 2 days incubation at 25 °C, symptoms became visible.

Characterization of bacterial isolates: Positive isolates were further characterized by flagella-staining (RUDOLPH & MAVRIDIS, 1989), hypersensitive response (HR) on tobacco (KLEMENT et al. 1964), inhibition of *Escherichia coli* (GASSON, 1980) and additional biochemical and physiological tests (LELLIOTT et al., 1966).

Inoculation of adult plants: Spring wheat plants grown in the greenhouse were inoculated in the water ripe stage. A bacterial suspension containing 10^7 cfu/ml was sprayed onto the ears until small water droplets appeared on the glumes. The inoculated plants were incubated in a humidity chamber with illumination (ca. 95% relative humidity, 18-22 °C). Screening for resistance:Twenty seven cultivars of winter wheat and 12 spring wheat varieties were tested in the greenhouse as described above. The spring wheat cultivars were also tested in the field in 1987. For evaluation the intensity of infection of each ear and the total number of infected heads were registrated until ripening. The varieties were scored according to the average number of spikelets per head which showed tpyical symptoms:

RESISTANT	≤2
MODERATELY RESITANT	3-5
MODERATELY SUSCEPTIBLE	6-9
SUSCEPTIBLE	10-12
STRONGLY SUSCEPTIBLE	≥13

RESULTS

Field symptoms of the disease were characterized by brown to dark brown lesions at the bases of glumes of unripe ears. In cases of heavy attack leaves and haulms showed longitudinal brown lesions. Several infected spikelets were either empty or contained shrivelled seeds and often showed dark discoloration at the basal end. In several cases *P.s.*pv. *atrofaciens* could be isolated from seeds, even after one year of storage.

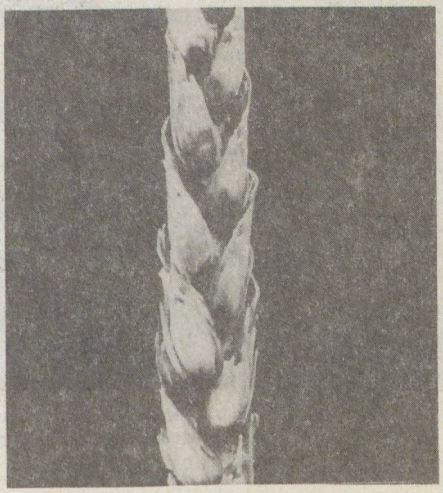


Fig. 1. Typical symptoms of basal glume rot of wheat after natural infection

Geographical distribution: The bacterium could be isolated in 1987 and 1988 from wheat and barley at several locations in West Germany. In wheat fields with high humidity the disease seemed to occur more frequently and in higher intensity.

Characterization of isolates: On the semi-selective medium of MOHAN & SCHAAD (1987) the bacteria formed light green, weakly turbid, domed, smooth, circular colonies of about 1.5 mm diameter within two days. After four days of growth the margin of

the raised colonies appeared less dense in translucent light. Yellow haloes were formed around the colonies due to acid later on. Additional characteristics of the isolates were: Levan- and HR-positive, motile rods with one to several polar flagella, negative for Gram-reaction, pectinase and arginine dihydrolase. Furthermore, the bacteria-free supernant inhibited growth of *E. coli*, but this activity was destroyed by autoclaving.

The bioassay on wheat seedlings by our isolates as well as an authentical strain (NCPPB no. 2612) led to brown lesions with dark brown margins, sometimes even browning of the entire haulms. A strain of *P. syringae* pv. *syringae* (NCPPB no. 2842) caused no visible symptoms on wheat seedlings.



Fig. 2. The Petri-plate bioassay: wheat seedlings showing dark brown spots on leaves and entire browning of haulms 3 days after inoculation with *P. syringae* pv. *atrofaciens*

Inoculation of adult wheat plants resulted in typical disease symptoms. Unripe ears showed initially water-soaked, dark green lesions, mainly at the glume basis, which turned brown later on. Also on leaves, predominantly at the point of attachment, and rarely on stalks, brown lesions developed out of initially water-soaked areas.

Resistance screening: The greenhouse test showed significant differences in the susceptibility of wheat cultivars, whereas in the field less significant differences were observed. This was because the warm and dry weather only allowed the development of weak symptoms.

Tab. 1. Reaction of the tested wheat cultivars after artificial infection with *P.s.* pv. *atrofaciens*

RESISTANT	MODERATELY RESISTANT	MODERATEL SUSCEPTIBLE	SUSCEPTIBLE	STRONGLY SUSCEPTIBLE
SW LS2918481	SW STAR	SW QUINTUS	SW KADETT	SW WALTER
WW BERT	SW SYROS	SW SELPEK	SW RALLE	SW KOLIBRI
WW DIPLOMAT	SW TAIFUN	SW TURBO	SW SAPPO	SW CARIBO
WW DISPONENT	WW BASALT	WW ADOLAR	WW KANZLER	
WW FALKE	WW GRANADA	WW APOLLO	WW OKAPI	
WW GÖTZ	WW KRONJUWEL	WW ARES		
WW HEIDUCK	WW OBERST	WW MILAN		
WW HERZOG	WW REIHER	WW MONOPOL		
WW MARKANT	WW SORBAS	WW URBAN		
WW REKTOR	WW SPERBER			
WW TUKAN				

WW = winter wheat, SW = spring wheat

Discussion

The survey revealed widespread occurrence of basal glume rot of wheat and barley in West Germany. The disease may have been overlooked for many years because it occurs predominantly under extreme conditions, with a humid microclimate. Also the symptoms are inconspicuous or are confused with Septoria glume blotch (*Septoria nodorum*). Lesions caused by *S. nodorum* usually affect the whole glumes or the upper part of young spikelets, whereas *P.s.*pv. *atrofaciens* tends to infect the lower third of the glumes. Also infections by *S. nodorum* occur earlier (at the heading stage) than by *P.s.*pv. *atrofaciens* which infect the glumes during the water ripe stage. Whereas *P.s.*pv. *syringae* occurs commonly on green leaves of many different plant species, including wheat and barley (LEBEN, 1981; FRYDA & OTTA, 1978), pv. *atrofaciens* is considered to be a specific pathogen of cereal grain (WILKIE, 1973; VASSILEV & KAROV, 1982).Other authors have reported it on isolates of *P.s* pv. *syringae* which cause disease of wheat, barley and other cereals (OTTA, 1974; ARSENIJEVIC, 1986). Differentation between these two pathovars is difficult (WILKIE, 1973) and may be only of academic importance. We used the wheat seedling bioassay to select only those strains of *P. syringae* which are pathogenic on wheat or barley. All the data available so far justify to call these strains *P. syringae* pv. *atrofaciens*.

While the pathogen could be isolated from seeds, even after one year of storage, the use of disease free seeds is one possibility for reducing the disease, as well as the cultiva-

tion of resistant or less susceptible cultivars (GUBOVA et al., 1982).

Although the cultivars "Rektor" and "Star" were scored as resistant or moderately resistant, respectively, both cultivars were very strongly affected by natural infection. These discrepancies may be due to different races of the pathogen, occurring in the field. In Bulgaria VASSILEV (1987) described four different races of *P.s.*pv. *atrofaciens*. Further investigations are necessary on this aspect.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

NATURE AND VARIATION OF FATTY ACID CLASSES OF ERWINIA AMYLOVORA

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Analysis and data of total cellular fatty acids by gas-liquid chromatography (GLC) has been used to support classification of bacteria (4,10). With the perfection and increasing use of capillary columns in GLC, fatty acid analyses may now be used as a primary taxonomic tool to place bacteria in their correct genus and, in some cases, species (1,5). Fatty acid composition, however, is biochemically variable. Composition for most bacteria can change depending on the substrate, temperatures of growth, and the physiological age of the bacterial cells. It is important, therefore, that the effects of these parameters be well understood and that the same culture conditions are used in separate analyses.

Erwinia amylovora (Burr.) Winsl. et al., the bacterium causing fire blight of pome fruits, can be identified in the laboratory by a combination of cultural, physiological, and pathological properties (7). Even though several selective culture media for diagnosis have been developed, none are truly definitive for absolute identification of E. amylovora. Initial research on fatty acid profiles of a large number of E. amylovora isolates grown on trypticase soy agar (TSA) showed a consistency of 49% saturated even-carbon chains (SECC), 28% unsaturated acids (UA), 10% cyclic acid (CA), 9% hydroxy substituted acids (HSA), and 1% saturated odd-carbon chains (SOCC)(8). More recently, 20 strains of E. amylovora were evaluated for fatty acid profiles after growing on 4 different media for various periods of time (1-6 days). The average fatty acid make-up was 41% SECC, 34% UA, 7% CA, 6% HSA, and 6% SOCC (2). Composition was affected mainly by growth medium and physiological age of cells. The analysis itself was effected by chromatograph sensitivity. Cyclic acids increased with physiological age on glucose yeast extract

carbonate agar (GYCA) and King's B medium (KB). On nutrient agar (NA) and TSA, the SOCC (Class B) were 3-4 times higher than on GYCA and KB media. As cells aged, the concentration of saturated acids (Class A & B) increased and the unsaturated acids (Class C) decreased, resulting in a shift of the ratios between those two groups from 1.2 to 1.7.

When nine streptomycin resistant (SR) and nine strep-susceptible (SS) strains of E. amylovora were grown on TSA for 3 days (9), the percentage of CA (Class F) was consistently lower (3.1% of total fatty acids) for the SR strains than for the SS strains (7.1%) (Figure 1). The reverse was observed for unsaturated acids, with SR strains with 37.5% and SS strains with 32.9% of total fatty acids (Table 1). These differences were reflected in the ratio of saturated to unsaturated acids, which for SR strains was 1.2 and for SS strains 1.5, and in the significantly different ratios of unsaturated to cyclic acids (SR strains 12.1 and SS strains 4.6). The significance of these large differences is presently not understood. Cyclic acids generally accumulate in bacterial cells as they age at the expense of other fatty acids. It is possible, therefore, that streptomycin resistant cells are physiologically more developed than the normal wild-type bacteria.

This report integrates all available information on cellular fatty acid class analysis of E. amylovora. The variability in the data is similar to that in previously published reports. To date, the GLC analysis has shown a remarkable homogeneity among a large number of world-wide isolates from numerous rosaceous hosts (8). The main deviations from the established library are a higher percentage of CA for 2 isolates from Rubus as compared to other host plants, and for 9 streptomycin resistant versus susceptible isolates.

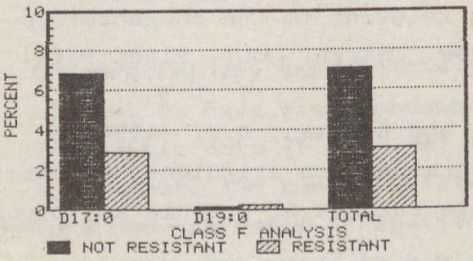
For the definite and conclusive identification of E. amylovora as the causal agent of fire blight, the fatty acid profiles library was invaluable when the recent occurrence of the disease in Egypt was being questioned (6). The further use of bacterial fatty acid analysis as a primary taxonomic tool is being developed in current studies, to include several physiologically (but not pathologically) related Erwinia species known as the "amylovora" group (3). However, before direct comparisons can be made between fatty acid profiles of any 2 bacterial cultures, it is essential that they be grown under the same conditions and be analyzed in chromatograms of comparable detail.

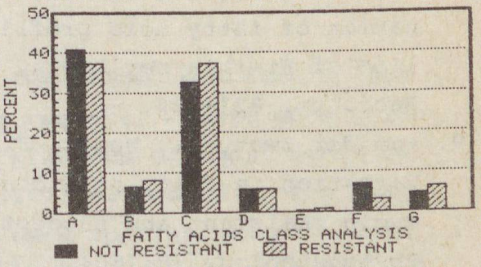
Table 1. Average percentages of total cellular fatty acids for 18 isolates of Erwinia amylovora

Isolates			Fatty Acid Classes*							Ratios**	
Number	Virulence	Streptomycin resistant	A	B	C	D	E	F	G	A+B/C	C/F
8	+	-	41.3	6.4	32.8	6.0	0.7	7.2	4.7	1.5	4.5a
6	+	+	37.9	8.8	36.4	5.9	0.6	3.6	5.8	1.3	10.1b
3	-	+	36.8	6.9	39.7	5.9	1.1	2.3	6.9	1.1	7.3b
1	-	-	39.8	8.0	34.2	5.4	0.5	6.6	5.3	1.4	5.2a
Totals											
9		-	41.1	6.5	32.9	6.0	0.6	7.1	4.8	1.5	4.6a
9		+	37.5	8.2	37.5	6.0	0.8	3.1	6.2	1.2	12.1b
14	+		39.8	7.4	34.3	6.0	0.7	5.6	5.2	1.4	6.1a
4	-		37.5	7.2	38.3	5.8	1.0	3.4	6.5	1.2	11.2b

*Classes: A = saturated even-carbon chains
B = saturated odd-carbon chains
C = unsaturated acids
D = hydroxy-substituted acids
E = branched chains
F = cyclic acids
G = unidentified acids

**Ratios: figures in columns followed by a different letter are statistically different at P = 0.05





	NOT RESISTANT	RESISTANT
D17:0	6.91	2.88
D19:0	0.20	0.25
TOTAL	7.11	3.13

	NOT RESISTANT	RESISTANT
A	41.08	37.53
B	6.54	8.17
C	32.91	37.49
D	6	5.89
E	0.64	0.80
F	7.11	3.13
G	4.78	6.18

Figure 1. Comparison of fatty acid cluster analysis of streptomycin resistant and susceptible isolates of Erwinia amylovora

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

RAPID BIOTEST FOR ESTABLISHING THE PLANT PATHOGENIC PROPERTIES OF BACTERIA

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The phytopathogenic properties of bacteria are most precisely determined on a host plant, but in many cases indicator plants are also used for a rapid testing. Several biotests are suggested for establishing the phytopathogenicity of bacteria belonging to different groups [1,4,6,7, 8,9].

The object of our study was to find a rapid, convenient and precise biotest method for a large number of phytopathogenic bacteria.

MATERIALS AND METHODS

Our preliminary experiments on the broad bean (Vicia faba L.) cv. A_1 have given promising results. Plants were grown in plastic pots (D = 14 cm) in mixtures of equal parts of loam, peat, and sand. Perlite (1:1) was added to the mixture and tap water irrigations were applied. Plants (2 in each pot) were grown in a glasshouse in the period October - June. Inoculation was made by a 1 ml syringe with a hypodermic needle No 20 at the 6-8 leaf stage into the conducting tissue of all petioles, so that the tissue and a part of the stem to be visibly wetted. Both our and collected strains (Table 1) presented by Drs B. Dragulev

(BMK), D.W. Dye, J.M. Young (ICMP), I.B. Koroleva (IMV), and Prof.Dr.sc. H.Kleinhempel (IP) were suspended in sterile distilled water to a concentration of 10^7 and then diluted (1:10) to 10^4 colony-forming-units/cm^3 (CFU/cm^3). The suspension densities were controlled

Table 1. Responses of Broad Bean (Vicia faba L.) Petioles to Selected Species of Acetobacter, Agrobacterium, Bacillus, Clavibacter, Erwinia, Escherichia, Gluconobacter, Pseudomonas and Xanthomonas

Species, collection No, and strain origin	Ratio of positive reaction strains from a species to total tested	Rate, type of response, and initial hour of manifestation
1	2	3
Acetobacter aceti 023 HIFFI, vinegar	2/5	2HR (24 h)
Acetobacter xylinum 072 HIFFI, vinegar	1/4	3HR (48 h)
Agrobacterium tumefaciens K10 HIA, grapevine		2HS (after 72 h)
Agrobacterium tumefaciens K11 HIA, peach	2/2	1N 1HS (after 72 h)
Bacillus pumilus 48 HIA apricot		1HR (24 h)
Bacillus pumilus T8 IP broad bean	4/5	4HR (24 h) 4N (after 72 h)
Clavibacter michiganensis I161a HIA, tomato	2/2	1N 3HS (after 72 h)
Clavibacter tritici 2626 ICMB, wheat	1/1	3N (after 72 h)
Erwinia carotovora 243 IP		4N 3HS (after 72 h)
Erwinia carotovora ENA 114 IP		4HR (24h) 4N 3HS (after 72h)
Erwinia carotovora 623 IMV, wheat		3HR (48h) 4N (after 72 h)
Erwinia carotovora 826 IMV, wheat	6/7	1N 2HS (after 72 h)
Erwinia herbicola 112YIP, apple	1/1	4N (after 72 h)
Erwinia sp. WK-6 IP, broad bean	1/1	4HR (24 h) 4N (after 72 h)
Escherichia coli K12 BMC	0/3	0
Gluconobacter oxydans 4339 NCIB	1/4	2HR (24 h)
Pseudomonas syringae:		
pv. atrofaciens V28 IIP6R, wheat	60/60	4HR (24 h)
pv. lachrymans C4a$_1$ HIA, cucumber	2/2	4N 4C (after 72 h)
pv. morsprunorum PM56 IP, pear	2/2	4HR (24 h)
pv. phaseolicola HB-1bIP, bean	2/2	4HR (24 h) 3HS (after 72 h)
pv. syringae 3023 ICMP, lilac	2/2	4HR (24 h)
pv. tabaci T1128a$_1$ IIP6R, tobacco	25/25	4HR(24h) 4N4C2HS (after 72h)
pv. tomato K3 HIA, tomato	2/2	2N 2HS (after 72 h)

Table 1 (cont.)

1	2	3
Xanthomonas campestris:		
pv. cerealis 1409 ICMP, bur-reed	1/1	4N (after 72 h)
pv. phaseoli C12a HIA, bean	10/10	4HR (48 h) 4N (after 72 h)
pv. pruni 67 HIA, plum	1/1	3N (after 72 h)
pv. translucens 5752 ICMP, barley	1/1	3HR (24 h) 3N (after 72 h)
pv. undulosa 5755 ICMP, wheat	1/1	4HR (24 h) 4N (after 72 h)
pv. vesicatoria C80 HIA, tomato	1/1	1HR (24 h) 4N (after 72 h)

Collections: BMC - Bulgarian Microbial Collection, Sofia, Bulgaria
HIA - Higher Institute of Agriculture, Plovdiv, Bulgaria
HIFFI - Higher Institute of Food and Flavour Industries, Plovdiv, Bulgaria
IP - Institut fur Phytopathologie, DDR-482, Aschersleben
IIPGR - Institute of Introduction and Plant Genetic Resources, Sadovo, Bulgaria
IMV - Institute of Microbiology and Virology, Kiev, USSR
ICMP - International Collection of Microorganisms from Plants, PDD DSIR Auckland New Zealand
NCIB - National Collection of Industrial Bacteria, Aberdin, Scotland

Rates	Affected part of petiole
1	1/3
2	1/2
3	2/3
4	whole

Types of responses

0 - no response
HR - hypersensitive (necrosis by 48 h)
HS - hyperplastic symptoms
N - necrosis in the site of infiltration or out of it after 72 h
C - chlorosis

through inoculations and counting on potato dextrose agar (PDA). The reactions were recorded in 12, 24, 48, and 72 h as well as on 9, 14, and 20 day after inoculation. The rates and types are given in Table 1 where the highest estimations made on the basis of triple experiments are shown.

RESULTS

Almost all phytopathogenic bacteria and certain acetic-acid bacteria used in the experiment showed a positive reaction (Table 1). The controls of water and Escherichia coli were negative. No reaction was established in one of Bacillus pumilus and Erwinia carotovora strains. A typical hypersensitive reaction (HR) by 24 h (rarely by 48 h) was observed in representatives of the genera Acetobacter, Bacillus, Erwinia, Gluconobacter, Pseudomonas, and Xanthomonas. The latter induces a rapid and reliable reaction in 10^{8} - 10^{9} CFU/cm^{3}. Some Pseudomonas strains cause HR of 10 CFU/cm^{3}. A delayed necrosis (N) was observed in Agrobacterium (K11) and Clavibacter (slight), and some strains of Erwinia (243, 112 Y), Pseudomonas (K_{3}), and Xanthomonas (1409, C12a). Hyperplastic symptoms (HS) on petioles were recorded in Agrobacterium, some strains of Clavibacter (N 161a), Erwinia (243, ENA 114, 826), and Pseudomonas (C $4a_{1}$, T $1128a_{1}$, K 3). In some Pseudomonas strains (C 4a, T $1128a_{1}$) a well-expressed leaf chlorosis is observed. In a severe infection (rate 4) leaves fall off. Some strains of Bacillus (T 8) and Xanthomonas (C 12a, 5752,5755) induce high spotting on leaves usually after 72 h.

DISCUSSION

Detached leaves of broad beans as a biotest for determining the pathogenicity of Erwinia, Pseudomonas, and Xanthomonas bacteria are suggested by Ovechnikova (1966). In our opinion, they are less reliable than the stem and petioles of the whole plant. Arsenievic (1973) reported a reaction of the stem and leaves of a broad bean plant, injected with P. syringae isolates, after 48 h. A more rapid reaction (after 24 h) of an undetached broad bean leaf to Bacillus and Erwinia was observed by Griesbach et al. (1979). Petioles have proved to be significantly more

sensitive than the other organs of broad bean plants as some bacteria (E. herbicola 112 Y) had not reacted, or others (Pseudomonas, Xanthomonas) with a slight reaction in the experiments of the above cited authors, have shown a clearly expressed positive reaction in our tests. The reactions are mainly of a hypersensitive type (Klement, 1982), occurring by 24 h. The delayed necroses are specific for Clavibacter, some representatives of Erwinia, Pseudomonas, and Xanthomonas. The hyperplastic symptoms formed by Agrobacterium, some isolates of Clavibacter, Erwinia, and Pseudomonas are probably due to some biologically active substances of the type of plant growth regulators.

The reactions of broad bean petioles to some species of Acetobacter, Agrobacterium, Bacillus, Clavibacter, Erwinia, Gluconobacter, Pseudomonas, and Xanthomonas are a reliable test for their pathogenicity in a large temperature range (5 - 35°C). Plants are well grown and easy to infiltrate with no requirement for special skill as is the case with the tobacco leaf.

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Proc. 7th Int.Conf.Plant Path. Bact., Budapest, Hungary, 1989

Plant Pathogenic Bacteria Z. Klement (ed.)

THE IMPACT OF MOLECULAR BIOLOGICAL STUDIES ON THE NOMENCLATURE OF PLANT PATHOGENIC BACTERIA

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Bacterial taxonomy includes three interrelated activities (Cowan 1968, Staley & Krieg 1984): classification, which involves the allocation of organisms among groups (taxa); nomenclature, the naming of taxa defined by classification; and identification, the process by which unidentified organisms are referred to taxa. Phenetic classification, based on overall similarities expressed by all available characters of the taxa, is the most generally useful.

The naming of taxa is governed by the International Code of Nomenclature of Bacteria (Lapage *et al.* 1975), which aims to maintain order and stability, and to avoid confusion and the unnecessary proliferation of names. Species have been accepted in the Approved Lists (Skerman *et al.* 1980), and in subsequent validations of taxa published in the *International Journal of Systematic Bacteriology*, only if there was a valid and legitimate name, a type strain, and a description by which the species could be identified. The formal naming of a taxon presupposes that it has been characterized by a range of methods, sufficient to classify it, and to distinguish it from all other named taxa. Published descriptions of taxa should be as complete as possible and are expected to include such information as morphological descriptions, physiological behaviour, biochemical reactions, chemical composition, and nucleic acid studies. The description of a given taxon will expand, becoming more detailed, as closely related taxa are distinguished and as new methods are applied. The Code also calls for the preparation of Minimal Standards, to which species descriptions should conform in future, which will include tests for generic identity, and tests which would distinguish the species from others. The intention of the Code in requiring adequate differentiation of species by modern descriptions was to provide an independent reference by which strains of species could be identified. The collected descriptions of taxa in compendia, such as *Bergey's Manual of Systematic Bacteriology*, makes it possible to identify any previously classified bacterium.

An essential function of classification is to provide a procedure by which known strains can be identified, and unknown or unclassified strains can be recognized as such (Truper & Kramer 1984). Identification may involve reference to the reactions of strains to a relatively small number of carefully chosen determinative (key) tests. Highly specific methods such as reactions to antisera, phage, or DNA probes may be very useful for the confirmation of the identification of specified organisms, but play a relatively small part in the classification of bacteria.

In recent years, molecular biological methods for analysing nucleic acids and oligonucleotides have been increasingly applied in taxonomic studies. These methods provide alternatives for a better understanding of the relationships of bacteria. However, it is too early to be confident that present methods and their interpretations give unambiguous results. For instance, the sequencing and comparison of conserved moieties in rRNA has led to claims that it is possible to infer phylogenies showing ancestral relationships of prokaryotes (Woese 1987). Interpretations of these data vary (Lake 1986), and it has been suggested that a wider range of comparisons of unrelated molecular structures should be made before phylogenies at this level can be proposed with confidence (Sneath 1980). It has been proposed (Palleroni 1984) that rRNA-DNA comparisons offer the prospect of defining taxa at, and above, the genus level. Within the genus *Pseudomonas* Migula 1894 it is possible to distinguish four rRNA-DNA groups which could be considered as separate genera or families. However, the genus in phylogenetic taxonomy has not yet been defined, and Palleroni (1984) refrained from proposing these taxa formally because they could

not be recognized using phenetic methods, because knowledge of many of the constituent species was incomplete. Although they have yet had little effect on nomenclature, these essentially cladistic methods have profoundly influenced taxonomic thinking.

Comparative studies of DNA-DNA homologies of the total bacterial genome are considered to indicate taxonomic differences at the species level. As yet the fragmentary nature of these studies, and the diversity of procedures and possible interpretations, limits our ability to use them for species classification (Denny *et al.* 1988). The genospecies (Wayne *et al.* 1987) has been defined as including strains with 70% or greater DNA-DNA relatedness and with a $\Delta\ T_m < 5\%$. Implicit in this definition is the requirement for extensive genetic comparison not yet seen in practice. Genetic studies, by any procedure, have been made on only a few identifiable plant pathogenic bacteria. If genospecies are to be established in nomenclature, then an orderly procedure which avoids introducing subjective synonymies is essential. It should involve genetic comparisons with all, or a wide range of, previously named species within a genus. It will be regrettable if a piecemeal approach to the creation of species leads to a disorganized and confusing nomenclature.

Recent developments, such as genomic fingerprinting (Hartung & Civerolo 1987) and restriction fragment length polymorphisms (RFLP) (Gabriel *et al.* 1989), offer powerful diagnostic tools for identifying bacterial strains. For RFLP analysis, the choice of probes is critical; some reveal little polymorphism, others much (Gabriel *et al.* 1989). Probes must be selected which identify a chosen ecological grouping of organisms, such as those sharing a common host range or which induce common symptom expression, and should be checked against a collection of strains from the widest geographical and temporal range. Probes give expression to a small proportion of the genome and do not give a measure of overall DNA-DNA homology.

An *ad hoc* committee recently met to examine and reconcile trends in modern approaches to bacterial systematics (Wayne *et al.* 1987). Relevant conclusions were:

phylogeny should determine taxonomy;

the complete genomic DNA sequence would be the reference standard;

nomenclature should agree with and reflect genomic information;

DNA reassociation (homology) approaches the sequence standard and represents the best applicable procedure at present; and

a distinct genospecies that cannot be differentiated from another genospecies on the basis of any known phenotypic property not be named until such differentiation is possible.

These conclusions are useful guiding principles for bacterial systematics although it is not yet clear to what extent a further reconciliation between the results of phenetic and cladistic analyses may be necessary. The recommendation that genospecies not be named unless there are phenotypic properties available for identification is consistent with earlier practice and ensures that it is possible to identify strains to species level in the laboratory using published descriptions.

A recent paper which does not follow these principles is that of Gabriel *et al.* (1989) who proposed the reinstatement of *Xanthomonas citri* and *X. phaseoli* as species. The two species differed in pectinase activity and in an RFLP analysis. The two species were distinguished from the type species, *X. campestris* (Pammel 1895) Dowson (1939), solely on the basis of RFLP analysis. No other differentiating comparative data were reported. It would therefore be impossible for another worker to make an identification, or to characterize an unknown strain using these descriptions.

A consequence of the extensive use of genetic probes could see the reinstatement of species, or the creation of new species, distinguished solely by probes. This procedure could result in descriptions of species which would be essentially cryptic, providing little or no account of distinguishing characters.

The *ad hoc* committee (Wayne *et al.* 1987) considered that an ideal taxonomy would involve a single formal system. It is possible that if many species are proposed which can be identified only by using molecular biological methods, then working bacteriologists will refer to informal nomenclatures.

Formal use of the term pathovar was established (Dye *et al.* 1980) in a special purpose nomenclature, at the infrasubspecific level, to satisfy the needs of plant pathologists for names of pathogens which do not meet the standards for higher taxa under the Code. It is recognized that this special purpose classification is a nomenclatural compromise imposed by our present state of knowledge. It is suggested that orderly nomenclature of plant pathogenic bacteria will be best served if more data are gathered, and an accurate overview of the relationships of organisms at the molecular level is obtained, before individual plant pathogenic species presently named as pathovars are reinstated.

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ISBN 963 05 5871 8 (A–B)
ISBN 963 05 5872 6 (A)

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