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

PLANT PATHOGENIC BACTERIA

PART B

EDITED BY
Z. KLEMENT



AKADÉMIAI KIADÓ, BUDAPEST

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edited by
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AKADÉMIAI KIADÓ, BUDAPEST 1990

MTA KIK 0 00003 74744 5

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 5873 4 Part B

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Printed in Hungary
by Akadémiai Kiadó és Nyomda Vállalat, Budapest

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SESSION 6

ERWINIAS

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

Plant Pathogenic Bacteria Z. Klement (ed.)

CREATION AND ANALYSIS OF MUTANTS OF ERWINIA AMYLOVORA ALTERED IN PATHOGENICITY

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INTRODUCTION

Erwinia amylovora causes fireblight, a necrotic disease of Pomoïdae. Especially, apple and pear trees are susceptible hosts of economic importance. In order to study the determinants of the pathogenicity of *E. amylovora* we choose the efficient approach which consists in mutagenizing a virulent strain by means of a transposable element.

Our tools for creating mutants altered in pathogenicity were as follows :

1. The *E. amylovora* strain CFBP1430 is considered as the reference virulent strain in France. It has been isolated from *Crataegus* in France in 1972. This strain shows a very stable virulence level on different host species of fireblight

2. The phage Mu derivative MuPR13 was used to mutagenize CFBP1430. During the infective process of a susceptible strain it is stably integrated into the genome because it is deleted for genes A and B which are necessary for further transposition.

   On the basis of resistance to chloramphenicol conferred by MuPR13, 6900 independent clones were recovered among which about 2.3% were auxotrophs. The diversity and the distribution of nutritional requirements are indicative of essentially random integration of MuPR13 into *E. amylovora* genome.

3. The first step of screening for mutants altered in pathogenicity was the assay on apple calli developed by Paulin and Duron (1986) by using calli from *in vitro* cultured roots of *Malus domestica cv.* "Golden delicious". After inoculation with virulent strains, there is production of ooze at the surface of the callus. The clones which did not induce the production of ooze were called Cal$^-$.

RESULTS

Out of more than 6000 chloramphenicol resistant clones, 85 Cal$^-$ clones were identified, among which 61 presented a unique insertion of Mu (either MuPR13, or the helper phage Mu*cts*62). 46 Cal$^-$ single mutants were further characterized on susceptible hosts : on the one hand, on apple and pear seedlings and on the other hand, on axenicly grown unrooted pear microcuttings. It appeared that the screening on apple calli was an efficient method since the major part of Cal$^-$ mutants were indeed altered in pathogenicity :

- Only 5 Cal$^-$ mutants proved to be as virulent as the parental strain : they are Pat$^+$ and Agr$^+$. "Agr" is from the French spelling of aggressive;
- 17 other mutants, although still able to induce symptoms in apple or pear seedlings, were less virulent than CFBP1430. This was shown both by a reduced proportion of plants showing symptoms, and by a lower mean length of necrosis. These mutants with attenuated virulence are designed Pat$^+$ Agr$^-$;
- At last, 24 Cal$^-$ mutants were completely avirulent : they were called Pat$^-$.

The two main classes Pat$^+$ and Pat$^-$ were similarly found after inoculation of unrooted pear microcuttings proving that the assay on this material is valid to study avirulent mutants of *E. amylovora*. Indeed Pat$^+$ mutants were able to produce stem necrosis and ooze production on axenicly grown pear microcuttings when Pat$^-$ mutants usually produced no symptom on these plants although they exceptionally caused local necrosis.

On the basis of the ability to induce hypersensitive response in Tobacco leaves the avirulent mutants split into two classes : 8 were able to induce the hypersensitive response and 16 were not.

MOLECULAR ANALYSIS

We were then able to identify a genomic cluster involved in determining pathogenicity of *E. amylovora* : (i) by cloning in pBR322 the mutated *Sal*I fragments of three avirulent mutants, including 2 HR$^-$ and 1 HR$^+$ mutants and (ii) by screening a genomic library of CFBP1430 constructed in pLA2917 for hybridization with radioactively labeled cloned fragments from the mutants.

The identified cluster covers more than 43 kb. It is composed of two contiguous segments the one involved in *h*ypersensitive *r*esponse and *p*athogenicity or *hrp* functions and the other in *d*isease *sp*ecific or *dsp* functions. At least 14 Pat$^-$ HR$^-$ mutants were located in the *hrp* region and all were complemented both for HR and for pathogenicity by cosmid pPV130 which covers about 32 kb of the genomic DNA from strain CFBP1430. The 8 Pat$^-$ HR$^+$ mutants were located in the *dsp* region at the right hand of the cluster. They were complemented for pathogenicity by cosmid pPV132 which contains a 27 kb long insert of genomic DNA. In addition, we have

got indications that some Pat$^+$ Agr$^-$ mutants could be located in this region. One Pat$^-$ HR$^-$ mutant, PMV6112 is located on a 3.9 kb long *Sal*I fragment common to cosmids pPV130 and pPV132 : it was complemented by both cosmids.

PROSPECTS

These studies are currently going on. About 20 more Cal$^-$ mutants are being characterized for their phenotypes *in planta*. The molecular analysis of the large virulence cluster should allow the number and the functions to be determined of the genes involved in *E. amylovora* pathogenicity.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

VIRULENCE FACTORS OF ERWINIA AMYLOVORA INFLUENCING DEVELOPMENT OF FIREBLIGHT SYMPTOMS

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(Summary)

The fireblight pathogen *Erwinia amylovora* was influenced in virulence tests on immature pear slices, in pathogenicity on pear seedlings or in its effect on lawns of cultivated pear cells by several factors, which can be connected to genetic or biochemical features of the bacterium: (i) Presence of a 29 kb plasmid common to all investigated *E. amylovora* strains; (ii) production of (L)-2,5-dihydrophenylalanine by a few strains; (iii) synthesis of extracellular polysaccharide to prevent defense reactions of the plant; (iv) other defined defects in the bacterial cell metabolism; (v) mutations in the bacterial genome affecting unknown pathways required for virulence. Genomic DNA of *E. amylovora* has no homology to cloned fragments encoding cellulase or pectate lyase in *E. carotovora* subsp. *atroseptica*. Expression of the pectate lyase gene in *E. amylovora* prolonged the bacterial growth period which was found to be limited to viable pear cells. This progress report attempts to review recent findings on these subjects.

INTRODUCTION

Mechanisms for virulence of the fireblight pathogen *Erwinia amylovora* are barely understood. The bacteriosis affects plants of the family of *Rosaceae* and is in many countries most destructive to apple and pear trees. Severe disease symptoms like blackening of shoots and ooze formation can be used to identify the causative pathogen *Erwinia amylovora* (Lelliot, 1967; Zeller, 1974). Ooze production in immature pears is an important standard assay which however is limited to the availability of fresh plant material. Further confirmation for the presence of the bacterium can be obtained with semi-selective agar plates (Miller and Schroth, 1972) or by immunofluorescence assays (Roberts, 1980), where monoclonal antibodies are preferable (Lin et al., 1987). In case of ambiguous results DNA hybridization with a specific DNA probe can give clear answers about the presence of *E. amylovora* in a sample collected from suspicious plants (Falkenstein et al., 1988).

Pathogenic properties of *E. amylovora* are affected by changes in production of extracellular polysaccharides (EPS). EPS is considered to prevent recognition of the pathogen by the host plant. The defense mechanisms otherwise induced by cellular components of the bacterium (Klement and Goodman, 1967) seem to be inactive in the

presence of EPS. The complexity of EPS includes sugar composition, linkage of the sugar molecules, substituents like pyruvate or acetate and the size of the polymer sugar chains. Even a change in the formation of structures of high order could disturb a balanced protection of bacterial cells against the induction of a hypersensitivity reaction of the plant.

Auxotrophic defects can also lead to altered virulence like other deficiencies affecting growth properties of the bacterium. Other plant pathogenic bacteria release toxins to the infected plant or secrete cell wall degradating enzymes. The *E. amylovora* strains E8 and E9 produce a plant cell damaging phenylalanine-derivative (Feistner, 1988) which was also discussed for its possible involvement in virulence. The *E. carotovora*-group has been studied for its release of pectate lyase, cellulase and proteases (Andro et al., 1984), although a similar principle has not been found for *E. amylovora*. As stated in the result section, some of our mutants are deficient in EPS-production, another is auxotrophic and *rec*A-mutants have poor growth properties. Synthesis of (L)-2,5-dihydrophenylalanine was not found for most virulent and avirulent *E. amylovora* strains and does not appear to be a general virulence factor. Homology of cloned cellulase and pectate lyase genes from *E. carotovora* subsp. *atroseptica* was not detected for genomic DNA of *E. amylovora*. Virulence factors can be limited so far to the production of EPS or to a good growth of *E. amylovora* on plant cells.

RESULTS

Attenuated virulence of plasmid-free E. amylovora-*strains*

Fragments of the 29 kb plasmid found in all *E. amylovora* isolates (Falkenstein et al., 1988) were cloned into pfd-plasmid (Geider at al. 1985) and the resulting plasmids transformed into *E. amylovora* Ea7/74, Ea1/79 (Falkenstein et al., 1988) and the new isolate Ea11/88. A cloned 4.4 kb *Pst*I-fragment was incompatible with the resident *E. amylovora* plasmid, which was subsequently lost in almost 100 % of the carrier cells. The newly introduced plasmid was not stable in *E. amylovora* and segregated out of the bacteria (Falkenstein et al., 1989). Plasmid-free strain Ea7/74 showed a delay of ooze production on pear slices, although virulence symptoms were like wild type after 10-14 days after inoculation. When the plasmid-free strains, labelled with a plasmid expressing bioluminescence, were inoculated on a lawn of pear cells, they spread more slowly than the corresponding wild type strain. The ability of plasmid-free strain Ea7/74 to produce fireblight symptoms on pear seedlings was also retarded compared to wild type (with W. Zeller, Dossenheim). On solidified medium with asparagine-sorbitol the plasmid-free strains Ea1/79 and Ea11/88 were non-mucoid. Wild type strain Ea7/74 is barely mucoid under these conditions. Additional growth defects of the three plasmid-free *E. amylovora* strains assayed by propagation in minimal medium or on agar were not observed with the exception that the mutants formed translucent colonies in minimal agar without thiamine and turbid colonies on the same agar with thiamine. Wild-type cells produced turbid colonies in both cases. Other investigators found a requirement for thiamine for their plasmid-free strain (Laurent et al., 1989),

which was not the case for the three *E. amylovora* isolates cured by us. It appears that removal of the 29 kb plasmid impairs the bacterial cell metabolism, a reason why plasmid-free *E. amylovora* strains may not occur in nature.

Creation of avirulent transposon-mutants

Using the mobilizable pBR-derivative pSUP201 (Simon et al., 1983) and a pTiC58-derivative with constitutive autonomous transfer, pGV3100 (Holsters et al., 1980), both carrying transposon Tn5 we have constructed a set of Kanamycin-resistant mutants of strain Ea 7/74. This strain replicates neither colE1-plasmids nor the Ti-plasmid of *Agrobacterium tumefaciens*. Out of 1600 mutants 11 were found to be strictly apathogenic on immature pears and on pear seedlings. The sites of the insertions were characterized and the mutants were classified according to the *Eco*RI-fragments with insertions of Tn5 into 6 groups. One of them was an auxotrophic mutant requiring leucine for growth. If pear slices were soaked with this amino acid virulence could be restored. The other mutants were investigated for specific defects which could explain avirulence. No differences to the wild type strain were observed for growth rates and the lag-phase in liquid medium. The hypersensitivity reaction in tobacco leaves as a non-host plant was also similar to the wild-type strain. The mutant strains normally swarmed on soft agar and grew well on pear slices. The mutated bacteria spread on a pear cell lawn and secreted protease like wild type. Besides their lack of virulence on immature pears and of pathogenicity on pear seedlings 8 of the mutants were not lysed by the *E. amylovora* phage 4L. This phage has affinity to EPS and lipopolysaccharides of the bacterium and indicates a possible change in EPS synthesis of most of the mutants. Analysis of the amount and the composition of EPS secreted by the strains showed indeed a deviation from wild type EPS as found in similar experiments by Steinberger and Beer (1988). One strain showed an increase of EPS production. Composition and structure of mutant EPS has to be further investigated in order to understand the avirulent phenotype.

Synthesis of dihydrophenylalanine by some E. amylovora *strains and its effects on plant cells*

When cultivated pear cells were embedded in B5-agar (with 2,4-D) they grew to a dense cell lawn. Inoculation with 10 µl of an overnight culture of most *E. amylovora* investigated affected growth of the cell lawn only to a slight extent, whereas inoculation with strains E8 and E9 caused a large zone of growth inhibition (Bernhard, F., Schwartz, T., Theiler, R., and Geider, K., submitted for publication). When pear cells were plated on a cellophane disk and then transferred to bacteria of strain E8 on $B5^+$-agar, areas of the pear cells adjacent to bacterial colonies stopped growth. This indicates the release of a membrane diffusible substance by the *E. amylovora* strain. During search for the nature of this compound we got the information from G. Feistner (1988) that *E. amylovora* strains E8 and E9 secrete (L)-2,5-dihydrophenylalanine into their environment.

Table 1: Dihydrophenylalanine production of various *E. amylovora* strains
Methanol extracts were assayed by HPLC and thin-layer chromatography.

strain	origin	pathogenicity	DHP	growth inhibition of pear cells
Ea7/74	Germany	virulent	-	-
Ea1/79	Germany	virulent	-	-
Ea5/84	Egypt	virulent	-	-
Ea11/88	Germany	virulent	-	-
Ea322	France	virulent	-	-
E8	USA	avirulent	+	+
E9	USA	virulent	+	+
P66	England	avirulent	-	-

As shown in Table 1 only strains synthesizing dihydrophenylalanine (DHP) had an affect on growth of pear cells surrounding the bacterial colony. DHP was shown to cause feedback inhibition in the Shikimi acid pathway (Fickenscher and Zähner, 1971) which can be competed with phenylalanine. It is likely that plants respond in a similar way, because the inhibitory effect of DHP was also released in the presence of phenylalanine, when DHP was directly applied to the pear cells. Only *E. amylovora* strains E8 and E9 synthesized DHP whereas most virulent and avirulent strains did not produce this compound under identical bacterial growth conditions. We think that DHP can help to improve positive strains in competition to other microorganisms and in attacking the host plants, but it is not a general virulence factor required for the development of fireblight.

Cellulase and pectate lyase genes from E. carotovora *subsp.* atroseptica *lack homology to genomic DNA of* E. amylovora

After cloning of DNA fragments encoding cellulase or pectate lyase from *E. carotovora* subsp. *atroseptica* we looked for their homology to other strains in the *E. carotovora* group and to strains of *E. amylovora*. The genomes of *E. carotovora* subsp. *carotovora* and of *E. chrysanthemi* had a significant homology in contrast to *E. amylovora* (Theiler, R., Schäfer, I., and Geider, K., submitted for publication). Both genes were expressed in *E. amylovora*, but the enzymes were not secreted in the bacterium. Release of the enzymes from *E. amylovora* occurred by spontaneous lysis in the bacterial population. We assayed the influence of these enzymes on the growth properties of *E. amylovora* using a lawn of pear cells on B5-agar. In an *E. amylovora* strain transposon Tn4431 (Shaw et al., 1987) was inserted into the chromosome and produced bioluminescence of the bacterium, when grown on pear cells. When this strain also carried DNA with the pectate lyase gene from *E. carotovora* subsp. *atroseptica*, bioluminescence was prolonged compared to control strains including strains with the cellulase gene. It is likely that *E. amylovora* does not have information

for cellulase and pectate lyase, but introduction of the pectate lyase can improve feeding of *E. amylovora* on pear cells.

CONCLUSIONS

Virulence of *E. amylovora* is influenced by several aspects. The development of fireblight symptoms relies strongly on extracellular polysaccharides produced by the bacterium. Changes in the amount and the structure of EPS can be the reason for avirulence. Avirulent strains can also rise from growth defects of the bacteria. Auxotrophy is one example. Insertion of transposon Tn5 into the *rec*A gene of *E. amylovora* results in UV-sensitive strains that have a low growth and survival rate which explain the avirulence of those mutants (Jahn, N. and Geider, K., unpublished). It could be possible that *E. amylovora* circumvents the plant defense reactions by secretion of EPS and is able to feed on the surface of the host plant cells thereby killing its source without the release of specific toxins.

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The work was supported in part by grants from Stiftung Volkswagenwerk (to P.B., R.T.) and DECHEMA (to T.S.).

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

Plant Pathogenic Bacteria
Z. Klement (ed.)

ELICITATION OF THE HYPERSENSITIVE RESPONSE BY ESCHERICHIA COLI CONTAINING A CLUSTER OF PATHOGENICITY GENES FROM ERWINIA AMYLOVORA

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Many phytopathogenic microorganisms, including *Erwinia amylovora* Burr. Winslow et al., trigger a rapid local necrosis of plant tissue termed the hypersensitive response (HR). Because the potential pathogen is localized, the HR is considered to be a mechanism of disease resistance (Klement, 1982). Bacteria that are not pathogenic to plants and most plant pathogens that cause rots, galls or wilts do not elicit the HR. Studies of several bacteria have indicated that the abilities to elicit disease in host plants and the HR in non-host plants are genetically related; mutants deficient in the ability to elicit the HR are deficient also in pathogenicity to host plants (Bauer, 1989; Steinberger and Beer, 1988; Niepold et al., 1985; Lindgren et al., 1986; Cuppels, 1986; Malik et al., 1987; Boucher et al., 1985).

In previous molecular genetic studies aimed at discovering the mechanism of pathogenesis of *E. amylovora*, we created mutants, termed Hrp$^-$ (Lindgren et al., 1986), that are deficient in pathogenicity and HR-eliciting ability (Steinberger and Beer, 1988). Plasmids and cosmids containing wild-type DNA from *E. amylovora* strain CFPB1367 were identified that restored pathogenicity and the ability of mutants to elicit the HR. In this preliminary report, we describe the identification of a region of chromosomal DNA of *E. amylovora* that includes all the genes necessary for elicitation of the HR in the saprophyte *Escherichia coli*, other non-phytopathogenic bacteria and non-necrosis-inducing phytopathogenic bacteria.

A cosmid, designated pCPP430, was identified based on its ability to restore Hrp functions to a Tn*10*-induced Hrp$^-$ mutant that had not been complemented by previously identified plasmids. The cosmid restored Hrp

function to 17 transposon-induced Hrp$^-$ mutants of *E. amylovora* and to two spontaneous Hrp$^-$ mutants. Two other distinct cosmids, pCPP440 and pCPP450, were identified from a library of wild-type DNA based on their hybridization to subclones of previously identified plasmids that complemented several Hrp$^-$ mutants. The cosmids pCPP440 and pCPP450 complemented all but one and two Hrp$^-$ mutants, respectively.

Based on its' ability to complement all our Hrp$^-$ mutants of *E. amylovora*, we hypothesized that pCPP430 might contain all the genes necessary to elicit the HR. This hypothesis was tested by infiltrating tobacco leaf panels with strains of *E. coli* containing pCPP430, pCPP440 or pCPP450. All three cosmids conferred on *E. coli* DH5 the ability to elicit the HR in tobacco leaves. The most rapid and strongest response was seen in leaves infiltrated with *E. coli* DH5(pCPP430). The response was more rapid and intense than that elicited by the wild-type strain CFPB1367. The response elicited by DH5(pCPP440) and DH5(pCPP450) was comparable, in intensity and rapidity of development, to that elicited by the wild-type *E. amylovora* strain. In contrast, *E. coli* containing previously identified cosmids that complemented many *E. amylovora* Hrp$^-$ mutants did not elicit the HR in tobacco.

Cosmid pCPP430 was transferred to other bacterial strains that lack the ability to elicit the HR, including strains of *Erwinia stewartii*, *Erwinia herbicola* and *E. coli*. All strains harboring pCPP430 elicited the HR. Strains naturally capable of eliciting the HR, including *E. amylovora*, *Erwinia nigrifluens*, *Erwinia rubifaciens* and *Erwinia tracheiphila*, generally elicited a more intense and rapid HR when they harbored pCPP430. These results indicate that pCPP430 contains all the genes necessary for elicitation of the HR, and that these genes are strongly expressed in all strains tested.

The elicitation of the HR by *E. coli* DH5(pCPP430) was very strong and developed several hours before collapse was apparent in adjacent tobacco leaf panels that had been infiltrated with *E. amylovora* CFPB1367. The cosmid vector of pCPP430 is pCPP9 (Bauer et al., 1989), which occurs in 4-6 copies per chromosome in *E. coli*. The increased gene dosage of the *hrp* genes in cells harboring pCPP430 may be responsible for the more rapid development of the HR.

Expression of the *E. amylovora hrp* gene cluster in *E. coli* contrasts with the results of Huang et al. (1988). Their *hrp* gene cluster from

Pseudomonas syringae pv. *syringae* was expressed well in *P. fluorescens*, but was expressed very weakly in *E. coli*. The differences in apparent levels of expression may be based on differences in promoter activity in foreign cells.

Preliminary mapping data indicate that pCPP430, pCPP440 and pCPP450 contain ca. 46 kb of *E. amylovora* chromosomal DNA. Based on complementation studies of mutants of *E. amylovora*, it is apparent that the *hrp* gene cluster includes genes located throughout the cloned region. It is also apparent from the size of the cluster that a relatively large number of genes are required.

The ability of *E. coli* harboring the *hrp* gene cluster of *E. amylovora* to elicit the HR provides an ideal system to further elucidate the genetics of the HR. Since the physiology and biochemistry of *E. coli* are understood better than those of any other bacterium, analysis of *hrp* gene products will be facilitated. Work is in progress to further characterize the genes of the *hrp* cluster, their regulation and their products.

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This work was supported in part by grant 86-CRCR-1-2225 from the Competitive Research Grants Office of the United States Department of Agriculture and grants from the Cornell University 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.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

THE REQUIREMENT OF DEPOLYMERIZING ENZYMES AND A HIGH AFFINITY IRON TRANSPORT SYSTEM FOR PATHOGENICITY OF ERWINIA CHRYSANTHEMI

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INTRODUCTION

Erwinia chrysanthemi 3937 incites a systemic soft rot disease on saintpaulia plants. This strain secretes a set of depolymerizing enzymes among which 5 pectate lyases (PLa-e), 1 pectin methylesterase (PME) and 2 endoglucanases (EGZ and EGY). It also secretes a catechol type siderophore, chrysobactin, which takes part in a specific high affinity iron transport system, induced under iron starvation (8, 9, 10). A number of reports have been shown that secretion of pectinases is essential for plant tissue maceration (6). We have recently shown, that the PLa, PLd, PLe, PME enzymes and the chrysobactin dependant iron transport system are required for the systemic development of the disease (3, 4, 8). In this report, the role of these different factors in the pathogenicity of *Erwinia chrysanthemi* 3937 is examined in further details (Fig.1).

MUTANTS ISOLATION

The genes encoding the PLa-e, PME, EGZ and EGY enzymes were cloned in *Escherichia coli*, then mutagenized by *in vitro* or *in vivo* insertions, using respectively the streptomycin and spectinomycin conferring resistant Ω interposon and the *lacZ* fusion forming Mud*II*1734 derivative that confers kanamycin resistance (2, 3). The mutated alleles were introduced back to *Erwinia chrysanthemi* 3937, and after marker exchange recombination, chromosomal mutants were selected. A collection of mutants lacking any one of the enzymes were isolated in this way. It should be noted that the mutant lacking the endoglucanase EGZ contains a *celZ::lacZ* gene fusion. A mutant (strain CB7) deleted of the 5 *pel* genes was also constructed.

Expression of the pectinase genes is induced by the presence of polygalacturonate in the growth medium (7,11). Expression of the *celZ* gene, encoding the major endoglucanase (EGZ), is induced during the late growth phase, and depends on DNA supercoiling (2) and extracellular conditions as oxygen level, osmolarity or iron supply.

Twenty two mutants affected in iron transport were isolated by Mud*II*1734 induced mutagenesis. They are affected either in the chrysobactin biosynthesis (Cbs^-) or in the secretion of this siderophore (Cbx^-) or in the uptake of the externally formed ferric:complex of the siderophore (Fct^- or Cbu^-) (8).

PATHOGENICITY OF THE MUTANTS

Pathogenicity of the mutants was assayed by inoculation on saintpaulia plants axenically grown in test tubes. When inoculated with the wild type strain, the plant was completely rotten in at most 15 days (3, 8).

The different responses obtained are presented in Figure 1. The *pelB* and *pelC* strains are as aggressive as the wild type phenotype, while the *pelA* and *pelD* mutants are less aggressive.The *pelE*, *pem* and Δ*pel* (*pel**) strains are non-invasive, but are still able to elicit a localized maceration. In most cases, the *celZ* mutant failed to induce any symptoms, while the *celY* appeared to be more aggressive than the wild type strain.The mutants were also examined for their ability to macerate isolated leaves from potted plants. The results showed in Table 1 match with those obtained with *in vitro* plants (Fig.1). It is worthy to note that the *celZ* mutant failed to macerate also isolated leaves while in the double *cel*$^-$ mutant, the *celY* mutation appeared to be dominant on the *celZ* one.

Otherwise, all the mutants unable to take up iron under iron starvation display a similar behavior: they were unable to give systemic symptoms on saintpaulia plants (see mutant Cbs$^-$ fig.1). Transfer of a 55 kb genomic DNA fragment from the wild type strain to the mutants restored the systemic virulence (8). An experiment of coinoculation of a couple of mutants including an uptake mutant (Cbu$^-$) that could stimulate the growth of a chrysobactin biosynthetic mutant (Cbs$^-$) in our bioassay (8) allowed to restore a systemic response. While the starting inoculum was the same for each mutant, their respective number, as scored at the end of infection appeared to be completely different: up to 80% of the living bacteria displayed a Cbs$^-$ phenotype. Furthermore, from this Cbs$^-$ population, a few clones Cbs$^+$ having recovered the wild type phenotype, including the pathogenicity, were isolated: such Kan^s revertants might have stemmed from a precise excision event of the mini-Mu element, not anymore detectable by Southern blot hybridization.

TABLE 1. Maceration assays on saintpaulia leaves with different mutants

strains	1 day	1 week	2 weeks
3937 (w.t.)	+/-	+	+/C
E-1002 (*celZ*)	-	-	-
E-2001 (*celZ*$^+$)	+/-	+	+/C
E-1005 (*celY*)	+	+/C	++/C
E-1007 (*celY, celZ*)	+/++	+/C	++/C
CB7 (*pel**)	+/-	+	+/++
$MgSO_4$ 10^{-2} (T)	-	-	-

- no maceration
+ localized maceration at infiltration zone
++ progressive
C complete maceration of the leaf

Leaves were infiltrated with bacterial suspension in $MgSO_4$ 10^{-2}M, then cut off and put on a wet filter paper. Incubation was at 30°C.

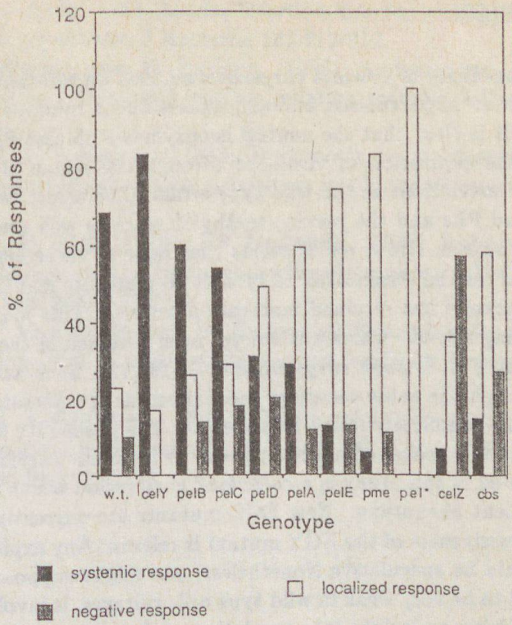

FIGURE 1. Distribution of responses induced by the mutant strains on saintpaulia plants

About 15-20 axenically grown saintpaulia plants were inoculated with each mutant strain as described previously (3, 8).

- w.t.: wild type strain 3937
- *celZ* and *celY*: endoglucanases Z and Y mutants respectively
- *pelA*, *pelB*, *pelC*, *pelD* and *pelE*: pectate lyases A, B, C, D and E mutants respectively
- *pel** (Δpel) :mutant containing chromosomal deletion including the 5 *pel* genes
- *pme*: pectin methylesterase mutant
- Cbs: chrysobactin biosynthetic mutant

AN APPROACH TO ASSESS "IN PLANTA" IRON CONDITIONS

The leaf intercellular fluid (IF) where the bacteria are supposed to develop during infection was analysed as a growth medium for bacterial cells. IF was prepared from healthy plants by centrifugation of leaves previously infiltrated with water under *vacuo*. When the wild type strain was grown in filter sterilized IF supplemented with glucose as a carbon source, a siderophore activity related to chrysobactin (scored by chemical and biological methods) was detected in the culture supernatant prepared at the stationary growth phase. No chrysobactin specific activity could be observed from IF cultures of a Cbs$^-$ mutant. This result suggests that the plant leaf IF is sensed by bacterial cells as a low iron medium since a catechol that displays chysobactin biological activity was produced in IF cultures.

DISCUSSION

The pathogenicity of *Erwinia chrysanthemi* 3937 on saintpaulia plants involves the external production of pectinases, endoglucanases and a functional high affinity iron transport system. It is clear that the neutral isœnzymes PLb and PLc do not play an important role in the expression of virulence through the whole plant. A double *pelB,pelC* mutant is still as virulent as the wild type strain. The acidic enzyme PLa, both basic enzymes PLd and PLe and the pectin methylesterase as well are required for the systemic spread of the disease. However, it seems that none of these enzymatic activities is absolutely essential for the strain 3937 to be able to macerate leaf tissue. The mutant lacking the 5 PL isœnzymes has retained maceration activity. This mutant might still produce a pectin degrading activity which has not yet been detected in the wild type strain as it was previously shown in *Erwinia chrysanthemi* EC16 (12). Such activity would be externally produced through the same secretory mechanism as the pectate lyases, since PL non secretory mutants completely failed to macerate. The possibility for the endoglucanase Z to be involved in early stages of infection needs to be more carefully looked at. Indeed, the *celZ* mutant used in this study is a *celZ::lacZ* gene fusion which might have generated this peculiar avirulent phenotype. New EGZ mutants are currently being constructed. The increased aggressiveness of the EGY mutant is relevant.Any explanation for this particular behaviour would be speculative.Nonetheless, one might suppose that this endoglucanase activity, found to be very weak in wild type cell cultures, is involved in some plant defense mechanism. Giving an insight into regulation of the expression of the *celY* gene might be worthy.

In addition, several lines of evidence suggest, that the expression of virulence through the plant vessels is dependent on iron availability. Production of the siderophore chrysobactin and its uptake as a ferric:complex by the infecting bacteria appears a prerequisite for successful invasion. Data not reported in this study have shown that the iron transport systems identified in strain 3937 are negatively regulated, iron acting as a corepressor. In this respect, we can wonder if the iron status of the plant could not be sensed by bacterial cells as a signal for iron assimilation not related genes to be expressed.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

ENDOGLUCANASES OF ERWINIA CHRYSANTHEMI, STRAIN 3937

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INTRODUCTION

Erwinia chrysanthemi (Erch) causes soft-rot disease of a wide array of plants. While being a Gram(-) bacteria, Erch secretes numerous enzymes in the external medium such as pectate lyases, pectin lyases, polygalacturonases, pectin methyl esterases, proteases and cellulases (9,18). Most of those activities are likely to be instrumental in the ability of Erch to macerate vegetal tissues. Although evidence has been collected that point to the importance of some pectate lyases (4,6,10,16) and cellulase (Aymeric et al., this volume), the influence of individual exoenzymes upon the pathogenesis awaits more clarification.

Cellulolytic organisms, i.e. capable of solubilizing crystalline cellulose produce several endoglucanases, β-glucosidases, and for a few exoglucanases (5,15). In contrast, Erch produces only two endoglucanases referred to as EGY and EGZ that are unable to degrade crystalline cellulose.

While detailed information about how a protein crosses a membrane has been provided by studies in E. coli, the crossing of both the cytoplasmic and the outer membranes that are required for the external secretion is not clear (18). In Erch, mutants (Out^-) impaired in the secretion of several enzymes including EGZ have been obtained, thereby suggesting the existence of a cellular secretion machinery (1,14,20). In this context, it seems likely that some motif should be present on the protein for its recognition and eventual secretion. In this review we will first cover the genetic and biochemical basic information on EGZ and EGY proteins. We will then show that both proteins can be considered as

true endoglucanases even though produced from an untrue cellulolytic organism. Last we will present some preliminary work aimed at identifying the information required for EGZ to be secreted.

THE EGY ENDOGLUCANASE

Genetic and regulatory aspects of the EGY production

As further work will eventually reveal, EGY is produced at an extremely low level and accounts for about 3% of the total endoglucanase activity. In this light, it is pretty ironic that the cognate gene *celY* was the one to be repeatedly found in genomic banks (3,17,19,21). The reasons for this are probably to be found in both the screening method used, i.e use of CMC plates stained by red congo, and some peculiar aspects of the regulation of EGZ production (see below). The *celY* gene was inactivated by inserting a miniMu::*lacZ* fusion forming element; the resulting *celY*::*lacZ* hybrid gene was recombined at the *celY* locus into the *Erch* chromosome. The *celY* gene was thereby located at 45' on the *Erch* chromosomal map (2). From that fusion a low level of β-galactosidase (9 U Miller) was synthesized indicating a very poor expression of the *celY* gene. As expected the overall endoglucanase activity contained in that *celY* mutant was barely reduced (2).

The nucleotide sequence of the *celY* promoter region was analyzed for identifying potential transcriptional signals (12). Approximately 130 nt. upstream the translational start codon, a consensus sequence for a NtrA dependent promoter was present. This promoter was shown to be functional by introducing a *celY* carrying plasmid in a pair of $NtrA^+$ and $NtrA^-$ strains of *E. coli*. No endoglucanase activity was found in the *ntrA* $celY^+$ strain grown in minimal medium. This showed that *celY* transcription can initiate at the NtrA dependent promoter. However, normal levels of endoglucanase were produced when cells were grown in rich medium. A possibility was the existence of an additional promoter that functions solely in rich medium. Preliminary data obtained from S1 mapping indicated that such indeed could be the case since a transcriptional start was identified that located approx. 241 nt upstream the translational start codon (12). These last experiments were performed in an *E. coli* strain

harboring the celY gene on a multicopy plasmid. Whether this putative promoter is also directing the celY expression in Erch needs to be checked

Biochemical aspects

Owing to its poor level of production, the exact location of EGY in Erch is unknown and the EGY protein was purified from E. coli where its amplification was possible. Its biochemical characteristics are given in Table 1 (8). Note that when produced in E. coli the EGY protein located into the periplasmic space. The comparison of the aminoacid sequence deduced from the nucleotide sequence and that of the purified protein indicates that EGY is first synthesized as a precursor with a NH2 terminal extension (12). The aminoacid sequence of that precursor resembles the typical signal sequence found in extracytoplasmic proteins.

TABLE 1

Biochemical properties of EGZ and EGY proteins

Property	EGZ	EGY
Specific activity : (nmoles glucose/mn/mg)		
Carboxymethyl cellulose	200	33
Avicell PH101	0.6	0.2
Methylumbelliferyl β-cellobioside	+	-
Optimum pH	7	5.5
Molecular weight	45 KDa	35 KDa
Isoelelectric point	4.5	8.2

The celY gene as a case of horizontal gene transfer

When the deduced aminoacid sequence was compared with that of other endoglucanases, a strikingly high level of homology was observed with an endoglucanase produced by Cellulomonas uda, a typical Gram(+) cellulolytic bacteria (12). Throughout the 280 AA that constitute the major part of

both enzymes, 164 identicals and 31 conservatives aminoacids could be identified. A similar level of homology was to be observed at the nucleotide sequence level: 57% of the codons were identical and 23% differed solely on the 3rd position (12). Given the phylogenetic distance between both Erch and C. uda, it is likely that the celY gene was recently transferred between both bacteria.The GC% of the nucleotide sequence would tend to support that C. uda acquired that gene from Erch.

THE EGZ ENDOGLUCANASE

Genetic and regulatory aspects of the EGZ production

The celZ gene could be isolated only from a λbased genomic bank. By using this clone as a probe it was found in all other genomic banks. By using a mini-Mu fusion forming element a celZ::lacZ gene was constructed and exchanged in the Erch chromosome at the celZ locus, thereby located at 10' on the chromosomal map (2). The level of β-galactosidase activity produced by this hybrid gene varied depending upon the host: in Erch approx. 300 U were found, reflecting a good expression while in E. coli it was less than 10 U. In addition, the amount of endoglucanase activity produced by an E. coli clone containing the celZ gene was not above that of an E. coli clone containing the celY gene. This observation provided us with an a posteriori explanation of why the celY gene had been detected as frequently as the celZ in the genomic banks. Altogether, these observations point to the probable existence of a positive effector in Erch that intervenes within the EGZ production and which is missing in E. coli.In Erch, the production of EGZ is occurring only when the cells reach the stationary phase. The basis for such a delayed regulation are unknown but anaerobiosis and growth yield have already been discarded at least as determining factors (Aymeric et al. in prep.). Of interest, however, is the fact that the celZ expression is sensitive to changes in DNA superhelicity (Aymeric et al. in prep.). Hence since DNA supercoiling is thought to change during the growth, an attractive hypothesis would be that those changes are part of the molecular basis for restricting the EGZ production to the stationary phase.

The EGZ protein as a typical endoglucanase

The EGZ protein was purified from the Erch supernatant (7). The biochemical characteristics are given in Table 1. Of great interest is the fact that the purified protein exhibits a CMC-ase specific activity higher than that of most endoglucanases found in cellulolytic organisms.

The computer aided comparisons of various endo- and exo-glucanases from cellulolytic organisms allow to picture those enzymes as being made up of two domains linked by an hinge region (15,22). The catalytic activity would reside in the domain that is predicted to adopt secondary structure. Mutageneses of several endo- and exo-glucanases have allowed to confirm that the secondary structure forming domain is indeed sufficient for conferring an enzymatic activity (11,23). The non-structured domain was attributed a role in substrate binding. The hinge region is characterized by its rich content in Pro and Thr residues (Figure 1). Such an organization was found in the EGZ protein, i.e. a structured NH2 domain (residues 1 to 288), and a non structured region (residues 323 to 388), separated by a Thr-Pro rich region (Figure 2;13). Moreover, a significant homology can be seen between EGZ and endoglucanases produced by various Bacillus and Clostridium (13). Interestingly, the homologous regions (approx. 50%) lie within the structured domain of the proteins (residues 177 and 288 of EGZ).

A mutated EGZ protein, EGZ3, was constructed by inserting an interposon that contains transcriptional and translational stop signals between the codons for residues 288 and 289 (Figure 2; Py and Barras, unpublished). When produced in E. coli, EGZ3 exhibited a reduced endoglucanase activity, i.e. 5-10% of the wild type enzymatic activity. This confirmed the prediction that in EGZ the catalytic site lies within the structured domain at the NH2 part of the protein. The mutated gene was recombined in the Erch chromosome at the celZ locus. Interestingly, the recovered activity was about 70% that of the wild type, that is 10 times more than in E. coli. A possibility is that in this latter EGZ3 is unstable.

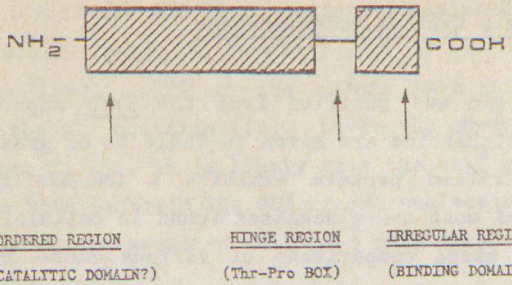

Fig.1 Structural model for cellulases (15,22)

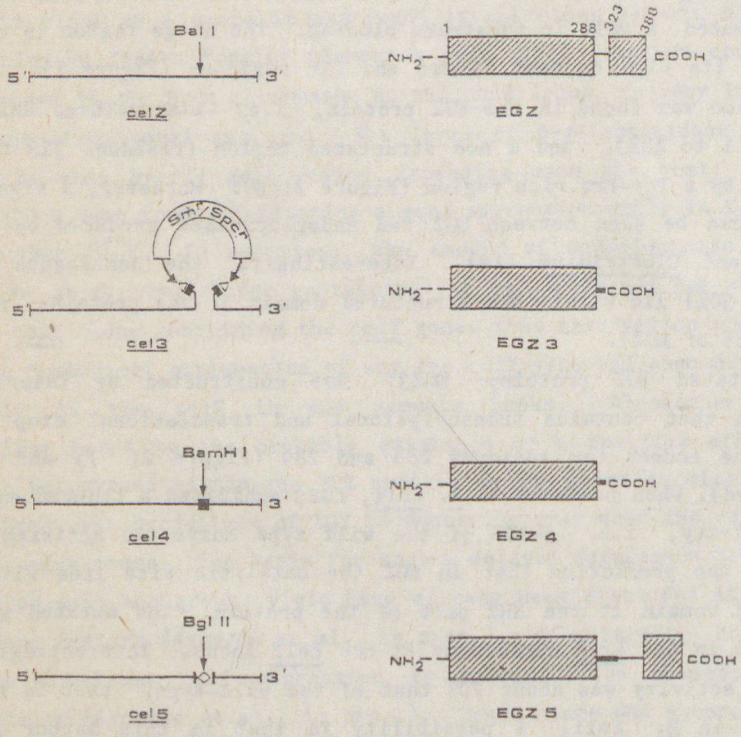

Fig.2 Construction of EGZ mutants. An interposon was inserted at the unique BalI site, giving rise to the EGZ3 protein. The excision of the interposon created a unique BamHI site at which a 12-mers linker was inserted in frame, giving rise to the EGZ5 protein.

The EGZ protein as a secreted protein

The comparison of both NH2 terminal ends of the purified protein and of the deduced aminoacid sequence revealed the presence of a signal sequence likely cleaved off during the crossing of the cytoplasmic membrane, at least in E. coli. The structure of this sequence, however, is pretty atypical in that it is very long (43 AA) and harbors negatively charged AA within the hydrophobic region (13). Its functioning as a signal sequence was nevertheless confirmed by fusing it to a signal sequence-less alkaline phosphatase and observing that the resulting hybrid protein located extracytoplasmically both in E. coli and Erch (13, unpublished).

As briefly exposed in the Introduction, the EGZ protein presents the peculiarity to be secreted through both the cytoplasmic and the outer membranes of Erch. The present working model invokes the existence of a cellular machinery that would recognize and assist a specific subset of proteins, among which is EGZ.

This implies that secreted proteins bear some motif, either sequential or conformational, that is instrumental in their selection by the secretion machinery. We previously mentioned that the homology existing between EGZ and endoglucanases of Bacillus is restricted to the first moiety of those proteins. Since most of the exoproteins from Gram(+) organisms are usually not secreted in Gram(-) bacteria (18), a possibility was that the secretion motif of EGZ would lie in the non-homologous region, i.e. at the COOH part of EGZ. The location of EGZ3 was therefore analyzed in Erch. The sole enzymatic activity could be used as a reporter of the EGZ location since no cross reaction could be obtained with the antiserum anti-EGZ (see below). It was found that 96% of the produced activity was located in the external medium (Table 2). This suggested that the secretion motif is contained in the catalytic domain.

That EGZ3 was secreted by following the "normal" pathway was checked by making use of an Erch outJ mutant (14). The results showed that, similarly to the parental EGZ, EGZ3 secretion was hampered by the outJ mutation. An unexpected observation, however, was that in the outJ mutant 10 times less EGZ (or EGZ3) activity was recovered than in the w.t. (Table 2). This is in disagreement with the initial observations (14). A possibility is that in the initial work the extracellular EGZ activity had been underestimated due to the omission in the culture medium of Triton X-100, a powerful agent for stabilizing EGZ (7). Hence, when compared,

both the EGZ activities trapped in the periplasm of the outJ mutant and secreted in the w.t. could have appeared as similar. If proved to be correct this hypothesis would uncover an as yet, not suspected link between EGZ production and secretion.

TABLE 2

Characterization of EGZ AND EGZ3 produced in Erch w.t and outJ

Strain	O.D.	Activity (U./O.D.)	Location	
			Cell	Supernatant
w.t.	2.8	14	0%	100%
$\underline{cel}_3$	2.6	10	4%	96%
outJ	3.7	1.3	88%	12%
outJ $\underline{cel}_3$	4.0	0.9	83%	17%

The lack of crossreactivity between EGZ3 and the anti EGZ antiserum brought up the question of the location of the epitopes in EGZ. A second mutant, referred to as EGZ5, derived from EGZ3 was therefore constructed in which the COOH part of EGZ was put back behind the catalytic region but separated by 8 additional aminoacids (Py and Barras, unpublished; Figure 2). As expected, a signal could be observed confirming the major EGZ epitopes are lying within the non-structured domain. It is interesting to note that computer based analysis had predicted this region to be the most antigenic.

CONCLUSION

Erch produced two unrelated endoglucanases from biochemical and genetic stand points. Both compare very well with endoglucanases from cellulolytic organisms. Recent results established a role for both EGZ and EGY in the plant bacteria interaction (see Aymeric et al., this volume). Here also, their role would differ drastically since EGZ appears to be part of the overall battery of cell wall degrading enzymes along with the

pectinases, while EGY could play a more subtle role in interfering with the plant defense mechanisms. Preliminary mutagenesis study of the EGZ protein suggested that the last 100 AAs are not essential either for its enzymatic activity or its secretion.

ACKNOWLEDGEMENTS

This work has been supported by CCE Biotechnology Program. Thanks are due to M. MAGNAN for technical assistance and to those, J.CATTANEO, B.CAMI, JL. AYMERIC, and MH BOYER, who contributed over the years to the "cellulase story" in Marseille.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

DEVELOPMENT OF DNA PROBES FOR DETECTION OF ERWINIA CAROTOVORA

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ABSTRACT

To develop DNA probes for detection of Erwinia carotovora, two probes were selected from a genomic library of E. carotovora in the Bluescribe plasmid vector. The first probe, 1 Kb in length, was obtained by selecting Erwinia DNA segments not found in Escherichia coli strains HB101, RR1, or DH5. Another probe, 6 Kb in length, was selected because E. coli DH5 transformants containing this segment of Erwinia DNA were able to grow on crystal violet pectate medium which is selective for pectolytic Erwinia. The probes were labelled with ^{32}P using a random primer and then used to probe lysed colonies or purified DNA of various E. carotovora strains fixed to Zeta-probe membranes. The first probe hybridized with only 63% of E. carotovora strains tested, whereas the other probe hybridized with all the strains. Restriction fragment length polymorphism analysis with the 6 Kb probe of several strains within serogroups revealed that while restriction patterns were identical among some strains within a serogroup, differences in pattern within a serogroup also occurred.

INTRODUCTION

The pectolytic Erwinia carotovora species is a serologically heterogeneous group of bacteria. Since many serogroups of E. carotovora subsp. atroseptica and E. carotovora subsp. carotovora occur in a single geographic region (De Boer 1983), serological detection and diagnosis of pectolytic erwinias is not possible with a single test. However,

detection of this group of bacteria on potato is required, for example, in order to assess the disease status of seed lots (Perombelon et al 1987). An alternate method to serological testing is the use of labelled DNA probes. DNA probes have already been made to several plant pathogenic bacteria (Thompson et al 1989), but their use for detecting Erwinia has not yet been reported. In this study two DNA probes to E. carotovora were investigated.

DEVELOPMENT OF PROBES

Two probes were selected from genomic libraries of E. carotovora in the Bluescribe plasmid vector which was propagated in E. coli DH5. One of the libraries consisted of total genomic DNA from four E. carotovora subsp. atroseptica strains and another of four E. carotovora subsp. carotovora strains.

One probe was selected by plating out the E. coli transformants of both libraries on crystal violet pectate medium which is selective for pectolytic Erwinia. Since untransformed E. coli colonies do not grow on this medium, only colonies that contained a segment of Erwinia DNA coding for the phenotypic characteristics that permit growth on the selective medium were selected. One positive colony, containing a 6 Kb insert, was selected from the E. carotovora subsp. carotovora library and used as probe A.

The second probe, 1 Kb in length, was obtained by selecting Erwinia DNA segments not found in Escherichia coli strains HB101, RR1, or DH5. This was done by plating a mixture of the two libraries onto a membrane filter. After colonies had grown up on the filter they were lysed and the filter blocked with E. coli DNA. The filter was then probed with ^{32}P-labelled Erwinia DNA which had also been pre-hybridized with E. coli DNA. In this way colonies from the library that contained common Erwinia DNA could be identified as giving the most intense radioactive spots on the filter. One colony was selected and used as probe B.

TESTING OF DNA PROBES

Both probes A and B were labelled with ^{32}P using a random primer and then used to probe lysed colonies of various E. carotovora strains fixed to Zeta-probe membranes. Probe B hybridized to all 40 strains from different serogroups (Table 1). Both subspecies, carotovora and atroseptica, were included among the strains. The 6 Kb probe A hybridized strongly to only 25 of the 40 strains tested. Subsequently this probe was subcloned and a smaller insert of 2 Kb selected. The subclones hybridized with the 40 strains in the same pattern as the parent probe (Table 1).

Restriction fragment length polymorphism (RFLP) analysis was made of purified DNA from E. carotovora cut with *Eco*RI. Analysis of several strains in serogroups I, III, and XVIII with probe A revealed that while restriction patterns were identical among some strains within a serogroup, differences in pattern within a serogroup also occurred. Probe B also gave variable restriction patterns with DNA from the 40 serogroups, but common bands were present in most strains.

Table 1. Reaction of probe A and B with E. carotovora strains representing forty different serogroups

Subspecies	No. of strains tested	Number of strains with positive reaction		
		Probe A (6Kb)	Probe A (2Kb)	Probe B
atroseptica	4	0	0	4
carotovora	36	25	25	36

DISCUSSION

Probe B appears to be the better of the two probes for detection of E. carotovora strains. It reacted with a wide range of different Erwinia strains in different serogroups and thus can be used for indexing of soft rot causing erwinias on potato. It did not react or reacted only very weakly with a number of unidentified bacteria isolated from potato field soil (unpublished data).

The practicality of using the DNA probe for potato tuber testing, however, still needs to be explored. Ideally the probe would be used in a non-radioactive detection protocol such as the strepavidin-biotin system. Furthermore enrichment of the target bacterium in selective medium may be required to attain adequate sensitivity in the same way as is done for enhancing sensitivity of serological detection (De Boer 1983). Alternatively, an enhancement procedure whereby the specific gene that is being probed is increased several fold, prior to testing, may be possible (Steffan and Atlas 1988).

RFLP analysis has been useful for identification of specific bacterial strains and has been applied to some plant pathogenic bacteria (Denny 1988). Probe A may be more useful than probe B for RFLP analysis of E. carotovora strains. Our preliminary results suggest that strains within serogroups can be classified as to RFLP banding patterns with probe A. On the other hand, strains in different serogroups gave very similar banding patterns in RFLP analysis with probe B.

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

Plant Pathogenic Bacteria
Z. Klement (ed.)

PLANT-INDUCIBLE GENES IN PLANT INTERACTING BACTERIA

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Introduction

Plant-bacteria interaction is a complex process which involves recognition steps between the interacting organisms. A primary step in the recognition can be the synthesis by the host of compounds acting as molecular signals for the invading bacteria and allowing the microbes to detect susceptible host cells. These plant compounds induce bacterial genes and determine the issue of the interaction. Such plant inducible genes have been identified in several species of plant-interacting bacteria. The purpose of this paper is to describe systems in which such recognition mechanism has been identified and to determine the role of plant-inducible genes in the interaction.

Plant-inducible genes in *Agrobacterium* and *Rhizobium spp.*

Agrobacterium tumefaciens causes crown gall disease on many dicotyledonous plants. Following infection of a wound site, *Agrobacterium* transfers a part of its Ti plasmid to the plant chromosome. The transferred region is called T-DNA and encodes the genes responsible for opine and phytohormone synthesis. Another region of the Ti plasmid encodes a set of genes needed for virulence (*vir*). The vir region contains at least 6 loci, *vir*A, *vir*B, *vir*G, *vir*D, *vir*C and *vir*E which are essential to T-DNA transfer (14). In the vegetative bacterium, only *vir*A and *vir*G are significantly expressed. However, when the bacterium is cocultivated with plant cells, expression of the *vir*B, *vir*C, *vir*D, *vir*E and *vir*G loci is induced from 10 to 300 fold whereas *vir*A expression is unaffected (14). The plant factors affecting *vir* gene expression have been identified as low molecular weight and diffusible phenolic compounds as acetosyringone and α-hydroacetosyringone whose the presence in plant tissues is increased when the plant has been wounded (13). The product of *vir*A is postulated to bind plant factor and acts as signal transducer. This step probably mediates the activation of *vir*G protein which

controls the expression of the other *vir* genes. When induced the *vir*B, *vir*C, *vir*D and *vir*E loci mediate the cleevage of the T-DNA and possibly other functions necessary for DNA transfer to the plant chromosome (15).

In *Rhizobium*, a soil microorganism which induces the formation of nitrogen-fixing root nodules on legumes, transcription of the nodulation (*nod*) genes can be induced or repressed by phenolic compounds found in root exudates (4). Similarly to the *vir*A gene of *Agrobacterium*, the *nod*D gene is constitutively expressed. Both the *nod*D gene product and phenolic plant factors are required for induction of the other *nod* genes. The *nod*D gene plays a role in host specificity since the *nod*D gene product of different species interacts with different plant factors (6). Different plant-inducing flavones have been identified from white clover and alfalfa (10,11). These compounds are chemically different of acetosyrinone and the other phenolic compounds stimulating *vir* gene expression in *Agrobacterium*.

Plant-inducible genes in *Xanthomonas campestris*.

Osbourn *et al.* (9) were first to present a method to identify plant-induced promotors inside a complete genome. They cloned, into a promotor-probe plasmid, *Xanthomonas campestris* chromosome fragments in front of a promotorless chloramphenicol acetyltransferase gene. The recombinant plasmids were transferred into the wild type strain and the recombinant bacteria containing a plasmid carrying a plant-induced promotor were selected by their resistance to chloramphenicol *in planta* and their sensitivity to this antibiotic *in vitro*. This elegant work allowed the identification of several plant-induced promotors but an extensive subsequent work is required to determine the role of the corresponding genes in pathogenicity.

Plant-inducible genes in *Erwinia chrysanthemi*.

Another method to identify plant-inducible genes has been used in *Erwinia chrysanthemi*, a pectinolytic enterobacterium which causes soft rot disease on many plant species by producing a set of depolymerizing enzymes which can degrade the plant cell wall (C. B. and F. V.G., submitted). The procedure requires a simple insertion mutagenesis and allows in a one-step experiment, both, the identification of plant-inducible promotors and the isolation of mutants which can be directly tested for their virulence on plants. The studied model is the interaction between *Erwinia chrysanthemi* strain 3937 and its host *Saintpaulia ionantha*.

To identify plant-inducible genes in *E. chrysanthemi*, the following strategy was used. First, insertion mutagenesis was performed using the MudIIPR3 transposon. This mini-Mu carries a constitutive chloramphenicol resistance gene and a promotorless neomycin phosphotransferase gene. Upon insertion, the truncated gene can fuse with *Erwinia*

promotors. Chloramphenicol resistant mutants obtained from the insertion mutagenesis were pooled and inoculated in *Saintpaulia* plant extract. During exponential growth phase, kanamycin was added to plant extract and the incubation was continued overnight. Two types of mutants survived to this treatment; those which constitutively expressed kanamycin resistance and those where the resistance gene was induced by the plant extract. Surviving mutants were purified on chloramphenicol plates and then tested on kanamycin plates for their sensitivity to this antibiotic *in vitro*. Using this procedure, 30 conditional mutants resistant to kanamycin only in the presence of plant extract were found. Up to now, 10 of these mutants have been characterized.

Inoculation of *E. chrysanthemi* strain 3937 on its host *Saintpaulia* can produce 3 types of responses: systemic, localized or negative (1). Mutants can be differentiated by their symptom distribution on plants. Half of the mutants failed most times to induce systemic response on plant. Three mutants caused a number about equal of systemic, localized and negative responses. The 2 other mutants usually provoked systemic disease and the distribution of their responses on plant did not differ significantly from the wild type strain. Consequently, the mutants were respectively grouped into 3 classes: strongly attenuated, slightly attenuated and virulent (Table 1). Therefore, 8 out 10 mutants with an insertion in plant-inducible genes were affected in virulence on *Saintpaulia* plant.

Among the depolymerizing enzymes produced by *E. chrysanthemi*, it is well established that the pectate lyase isoenzymes, specially PLa, PLd and PLe, play an essential role in pathogenicity on *Saintpaulia* plants (1). Mutations in the corresponding genes *pel*A, *pel*D and *pel*E cause an important reduction of the bacterial virulence. Because of their essential role in the plant-bacteria interaction, depolymerizing activities were tested in each mutant. All mutants retained the ability to degrade *in vitro* the substrates of proteases, cellulases and pectinases. No mini-Mu was inserted into the *pel*A, *pel*D and *pel*E genes and the pectin methylesterase gene (*pem*) .Electrofocusing of the culture supernatant of the mutants grown in the presence of polygalacturonate showed that the 5 pectate lyase isoenzymes were secreted by the mutants. However, the amount of the acidic pectate lyase PLa was less abundant in strain RH7005 suggesting that strain RH7005 contains an insertion in a regulator gene of *pel*A. Furthermore, when inoculated on potato tubers, all mutants retained the ability to macerate the plant organ. Thus, the reduced virulence of some mutants on the host cannot be explained by a loss of the maceration ability.

The degradative products of pectin, a major constituant of the plant cell wall, are polygalacturonate and galacturonate. These pectin degradative products can be utilized by *E. chrysanthemi* as the sole carbon source (7). All mutants can catabolize these compounds except strain RH7009 which is defective in galacturonate catabolism. Since strain RH7009 is

Table 1. Characterization of mutants.

strain	virulence on host	relevant characteristic
RH7001	strongly attenuated	no growth with EDDA
RH7003	strongly attenuated	
RH7004	strongly attenuated	
RH7005	strongly attenuated	reduced secretion of PLa
RH7006	strongly attenuated	
RH7002	slightly attenuated	no growth with EDDA
RH7008	slightly attenuated	3 mini-Mu insertions
RH7009	slightly attenuated	deficience in galacturonate catabolism
RH7007	virulent	
RH7010	virulent	

attenuated in virulence, this suggests that galacturonate catabolism is an important function during pathogenesis.

In addition to depolymerizing activities, others requirements are essential to virulence: the integrity of the lipopolysaccharide (LPS) molecules (12) and a functional iron assimilation system (5). The LPS composition of the mutants was analyzed but no detectable alteration was noticed. However, 2 strains (RH7001 and RH7002) do not grow in the presence of ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDA), a strong iron chelator, suggesting that these 2 mutants are affected in iron assimilation.

Biochemical evidence for the existence of plant-inducible genes in other plant pathogenic bacteria.

The pathogenicity factors of *E. amylovora*, the agent of fire blight are rudimentarily understood. Unlike *E. chrysanthemi*, *E. amylovora* does not produce depolymerizing enzymes. Exopolysaccharides are essential to pathogenicity but have no toxic effect on plant cells. However, recent works indicate that a toxin could be involved in the fire blight disease. Furthermore, this toxin production would be induced in the presence of plant cells (2, 3). Indeed, a low molecular weight product with toxic effect on plant cells was detected in fluids

from the interaction between a suspension-cultured apple cells and a virulent *E. amylovora* strain. Fluids from the interaction with an avirulent capsulated strain have no toxic effect. When bacterial and plant cells are in contact, a lag phase of about 4 h is required before the beginning of apple cell death. However, this delay can be abolished by preincubating the bacterial cells with host cells, protoplasts or intracellular plant fluids. The plant factor abolishing the lag phase is a low molecular weight phenolic. Clorogenic acid, a phenolic found in apple, which mimics the effect of the plant factor could *in vivo* activate the toxin production(2, 3). Even if the genes involved in toxin synthesis have not been identified yet, these results of Cooper *et al.* (2, 3) on the biochemical characterization of a plant-inducing factor of *E. amylovora* virulence suggest that plant-inducible genes do exist in *E. amylovora*.

Indirect evidence for the existence of plant-inducible genes in a fluorescent *Pseudomonas* strain has been brought by the work of Van Outryve *et al* (16). Infectivity titration (i.e. the number of cells needed to cause symptoms) of a *Pseudomonas* strain causing soft rot on witloof chicory leaves decreases when the bacterium was grown in the presence of chicory tissues or an extract of leaves tissues before its inoculation on plants. Preliminary characterization of the plant extract-inducing factor reveals that the compound is heat stable and has a molecular weight lower than 10000 d (16).

Conclusions

The existence of plant-inducible genes in plant-interacting bacteria has been established by different ways, regulation studies of genes known to be essential to plant-bacteria interaction, identification of genes induced *in planta* or by plant extract, identification of plant factors which induce bacterial virulence. Different plant compounds regulate bacterial genes. These compounds seem to be specific of each interaction. However the plant factors share common characteristics, they are low molecular weight diffusible phenolic compounds. Since plant-inducible genes are found in bacterial species as different as *E. chrysanthemi*, *E. amylovora*, X. *campestris*, *Rhizobium spp.*, A. *tumefaciens* and *Pseudomonas sp.*, plant signal recognition mechanism could be a general process in plant-bacteria interactions.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

EVIDENCE FOR GLOBAL REGULATION OF THE EXPRESSION OF PATHOGENICITY GENES IN SOFT-ROT ERWINIAS

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INTRODUCTION

Erwinia carotovora subsp. *carotovora* (*Ecc*) and the related pathogens *E. carotovora* subsp. *atroseptica* (*Eca*) and *E. chrysanthemi* (*Echr*) incite soft-rot disease in many varieties of plants. Substantial biochemical and genetic evidence has established that pectolytic enzymes synthesized and secreted by the soft-rot *Erwinias* play a major role in the development of soft-rot disease (Daniels et al., 1988). Soft-rot *Erwinias* also produce extracellular pectin methyl esterases (PME), proteases, cellulases, and xylanases. Although genes for several of these enzymes have been cloned, their role in pathogenicity have not yet been established (Daniels et al., 1988). A mutant of *Ecc* defective in the synthesis of PME, which we isolated using marker exchange mutagenesis, shows pathogenicity similar to that of a wild type strain (unpublished result). We have shown that a mutant impaired in the synthesis of UDP-glucose pyrophosphorylase is avirulent (Jayaswal et al., 1985). At present little is understood regarding the regulation of virulence genes in the soft-rot *Erwinias*. We have recently reported the existence of a global mechanism which regulates expression of several extracellular enzymes, including the pectolytic enzymes, in the soft-rot *Erwinias* (Chiu et al.,1989).

RESULTS

Characterization of pectolytic avirulent mutants of *Ecc*:

To understand the overall genetic basis of soft-rot disease development, we employed transposon mutagenesis to isolate independent mutants of *Ecc* which are impaired in their pathogenicty (Handa et al., 1987). Among the 63 pathogenicity deficient mutants that we isolated, 4 mutants (AH2024, AH2552, AH2819, AH4334) were not impaired in synthesis or secretion of pectate lyase (PL) or

polygalacturonase (PG) (Handa et al., 1987). The isoelectric profiles of secreted PL isozymes and PG in these prototrophic mutants were similar to that of the wild type parent strain *Ecc* AH2 (unpublished data). Mutants AH2024, AH2819 and AH4334 each have a single Mud1 insertions at different locations (Handa et al., 1987). Mutant AH2024 has two separate Mud1 insertions. These results, for the first time, indicated presence of virulence genes other than those involved in the production and secretion of pectolytic enzymes in soft-rot *Erwinias* (Handa et al., 1987).

We have investigated the relationship between the *in planta* bacterial growth and the production of soft-rot symptoms in the potato tuber by the wild type and mutant strains under aerobic and anaerobic conditions. Our results indicate that a threshold level of about 1×10^8 bacteria are required to cause soft-rot disease symptoms both under aerobic and anaerobic conditions (unpublished data). However, under anaerobic conditions the host resistance to bacterial growth is less effective, resulting in a higher final bacterial concentration and more severe soft-rot disease. Effects similar to anaerobic conditions can be obtained by inhibiting protein synthesis using cycloheximide. Our results indicate that under aerobic conditions our pectolytic avirulent mutants increase 10^3 to 10^5-fold (i.e. 10 to 17-doublings) over the initial inoculum *in planta* and anaerobic conditions enhance their *in planta* growth by 10 to 100-fold. However, growth of these pectolytic avirulent mutants is arrested before reaching the threshold bacterial levels required for soft-rot development. The pathogenic wild type strain undergoes another 4 to 6-doublings to reach the final concentration of about 5×10^9cfu/inoculation site (unpublished data). Pretreatment of the potato tuber with cycloheximide or inoculation of the potato tuber with more than 10^8 bacteria can partially restores pathogenicity to these mutants. Overall, it seems that the Mud1 insertions have inactivated genes in these mutants which are involved in overcoming host resistance to bacterial growth.

Molecular cloning of genes which complement mutations in pectolytic avirulent mutants of *Ecc*:

We have isolated recombinant cosmid clones which restore the pathogenic phenotype to mutants AH2552, AH2819 and AH4334. To isolate these cosmid clones, a 3 Kb *Bam*H1-*Eco*R1 fragment containing the right end of Mud1 was purified from pPO1681, a derivative of MudII 7701-301, and cloned into pUC19. This 3 Kb DNA fragment was used to screen genomic DNA clone banks constructed from the total DNA of the pectolytic avirulent mutants AH2552, AH2819, and AH4334 in a bacteriophage vector EMBL3. Several plaques which hybridized to the Mud1 probe were identified and purified for each of the mutants. After establishing partial

restriction maps of inserts in these recombinant clones, DNA fragments containing the sequences flanking the right end of the Mud1 insertions were identified and subcloned into pUC19. The DNA sequences which flanked the Mud1 insertions were used to screen a genomic library of wildtype Ecc AH2 total DNA constructed in cosmid vector pLAFR3. Hybridizing cosmids were selected for mutants AH2552, AH2819, and AH4334. The ability of selected cosmid clones to complement their respective mutations was demonstrated after mobilization of these cosmid clones into the appropriate mutants using triparental crosses (unpublished results).

Molecular cloning of DNA fragments which impart avirulent phenotype to wild type *Erwinias* and inhibit synthesis of plant cell-degrading enzymes:

Although the cosmid clone 2552-462 (Fig. 1) restores the pathogenic phenotype to mutant AH2552, two adjacent DNA fragments from this cosmid when cloned into pBR322 (a multicopy plasmid), impart an avirulent phenotype to the parental pathogenic strain, *Ecc* AH2 (Fig. 2). Plasmids pEcc 0.6 and pEcc 5.0 containing the 0.6 Kb *Eco*R1-*Pst*1 and the 5.0 Kb *Pst*1-*Sal*1 fragments, respectively, do not complement mutation in AH2552. Both pEcc 0.6 and pEcc 5.0, when present in a pathogenic strain of *Ecc*, have no apparent effect on bacterial growth but greatly inhibit the accumulation of PLs, PG, PME, cellulase, and protease activities (Table 1). Based on isoelectric focusing analysis of culture fluid and bacterial extracts, the production of all PL isozymes is inhibited in *Ecc* AH2 containing either pEcc 0.6 or pEcc 5.0. The effects of pEcc 0.6 and pEcc 5.0 on *Eca* and *Echr* are similar to those

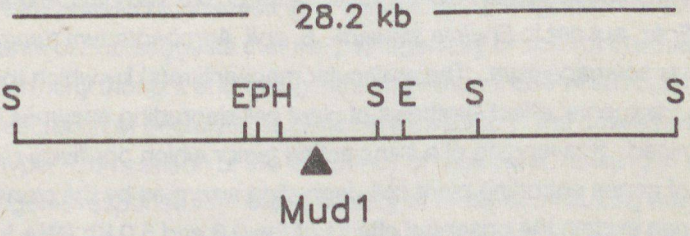

Fig. 1 The partial restriction map of cosmid 2552-462 which restores pathogenicity to the pectolytic avirulent mutant AH2552. The 0.6 Kb *Eco*R1-*Pst*1 and 5.0 Kb *Pst*1-*Sal*1 fragments subcloned in pBR322 are designated as pEcc 0.6 and pEcc 5.0, respectively. Also shown is the site of Mudl-insertion in mutant AH2552.

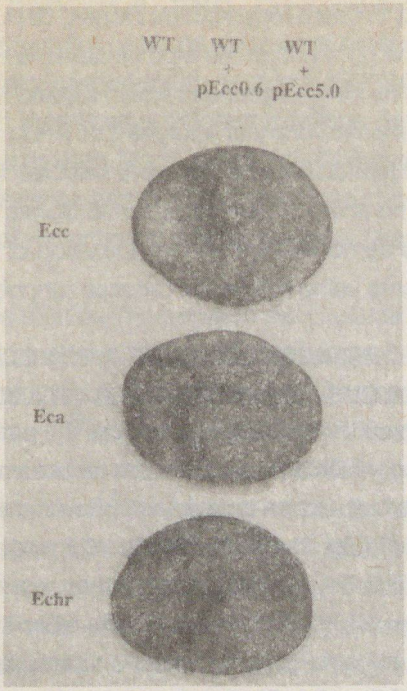

Fig. 2. Phenotype of wild type strains of *Ecc* AH2, Eca 12 and *Echr* EC16 containing plasmids pEcc 0.5 and pEcc 5.0. Potato slices were inoculated with ten micro-liters of overnight cultures of different strains and incubated in petri dishes at 30° C for 24 hours. A wet filter paper was placed in the petri dish to maintain high humidity.

on *Ecc* (Chiu et al., 1989). Pathogenic strains of *Eca* and *Echr* containing either pEcc 0.6 or pEcc 5.0 become avirulent (Fig. 2) and defective in production of PLs, PG, PME, cellulase(s) and protease(s), but show no effect on bacterial growth rates (Chiu et al., 1989).

Based on Southern analysis, pEcc 0.6 and pEcc 5.0 do not hybridize to each other. Also, these plasmids do not encode a PL, PG, PME, protease, or cellulase. DNA sequences which hybridize to inserts present in pEcc 0.6 and pEcc 5.0 are present in *Eca* and *Echr*, but not in *Erwinia stewartii, E. coli, Agrobacterium tumefacien,* or *Pseudomonas solanacearum.* The molecular mechanism(s) by which the 0.6 and 5.0 Kb DNA fragments affect synthesis of plant cell-degrading enzymes have not yet been determined. Scavenging of a *trans*-acting factor which positively regulates expression of genes encoding plant cell-degrading enzymes by the cloned DNA sequences can explain the observed effects of the 0.6 and 5.0 Kb DNA fragments. However, other more involved mechanisms should also be considered. The side-by-side presence of two DNA sequences which cause similar effects could be due to repeats of the same DNA sequences within a gene or an operon or due to two adjacent operons containing an identical regulatory DNA sequence and transcribed

Table I. Effects of pEcc 0.6 and pEcc 5.0 on the bacterial growth and enzyme production in *E. carotovora* subsp. *carotovora*

Parameter	WT	WT + pBR322	WT + pEcc 0.6	WT + pEcc 5.0
Bacterial growth				
Doubling time(h)	3.23	3.28	3.21	2.60
Maximum A_{600}	1.34	1.41	1.52	1.26
Pectate lyase	51.30	36.40	0.16	2.36
Polygalacturonase	2.62	2.10	0	0
Cellulase	+	+	UD	UD
Protease	+	+	I	I
Pectinesterase	+	+	I	I

Bacterial growth was determined in M63 medium containing 0.4% glucose. PL and PG activities (units/A_{600}) were determined by monitoring the increase in A_{230} and using the arsenomolybdate assay for reducing sugars, respectively (Reid and Collmer, 1985). Production of cellulase, protease and PME were determined using plate assays (Teather, R.M. and P.J. Wood (1982); Hugenholtz J. et al. (1987); Zimmerman, R. E.(1978). UD and I represent undetectable and inhibited, respectively.

in opposite direction. Experiments are in progress in our laboratories to determine the mechanism by which these DNA sequences effect expression of virulence genes in the soft-rot *Erwinia* species.

CONCLUSIONS

Analysis of avirulent *Ecc* mutants resulting from Mud1-transposon mutagenesis (Handa et al., 1987) has led to interesting observations. The isolation of avirulent pectolytic mutants of *Ecc* suggests that the pathogenicity of soft-rot *Erwinias* is more complex than formerly thought and that genes other than those which control synthesis and secretion of pectolytic enzymes are involved in the development of the soft-rot phenotype. Comparison of the frequency of mutations resulting in an impaired pathogenic response with the frequency of mutations causing auxotrophy indicates over 100 *Ecc* genes can influence pathogenesis in the potato tuber. The effects of cycloheximde on development of the soft-rot phenotype and characterization of the pectolytic avirulent mutants suggests that induced host-resistance plays an important role during pathogenesis.

The coordinate expression of virulence genes and the presence of sensory transduction mechanisms have been demonstrated in several human pathogenic bacteria and in one phytopathogenic bacterium (Miller et al., 1989). Although the pathogenicity of soft-rot *Erwinias* is known to be influenced by several environmental factors, including temperature, humidity, and oxygen tension, the presence of a sensory transduction mechanism regulating expression of pathogenicity genes in *Erwinias* has not yet been shown. Our results demonstrate the presence of mechanism in soft-rot *Erwinias* which coordinately regulates expression of several pathogenicity genes. Whether this mechanism also represents a sensory transduction mechanism in soft-rot *Erwinias* remains to be established.

Acknowledgement: This research was supported by a USDA CRGO grant (85-CRCR-1-1595) to AKH.

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

Plant Pathogenic Bacteria
Z. Klement (ed.)

EXPRESSION OF BACTERIAL PATHOGENICITY- AND PLANT DEFENSE-RELATED GENES IN POTATO SOFT ROT

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ABSTRACT

Potato tuber slices and *Erwinia carotovora* subsp. *carotovora* (Ecc) were used to study *in planta* regulation of bacterial pathogenicity-related (BPR) and plant defense-related (PDR) genes in soft rot interaction. Northern blot analysis showed that induction of mRNAs for BPR genes including endo- and exo-pectate lyases (PLs) and endo-polygalacturonase (PG) occurred within 3 hr reaching maxima between 6 and 12 hr after inoculation. These genes are expressed sequentially; exo-PL, endo-PL, and then endo-PG. The induction kinetics for these BPR genes are correlated closely with soft rot development. Host defense responses were monitored by following phenylalanine ammonia lyase (PAL) and hydroxymethylglutaryl CoA reductase (HMGR) enzyme activity and mRNA levels. While induction of PAL enzyme activity and mRNA by Ecc is rapid and transient with maxima at 6 and 9 hr, respectively, HMGR induction is slower and persistent through 22 hr. Ecc activates a HMGR isogene distinct from that induced by wounding, indicating that plants respond to soft rot bacteria actively and specifically. PAL expression is activated by Ecc endo-PL but not endo-PG or exo-PL, presumably by releasing endogeneous signals from plant cell walls.

INTRODUCTION

Soft rot incited by *Erwinia* species is a destructive important plant disease spread worldwide. It is characterized by maceration and degradation of plant tissues resulting from the action of a series of plant cell-degrading enzymes (CDEs) such as pectic enzymes, cellulases, and proteases produced by soft rot *Erwinia*. Genes encoding these enzymes are called BPR genes since their role in pathogenecity is not fully understood. Recently, most research has focused on molecular genetics of these genes. Expression of cloned genes in *E. coli* and site-specific mutagenesis in *Erwinia* provide support for the hypothesis that some pectic enzymes are responsible for maceration (Kotoujansky, 1987). Results of *in vitro* studies indicate that these enzymes are subject to substrate induction and catabolite repression (Collmer et al., 1982; Kotoujansky, 1987; Reverchon and Robert-Baudouy, 1987). In spite of great progress, several key questions in the soft rot interaction remain unanswered: 1) Are CDEs regulated *in planta*? If yes, is *in planta* regulation related to pathogenesis? 2) How do plants respond

to the attack of the soft rot *Erwinia*? 3) What is the mechanism of plant-bacterial communication?

To address these questions, we developed a system to study simultaneously *in planta* expression of bacterial and plant genes. This system involves inoculation of tuber slices with Ecc separated from the host by a biologically-inert polysulfone membrane. This arrangement permits reproducible development of tissue maceration and allows rapid and quantitative isolation of bacteria free of plant cells and debris and isolation of bacteria-free plant cells (Yang et al., 1989). In this paper, we use this system to analyze *in planta* regulation of several BPR and PDR genes in potato-Ecc interaction. *In planta* induction kinetics of mRNAs encoding endo-PL, endo-PG, and exo-PL from Ecc strain EC14 are described. Endo-PL production is mediated by Ecc DNA cloned in plasmid pDR1 and endo-PG and exo-PL is mediated by DNA cloned in pDR30 (Roberts et al., 1986). Earlier work showed that combination of pDR1 and pDR30 was required for *E. coli* to macerate plant tissues (Roberts et al., 1986).

Probes from two PDR marker genes, PAL and HMGR, were used to monitor plant defense responses. PAL is a key enzyme involved in the synthesis of phenolics and lignins. HMGR is a key enzyme involved in the synthesis of sesquiterpenoid phytoalexins. These compounds have been implicated in the defense of tubers against soft rot *Erwinia* (Ghanekar et al., 1984). Although activation of defense-related genes by pathogenic fungi has received much attention (Collinge and Slusarenko, 1987), very little is known about the induction of these genes by pathogenic bacteria. Generally, fungal cell walls contains elicitors of defense gene activation. In this paper, we also investigate bacterial gene products that induce plant defense response.

MATERIALS AND METHODS

The conditions described in this paper for bacterial strains, tuber origins, culture, inoculation, RNA isolation, Northern hybridizations, and enzyme activity, are the same as described by Yang *et al.*, 1989 for experiments with endo-PL and PAL. The results and discussion section is amended to include procedures and results concerning exo-PL, endo-PG, and HMGR not included in the earlier paper. Results of endo-PL and PAL are compared here with this new information. HMGR enzyme activity was assayed using the procedure of Stermer and Bostock (1987)

RESULTS AND DISCUSSION

Regulation of Bacterial Pathogenicity-related Genes. Expression of endo-PL, endo-PG, and exo-PL in Ecc was studied under compatible conditions using the membrane-separated system (Yang et al., 1989) or in culture using polygalacturonic acid (PGA) as inducer. Levels of mRNAs were analyzed by Northern blot hybridization. Mobilized RNA was first hybridized with endo-PL DNA probe as described (Yang et al., 1989) and rehybridized with exo-PL or endo-PG DNA probe (1.1-kb *Bam*HI-*Bgl*II or 600-bp *Bgl*II-*Bam*HI fragment of pDR30, respectively, Roberts et al., 1986). Transcripts of endo-PL, exo-PL, and endo-PG were 1.4, 1.8, and 1.3 kb, respectively.

Although levels of all three transcripts increased within 3 hr and dropped to basal levels by 33 hr after inoculation, inductions peaked at different times with exo-PL at 6 hr, endo-PL at 9 hr, and endo-PG at 12 hr (Fig. 1A) indicating that their expression is sequentially regulated. Production of pectic enzymes in rotting tissues has been detected (Kotoujansky, 1987). However, our results provide the first concrete evidence that these enzymes are regulated *in planta*. The strong correlation between their induction kinetics and soft rot development (Fig. 1A) indicates that their regulation plays an important role in soft rot pathogenesis.

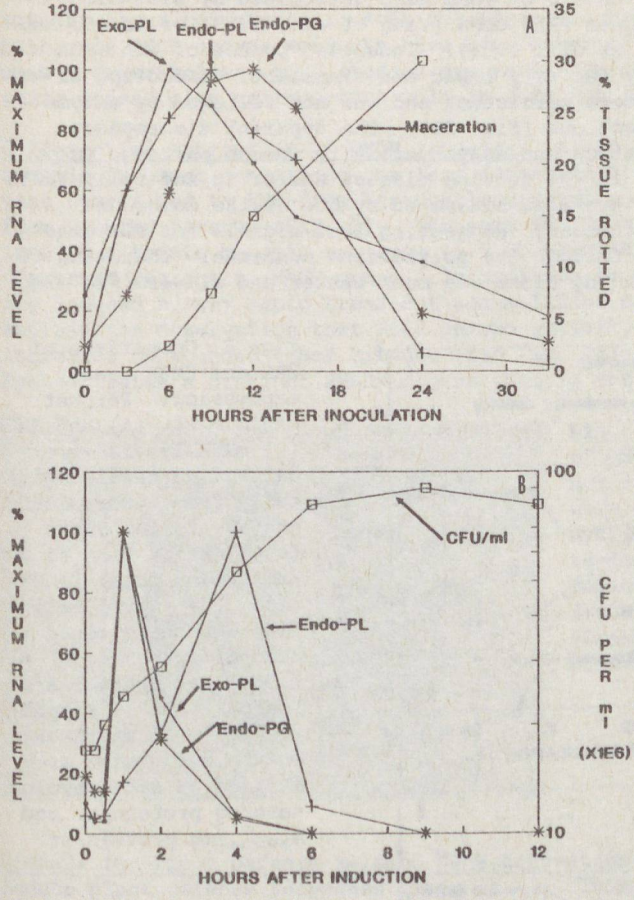

Fig. 1. *In planta* and *in vitro* regulation of endo-PG, endo-PL and exo-PL. Bacteria were harvested at times indicated after tuber slice inoculation with Ecc using the membrane-separated sysyem (A) or after resuspension of Ecc cultures in PGA-containing medium (B). mRNAs were detected by Northern blot hybridization (see text). Percent maximum mRNA levels were determined densitometrically from autoradiographs of the Northern blots. Percent tuber macerated was determined by weight loss before and after washing off rotted tissues. Number of bacteria was determined spectrometrically.

Sequential induction of pectic enzymes suggests that *in planta* induction is not controlled by a global mechanism. Interestingly, kinetics of *in vitro* induction did not follow the *in planta* sequential induction (Fig. 1B). Both endo-PG and exo-PL mRNAs were induced to maxima at 1 hr by PGA and decreased to basal levels by 4 hr while endo-PL mRNA increased to maximum levels by 4 hr and returned to basal levels by 6 hr. These results suggest that mechanisms of *in planta* and *in vitro* regulation are different and point out the need for understanding *in planta* regulation. The significance of the *in planta* sequential induction is not understood. Successful establishment by bacteria may require that these enzymes be sequentially

regulated. Higher basal levels and early induction of exo-PL suggest its role in releasing inducers of pectic enzymes. Unsaturated digalacturonates, products of exo-PL, are the most effective inducers among various monomers, dimers, and polymers (Collmer et al., 1982). Alternatively, separate and sequential induction may allow an omnivorous pathogen such as Ecc to mobilize expression of CDEs in a flexible manner to accomodate pathogenesis on a wide variety of hosts.

Activation of Plant Defense-related Genes. Expression of PAL and HMGR genes were monitored by mRNA levels and enzyme activity. Total RNA was isolated from Ecc-challenged tuber tissues in either direct-contact or membrane-separated systems. Levels of mRNA were determined by slot blot hybridization to labeled bean PAL5 cDNA (Yang et al., 1989) or tomato HMGR genomic sequences (Cramer et al., 1989). Induction by Ecc of PAL mRNA with subsequent enzyme activity was very rapid and transient. Induction of HMGR mRNA was much slower and more persistent and was not followed by enzyme activity in compatible reactions (Fig. 2B). The apparent discrepancy between HMGR mRNA accumulation and enzyme activity was in part due to enzyme inhibitors present in the rotting tissues and/or in Ecc cells (data not shown). PAL and HMGR are also activated by Ecc in the membrane-separated system (data not shown), suggesting that signals for PDR gene activation are diffusible through the polysulfone membrane. Induction of PAL and HMGR mRNAs by wounding alone was much weaker and slower (Fig. 2A and 2B).

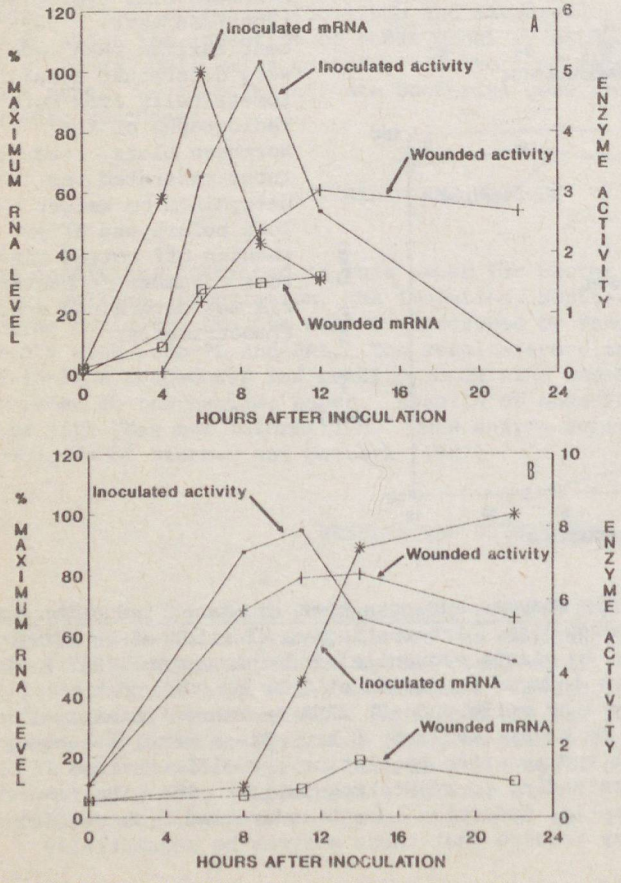

Fig. 2 Comparison of PAL and HMGR gene expression. Percent maximum PAL (A) and HMGR (B) mRNA levels were determined densitometrically from autoradiographs of slot blots (see text). PAL (A) and HMGR (microsomal fraction) (B) enzyme activities were determined according to Yang et al. (1989) and Stermer and Bostock (1987), respectively. PAL and HMGR enzyme activities are defined as nmol mevolonate/mg protein/hr and A_{290nm}/mg protein/hr.

These results demonstrate that Ecc triggers active plant defense responses. This is quite suprising since soft rot *Erwinia* have been considered to be opportunistic pathogens or saprophytes (Perombelon and Kelman, 1980). Activation of PAL and HMGR genes by Ecc suggests that these genes play an important role in soft rot resistance. This is consistent with the finding that large quantities of phenolics and sesquiterpenoid phytoalexins accumulate in tubers following *Erwinia* infection (Ghanekar et al., 1984). There is other circumstantial evidence that these PDR genes are involved in soft rot resistance. Induction of PAL is inhibited by hypoxic conditions which favor soft rot development (Vayda & Schaeffer, 1989). In our laboratory, tubers treated with arachidonic acid, an elicitor of HMGR expression, exhibit increased resistance to soft rot (data not shown). In addition, inhibitors of HMGR enzyme activity have been detected in Ecc and rotting tissues. Nevertheless, more studies are needed to clearly show that these genes indeed play an important role in soft rot resistance.

Differential Expression of HMGR isogenes. A 700 bp *Ava*I-*Eco*RI tomato genomic fragment containing the less conserved 5'-portion of tomato HMGR gene hybridized to RNA from Ecc-challenged tuber tissues but not to RNA from wounded tissues (Fig. 3). The same RNA from wounded tissues hybridized to a tomato genomic sequence (1.5 kb *Eco*RI-*Hind*III) that contains more conserved regions of HMGR genes. In addition to delayed induction, wounding induced a very rapid transient accumulation of HMGR mRNA. This may explain the observation that HMGR enzyme activity increases preceded mRNA increases in wounded tuber tissues (see Fig. 2B). These results show that Ecc activates a distinct HMGR isogene that is not induced by wounding.

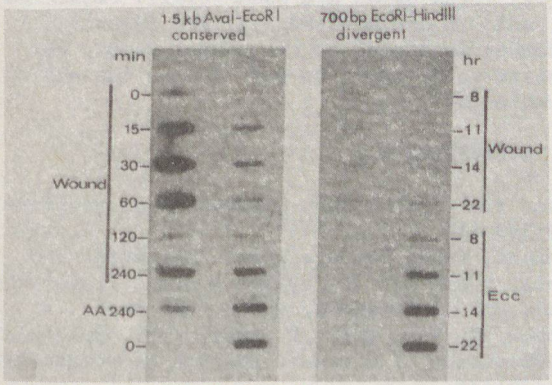

Fig. 3. Differential induction of HMGR mRNAs by wounding and Ecc inoculation. mRNA levels were determined by slot blot hybridization to a conserved region (left) or a divergent region (right) of HMGR DNA sequences cloned from tomato (Cramer et al., 1989). Times of treatment are indicated.

Signals for Plant Defense-related Gene Activation. Since *E. coli* does not induce plant defense responses (Yang et al., 1989), we use *E. coli* strains containing cloned Ecc genes to determine if a specific Ecc gene product(s) is responsible for activation of plant defense-related genes. Total RNA was isolated from tuber tissues inoculated with *E. coli* containing pDR1 or pDR30. Expression of the PAL gene was measured by slot blot hybridization. As shown in Fig. 4, PAL mRNA was induced by endo-PL encoded by pDR1 but not by exo-PL and/or endo-PG encoded by pDR30, suggesting that PAL is activated by some specific elicitor released by endo-PL. Davis et al. (1986) found that oligogalacturonides released from soybean cell wall by Ecc pectate lyase were able to elicit phytoalexin accumulation in soybean. The most active molecules were decagalacturonides. Degradation products of pectic

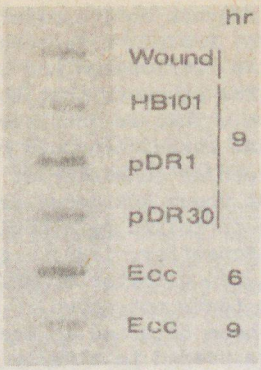

Fig. 4. Activation of PAL mRNA by pectic enzymes. PAL mRNA was detected by slot blot hybridization (see text). Tuber slices were inoculated with minimal medium plus glycerol, *E. coli* HB101, HB101 containing pDR1 (endo-PL) or pDR30 (endo-PG and exo-PL), or Ecc.

enzymes have also been proposed as inducers of these enzymes (Collmer et al., 1982). It appears that pectic enzyme inducers are different from phytoalexin elicitors since they are likely to be smaller oligomers that can be transported to bacterial cells (Collmer et al., 1982). Nonetheless, further studies are needed to understand the apparently conficting role of pectic enzymes as pathogenicity factors and defense response elicitors.

We have studied expression of both BPR and PDR genes in the soft rot interaction using an Ecc-potato system. Several important conclusions regarding mechanisms of bacterial soft rot interaction can be drawn from these studies: 1) Expression of Ecc BPR genes including endo-PL, endo-PG, and exo-PL is induced *in planta*; their induction kinetics are closely correlated with soft rot development. 2) These genes are sequentially regulated *in planta* in the order of exo-PL, endo-PL and endo-PG. 3) Ecc is able to trigger active plant defense responses monitored by two marker genes, PAL and HMGR. 4) Defense gene activation is at least in part due to the action of certain CDEs produced by the bacterium.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

IN PLANTA COMPETITION AMONG CELL-DEGRADING ENZYME MUTANTS AND WILDTYPE STRAINS OF ERWINIA CAROTOVORA

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For release of genetically-modified bacteria for agricultural applications, environmental impact may be predicted to some extent by comparing altered organisms with wildtype organisms in model habitats and non-habitats for survival, colonization, genetic exchange, and perturbation of community structure or function. In our system, impacts of partially-disarmed pathogens are compared with wildtype in soil and water (non-habitats) and in plant tissue (a natural habitat). In soil and aquatic microcosms, genetically-altered strains of *Erwinia carotovora* subsp. *carotovora* (Ecc) did not survive or affect, in the long-term, community structures or functional groups differently than the wildtype pathogen (Orvos, 1989; Scanferlato et al., 1989). However, Ecc does not colonize these habitats. To study colonization, we discuss here competition among a cell-degrading enzyme mutant and an antibiotic resistance-marked wildtype Ecc in potato tuber tissue.

MATERIALS AND METHODS

The Ecc strains used in these studies are listed in Table 1; they were maintained at 30°C on Plate Count Agar (PCA) or Trypticase Soy Broth (TSB) (both from Difco, Detroit, MI) with the appropriate antibiotic addition as necessary (30 µg/ml kanamycin or 150 µg/ml rifampin).

Table 1. *Erwinia carotovora* subsp. *carotovora* used in this work

Number	Remarks
L-543	EC14 isolated from calla lily (Roberts et al., 1986)
L-863	Spontaneous rifampin-resistant mutant of L-543
L-833	Low phosphate plasmid-cured EC14/pVS20, Kan^R (Scanferlato et al., 1989)
L-864	Spontaneous rifampin-resistant mutant of L-833
L-872	Low phosphate plasmid-cured EC14/pDRT4, pectate lyase-deficient, Kan^R (Roberts et al., 1986)

Plasmids containing altered genes for pectate lyase production were introduced into EC14 from *Escherichia coli* by triparental mating using plasmid pRK2013 as the helper plasmid (Allen et al., 1986). Several serial transfers using Torriani's (1960) low phosphate broth cured the plasmids and force recombination with the chromosome. The genetically engineered Ecc strains L-833 and L-872 were constructed in this manner.

Whole *Solanum tuberosum* cv. Russet Burbank tubers were purchased locally and prepared as described by Roberts et al. (1986). The tubers were held at least 24 hr at room temperature before any treatment including disinfesting.

Growth curves of L-543, L-863, and L-872 were performed in tubers or TSB plus the appropriate antibiotic. TSB optical densities were read at 550_{nm} on a Spectronic 20D colorimeter. Plate counts, for TSB or tubers, were determined on PCA amended with the appropriate antibiotic.

The inoculum was prepared from overnight broth (TSB plus appropriate antibiotic) cultures grown on a rotary shaker at 30°C. Optical densities were standardized to give about 3 X 10^5 CFU/ml using sterile water to make dilutions. Combinations of organisms were made. Ten inoculation sites were made in the top of each dried disinfested tuber with a sterile 200-1000 µl pipet tip. Fifty µl of inoculum were added into each wound. Two tubers were prepared for each treatment. The tubers were incubated hypoxically as described by Antonov et al. (1989).

One tuber of each pair was used to determine the numbers of bacteria present. A number 9 cork borer was used to take a tissue sample from three inoculation sites per tuber. Each sample was placed in a separate 17x100 mm polystyrene snap-cap tube and 1.0 ml water was added. The samples were vortexed vigorously, diluted serially, and plated separately in duplicate. Maceration was estimated by weight loss from the remaining tuber. The tuber was weighed and the inoculation sites were washed with a stream of water from a squirt bottle to remove macerated tissue. The tuber was inverted on paper towels placed over a finger rack and reweighed after air-drying to estimate the amount of tissue loss.

RESULTS AND DISCUSSION

Growth curves. In TSB no significant difference in growth curves was observed among Ecc strains L-543, L-863, and L-872. All three strains macerated tubers. *In planta* growth curves indicated that successful maceration is dependent on the original inoculum population (Fig. 1). At low inoculum levels ($\leq 10^3$ CFU) bacterial cells were bound tightly enough to the tuber to reduce plate counts. At 24 through 48 hr, maceration occured in ≤50% of the inoculation sites. At higher inoculum levels, no reduction in plate counts was observed presumably since the inoculum population greatly exceeded the binding capacity of the wound. At inoculum levels $\geq 10^6$ CFU, 100% of the inoculation sites macerated.

In planta competition. In tubers inoculated with two strains of Ecc some variation in results was observed (Fig. 2A and B). In one experiment where the initial inoculum levels were about 10^6 CFU/site, equivalent growth curves for L-863 and L-872 were observed (Fig 2A) and maceration occured at all inoculation sites. In another experiment, with lower inoculum levels (10^5 CFU/site), L-863 grew significantly more slowly than L-872 (Fig. 2B) and maceration did not occur in any inoculation site by 24 hr.

Because L-863 had been used as an antibiotic resistance-marked (Rif^r) "wildtype" strain in some release experiments (Scanferlato et al., 1989), we were interested in comparing its *in planta* growth to the parental strain L-543. In this experiment, initial inoculum levels of $\geq 10^5$ CFU/site were

used and maceration developed in all Ecc-inoculated sites. Strain L-543 produced 2-fold fewer cells (10^8 CFU/g) than the genetically-engineered strain L-872. This is not unexpected because although L-872 is deficient through chromosomal recombination with plasmid pDRT4 for production of one extracellular pectate lyase, the parental strain may produce enough pectate

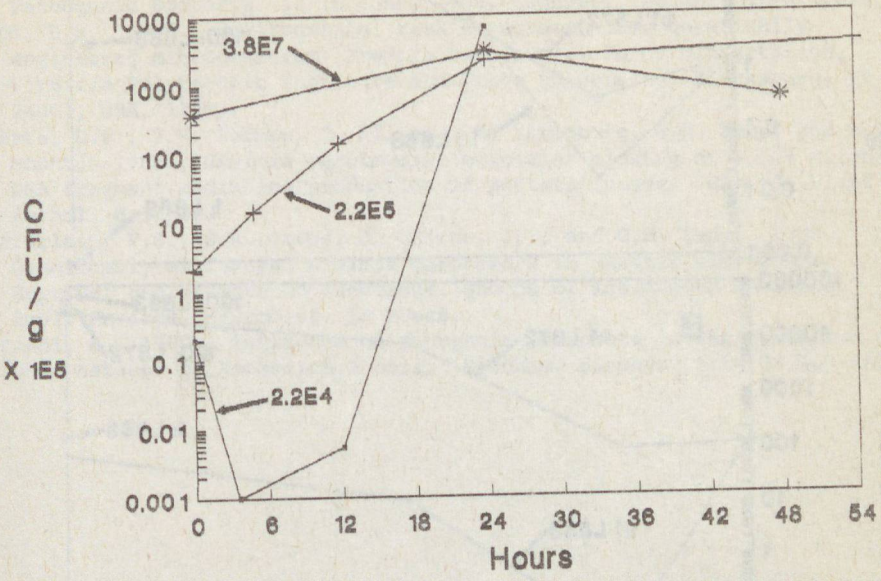

Fig. 1. Growth curves of *Erwinia carotovora* subsp. *carotovora* strain L-872 in tubers of *Solanum tuberosum* cv. Russett Burbank. Tubers were inoculated with 50 µl of TSB containing various populations of bacteria. Bacterial populations were assayed on PCA fortified with 30 µg/ml of kanamycin.

lyase to make up for any loss (Scanferlato et al., 1989). However, L-863 produced 10-fold fewer cells (10^7 CFU/g) than L-872 or L-543 from mixed paired inocula. We speculate that the *rif* lesion in L-863 may interfere with the RNA synthesis rate required for successful pathogenesis. Variable results may result from the interaction of the L-863 with different batches of tubers since we have a large variation in susceptibility to maceration among batches.

We conclude that although Rif^r mutants may be useful for survival studies in evaluation for planned releases, they may be less useful for colonization studies.

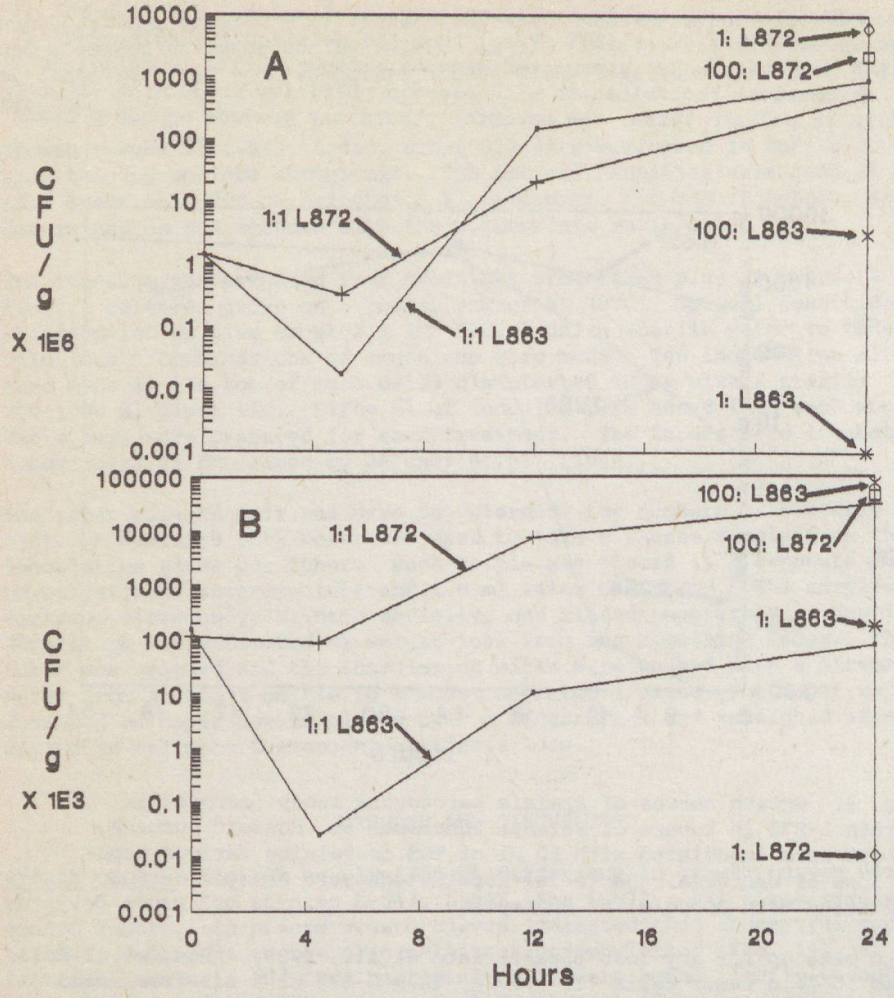

Fig. 2. Growth curves of *Erwinia carotovora* subsp. *carotovora* strain L-872 and L-863 in tubers of *Solanum tuberosum* cv. Russet Burbank. Tubers were inoculated with 50 µl of TSB containing inocula with approximately equal populations of bacterial strains in 1:1 mixtures. Single points with 100:1 and 1:100 levels are indicated at 24 hr. Bacterial populations were assayed on PCA fortified with 30 µg/ml of kanamycin or 150 µg/ml of rifampin. A and B represent the range of variation observed among experiments.

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

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INDUCTIONS OF PECTIN LYASE AND A SOS FUNCTION (UMUC PROTEIN) IN VARIOUS PLANTS

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Abstract

Using two types of gene fusions (umuC::lacZ and pnl::lacZ) in Erwinia carotovora subsp. carotovora, both UmuC protein and pectin lyase were found to be simultaneously induced by cultivation in the presence of mitomycin C and by inoculation into various plants. Thus, it was suggested that the production of pectin lyase is controlled as one of SOS functions and that the level of its activity is high enough to assist the degradation of pectin.

Introduction

Pectin lyase (E. C. 4.2.2.10, PNL) cleaves the pectic substance of high methoxyl content in a trans-eliminative manner, while pectate lyase (E. C. 4.2.2.2, PL) cleaves the one of low methoxyl content. Although either one of these pectinases has been shown to macerate plant tissues, neither one of them can digest the natural pectic substance in plants (ie. pectin) to smaller subunits (Tsuyumu & Ohiwa, 1987). However, the combination of these enzymes can lead to its complete digestion so that these pathogens may be able to obtain energy from it.

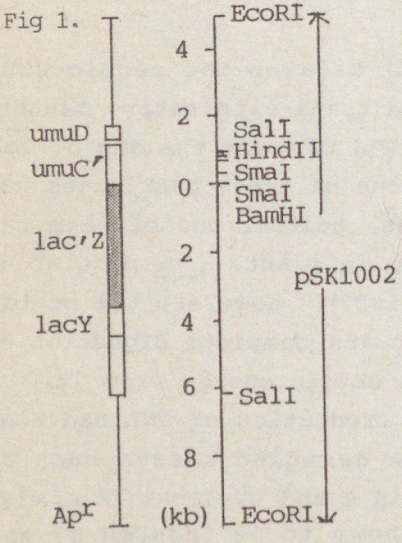
In soft-rotting Erwinia species, production of PNL had been shown to be inducible only by DNA damaging agents such as nalidixic acid, bleomycin, mitomycin C and UV light (Kamimiya et al, 1972), while PL had been shown to be induced by the metabolic product(s) of pectic substance (Tsuyumu, 1977). Due

to its unusual induction mechanism, the production of PNL by these bacteria has not been known until 1972. Thus, although the role of PL in the pathogenicity has been clearly demonstrated from both genetic (Chatterjee and Starr, 1977) and biochemical studies (Basham and Bateman, 1975), such a role of PNL has been remained to be studied. Previously, we have reported the presence of DNA-damaging agents in various plants and have suggested that these agents may be responsible for the induction of PNL at the infection sites of soft-rotting Erwinia (Tsuyumu et al). Our data confirmed this possibility and supported our hypothesis that PNL may be one of the SOS functions (Tsuyumu and Chatterjee, 1974).

Materials and Methods

E. carotovora subsp. carotovora strain HMgal9 (lacZ$^-$), of HMgal9(lacZ::pnl) and of HMgal9/pSK1002 (lacZ::umuC, a generous gift from Dr. Y. Oda)(Oda et al, 1984) were used. The gene product of umuC has been reported to be responsible directly for mutagenesis and to be regulated by the recA and lexA genes (Shinagawa et al, 1983). The origin of umuC is Escherichia coli and its location on pSK1002 is shown in Fig. 1 (Oda et al, 1984).

Fig 1.



To see the effect of addition of mitomycin C on the inductions of two fused genes , one ml of overnight culture of each bacterial strain in YP medium was inoculated into 5 ml of fresh YP medium with or without mitomycin C (2 μg/ml, final). After 5 hours incubation, the supernatants after centrifugation of the sonicated cell suspensions were

used for β-galactosidase assay. The activities of β-galactosidase were also assayed in macerated plant tissues. One hundred ul of overnight bacterial suspensions were placed onto the cut plants which were placed on the filter papers (absorbed 2 ml sterile water) in sterile petri dishes. The macerated tissues were collected into sterile micro-centrifuge tubes. They were centrifuged with or without sonication treatment, and the supernatants were used for β-galactosidase assay. The activity of β-galactosidase was assayed by measuring the appearance of the absorbance at 420 nm due to the cleavage of o-nitrophenyl-β-D-galactopyranosido.

Results and Discussions

Induction by mitomycin C. As shown in Table 1, after the growth in YP + MMC, all three bacterial strains were lysed (see the decrease in the absorbance at 600 nm), and the activities of β-galactosidase in both HMgal9/pSK1002 and HMgal9 (pnl::lacZ) were high considerably compared to the basal activities after the growth in the absence of MMC. Simultaneous inductions of PNL and the SOS function indicates that the induction of PNL is also controlled with lexA and recA.

Table 1. Effects of MMC on the induction of β-galactosidase.

Strains	MMC	OD_{600}	β-galactosidase*	
			Ext.	Total
HMgal9	-	0.502	-	-
	+	0.066	-	-
/pSK1002	-	0.525	0.30	1.45
	+	0.070	11.40	12.45
pnl::lacZ	-	0.500	0.25	0.20
	+	0.074	4.05	6.65

* Both extra-cellular (Ext.) and total activities of β-galactosidase were assayed. The activity of B-galactosidase was expressed by units/ml. One unit was defined as the activity required to increase the absorbance of 1.0 at 420 nm in one hour.

However, the efficiency of induction in pnl fusion was lower compared to the one in umuC-fusion. This difference may be partly due to the fact that umuC-fusion exists on the plasmid. For future quantitative comparison, this has to be marker-exchanged into umu operon on the chromosome of HMgal9. Also, it is noteworthy that activated recA protein of E. carotovora subsp. carotovora could control the expression of umu operon of Escherichia coli. This indicates that the regulatory system of SOS functions seems to be well conserved at least in these bacteria. It should also be pointed out that most of the fused proteins were found extra-cellularly probably due to the extensive cellular lysis.

Activities in macerated plant tissues. Comparing the induced level of B-galactosidase in the medium containing MMC, the activities in the saps of the tissues which were macerated by the inoculations with HMgal9/pSK1002 and with HMgal (pnl::lacZ) were considerably higher. Also, the activities in these saps were higher than those of the plants macerated with HMgal9 except in the cases of spinach and celery. The high activities of B-galactosidase in these plants may be due to the activity of B-galactosidase released from these plant cells by maceration or to the contaminations with β-galactosidase producing microorganisms. However, considering the data

Table 2. Activities of β-galactosidase in macerated plant tissues.

Plants	HMgal9	/pSK1002	pnl::lacZ
Potato	8.4*	80.8	50.3
Radish	1.1	16.1	4.6
Spinach	31.6	19.9	21.4
Lettuce	2.3	8.1	4.3
Celery	7.3	7.8	5.4
Carrot	2.0	12.9	5.5
Chin. cabbage	0.9	3.9	3.2

* Total activity of β-galactosidase was expressed as in Table 1.

that the specific activities of β-galactosidase increased after cultivation of these gene-fused strains in the presence of hot-water extracts of the plants including spinach and celery (to be published elsewhere), β-galactosidase at the macerated tissues may in fact be high even in these plants. Thus, these data seem to support our previous hypothesis that DNA damaging agents are widely distributed among plants and they are responsible for the induction of PNL (Tsuyumu et al). High PNL activity at the infection site should be possible at the degradation of pectin.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

HOMOLOGY BETWEEN DNA OF TEMPERATE AND VIRULENT PHAGES OF ERWINIA HERBICOLA

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ABSTRACT

Most of the strains of Erwinia herbicola pv. milletiae, a causal agent of bacterial gall of wistaria (Wistaria floribunda DC.)(Goto et al. 1980), were shown to be lysed by cultivation in the presence of mitomycin C. Small number of virulent phages were isolated from the concentrated lysates. These virulent phages lysed not only pv. milletiae strains but also some pathogenic and nonpathogenic E. herbicola strains which were isolated from other plants. DNA of a temperate phage showed homology with total DNA of pv. milletiae strains and of some other E. herbicola strains.

INTRODUCTION

Virulent phages are often isolated from the sites which are infected with plant pathogenic bacteria. Considering the immobile nature of phage, we have speculated that these virulent phages may be the mutants of the temperate phages in these bacteria. This possibility was tested in E. herbicola pv. milletiae as a model system.

MATERIALS AND METHODS

E. h. pv. milletiae were isolated from the gall of wistaria. Other pathogenic or non-pathogenic herbicola strains were isolated from various plants. Virulent phages were isolated

either from gall or from the concentrated lysate after the treatment with mitomycin C (MMC, 3.3 µg/ml, final). Each phage was purified by several single plaque isolations. Purification of the lytic agent from the MMC-lysate was performed by sedimentation with polyethylene glycol-NaCl, DNase treatment, followed by ultracentrifugation in cesium chloride as in the purification of Rhambda phage (Maniatis et al. 1982). DNA manupulations were also done following the procedures of Maniatis et al. DNA Dot hybridization on nylon membrane was performed according to the procedures in Amersham handbook.

RESULTS AND DISCUSSIONS

Cellular lysis by MMC. Since the optical densities of the cultures of six pv. milletiae strains dropped (data not shown), cellular lysis seems to be induced. Although these lysates did not lyse the strains from which they were obtained, some of them lysed other strains (Table 1).

Table 1. Lytic reactions of E. h. pv. milletiae strains to MMC-lysates

	Strains of pv. milletiae									
M-C Lysate	S1	S2	S3	S4	S5	S6	M1	M2	M3	M4
S1	–	–	–	–	–	–	–	–	–	–
S2	–	–	–	–	–	–	–	–	–	–
S3	–	–	–	–	–	–	–	–	–	–
S4	–	–	–	–	–	–	–	–	–	–
S5	–	–	–	–	–	–	–	–	–	–
S6	–	–	–	–	–	–	–	–	–	–
M1	+	+	+	+	+	+	–	+	+	+
M2	–	–	–	–	–	–	+d	–	–	–
M3	+	+	+	+	+	+	–	–	–	–
M4	+	+	+	+	+	+	–	+d	–	–

Symbols: +, clear lysis; +d, turbid lysis; -, no lysis.

When the lysates were precipitated with polyethylene glycol-NaCl and placed onto the layer of the same bacterial strains from which the lysates were obtained. Few small but clear plaques were often observed in the spotted area. They were picked and were purified by several single plaque isolations. These virulent-like phages isolated *in vitro* and the phages isolated from the galls lysed all the tested pv. *milletiae* strains (Table 2) and some of the other *E. herbicola* strains from different plants (Table 3). However, they did not lyse *E. chrysanthemi*, *E. carotovora* subsp. *carotovora*, *E. rhapontici*, *Agrobacterium tumefasciens*, *A. rhizogenes* and *Escherichia coli* at all (data not shown). Virulent phages obtained from the lysates of other groups of *E. herbicola* lysed all the strains of pv. *milletiae* (Table 4).

Table 2. Host range of virulent phages isolated from pv. *milletiae* strains

	Virulent phage					
Strain	EMSV-S1	EMSV-S2	EMSV-S4	EMSV-S5	EMSV-M1	EMSV-M3
S1	+[1]	+	+	+	+	+
S2	+	+	+	+	+	+
S3	+	+	+	+	+	+
S4	+	+	+	+	+	+
S5	+	+	+	+	+	+
S6	+	+	+	+	+	+
M1	+	+	+	+	+	+
M2	+	+	+	+	+	+
M3	+	+	+	+	+	+
M4	+	+	+	+	+	+

Virulent phages EMSV-S1 and EMSV-M3 were isolated from the gall infected with strain M3, while the others were isolated from *in vitro* cultures of corresponding pv. *milletiae* strains.

Table 3. Lytic reactions of other E. herbicola strains to the virulent phages isolated from pv. milletiae

Strain	Virulent Phages					
	EMSV-S1	EMSV-S2	EMSV-S4	EMSV-S5	EMSV-M1	EMSV-M3
CEh801	+	+	+	+	+	+
CycEh 1	+d	+	+d	+d	+d	+d
ZEh1-2	−	−	−	−	−	−
ZEh 5	−	−	−	−	−	−
ONEh6-2a	−	−	−	−	−	−
ONEh6-2b	+d	+d	+d	+d	+d	+d
ONEh7-1	−	−	−	−	−	−
ONEh 3	−	−	−	−	−	−
OKC 3S0	+	+	+	+	+	+
OKEh 4	−	−	−	−	−	−
KuwaEh	+	+	+	+	+	+
PNEh4-1	+	+	+	+	+	+
CANAEh	+d	+d	+d	+d	+d	+d
FIGEh 6	+d	+d	+d	+d	+d	+d
Clover8103	−	−	−	−	−	−
Mary 2	−	−	−	−	−	−
Chry 19	+	+d	−	−	+d	+
CornEh 1	−	−	−	−	−	−
CarEh17	−	−	−	−	−	−
GVEh 4	−	−	−	−	−	−

Table 4. Lytic reactions of pv. milletiae by virulent phages isolated from other E. herbicola

Virulent Phages	pv. milletiae strains									
	S1	S2	S3	S4	S5	S6	M1	M2	M3	M4
EHV-C	+	+	+	+	+	+	+	+	+	+
EHV-F	+	+d	+d	+	+d	+d	+d	+	+d	+d
EHV-O	+	+	+	+	+	+d	+d	+	+	+d

EHV-C, EHV-F and EHV-O were isolated in vitro from the strains of Clover8103, FIGEh6 and OKC380, respectively.

These results suggest that all of the tested strains of pv. milletiae and of some pathogenic and non-pathogenic E. herbicola strains may be lysogenic with similar temperate phages which are distinguishable in terms of their immunity. In fact, it was shown by DNA dot hybridization that the DNA of a temperate phage from pv. milletiae strain M1 showed homology with total DNA of all strains of pv. milletiae and of the above mentioned strains of E. herbicola but not of other pathogens including soft-rotting Erwinia (Fig. 1).

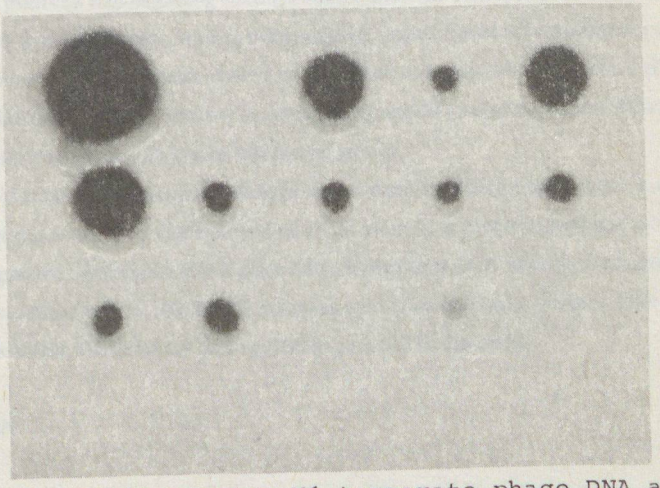

Fig. 1 DNA dot blotting using M1 temperate phage DNA as probe

Top row: (from left to right) DNA of M1 temperate phage (control), Ech12 (a temperate phage of E.chrysanthemi, D12), pv. milletiae M1, M2 and M3

2nd row: DNA of pv milletiae M4, S1, S5, S6 and E.herbicola, FIGEh-6

3rd row: DNA of E.herbicola, OKC380, Clover8103; E.carotovora subsp. carotovora, EC1; E.chrysanthemi, EC16, EC183

4th row: DNA of E.chrysantemi, ALE829P; E.carotovora, T29; E.chrysanthemi, D7; Xanthomonas campestris pv. citri 86, 87

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

Plant Pathogenic Bacteria
Z. Klement (ed.)

PECTINOLYSIS REGULATION IN ERWINIA CHRYSANTHEMI

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Erwinia chrysanthemi is a phytopathogenic bacterium that incites soft rot disease of many plant species. It secretes a group of plant cell wall-degrading enzymes that macerate the colonized plant tissues and provide a nutritional base for the invading bacteria. Among these enzymes, pectate lyases are considered central to pathogenesis by *E. chrysanthemi*. Synthesis of these pectate lyases is inducible in the presence of polygalacturonate, galacturonate and is sensitive to catabolite repression exerted by glucose. Furthermore, pectate lyases are synthesized during the end of exponential growth.

Various mutations affecting pectate lyase synthesis were isolated by different approaches. Among them, insertions in *kdgR* gave a constitutive expression of all genes involved in pectin degradation. Insertions in *pecS* provoked a depressed expression of *pel* genes only. Mutations in *pecA* strongly decreased pectate lyase synthesis and insertions in *pecI* affected the induction ratio of pectate lyase synthesis. Other regulatory mutations are less identified. The nature of each regulatory gene will be discussed.

INTRODUCTION

The pathogenicity of *Erwinia chrysanthemi* is chiefly due to the action of pectate lyases, which allow the bacteria to degrade plant cell walls (Chatterjee and Starr, 1980). *Erwinia chrysanthemi* strains (particularly the strains used in this study: 3937 and B374), contain five independent genes encoding pectate lyases *pelA*, *pelB*, *pelC*, *pelD* and *pelE*. These genes are distributed into two clusters on the bacterial chromosome, *pelB*, *pelC* and *pelD*, *pelE*, *pelA* (Fig .1). Synthesis of pectate lyases is inducible in the presence of polygalacturonate (PGA) or galacturonate. However, PL induction is not by these compounds themselves, but by their catabolic products. The true inducer produced from galacturonate is 2-keto-3-deoxygluconate (KDG) (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1987), the inducers produced from PGA are KDG and 2-5-diketo-3-deoxygluconate (DKII) or DKI (Condemine et al. 1986). In addition, PL synthesis is sensitive to catabolite repression, and is also regulated by growth phase (Hugouvieux-Cotte-Pattat et al. 1986). Finally, PL synthesis is dependent on oxygen pressure (PO_2) and temperature (M. Perombelon, pers. comm.). For a better understanding of PL regulation in *Erwinia chrysanthemi*, we isolated various mutants altered for PL production.

RESULTS

Isolation of mutants showing depressed PL synthesis

Two main approaches were used to select constitutive mutants: the first one was to detect clones producing PL activity after growth in absence of inducers, the second approach took advantage of the properties of *lacZ* gene fusions. Regulatory mutants synthesizing ß-galactosidase at a higher level than that of the parental fusion strain were isolated by growth on lactose minimal medium.

The selected mutants (*kdgR, pecS, gpi, cri*) produced PL at a higher level than the wild type strain in the absence of inducer (Table 1). However, they showed various depressed levels and different responses to the presence of inducer or glucose in the culture medium. Theses mutants were also analyzed for expression of other genes involved in pectin degradation and for genes independent of pectinolysis such as genes encoding proteases or cellulases (Table 1). Briefly, depressed mutants fell into four classes based on their phenotypic pattern (Table 1): the *kdgR, pecS, gpi* and *cri* mutants.

(a) The *kdgR* negative regulator gene

Using the two approaches described above, various *kdgR* mutants were found after chemical mutagenesis or insertion mutagenesis (Tn5 or Mu*lac* insertions) both in B374 and 3937 strains (Hugouvieux-Cotte-Pattat et al. 1986, Condemine and Robert-Baudouy 1987) . Moreover, *kdgR* mutations could be obtained spontaneously by selection of clones able to grow on KDG as sole carbon source, due to the derepression of the *kdgT* encoded transport system. The *kdgR* mutations led to a constitutive expression of all the genes involved in pectinolysis: *pem, pelA-E, ogl, kduD, kduI, kdgT, kdgA* and *kdgK*. In the *kdgR* mutants, pectate lyases are expressed at a high level but a higher level can be obtained when inducer (KDG or DKII) accumulated, such as in *kdgK* or *kduD* mutants. This phenomenon of "superinduction" of pectate lyase indicates that other regulatory genes are involved in pectate lyase regulation. Electrofocusing of pectate lyase revealed that PLa, PLd and PLe synthesis was more increased in *kdgR* mutants than PLb and PLc. The *kdgR* gene was located on the chromosomal map of *E. chrysanthemi* B374 or 3937 near the *trp* marker (Fig.1). In vivo cloning of the *ogl* gene of strain 3937, using the RP4::miniMu system, allowed us to select an R' plasmid, pROU2, which also carried the genes *kdgR* and *kduD* (Reverchon and Robert-Baudouy 1987).Complementation of the *kdgR* mutations by the *kdgR* wild-type allele present on the plasmid in addition to the fact that genes involved in pectinolysis are expressed constitutively after Tn*5* or Mu*lac* insertion demonstrated that *kdgR* acts as a negative regulator. Comparison between the 5' end nucleotide sequences of three genes regulated by *kdgR (pelE, kdgT, ogl)* revealed the existence of a highly conserved sequence which could correspond to a KdgR-binding site (Reverchon et al. 1989). Binding of a repressor to such a site would prevent transcription by steric hindrance.

(b) The *pecS* regulatory gene

Two *pecS* mutants were obtained by Mu*lac* insertions in strain 3937. In these mutants, pectate lyase

TABLE 1: Characterization of *E. chrysanthemi* mutants affected for PL synthesis

Class of mutation	Designation of prototype strain	Nature of mutation	Derivative from	Growth conditions C. source-inducer	PL activity (a)		KDGDH activity	PME activity	cellulase activity	protease activity
wild-type	3937	-	-	Glycerol-None Glycerol-PGA Glucose-PGA	0.03 1.40 0.65	(8)	18 34	- ++	+	+
wild-type	B374	-	-	Glycerol-None Glycerol-PGA Glucose-PGA	0.12 1.02 0.45	(7)	15 32	- ++	+	+
kdgR	A902	Tn5 insertion	3937	Glycerol-None Glycerol-PGA Glucose-PGA	1.56 1.66 0.50	(2)	1425 1840	+++ +++	+	+
pecS	A1524	Mu insertion	3937	Glycerol-None Glycerol-PGA Glucose-PGA	1.74 4.60 0.68	(5)	20 37	+ +++	+	+ -
gpi	P45	NTG	B374	Glycerol-None Glycerol-PGA Glucose-PGA	1.16 1.10 0.56	(1)	15 28	++ ++	+++	+
cri	P57	NTG	B374	Glycerol-None Glycerol-PGA Glucose-PGA	0.47 3.21 1.73	(6)	17 35	+ +++	+++	+++
pecA	A1294	spontaneous	B374	Glycerol-None Glycerol-PGA Glucose-PGA	0.02 0.06 0.01					
pecL	A1125	Mu insertion	3937	Glycerol-None Glycerol-PGA Glucose-PGA	0.11 0.20 0.05			- +		
pecI	A1114	Mu insertion	3937	Glycerol-None Glycerol-PGA Glucose-PGA	0.18 22 0.9			- +++++		

Specific activities were expressed in µmol of product formed per min per mg of dry bacterial weight for PL, in nmol of product formed per min per mg of dry bacterial weight for KDGDH (*kduD* gene product). Expression of KDGDH is representative of that of all enzymes involved in pectinolysis except PL and pectin methyl esterase (PME) synthesis. Cellulase, protease and PME activities were determined by a semi quantitative method. (a): PL specific activity in stationary phase / PL specific activity in exponential phase.

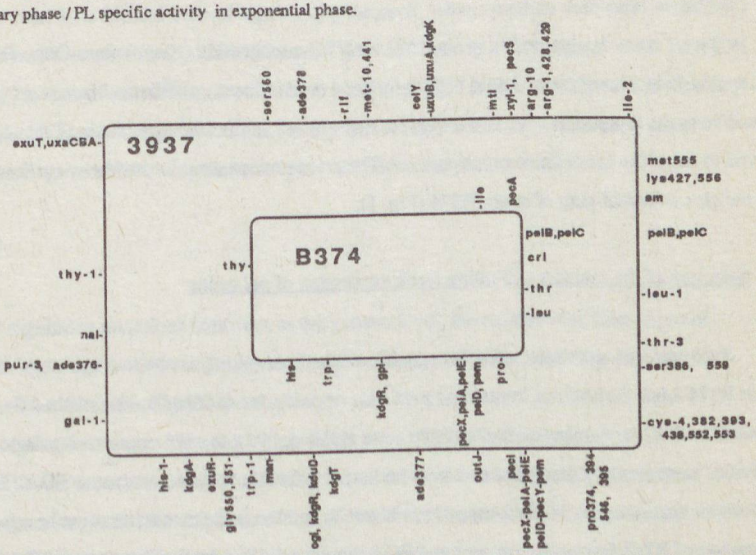

Fig.1. Genetic linkage maps of the *Erwinia chrysanthemi* 3937 and B374. The distance between any two neighbouring markers on the genetic maps reflects the cotransfer frequencies found by RP4::miniMu transfer. Maps of 3937 and B374 were from Hugouvieux-Cotte-Pattat et al. (1989) and from Schoonejans and Toussaint (1983) respectively.

synthesis was derepressed but remained inducible by PGA (Table 1). In contrast to *kdgR*, *pecS* mutations did not affect expression of other genes of pectinolysis. Electrofocusing of pectate lyase isoenzymes revealed that PLb and PLc synthesis strongly increased in *pecS* mutants whereas PLa, PLd and PLe were not or weakly affected by this mutation. This new type of mutations has been located close to the *xyl* marker (Fig. 1) and so appeared different from *kdgR* mutations. Since inactivation of *pecS* by insertion caused a constitutive PL synthesis, we believed that such mutations defined a second negative regulatory gene whose product specifically repress *pel* genes.

(c) The *gpi* mutation

The *gpi* mutants were obtained by NTG mutagenesis in strain B374 (Hugouvieux-Cotte-Pattat et al. 1986). In these mutants, the same level of PL was found in the presence or absence of an inducer showing a constitutive expression of *pel* genes (Table 1). Moreover, *gpi* mutations led to a synthesis of PL and cellulase independent of the growth phase. This probably results from the loss of the control of mechanism regulating the temporal activation of PL and cellulases, whose synthesis is coincident during growth. Electrofocusing revealed that three PL isoenzymes, PLa, PLd, PLe were strongly affected by the *gpi* mutations. The *gpi* mutations were located near the *trp* marker on the B374 chromosome and thus were approximatively at the same locus as *kdgR* mutations. Although *kdgR* and *gpi* mutations have similar effects on *pel* expression and are situated near the same locus, they do not possess the same action on other genes involved in pectinolysis. *kdgR* controls all pectinolysis genes whereas *gpi* action is restricted only to *pel* genes. However, we cannot exclude the possibility that they are two different mutated alleles of the same regulatory gene.

(d) The *cri* mutations

In the *cri* mutants obtained in strain B374 by NTG mutagenesis (Hugouvieux-Cotte-Pattat et al. 1986), PL synthesis increased about 3-fold both in induced or uninduced conditions. Moreover PL synthesis was less sensitive to the repression observed in presence of glucose in the wild-type strain (Table 1). So the *cri* gene seems to act at the catabolite repression level. The *cri* mutations were located between the *thr* and *ile* markers on the chromosomal map of strain B374 (Fig. 1).

Selection of the mutants exhibiting weak expression of *pel* genes

Such mutants were selected using specific method permitting to obtain a low expression of *lac* genes from B374 *kdgK* derivatives, containing *pelE::lac or pelD::lac* fusions. By this method five spontaneous mutants with a low ß-galactosidase activity were isolated, in these *pecA* mutants ß-galactosidase and PL activities were strongly decreased and were no longer inducible by galacturonate or PGA (Table 1). The hypothesis that inducers of PL synthesis could not be formed in these mutants must be rejected since formation of KDG from galacturonate and PGA involves distinct catabolic pathways. The existence of such mutants may be explained by alteration of positive regulatory genes governing the expression of all *pel* genes. The *pecA* mutations were found to cotransfer with the *ile* marker of B374.

Regulatory mutants with decreased PL synthesis were also obtained from strain 3937 by screening for Mu*lac* insertions induced in the presence of PGA (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1989). These mutants were called *pecL*, they showed a phenotype very similar to *pecA* mutants. Chromosomal location of these insertions is currently under investigation to determine if *pecA* and *pecL* are different or similar genes. The fact that Mu insertions could provoke a decrease in PL synthesis suggests that positive regulation of *pel* genes is superposed to the negative regulation mediated by *kdgR* and *pecS* genes.

Characterization of the *pecI* mutant altered in induction of pectinolytic enzymes.

Among PGA-induced Mu*lac* fusions obtained in *E. chrysanthemi* strain 3937 (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1989), one mutant named *pecI* showed a very high induction of both PL and pectin methylesterase (PME), in presence of PGA, with no or very little alteration of the uninduced levels of these enzymes (Table 1). Chromosomal mapping of this mutation showed that *pecI* was located near the *pem-pelDEA* cluster.

The genes *pecX* and *pecY* adjacent to the *pem* and *pelD,E,A* genes.

The genetic organization of the *pem-pelD, E, A* region in the two *E. chrysanthemi* strains B374 and 3937 has been analyzed by both subcloning procedures and insertion mutagenesis with miniMu*lac* phage (Reverchon et al. 1986). Insertions resulting in a strong decrease in PLa synthesis were found in a locus adjacent to *pelA*, these insertions defined a novel regulatory gene *pecX* whose product seems to specifically activate *pelA* expression (Reverchon et al., 1986). The *pecX* gene is currently being sequenced (S. Favey, pers. comm.). In the same chromosomal fragment of *E. chrysanthemi* 3937 a gene called *pecY* was found between the *pem* and *pelD* genes. This gene could act as a negative regulator of *pem* expression since a miniMu insertion in *pecY* gave a higher expression of *pem* (M. Boccara, pers. comm.).

CONCLUSIONS

The isolation of regulatory mutations confirms, at the genetic level, the existence of three types of regulation affecting PL synthesis which were suggested by physiological studies (catabolite repression, growth phase dependence and induction by PGA derivatives).

In strain B374, regulatory mutants affecting PL synthesis were obtained either spontaneously or after chemical mutagenesis. It was therefore difficult to identify the nature of the mutated genes. However, localization of the mutations revealed the existence of four different loci involved in PL regulation: (i) *cri* near the *thr* marker, (ii) *kdgR* and *gpiR* near *trp*, (iii) *pecA* near *ile* and (iv) *pecX* near the *pelA, D, E* cluster.

In strain 3937, insertions in six genes affecting pectinolysis regulation were obtained: (i) *kdgR* which defines a negative regulatory gene controlling expression of all genes involved in pectin degradation . (ii) *pecS* which defines a second negative regulatory gene controlling only *pel* genes. (iii) *pecL* insertions which strongly decrease PL synthesis and suggest the existence of a positive regulation controlling expression of all *pel* genes. (iv) *pecI* insertions which increase the induction ratio of *pel* and *pem* genes. (v) *pecX* insertions which define a specific positive regulation for *pelA* gene. (vi) Finally *pecY* insertions which define another negative regulatory gene affecting specifically *pem* expression (M. Boccara, pers. comm.). For strain 3937, these different insertions define three distinct loci involved in pectinolysis regulation: (i) *kdgR* near *trp*, (ii) *pecS* near *xyl* (iii) *pecI*, *pecX* and *pecY* near the *pem-pelD, E, A* cluster. *pecL* insertions remain to be located (Fig.1). In addition, expression of *pecI* and *pecL* are themselves induced in presence of PGA (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1989). There is therefore a complex regulatory circuit with various regulatory genes, the expression of which can vary, regulatory genes probably activating or repressing each other.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

ECOLOGY AND PATHOGENICITY OF SOFT ROT ERWINIAS: AN OVERVIEW

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INTRODUCTION

Soft rot erwinias comprise species belonging to the genus Erwinia which produce large quantities of extracellular pectic enzymes and rot plant tissue. They include E. carotovora, E. chrysanthemi, E. rhapontici, E. cypripedii and probably also the recently established species, E. ananas (Lelliott & Dickey, 1984). E. carotovora is further subdivided into E. carotovora subsp carotovora (Ecc), E. carotovora subsp atroseptica (Eca) and E. carotovora subsp betavasculorum (Lelliott & Dickey, 1984) and some workers recognise six E. chrysanthemi (Echr) pathovars (Dye et al., 1980). E. rhapontici, E. cypripedii and E. ananas are not pectolytic in vitro but since they can rot plant tissues they are listed as soft rot bacteria. In contrast, although E. salicis and E. rubrifaciens are pectolytic, they do not cause rotting in their natural or in other hosts and therefore are not considered as soft rot erwinias.

Only Ecc, Eca and Echr of the soft rot erwinias have been extensively studied because of their economic importance as pathogens of several crops of which potato is the most important. This overview will only discuss those three bacteria with particular reference to potatoes. Since their ecology has recently been reviewed (Pérombelon & Kelman, 1980; Pérombelon, 1988), emphasis will be placed on pathogenicity which will be considered in ecological terms assuming that soft rot erwinias are essentially opportunistic pathogens.

SYMPTOMS

Although infection by soft rot erwinias usually results in maceration and rotting especially of parenchymatous tissue of storage organs (tubers),

symptoms on the growing plant are diverse. They range from soft rot of the stem and leaves to stunting, wilting and desiccation of the plant with vascular browning which can also occur in different combinations and variations. Environmental conditions, especially humidity, determine the type of symptoms; a soft rot tends to occur under wet conditions while wilting and desiccation predominates under dry conditions (Pérombelon & Kelman, 1987).

ECOLOGY

Distribution

The three soft rot erwinias (Ecc, Eca and Echr) have a worldwide distribution but with contrasting host ranges which tend to be reflected in their temperature characteristics and geographical distribution (Pérombelon & Kelman, 1980). Host specificity is not well developed and the reported limited degree found mostly under field conditions can be attributed more to their geo-climatic distribution and that of their hosts than to an intrinsic genetically-based character. It is notable that the number of different crops suffering from frequent and severe attacks is markedly lower than the actual number of host species that can become infected. Ecc strains are widely distributed in both the temperate and tropical zones and are pathogenic to a wide range of plants. In contrast, Echr is pathogenic to a wide range of plants in tropical and subtropical regions and is also found in certain glasshouse crops grown in temperate regions as well as in a few field-grown crops such as maize, dahlia etc. The host range of Eca is restricted mostly to potatoes which have until recently been grown in temperate regions. Its occasional detection on other crops probably indicates chance contamination from sources associated with potatoes.

Sources, survival and spread

Several sources of the bacteria, in addition to infected plants and seed, have been identified and their relative importance evaluated. In temperate regions, Ecc and Eca and to a lesser extent Echr, are widely distributed in the environment. They can be airborne on insects which have been in contact with rotting vegetable matter or in aerosols produced by rain impaction on diseased plants, contaminated surface water and when potato haulm is pulverised prior to harvest (Pérombelon & Kelman, 1980).

The predominant subspecies is Ecc and they can survive and multiply after deposition on leaves when the surface is wet. However, high populations develop only when the leaves senesce and rot on the soil surface from where they can be washed into the soil.

The capacity of erwinias to survive in soil has always been a controversial subject. In vitro work in unsterile soil suggests that longevity of Ecc and Eca is more temperature than moisture dependent and is restricted to only a few days at >20°C (Pérombelon & Hyman, 1989). Plant pathogenic erwinias are adapted to grow in nutrient rich plant tissues and may not be able to adapt their endogenous metabolism to allow them to compete successfully in a relatively nutrient poor soil environment especially at high temperatures. However, the repeated isolation of low numbers of erwinias from field soil (McCarter-Zorner et al, 1985; Pérombelon & Hyman, 1989), suggests that they have some survival ability but they are repeatedly introduced from a wide range of sources. The longevity of Ecc tends to be greater than that of Eca, especially in rhizosphere soil.

Most streams and rivers flowing through arable land in both temperate and tropical regions are contaminated with up to 10^6 cells/l erwinias at (McCarter-Zorner et al., 1984; Pérombelon & Hyman, 1987). The bacteria are believed to have an allochthonous origin being washed in from surrounding land, in drains and run off water.

The most important sources of inoculum are contaminated potato seed tubers and farm machinery (Pérombelon & Elphinstone, 1986). When seed tubers rot, the bacteria are liberated in large numbers in the soil and are transmitted to progeny tubers by soil water. Similarly, when crops are harvested and graded, wounds are unavoidable and they become infected by bacteria smeared on machinery by rotted tubers. Eca and Echr tend to predominate in this situation in contrast to contamination which originates from the other sources.

ETIOLOGY

The level of contamination of potato tubers by soft rot erwinias depends largely on environmental conditions and wet soil conditions favour survival, growth and transmission of the bacteria. However, although lenticels of most potato tubers and stems of healthy potato plants are often latently infected in large numbers with more than one erwinia type, the incidence of rotting in store and blackleg in the field are low

(Pérombelon & Kelman, 1980; Pérombelon et al., 1987). Apparently plant 'resistance' must be high enough to prevent multiplication of the bacteria to the critical level (10^7 cells/site) at which symptoms develop. Resistance breaks down and the bacteria can grow more freely and produce their cell wall dissolving enzymes to cause a rot when $p.O_2$ is lowered and host tissue water status (a_w) is high, in other words when the host is under stress.

The incidence of blackleg symptoms which is the extension of rotting of the mother tuber to the stem is related to the number of bacteria on the seed tuber (Pérombelon et al., 1989) and high soil moisture levels which favour early rotting of the seed tuber also favour blackleg expression.

PATHOGENICITY

Latent infection of potato by more than one erwinia subspecies is common and experiments have shown that temperature exerts a differential effect on the dominance of each form as disease develops. For example, Eca caused blackleg in the field only when mean weekly temperatures were <25°C whereas the opposite was true for Echr, even when both were present in equal numbers on the seed tubers (Perombelon et al., 1987). Similar results were obtained when tubers were inoculated with equal numbers of Ecc and Eca and Ecc and Echr except that Ecc was found to be not pathogenic at both low and high temperatures.

The influence of temperature on pathogenicity may be due to an effect on pectate lyase (PL), which is an important pathogenicity determinant. PL production of Eca was four times greater at 27° than at 30°C although total protein production and cell growth were comparable at both temperatures (Lanham & Pérombelon, 1989).

Other pathogenicity determinants may be implicated and recent molecular work has revealed a role for siderophores in Fe uptake (Expert & Toussaint, 1985), LPS (host recognition or enzyme secretion) (Toth et al., 1989) and certain unidentified genes which are expressed only by the host tissue (F. Van Gijsegem, personal communication). However, it is not known to what extent these characters are specific to erwinias and not also present in non-pathogenic bacteria. Many saprophytic bacteria, eg. Bacillus spp. Clostridium spp. Pseudomonas spp., associated with potatoes are pectolytic and can rot plant tissues in vitro but are not considered to be potato pathogens. They can grow as well as erwinias when anaerobic conditions have reduced tissue resistance but do not cause disease in the

field. It is possible that ecological factors such as temperature and $p.O_2$ tend to favour the preferential growth and pectic enzyme production of the erwinias which could be viewed therefore, as opportunistic pathogens in the case of potatoes.

The hypothesis implies that competition modulated by temperature determines whether an erwinia will predominate and cause disease. Although its survival in nutrient poor and microbiologically rich environments as soil is more restricted than that of Ecc, Eca outgrows and Echr in potato tissue at low temperatures (Pérombelon et al., 1979; Pérombelon, unpublished data) because of its greater pectic enzymes production and growth rate. The reverse is true at high temperatures. The same argument can be extended to explain why tuber rots in hot countries are caused primarily by bacteria which can grow well and produ pectic enzymes at high temperatures (>30°C), Bacillus spp., Clostridium spp. (Lund, 1979). Conversely, the failure of Ecc to cause blackleg in several hot countries such as Israel and Peru may be explained by its failure to compete with other pectolytic bacteria in the rotting mother tubers. Ecc can cause blackleg when inoculated in mother tubers in the absence of competition in controlled environmnents. Failure to compete with saprophytic pectolytic bacteria could also explain why more, if not all, plants in the field grown from potato seed tubers heavily contaminated with Eca do not develop blackleg under favourable conditi (Pérombelon et al., 1989). Thus, the greater the initial number of Ecc present on the seed, the more likely that the bacterium will predomina when the mother tuber rots and cause the disease when large numbers in the stems. Similarly, competition by Ecc in mother tubers can affect numbers of Eca or Echr also present resulting in lower blackleg incide at temperatures favourable to the different erwinias (Pérombelon et al 1987).

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

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OCCURRENCE OF ERWINIA SPP. IN SWEDISH SURFACE WATER AND THE RISK OF TRANSMITTING DISEASE BY IRRIGATION

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In Sweden two types of soft rot symptoms can be seen on the potato haulm a) **blackleg**, the basal soft rot that originates from the mother tuber and b) **stem rot**, a local soft rot that is separated from the stem base. Blackleg has, in Sweden been identified as **Erwinia carotovora** subsp. **atroseptica (Eca)** and from the stem rots both **Eca** and **E. carotovora** subsp. **carotovora (Ecc)** have been isolated. To ascertain how common pectolytic **Erwinia** spp. are in water used for irrigating potatoes, ten samples from south and central Sweden were collected from streams, lakes and water reservoirs during July and August 1988. The samples were analysed immediately after collection and were plated on crystal violet pectate medium. Ecc was isolated in seven of the ten samples and in one stream Ecc was found together with **E. chrysanthemi (Echr)**, also found in the same stream 1976.

An irrigation experiment has been carried out in a growth cabinet in order to estimate the risk for progeny tubers to be infected after being exposed to contaminated water. Meristem based, potted potato plants were, from the time of tuber formation, irrigated with artificially Eca- contaminated water of different concentrations. The plants were irrigated with contaminated water six times with ten days interval. After haulm killing the progeny tubers were harvested and no visible soft

rots were observed. Half of the tubers from each treatment were analysed directly for latent Eca- infections with specific antiserum using ELISA. The other half were analysed after six months storage. The results show that tubers irrigated with the highest concentration of bacteria were latent infected with Eca. A similar irrigation experiment has been carried out in the greenhouse with tomato plants and one of the Echr-isolates found 1988. Tomato plants with wounded and unwounded roots were irrigated with Echr- contaminated water. The results show that nearly all tomato plants became infected with Echr and that wounding of roots was not necessary to get infection.

The results from the experiments indicates that irrigation water may be a source of transmitting soft rot Erwinia spp. to healthy plants.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

INCIDENCE OF ERWINIA-CAUSING SOFT ROTS IN IRRIGATION WATER IN VALENCIA (SPAIN)

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The presence of Erwinia carotovora subsp. atroseptica (Eca) E. c. subsp. carotovora (Ecc) and E.chrysanthemi (Ech) was analysed in the irrigation water from downstream sites of the Turia river in Valencia (Spain). The most efficient detection was obtained by direct plating on CVP medium or anaerobic enrichment in selective medium and plating on CVP. Erwinia were isolated from all irrigation water samples and in water samples taken upstream the Turia river, about 200 km away from the intensive vegetable growing area of Valencia. Populations of erwinia ranged from 0.1 to 1000 cfu/ml with maximum concentrations detected in June and July. The Ecc was the predominant species isolated, but occasionally, Eca and Ech were also isolated. The bacteria were identified by standard bacteriological methods and by western blot of protein bands and their serological determination with antisera of Ecc and Eca. The serogroups of the strains isolated from water were compared with the serogroups which occurred frequently in the same area. The pathogenicity of the erwinia strains to different hosts was studied.

INTRODUCTION

The ecology of the erwinias causing soft rots has been extensively studied in recent years, in order to find the inoculum sources. Frequent presence of erwinias, partic-

ularly of Ecc, has been observed in sewers, irrigation ditches, streams, rivers, reservoirs, lakes, sea, rain and even snow from different zones (Pérombelon, 1981; McCarter-Zorner et al. , 1984, Olsson, 1984; Jorge and Harrison, 1986; Franc et al. , 1986a; Franc et al. , 1986b). In the intensive vegetable growing area of Valencia, frequent attacks of Ecc occur on horticultural and ornamental crops. This is an approach to study the presence and incidence of erwinias in water from irrigation ditches in that area and to evaluate the significance of this inoculum source.

MATERIALS AND METHODS

Twenty freshwater samples were analysed. Sixteen were collected in the horticultural area of Valencia from irrigation ditches from the Turia river. Four samples came from creeks close to the source of the Turia, located more than 200 km away from the area of intensive horticultural crops. Water samples were collected in sterile glass flasks, maintained at 4°C until processing within the following 24 h. Sample temperature was measured at the collection site, and pH was read in the laboratory after a previous filtering to eliminate gross elements.

Initially, a study of the efficacy of a number of erwinia detection methods was performed, using four water samples from different sites. Direct plating on King B medium (King et al. , 1954) and on the selective CVP media, erwinia selective (Cuppels and Kelman, 1974) was compared. Membrane filtration, 0.45 µ was made, plating the membrane on different media: Glucose-Tryptone (Sartorius), Standard TTC (Sartorius), Cetrimid (Sartorius), King B and CVP. The enrichment on D-PEM medium (Burr and Schroth, 1977) was also compared, followed by plating on CVP medium. In a second stage, the analyses of samples were conducted by directly plating 50 µl of the sample and of three dilutions of the water sample (1/10, 1/100 and 1/1000) on King B and CVP media. A parallel enrichment of 50 ml of water samples on

50 ml of D-PEM, incubated anaerobically for 48 h, was done. From this, 50 µl were subsequently plated on CVP.

Plates were incubated at 27°C and 48 and 72 h after, counting of colonies morphologically characteristics of Erwinia was made, followed by characterization. The purified isolates were biochemically characterized as Eca, Ecc, or Ech by the following tests: growth, at 37°C, acidification of lactose, maltose, α-methyl-glucoside and trehalose, production of sucrose-reducing substances, presence of phosphatase and sensitivity to erythromicin, according to the biochemical tests described by Cother and Sivasitham-param (1983). Some of the strains were selected for characterization by electrotransfer of protein bands from a polyacrylamide gel containing SDS to a hydrophobic polyvinylidene difluoride membrane (western blot) and their serological determination by indirect ELISA (immunoblotting) using antisera of Eca or Ecc, according to the method of Alarcón et al. (submitted to the J. Appl. Bacteriol.). By double immunodiffusion, 43 strains representative of different samples were analysed, with two antisera produced from two strains of serogroups I and III of De Boer et al. (1979), according to the procedure described by Allan and Kelman (1977). Inoculations of 60 representative strains, in potato slices, carrot slices, and lettuce leaves, were made with approximately 10^9 cfu/ml. Inoculations were read 48 h, and 7 days later.

RESULTS AND DISCUSSION

Initial comparison of efficiency of different methods in detecting erwinias in irrigation water showed that the best results were obtained with direct plating on CVP medium, which allowed Ecc isolation from three of the four samples analysed, and Eca isolation from one sample. With D-PEM enrichment, Ecc was also isolated in the three samples, but Eca was not. By direct plating on King B medium, or with membrane filtration and plating on various media, it was not

possible to isolate erwinias in any case. The results of the analyses of erwinia incidence in 20 water samples shows that presence of erwinias was universal, both in ditch irrigation water, and in water from the Turia river, near its source. Ecc population ranged from 1 and 1000 cel/ml. Isolates with biochemical characteristics of Eca but growing at 37°C, and Ech were also isolated together with Ecc in two different samples.

A one year quantitative study on the evolution of erwinias population in irrigation water from a ditch in Valencia, showed that Ecc was isolated in all the samples analysed since November until October of the following year. Ecc populations ranged from 800 to 1000 cfu/ml in June and July, and 80 cel/ml in September. In addition, "Eca" type but growing at 37°C, (10 cfu/ml) was isolated in November. Biochemical characterization of some of the purified erwinia cultures from the water samples, showed that 185 were classified as Ecc, 8 as "Eca," and 29 as Ech. Characterization of protein bands by western blot, and subsequent indirect ELISA showed that 15 Ecc strains studied could be classified as the reference strain 194. 43 Ecc and "Eca" type strains isolated from water could not be classified into serogroup I, nor into III of De Boer (1979). The Eca strains commonly isolated from potato in the area, grew at 37°C and are from serogroup I, but not those isolated from water. Ecc isolates from the same area could neither be classified into serogroup I, nor into III which are the most frequent in other zones (De Boer et al. , 1979); however, serotyping of unclassified isolates will soon be performed. All of the isolates studied are able to cause soft rots on potato, and carrot slices and some of them on lettuce leaves. Studies have been initiated on the influence of erwinias in irrigation water on incidence of blanking and blackleg on potatoes grown under greenhouse and irrigated with water containing erwinias. The initial result show evidence of the influence of the irrigation water in potato blanking.

These results show a frequent presence of Ecc in waters from rivers and from irrigation ditches in the Valencian Community, which may reach high populations of this bacterium, even up to 1000 cell/ml, and occasional presence of Eca and Ech. The serological characteristics of the erwinia isolates from water are being studied. Our conclusions confirm that, like in the U.S.A., Scotland, or Sweden, (McCarter-Zorner et al. 1984; Olson, 1984) the water from rivers and from the numerous irrigation ditches existing in Valencia can become an inoculum source of erwinias particularly Ecc, for any crops, throughout the year.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

CHARACTERISTICS OF SOME ERWINIA SOFT ROT STRAINS FROM YUGOSLAVIA AND USA

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Strains of Erwinia carotovora (Ec) isolated from lettuce, paprika and cactus in Yugoslavia, belong primarily to E.c. subsp. carotovora (Ecc), based on physiological tests. However, strains of Ec isolated from sunflower in Yugoslavia and North Dakota and from sugar beet grown in North Dakota were variable and had biochemical and fatty acid characteristics similar to both Ecc and E.c. ssp. atroseptica (Eca).

INTRODUCTION

Strains of Erwinia carotovora which originated from different hosts, demonstrated many characteristics in common, but also, some differences. That was a base for separating two subspecies: E.c. subsp. carotovora and E.c. ssp. atroseptica. Erwinia carotovora ssp. betavasculorum was described later on Ec. ssp. wasabiae - a parasite of Japanese horseradish (Eutrema wasabi) (Goto and Matsumoto, 1987) quite recently.

During the studies of E. carotovora as a sunflower parasite, a few authors (Arsenijević, 1970; Arsenijević and Maširević, 1987, 1988; Fucikovsky et al., 1978; Richeson, 1981; Gudmestad et al., 1984) found that strains of this bacterium differed in biochemical aspect from E.c. ssp. carotovora, for instance in the utilization of sucrose. So, the strains of E. carotovora, originating from sunflower are more similar to E.c. ssp. atroseptica than to E.c. ssp. carotovora.

The observed differences moved us to compare strains of E. carotovora isolated from sunflower in Yugoslavia and the USA, and with strains of E. carotovora originating from other hosts. It was shown that the strains of E. carotovora isolated from sunflower in Yugoslavia were specific in several characteristics.

MATERIAL AND METHODS

Carbohydrate utilization

Carbohydrate utilization was performed using the Biolog Gramnegative microplate system (Biolog. Inc., Hayward, CA). Twenty-four hour cultures of each Ec strain were harvested from nutrient agar plates grown at 23 ^{o}C. Bacterial suspensions of each strain were set against a turbidity standard of OD 0.22-0.25 at 590 nm. Microplates were incubated at 23 ^{o}C for 24 h and color changes (indicating a positive test) were determined with a Biotek EL-311 microplate reader set at 605 nm.

Fatty acid analysis

All bacterial cultures were grown at room temperature in shake culture for 24 h in nutrient broth. Cells were prepared for fatty acid extraction by a method similar to that of Suzuki and Komagata (1983). Following incubation, cells were subjected to centrifugation at 9150 X g for 15 min. The broth was then decanted and the cells were washed by resuspending the pellet in 150 ml of sterile double-distilled water and agitating the centifuge bottle for 2 min. The cells were then recentrifuged. The water was decanted, the wash procedure repeated, and the bacterial cells recentrifuged. Approximately 1.0 ml sterile double -distilled water was added to the bacterial pellet and stirred: 1.0-2.0 ml of the bacterial slurry was placed in a 5 ml Wheaton serum vial. This vial was sealed with laboratory film and frozen at -83 ^{o}C for 24 h. The cells were then subjected to freeze-drying for 24 h on an FTS Systems freeze-dryer (Model No. FDX-1-54A-0).

Fatty acid determination

Fatty acid composition was determined by gas-liquid chromatography procedures similar to that described by Miller (1982). Lyophilized bacteria (5 mg) were placed in a 13 mm X 100 mm screw cap test tube with a Teflon-lined cap. Saponification was accomplished by adding 1.0 ml of a 7.5 M NaOH - 50% methanol (1:1 v/v) reagent, vortexing for 5-10 sec, and heating at 100 ^{o}C in a water bath for 5 min. The tube was then vortexed for 5-10 sec and returned to the water bath for an additional 25 min. After cooling to room temperature, the saponificate was acidified and methylated by adding 2.0 ml of a 6N HCl - 100% methanol (13:11 v/v) solution and heating for 10 min at 80 ^{o}C. The tube was then cooled under cold running tap water.

The fatty acid methyl esters were extracted by adding 1.25 ml of hexaneethyl ether (1:1) and rotating the tube end over end for 10 min. The tube was then uncapped and the aqueous (lower) phase was removed with a Pasteur pipet and discarded. The remaining extract was then washed by adding 3.0 ml of 0.3 M NaOH and rotating the tube end over end for 5 min. Two-thirds of the organic extract (upper phase) was transferred witha Pasteur pipet to a glass sample vial with a Teflon- lined cap.

Fatty acid methyl esters were analyzed with a Shimadzu model 14A gas chromatograph equipped with a flame-ionization detector. A 30 m X 0.25 mm I.D. fused silica capillary column (Supelco SPB-1, film thickness 0.25 µm) was used with helium as the carrier gas. The temperature program was initiated at 150 ^{o}C and increased at a rate of 4 ^{o}C/min, to a final temperature of 250 ^{o}C. Fatty acids were identified by comparing their retention times with those of a fatty acid methyl ester (FAME) standard (Supelco 4-7080, Bacterial Acid Methyl Ester mix CP). Identity of peaks not found in the FAME standard was determined by calculating equivalent chain length plot derived from the FAME standard.

RESULTS

Carbohydrate utilization

Two observations stand out in this study. First, the E. carotovora (Ec) strains recovered from sunflower grown in Yugoslavia are biochemically unique. For example, Ec sunflower strains from Yugoslavia utilize D.L lactic acid and succinamic acid similar to E. carotovora ssp. betavasculorum. However, Ec strains from other hosts do not utilize these two carbohydrate sources. In addition, Ec sunflower strains from Yugoslavia utilize maltose, turanose and many were able to utilize psicose, glycogen, L-serine and D-sorbitol. All of these carbohydrates are rarely, if ever, utilized by E. carotovora from other hosts. This suggests that E. carotovora strains recovered from sunflower in Yugoslavia may constitute a separate subspecies (ssp. helianthum?). Secondly, strains of E. carotovora recovered from hosts other than potato, tend to be more variable in their carbohydrate utilization when comparisons are made among strains within a host. For example, Ec strains from sugar beet, sunflower, paprika, and lettuce have variable reactions among the strains for their ability to utilize γ-hydroxy butyric acid, D-saccharic acid, tween 40, D-galactose, L-glutamic acid and D-galactose. Ec strains from other hosts tend to be either positive or negative in their utilization of these carbohydrate sources.

Tab. 1. Percentages of Fatty Acids for Strains of E. carotovora from Various Hosts

Host origin of Ec strains	No. of Strains Tested	% F.A. 12:0 $\bar{X}$	SD	% F.A. 14:0 $\bar{X}$	SD	% F.A. 16:0 $\bar{X}$	SD	% F.A. 16:1 $\bar{X}$	SD	% F.A. 18:1 $\bar{X}$	SD
Lettuce	4	5.69	0.15	0.87	0.06	29.36	1.4	31.73	0.82	18.98	0.15
Paprika	4	5.47	0.26	0.97	0.13	28.95	2.05	30.83	0.24	21.94	2.01
Aloe	2	5.68	0.15	0.91	0.01	28.38	0.53	30.79	0.45	21.82	1.65
Sunflower (USA)	4	4.48	0.07	1.22	0.15	34.10	0.96	32.57	0.57	16.37	1.41
Sunflower (Europe)	22	5.99	0.24	1.62	0.33	32.95	2.13	32.11	0.88	15.14	1.41
Sugar beet Ecc	12	5.67	0.24	1.48	0.23	33.36	1.24	30.84	0.89	16.28	1.17
Sugar beet E. c. ssp. betavasculorum	5	6.43	0.28	1.10	0.33	31.43	1.46	32.27	0.26	16.67	1.65
Sugar beet hybrid*	6	4.07	2.95	2.37	2.00	27.59	5.16	32.19	1.54	17.65	4.14
Ecc	6	5.60	0.33	1.08	0.27	30.68	2.97	30.95	1.21	19.66	2.62
Eca	3	5.17	0.26	1.07	0.26	33.58	2.55	35.06	0.71	13.93	0.57
Potato (Europe)	12	5.12	0.20	1.13	0.24	33.63	1.42	33.87	0.91	13.64	0.99
Potato hybrid (US)[1/]	3	5.22	0.22	1.40	0.39	36.41	3.78	31.92	1.29	13.64	2.22

*Strains have characteristics in common with E. carotovora ssp. carotovora and ssp. atroseptica based on carbohydrate utilization.

Fatty acid analysis

The fatty acid methyl esters examined in this study were dodecanoic acid (12:0), tetradecanoic acid (14:0), hexadecanoic acid (16:0), cis-9-hexadecanoic acid (16:1) and trans-9-octadecanoic acid (18:1). Fatty acid rations were also studied as per De Boer and Sasser (1986). The fatty acid rations examined were 12:0/14:0, 16:0/12:0 and 16:1/18:1. The most useful fatty acid ratio to differentiate E.c. ssp. carotovora from ssp. atroseptica was the 16:1/18:1 ratio. The mean ratios of these fatty acids were 1.59 and never more than 1.87 (never 1.87) for Ecc and 2.52 (never less than 2.43) for Eca. The ratios of this fatty acid combination (16:1/18:1) indicate that the Ec strains recovered from lettuce, paprika, Aloe, sunflower (USA strains) and sugar beet belong to the subspecies carotovora. The 16:1/18:1 ratio for the Yugoslavian Ec strains was intermediate of ssp. carotovora and ssp. atroseptica (Table 1). All Ec strains recovered from potato in Europe belonged to the subspecies atroseptica.

DISCUSSION

Strains of Erwinia carotovora isolated from lettuce, potato, cactus (Aloe) and sunflower grown in Yugoslavia and from potato, sugar beet and sunflower, three among four most important and most interesting crops in North Dakota, USA, were characterized biochemically. Five major fatty acids were identified in these E. carotovora strains from various hosts. They were dodecanoic acid (12:0), hexadecanoic acid (16:0), 9-hexadecanoic acid (16:1), 3-hydroxytetradecanoic acid (3-OH 14:0) and 9-octadecanoic acid (18:1). Minor fatty acids present (2% of the total fatty acid content) were tridecanoic acid (13:0), tetradecanoic acid (14:0), pentadecanoic acid (15:0), heptadecanoic acid (17:0) and octadecanoic acid (18:0). Ratios of various fatty acids and traditional biochemical tests aided in delineating the subspecies E. carotovora recovered from each host. Potato strains of E. carotovora were easily delineated into either E. carotovora ssp. carotovora (Ecc) or E. carotovora ssp. atroseptica (Eca) with little variability. Strains of E. carotovora isolated from lettuce, paprika and cactus in Yugoslavia belong primarily to Ecc based on these tests. However, strains of E. carotovora isolated from sunflower in Yugoslavia and North Dakota and from sugar beet grown in North Dakota were variable and have biochemical and fatty acid characteristics similar to both Ecc and Eca. Finally, these

investigations show that E. carotovora sunflower strains isolated in Yugoslavia, may represent a new taxon in E.c. group.

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

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FIMBRIAE IN ADHESION OF ERWINIA CAROTOVORA SUBSP. CAROTOVORA TO PLANT SURFACES

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INTRODUCTION

Erwinia carotovora subsp. carotovora is an important soft rot pathogen of potato and other plants. The role of bacterial cell wall structures in the pathogenesis of soft rot has not been extensively studied. Many of the bacteria associated with plants possess binding organelles, fimbriae, which seem to be important in some associative and pathogenic bacterium-plant interactions (Korhonen et al. 1986). The potato pathogen we are studying, E. carotovora subsp. carotovora 312, produces type 1 fimbriae (Fig. 1; Korhonen et al. 1987), which bind to mannose-containing oligosaccharides and occur widely in all enterobacterial species. To evaluate the possible role of fimbria-mediated adhesion in pathogenesis, we used transposon Tn5 mutagenesis to make non-fimbriated mutants of E. carotovora.

TRANSPOSON MUTAGENESIS

The transposon Tn5, which codes for kanamycin resistance was introduced into E. carotovora subsp. carotovora 312 by using the plasmid vector system pSUP2021 (Simon et al. 1983). The donor strain Escherichia coli SM10(pSUP2021) and the recipient were mated on membrane filters on Luria broth for 48 h at 28°C. After incubation, kanamycin-resistant

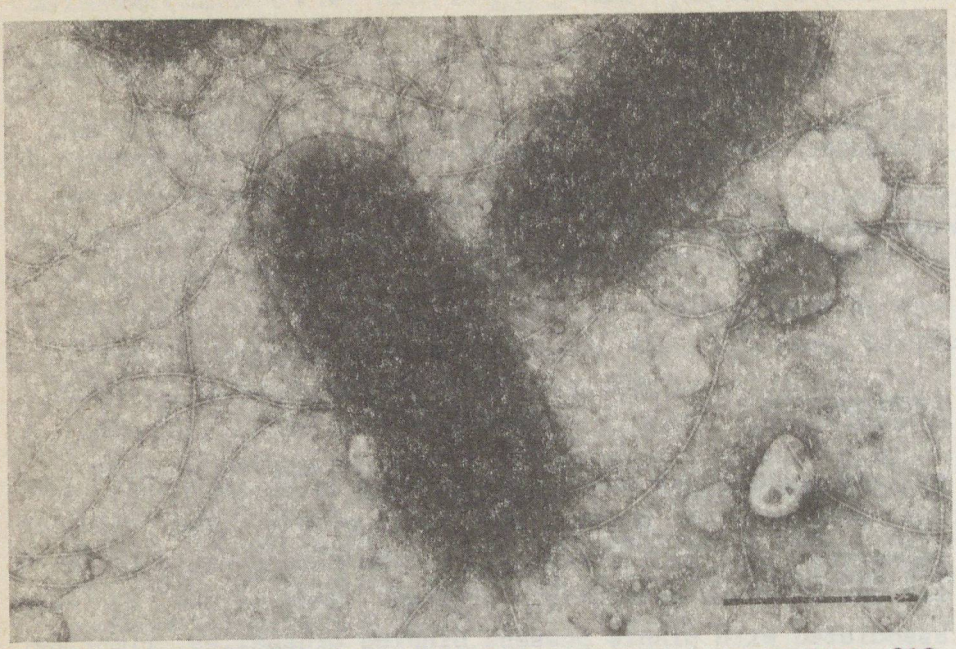

Fig. 1. Cells of E. carotovora subsp. carotovora 312 carrying type 1 fimbriae and flagella. Bar represents 1 μm

E. carotovora transconjugants were selected on minimal agar, and the mutants were screened immunologically by colony blotting (Väisänen-Rhen et al. 1985) with an antiserum prepared against purified type 1 fimbriae (Korhonen et al. 1987) and biotin-avidin-peroxidase. Pathogenicity of the non-fimbriated mutant 312/2.14 was tested by stabbing bacterial cells into tubers and incubating for 48 h at 28°C in moist chambers. The pathogenicity as assessed by this procedure was not altered in the non-fimbriated mutant 312/2.14.

ADHESION TO TOBACCO LEAVES

Bacterial cells (10^{10} per ml), grown in static malate-glucose broth for 48 h at 28°C, were incubated at room temperature for 1 h with pieces of tobacco leaves in phosphate-buffered saline (PBS, pH 7.2). After washing, the

subsequent treatments for scanning electron microscopy were according to Haahtela et al. (1988). A Jeol JSEM-820 scanning electron microscope operating at 10 kV was used to examine the samples.

Adhesion of bacteria to tobacco leaves was highly correlated to fimbriation. The type-1-fimbriated strain 312 adhered strongly to the leaves (Fig. 2a). The bacteria were found evenly spread over the whole leaf surface. The non-fimbriated mutant 312/2.14 adhered only poorly, showing hardly any adherent cells on tobacco leaves (Fig. 2b).

ADHESION TO POTATO TUBERS

Adhesion of the strain 312 to potato tubers was studied in similar experiments. Slices from potato tuber surfaces were incubated with bacteria, and after washing the slices were examined by scanning electron microscopy. Effect of α-methyl-D-mannoside, a specific receptor analogue of type-1-fimbrial binding, was also tested.

The fimbriated cells adhered in high numbers evenly over the potato tuber surface (Fig. 3a). The adhesion was inhibited by -methyl-D-mannoside (Fig. 3b). The non-fimbriated mutant adhered in only low numbers (Fig. 3c).

DISCUSSION

Fimbriae and bacterial adhesion have not been a subject for intensive studies in plant pathology. Our results show that the potato pathogen E. carotovora subsp. carotovora has the capacity to adhere to potato tubers and plant leaves and that fimbriae are involved in this process. We are currently evaluating the biological significance of this adhesion system in pathogenicity tests that mimic the early events in bacterial invasion into host plants. Possibly adhesion to plant surfaces increases the pathogenic potential of E. carotovora subsp. carotovora by allowing colonization of

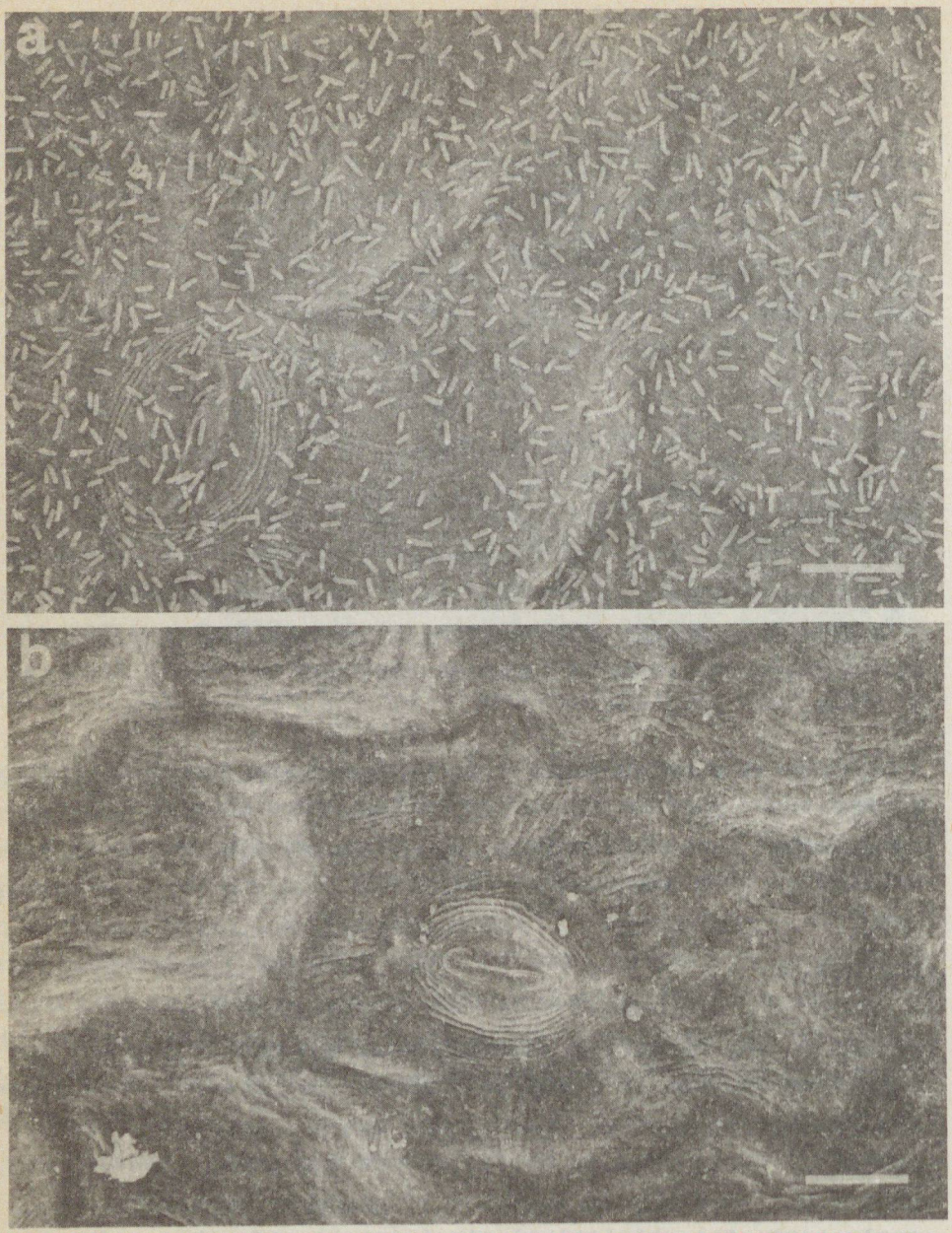

Fig. 2. Scanning electron micrographs of Nicotiana tabacum leaves with adherent E. carotovora subsp. carotovora 312 bacteria. In A, adhesion of the fimbriated wild-type strain 312, and in B, adhesion of the non-fimbriated mutant strain 312/2.14. Note strong and unlocalized adhesion in A and poor adhesion in B. Bars represent 10 μm

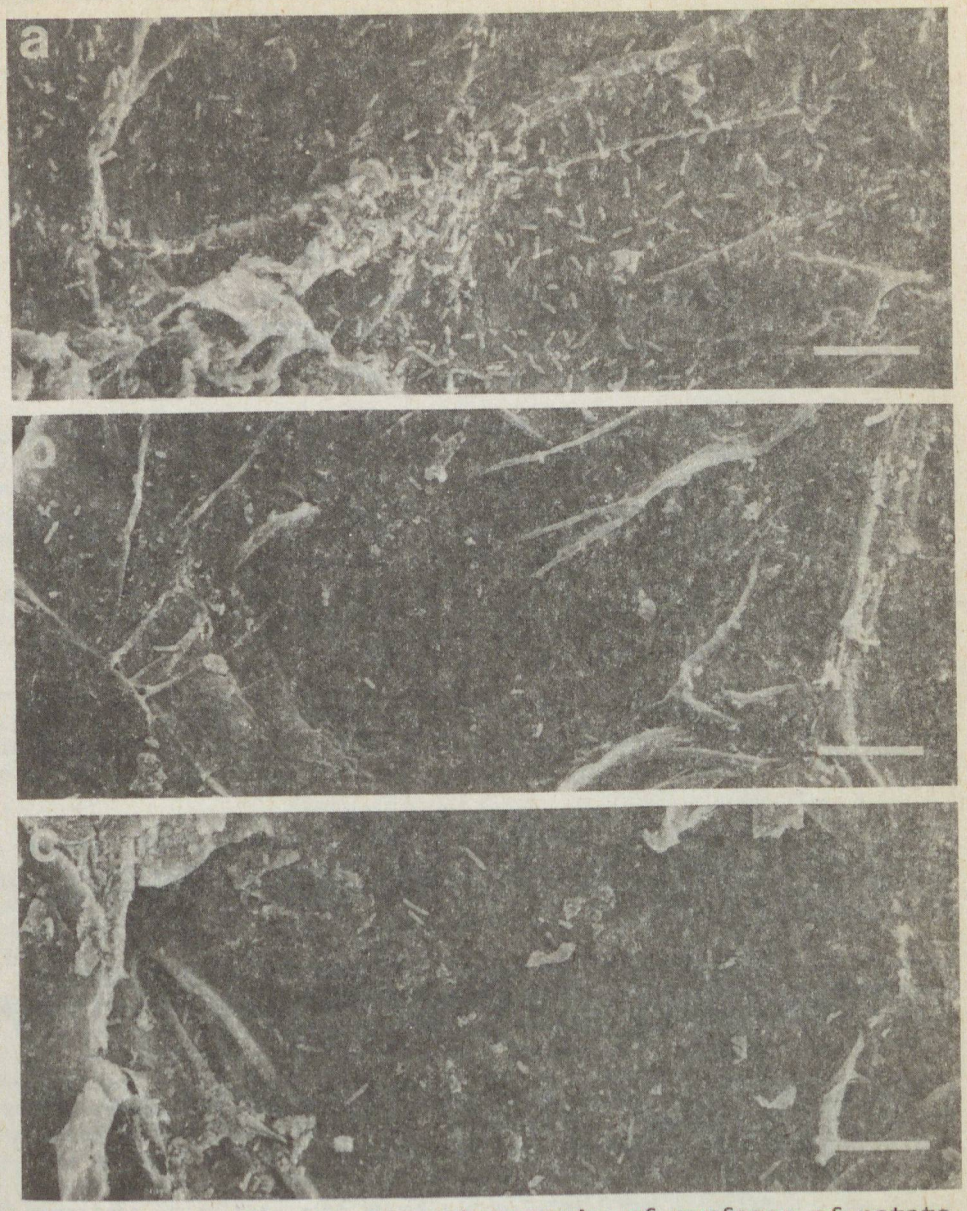

Fig. 3. Scanning electron micrographs of surfaces of potato tubers with adherent E. carotovora subsp. carotovora 312 bacteria. In A and B, adhesion of strain 312 in the absence of and in the presence of α-methyl-D-mannoside, respectively. In C, adhesion of the mutant strain 312/2.14. Note the strong and unlocalizated adhesion in A, inhibition of adhesion by the receptor analogue in B, and poor adhesion of strain 312/2.14 in C. Bars represent 10 μm

plant surfaces and efficient access to sites of invasion. Examples supporting this hypothesis exist: fimbriae have been shown to be involved in adhesion and pathogenicity of Pseudomonas species to bean (Romantschuk and Bamford 1986) and to corn (Vesper 1987).

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

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POTATO TUBERS: HYPOXIC RESISTANCE TO SOFT-ROT

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Potato (*Solanum tuberosum*) tubers become more susceptible to soft rot, caused by *Erwinia*, when subjected to hypoxic conditions (reviewed in [1]). Such conditions may prevail during storage, in transportation, or upon planting of seed tubers and can result in severe losses. It is not known what factors mediate disease resistance although isoprenoid pathway products have been implicated as well as wound healing gene products [2-5]. Hypoxic incubation causes global inhibition of tuber protein synthesis including phenylalanine ammonia-lyase (PAL), a key enzyme in the isoprenoid pathway [6,7].

We confirm that hypoxic stress induces a transient increase in susceptibility to soft rot, but that prolonged hypoxic stress renders tubers more resistant to soft rot. This resistance is correlated with the synthesis of novel proteins which occurs after prolonged hypoxic stress [7].

MATERIALS AND METHODS

Origin of bacterial strains, gene probes and tubers. The origin of *Erwinia carotovora* subsp. *carotovora* (Ecc) strain EC14 has been reported [8]. Tubers of potato cv. Russet Burbank were purchased at local Blacksburg supermarkets.

Inoculation procedures. Ecc was cultured at 30°C on plate count agar (PCA; Difco, Detroit, MI) or aerated LB broth. Tubers were washed, rinsed, air-dried, and incubated overnight at room temperature. Immediately prior to inoculation, tubers were surface-disinfested briefly in 0.5% sodium hypochlorite, rinsed in deionized water, and air-dried in a laminar flow hood. Two inoculation methods were used. The eye-of-the-needle method [8] was used to deliver colonial growth from PCA, containing about 1 x 10^6 CFU, to a depth of 1.0 cm into the tuber. The pipet-tip method [7] delivers 75 µl of overnight LB culture with about 1 x 10^7 CFU. Ten inoculations per tuber were made by one of these methods.

Hypoxic Conditions. Whole tubers were supported on a zinc-coated wire grid above a layer of water in a plastic box covered with a close-fitting lid. To maintain a high relative humidity, the top and walls of the box were lined with dampened paper towels. Argon (Ar) gas bubbled into the water displaces O_2, N_2, and CO_2 in the air by its greater specific weight. O_2 concentrations are monitored from within the box using a portable gas monitor (Model EXOTOX 50 OF CH, Neotronix Inc., Gainesville, GA). To allow

direct measurement of gases, the battery-powered monitor was activated from outside the box by a gas-tight plunger system constructed using a cut-off 1.0-ml syringe barrel inserted through the wall of the plastic box and sealed with silicone glue. Two syringe plungers placed in the barrel piston-to-piston manipulate the on-off switch of the gas monitor. The digital readout is visible through the plastic box walls.

Inhibition of protein synthesis. Cycloheximide (Sigma, St. Louis MO) in aqueous solution (75 µl of 10 mg/ml) was presented to tubers at the sites of inoculation by the pipet-tip method.

RESULTS AND DISCUSSION

Hypoxic conditions. The O_2 concentration in the incubation boxes was measured at various times as air or Ar bubbled into the boxes (Fig. 1). With Ar (≥58cc/min), O_2 concentration was reduced to 2% by 8 hr. With air (≥100cc/min), tuber respiration decreased the O_2 to 20.7% by 8 hr.

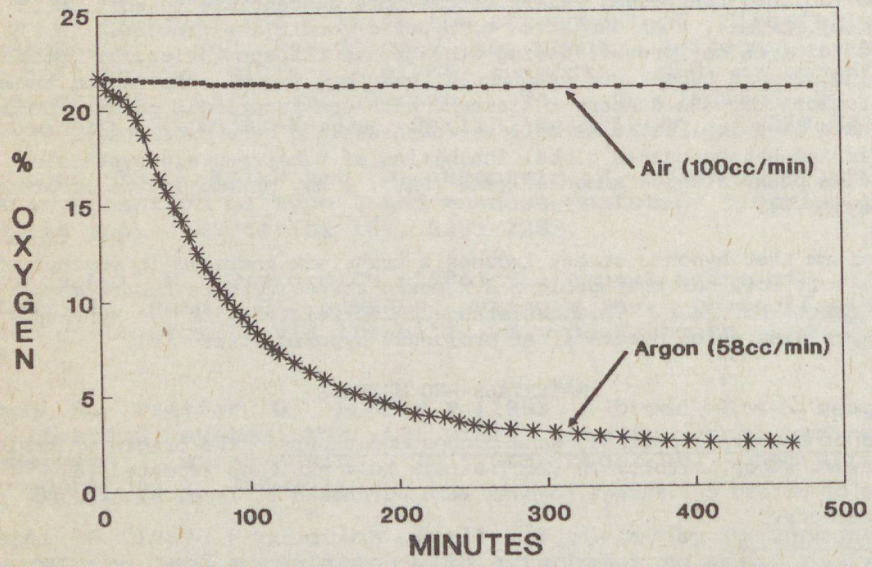

Fig. 1. O_2 concentrations measured with a portable gas monitor in the incubation boxes (see text) over time

Hypoxic resistance. Tubers incubated aerobically prior to inoculation with Ecc, rotted much more when incubated in hypoxic than in aerobic conditions (Fig. 2) in agreement with previous observations (reviewed in [1,9,10]). Increased maceration may be due to impaired host resistance in low O_2 correlating with the hypoxic inhibition of aerobic protein synthesis documented by Vayda and Schaeffer [7].

Vayda and Schaeffer [7] also reported that after prolonged hypoxic incubation, a set of proteins was synthesized in response to wounding that was different from those synthesized aerobically. To determine if some of these proteins function in disease resistance, tubers were incubated in hypoxic conditions for various times before inoculation with Ecc (Fig. 2).

After inoculation, hypoxic incubation was continued and tuber weight lost to maceration was measured. Hypoxic preincubation for 48 hr resulted in a significant decrease in macerated tissue compared to tubers preincubated aerobically. Hereafter, we refer to this reduced maceration as "hypoxic resistance." The amount of maceration was similar to the low level of rot found in aerobically preincubated tubers that are incubated aerobically after inoculation. We refer to that reduced level of maceration as "aerobic resistance."

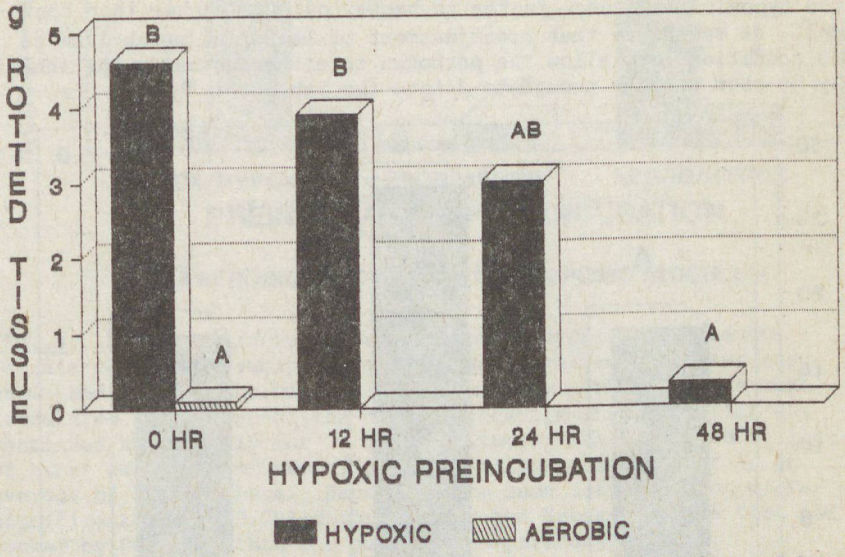

Fig. 2. Influence of hypoxic preincubation on maceration. Tubers were preincubated hypoxically for various times, inoculated by the eye-of-the-needle method with *Erwinia carotovora* subsp. *carotovora*, and incubated at 30°C in a closed plastic box with bubbling Ar (≥58 cc/min) for 70 hr. Maceration was estimated by loss of tuber weight after removing rotted tissue. Each bar is an average of five tubers. Bars with the same letter do not differ significantly at $p \leq 0.01$ determined by the Kruskal-Wallis test and ranked by Dunn's method for multiple comparisons. The "aerobic" bar represents maceration of tubers incubated aerobically for 70 hr after inoculation

Role of protein synthesis in hypoxic resistance. Cycloheximide applied before hypoxic preincubation allowed more maceration than when it was applied after hypoxic preincubation with the inoculum (Fig. 3). This supports the hypothesis that proteins are involved in hypoxic resistance. However, because cycloheximide applied before hypoxic preincubation had the greatest effect we conclude that proteins involved with hypoxic resistance are preformed rather than induced by inoculation. This is in contrast with the induced proteins believed to be active in aerobic responses to the pathogen [11,12]. If hypoxic resistance proteins are preformed, they may function directly as inhibitors or indirectly by catalyzing production of inhibitors.

Inoculum preparation vs. hypoxic resistance. In the experiments described above, Ecc inoculum was prepared "aerobically" as colonial growth on PCA plates or in broth. To determine the effect of hypoxic incubation on the inoculum, plates streaked with Ecc were preincubated with the tubers to be inoculated. Hypoxic inoculum overcame hypoxic resistance in tubers preincubated hypoxically, inoculated, and incubated hypoxically (Fig. 4). However, hypoxic inoculum did not affect maceration of aerobically preincubated tubers that were inoculated and incubated aerobically. Finally, hypoxic inoculum was less effective in maceration of aerobically preincubated tubers that were inoculated and incubated hypoxically. We conclude that the hypoxic resistance factor is bacteriostatic rather than bacteriocidal. We speculate that preadjustment of bacterial metabolism to hypoxic conditions may allow the pathogen to either detoxify the inhibitor quickly or grow rapidly enough to dilute the inhibitor.

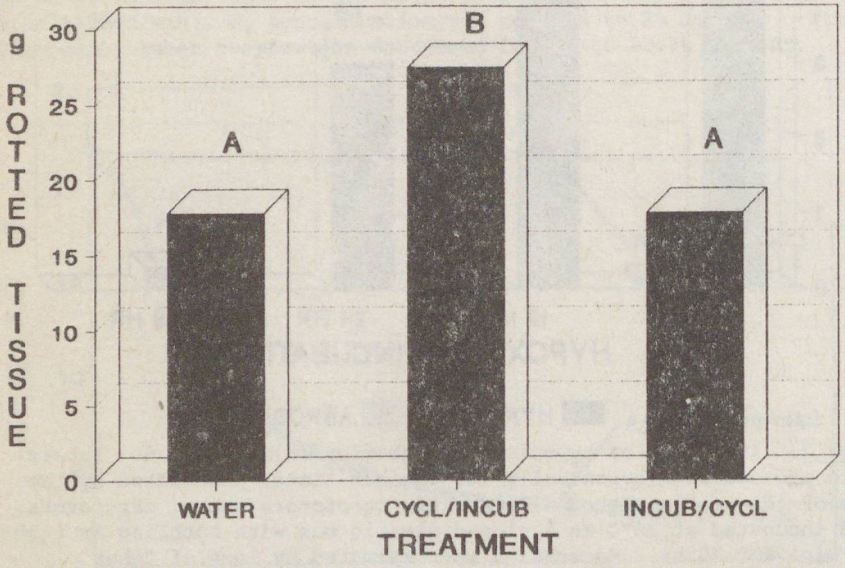

Fig. 3. Cycloheximide inhibition of hypoxic proteins synthesis. Tubers were treated with cycloheximide before or after 48 hr preincubation in Ar (≥58 cc/min). One group of tubers was treated with water after hypoxic preincubation as a control for the cycloheximide treatments. Whole tubers were inoculated with *Erwinia carotovora* subsp. *carotovora* by the pipet-tip procedure and incubation was continued at 30°C for 70 hr in a closed plastic box with bubbling Ar 70 hr. Maceration was estimated by the loss of tuber weight after removing rotted tissue. Each point is an average of seven tubers. Bars with the same letter do not differ significantly at $p \leq 0.001$ determined by the Kruskal-Wallis test and ranked by Dunn's method for multiple comparisons.

Ecological role of hypoxic resistance. We speculate that hypoxic resistance is important to survival of tubers in soil under hypoxic conditions. We believe that the phenomena of hypoxic susceptibility and hypoxic resistance reflect the shift of tuber metabolism from aerobic to hypoxic resulting in a "window" of susceptibility between the cessation of aerobic protein synthesis and the start of hypoxic protein synthesis. Should infection not occur during this window, the tuber will be resistant to soft-rot

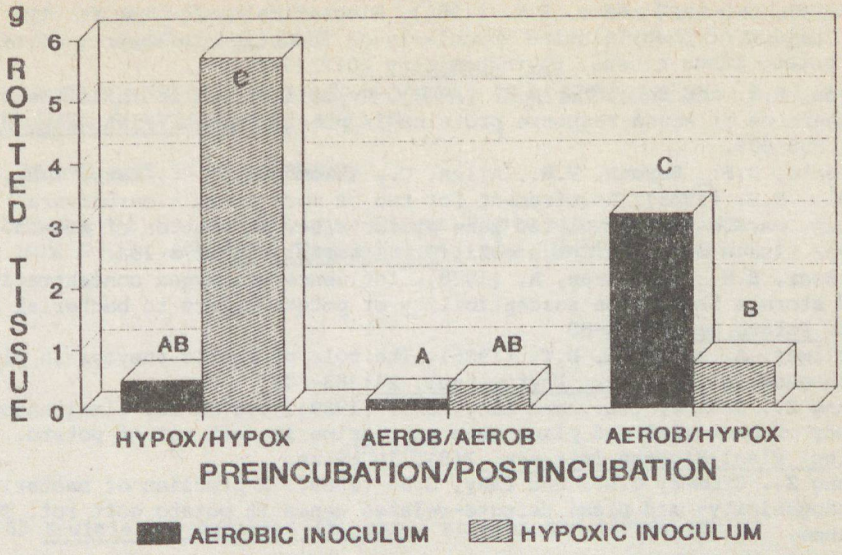

Fig. 4. Effect of inoculum preparation on hypoxic resistance. *Erwinia carotovora* subsp. *carotovora* was streaked on plates and incubated at 30°C aerobically or hypoxically. Whole tubers were inoculated by the eye-of-the-needle procedure and incubation was continued hypoxically for 70 hr. Maceration was estimated by loss of tuber weight after removing rotted tissue. Each point is an average of seven tubers. Bars with the same letter do not differ significantly at $p \leq 0.001$ determined by the Kruskal-Wallis test and ranked by Dunn's method for multiple comparisons

upon the development of the hypoxic resistance proteins. If hypoxic resistance is useful in breeding for disease resistance is not currently known. We suspect that the importance of hypoxic resistance has not, however, been considered for crop improvement schemes.

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

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FURTHER BIOCHEMICAL AND SEROLOGICAL CLASSIFICATION OF ERWINIA CHRYSANTHEMI STRAINS

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ABSTRACT

From 65 Erwinia chrysanthemi (E. chr.) strains the biovar and/or serogroup/flagellatype were determined using phenotypic biochemical tests and indirect immunofluorescence. Data of 41 strains described before were included for comparison. In total 106 strains from 34 hosts were studied.

It appears that strains from 'warm' hosts (grown in (sub)tropical areas or in greenhouses at higher temperatures) do grow at 39°C and generally show strong pectinolysis at 37°C. Many of them belong to biovar 3. Strains from 'cold' hosts (grown in temperate areas or at lower temperatures in greenhouses) do not grow at 39°C and do not show moderate pectinolysis at 37°C. Many of them belong to biovar 1, 5 and 7. One host indeed may harbour several biovars and one biovar may occur in several hosts. The biovar concept therefore appears more adequate than the pathovar concept for describing differences in strains of E. chr.

There was no correlation between original host and somatic (O) serogroup/flagellar (H) serotypes, of strains. Most strains of the seven biovars belong to serogroup 1 - flagellatype 1 (O1 : H1). Many strains with unknown serogroup (O?) and/or flagellatype (H?) were found. For reliable classification and identification sera should be produced by one central laboratory.

INTRODUCTION

Erwinia chrysanthemi Burkholder, Dimock & McFadden (E. chr.) is a gram-negative, pectolytic bacterium belonging to the enterobacteriaceae family. It has a very large and diverse host range, comprising ornamentals and food crops grown in (sub)tropical or temperate areas as well as in greenhouses (Bradbury, 1986). Strains from different hosts show different pathological, biochemical and serological characteristics. Several attempts have been made to subdivide E. chr. on the basis of these differences. The most recent subdivision into 7 biovars, 3 serogroups (based on cell-wall-(O-)antigens) and 3 flagellatypes (based on flagella-(H-)antigens) was developed by Samson & Nassan-Agha (1978) and Samson & al. (1987).

Janse & Ruissen (1988) classified 41 E. chr. strains, mainly isolated in the Netherlands from different hosts, using Samson's biovar and serogroup/flagellatype scheme. In their study it was found that strains from plants growing in temperate areas usually did not grow at 39°C, did not or only weakly hydrolyzed pectin at 37°C and did not infect Philodendron erubescens after artificial inoculation.

In this article we describe biochemical and serological classification of an additional 42 strains isolated in the Netherlands from Aglaonema, Cichorium, Ctenanthe, Erynchium, Dieffenbachia, Gymnocalicium, Kalanchoë, Phalaenopsis and potato. Erynchium, Gymnocalicium and Scindapsus were described as new hosts before (Janse, 1980, Janse & al. 1986, 1987). Ctenanthe lubbersii is mentioned here as a new host for the first time. Furthermore 23 strains of the NCPPB collection from 18 different hosts and different parts of the world were studied. The 41 strains described by Janse & Ruissen (1988) are included for comparison.

MATERIAL AND METHODS

The 106 bacterial strains used and compared in this study are listed in Table 1. For biovar determination the tests mentioned in Table 2 were used according to Janse & Ruissen (1988) with one modification: utilization of carbon sources was determined by acid/alkali formation in liquid Ayers & Rupp medium with 0.005% w/v bromthymolblue instead of determination by growth on the same medium solidified with agar.

Serogroup and flagella type was determined as described by Janse & Ruissen (1988) using the same seven antisera, viz. 4 against serogroup 1 (O1) and flagellatype 1 (H1), one against O1 : H2, one against O2 : H3 and one against O3. Indirect immuno-fluorescence, using sera in a 400-fold dilution was performed as described in the same article.

Table 1. Origin of 106 Erwinia chrysanthemi strains used and compared in this study

Original host	Location	Strain number
Aechmea fasciata	Netherlands	PD[1]382
Aglaonema 'Silver Queen'	Netherlands	PD430,827,920*,1099
	UK	PD872=NCPPB[2]3274
Ananas comosus	Malaysia	PD862=NCPPB1125, PD871=NCPPB551
Begonia bertinii	Netherlands	PD823=NCPPB2421
Brassica chinensis	Malaysia	PD852=NCPPB3193
Chrysanthemum morifolium	UK	PD687=NCPPB402, PD693=NCPPB2339
Cichorium intybus	Netherlands	PD682=82/21, PD787,788,806,1036, 1233, 1237*
Colocasia esculenta	Br.Salomon Is.	PD830=NCPPB2929
Ctenanthe lubbersii	Netherlands	PD1084,1086
Dahlia sp.	Netherlands	PD855=NCPPB1955
	Romania	PD858=NCPPB1385
Daucus carota	USA	PD844=NCPPB2899
Dianthus caryophyllus	UK	PD846=NCPPB1111, PD718=CFBP[3]1200 = NCPPB453, PD863=NCPPB393
Dieffenbachia sp.	USA	PD690=NCPPB2976
	Netherlands	PD267,345,663,722*752*782*797,1073*
Erynchium alpinum	Netherlands	PD753*,783,784,785,786,789
Euphorba pulcherrima	UK	PD868=NCPPB2148
Gymnocalicium mihanovichii	Netherlands	PD826
- - 'makan'	Netherlands	PD1130
- - 'optima rubra'	Netherlands	PD1131,1132

Kalanchoë blossfeldiana[4]	Netherlands	PD481,551,554,593,594,598,664, 665,677=86/122,720*,721*,1325*
Musa paradisiaca	Malaysia	PD864=NCPPB2512
	Colombia	PD865=NCPPB2511,PD869=NCPPB2513
Orchid sp.	Sri Lanka	PD856=NCPPB3211
Oryza sativa	Japan	PD866=NCPPB3090
Parthenium argentatum	USA	PD845=NCPPB1849
Pelargonium capitatum	Comoro Is.	PD859=NCPPB898
Pelargonium zonale	UK	PD857=NCPPB2302
Phalaenopsis sp.	Netherlands	PD813-818
Philodendron sp.	USA	PD692=NCPPB533[5]
Philodendron erubescens 'Emerald King'	Netherlands	PD471,487
Physcius filicifolia	UK	PD860=NCPPB3306
Saccharum officinarum	Australia	PD870=NCPPB569
Saintpaulia ionanthe	France	PD861=NCPPB2521
Scindapsus pictus	Netherlands	PD550,552,553
Solanum tuberosum	Netherlands	PD226,472,482,483,484,490,499, 581,676=84/924,695=81/310,701= CFBP2015,719*,751*,956*,1008*, 1022*,1077*,1097*,1098*
Zea mays	USA	PD688=NCPPB2538
	Egypt	PD686=NCPPB1066
	Italy	PD691=NCPPB2347
	Malaysia	PD689=NCPPB=2476

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* strain not biotyped

1) PD, culture collection Plant Protection Service, Wageningen, the Netherlands

2) NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK

3) CFPB, Collection Francaise de Bactéries Phytopathogènes, Angers, France.

4) different cultivars, also see Janse & Ruissen, 1988

5) this strain proved to be Klebsiella aerogenes, see Janse & Ruissen, 1988, excluded

RESULTS

Results of biovar determination of 88 E. chr. strains are mentioned in Table 2 and 3. Generally strains could be classified easily. Biovar 3 was most commonly found in hosts from warmer climates or in those cultivated in greenhouses. Strains from Pelargonium were placed in biovar 6, but NCPPB 2302 did not grow at 39°C and NCPPB 898 produced acid from D(-)arabinose. Strains from hosts growing in (sub)tropical areas or at higher temperatures in greenhouses generally did grow at 39°C and showed strong pectinolysis at 37°C. However strains from Ananas, Brassica, Saccharum and Saintpaulia only

Table 2 Results of tests used for biovar determination

Test	biovar 1 (5)*	2 (6)	3 (34)	4 (3)	5 (19)	6 (4)	7 (17)
Growth at 39°C	-	+[1]	+	+	+/-[4]	+	-
Arginine degradation	+	-	-	-	+	-	+/-[6]
Cis-aconitate	+	-	V	-	V	V	-
D(-)arabinose	-	+	+	+	-	-	-
5-Ketogluconate	-	-	-	+	-	-	-
Inulin	+	-	-	-	+	-	+
Mannitol	+	+	+	-	+	+	+
Melibiose	+	-	+	+	+	+	-
Raffinose	+	-	+	+	+	+	-
D(-)tartrate	+	-	-	+	-	-	V
y-aminobutyrate	-	-	V	-	-	+	-
L-proline	-	-	V	-	-	+	-
Pectinolysis at 37°C	+[2]	++	+++/+[3]	d	+/w	+[5]	d/w/+

+ = 90-100% of strains positive; - = negative; ++ = strong positive; +++ = very strong positive; w = weak; d = doubtful; V = variable; * = number of strains tested

1) Some Dieffenbachia strains negative

2) PD823, Begonia, +++

3) Strains from Ananas, Brassica, Saccharum and Saintpaulia, +

4) Strains from Cichorium, Dianthus, Erynchium and potato negative

5) PD 1849 Parthenium, +++

6) Strains from Kalanchoë and one potato strain PD 472, negative

showed weak or moderate pectinolysis at 37°C. In biovar 5 there was a clear separation between 'warm' (Daucus from Malaysia, Euphorbia and Chrysanthemum) and 'cold' strains (Cichorium, Dianthus, potato and Erynchium). PD 823 from Begonia was an exception in being negative for growth at 39°C and strong positive for pectinolysis at 37°C. Dieffenbachia strains were either positive for growth at 39°C or negative (PD 267, 345).

Table 3 Classification of Erwinia chrysanthemi strains from different hosts and localities into biovars

Biovar	Host
1	Begonia, Dahlia, Dianthus (NCPPB 393)
2	Colocasia, Dieffenbachia
3	Aechmea, Aglaonema, Ananas, Brassica, Ctenanthe, Gymnocalicium, Orchid, Oryza, Phalaenopsis, Philodendron, Physcius, Saccharum, Saintpaulia, Scindapsus, Zea
4	Musa
5	Chrysanthemum (NCPPB 402), Cichorium, Daucus, Dianthus (NCPPB 1111), Euphorbia, Solanum, Erynchium
6	Chrysanthemum (NCPPB 2339), Parthenium, Pelargonium
7	Kalanchoë, potato

Classification of 106 E. chr. strains into serogroups and flagellatypes is mentioned in Table 4. The combination O1 : H1 was most common in 6 of the 7 biovars. There was no clear correlation with biovar or host (except for O3:H? present in Musa, biovar 4 strains only). Unknown flagellatypes were found in strains from at least 12 different host species and unknown serogroups in strains from 7 host species.

Table 4 Serogroup and flagellatype of 106 Erwinia chrysanthemi strains

Serogroup/ flagellatype	Biovar	Host
O1 : H1	1	Dahlia, Dianthus
	2	Dieffenbachia
	3	Aechmea, Aglaonema, Gymnocalicium, Phalaenopsis
	5	Cichorium, Dianthus, Erynchium
	6	Pelargonium capitatum
	7	Kalanchoë, potato
O1 : H2	5	Chrysanthemum, Cichorium, Daucus, Euphorbia
O1 : H?	2	Colocasia, Dieffenbachia
	3	Aglaonema, Orchid, Philodendron, Physcius, Saintpaulia, Scindapsus, Zea
O2 : H3	6	Parthenium
O3 : H?	4	Musa
O? : H1	3	Ananas, Brassica, Ctenanthe, Zea
	6	Chrysanthemum
O? : H2	6	Pelargonium zonale
O? : H?	3	Oryza, Saccharum, Zea

DISCUSSION

Our results show that strains of E. chr. can be easily classified into biovars. Cis-aconitate proved to be a variable test for strains which should give a positive result according to the scheme of Samson & al. (1987). Growth on y-aminobutyrate and L-proline was variable for strains belonging to biovar 3. D(-)tartrate was variable in biovar 7. As was found in earlier studies (Samson & al., 1987; Janse & Ruissen, 1988) one biovar

may occur in more than one host (biovar 3 as extreme). Only biovar 4 (Musa strains) appears to be an exception and possibly deserves another than biovar status as it is serologically distinct and also its fatty acid profile is quite different from that of the other biovars. (Dr. D.E. Stead, personal communication). In one host more than one biovar may occur. In our work this was the case for Dianthus (biovar 1 and 5), potato (biovar 5 and 7), Chrysanthemum (biovar 5 and 6). Samson & al. (1987) described this phenomenon for potato (biovar 1, 3 and 7), tomato (biovar 1 and 5) and Cichorium (biovar 1 and 5). It is concluded that the biovar concept at present is more adequate for describing differences between strains of E. chr than the pathovar concept.

Again we found that E. chr. strains could be divided in 'cold' (from hosts of temperate areas or cultivated at lower temperatures in greenhouses) and 'warm' strains (from hosts of (sub)-tropical areas or grown at higher temperatures in greenhouses). This division is based in the present study on growth at 39°C and strong pectinolysis at 37°C. For pectinolysis, however, there was not a 100% correlation, since some strains of biovar 3 and 'warm' strains of biovar 5 showed only moderate pectinolysis at 37°C. Apparently 'warm' strains do not always express pectic enzymes with a high temperature optimum on artificial medium or they do not always need them for pathogenesis. This should be elucidated further, since this difference certainly will have epidemiological and taxonomical significance. Differences in pectic enzyme constellation between strains have been reported (Garribaldi & Bateman, 1971, Ried & Collmer, 1986). Unfortunately not too much has been published about temperature optima of these enzymes, contrary to other enzymological, immunological, electrophoretic and genetic data.

On the basis of this study probably other serogroups and/or flagellatypes can be defined by preparing sera against O? and/or H? strains. No correlation was found between original host and serogroup/flagellatype. This was also described for lipopolysacccharide (LPS) which forms the basis of somatic antigenic reactions (Bradshaw - Rouse & al., 1988). These authors also showed that somatic antigenic reaction of E. chr. strains was correlated with sugar composition of LPS. To standardize serological classification of strains, especially important in epidemiology, sera

should be produced in one central laboratory. The laboratory of Dr. Samson (Angers, France) has a lot of experience in this matter.

Acknowledgements: We wish to thank Dr. R. Samson, Angers, France for supplying antisera, Dr. D.E. Stead, Harpenden, U.K., for NCPPB strains of Erwinia chrysanthemi and miss B.E. Spit for skilfull technical assistance.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

RESISTANCE TO BLACK LEG IN POTATO

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The black leg is one of the most harmful bacterial diseases of potatoes. Susceptibility to this disease in the USSR averages 1-5%, in some years it can reach 40-50% (Belova, 1964). In Leningrad region the losses of potato tubers due to the disease can account to 30% and more (Bushkova, 1977).

Foreign researchers also note high harmfulness and spread of the black leg disease. In Federal Republic of Germany, as M. Münzert (1975) believes, the losses of tubers infected with the pathogen during storage amount to 40-69% of total losses from rots and these losses reach 700-900 thousand tons, or 15 mln DM. D. Little (1976) attributes the black leg disease to the most harmful disease of potatoes in the USA. The decrease of yield reaches up to 15-25%. M. Stanghellini, J. Meneley (1975) recorded even more substantial losses - up to 40%. Analogous situation can be observed in other potatogrowing countries of the world.

The main method to control potato bacterial diseases is the selection and development of resistant varieties. However, many countries do not possess varieties resistant to black leg. At the same time, according to investigations by the authors (1985, 1987), the world collection of VIR that numbers more than 10 thousands of species, varieties and hybrids includes valuable initial material for breeding for resistance against bacterial diseases.

During the period of 1985-1988 the authors differentiated the botanical and varietal diversity of the world collection of potatoes by the resistance to black leg in order to obtain highly resistant forms, suitable for their further use in breeding.

Research methods. To evaluate potato material we used 2 laboratory methods of artificial inoculation of stems and tubers. 1) Inoculation of stems. We used the method by Schneider Yu. I. (1965), i.e., we cut 6 stems per sample in the field (4 - for screening and 2 - as control ones) and placed them into 1 litre glass pots with tap water. The stems were cut during the stage of full emergence-beginning of flowering prior to their hardening. Then the stems were inoculated at their middle part with the help of a medical syringe. The concentration of bacterial suspension was 10 mln cells per 1 ml of solution. After 3-5 days we evaluated the stems against the 5-point scale, where 1 point is the very low resistance with complete rotting and dying off of leaves and stems, 5 - very high resistance without visible symptoms of damage to leaves and stems. 2) Inoculation of tubers was performed using the authors' method. 3-5 externally healthy tubers per sample were cleaned with a brush in running water and after drying were sterilized in 96% ethyl alcohol. Then tissue cylinders were cut out of the tubers with an auger (130 mm-long with a piston 15 mm in diameter). These cylinders were cut with a scalpel into disks (h = 10 mm). Minimum number of 20-25 disks + 5 as control ones were taken per sample. Then these disks were weighed precisely up to the second digit. Prior to inoculation we kept the disks in sterile water so that they would not get dry and then placed them evenly in sterile Petri dishes (8-10 pieces per dish) on moistened filter paper. On each disk we dropped 0.1 ml of suspension by means of a Pasteur pipette. On control disks we dropped sterile water. Inoculum concentration was 100 mln cells per 1 ml. All the potato material in Petri dishes was incubated in a thermostat at +25-27 ^{o}C for 24-26 hours The next day the decayed tissue was washed away and the remaining healthy tissue was dryed at the room temperature and weighed. The degree of injury for each replicant was determined according to the weight difference before inoculation and after a 1 day incubation with calculation of the average percentage. Evaluation of potato resistance was done against the 9-point scale, adopted in the International Comecon List of Descriptors (1984): 1 point - very low and 9 - very high resistance. Then the susceptibility of the examined samples was compared with that of the standard varieties, Gatchinskii (relatively resistant - 7 points) and Stolovii 19 (susceptible - 3,5 points). Thus, potato samples with 1, 3, 5 points damage we regarded as susceptible, while these with 7, 9 points as resistant ones. We conducted artificial inoculation of tubers in a dust-proof chamber BP-4-004, which ensures sterile conditions. Using this method mass-testing of potato material can be carried out in a short period of time (several days) during a year.

The present paper deals mainly with the evaluation of tuber material. The samples evaluated as resistant in September-November (7, 9 points) were additionally tested in April-May prior to their planting in the field. In some cases we turned to inoculation of potato stems also.

In both methods we used the mixture of 2-3 high-virulent strains of the black leg bacteria Erwinia carotovora var. atroseptica, received from A.M. Lazarev (Institute of Plant Protection).

Results. Phytopathological evaluation of tubers for resistance to black leg in different species, hybrids and varieties of potato has indicated to a possibility of purposeful search for forms with a higher level of resis - tance to the disease.In the years of research we screened more than 250 samples of tetraploid polymorphous specy Solanum andigenum Juz. et Buk. and a series of other potato forms (Table 1). Complete immunity (9 points) was recorded in the following samples: Columbian - kk-15375, 15379a, 15415 - var. mammiforme, k-15543 - var.tocanum forma rubriculum, k-16153 - var. tocanum, kk-16345, 17720, 15686 - ssp. Colombianum, as well as a South-Peruvian form - k-1763 - var. ccompetillo, Peru-Bolivian - k-4615 - Criolla and a sample from Equador - k-10398 - Chola.

Relative resistance (7 points) was found in the samples: kk-15989, 15646, 18972, 15632, 16152, 5622, 1701, 1751, 15553, 15379b, etc.

In order to check genetic characters of the sources of high resistance to black leg of S. andigenum we tested the resistance of their self-progeny (I_1) (Table 2). It can be seen that in self-progeny of highly resistant forms as well as of relatively resistant ones segregation was observed. Resistant forms segregated a considerable quantity of forms with 9- and 7-points resistance; it was also characteristic of the relatively resistant forms. It means that the inheritance of resistance to potato black leg is a complex polygenic character. We intend to extend the work in terms of quantity and quality (testing up to I_2, I_3, etc. progenies) with consequent analysis of dialel crosses or employing other genetic method.

The following bred varieties of cultivated potato possess a higher level of resistance to black leg (7 points): CFP-69-1, CIP (k-18598), 503-31, CIP (k-18601), etc. from Peru, Siro (Netherlands), Tempora (Federal Republic of Germany), Gatschinskii, Polonina, Letnii 92 (USSR), etc.

The investigations conducted indicate that the purposeful search for the forms with the highest resistance to the black leg pathogen among the specific and varietal diversity of potato is possible and is promising.

Table 1. Resistance (susceptibility) to black leg in species, hybrids and varieties of potato

Species, hybrid, variety	Year	Total samples studied,	Resistant						Susceptible	
			points							
			9		7		5		3.1	
		pcs	pcs	%	pcs	%	pcs	%	pcs	%
S. andigenum	1985	27	6	22	8	30	13	48	-	-
	1986	23	1	4	21	92	2	4	-	-
	1987	145	10	7	72	50	35	24	28	19
	1988	66	10	15	42	64	8	12	6	9
Intraspecific hydrids	1987	47	5	11	24	51	15	32	3	6
S. andigenum	1988	32	-	-	7	22	25	78	-	-
Primitive cultivated	1985	96	26	27	14	14	15	16	41	43
	1986	21	2	10	-	-	3	14	16	76
species	1987	14	-	-	3	21	6	43	5	36
	1988	18	-	-	-	-	3	17	15	83
Interspecific hybrids of	1985	33	2	6	14	42	15	46	2	6
	1986	30	-	-	7	23	17	57	6	20
different origin	1987	213	2	1	25	12	84	39	102	48
	1988	276	1	0.3	62	22.5	171	62	42	15.2
Bred varieties	1985	27	2	8	7	26	12	44	6	22
	1986	11	-	-	2	18	7	64	2	18
	1988	110	-	-	27	25	63	57	20	18

Table 2. Segregation pattern in self-progeny (I_1) of S. andigenum samples by their resistance to black leg

Sample, VIR Cat. N°	Seedlings planted in field,	Seedlings with tubers obtained,	Resistant		Susceptible
			points		
	pcs	pcs	9	7	5, 3, 1
Immune (9 points)					
k-15650	37	25	5	13	7
15686	29	12	1	10	1
16153	24	9	1	4	4
17720	19	9	1	2	6
Relatively resistant (7 points)					
15379b	55	3	0	3	0
15553	27	11	0	4	7
1701	18	7	2	4	1
16345	17	3	1	2	0

The screened samples can be used at the early stages of breeding, for interspecific hybridization in the first place, while creating resistant hybrids of complex interspecific origin and, eventually, of immune potato varieties.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

A GENERALIZED TRANSDUCTION SYSTEM FOR ERWINIA CAROTOVORA AND THE USE OF PHAGES TO ISOLATE REDUCED VIRULENCE (Rvi-) MUTANTS ON POTATO

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ABSTRACT

The uses of bacteriophages (phages) in Erwinia biology are outlined. After the isolation and characterisation of novel Erwinia phages, they were used to isolate phage resistant mutants of Erwinia carotovora subsp. atroseptica SCRI1043 and their effects on virulence in potato plants were studied. One phage resistant mutant, SCRI1043/A5/22, which showed reduced virulence in potato plants has been partially characterised. The production of a generalized transduction system in Erwinia carotovora subsp. carotovora SCRI193 is also described. A phage capable of generalized transduction, ØKP, has also been characterised and is briefly described.

INTRODUCTION

The soft rot erwinias, Erwinia carotovora subsp. carotovora (Ecc) and Erwinia carotovora subsp. atroseptica (Eca) are economically important pathogens of potato and other crops worldwide (Perombelon 1987). Both cause soft rot in potato tubers, and Eca also causes the disease blackleg in the field. Work is presently being carried out to study pathogenicity of erwinias in potato. Several factors are believed to be involved in pathogenicity and virulence, including the production of a number of extracellular enzymes (Kotoujansky 1987). Recent attention has focussed on the role of the bacterial cell surface in pathogenicity. Work has been carried out on the selection of bacteriophage and bacteriocin resistant

mutants, as a method of screening for reduced virulence (Rvi-) or avirulent phenotypes due to cell surface alterations. Erwinia chrysanthemi (Echr) mutants have been shown to have an altered cell surface and show a reduced virulence in their host Saint paulia, implying a role for the cell surface in pathogenicity and virulence (Expert et al 1985, Schoonejans et al 1987).

In this paper we report the isolation of phages and the production of phage resistant mutants to allow a study of the role of the Eca bacterial cell wall on virulence in potato. We also report the finding of a phage capable of generalized transduction in Ecc. Strains used in these studies include Erwinia carotovora subsp. atroseptica SCRI1043 (selected for its susceptibility to phage action and used in the study of blackleg virulence) Erwinia carotovora subsp. carotovora SCRI193 (used in the study of enzyme secretion) and Erwinia chrysanthemi NCPPB1066 (used in the study of L-asparaginase production).

RESULTS AND DISCUSSION

Phage isolation and characterisation. 34 phages were isolated, by enrichment, from sewage and classified into 5 morphological groups by electron microscopy. Phages were also characterised by plaque morphology and U.V. and heat inactivation (data not given).

The host ranges of these phages were studied extensively and are summarised in Table 1. Since Eca SCRI1043 was selected for its susceptibility to phage infection it was found, as expected, that the majority of phages were isolated to this strain. These phages had a very extensive host range amongst Eca strains but also showed infection in a small number of Ecc strains.

Pathogenicity and phage resistance. 40 mutants of Eca SCRI1043 resistant to phage ART-5 were tested in planta as described by Hinton et al 1989. Of the 40 mutants one, named SCRI1043/A5/22 (A5/22) did not cause blackleg symptoms but instead gave a slight necrosis around the inoculation site. This mutant had

TABLE 1

Phage isolates	No. phages isolated	No. sensitive strains / strains tested			
		Eca	Ecc	Echr	Other Erwinias
Eca SCRI1043 phages	25	47/58	3/60	0/5	0/27
Ecc SCRI193 phages	5	4/58	7/60	0/5	1/27
Echr NCPPB1066 phages	4	-/58	-/60	1/5	3/27

Table showing, in summarised form, the host ranges of isolated Erwinia phages.

TABLE 2

	Mutants					
	1043 (wt)	A5/8	A5/12	A5/22	A5/33	Other Mutants
Phage						
ART-1	+	-	-	-	-	-
ART-2	+	(+)	+	(+)	(+)	+
ART-3	+	(+)	+	(+)	(+)	+
ART-4s	+	(+)	+	(+)	(+)	+
ART-4L	+	-	-	-	-	-
ART-5	+	-	-	-	-	-
ART-6	+	+	(+)	-*	(+)	+
MIK-1	+	-	-	-	-	-
MIK-2	+	-	-	-	-	-
MIK-3	+	-	-	-	-	-
MIK-4	+	-	-	-	-	-
MIK-5	+	-	-	-	-	-

Analysis of variety of phage-resistant mutants of Eca SCRI1043 and their susceptibilities to a range of Eca SCRI1043 phages. + = susceptible, - = resistant and (+) = reduced efficiency of plating. * ART-6 resistant A5/22.

reduced virulence (Rvi-) when tested in duplicate on a further 5 occasions. The mutant was prototrophic and was found to be as wild type for growth rate (in liquid minimal medium) and extracellular enzyme production.

Although other phage resistant mutants obtained showed cross resistance to a range of Eca phages, mutant A5/22 was the only mutant to show total resistance to ART-6 (Table 2), perhaps suggesting a correlation between resistance to ART-6 and reduced virulence. Adsorption experiments showed that ART-5 did not adsorb to A5/22.

From these data it appears that A5/22 is altered in its cell wall structure and differs from the wild type bacterium in phage resistance and virulence only. Many phages appear to use the same receptor and resistance to these phages seems to depend on the inability of phages to adsorb to the cell surface, probably due to the absence or alteration of the phage receptor. Two possibilities for the identity of this structure are outer membrane protein (OMP) or lipopolysaccharide (LPS), and both are presently being characterised in the mutant.

Recent work has concentrated on complementing the A5/22 mutant in an attempt to clone the relevant wild type gene, and use this to identify the corresponding product.

Generalized transduction. From the work on phage isolation it was possible to attempt to identify a phage capable of generalized transduction. This would have enormous utility in the study of both pathogenicity in Eca SCRI1043 and extracellular enzyme secretion in Ecc SCRI193.

Only one phage from over 30 tested proved to be capable of generalized transduction, ϕKP on Ecc SCRI193. ϕKP was capable of transducing a range of chromosomal markers, and plasmid pHCP2, at frequencies ranging between 10^{-8} and 10^{-6}. These frequencies were improved by U.V. inactivation of the phage lysate. Restriction analysis of ϕKP DNA suggested the genome size of the phage is approximated to 46Kb.

ϕKP was found to have a limited host range. It was able to infect Ecc SCRI193, on which it was originally isolated, but

from over 200 Erwinia strains (60 being of the subspecies carotovora) only one other strain, Ecc ATCC39048 showed susceptibility to the phage.

ϕKP is now being exploited in enzyme secretion studies in Ecc SCRI193.

CONCLUSION

Studies to date have led to the isolation of several phages able to infect Eca, Ecc and Echr strains.

In studying pathogenicity of Eca, a phage resistant mutant has been isolated which exhibits reduced virulence in potato plants. This reduced virulence appears to be due to an alteration in the cell surface of the mutant possibly OMP or LPS. These possibilities are presently being investigated. Work also in progress is the complementation and cloning of the relevant gene(s) to allow identification of products presumably important for phage susceptibiliy and virulence.

ϕKP, a phage capable of generalized transduction, is already being exploited in the analysis of enzyme secretion in Ecc SCRI193.

ACKNOWLEDGEMENTS

This work was supported by CEC/BAP contract No. BAP-0191-UK(H1) and Warwick University Research and Innovations Fund, and was done under MAFF licence number PHF 248/37(27), Plant Pests (Great Britain) Order 1980. I.K.T. was supported by an SERC research studentship.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

BACTERIAL SOFT ROT OF HYACINTHS AND TRIALS OF ITS CONTROL

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ABSTRACT

Bacterial soft rot of hyacinth bulbs is one of the factors limiting production of this plant in Poland. Investigations on the etiology of this disease showed that the bacteria causing the soft rot of hyacinths belongs to the species Erwinia carotovora subsp. atroseptica.

Among cultivated hyacinth varieties there are differences in susceptibility to this disease. Investigations conducted in the laboratory showed that the most susceptible varieties were: Colosseum, Jan Bos, Anne Marie, Gypsy Queen, Blue Jacket, Blue Giant and Carnegie, while the varieties: Grow Princess, Lady Derby and Scarlet Pimpernel appeared more resistant.

Laboratory experiments on the effectiveness of several compounds used protectively against soft rot of hyacinths showed that technical streptomycin (75% of a.i.) as well as S-0208 (Sumitomo), both in concentrations of 0.1% and 0.3% completely inhibited the disease development. Copper compounds (Miedzian 50, Champion WP) in concentrations of 0.5% and 1% did not protect bulbs against soft rot.

INTRODUCTION

Soft rot caused by Erwinia carotovora subsp. atroseptica is one of the three known bacterial diseases occurring on hyacinths. The disease was first noted by Heinz in 1889 (6). In Poland, the disease was first observed in 1988, however it seems that soft rot was one of the factors limiting earlier hyacinth production.

This paper reports the results of investigations on some aspects of disease epidemiology and trials of its control.

MATERIAL AND METHODS

The bulbs of hyacinths showing symptoms of soft rot obtained from commercial plantations near Warsaw, Poland were examined.

Isolation of bacteria

The bulbs were disinfected by soaking in 70% ethanol for 1 minute. The scabs from the infested tissue were taken and macerated in 1 ml of sterile water. Macerate was seeded on nutrient agar enriched with 5% saccharose. After 48 hours of incubation at 26 ^{o}C the growth of bacteria was observed.

Pathogenicity tests

The base, apical and lateral parts of healthy bulbs were inoculated by pricking with an entomological needle dipped previously in a water suspension of tested bacterial isolate. The same bacterial suspension was placed on 0.5 cm thick disks obtained from the bulbs cut transversely. The tests were conducted in a high humidity chamber at a temperature of 26 ^{o}C. Observations of disease symptoms were made after 48 h.

Identification of isolated bacteria

Isolated bacteria were identified using physiological and biochemical tests described in the monographs and keys specified by Graham (3), Dye (2), Lelliott and Stead (4), Thomson et al. (8), and Dickey (1).

Varieties susceptibility

Susceptibility of ten hyacinth varieties was estimated in laboratory conditions. In three separate series of experiment hyacinth scales were used. For each variety in each series 20 scales (5 in 4 replicates) were tested. The hyacinth scales were inoculated by pricking with an entomological needle immersed in E.carotovora subsp. atroseptica growht and then placed in moist chamber. After 48 and 72 hours the size of a necrose developed around the wound was measured. The data was subjected to analysis of variance; for separation of means the Duncan's multiple range t-test at 5% significance was used.

Bactericide effectiveness

Hyacinth bulbs were disinfected according to the method used for pathogenicity tests and then cut transversely. Obtained discs were dipped in a

suspension of a tested bactericide (10 discs in 4 replicates) and placed on filter paper. After 6 hours the discs were inoculated by spraying with a water suspension of Erwinia carotovora subsp. atroseptica using an atomizer. Two kinds of control were provided; discs treated only with water and discs only inoculated. After one, three and seven days the necroses were evaluated using the 7-degree scale (0 - healthy, 6 - total rotted). Results obtained were statistically analysed using the method described above.

RESULTS AND DISCUSSION

Colonies of the bacteria isolated from the infected bulbs were grayish-white, round, smooth, opaque and mat.

All isolates were pathogenic to hyacinth bulbs and caused symptoms of soft rot similar to those observed in nature. The check bulbs treated with sterile water remained healthy and did not show any discoloration.

Bacteria causing soft rot of hyacinths were identified according to their biochemical and physiological characteristics as Erwinia carotovora subsp. atroseptica. The positive characteristics were found: acid production from lactose, glucose, salicin, maltose, trehalose; potato soft rot, fermentative metabolism of glucose, gelatine liquefaction, growth in 5% NaCl, reducing substances from sucrose, nitrate reduction, catalase production. The negative characteristics found were: reaction to Gram Stain, pigment on King B and NA, oxidase, growth at 36 ^{o}C, indole, levan, spore production, starch hydrolysis.

Among investigated hyacinth varieties significant differences in susceptibility to the soft rot were found (Table 1). The most susceptible were: Collosseum, Jan Bos, Anne Marie, Gypsy Queen, Blue Jacket, Blue Giant and Carnegie, while Grow Princess, Lady Derby and Scarlet Pimpernel appeared more resistant. The necroses of the first group of varieties, after 48 and 72 hours were approximately twice as high if compared with the group showing higher resistance.

Table 2 presents the results of experiments on evaluation of some chemicals against soft rot of hyacinths. Streptomycin and S-0208 (Sumitomo) showed excellent protective efficiency. The bactericide S-0208 looks very promising because the use of the antibiotics as pesticides is prohibited in Poland. The high effectiveness of this bactericide was also demonstrated in control of soft rot of cabbage caused by Erwinia carotovora (7) and fire-blight caused by Erwinia amylovora (5). Copper preparations (Miedzian 50

Table 1 Susceptibility of hyacinth varieties to bacterial soft rot (Erwinia carotovora subsp. atroseptica)

Variety	Size of necrosis (in cm) After 48 h	After 72 h
Colosseum	1.8 d	2.7 d
Jan Bos	1.8 d	2.6 d
Anne Marie	1.6 d	2.5 d
Gypsy Queen	1.4 cd	2.4 cd
Blue Jacket	1.6 d	2.4 cd
Blue Giant	1.3 cd	2.3 cd
Carnegie	1.0 bc	2.0 c
Grow Princess	0.8 b	1.4 b
Lady Derby	0.8 b	1.3 b
Scarlet Pimpernel	0.8 b	1.3 b
Check (water)	0.0 a	0.0 a

Notice: Means followed by the same letter(s) do not differ significantly at 5% level of probability; evaluation of significant differences was made separately for each time of evaluation.

Table 2 The mean rating of soft rot (Erwinia carotovora subsp. atroseptica) development on hyacinth bulbs after protective treatment with chemicals

Treatments		Time of evaluation 1 day	After 3 days	7 days
Champion WP	0.5%	0.00 a	2.50 c	4.68 b
Champion WP	1.0%	0.00 a	2.55 c	4.33 b
Miedzian 50	0.5%	0.00 a	1.88 b	4.48 b
Miedzian 50	1.0%	0.00 a	1.98 b	4.50 b
Streptomycin	0.1%	0.00 a	0.00 a	0.00 a
Streptomycin	0.3%	0.00 a	0.00 a	0.00 a
S 0208	0.1%	0.00 a	0.00 a	0.00 a
S 0208	0.3%	0.00 a	0.00 a	0.00 a
Check (water)		0.00 a	0.00 a	0.00 a
Check (inoculation only)		1.95 a	5.35 d	5.90 c

Notice: see Table 1.

and Champion WP) did not protect hyacinths against bacterial infection. The necrosis development on discs treated with them was very high after 7 days, however it was significantly lower if compare with non-treated discs. Thus, it seems that bactericides based on copper compounds can be used in rotation with other pesticides in control of bacterial diseases of ornamentals.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

PSEUDOMONAS SYRINGAE PV. ATROFACIENS - THE AGENT OF BACTERIOSIS OF CEREALS IN UKRAINE

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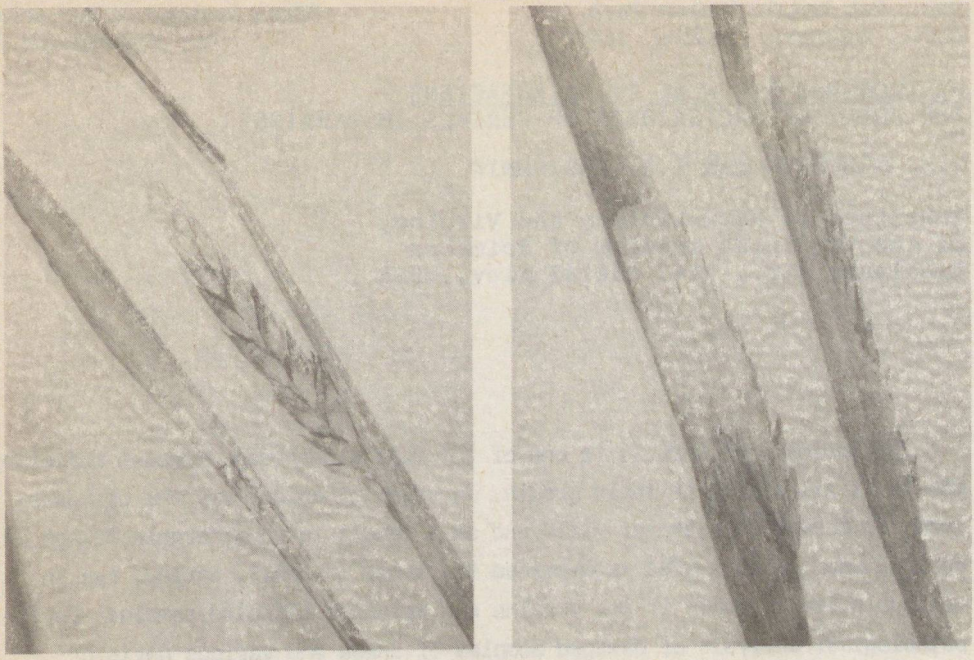
Bacteriosis of cereals is one of the most widespread diseases infecting plants at all stages of their growth. In years of epiphytoty the diseases lead to marked decrease of yield and as a result to considerable damage in agriculture. In 1971-1986 we examined the crops of wheat, barley, rye in various climatic zones in the Ukraine and selected affected samples. Phytopathological analysis of damaged samples of seeds and various parts of vegetating plants revealed bacterial infection on seeds, seedling leaves and various parts of vegetative plants. Bacteriosis was registered on all three cultures in all examined regions. The rising temperature and air humidity markedly influenced its spread in nature.

Under natural conditions the agent causes weak spot formation on leaves and seldom, rotting of node of bushing out that leads to seedling death. Later on, at stages of bushing out and ear formation spots formed on plant leaves and stems and at milky stage - on ear scale. At this stage wheat ear is mostly infected; rye developed leaf spots. In infected plants the ear length shortens or it is not formed at all. Infected seeds give inhibited plants. At the early stage of growth the damaged tissues develop small, watery, transparent, whitish or light-gree spots. Later on they increase in size and spread along leaf or stem acquiring yellowish or brown colour. The harmful action of bacteria influences seed germination or causes different levels of damage in plants. As a result yield decreases and its quality deteriorates. The plant inoculation with bacterial suspension leads to spot formation of various colouring similar to that in nature. Light-brown or brown spread spots was the most frequent. The plant was often stunted and did not form ears.

When studying species resistant to bacteriosis on small test plots, we established that in the Ukraine the following species of winter wheat:



A B

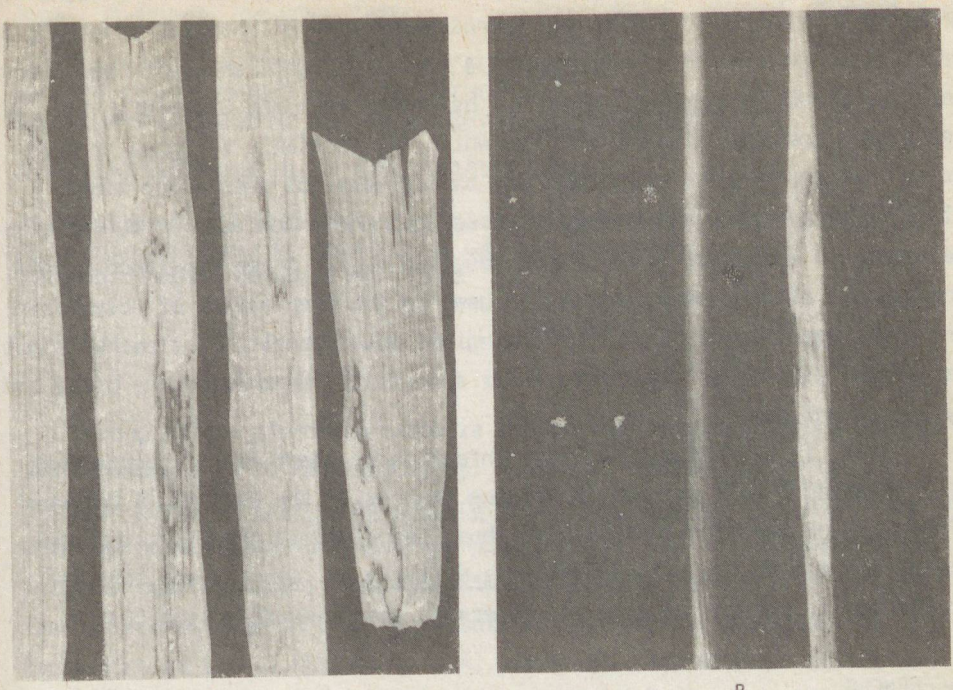
Fig. 1. Plants of wheat infected with Pseudomonas syringae pv. atrofaciens
A - natural infection
B - artificial infection

Priboi, Volna, Polesskaya 71, Ivanovskaya 6, Veselopodolyanskaya 80, Sche raya, Mironovskaya yuvileynaya 10, 25 were less damaged. Among rye specie such as: Ukrainskaya tetra, Polesskaya tetra, Belorusskaya 23, Verhnyach- skaya 32, Desnyanka 2, Slavutich were more resistant to bacteriosis; amon barley species such species as Donetsky 4, Nutans 518, and also Elgina, Chernomorets, Vinnitsky 7, Karlsberg, Dnepropetrovsky 425, Abava, Donetsk and 8, Slavutich, Ustinovsky 70 were less damaged.

At artificial crossing infection isolates from all three cultures infected rye, wheat, barley, oats, millet, sorghum, sudan grass and also lilas, the hostplant of Pseudomonas syringae.

A study of morphological, cultural-biochemical, pathogenic and sero- logical properties (70 tests) or isolates from wheat, barley and rye enab characterizing them, to establish strain heterogeneity limited to species

A study of serological properties of the agent of bacteriosis in whe barley and rye by agglutination method, precipitation and ELISA-method revealed that identical (by biochemical characteristics) isolates from th



A B

Fig. 2. A - leaves of rye infected with bacterial leaf blight
B - barley stem inoculated by Pseudomonas syringae pv. atrofaciens



Fig. 3. Cell of Pseudomonas syringae pv. atrofaciens
under electron microscope

cereals appeared to be heterogeneous in serological aspects. The strains under study were divided into four serological groups (4-6, 11) according to group-specific antigenic complexes by a serogrouping of phytopathogenic bacteria of genus Pseudomonas worked out by Pastushenko and Simonovich (1). We isolated a group of strains from rye which was initially assigned to serogroup 1 where cultures from wheat and barley were not presented. Among other serogroup isolates belonging to serogroup 1 is dominant on rye.Strains belonging to serogroup 5 are seldom observed on all three mentioned plants. The members of this serogroup can be used in the preparation of polystrain serum for agent identification. Serological heterogeneity of strains is not associated with their virulence, their specificity depends on the place and type of plant damage.

Thus, it was established that infection by identical microorganisms is widepsread in all cereals in various regions of the Ukraine. A comparative study of isolated strains with those described in the literature permitted identifying them as Pseudomonas syringae pv. atrofaciens that is completely in accordance with the generally used classification (2).

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

Plant Pathogenic Bacteria Z. Klement (ed.)

THE EFFECT OF NITROGEN NUTRITION SOURCES ON THE DEVELOPMENT OF THE PATHOGENIC AND NON-PATHOGENIC BACTERIA ERWINIA CAROTOVORA

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Among potato affecting bacteria the microorganisms of genus Erwinia are considered to be most injurious. A typical representative of this pathogen group is Erwinia carotovora subsp. atroseptica (E.c.a.), a causal agent of potato plant blackleg and potato tuber soft rot. Isolating bacteria in pure culture it was noted that a considerable part is characterized as non-pathogenic properties but they are mostly characterized by properties as E.c.a. (Lazarev, 1984). Although comprehensive literature is available concerning this pathogen group, the influence of nitrogen nutrition sources on the development of pathogenic and non-pathogenic bacteria E.c.a. has not been adequately investigated. Therefore the aim of our experiments was to investigate the effect of nitrogen nutrition on growth of microorganisms E.c.a. with different pathogenicity.

MATERIALS AND METHODS

To investigate the microorganism development in liquid medium the Omelyansky synthetic mixture of following content was used (g/l): K_2HPO_4 - 1.0 $MgSO_4$ - 0.5 $CaCl_2$ - 0.1 $(NH_4)_2HPO_4$ - 1.0 NaCl and $FeSO_4$ - traces, glucose - 10.0, distilled water - 1000 ml pH 7.0 (Omelyansky, 1940). This medium served also as a base for composing a number of subsequent mineral media. The organic medium, MPB, peptone water with nitrate or sodium nitrite were used to compare with mineral media. The experiments were conducted in glass 50 ml vials with 20 ml of culture medium to investigate gaseous products (CO_2, O_2, H_2, N_2O) of bacterial activity, as we have shown earlier (Lazarev, Chernyaeva, 1986). The pathogenic (P884, S78, G784) and non-pathogenic (P880, S853) strains E.c.a. served as test-organisms.

Table 1 The effect of nitrogen nutrition sources on the gas-exchange of pathogenic and non-pathogenic strains of Erwinia carotovora subsp. atroseptica and on medium reaction

Nutrient medium	O_2 utilization		CO_2 release		H_2 release		pH of the medium	
	mg/ml of cultural medium ($\times 10^{-2}$)							
	S78 ** / G784	P880 / S853	S78 / G784	P880 / S853	S78 / G784	P880 / S853	S78 / G784	P880 / S853
$(NH_4)_2HPO_4$ *	15.3 / 17.4	36.2 / 44.0	17.1 / 15.0	79.8 / 83.6	0.78 / 0.75	2.90 / 3.00	4.73 / 4.66	5.40 / 5.47
$NaNO_3$ *	13.3 / 17.8	15.6 / 18.8	12.3 / 11.2	19.3 / 18.2	0	0	5.16 / 5.18	5.47 / 5.40
$NaNO_2$ *	14.2 / 14.9	17.0 / 15.3	9.7 / 9.7	19.8 / 15.0	0	0	5.11 / 5.22	5.43 / 5.45
N source is absent *	13.8 / 9.9	15.6 / 16.7	1.9 / 1.9	3.5 / 3.8	0	0	6.43 / 6.50	5.38 / 5.02
MPB	19.2 / 23.8	30.2 / 32.3	22.5 / 22.5	32.5 / 32.2	+	+	5.81 / 5.82	6.52 / 6.65
NPB + $NaNO_3$	20.9 / 18.8	20.9 / 21.3	34.3 / 30.5	24.7 / 29.5	0	0	6.55 / 6.48	6.10 / 6.20
MPB + $NaNO_2$	23.1 / 19.9	21.3 / 24.6	33.8 / 32.5	26.2 / 29.9	0	0	6.28 / 6.32	6.00 / 6.27

* - the basal layer - Omelyansky's medium;

** - S78 and G784 - pathogenic strains, P880 and S853 - non-pathogenic strains;

(+) - H_2 release

RESULTS

The results of investigations represented in Table 1 show that E.c.a. strain pathogenicity and the culture medium composition have a great effect on respiration rate and medium oxidation level. On all compositions of mineral origin and MPB the respiration occurred more energetically with non-pathogenic microorganisms. In the Omelyansky medium non-pathogenic bacteria utilize O_2 2-3 times and release CO_2 4-5 times more than pathogenic ones. Although the pathogenic bacteria was characterized by a weaker development they produced acetic acid 3-3.5 times more and oxidized a medium better than the non-pathogenic ones. Thus, its proportion fluctuated from 0.118-0.138% for non-pathogenic to 0.415-0-455% for pathogenic strains. In substituting ammonium for nitrate nitrogen form it was observed, that non-pathogens increased acid production (0.293-0.309%) and pathogens declined acetic acid production (0.305-0.374%).

The data in Table 1 show that the bacteria E.c.a. on the Omelyansky medium release molecular hydrogen, its production by non-pathogenic strains being 4 times higher than by pathogenic ones. In spite of the glucose availability in the medium as a donor of H_2, the presence of $NaNO_3$ or $NaNO_2$ inhibited the production of this gas. Therefore, in the course of denitrification by microorganisms the bacteria lose their ability to release H_2. The hydrogen production was not noticed on nitrogen-free medium that is probably releated to slow growth of strains. The pH of 6.8 (control; non-plated) changed to 6.4-6.5.

When the MPB as a nutrient medium was used a more intensive gas exchange was registered than in non-pathogenic strains. But in variants such a significant difference in respiration activity between pathogenic and non-pathogenic organisms was absent in comparison with the Omelyansky medium. The addition of $N\text{-}NO_3$ or $N\text{-}NO_2$ to the organic medium decreases this difference to a greater extent between the above mentioned bacteria groups. In this case the pathogenic strains were observed to decrease and non-pathogenic strains to increase gas exchange.

Besides, we found that with $NaNO_3$ nitrogenous oxide appeared without nitrite demonstrating E.c.a. ability to assure "nitrate respiration" followed by the reduction of NO_3 to NO_2 and then N_2O. Nitrous oxide production by a number of microorganisms particularly E.c.a. was pointed out in the literature (Bleakley, Tiedje, 1982).

It is known that pathogenic and non-pathogenic organisms of E.c.a.

Table 2 The effect of various C- and N-nutrition sources on the gas exchange of bacteria Erwinia carotovora subsp. atroseptica

Gaseous characteristics of bacteria	Medium (C and N source)*					
	$(NH_4)_2HPO_4$ + glucose	1/2 $(NH_4)_2HPO_4$ + 1/2 $NaNO_3$ + glucose	$(NH_4)_2HPO_4$ + glycerol	$(NH_4)_2HPO_4$ + citrate Na	$(NH_4)_2HPO_4$ + peptone	MPB
P884/G784**						
utilized: O_2%	4.5 / 5.5	2.8 / 3.1	3.2 / 4.9	7.9 / 8.0	4.4 / 2.9	7.1 / 6.4
released: CO_2%	19.5 / 12.9	9.1 / 6.6	4.9 / 3.5	28.7 / 27.2	1.6 / 1.6	15.2 / 14.5
H_2%	0.98 / 1.04	0	0	0	0	1.00 / 0.81
P880/S853						
utilized: O_2%	11.8 / 11.8	7.9 / 2.0	9.1 / 5.9	11.7 / 5.5	8.6 / 4.2	7.2 / 7.2
released: CO_2%	60.5 / 29.6	10.5 / 13.1	12.3 / 15.6	33.2 / 32.7	5.2 / 3.2	21.2 / 20.6
H_2%	4.16 / 7.70	0	0	0.71 / 1.22	0	3.81 / 3.43

* - the basal layer - Omelyansky medium;

** - P884 and G784 - pathogenic strains;

P880 and S853 - non-pathogenic strains

ferment carbon nutrition sources to varied extents and rates (El-Khatib, 1977; Lazarev, 1984; et others). It is shown that these bacteria, growing in glycerol and inositol, do not produce any gas. Therefore, the following series of experiments was conducted on the Omelyansky medium using both different nitrogen and carbon nutrition sources, and also for composition with MPB was applied. From results of these studies (Table 2) it can be seen that non-pathogenic strains E.c.a. produce more active gas exchange in all media than pathogenic ones. The experimental data on CO_2 production show that in a medium with glycerol microorganisms grow quite slowly in comparison with the Omelyansky medium. The presence of CO_2 in all variants and absence of H_2 in some suggest that the gas, accumulating in Durham tube during fermenting is hydrogen. In variants with citrate Na active growth of pathogenic

bacteria was noted but nevertheless as opposed to non-pathogenic bacteria they do not produce H_2. Hydrogen is absent when the strains utilize peptone as a carbon and nitrogen source, that is, during the ammonification of proteins. The hydrogen production by microorganisms on MPB demonstrate the presence of carbohydrates in this medium.

Thus, it was found that forms of nitrogen and carbon nutrition in medium strongly influence the respiration process and the activity of life of different pathogenic bacteria strains E.c.a. In the presence or reduced form of nitrogen nutrition in cultural medium these microorganisms grow, utilizing oxygen intensively and releasing carbon dioxide and hydrogen. In the presence of $N\text{-}NO_3$ and $N\text{-}NO_2$ gas exchange indices are significantly transformed: the transpiration is inhibited (in non-pathogenic strains to a greater degree), the ability to produce hydrogen is lost and nitrous oxide appears as the products of the activity of life that demonstrates "the nitrate respiration" of microorganisms E.c.a.

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SESSION 7

AGROBACTERIA

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

Plant Pathogenic Bacteria Z. Klement (ed.)

CHARACTERIZATION OF THE AGROBACTERIUM TUMEFACIENS STRAINS OF DIFFERENT NATURE

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The bacterial diseases of plants, in particular, bacterial canker caused by A. tumefaciens is widespread and is extremely harmful (1).

A tumefaciens injures nearly all sorts of grape, fruit trees, ornamental cultures, various species of trees and bushes grown in forests. Despite the great developments of A. tumefaciens genetics, molecular biology and gene engineering many questions on taxonomic position and biology of the inducer still remain to be elucidated.

The objective of this work is a comparative study of biological properties of A. tumefaciens virulent and avirulent strains isolated from diseased plants from various climatic zones of the USSR and some other countries.

The subject of the study is strains of A. tumefaciens (92), A. radiobacter (2), A. rubi (1) received from the Collection of Plant Pathogenic Bacteria of the Institute of Microbiology and Virology, UkrSSR Academy of Sciences and Armenia (Dr. Nagapetyan), Georgia (Prof. Palavandashvili), Krasnodar region (Dr. Samus), Moldavia (Dr. Lemanova), Moscow (Dr. Chumaevskaya), Greece (Prof. Panagopoulos), Czechoslovakia (Prof. Kotsur) and USA (ATCC).

MATERIALS AND METHODS

Bacteria were isolated from tumour-affected tissues of plants (2). Pathogenic properties of bacteria were tested with indicator-plants under the field, greenhouse and laboratory conditions (3). The culture, morphological, physiological and biochemical properties of A. tumefaciens by methods reported earlier (2, 4, 5, 6, 7, 8, 9).

Presence, quantity and location of flagella on A. tumefaciens cells were studied by YEMV-100A electron microscope at 8000-12000 magnification. The quantitative content of polyamines in bacterial cells was defined by the modified method (10), the antigenic properties of cells - by the methods (11), the matters of exocellular nature characterized by antigenic activity was isolated accordingly (12).

RESULTS AND DISCUSSION

Virulent properties of A. tumefaciens strains were studied with seven types of plants: tomato, sunflower, tobacco, haricot bean, horse bean, calanchoe, beat, and on disks of carrot root-crops. A significant percent of strains caused pathologic process in sunflower (94%), tomato (90.2%) beet was most resistant (6.6%).

In spite of the fact that A. tumefaciens affects almost all dicotylendonous plants (19), some strains show high specificity and are virulent to certain plant species only.

The tumour formation depends on the stage of development and age of plants. Thus, when A. tumefaciens strains 8628, 8996 infected root-neck of one-month old tomatoes, tumours formed but in case of infection in three-month old plants tumours were not detected in any of the large number of the plants. However, infection introduced in apexes of three-month old tomato plants leads to tissue spreading and tumour formation. The tissue of tomato fruit in the infection site necrotizes and acquires brown colour, but no tumour forms; these results do not contradict other literature data (14) on other plants.

The strains studied, mainly, did not differ from other strains described in the literature by a number of properties. There is no unified conception concerning the number of flagella and their location on cells (15). By means of electron microscopy it was shown that sometimes subpolar flagellation can be detected with A. tumefaciens cells. The majority of the A. tumefaciens strains, however, possess peritrichal cells, irrespective of virulence, source and age of culture. The presence of a large number of fimbria and well-shaped microcapsula was noted. When the A. tumefaciens strains were cultivated in the meat-peptone broth, and the Omelyansky medium the differences were not detected. It is shown that acid production by microorganisms during growth on media with carbohydrates is a stable sign, with some exception. All A. tumefaciens strains utilize amino

acids as a source of nitrogen nutrition, produce 3-keto-lactose, oxidase, use sodium citrate, grow at t^{o} = 35 ^{o}C.

The cultures studied did not produce gelatinase, indole did not reduce nitrate; varied properties - tirosinase and H_2S production, growth at 2 and 3% of NaCl in the medium, growth on potato agar at 38 ^{o}C.

The A. tumefaciens strains isolated from different plants, in different regions, belonging to different biotypes possess similar contents of polyamines. The polyamines in cellular extract from the strains are represented by spermidine and trace amount of putrescine.

The identical content of polyamines was found for cells of other Agrobacterium strains: A. radiobacter and A. rubi. Spermine was detected in no bacterial strains studied. Results of direct and cross reactions of agglutination and double diffusion in agar with antisera to 12 A. tumefaciens strains indicate the antigenic heterogeneity of agrobacteria.

The A. tumefaciens strains studied were divided into 6 serological groups, irrespective of biotype. It should be noted that some agrobacterial strains were not included in these 6 serogroups. Apparently, there are many more serogroups than we described.

Antiserum against exocellur substance of polysaccharide nature of the A. tumefaciens strain Ag 57 is characterized by definite specificity. The agglutination reaction on glass showed that agglutinines formed only with agrobacteria, but not with representatives of the genera Erwinia, Pseudomonas and Xanthomonas. The number of agglutinated strains, however, was 60%.

A polystrained antiserum was prepared in vitro by mechanical mixing of monoantisera against representatives of six serogroups. This polystrained antiserum agglutinated all studied agrobacteria, except A. rubi. Phytopathogenic bacteria of other genera, Erwinia, Pseudomonas, Xanthomonas, and bacteria of the genus Rhizobium were not agglutinated by this antiserum.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

ATTACHMENT OF AGROBACTERIUM RHIZOGENES TO HOST CELLS

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INTRODUCTION

Infections of some dicotyledenous plants with Agrobacterium rhizogenes result in hairy root disease. The mechanism of pathogenesis of this bacterium is the same as that of the closely related A. tumefaciens- the transfer of bacterial plasmid DNA to the plant host cell where this foreign DNA becomes integrated into the host chromosomes. This DNA codes for enzymes involved in plant hormone synthesis, auxin in the case of A. rhizogenes and auxin and cytokinin in the case of A. tumefaciens. It is the unregulated expression of these genes which results in the formation of hairy roots or crown gall tumors(1). In the case of A. tumefaciens the transfer of DNA from the bacterium to the plant cell requires bacterial attachment to the host cell surface. Bacterial mutants which fail to bind to plant cells are avirulent(2). The role of bacterial attachment in the induction of hairy root disease has received little attention.

MATERIALS AND METHODS

Bacteria were grown in Nutrient broth(Difco) at 25°. The transposon Tn5 was introduced into A. rhizogenes by conjugation from E. coli 1830 of the plasmid pJB3JI which carries Tn5 as previously described(3). Transconjugates containing Tn5 were selected on minimal medium containing 60ug/ml neomycin and screened for their ability to bind to carrot cells.

Carrot suspension cultures were grown in Murashige and Skoog(MS) medium. Early stationaly phase cells were diluted to 2-5x10^5 cells/ml and 2x10^6 bacteria/ml were added. Attachment of the bacteria to carrot cells was observed in the light microscope after 4 and 24 hours incubation. The time course of bacterial attachment was examined as previously described using 2x10^5 carrot cells/ml and 2x10^3 bacteria/ml(4).

In order to be certain that the mutant phenotype was due to the insertion of Tn5, the EcoRl fragment containing the transposon was cloned in E. coli. The cloned DNA containing Tn5 was then used to replace the wild type DNA in the parent strain A. rhizogenes A4PC by marker exchange and the phenotype of the resulting bacteria examined(5). For all four of the mutants studied the phenotype of the products of marker exchange was the same as that of the original mutant suggesting that the phenotype was due to a single mutation caused by the insertion of the transposon.

CHARACTERIZATION OF BINDING OF A. TUMEFACIENS AND A. RHIZOGENES TO HOST CELLS

The binding of biotype I strains of A. tumefaciens to plant cells has been studied extensively. It is not strongly dependent on the composition of the medium. The bacteria bound to carrot suspension culture cells in 4% sucrose, in Murashige and Skoog(MS) medium, in the presence of chelating agents, and in 0.25 M NaCl(2,6). In contrast the binding of A. rhizogenes to carrot cells was markedly affected by the composition of the medium(7). Although about 20% of the bacterial inoculum bound to carrot cells after 2 hours incubation in 4% sucrose, only about 2% of the bacteria bound in media with an ionic strength of 0.09M or greater(Table 1). In the light microscope bacterial binding to carrot cells was evident after 4 hours in 4% sucrose. Very few bound bacteria were detected after 4 hours in MS medium. A small amount of binding of the bacteria to carrot cells in MS medium was observed in the light microscope after 24 hours incubation. This binding consisted of about 5-10 bacteria bound to the average carrot cell and represented binding of less than 1% of the bacteria

present. It was difficult to determine if this binding was significant or if it represented nonspecific sticking of the bacteria.

The comparison of the attachment properties of A. tumefaciens and those of A. rhizogenes suggests that these two species of bacteria may use different mechanisms in binding to plant cells. It is unclear whether the binding of A. rhizogenes involves two different mechanisms: one using ionic charge and the other slower less efficient mechanism insensitive to ionic strength or whether the binding seen at higher ionic strength represents nonspecific binding. In order to examine this question and the role of the low and high ionic strength binding in bacterial virulence, bacterial mutants which were altered in binding to carrot cells were obtained.

Table 1. Effect of the medium on attachment of Agrobacterium strains to carrot cells.

Bacterial strain	Percent bacterial inoculum bound in: 4% sucrose	MS	0.05 M NaCl	0.25 M NaCl
A. tumefaciens				
A6	60±5	50±7	55±9	54±8
C58	30±5	24±6	28±8	30±6
A. rhizogenes				
A4PC	15±4	0±2	1±2	0±0
15834	19±6	0±1	1±1	3±3

$2\text{-}3 \times 10^3$ bacteria/ml were incubated with $2\text{-}5 \times 10^5$ carrot cells/ml. The number of attached and free bacteria was measured after 2 hr of incubation. The ionic strength of MS is 0.09 M. Numbers are given as mean ± standard deviation of a minimum of three experiments.

NONATTACHING MUTANTS OF A. RHIZOGENES

Transposon mutants of A. rhizogenes were screened individually for lack of attachment to carrot cells using a microscopic assay for attachment. Four mutants which showed altered binding were obtained. All four of the mutants showed reduced binding in 4% sucrose when number of bacteria bound was measured after 2 hours. However, one of the mutants, Att-325, showed increased binding to almost the wild type level after 4 hours incubation with carrot cells suggesting that it was delayed in attachment rather than unable to attach at all. The other 3 mutants showed no increase in attachment with increasing time of incubation with carrot cells in 4% sucrose.

Bacterial binding to carrot cells in MS medium was also examined. Since the binding of the parent strain in MS medium was very low(about 2% of the bacterial inoculum bound in 2 hours), it was very difficult to detect reduced binding of the mutants. Nevertheless two of the mutants(Att-310 and Att-325) showed less than 0.7% of the inoculum bound after 2 hours in MS medium. The other two mutants(Att-312 and Att-323) showed a slight but not significant reduction in binding in MS medium. Microscopic examination of binding in MS medium after 24 hours incubation suggested that mutant Att-310 was definitely reduced in binding. No reduction in the binding of the other three mutants was detectable in the light microscope.

Virulence of the mutants was tested on leaves of Bryophyllum diagremontiana and on carrot root discs. All four mutants were avirulent on both hosts.

CONCLUSIONS

The binding of A. rhizogenes to carrot cells appears to differ from that of A. tumefaciens in its sensitivity to ionic strength. The inhibition of attachment of A. rhizogenes by salt suggests that ionic or charge bonding plays a role in the attachment of these bacteria to carrot cells. This binding may play a role in the

transfer of DNA from the bacteria to host cells since mutants which failed to bind to carrot cells at low ionic strength were avirulent. It is difficult to determine the role of the small amount of bacterial binding seen at high ionic strength from the mutants analysed. All four of the mutants were avirulent and were reduced in binding at low ionic strength. Two of the mutants appeared to be reduced in their ability to bind to carrot cells at high ionic strength while the other two mutants appeared to be unaltered in binding in MS medium suggesting that there are both genes required for binding at both high and low ionic strength and that there are genes required only for binding at low ionic strength. It appears that the ability of the bacteria to bind to carrot cells in 4% sucrose is needed for virulence. However, in the absence of mutants which showed normal attachment in 4% sucrose and reduced attachment to carrot cells in MS medium the role of the bacterial binding seen at high ionic strength can not be determined. The avirulent mutant Att-325 which is delayed in attachment rather than simply unable to bind is intriguing and may suggest that there is an obligatory time sequence of events required for bacterial attachment and DNA transfer.

ACKNOWLEDGEMENTS

This research was supported by U. S. Department of Agriculture competitive research grant 85-CRCR-1-1902.

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

Plant Pathogenic Bacteria
Z. Klement (ed.)

ISOLATION OF AGROBACTERIUM TUMEFACIENS FROM APPLE ROOTSTOCKS WITH GROWN GALL DISEASE

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ABSTRACT

Efforts to control crown gall on apple rootstocks have been largely unsuccessful and attempts to understand the disease have been hampered by the inability to isolate *Agrobacterium tumefaciens* from rootstock tumors. New methods of isolation were therefore employed using opines as the sole carbon and/or nitrogen source in isolation media. Nine strains of *A. tumefaciens* have been isolated using opine media which were able to infect Emla 7 and domestic seedling rootstocks in greenhouse studies. Spontaneous mutants to antibiotics of the two most virulent strains, B49C (100 mg L^{-1} rifampicin) and D10B (50 mg L^{-1} kanamycin) were field tested in 1988 on Emla 7, Mark, and domestic seedling rootstocks. The incidence of infection was as much as 64% higher than on water treated controls. Eighty-two per cent of the strains isolated on rifampicin medium from trees inoculated with B49C were pathogenic as were 45% of the strains isolated on kanamycin from trees inoculated with D10B.

INTRODUCTION

Crown gall is a serious disease of apple rootstocks in Washington nurseries. Attempts to control the disease with chemical treatments or by using *Agrobacterium radiobacter* strain K84, which has been used widely to control crown gall in other woody plants (Kerr, 1980, Moore, 1977, 1988) have failed. A new rootstock (Mark) currently used by growers is especially susceptible to crown gall and has increased the need for understanding the nature of the disease.

A necessary step in establishing control of crown gall in apple is isolation of the causal agent, and demonstrating that this organism can reinfect in the field. Although numerous efforts have been made to isolate such pathogens, these attempts have met with failure (for a summary see Rossignol and Dion, 1985).

The purpose of this study was to use new methods of isolation from apple rootstock tumors in order to obtain and characterize *A. tumefaciens* strains that are consistently virulent in the field.

MATERIALS AND METHODS

Isolations. Bacteria were isolated from tumors or roots of field grown apple seedling and Emla 7 rootstock using minimal salts medium containing (g L^{-1}) K_2HPO_4, 10.5; KH_2PO_4, 4.5; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.01; $FeSO_4$, 0.005; $MnCl_2$, 0.002; Nobel agar, 20; octopine or nopaline, 0.15.

Pathogenicity testing. Bacteria were grown for 48 hours on mannitol-glutamate medium (Keane et al., 1970) with 1 g L^{-1} yeast extract (MGY), then inoculated to wounded stems of tomato (*Lycopersicon esculentum* c.v.'Bonnie Best'). Those strains that induced tumor formation four weeks after inoculation were tested on seedling and Emla 7 rootstock grown in pots in the greenhouse and Emla 7 plantlets maintained in tissue culture (Kyte, 1987).

Strain identification. Pathogens were identified biochemically using standard tests (Moore et al., 1988).

Sensitivity to Agrocin 84. *Agrobacterium radiobacter*, strain K84, which produces the antibiotic Agrocin 84, was spotted in the center of mannitol-glutamate agar plates and sensitivity of apple isolate strains to that compound was tested by the method of Stonier as modified by Cooksey and Moore (1982).

Opine utilization. The ability to utilize opines as sole carbon and nitrogen source was tested according to Bouzar and Moore (1987).

Field testing. Spontaneous mutants of B49C to rifampicin (100mg L^{-1}) of B49C and kanamycin (50mg L^{-1})of D10B were grown on the appropriate antibiotic amended MGY media and used to make suspensions of 10^7 cfu ml^{-1} Root-pruned Emla 7, Mark and domestic seedling rootstocks, 100 trees per site, were dipped into the suspension and planted in a random block design at two nurseries in Washington in April, 1988. All of the trees were dug in October, 1988 and examined for the presence of tumors. Isolations were made from tumors and roots on antibiotic amended MGY media.

RESULTS

Nine strains were isolated on opine media which were pathogenic when inoculated to seedling and Emla 7 rootstocks in the greenhouse. (Table I and Fig. 1). Four of these strains, D10B, B49C, B74A

TABLE I. Characteristics of *Agrobacterium tumefaciens* strains isolated from apple rootstocks

Strain	Host[a]	Biovar	Sensitivity to Agrocin 84	Opine utilization
B47A	seedling[b]	II	-	mannopine and nopaline
B49C	"	"	"	"
B49E	"	"	"	"
B72B	"	"	"	nopaline
B72C	"	"	"	mannopine and nopaline
B74A	"	"	"	mannopine
B79A	"	"	"	"
BI00B	"	"	"	nopaline
D10B	Emla 7	"	"	"

a) Plant host from which bacteria were isolated b) Rootstocks grown from seed

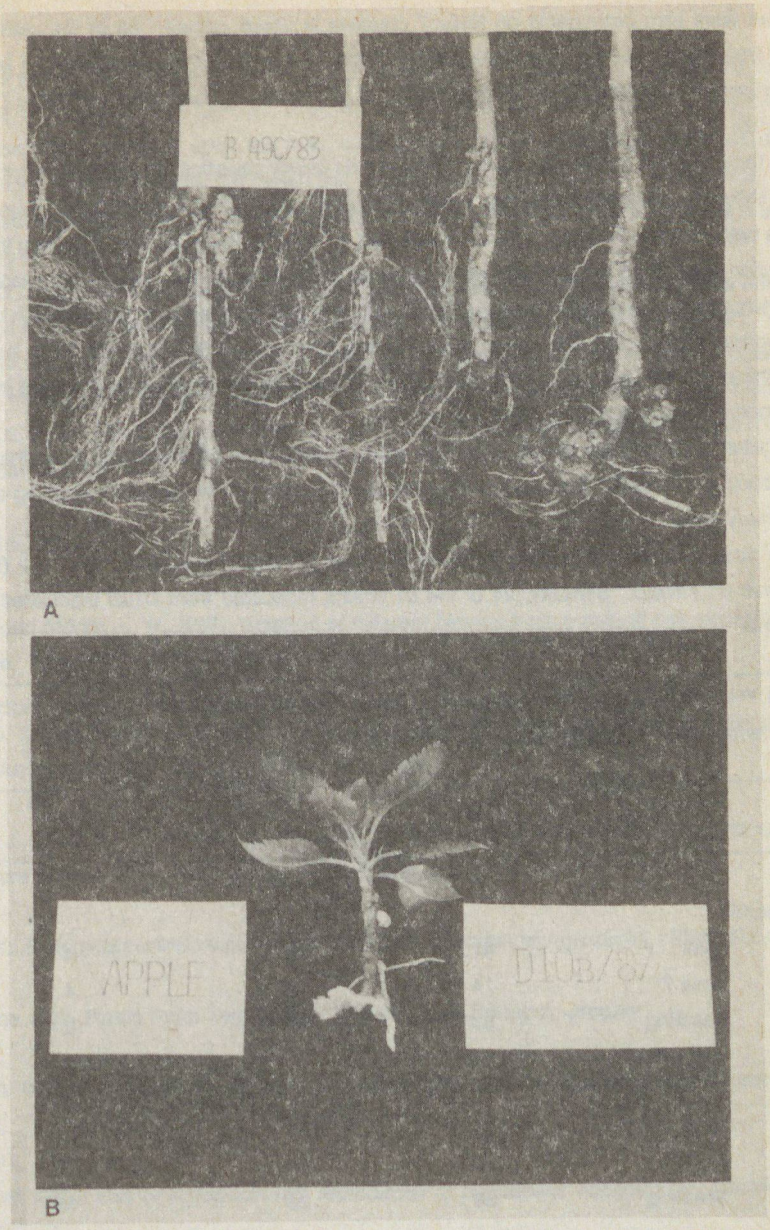

Fig. 1. a) Emla 7 (on the left) and seedling (on the right) rootstocks six months after being infected with B49C in greenhouse pot trials and b) Emla 7 plantlet grown in tissue culture four weeks after being inoculated with D10B.

and B79A were also pathogenic on Emla 7 plantlets in tissue culture. All of the pathogens were isolated from tumors rather than roots. Eight of the 38 *Agrobacterium* strains (21%) isolated from seedling tumors were pathogenic, whereas only 1 of 172 (0.6%) of the isolates from Emla 7 tumors was pathogenic.

Even though the *A. tumefaciens* were similar in their biovar classification and insensitivity to Agrocin 84, they differed in the opines that they could utilize and four of the nine strains had the unusual ability to utilize both mannopine and nopaline. There was no direct correspondence between the opines in the isolation media and the opine(s) which were utilized by the purified strains. For example, even though octopine was one of the opines used as a carbon and nitrogen source in isolation media, none of the isolates tested used this opine. However two mannopine utilizing strains were isolated, even though this opine was not provided in the media and some of the nopaline strains were isolated from plates containing only octopine (data not shown).

The incidence of infection in field sites was consistently higher on inoculated trees than on water-treated controls (Table II). Mark rootstock exhibited higher infection rates than seedling or Emla 7 at both nursery sites.

Of 74 strains recovered from galls from the field on B49C inoculated trees, 61 (82%), were pathogenic on tomato. Similarly, 24 of the 61 strains recovered from D10B inoculated trees were pathogenic.

TABLE II. Percent of three rootstocks showing tumor formation at two field sites after inoculation with *Agrobacterium tumefaciens* strains B49C and D10B.

	D10B[a]	B49C[a]	Controls[b]
Site 1			
Rootstock:			
Mark	41	20	4
Emla 7	14	17	4
Seedling	31	35	0.4
Site 2			
Mark	85	85	21
Emla 7	43	33	12
Seedling	38	28	13

a) 100 rootstocks root-pruned and dipped in 10^7 cfu/ml of a bacterial suspension prior to planting
b) 100 rootstocks root-pruned and dipped in water prior to planting

DISCUSSION

The use of opines in isolation media was an effective method for obtaining *A. tumefaciens* from both seedling and Emla 7 rootstock tumors. Even so, there was a marked difference in the percent of pathogens isolated depending upon the host, with a much higher percentage being isolated from seedling tumors. Growers report that crown gall is more severe in Emla 7 than is seedlings, yet only one of 172 isolates from this host was pathogenic. Modifications of the existing selective media may be necessary to isolate pathogens in the future.

Both B49C, isolated from a seedling tumor, and D10B, from an Emla 7 tumor, were effective in giving a high incidence of infection on seedling, Emla 7 and Mark rootstocks in the field. Whether these strains are representative of strains which cause natural infections in apple rootstocks is unknown. If these strains, along with the other seven pathogens isolated in this study, are typical, their insensitivity to Agrocin 84 could explain the lack of biological control of crown gall in apple rootstocks by A. radiobacter strain K84.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

ANALYSIS OF GC-FAME FOR IDENTIFICATION OF AGROBACTERIUM ISOLATES FROM NATURE

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ABSTRACT

Gas-chromatographic analysis of fatty acid methyl esters (GC-FAME) was performed on Rhizobiaceae strains to determine whether putative agrobacteria isolated from diverse geographic regions and host plants could be identified rapidly. The accuracy of the method was evaluated using database versions 1.1 and 3.0 of the Hewlett-Packard Microbial Identification System (MIS). Cluster analysis was performed on GC-FAME data to assess relationships among strains. GC-FAME identified 44/52 strains (85%) as *Agrobacterium*. The mean value of the ID coefficient with version 3.0 was 0.581 compared to 0.344 obtained with version 1.1. The number of strains with "no match to database" also dropped from 7 to zero upon analysis with version 3.0. Cluster analysis of ID coefficients, based on "fit to the taxon", generated three major groups of *Agrobacterium* strains. Two strains of *Rhizobium meliloti* were misidentified as *Agrobacterium*, due in part to the diversity of fatty acids within the membranes (coefficients ranged from 0.001 to 0.897) of *Agrobacterium* strains. This diversity of membrane fatty acids highlights the need for a more complete database of fatty acid profiles from members of the Rhizobiaceae before GC-FAME can be used for rapid identification of *Agrobacterium*.

INTRODUCTION

Members of the genus *Agrobacterium* are classified typically on the basis of cultural, morphological, physiological, and pathological tests. These tests are often laborious, time-consuming, and some require days to weeks before they can be read. Our ecological studies of phenonmena such as survival and dispersal of *Agrobacterium* spp. have been hampered by these time-consuming methods of identification, and we have been compelled to search for more rapid and accurate means of identification. One line of research resulted in the development of antisera against 50 S ribosomal subunits (Bouzar, et al., 1986) which proved useful for identification of *Agrobacterium* isolated from a natural habitat (Bouzar and Moore, 1987). The objective of the present research is to determine whether gas chromatography fatty acid methyl ester (GC-FAME) analysis, used successfully with other bacteria (Drahos, et al., 1986; Hemming,

1988; Miller, 1982), would prove accurate and rapid for identification of naturally-occurring *Agrobacterium* strains.

MATERIALS AND METHODS

Strains and culture conditions

Seventy-seven putative strains of *Agrobacterium* and two strains of *Rhizobium meliloti* were selected for gas-chromatographic analysis of their fatty acids. Strains were streaked first for purity, and single colonies were selected for culture on trypticase soy broth agar (TSBA). The cultures were incubated at 27°C for 24 ± 2 hours before extracting their membrane fatty acids.

Extraction of fatty acids

Bacteria were harvested and processed according to the method prescribed by Miller (1982). Briefly, a heaping loopful of cells was scraped gently from the agar surface with a 4mm inculating loop and placed in a screw cap test tube. Fatty acids were extracted from whole bacterial cells by saponification with sodium hydroxide in aqueous methanol and acidified with hydrochloric acid in methanol to methylate the freed fatty acids. The methylated fatty acid esters were extracted with an equal voume mixture of hexane and methyltert butyl ether and washed with sodium hydroxide to remove residual acid and reagent.

Gas-chromatographic analysis of fatty acids

Fatty acid analyses were performed with a Microbial Identification System (MIS) gas chromatograph (Hewlett-Packard 5898A) equiped with a 50mm x 0.2mm methyl silicone fused silica capillary column. Hydrogen gas was used as a carrier to increase sensitivity of resolution. Data were collected using version 1.1 of the MIS data base and compared to the hypothetical mean-organism for the taxon *Agrobacterium*. Based on this comparison, a similarity coefficient (ID coefficient) was calculated for each unknown strain. An ID between 0.6 and 1.0 illustrates an excellent match. All data were anaylyzed later with a new version 3.0 MIS data base updated with data from additional *Agrobacterium* strains. The GC-FAME data were subjected to cluster analysis to assess visually the relationships among strains.

RESULTS

Forty-four of 52 (85%) reference and putative *Agrobacterium* strains were identified as *Agrobacterium* by GC-FAME using the Hewlett-Packard MIS. An additional 27 strains could not be

compared to the MIS database because of the strict MIS requirement that test strains be grown on TSBA. The 27 strains either grew poorly or peak matching at sufficient sensitivity was too low for reliable analyses. This problem should be overcome with recent software now available from Microbial ID, Inc. This software will permit construction of a personalized database using media of one's choice.

The recently expanded database (H.P.'s Version 3.0) improved the ability to identify *Agrobacterium* strains (Table 1). The mean value of the ID coefficient with verison 3.0 was 0.581 compared to the lower 0.344 obtained with verison 1.1. In addition, the number of "no match" (nonidentity) changed from 7 to 0.

Table 1. Partial list of strains examined by GC-Fame and analyzed by comparison to both database versions 1.1 and 3.0

Strain	I.D Coefficients for fit to *Agrobacterium* Ver. 3.0	Ver 1.1	Host Plant	Geographic Origin
N 2/73	0.876	0.027	Raspberry	OR
C 5/73	0.782	0.038	Mt. Ash	OR
G 26/79	0.723	0.019	Weeping Willow	TX
5/2	0.530	no match	Grape	Hungary
S 7/73	0.827	0.029	Lippia	AZ
B 49C/82	0.026	no match	Apple	WA
T 3/73	0.041	no match	Rose	MO
J6/79	0.596	0.652	Apple	Libya
I 13/80	(0.760)	(0.371)		Bolivia
C58	0.539	0.121		NY
M 50/79	0.873	0.810	Cottonwood	OK
CG 62	0.371	0.420	Olivette NOIR (grape)	NY
YA 15	0.104	0.049	alfalfa (*R. meliloti*)	OR
F 15/79	0.452	0.492	Gypsophila	FL

( ) parentheses indicate strain identified to Enterobacter (or *Erwinia herbicola*) rather than *Agrobacterium*.

There was a wide diversity of fatty acid compositions within the membranes of the *Agrobacterium* strains examined (ID coefficients ranged from 0.001 to 0.897). Both strains of *Rhizobium meliloti* in this study were identified as *Agrobacterium*, demonstrating again the close relationship of these genera. These findings highlight the need for further comparative examination of strains of Rhizobiaceae for distinction between *Agrobacterium* and *Rhizobium* by GC-FAME procedures.

Cluster analysis on the basis of fit to taxon (as represented by the fatty acid profile ID coefficients) identified three major groups of *Agrobacterium* strains. Representative strains of each group can now be chosen for more extensive characterization and comparison to see if they correspond to the current biovar phenotypes.

Gas-chromatographic identification of methlyated fatty acids from the membranes of bacteria is rapid. Using this procedure, fifty samples could be prepared per hour. Samples could be stored safely up to 2 weeks under refrigeration before analysis. Based on a thermal gradient run requiring approximately 25 minutes/per sample, 48 samples could be processed unattended every 24 hours.

SUMMARY

GC-Fame identified rapidly and correctly 44/52 (85%) test strains as *Agrobacterium*. Twenty seven additional strains could not be compared to the MIS database because they failed to grow on TSBA, but new software is now available which will allow construction of personalized databases using media of one's choice. This approach appears promising as evidenced by the improved ability to identify *Agrobacterium* strains with Hewlett-Packard's recently expanded database (Version 3.0).

Fatty acid composition within membranes of the *Agrobacterium* strains was widely diverse (ID coefficients from 0.001 to 0.897). However, only three major groups of *Agrobacterium* strains were generated by cluster analysis of the ID coefficients derived from this study

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

Plant Pathogenic Bacteria Z. Klement (ed.)

INFECTION PROCESS VARIATIONS BY BIOVAR 1 AND 3 STRAINS OF AGROBACTERIUM TUMEFACIENS IN CHANCELLOR GRAPE STEM TISSUE

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INTRODUCTION

Crown gall incited by Agrobacterium tumefaciens (AT) was extensively studied by Smith and Townsend (1911) and Braun (1982). The disease occurs world-wide and is characterized by neoplastic proliferation of cells at the site of the inoculation. In 1973, Panagopoulos and Psallidas described a new group of AT isolates obtained mainly from grapevine which was designated biovar 3. Further studies confirmed the dominance of this biovar in Vitis vinifera in different grape growing regions (Bazzi et al, 1987; Burr et al., 1987). AT biovar 1 was detected in some grape cane cuttings but to a lesser extent than biovar 3 (Burr and Katz, 1984). Burr et al. (1987) suggested that tumors are induced in grapevine only by biovar 3. We, however, noted that biovar 1 strains are able to form tumors on grapes as well as biovar 3. This research was undertaken to study the anatomical variations in tumors induced by AT biovars 1 and 3 on grape stem tissue.

MATERIALS AND METHODS

Bioassay for tumor development of grape stem tissue: Virulent strains FA1 and Ag63, biovars 1 and 3 of AT respectively, were used during this study. FA1 was isolated from Chancellor grape xylem vessel fluids in our laboratory. Ag63 was isolated by Panagopoulos and Psallidas (1973) from grape. Inocula containing 10^8 CFU/ ml were prepared from 48 hr-slant cultures and used to test pathogenicity. Third internode segments, 2 to 3 cm in length, taken from Chancellor, a highly susceptible grape cultivar to AT, were surface sterilized and placed

vertically in a 2.5 x 6.0 cm screw cap vial containing 10 ml of WPM, a Murashige-Skoog medium modified by Lloyd and McCown (1980). Inoculations were performed by placing a 10 ul droplet of bacterial suspension on the exposed cross-section surface and incubated at 28°C.

Light microscopy:

The sections we examined were taken from tissue immediately below the inoculated surface. These were embedded for light microscopy in JB-4, a water soluble plastic (Polysciences, Warrington, PA), Tarbah and Goodman (1988). Controls were inoculated with distilled water.

RESULTS

Large tumors were apparent within two weeks in tissue inoculated with FA1 (Fig. 1). We noted a significant difference in both the size and the rate of tumor development induced by FA1 and Ag63 respectively. Whereas FA1-inoculated surfaces of the stem segments developed tumors within two weeks, Ag63 inoculations caused no macroscopic evidence of tumors at this time period. Instead, a necrosis was commonly noted at the tip of some inoculated stem segments (Fig. 1). Tumor development became apparent 4 to 5 weeks after inoculation.

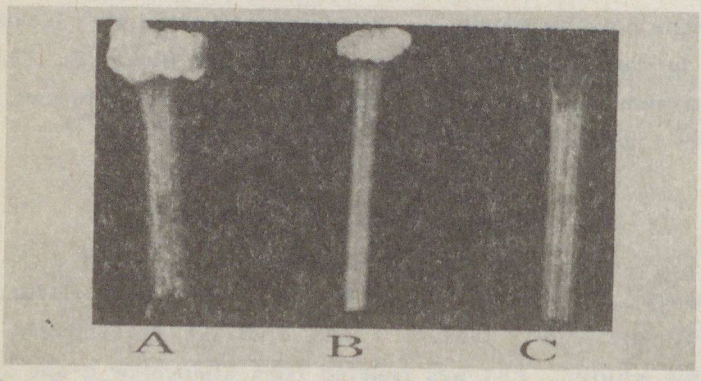

Fig. 1 Bioassay for tumor development and Necrosis on Chancellor stem tissue inoculated with FA1 and Ag63 respectively. A, with FA1; B and C with Ag63 isolate.

Major differences were observed microscopically between FA1 and Ag63 regarding the stimulation of cell division. At 120 hr after inoculation with Ag63, intensive cell division occured in the cambium, ray and phloem cells, as well as in the starch sheath (Fig. 2). Both the rate of cell division and size of dividing cells induced by the two inocula were also at variance. With FA1, tissue stimulation progressed more gradually resulting in the eventual division and expansion of large numbers of cells (Fig. 3). In tissue inoculated with Ag63, cells divided more rapidly, were much smaller (Fig. 2) and frequently became necrotic (Fig. 4). Furthermore, centers of growth stimulation (concentric whorls

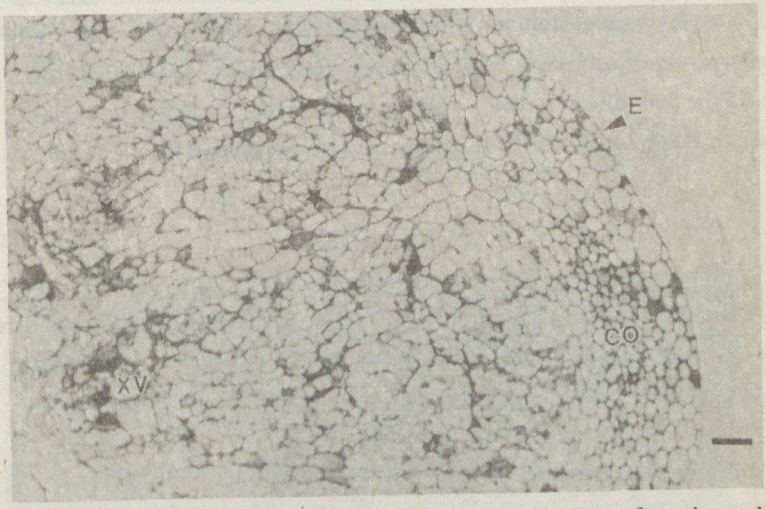

Fig. 2 Cross-section of Chancellor stem tissue 120 hr after inoculation with Ag63. Note whorls of small cells (stars) surrounded by larger hypertrophied ones. E, epidermis; CO, cortex; XV, xylem vessels. (bar = 100 µm)

of cells) induced by Ag63 were apparent at 120 hr after inoculation (Fig. 2), whereas a comparable condition was detected following FA1 inoculations after 240 hr (Fig. 5). In wounded, non-inoculated stem tissue active cell division corresponding to wound healing ceased in the vascular cambium and the interfascicular parenchyma, after 98 hr (Souissi and Goodman, in press).

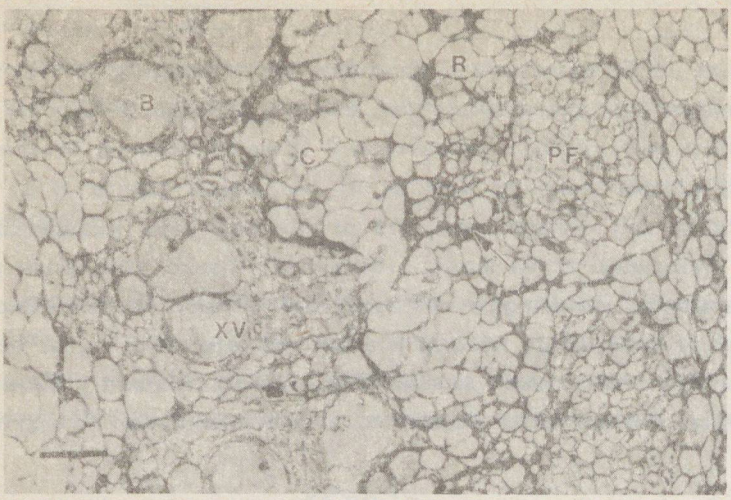

Fig. 3 Stem tissue 120 hr after inoculation with FA1. Ray (R) parenchyma and cambium (C) continue to enlarge and to divide, phloem fibers (PF) are discernible, and phloem sieve elements are being crushed (arrow). Note bacteria (B) in the xylem vessels (XV). (bar = 100 μm)

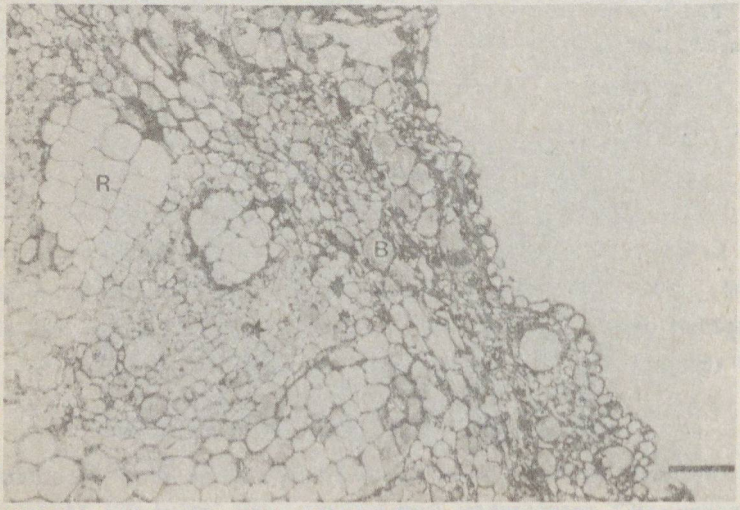

Fig. 4 Chancellor stem tissue 120 hr after inoculation with Ag63. Transformation has occurred, note hypertrophied ray (R) parenchyma and a massed mixture of vascular bundle cells (star). Epidermal and cortical regions have collapsed and many are filled with bacteria (B).
(bar = 100 μm)

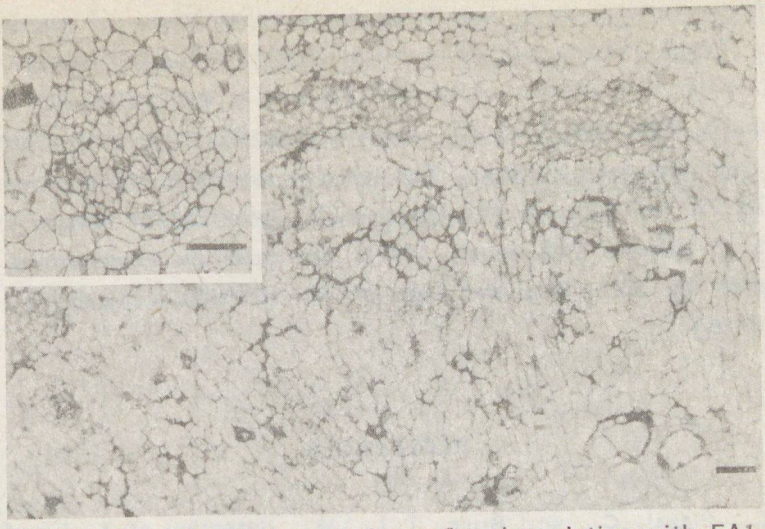

Fig. 5 Chancellor stem tissue 10 days after inoculation with FA1. Growth centers developed (insert, bar = 100 μm) in the tumorigenic tissue.

DISCUSSION

Burr et al, 1987 reported that AT biovar 3 is the only strain that initiates tumors on grapevine. Our results indicate that biovar 1 strains also are tumorgenic on grapes, at least following artificial inoculation (Fig. 1).

Tumor development in Chancellor stem tissue inoculated with AT, revealed a number of variations between biovar 1 and 3 concerning the transformation of normal cells to tumor cells. FA1 appears to react uniformily allowing the transformation of a larger number of cells, as indicated by a greater number of centers of growth stimulation and surrounding hypertrophied cells. A perception exists from an earlier report (Kupila, 1963) and our observations, Fig. 2, 5, that the whorls of small successively dividing cells, are the centers of activity of cells that have actually been transformed. In addition, it appears that the larger cells surrounding these whorls may be responding hypertrophically to the potentiated levels of auxin and cytokinin synthesized under the direction of Ti plasmid genes. Ag63 which is devoid of the cytokinin gene seems to be more destructive and violent, resulting in either

self-limiting tumors or in the subsequent development of necroses (Fig. 1, 4). Large numbers of cells were crushed and destroyed as early as 120 hr after inoculation, while with FA1 cells were still enlarging and dividing at this time (Fig 3). Similar results have been reported by Tarbah and Goodman (1988). The level of auxins in tissues transformed either by FA1 or Ag63, or the absence of a gene coding for the cytokinin in Ag63 Ti plasmid (Ma et al, 1987), are possible explanations for the differences in both size and rate of cell division induced by the two strains of AT.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

INHIBITION OF TUMOR GROWTH INDUCED BY AGROBACTERIUM TUMEFACIENS THROUGH METHYL JASMONATE

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ABSTRACT

Jasmonic acid (JA) and methyl jasmonate (JA-Me) are cyclic fatty acids with prostaglandin-like structure.These compounds are known to be widespread in many plants, the biosynthess of which comes from linolenic acid. JA and JA-Me show a variety physiological properties in plants i.e. stimulate leaf senescence, inhibit lycopene accumulation and stimulate ethylene formation in tomato fruits, inhibit cell division and are probably a new group of plant hormones.

Experiments with application of JA-Me in lanolin paste at a concentration of 1% on wounded and inoculated by *Agrobacterium tumefaciens* stems of 4 week old tomato plants showed a lack of tumor growth during 4-5 weeks. After that time the appearance of small tumors was observed. It is suggested that methyl jasmonate affected pathogenesis of crown gall.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

THE DETECTION IN SITU OF AGROBACTERIUM TUMEFACIENS BIOVAR 3 IN GRAPEVINE

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INTRODUCTION

Agrobacterium tumefaciens (Smith and Townsend) Conn. biovar 3 (AT3) is considered to be the main causal agent of grapevine crown gall (Kerr and Panagopoulos, 1977; Burr and Katz, 1983).

The symptoms of the disease on trunks, canes and, occasionally, on roots consist of tumors varying in size, which can cause marked changes in the growth of infected plants (Schroth et al., 1988). Agrobacteria unevenly colonize the xylem tissue of the host plant (Lehoczky, 1968; 1978) and, when conditions do not favour the development of visible symptoms, there can be latent survival for a long period; there is therefore a serious risk of using asymptomatic infected material for propagation.

The relative distribution of AT3 in the various parts of the plant cannot be determined with the current indexing procedures for grapevine cuttings, based on the analysis of vacuum grape extracts (Tarbah and Goodman, 1986; Bazzi et al., 1987). Therefore, to study the endophytic movement of the pathogen, an immunofluorescent staining (IFAS) procedure was set up for the *in situ* detection of bacterial antigens in serial sections of grapevine tissue.

* Research supported by grant No. 88.02123.06 from Consiglio Nazionale delle Ricerche, Rome - Italy, under Italy-USA bilateral research project "Grape Crown Gall (*Agrobacterium tumefaciens*): ecology, diagnosis and control".

MATERIALS AND METHODS

Cane infiltration

Dormant healthy cuttings cv. Albana (20 cm long, with 2 nodes) were infiltrated, using a low vacuum pressure (21 KPa), with 0.5 ml of a suspension containing 108 CFU.ml-1 of the AT3 strain, IPV-BO FC214. The inoculum was grown on 523 medium (Kado *et al.*, 1972) for 48h at 28°C. The contaminated canes were kept in sterile perlite in a greenhouse for 3 months. Water-infiltrated canes were used as controls.

Antiserum

The conventional polyclonal antiserum to the AT3 strain, IPV-BO FC214 (Bazzi *et al.*, 1988) was used for immunofluorescent staining.

Tissue preparation and *in situ* detection

Samples were collected each fortnight after shoot emergence. Tissue was taken from the cuttings at different levels: half-way between the nodes, in the nodal region and along the shoots as they developed. Small sectors were cut from cross-sections approximately 2 mm thick, taking care to remove the bark. These samples were fixed, dehydrated and embedded in paraffin using the method described in Table 1 (Hockenhull, 1978) with some changes as regards time and temperature.

Thin sections, 10-15 µm thick, were cut from the samples embedded in paraffin (Histosec), placed on slides coated with Kaiser's adhesive and left to stretch for a few minutes on a heated plate (50°C) and then placed in an oven for 4 hours at 50°C. The sections were then dewaxed (Tab.1, step 6) and IFAS was carried out directly on the slides.

The preparations were observed with a III RS Zeiss microscope.

Table 1: Tissue preparation for AT3 in situ detection from infected grapevine cuttings, using IFAS.

Step	Treatment	°C	Duration
1. Fixation:	Formalin 10% in PBS + Ethanol 70%; 1:1; pH 7.5	4	overnight
2. Dehydration:	tert-Butanol: Ethanol 96%: H2O 6 steps at different proportions (v:v:v)		
	a. 10:40:50	room	2h
	b. 20:50:30	"	2h
	c. 35:50:15	"	2h
	d. 55:45:0	"	2h
	e. 100:0:0	29	4h
	d. 100:0:0	29	overnight
3. Infiltration:	liquid paraffin + tert-Butanol 1:1	29	8h
	liquid paraffin + Histosec 1:1	65	overnight
	Histosec I	65	10h
	Histosec II	65	overnight
4. Embedding in Histosec		65	
5. Sectioning with microtome			
6. Dewaxing:			
	a. Immersion in Xylol (x2)	30	10 min
	b. Immersion Isopropanol abs.(x2)	room	5 min
	c. Immersion in Ethanol 96%	room	3-5 min
	d. Immersion in Ethanol 70%	room	3-5 min
	e. Immersion in PBS (x2)	room	3-5 min
7. Immunofluorescent staining (IFAS)			

AT3 reisolation

While preparations were made for *in situ* detection, isolations on RS medium (Roy and Sasser, 1983; Burr et al., 1987) were also attempted from callus formed at the basal end, as previously reported (Burr and Katz, 1984), roots, nodal and internodal regions and green shoots. Plates were incubated at 28°C for 5-7 days and bacterial colonies with red centres were serologically identified. The pathogenicity of some of these was also tested on *Nicotiana glauca*.

RESULTS AND DISCUSSION

The first sampling was carried out 8 weeks after inoculation and the subsequent transplanting of the cuttings. All the cuttings had developed normal root systems although those infiltrated with AT3 had a marked callus. AT3 reisolation was successful from callus, roots and from the nodes. No reisolation was possible from the internodal region or from the base of the shoots. Observations at 10 weeks confirmed these data; moreover a few colonies were isolated from the internodal area and from the lower shoots. These results were subsequently repeated: constant reisolations were made from callus, roots and nodal areas and, occasionally, from internodal areas and shoots.

In situ detection permitted the visualization of agrobacteria in the sections taken from the nodes. They were randomly distributed in the newly formed xylem tissue: high concentrations of fluorescent bacterial cells could be seen in the lumen of some of the youngest vessels whereas others remained free. The intensity of plant tissue fluorescence did not interfere with observation of the bacteria which could be clearly distinguished against the dark background of the vessel lumen. In other systems, however, the bacteria were only observed attached to the walls within the vessel lumens (Stefani, 1989). This difference may be due to the shorter time involved in the preparation procedure described above and the different morphology of the tissue examined. The time required to prepare the sections for IFAS is approximately 4 days, using strongly lignified material. An analysis of sections taken from green shoots required only about 60 hours.

As with other bacterial antigens (Hockenhull, 1979; Stefani, 1989), this technique for AT3 *in situ* detection does not mask its antigenic determinants. A conventional polyclonal antiserum must be used since even with highly specific monoclonal antibodies the bacterial cell fluorescence is very weak. A comparative study of *Agrobacterium* biovars clearly revealed the differences in

antibody reactivity, depending on the serological assay used, IFAS and/or ELISA (Bazzi *et al.*, 1988).

The experiments were very useful for the study of the relative distribution and dynamics of AT3: the greatest concentration was always observed in the nodal xylematic region, probably due to the complexity of this vascular tissue. The major presence of the pathogen in the younger xylem vessels, close to the cambium, indicates an active colonization of these Spring-formed transport elements, from the vessels present in the dormant cuttings and invaded at the time of the experimental infiltration.

Moreover, the discovery of germs in green shoots indicates a very early transfer to the newly-formed organ, facilitated by the greater bacterial concentration at a nodal level and by its localization in the larger xylem vessels.

Attempts were also made to isolate germs from Spring shoots of naturally infected plants and a pure culture of AT3 was obtained. This is further confirmation of the hypothesis that bacteria can infect the new shoots during their development thus casting doubts on the phytosanitary usefulness of bringing up new suckers from infected trunks.

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SESSION 8

SEROLOGY

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

Plant Pathogenic Bacteria Z. Klement (ed.)

RELATIONSHIPS AMONG PHYTOPATHOGENIC BACTERIA DISTINGUISHED WITH MONOCLONAL ANTIBODIES

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ABSTRACT

A series of monoclonal antibodies (mAbs) was developed for *Xanthomonas campestris* pathovars (*campestris*, *dieffenbachiae*, *citri*, *oryzae*, *begoniae*, and *pelargonii*), *Clavibacter michiganensis* subsp. *michiganensis*, and several *Erwinia* sp. from widespread geographical origins. The mAbs were useful for detection of taxon-specific epitopes for diagnostic purposes, and for ecological and epidemiological studies. MAbs generated to pathovars of *X. campestris* showed that some pathovars (*campestris*, *dieffenbachiae*, *citri*) are serologically heterogeneous whereas others (*oryzae*, *begoniae*, *pelargonii*) are homogeneous. In particular, Asiatic (Pathotype A) strains of *X. c. citri* were serologically distinct from strains in Pathotypes B and C from Argentina and Brazil, respectively, as well as from strains recovered from disease outbreaks in Mexico and Florida. Strains from Florida were serologically heterogeneous; positive reactions with one mAb differentiated the most aggressive strains from mildly aggressive strains. Likewise, mAbs distinguished aggressive Asiatic strains of *X.c. oryzae* from mildly agressive strains of *X.c. oryzae* originating in continental U.S.A. A mAb specific for *C.m. michiganensis* did not react with avirulent *Clavibacter* sp. isolated from tomato plants showing symptoms of bacterial canker. In order to localize surface antigens, cell structure was examined by immunoelectron microscopy. Epitopes detected by taxon-specific mAbs were associated with surface structures of different biochemical nature for the various bacterial plant pathogens.

INTRODUCTION

Epidemiological studies of diseases caused by phytopathogenic bacteria have been limited in the past by inefficient methodology that did not permit a rapid and accurate identification of the bacteria affecting the plant tissues. With the development of hybridoma technology by Kohler and Milstein in 1975 (8), it became possible to

identify unique antigenic determinants (or epitopes) on cell surfaces using highly specific monoclonal antibodies (mAbs). Selected antibodies then can be produced in large quantities to make standard reagents that now permit a researcher to identify the phytopathogen in question, to separate it from saprophytes, to identify unique strains and to trace their movement through the field. Such tools are particularly relevant to tropical agriculture where bacterial diseases are prevalent and often limit crop production.

Monoclonal antibodies that detect taxon-specific epitopes of several phytopathogenic bacteria have been produced and characterized (1,3,4,5). In early studies with xanthomonads (1), two antibodies (X1 and X11) were generated that detected common epitopes on the surfaces of all xanthomonads, both pathogenic and nonpathogenic. Later, mAbs generated to surface antigens of various pathovars revealed serological differences that can be used in taxonomic and epidemiological studies.(4,9).

Pathovar-specific mAbs for *Xanthomonas campestris*

Monoclonal antibodies generated to strains of *X.c. campestris* showed that the pathogen is serologically heterogeneous. There is no common epitope that will identify all strains of the black rot pathogen; however, a panel of mAbs designated X9, X13, X17, X21 reacts with 98% of the virulent strains. A positive reaction with two or more of these mAbs is generally indicative of a typical, virulent black rot organism. If only one of the four mAbs reacts, the strain is generally less virulent or produces atypical symptoms that develop slowly. Avirulent xanthomonads recovered from crucifer leaf surfaces and crucifer seeds reacted only with X11 and/or X1. Considering over a thousand Xcc strains tested to date, we now see repeating patterns from strains isolated from widely separated geographical origins.

Several other pathovars, also exhibit heterogeneity with respect to surface antigens. Pathovar-specific mAb Xcd-7, made to *X.c. dieffenbachiae*, reacted with only 34% of 323 strains of this pathovar tested. Other mAbs, Xcd-1 and Xcd-3, reacted with 98% of the strains, but they also detected other pathovars, *phaseoli* and *alfalfae*. A similiar observation was made for *X.c. vesicatoria*.

In the case of *X.c. citri* a somewhat different situation is revealed. All strains of the pathogen that cause the severe (Asiatic) form of the disease reacted with one mAb (A1) generated to a surface antigen of a Pathotype A reference strain (XC 62).

Pathotype A strains thus appear to be serologically similar. Other mAbs reveal the heterogeneity of bacterial strains associated with different forms of citrus canker; mAb B1 reacts only with one reference strain isolated from the mild form of canker in Argentina; mAb B2 reacts with all slow-growing Argentine B strains, with a pathotype C strain from *Citrus aurantifolia* (lime) in Brazil and with a strain associated with bacteriosis of lime in Mexico; mAb C1 reacts only with the Brazilian strain. These B and C mAbs reveal common epitopes on strains that cause the mild Argentine, Brazilian, and Mexican forms of citrus canker. Nevertheless, the B and C strains are not identical because mAbs B1, B2, and C1 react selectively with some of these strains.

In contrast to the relative uniformity of the Pathotype A strains that cause the severe form of citrus canker, strains associated with the Florida citrus leaf spot disease are heterogeneous and share certain epitopes with other pathovars of *X. campestris*. None of the xanthomonas strains isolated from Florida nurseries in 1984-1986 reacted with the *citri*-specific mAbs, except for two Pathotype A strains that reacted with mAb A1. In 1987 more strains were found that reacted with this antibody. All Florida strains reacted with *Xanthomonas*-specific mAbs, X1 and X11, and some also reacted with mAbs Xcd-1 and Xcd-3, made to *X.c. dieffenbachiae*. One mAb (F1) made to the Florida strains reacted with *X.c. alfalfae*. The relationship of the Florida strains to *X.c. alfalfae* also has been implicated by genetic analysis (6).

Despite the heterogeneity among the Florida citrus strains, distinct reactivity patterns have been repeatedly found among strains isolated from selected nurseries. Some patterns were associated with less aggressive strains and others with the most aggressive strains. Weakly virulent and avirulent xanthomonads found on the leaf surfaces of citrus in Florida and Mexico also strongly react with *Xanthomonas*-specific mAbs, X1 and X11, but react with none of the other mAbs that identify pathogenic reference strains. By using a panel of mAbs the pathogens can be separated from avirulent strains, and the ecological role of avirulent xanthomonads on leaf surfaces of crops can be examined.

A different situation was revealed by mAbs generated to *X. campestris* pathovars *pelargonii, begoniae,* and *oryzae*. For each of these pathovars a single pathovar-specific mAb was generated that detected all strains of the pathovar tested to date; mAb Xcp-1 was positive with 76 strains of *X.c. pelargonii*, mAb Xcb-1 with 26 strains of *X.c. begoniae*, and mAb Xco-1 with 178 strains of *X.c. oryzae*. These three mAbs react only with their respective pathovars of *X. campestris* but not with other xanthomonads or other genera or species tested.

Recent outbreaks of a disease that resembled bacterial blight of rice were reported in 1987 in Texas and Louisiana (7). The pathogenic strains associated with this disease reacted with the *X.c. oryzae*-specific mAb, Xco-1 (3). A mAb generated to the Texas strains, designated Xco-5, separated the Asian from the U.S. strains, and this mAb also reacted weakly with *X.c. oryzicola*. The serological characteristics of the U.S. strains along with genetic and other differences indicate that the strains associated with the U.S. form of bacterial blight are distinct from the Asian strains even though the former share a common epitope with Asian *X.c. oryzae* strains. (3,7), The two *Xanthomonas*-specific mAbs, X1 and X11 reacted with all pathogenic and non-pathogenic strains. A panel of mAbs can thus be used to separate avirulent xanthomonads from the virulent strains of *X.c. oryzae* on rice leaves and seed.

Species-specific mAbs also have been generated for *Clavibacter michiganensis* ssp. *michiganensis*; mAb Cm-1 reacted with all but one of 88 virulent strains but did not react with 13 avirulent strains otherwise resembling *C.m. michiganensis* isolated from tomato plants and seed. This antibody did react with one strain of questionable virulence that caused mild chlorosis on leaves but no stem cankers.

Epitopes detected by taxon-specific mAbs were associated with surface structures of different biochemical nature for the various bacterial plant pathogens. Two lipopolysaccharide (LPS) epitopes associated with virulent strains of *X.c. campestris* were evenly distributed on the surface of these strains. Epitopes detected by mAb A1 that reacts with all Pathotype A strains of *X.c. citri* were labeled in a pattern resembling LPS-labeled epitopes. Another mAb (A2) reacted with the bacterial flagella. The mAb Cm-1 that detected *C. m. michiganensis* was of high molecular weight in Western blots and reacted with an epitope on cells of virulent cultures that appeared to be associated with extracellular polysaccharide.

Detection of surface antigens of phytopathogenic bacteria by taxon-specific mAbs has a number of practical applications in field studies. Selected mAbs can be used to a) trace the geographical distribution of bacterial pathogens, b) follow the movement of marked strains in field plots, c) study differences in environmental fitness of selected strains, and d) detect phytopathogenic bacteria on seed and other plant parts.

The ease of generating taxon-specific mAbs appears to be inversely related to the heterogeneity of the host-range of the bacterial plant pathogen. For certain

pathovars of *X. campestris*, such as *campestris*, *dieffenbachiae*, and *citri*, that affect several host genera, a single mAb was not found that reacted with all strains of the pathovar, and a panel of mAbs was needed to identify the pathovar. In these cases, distinct serological subgroups were apparent, and serologically different strains sometimes varied with respect to virulence on the respective host.

In cases where the pathovar generally infects a single host (rice, geranium, begonia), pathovar specific mAbs were easily generated. For example, a single fusion was required to produce mAbs for *X.c. oryzae*, and subsequent fusions resulted in production of mAbs of the same specificity, indicating that strains of this pathovar share a common epitope. A similar observation was made for *X.c. pelargonii*, *X.c. begoniae*, and *C.m. michiganensis*, each of which has a relatively restricted host-range.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

ASSESSMENT OF LATENT BACTERIAL RING ROT INFECTIONS IN SEVERAL POTATO CULTIVARS

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ABSTRACT

Corynebacterium sepedonicum was detected by immunofluorescence and enzyme-linked immunosorbent assay in symptomless potato stems and tubers, grown from inoculated seed pieces, of all 27 potato cultivars that were tested. Since the cultivars differed greatly in degree of symptom expression, they were categorized into four classes on the basis of the percentage of progeny tubers with symptoms. Mean C. sepedonicum population densities in symptomless stems and tubers ranged from 10^2 to 10^{10} for different cultivars. The bacterial density in stems of three cultivars, representing different symptom expression classes, was monitored at two-week intervals during the growing season. The bacterial population increased most rapidly and uniformly in the cultivar that expressed symptoms most readily, but C. sepedonicum was detected in some stems of all three cultivars at all sampling dates. Thus the ability to detect latent ring rot infections by serological tests was independent of the tendency of the cultivar to express symptoms. In practice, post harvest serological tests were useful in detecting latent ring rot infections in potato lots that were considered ring rot-free on the basis of visual inspection.

INTRODUCTION

Many potato cultivars express severe foliage and tuber symptoms when infected with the bacterial ring rot causal agent Corynebacterium sepedonicum (Spieck. & Kotth.) Skapt. & Burkh. [syn. Clavibacter michiganense subsp. sepedonicum (Spieck. & Kotth.) Davis et al]. Some

cultivars such as Teton, Merrimack, and Saranac, however, are considered resistant since they do not, or only very occasionally, express ring rot symptoms (Bonde et al 1947). The resistant cultivars, however, are susceptible to infection by C. sepedonicum. Consequently these cultivars serve as reservoirs of inoculum that are not detected by potato field and bin inspections.

Recently serological procedures have been developed by which symptomless bacterial ring rot infections can be detected (De Boer and McNaughton 1986). However, it has not been determined whether C. sepedonicum also can be detected in cultivars that normally do not develop ring rot symptoms. In this study we compare incidence and population densities of the bacterial ring rot pathogen in cultivars that express ring rot symptoms well and those that do not.

SYMPTOM EXPRESSION CLASSES

Twenty-seven potato cultivars were grown from artificially inoculated seed pieces and the degree of symptom expression rated at harvest. The cultivars were categorized into four classes on the basis of the percentage of progeny tubers that expressed symptoms. Cultivars in which 50-100% of the tubers developed ring rot symptoms were placed in class 1, those in which 25-49% developed symptoms were placed in class 2, and those in which 10-24% and 0-9% developed symptoms were placed in class 3 and 4, respectively. The mean percentage of tubers developing symptoms in each class are shown in Table 1.

The degree of foliage expression including incidence and severity of symptoms were rated on a scale from 0-10, with 10 being severe symptoms on all plants. The mean foliage symptom expression index was lower in classes 3 and 4 in comparison to classes 1 and 2 (Table 1).

Table 1. Mean percentage of tubers that expressed bacterial ring rot symptoms and foliage symptom expression index for each class of potato cultivars.

Class	No. of cultivars	Mean of percent symptomatic tubers	Mean foliage symptom expression index
1	9	64.4	7.3
2	8	40.6	7.1
3	5	17.2	3.2
4	5	7.0	2.3

PRESENCE OF CORYNEBACTERIUM SEPEDONICUM IN POTATO STEMS AND TUBERS

The population density of C. sepedonicum was monitored throughout the growing season in the cultivars Red Pontiac, Russet Burbank, and Desiree, which represent the classes 1, 3, and 4, respectively. Potato plants were grown from inoculated seed, and stems were assayed at ground level by a quantitative indirect immunofluorescence procedure (De Boer and Hall 1988). The bacterium was detected at the first sampling date 26 days after planting and the population densities subsequently increased in all three cultivars (Table 2).

Table 2. Population density of C. sepedonicum in potato stems of three potato cultivars during the growing season.

Days after planting	Mean population density (cells/g of tissue)		
	Red Pontiac	Russet Burbank	Desiree
26	5.7 x 104	2.5 x 104	4.1 x 104
40	2.0 x 107	1.8 x 104	1.5 x 104
54	4.6 x 108	1.2 x 104	9.7 x 104
67	5.4 x 108	2.9 x 105	1.1 x 107
82	3.3 x 109	4.1 x 108	2.7 x 107
96	3.1 x 1010	1.8 x 109	1.3 x 109
110	4.5 x 1010	3.6 x 109	4.7 x 104

The presence of C. sepedonicum was also determined in potato stems and tubers in various other cultivars. The number of stems and tubers with the bacterium did not vary greatly among symptom expression classes and population densities were in the same order of magnitude (Table 3).

DISCUSSION

Although potato cultivars vary widely in their tendency to express bacterial ring rot symptoms, all the cultivars tested supported high populations of the bacterium. Furthermore, the incidence of C. sepedonicum in stems and tubers of cultivars in the four classes did not differ greatly.

Table 3. Incidence and population density of C. sepedonicum in potato tubers and stems as determined by immunofluorescence.

Class	Incidence (Percent)	Population range (Cells/g of tissue)
Stems		
1	57	$2.1 \times 10^7 - 3.8 \times 10^{10}$
2	43	NA*
3	47	$1.4 \times 10^7 - 3.1 \times 10^{10}$
4	50	$5.8 \times 10^6 - 7.8 \times 10^{10}$
Tubers		
1	41	NA
2	41	$2.5 \times 10^6 - 5.2 \times 10^8$
3	15	$3.2 \times 10^5 - 3.4 \times 10^8$
4	22	$2.9 \times 10^2 - 1.2 \times 10^7$

*NA - Not Available

Previous studies have shown that serological testing of composite tuber samples is effective for detecting symptomless bacterial ring rot infections in cultivars that normally do express symptoms (De Boer et al 1989). The data presented here suggests that ring rot infections can also be readily detected by serological tests in cultivars that usually do not express symptoms. Thus by serological screening of these cultivars for freedom of bacterial ring rot in place of field inspection, spread of ring rot by them can be avoided.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

THE APPLICATION OF THE COMBINED USE OF IMMUNOFLUORESCENCE MICROSCOPY AND DILUTION-PLATING TO DETECT PSEUDOMONAS SYRINGAE PV. PISI IN PEA SEEDS

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INTRODUCTION

Pseudomonas syringae pv. *pisi* causes bacterial blight in peas (Boelema, 1972) and has recently become a serious threat to pea crops in Europe (Stead, 1987). The use of disease-free seeds is important to prevent establishment of this pathogen in Europe. Therefore, methods to detect *P. s.* pv. *pisi* in pea seeds are needed.

Van Vuurde & Van den Bovenkamp (1987) described a detection method for *Pseudomonas syringae* pv. *phaseolicola* using immunofluorescence microscopy (IF) as a screening method. IF-positive subsamples are confirmed by dilution-plating, followed by a pathogenicity test. IF-negative subsamples are not plated. Taylor and Dye (1972) described isolation of *P. s.* pv. *pisi* from disease lesions on 5% sucrose nutrient agar (SNA) or King's medium B (KB; King et al., 1954). Mohan & Schaad (1987) described KBC medium (a modification of KB), containing boric acid, cephalexin and cycloheximide, for detecting *P. s.* pv. *syringae* in contaminated bean seed. These authors suggested that the KBC medium could also be useful for assaying *P. s.* pv. *pisi* in pea seeds. Identification of suspected colonies from dilution-plates is possible by using serology, specific bacteriophages and/or pathogenicity tests (Taylor, 1972). Also homoserine utilization may be a useful property for identification of *P. s.* pv. *pisi* (Hildebrand, 1972).

The aim of this study was to develop, improve and evaluate a detection method for *P. s.* pv. *pisi* in pea seeds on the basis of the combined use of IF and dilution-plating, followed by additional confirmation tests, analogous to the detection method for *P. s.* pv. *phaseolicola* (Van Vuurde & Van den Bovenkamp, 1987).

MATERIAL AND METHODS

Sample treatment and extraction time

Seed samples were all naturally infected and treated as described by Van Vuurde & Van den Bovenkamp (1987) for *P. s.* pv. *phaseolicola*, viz. the seed samples were divided into 5 subsamples of 1000 seeds, unless otherwise stated. Sterile tap water was added to each bag in a quantity corresponding to 2.0 ml multiplied by the weight in g of 1,000 seeds. The subsamples were incubated at 4 - 6 °C and briefly shaken by hand after 4 hours (hr). After 6, 24 and 30 hr a sample was taken for IF and dilution-plating. Three serial dilutions were always plated in duplo: an

undiluted extract (0 dilution), a 10x diluted extract (-1 dilution) and a 100x diluted extract (-2 dilution). In separate experiments concentration of seed leachates was done by centrifuging the samples for 10 min at 12,000 *g* after 6 hr soaking (for IF) and 24 hr soaking (for dilution-plating) of the seed lots. In these cases the pellets were resuspended in a volume 40x or 10x smaller than the original volume. The concentrated pellet was plated as well as two serial (tenfold) dilutions from this pellet.

Antisera and immunofluorescence microscopy

For screening seed samples, a quality-tested polyclonal rabbit antiserum was used at a working dilution of 1:5,000 in indirect IF. Indirect IF was carried out as described by Van Vuurde et al. (1983).

Media

Four media were used: KB, SNA, KBC and SNAC, which is SNA with the same antibiotics in the same concentrations as in KBC.

Identification tests

Isolates were considered to be *P. s.* pv. *pisi* isolates when colonies compared to the reference strains, were typical on the media, when isolates had no oxidase activity, utilized homoserine, reacted with the antiserum at a 1:5,000 dilution in indirect IF and when isolates showed typical symptoms on susceptible pea plants. The latter test was considered to be the most important. Pathogenicity tests were done by inoculating young plants of cv.'s Kelvedon Wonder and Maxi into the main stem at the junction with the stipules at the two youngest nodes (Taylor et al., 1989). All reference strains of pv. *pisi* produced an area of water soaking which spread from the site of inoculation.

RESULTS

Extraction time

Table 1 shows that the means of the logarithms (log) of the number of fluorescent cells per subsample was less after 24 hr and 30 hr soaking than after 6 hr soaking. Also less subsamples were found positive (meaning that fluorescent cells were found) in IF when pea seeds were soaked for 24 hr or 30 hr. The percentages of positive subsamples in IF after 6 hr, 24 hr and 30 hr soaking were 76%, 67% and 38% resp. (n=45).

Table 1 The effect of different soaking times on the results in immunofluorescence microscopy (IF) and dilution-plating

	soaking time (hr)		
	6	24	30
Mean logarithms of the colony forming units of pv. pisi per ml in dilution-plating (n = 320)	0.28 (a)[x]	0.38 (a,b)	0.49 (b)
Mean logarithms of the number of fluorescent cells per ml in IF (n = 45)	2.1 (a)[x]	1.4 (b)	0.8 (c)

[x] Means followed by different letters between brackets are significantly different (P=0.05) by Student's t-test

In dilution-plating the means of the log pv. *pisi* colony forming units (cfu's) per ml was higher at 30 hr soaking than after 6 hr soaking (Table 1). This was consistent for all media; no interaction was found between media and soaking time. The percentages of positive subsamples in dilution-plating for 6 hr, 24 hr and 30 hr did not differ significantly. They were resp. 24%, 21% and 21% (n=180).

Media

Table 2 shows that means of the log of the pv. *pisi* cfu's per ml were significantly higher for SNAC than for KB at all dilutions. In this respect no differences were found between SNAC, KBC and SNA. When considering the number of positive subsamples, SNAC clearly gave more positive subsamples at dilutions -1 and -2. However, as a whole no differences were found between SNAC, SNA and KBC in actual positive subsamples per seedlot. These media gave on an average all 1.2 positive subsamples per seedlot (n=27). Only KB gave a significantly lower amount of positive subsamples per sample (0.8).

Table 2 The performance of SNA, SNAC, KB and KBC at three dilutions of the seed leachate

Medium	Dilution		
	0	-1	-2
SNA	29 (0.60)[x]	16 (0.39)	7 (0.19)
SNAC	31 (0.62)	23 (0.48)	16 (0.35)
KB	17 (0.43)	12 (0.30)	9 (0.19)
KBC	30 (0.46)	16 (0.37)	8 (0.22)

[x] Results of 45 subsamples tested at three extraction times: 6, 24 and 30 hr. Figures are resp. the number of positive subsamples and, between brackets, the means of the log cfu's of pv. pisi per ml. The results between brackets should only be compared within a dilution (within a column). The means between brackets are significant at 95% probability when differences within the column are larger than 0.17, 0.17 resp. and 0.16 for dilutions 0, -1 and -2 (n = 240).

The ratio of pv. *pisi* colonies to the total amount of colonies can be found in Table 3. In general, the pv. *pisi* colonies as percentage of the total amount of colonies arelower at 30 hr and 24 hr than at 6 hr for the media SNA, SNAC and KBC, due to relatively higher saprophyte numbers. However, KB showed a higher percentage at 30 hr compared to 24 hr and 6 hr soaking. The dilution effect was also striking . For SNA, and KB to a lesser amount, the percentages were highest at the 0 dilution, whereas the opposite was found for KBC and SNAC.

Table 3 The average amount of Pseudomonas syringae pv. pisi colonies as percentage of the total amount of colonies

soaking time	medium											
	SNA			SNAC			KB			KBC		
dilution	0	-1	-2	0	-1	-2	0	-1	-2	0	-1	-2
6 hr	44%	10%	3%	91%	99%	97%	8%	2%	1%	76%	91%	100%
24 hr	33%	15%	5%	85%	90%	96%	5%	7%	3%	47%	63%	88%
30 hr	31%	15%	5%	76%	81%	91%	25%	12%	5%	37%	44%	65%

Concentration experiments

The effect of concentrating the seed leachate in IF can be found in Table 4. In two separate experiments, concentration proved to decrease (at 95% probability) the log number of fluorescent cells significantly as well as the percentage of positive subsamples. This was due to disturbance of reading of IF-slides by seed debris. Moreover, in experiment 1 and experiment 2 we have found 4 and 5 subsamples which were negative when slides were prepared of the concentrated leachate, but which were positive when no concentration was used. On the other hand we have found only one sample (in experiment 2) which was positive after concentration and negative when no concentration was used.

Table 4 The effect of concentration of seed leachates in immunofluorescence microscopy

	no concentration	10x concentration	40x concentration
	x		xx
experiment 1	1.9 ± 0.4 (97%)	1.2 ± 0.8 (86%)	n.t.
experiment 2	2.6 ± 1.2 (88%)	n.t.	1.2 ± 1.3 (71%)

x figures represent the mean log number of fluorescent cells per ml plus or minus the standard deviation; between brackets the percentage of positive subsamples (n = 24 for experiment 1; n = 36 for experiment 2)

xx n.t. = not tested

In dilution-plating, concentration of the seed leachate did not affect the recovery of *P. s.* pv. *pisi*. When seed leachates of 10 different seed samples (two replicates in duplo) were compared, the recovery of colonies was 0.33 ± 0.75 log cfu's per ml for non-concentrated leachates and 0.36 ± 0.67 log cfu's per ml for 40x concentrated leachates. On one occasion (one replicate of one sample) the non-concentrated leachate gave a positive result whereas concentration gave a negative result. On the other hand the contrary was found on two occasions, i.e. a positive result for concentrating and negative for not concentrating.

DISCUSSION AND CONCLUSIONS

Analogous to the detection method developed for *P. s.* pv. *phaseolicola* in bean seed (Van Vuurde et al., 1983; Van Vuurde & Van den Bovenkamp, 1987) an assay was developed for *P. s.* pv. *pisi* in pea seeds. Although more fluorescent cells were found in IF after 6 hr soaking than after 24 hr or 30 hr soaking, we have found in dilution-plating the highest numbers of colonies at 30 hr soaking. This apparent contradiction can be explained by the fact that dead cells die off during the process of soaking the pea seeds. Only viable cells will eventually survive and multiply. An indication for this fact is that after 24 hr and 30 hr soaking higher numbers of partially stained, frayed cells were observed than after 6 hr soaking. In general, KB was not satisfactory for isolation. Since indexing seed lots with SNAC gave more positive subsamples at dilutions -1 and -2 (Table 2) and resulted in relatively the highest amount of pv. *pisi* colonies as percentage of the total amount of colonies, SNAC is advised for isolation of *P. s.* pv. *pisi* from

pea seeds. Concentration of seed leachates is less favourable for IF. For dilution-plating, the effect of concentration was not clear-cut. In some cases it may be helpful. However, a concentration step is usually less suited for routine purposes.

As a result of this study we suggest to use the following detection assay. Incubate seedlots at 4 - 6 °C. Take a sample for IF after 6 hr of incubation. When IF-positive (sub)samples are found, take again a sample after 24 hr. Plate this sample, and two serial (tenfold) dilutions from this sample, in duplo on SNAC. Incubate for 3 days at 28 °C. IF-negative (sub)samples should not be plated. Confirm "Levan-type" colonies by pathogenicity testing as described by Taylor et al. (1989) and check for oxidase activity, homoserine utilization and reaction with a quality-tested antiserum in IF. This detection assay has already been used in the Netherlands since 1986 for routine indexing of pea seedlots. Research is now in progress to study the correlation between laboratory and field results.

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

Plant Pathogenic Bacteria
Z. Klement (ed.)

IDENTIFICATION OF PSEUDOMONAS CICHORII BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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ABSTRACT

Polyclonal antibodies raised in New Zealand white rabbits against heat-killed cells of a tomato pathogen, *P. cichorii*, were used in a double-antibody form of enzyme-linked immunosorbent assay (ELISA). Immunoglobulin G (IgG) purified by ion-exchange chromatography at a concentration of 1.0 µg/ml and IgG-alkaline phosphatase conjugate at 0.5 µg/ml enabled detection of heat-killed antigen at concentrations of 10^3-10^4 colony forming units/ml. The serological relationships of *P. cichorii* and other species were studied using a total of 48 species of *Pseudomonas, Corynebacterium, Erwinia* and *Xanthomonas*. *P. cichorii* was serologically distinct from non-phytopathogenic *Pseudomonas* spp; *P. aeruginosa, P. fluorescens, P. cepacia* and bacteria of other genera, *Corynebacterium, Erwinia* and *Xanthomonas*. Serological heterogeneity existed between isolates of *P. cichorii*, but *P. cichorii* could be identified from all other species.

INTRODUCTION

Three *Pseudomonas* species are the main pathogens of tomato in New Zealand. *P. cichorii* (Swingle 1925) Stapp 1928 is the causal agent of stem bacteriosis, the symptoms are similar to another disease, stem necrosis, which is caused by *P. corrugata* (Scarlett *et al.* 1978). Another more important pathogen which causes bacterial speck is *P. syringae* pv. *tomato* (Okake 1933). The identification of these species has been primarily based on biochemical characterisation and pathogenicity tests, which can be slow or tedious. It was necessary to develop a method which will assist rapid identification of these pathogens. The enzyme-linked immunosorbent assay (ELISA) has been shown to be highly sensitive and specific for detection of plant viruses (1, 2) and plant pathogenic bacteria (3). The ELISA was developed for

identification of these *Pseudomonas* species. Results of serological studies of *P. cichorii* are presented in this paper.

MATERIALS AND METHODS

Production of antibodies and conjugate. Pure colonies of *P. cichorii*, strain PC414 cultured on Nutrient Agar at 27°C for 24-48 h were washed with phosphate buffered saline (PBS). Bacterial suspensions were centrifuged at 10,000 g for 15 min in a Sorvall RC 2B centrifuge using an SS34 rotor. The bacterial pellet was resuspended in sterile PBS and the concentration adjusted to 4×10^8 CFU/ml using a spectrophotometer at 420 nm (Shimadzu UV-VIS 110-2). The bacteria were heat-killed in a water-bath at 100° for 1 h.

An emulsion of a 1.5 ml aliquot of heat-killed bacterial suspension and 1.5 ml of Freund's incomplete adjuvant (Sigma, F5506) was injected subcutaneously into New Zealand white (Chinchilla X) rabbits. The injection was repeated at weekly intervals for four weeks. Three additional intradermal and subcutaneous injections were applied weekly 9 weeks after the first injection. Antiserum obtained two weeks after the final subcutaneous and intradermal injection was used for preparation of purified antibodies. The antiserum had a reciprocal agglutination titre of 256.

The IgG fraction of the antiserum was separated from serum albumin by ammonium sulphate precipitation, and then purified by ion-exchange chromatography (4). Sterile distilled water (4.5 ml) was added to 0.5 ml of antiserum and the protein precipitated by drop-wise addition of five ml of saturated ammonium sulphate. After incubation at room temperature for 60 min, the protein was pelleted by centrifugation at 3500 g for 10 min. The protein pellet was resuspended in 1 ml of half strength (0.5 X) PBS and dialysed against 2 L of 0.5 X PBS at 5° for 2 h. Dialysis was repeated in fresh 0.5 X PBS at 5° overnight.

The protein fraction was then passed through a diethylaminoethyl (DEAE)-cellulose (Whatman DE-52) ion-exchange column. Immunoglobulin G fraction eluted from the column was monitored with a spectrophotometer (Pye-Unicam SP8-100) set at 280 nm. The purified Immunoglobulin G solution was stored in 0.5 ml aliquots at -20°C. An aliquot of 0.5 mg of IgG was labelled with 0.5 mg of alkaline phosphatase (Sigma P5521 type VII-S) using a one-step glutaraldehyde conjugation method (5).

The double antibody sandwich ELISA procedures. The double antibody sandwich ELISA (4) was used for serological studies of *P. cichorii*. Antibody, antigen, conjugate and enzyme substrate were added at 200 µl per well at successive stages into

polystyrene microtitre plates (Nunc-certified Immuno-plates). Antibody, antigen, and conjugate were incubated at 5° overnight. The plates were covered with a microtitre plate lid and placed in sealed plastic bags to minimise evaporation from the outer wells.

Immunoglobulin G and conjugate were used at 1.0 μg/ml and 0.5 μg/ml respectively. Plates were washed three times with PBS-Tween buffer, with 3 min soaking between washes. Enzyme substrate, p-nitrophenylphosphate (Sigma 104 lot 72F-60891 5 mg/tablet), freshly prepared at a concentration of 0.5 mg/ml in 1 M diethanolamine (Sigma D-8885) buffer was used. The reaction was allowed to proceed at room temperature, and the absorbances recorded with an EIA reader (Biotek Instrument Inc. EL307) at 405 nm. An ELISA index (EI), which is the ratio of the sample absorbance to the control absorbance was calculated.

Antigen preparation: Cultures of bacteria were obtained from Plant Diseases Division Culture Collection, Department of Scientific and Industrial Research, Auckland, New Zealand (PDDCC, DSIR). These cultures had been isolated at various times from a diverse range of host plants and countries (6). They were selected to include species designated as Group I, II and III phytopathogenic *Pseudomonas* spp. (7); and Group IV and V, the saprophytic or non-pathogenic *Pseudomonas* spp. Bacteria from genera such as *Corynebacterium*, *Erwinia*, and *Xanthomonas* were also tested. The original PDDCC numbers assigned to these species were used throughout this study.

Suspensions *P. cichorii* and the test isolates were prepared as described for preparation of immunogen. A turbid bacterial suspension was prepared and the absorbances adjusted to give approximately 10^8 CFU/ml. Heat-killed suspensions of bacteria at 10^5, 10^6 and 10^8 CFU/ml were tested in triplicate.

RESULTS

Anti-PC414 IgG was highly reactive with its homologous and heterologous antigens, with EI's of 16.41 - 20.82 at 10^8 CFU/ml (Fig. 1a). Differences among *P. cichorii* isolates became apparent at 10^5 and 10^6 CFU/ml, as a wider range of EI's were obtained. This difference in response suggests heterogeneity amongst *P. cichorii* isolates.

Most of the Group I *P. sy.* pv. *tomato* isolates were readily differentiated from *P. cichorii* as few cross-reactions were observed (Fig. 1a). However, one isolate, 2843 reacted more strongly than other *P. sy.* pv. *tomato* isolates at 10^6 and 10^8 CFU/ml. It

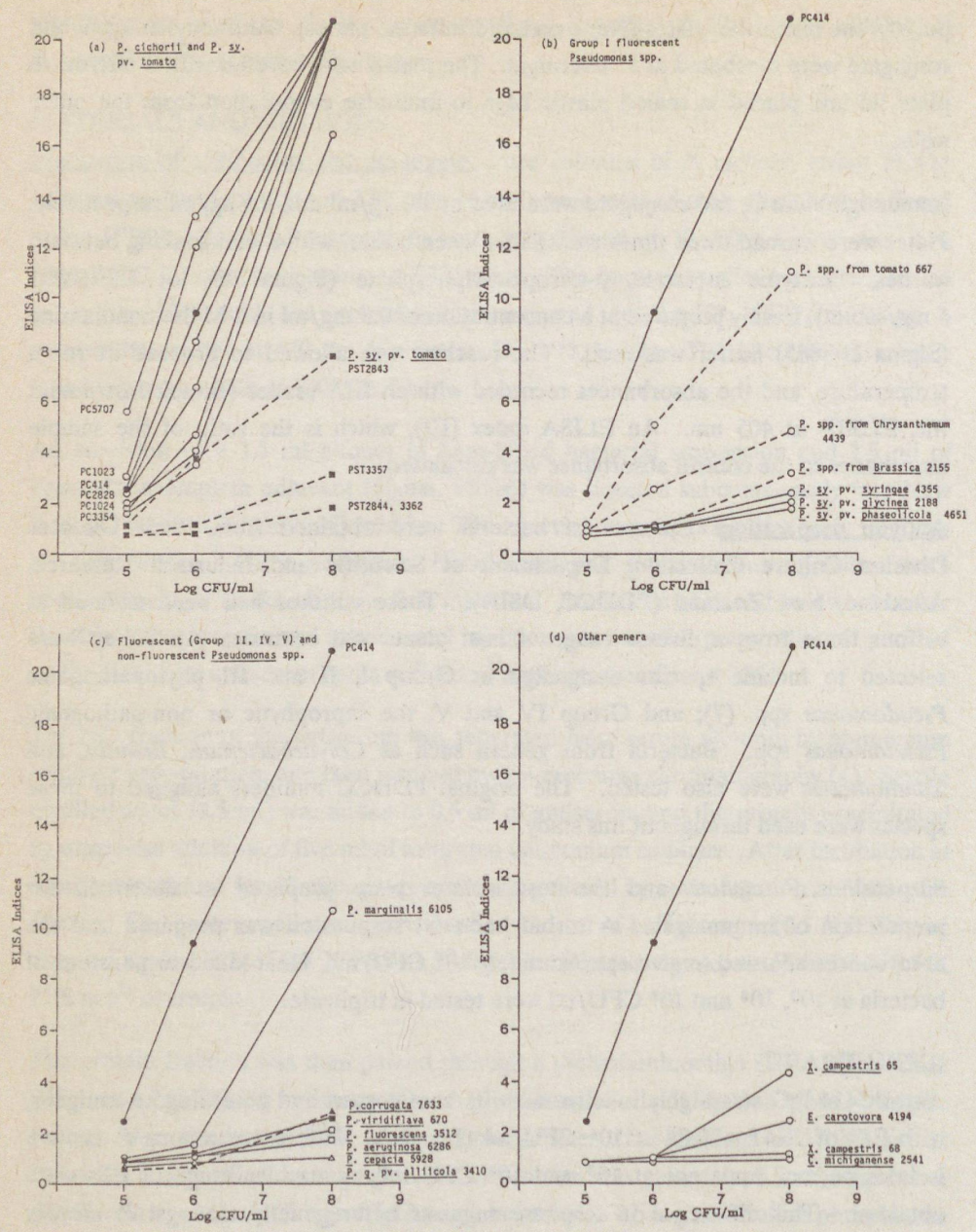

Figure 1 a-d Specificity test of anti-Pseudomonas cichorii PC414 IgG and conjugate against heat-killed bacteria at concentrations of 10^5, 10^6 and 10^8 CFU/ml. IgG and conjugate concentrations were at 1.0 and 0.5 µg/ml respectively. Graph of homologous PC414 was included in all figures for comparison.

probably contained more antigenic components similar to those of *P. cichorii* than other *P. sy.* pv. *tomato* isolates.

All other bacteria tested were easily differentiated from *P. cichorii* isolates because of their comparatively low reactions. At 10^8 CFU/ml, EI's obtained with PC414 were about 5 - 20 times higher than readings obtained with most bacteria. Low response curves of species representative of the grouping by Lelliot *et al.* (7) are illustrated in Fig. 1b-c. They included pathovars of *P. syringae* Group (Fig. 1b); Group II *P. viridiflava*, Group V *P. fluorescens* and *P. aeruginosa* (Fig. 1c). Little reaction occurred with the non-fluorescent *P. corrugata* and *P. gladioli* pv. *alliicola* (Fig. 1c). Bacteria from other genera, i.e. *Corynebacterium*, *Erwinia*, and *Xanthomonas* were easily differentiated because of their weak reactions (Fig. 1d).

Some cross-reactions were observed with a *Pseudomonas* species from tomato, 667 at 10^8 CFU/ml; but the low EI's allowed differentiation from *P. cichorii* at 10^5 - 10^6 CFU/ml (Fig. 1b). The Group IV bacterium, *P. marginalis* pv. *marginalis* probably has some antigens in common with *P. cichorii*, but the cross-reaction was distinguished from the homologous reaction by EI's of less than 1.5 at 10^5 and 10^6 CFU/ml (Fig. 1c).

The degree of relatedness of other species to *P. cichorii* can be represented by the percentage homology. This was obtained as a ratio of the EI's of other bacteria over the EI's of the homologous antigen, PC414, at a bacterial concentration of 10^6 CFU/ml. *P. cichorii* isolates shared common antigens and therefore had a high degree of homology. Other than isolates *P. sy.* pv. *tomato* 2483, anti-PC414 IgG had little antigenic affinity for most bacteria, as their low homology values were mainly below 24%.

DISCUSSION

The tests demonstrated the specificity of ELISA for differentiation of *P. cichorii* from other *Pseudomonas* spp. and species of other genera. There was little serological relationship between and *P. cichorii* with other fluorescent, saprophytic *Pseudomonas* spp., non-fluorescent *Pseudomonas* spp. and bacteria from other genera, even though these were isolated from tomato. The high specificity has also been demonstrated with *P. syringae* pv. *tomato* and *P. corrugata* using ELISA (8).

Strong cross-reactions with some bacteria usually can be distinguished from the homologous reactions by comparing the response curves. The ELISA also demonstrated serological diversity within *Pseudomonas* species. This heterogeneity

has also been shown with *P. sy.* pv. *tomato* where a range of reactions were obtained with different isolates (8). This heterogeneity will make serological identification less clear cut. However, additional knowledge of the hosts from which the bacteria were isolated, and use of a few selected cultural and biochemical tests of these isolates will assist identification of these species.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

MAGNETIC IMMUNOISOLATION OF XANTHOMONAS CAMPESTRIS PV. PELARGONII

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Detection of plant pathogenic bacteria in natural habitats oftentimes is difficult. Low concentrations of the target organism coupled with high populations of nontarget organisms hinders the recovery process and results in less precise determinations of pathogen populations and incidence on host tissue. Increasing the sensitivity of detection has been a major concern to phytobacteriologists concerned with quarantine restrictions and also with ecological and epidemiological studies. Over the years detection sensitivity has been increased by a number of procedures such as the use of selective media (Fahy & Persley 1983; McGuire et al. 1986), serological assays (Schaad 1979), use of host tissue for the selective enrichment of a target organism (pathogenic to that plant species) (Jones et al. 1981), and many other procedures. One new approach is immunoisolation in which antibodies specific to the target organism bond to a solid phase (van Vuurde 1987); the target organism attaches to the antibody, whereas the nontarget organism remains unattached. The nontarget species are washed away while the target organism remains attached to the antibodies which continue to be attached to the solid phase.

Recently, immunomagnetic separation has been developed as a procedure for the recovery of components from crude extracts (Reactifs IBF Manual 1979). This has opened up new possibilities for separation of chemicals and cells from mixed suspensions. It was the intention of this study to formulate a procedure for the concentration and selective isolation of phytopathogenic bacteria from samples using magnetic immunoisolation.

MATERIALS AND METHODS

Bacterial strains. All strains were maintained on silica gel (Sleesman & Leben 1978) for long term storage. For experiments, the bacteria were

grown on trypticase soy agar (TSA) for 24 h at 27 C. Erwinia carotovora pv. atroseptica strain 161, Xanthomonas campestris pv. pelargonii strain 272, and X. campestris pv. begoniae strain 206 were used in the studies. Cells were harvested from the plates, suspended in sterile one-quarter strength Ringers solution containing 0.1% peptone and Tween 20 (RPT), and adjusted to approximately 10^8 colony forming units (cfu)/ml by adjusting the optical density of the suspension to 0.15 O.D. with a spectrophotometer at 600 nm. The suspensions were then serially diluted in RPT to the desired concentration.

Antisera. Antiserum used in the studies was prepared by injecting whole cells of X. campestris pv. pelargonii strain 272 into New Zealand White rabbits. The antiserum used was purified IgG (1 mg/ml) using the procedure of Tobias et al. (1982).

Magnetic particles. The Biomag particles (Advanced Magnetics, Inc., 61 Mooney St., Cambridge, MA 02138) are superparamagnetic. The particles used in these studies have goat anti-rabbit antibodies covalently bound to the surface of the particle. The core is composed of iron oxide. The particles range in size between 0.5 and 1.5 um. The particles are suspended in a solution which contains sodium azide. Prior to use, the suspension must be washed by centrifuging at 12000 r.p.m. in an Eppendorf centrifuge, removing the supernatant and washing in sterile RPT. This procedure is repeated three times.

Magnetic immunoisolation (MII). Developing the procedure requires the selection of optimal antibody concentrations, target organism concentrations, buffer selection and incubation conditions, washing conditions to remove non-target organisms but minimize loss of the target organism and retrieval of the target organism. The following is a description of the procedure used presently (Fig. 1). The bacterial suspension is treated with the polyclonal antibody for approximately 60 min at room temperature. After treatment the suspension is centrifuged for 10 min at 12,000 r.p.m. in an Eppendorf microcentrifuge. The supernatant is removed and the pellet is resuspended in sterile RPT. The suspension (0.5 ml) is placed in a 24 well culture plate (Costar) and the suspension containing the magnetic beads is added to reach a final concentration of 10% (v/v). The suspension (0.5 ml) is placed in a 24 well culture plate (Costar) and then the Biomag suspension is added to reach a final concentration of 10% (v/v). The suspension is incubated for 1 h at room temperature and then it is treated with a super

magnet (14 mm diameter and 7 mm thickness) by placing it directly beneath the well and pulling the Biomag beads toward the bottom of the well. The supermagnet (Bakker Madava, Reebommel 2 Industrieterrein Ekkersrijt, 5691 MX Son, Nederland) is an alloy of neodimium, boron and iron. After exposure to the magnet for 3 min, the liquid is removed by placing the plate, which is still exposed to the magnet, on approximately a 15 degree angle and carefully aspirating away the liquid. The magnet is removed from beneath the well and 1 ml of sterile RPT is added to the well and the contents of the well are resuspended by gentle pipetting. The magnet is again placed beneath the suspension and the procedure is repeated. After three washes, the magnet is again removed and the contents of the well are resuspended in 0.5 ml RPT and the suspension is vortexed and plated directly on TSA. After incubation at 28 C for 72 h, X. campestris pv. pelargonii colonies are counted. In the first test, cell suspensions (10^3 cfu/ml) consisting of either X. campestris pv. pelargonii, X. campestris pv. begoniae, or E. carotovora pv. atroseptica were treated with a 1:30 dilution of a purified IgG preparation (1 mg/ml). The suspensions were then treated according to the MII procedure and compared with control plates where aliquots were plated from suspensions of the three bacteria where no washing procedure was done. The percent recovery was determined as (cfu in treatment)/(cfu in control)*100.

In a second test, two concentrations of X. campestris pv. pelargonii (10^3 and 10^4 cfu/ml), 3 concentrations of E. carotovora pv. atroseptica (10^3, 10^5, and 10^7 cfu/ml), and 2 concentrations of the above antisera were tested in a 2 x 3 x 2 design using the MII procedure. The experiment was replicated three times over time.

RESULTS AND DISCUSSION

In preliminary experiments, IgG purified antibody concentrations were tested to determine the optimal concentration for the MI procedure. Generally, the range from 30 to 480-fold dilution had little effect on the recovery of X. campestris pv. pelargonii. A 1:10 Biomag to test suspension ratio was more effective in binding the target bacterium than a 1:100 concentration and was used in all experiments.

MII resulted in a significantly ($p=0.05$) higher percent recovery of X. campestris pvs. pelargonii and begoniae where anti X. campestris pv. pelargonii polyclonal antibodies were used, compared to when they were omitted (Table 1). However, E. carotovora pv. atroseptica had a very low

recovery with or without the addition of the anti X. campestris pv. pelargonii antibodies. These results were useful for demonstrating the potential benefit of magnetic immunoisolation. The reason that X. campestris pv. begoniae and the homologous reaction reacted similarly is not known. However, similar surface antigens may exist which are responsible for similar results.

Table 1. The effect of the magnetic immunoisolation procedure on recovery of three plant pathogens using antisera produced against Xanthomonas campestris pv. pelargonii.

Bacterium	Antibody	Biomag beads	Recovery[a] (%)
X. campestris pv.	-	+	2.3
pelargonii	+	+	33.6
X. campestris pv.	-	+	5.6
begonia	+	+	25.2
Erwinia carotovora	-	+	3.2
pv. carotovora	+	+	1.6

[a]Recovery is ((cfus/ml) in treatment)/(cfus/ml) in control)) x 100.

In studies where mixed suspensions of E. carotovora pv. atroseptica and X. campestris pv. pelargonii were subjected to the MII procedure, recovery efficiency of X. campestris pv. pelargonii ranged from 28.1- 39.6% and 6.3-30.6 where no E. carotovora pv. atroseptica 10^5 cfu/ml or E. carotovora pv. atroseptica was mixed with 10^3 cfu/ml of X. campestris pv. pelargonii. In 2 or 3 tests, X. campestris pv. pelargonii was isolated from treatments where it was mixed with 10^7 cfu/ml of E. carotovora pv. atroseptica. In these tests, 99.8-100% of the E. carotovora pv. atroseptica cells were eliminated from all treatments where Erwinia was added.

The successes with MII for isolation of X. campestris pv. pelargonii in mixed populations and success in the isolation of the bacterium from contaminated leaf surfaces (Jones and van Vuurde, unpublished) indicate that this procedure may be used successfully in ecological type studies. Further work is needed to reduce the saprophyte to pathogen ratio. One modification, which has worked well, is to dip the magnet into the top of the suspension

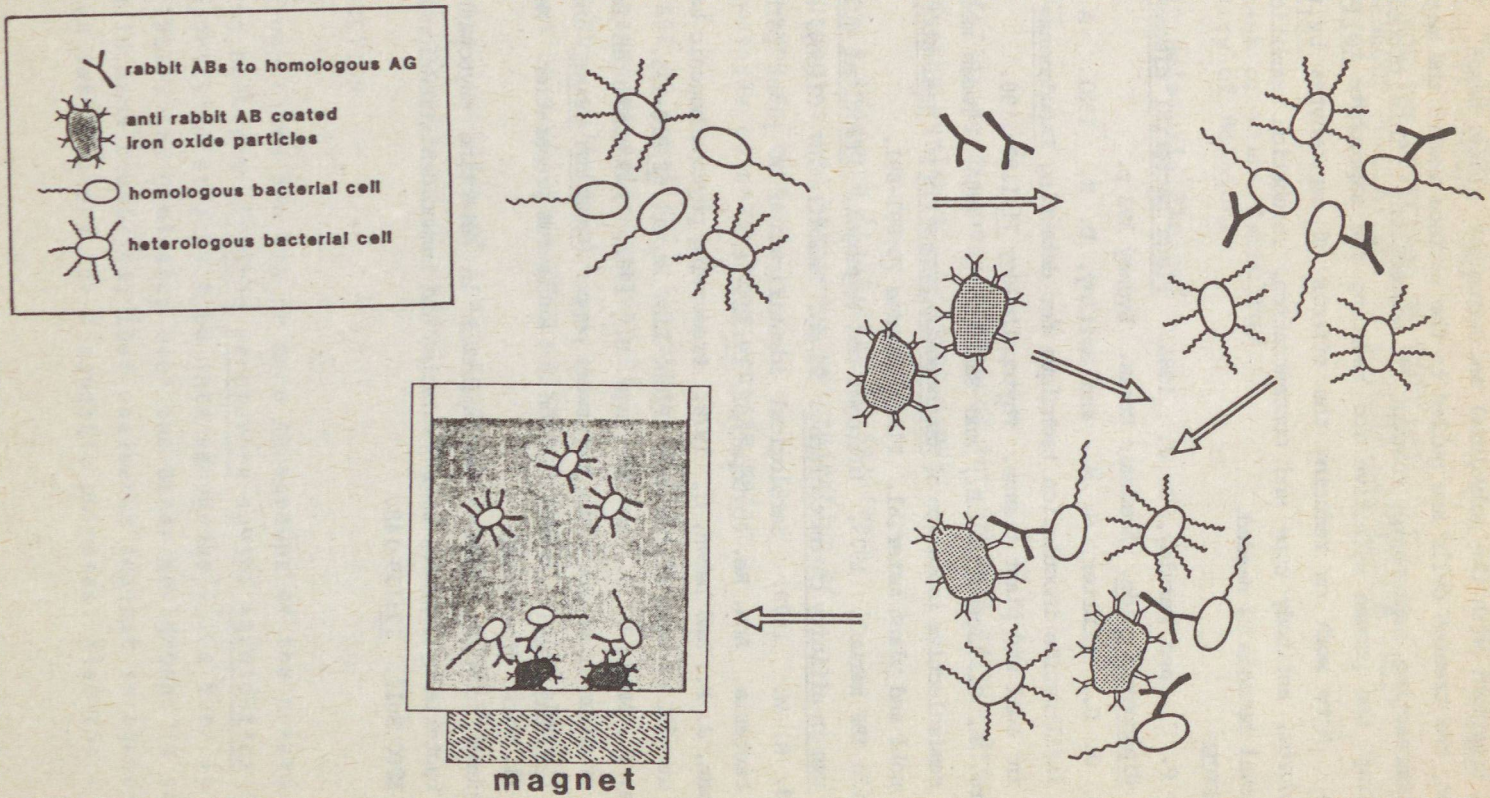

Fig. 1. Schematic representation of magnetic immunoisolation in a suspension of homologous and heterologous bacterial cells

after treatment with the polyclonal antiserum and biomag beads. In this manner, the treated cells are pulled to the surface toward the magnet and the nonreacting saprophytes remain in suspension. This procedure of 'fishing' has proven effective for reducing the saprophyte to pathogen ratio. More work on reducing the effects of saprophytes by washing procedures, antibody type and concentration, incubation conditions and retrieval methods is needed.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

METHODS FOR THE DETECTION OF SEROLOGICALLY HETEROGENEOUS POPULATIONS OF ERWINIA CAROTOVORA SUBSP. ATROSEPTICA

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ABSTRACT

The following diagnostic methods for Erwinia carotovora have been adopted during the present study:

-Protein A sandwich ELISA (PAS-ELISA) for
  -subspecies and serogroup determination of
    -pure cultures
    -single colonies from pectate media

-SDS-PAGE of total cellular proteins for
  -subspecies determination of
    -pure cultures
    -single colonies from pectate media

The applicability of the methods to diagnosing plant samples directly is discussed.

INTRODUCTION

Although there are ten or more serogroups of the potato blackleg pathogen, Erwinia carotovora subsp. atroseptica (Eca), the majority of strains type into serogroup I (De Boer et al. 1979, 1987). Its dominance over the other serogroups is so strong in many countries, that antiserum against serogroup I is often satisfactory for diagnostic purposes. Also the

commercially available kits for the detection of Eca have so far been specific to serogroup I. In Finland, however, a significant proportion of Eca strains type into the other serogroups. Of the 2330 Eca isolates typed during 1983-1988, only 74 % belonged to serogroup I, while the rest typed into serogroups XXXV, XLI, and XLIII, and 1 % of isolates remained untyped (P. Harju & J. Kankila, manuscript in preparation). Due to the serological heterogeneity of the Finnish Eca population, reliable screening for Eca in seed potatoes has been possible only by isolating colonies from pectate media for biochemical tests, which are too laborious for large scale routine testing.

Two approaches towards an Eca-specific diagnostic scheme were chosen. One was to search for an Eca-specific bacterial component that could be identified immunochemically or by other rapid methods. The other approach was to prepare a mixture of antibodies that would detect a sufficiently high percentage of the population.

MATERIALS AND METHODS

Electrophoresis of bacterial proteins

The buffer system of Laemmli (1970) and LKB Midget mini-gel device was used for the polyacrylamide gel electrophoresis (SDS-PAGE) of both total cellular proteins and outer membrane proteins (OMPs).

Pure cultures were prepared for analysis of total cellular proteins by suspending an overnight slant culture (Oxoid nutrient agar) to 4 ml sterile distilled water to reach a cell density of about 10^8 / ml, and pelleting 0.5 ml of the suspension in a microfuge. The pellet was suspended in 0.05 ml sample buffer and boiled for 5 min in a waterbath.

Samples from pectolytic colonies growing on Stewart's (1962) medium were prepared simply by picking a single colony with a pipette tip, suspending it in 0.05 ml sample buffer and boiling as above.

Outer membrane proteins were isolated by breaking the cells by sonication and solubilizing the OMP fraction by the method of Nurminen et al. (1976).

Protein A sandwich ELISA

PAS-ELISA (Edwards & Cooper 1985) is one of the several indirect ELISA procedures developed in order to avoid the production of a separate enzyme conjugate from each different antiserum.

The following modifications were made to the procedure described by Edwards & Cooper (1985). The coating and detecting antibodies were crude antisera produced against whole cells of Eca, and diluted mainly 1:1000 in PBS-Tween. Antisera were applied to the plates either singly or as a mixture of antisera produced against serogroups I, XXXV, and XLI. The sample buffer contained 2 g/l Bovine Albumin in PBS-Tween. 0.15 ml sample buffer was applied to the wells, followed by 0.05 ml of bacterial suspension. Protein A - alkaline phosphatase conjugate (Sigma) was diluted to 1 microgram/ml.

RESULTS AND DISCUSSION

As evaluated by visual inspection of SDS-PAGE gels, there is no significant variation in the OMP or total cellular protein patterns between different Eca serogroups. The most abundantly occurring OMPs have molecular weights of approximately 36 and 39 kd, as earlier demonstrated by Saarilahti & Palva (1986). The position of these major OMPs in the total protein patterns can be used to differentiate Eca and Ecc, since the corresponding proteins of Ecc have different MWs. There is some variation in protein patterns within the subspecies Ecc, but they are always clearly different from those of Eca.

There is no reason to pure cultivate the isolates for the total protein analysis, since a single colony on the isolation medium yields protein sufficiently. The protein concentrations of this kind of samples are prone to vary, but

the major proteins are distinguishable in a wide range of concentrations. The result of the analysis can, of course, be confirmed by measuring the positions of protein bands or by laser densitometry, but with little practice one can determine the subspecies of a sample by looking at the gel (see Fig. 1).

The total protein analysis has been used in our lab for example to confirm the subspecies of serologically untypable strains. If such strains are non-Erwinia, it is readily revealed by the protein "fingerprint". For large scale diagnostics the method may be considered too laborious, although the availability of mini-gel devices has relieved the work load.

The attempts to produce an Eca-specific antiserum by using OMP preparations as immunogens have not been successful so far. Obviously the lipopolysaccharides remaining in the protein preparations render the antisera serogroup specific. Even if the proteins were further purified, they could be weakly immunogenic.

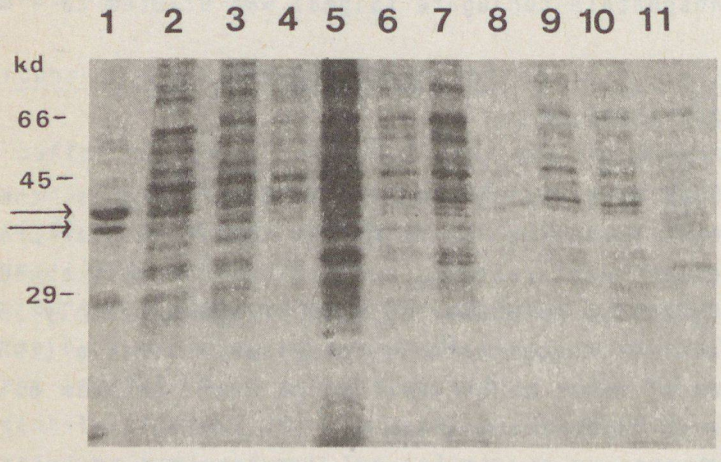

FIGURE 1. SDS-PAGE of outer membrane proteins and total cellular proteins in a 12 % mini-gel. Lane 1: OMP of strain 64, serogroup XXXV, major OMPs indicated by arrows. Lanes 2-11: total proteins of single colonies from Stewart's medium; lane 2, strain 802, Eca serogroup I; lane 3, 365, Eca I; lane 4, 64, Eca XXXV; lane 5, 281, Eca XLI; lane 6, 432, Eca XLI; lane 7, 10, Eca XLIII; lane 8,408, Ecc V; lane 9, 70, untyped Ecc; lane 10, 107, Ecc IX; lane 11, 2002, non-pectolytic strain isolated from potato on Stewart's medium.

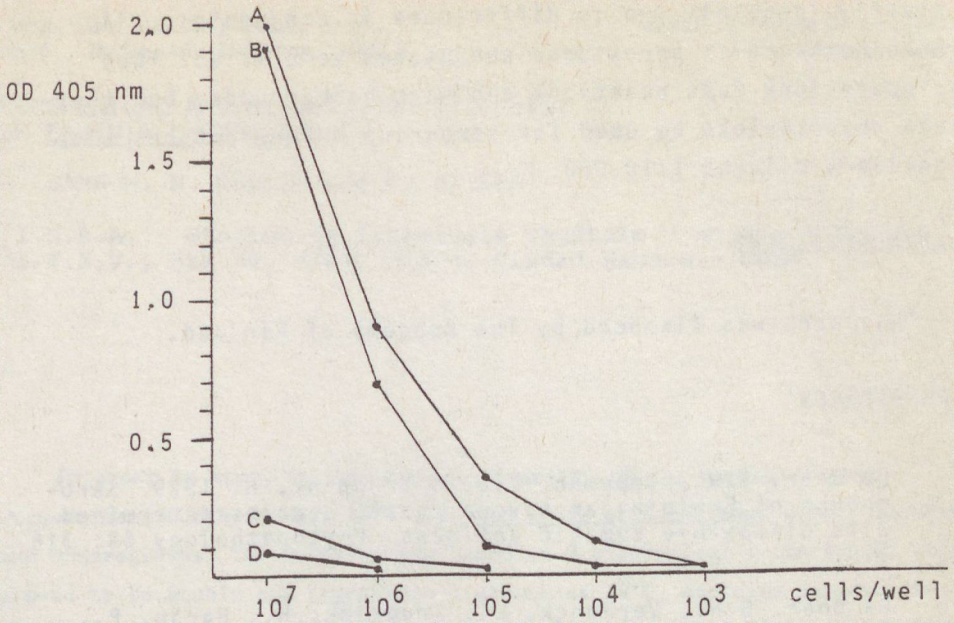

FIGURE 2. Reactions of various bacteria in PAS-ELISA with mixed Eca antisera. A = 64, Eca serogroup XXXV; B = 169, Eca I; C = 107, Ecc IX; D = 2002, non-pectolytic bacterium isolated from potato. Dilution of each antiserum (I, XXXV, XLI) was 1:1000. Values represent means of two determinations per sample.

PAS-ELISA with a mixture of three antisera can detect approximately 97 % of the Finnish Eca population, in contrast to the approximately 75 % detected by serogroup I -specific ELISA. The detection limit of the method is close to 10^5 cells per sample well (Fig. 2). The procedure is being tested with plant material and further developed to increase sensitivity and specificity. However, the current sensitivity, obtained with an antiserum dilution of 1:1000, is sufficient when using pure cultures or colonies from isolation media as sample material. Consequently, PAS-ELISA with single antisera has already been routinely used for serogroup determinations, and large quantities of antisera have been saved, as compared to Ouchterlony tests or producing conjugates of each antiserum.

It was found important that positive controls are included in the assay, since the rate of color development varied

greatly, probably due to differences in conjugate quality. Also horseradish peroxidase conjugates were tried. They produce very fast reactions and high backgrounds, but they can nevertheless be used for serogroup determinations with antisera diluted 1:10 000.

ACKNOWLEDGMENT

This work was financed by The Academy of Finland.

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

Plant Pathogenic Bacteria
Z. Klement (ed.)

BIOCHEMICAL AND SEROLOGICAL DIVERSITY OF ERWINIA CHRYSANTHEMI

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In order to study the phenotypic diversity of the species *Erwinia chrysanthemi* Burkh. *et al.* 1953 (2), two types of tools were used : biovars and O-serogroups. The biovars were based on 9 biochemical criteria (6) that proved to be stable and repeatable : growth at 39°C, arginine dihydrolase (ADH), D(-) arabinose, 5 ketogluconate, inuline, mannitol, melibiose, raffinose and D(-) tartrate. The O-serogroups were established by indirect immunofluorescent staining, showing fluorescent bacterial bodies, or fluorescent flagella, or both. Four serogroups were already described (5). Eight more are being published elsewhere. No cross-reaction was observed between the 12 serogroups. Flagella types were designated by a letter as to avoid confusion with the numbers of O-serogroups. A total of 192 strains of *E. chrysanthemi* were studied. They originated from 27 host-plants (including Banana, Carnation, Dieffenbachia, Kalanchoe, Maize, Potato, Saintpaulia, Tomato and Sunflower) and from different locations (the five continents).

The identity of the strains was checked to confirm the species : pectolytic, fermentative on Hughand Leifson, oxidase negative, nitrates reduced to nitrites, starch negative, lecithinase generally positive, malonate positive, trehalose negative, indole positive, production of gas from glucose (4).

When classifying the 192 strains on the basis of the 9 physiological criteria, we only encountered 9 combinations of the tests (table 1). The strains showed an uneven distribution into the 9 biovars. The biggest group fell into biovar 3 (49 %), then came biovar 1 with 24 % of the strains (table 2). Thus, 73 % of the strains belonged to only two biovars : 3 and 1.

TABLE 1 : Definition of the 9 biovars of Erwinia chrysanthemi

BIOVAR	39°C	ADH	D-arabinose	5keto-gluconate	inuline	mannitol	melibiose	raffinose	D-tartrate	REFERENCE STRAINS
1	-	+	-	-	+	+	+	+	+	NCPPB 453
2	+	-	+	-	-	+	-	-	-	NCPPB 2976
3	+	-	+	-	-	+	+	+	-	NCPPB 2538
4	+	-	+	+	-	-	+	+	+	GRANADA 142
5	+	+	-	-	+	+	+	+	-	NCPPB 402
6	+	-	-	-	-	+	+	+	-	NCPPB 516
7	-	+	-	-	+	+	-	-	+	CFBP 2015
8	+	+	+	-	-	+	+	+	-	NCPPB 2546
9	-	-	-	-	+	+	-	-	+	DINESEN EKII

As for the O-serogrouping, 21 polyclonal antisera were utilized by immunizing rabbits with formalized whole cells, injected intraveinously with 0.5 ml, 1 ml, 2 ml and 2 ml of suspension within 2 weeks. For each of the 12 serogroups, we used respectively : for serogroup 1, strains CFBP 795, 2013, 1495, 1888 and NCPPB 2976 with flagella a, and NCPPB 402 with flagella b ; for serogroup 2, NCPPB 1861 with flagella c ; for serogroup 3, CFBP 1451 (= Granada 142) ; for serogroup 4, CFBP 1502 with flagella d ; for serogroup 5, CFBP 1496 with flagella e ; for serogroup 6, CFBP 1596, NCPPB 2538 (pathotype *zeae*) and NCPPB 1065 with flagella a ; for serogroup 7, NCPPB 1121 with flagella a ; for serogroup 8, CFBP 1531 (= Kelman W1-1) with flagella a ; for serogroup 9, CFBP 1277 (= Kelman C1B1) with flagella e ; for serogroup 10, CFBP 1528 (= Kelman W3-20) with falgella a ; for serogroup 11, CFBP 1885 and SF 132-2 with flagella a ; for serogroup 12, C94 (Rivera) with flagella d. Some of the serogroups correspond to those of Dickey *et al.* (1987) (1).

When serogrouping the 192 strains, we found 68 % of them in the O-serogroup 1 and 10 % in the serogroup 6 (table 3). The other strains were scattered into the other ten serogroups. Ten strains remained non typed.

TABLE 2 : Distribution of 192 E. chrysanthemi strains into the 9 biovars and their host-plants of origin

BIOVAR	NUMBER OF STRAINS	%	HOST PLANTS
1	46	24	6 : Dahlia, Dianthus, Cynara scolymus, Cichorium intybus, Lycopersicum esculentum tuberosum
2	10	5	3 : Dieffenbachia, Musa, Lycopersicum esculentum
3	95	49	17 : Ananas, Aranda, Brachiaria, Cynara scolymus, Dioscorea, Euphorbia pulcherrima, Ipomea batatas, Musa, Nicotiana tabacum, Oncidium, Panicum, Pelargonium capitatum, Philodendron Saccharum officinarum, Saintpaulia, Solanum tuberosum
4	5		2 : Musa, Zea mays
5	19	10	4 : Cichorium intybus, Chrysanthemum maximum and morifolium, Lycopersicum esculentum
6	2		1 : Parthenium
7	4		2 : Cichorium intybus, Solanum tuberosum
8	7		1 : Zea mays
9	4		1 : Kalanchoe

The majority of the strains, belonging to serogroup 1, originated from 20 host-plants out of 27, and from any location. As for the flagella, the H-antigens overlapped the O-serogroups. Flagella-type a was dominant (79 % of the typed flagella). It was common to many strains over all the O-serogroups.

Considering the host of origin, there was no correlation between serogroups and host-plants. As for the biovars, in some cases (*Dianthus, Dieffenbachia* for instance), the isolates issued from the same host were homogeneous. But we generally found, in one biovar, several host-plant origins. In the other hand, the 41 *Solanum tuberosum* strains (table 4) belonged to biovars 1, 3 and 7. It was obvious that strains of biovars 1 and 7 came from Europa, temperate countries, and that biovar 3 strains came from tropical or subtropical countries. A correlation appeared between biovars and geographic origin of the strains. The temperate origin

TABLE 3: Distribution of 192 E. chrysanthemi in 12 O-serogroups, and corresponding flagella-types

O-SERO GROUP	NUMBER OF STRAINS	%	H-ANTIGENS a	b	c	d	e	NT	NUMBER OF HOST-PLANTS	GEOGRAPHIC ORIGIN
1	131	68	80	17	-	3	-	20	20	Africa America Europa
2	4		-	-	2	-	-	2	2	Italia USA
3	5		-	-	-	-	-	5	2	Colombia Cuba
4	1		-	-	-	-	-	1	1	France
5	2		-	-	-	-	1	1	1	France
6	19	10	10	-	-	3	-	6	3	America Asia Australia Europa
7	2		2	-	-	-	-	-	2	India Malaysia
8	3		2	-	-	-	-	-	3	Asia USA
9	3		-	-	-	-	1	2	1	Italia USA
10	2		2	-	-	-	-	-	2	Australia USA
11	9		8	-	-	-	-	1	3	F. Guyana Senegal
12	1		-	-	-	1	-	-	1	Cuba
NT	10		3	-	-	1	-	6	5	Africa America Asia Australia Europa

NT : serogroup or flagella-type not determined

TABLE 4 : Biovar heterogeneity of E. chrysanthemi from Potato and Maize; comparison with their geographic origin.

BIOVAR	Solanum tuberosum	NUMBER OF STRAINS	Zea mays	NUMBER OF STRAINS
1	France Switzerland	24		
3	Australia Brazil Cuba Peru	14	Africa America Asia Australia Europa	34
4			Cuba	1
7	France Switzerland	3		
8			India USA	6
TOTAL STRAINS	41		41	

must be linked to the criterium "no growth at 39°C". Strains of biovar 1, 7 and 9 are temperate strains (3). The reciprocal is true : no strain of biovar 1, 7 or 9 was found in tropical countries. Nevertheless, the distribution of biovar 3 is not restricted to tropical countries as we found it on *Zea mays* in Europe (table 4). It is worldwide.

This is a demonstration that the pathovar system should not be used in naming subspecies of *Erwinia chrysanthemi*. The physiological properties leading to biovars give acute separations that, associated to O-serogrouping, account for the diversity of the species.

ACKNOWLEDGEMENTS

We sincerely thank Dr. O. Cazelle (CH), Dr. R. Grimm (CH), J. Vogelsanger (CH), Dr. M. Perombelon (GB) and Dr. J.C. Girard (FR) for sending us strains.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

SPECIFICITY OF ANTIBODIES AGAINST ERWINIA CHRYSANTHEMI IN DAS-ELISA

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INTRODUCTION

Erwinia chrysanthemi Burkholder (Ech) is a pectinolytic plant pathogenic bacterium that causes diseases in a wide range of plant species (Janse and Ruissen, 1988). Serological methods play an important role in detection, identification and classification of Ech (Samson 1987, Janse and Ruissen, 1988 and Beguin, 1987). Polyclonal antibodies produced against whole cells of Ech have been commonly employed in these tests. However, the application of antibodies directed to a bacterial cell which possesses many different antigenic sites may have disadvantages. The chance on cross-reactions with polyclonal antisera (pca) containing antibodies against various epitopes is higher than with monoclonal antibodies (mca) produced against one specific epitope.

In this study the specificity of polyclonal antisera produced against whole cells of an Ech-strain isolated from potato (*Solanum tuberosum* L.) is investigated and compared with that of monoclonal antibodies (mca) to Ech in double antibody sandwich (DAS)-ELISA. One hundred isolates of plant pathogenic bacteria and 900 bacterial isolates from potato peels were analysed for cross-reactions. Strategies to improve DAS-ELISA as a detection method for Ech are discussed.

MATERIALS AND METHODS

Bacterial isolates. Strains of plant pathogenic bacteria of the IPO culture collection, belonging to the genera *Erwinia*, *Pseudomonas*, *Xanthomonas*, *Agrobacterium* and *Clavibacter*, were tested in concentrations of c. 10^{10} cells/ml in DAS-ELISA.

The collection of 900 potato peel isolates was obtained as follows: Peelings of the cortex and outer parenchymatic tissue of ten cultivars of seed potatoes produced on three soil types were macerated in a press. The diluted extracts (10^{-1} and 10^{-2}) of each tuber peeling were incubated on trypticase soy agar (TSA, BBL) and Standard Nährbouillon Agar (NBA, Merck) at 1/6 of the prescribed concentration (Trolldenier, 1972) for 24 h at 27 °C and a subsequent 24 h at 6 °C.

For all assays the bacteria were grown on TSA for 24 h at 27 °C.

Production of antibodies. Antisera 8276B and 8575E against whole cells of Ech (IPOnr. 502) were produced by the method as recommended by Lazar (1972). Antiserum 8151 was produced against glycoproteins of Ech extracted as described by Lazar (1968). For antiserum 8152D soluble antigens of Ech that were precipitated with antibodies (no. 7474 directed against whole Ech cells) in Ouchterlony double diffusion served as the antigen. Antisera to *Xanthomonas campestris* pv. *begoniae* (Xcb) and *E. carotovora* subsp. *atroseptica* (Eca) and potato virus Y (PVY) served as controls. Antisera to Xcb and Eca were prepared against whole cells. The potato virus Y (PVY)-antiserum was produced following the method of Maat (1981). The mca 2A4 against Ech (502) was produced by Dr. S.H. De Boer with Balb C mouse following the method of Köhler and Milstein (1975). It was proven that 2A4 is directed to epitopes on the lipopolysaccharides (LPS) of Ech (P.M. Boonekamp, pers. comm.)

The antisera were absorbed by suspending bacterial slime in crude antiserum (slime of one full-grown plate/ml antiserum) and incubating the suspension for 30 min. at 37 °C. The (precipitated) cells were removed by centrifugation (10,000 g, 10 min) and the procedure was repeated till no visible precipitation was observed anymore after the incubation. Absorption was checked with Ouchterlony double diffusion.

Immunoassays. DAS-ELISA was performed essentially as described by Clark and Adams (1977). Occasionally 5% defatted milk powder was added to the diluted antibody conjugate. In a first screening high concentrations (c. 10^{10} cells/ml) of the bacterial isolates from potato, grown on TSA and NBA, were tested in DAS-ELISA with an Ech-antiserum (8575E) produced against whole cells of Ech. In a second test ten-fold dilutions of bacterial suspensions with OD_{620}-values of 0.8 (c. 10^5 - 10^9 cells/ml) of 21 isolates which reacted positively together with 27 isolates which reacted negatively, were tested with four polyclonal antisera to Ech and one mca to the LPS of Ech.

ElISA with a coating of the antigen was performed as follows: Serial

dilutions of test strains in sodium carbonate buffer (0.05 M CO_3^{2-}, pH 9.6) were coated for 24 h at 6 °C onto wells of microplates (Inotech, 11041E). The plates were washed with tap water for 40 sec and incubated for 2 h at 27 °C with antibodies against Ech, conjugated with alkaline phosphatase. The plates were washed and incubated for 1 h with 0.75 mg/ml p-nitrophenyl phosphate dissolved in substrate buffer (9.7% v/v diethanolamine in demineralized water, pH 9.8). The absorbance was measured at 405 nm on a EAR-400 ELISA-reader (SLT).

Ouchterlony double diffusion (ODD) was performed as described by Ouchterlony (1958) and immunofluorescence colony staining was performed by the method of Van Vuurde (1987).

RESULTS

No cross-reactions with Ech antiserum 8276B occurred in DAS-ELISA with 100 different strains of plant pathogenic bacteria belonging to the genera *Erwinia*, *Pseudomonas*, *Xanthomonas*, *Agrobacterium* and *Clavibacter*.
In the second screening of the isolates from the potato peels, consistent positive reactions were obtained with 16 isolates with the pca 8276B, 8575E, 8152D and the mca 2A4, 12 of them reacted with 8151D. Detection levels of the different cross-reacting isolates varied between 10^6 and 10^9 cells/ml, also dependent on the antisera used.

These 16 isolates were tested for reactions with pca to other plant pathogens, viz. Eca, Xcb and PVY, to determine whether the cross-reactions were specific for Ech antibodies. Fourteen of the sixteen isolates reacted with the Xcb and the PVY pca. Two isolates (A254 and A256) specifically cross-reacted with the Ech antiserum. Only seven isolates reacted also with the Eca antiserum, probably because of the weak activity (determined with the homologue Eca).

The two isolates (A254 and A256) that only reacted with the Ech antisera were partially identified on the basis of fatty acid profiles as pectinolytic *Pseudomonas* spp. They were isolated from the same potato tuber. These two isolates also cross-reacted with Ech antiserum in the other serological methods used: IFC, ODD and an ELISA with a direct coating of the antigen. None of the other isolates reacted in any of these tests.

The reactions of the 16 non-specific cross-reacting isolates with the antisera against Ech were sufficiently reduced by the addition of 5% defatted milk-powder to the enzyme-conjugate. This blocking agent has proven to be very effective in reducing background and non-specific

reactions in several serological techniques (Johnson et al., 1984).

In order to reduce the specific cross-reaction of A254 and A256, the antiserum to Ech (8575E) was adsorbed with A254. As a consequence, the titre of the antiserum dropped from 32 to 8 in ODD.

DISCUSSION AND CONCLUSIONS

No cross-reactions were found with 100 strains of phytopathogenic bacteria with an Ech antiserum to whole cells.

A strong non-specific reaction in DAS-ELISA was found with 14 of 900 bacterial isolates, isolated from potato peel extracts, both with pca to different preparations of Ech antigens, with the mca 2A4 to Ech and with antisera to other plant pathogens.

These isolates did not react in any other serological test including other formats of ELISA. This observation makes it unlikely that a serological binding of an antibody with an antigen is involved in this type of reaction. It is also unlikely that protein A or similar type of components on the cell wall of the isolates are responsible for the non-specific cross-reactions. The possibility of cross-reactions caused by non-specific reacting organisms in potato peel extracts should be considered, realising that this type of reaction was obtained with c. 1.5% (14 of 900) of the tested isolates. The addition of 5% milkpowder to the conjugate-buffer has been shown to be an adequate method for the reduction of the non-specific reactions.

The significance of the specific cross-reaction with the two pectinolytic *Pseudomonas* isolates for the detection on Ech in practice is still unknown. It has not been investigated whether they will be able to build up high enough densities on potato tubers to interfere with DAS-ELISA results. The pectinolytic property of the two *Pseudomonas* spp. makes it probably for them to grow rapidly in an elective medium containing polygalacturonic acid (PGA). Media with PGA are used for isolation or enrichment of *Erwinia* spp. (Meneley and Stanghellini, 1975). Under these conditions the presence of the cross-reacting *Pseudomonas* in a test sample can easily give rise to false positive results in DAS-ELISA for Ech.

With pca and mca to Ech a specific cross-reaction was only found with one type of organism (2 isolates). This result indicates that the pca to whole cells of Ech are comparable with a mca prepared against the LPS of Ech with respect to the specificity. This specificity is considered to be reasonable but not optimal. To improve the specificity of the Ech-antisera,

a pca was absorbed with one of the *Pseudomonas*-isolates. The successful absorption resulted also in a four-fold decrease in titre against Ech in ODD. Recently, a start was made with research on the characterization of antigens specific for Ech as a basis for the production of more typical antibodies.

ACKNOWLEDGMENTS

I wish to express my appreciation to Dr. R.L. Forster (University of Idaho, U.S.A.), H.A. Underberg and Dr. J.W.L. van Vuurde for their critical reading of the manuscript, to G.C. Gussenhoven for his skilful assistance and to Ir. J.D. Janse (PD) for the identification of the bacterial strains.

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

Plant Pathogenic Bacteria
Z. Klement (ed.)

IMMUNOSTAINING OF COLONIES FOR SENSITIVE DETECTION OF VIABLE BACTERIA IN SAMPLE EXTRACTS AND ON PLANT PARTS

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INTRODUCTION

A new approach combining the advantages of isolation and serology has led to the development of the technique referred to as immuno-isolation. It is based on the selective trapping of target bacteria from the sample onto a solid phase coated with homologous antibodies (Van Vuurde and Van Henten, 1983) and can be used to enhance detection of the target bacterium in high saprophyte to pathogen ratios (S/P). The use of antibody-conjugated magnetic particles has shown its potential for effective selectively reducing this ratio (Jones and Van Vuurde, this book). Further reduction can be achieved by plating the trapped bacteria on a semi-selective medium.

The reliable isolation of a target bacterium present in low concentrations in complex substrates like soil or roots still may not be possible due to the abundance of saprophytes (10^6 to 10^8 cfu per g). Even when the S/P can be reduced a thousand times, the following factors limit sensitive detection: (a) traditional dilution plating has a maximum of 100 to 500 colonies per agar plate which can be counted accurately and (b) similar appearance of microbial colonies of different species. Additional identification procedures are then needed. This will reduce the speed, the detection level and the quantitative reliability and increase the labour of the test, making it less suitable for large-scale application.

A real breakthrough in the consistent detection of low levels of the target bacterium, e.g., for population dynamics studies in the rhizosphere or soil, will only be possible in plating when the number of colonies per agar plate can be greatly increased. With pour plating, up to 6 ml of a sample can be mixed with the agar medium in a standard Petri dish with a capacity of 100,000 to 1,000,000 single colonies in the agar (Fig. 2).

However, the use of pour-plating to optimize isolation can only be exploited fully in combination with colony differentiation techniques. Addition of antiserum during pour-plating to obtain immunoprecipitation around the colonies of the target bacterium showed its potential (Van Vuurde, 1987), but small colonies (< 1.0 mm) often gave a false negative result.

This paper describes the development of techniques for immunological staining of colonies of the target bacterium directly in agar medium. Furthermore, the potential of immunostaining of colonies for *in situ* detection of bacteria is demonstrated.

PROCEDURES FOR IMMUNOSTAINING OF COLONIES

Immunofluorescence colony staining (IFC). The principle and application of IFC staining are given in Fig. 1 and are described briefly below and more detailed in the APS Handbook on Serology (Van Vuurde, in press). The test sample is incubated in a suitable (selective) medium until pinhead sized colonies are formed (usually 24 to 48 h), after which the ager layer is dried into a thin film with a warm air blower. Target colonies in the film

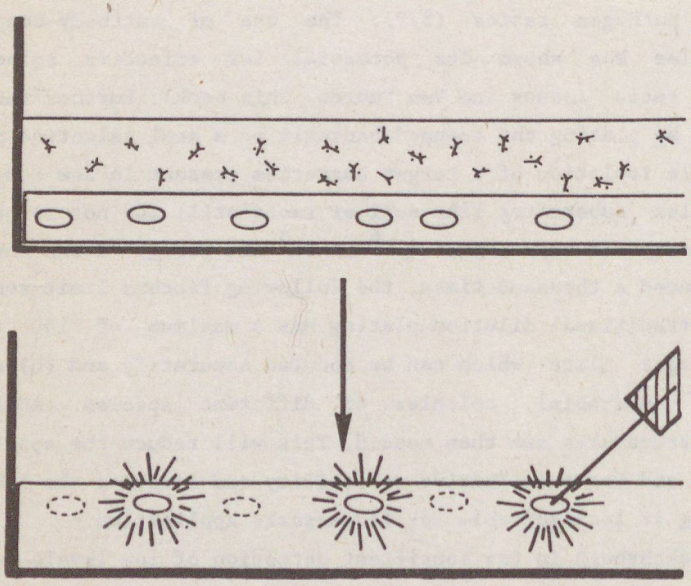


Fig. 1. Scheme of immunofluorescent colony staining. Top: Incubation of dried pour plate with FITC-conjugated antibodies. Bottom: Fluorescent colonies between non-fluorescent colonies under blue light (490 nm). Isolation from IFC positive colony with fine needle.

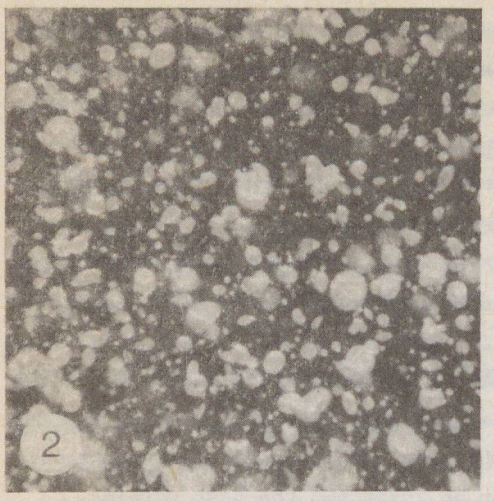
are stained with homologous antibodies conjugated with fluorescein isothiocyanate (FITC), prepared according to the method of Allan and Kelman (1977). The stained preparation is observed with incident blue light at 490 nm with a 4x objective. The incubation conditions for staining (2 h to overnight at 27 °C) and for the conjugate dilution (usually a two-fold higher concentration than for staining bacterial cells in direct IF) should be determined by titration for the specific microscope system in use.

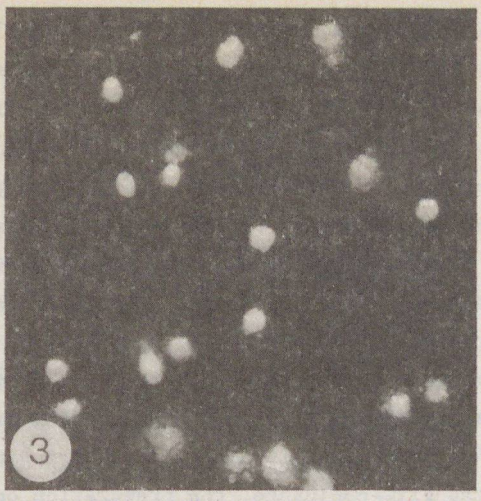
The microcolony assay can be used for fast growing bacteria in order to have the test result available the next day. Growth of the bacteria in the 0.5 to 1.0 mm thick agar layer is stopped after 6 to 8 h when microcolonies of at least 5 to 50 cells are formed. This can be inspected microscopically with a long working distance objective 40x (Olympus LWDCDPL 40x/0.55 LWD 1.9 mm). After drying the agar and incubation overnight with the FITC-conjugate, the preparation can be inspected the next morning.

For detection of target bacteria *in situ*, stem or tuber pieces of 0.1 to 1.0 mm thick (J.W.L. van Vuurde and Ph.M. de Vries, unpublished), or other suitable plant parts such as roots (Underberg and Van Vuurde, this book) were incubated in agar for one to two days. After incubation the fresh or dried preparations were stained as done for colonies in agar.

Immuno-enzyme colony staining. Antibodies conjugated with alkaline phosphatase, prepared for double antibody sandwich ELISA (Tobias et al., 1982), were used with a precipitating substrate as used in dot immuno-binding assays (Zutra et al. 1986) based on naphthol AS-MX phosphate and fast red TR salt (J.W.L. van Vuurde and N.J.M. Roozen, unpublished). The dried agar layer with bacterial colonies was prepared as for IFC-staining. Incubation with various dilutions of the enzyme conjugate and in the next step with the substrate was done as for the dot immuno-binding assay. Preparations were inspected with a stereo microscope at 6 times magnification and dark or bright field illumination.

Immunogold colony staining. Antibodies were conjugated with gold beads of 30 nm. Further data on the conjugation and staining procedure are given in the paper of Underberg and Van Vuurde in this book. Dried and not dried agar layers with bacterial colonies as prepared for IFC were incubated with dilutions of the gold conjugate. During the staining, the preparations were regularly observed with the same stereo microscope system used for immuno-enzyme colony staining and with a compound microscope with epipolarized light and 4x objective.







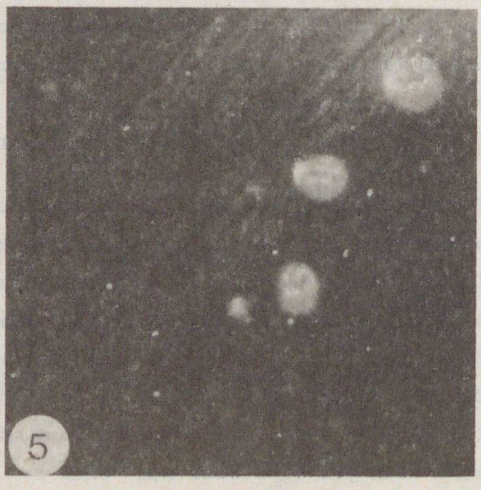


Fig. 2. Dried unstained part of pour plate of potato tuber extract (stereo microscope, dark field illumination, plate area ca 1 cm^2).

Fig. 3. IFC-positive colonies (< 1% of the total number of colonies) in similar pour plate as in Fig. 2 after staining with anti-Ech FITC conjugate (incident blue light 490 nm, obj. 4x, plate area ca 0.3 cm^2).

Fig. 4. IFC-positive colonies in potato stem coupe in agar medium stained with anti-Ech FITC conjugate (microscope data as for Fig. 3).

Fig. 5. Colonies of Eca in pour plate, stained (red) after incubation with alkaline phosphatase conjugated anti-Eca serum and incubation with a precipitating substrate based on naphthol and fast red (pure culture, microscope data as for Fig. 2).

RESULTS

IFC was sucessfully applied for the detection of 10^2 cfu per ml of *Erwinia chrysanthemi* (Ech) and *E. carotovora* subsp. *atroseptica* (Eca) in the extract of potato peel (Fig. 3) and in cattle manure slurry (J.W.L. van Vuurde, M.W.Bos, and N.J.M. Roozen, unpublished). To reduce the costs, incubation and staining were done in a 16 mm diameter well of a tissue culture plate, using 100 μl sample suspension in 300 μl agar medium. Concentrations of saprophyte colonies of ca 5,000 per cm^2 in the agar did not affect the detection of Ech in potato peel extract. Reisolation of Ech was possible from various IFC-positive colonies, but the percentage of successful reisolation for the various samples was inversely related to the density of the saprophyte colonies in the agar. Preliminary experiments with indirect IFC, using FITC conjugated second antibodies, gave less specific results for Ech and resulted in a higher background. With the microcolony technique, Ech could be detected after 6 to 8 h growth in agar.

IFC also enabled the detection of *Xanthomonas campestris* pv. *begoniae* (Xcb) in washing of begonia leaves (J.W.L. van Vuurde and C.E. Winterswijk, unpublished) and seedborne *Pseudomonas syringae* pv. *phaseolicola* and *Clavibacter michiganensis* subsp. *michiganensis* (Franken and Van Vuurde, in press).

Use of IFC for *in situ* detection of bacteria at low magnification was succesful for the various materials tested as shown for Ech in potato stem pieces in Fig. 4, and for Ech on roots in the paper of Underberg and Van Vuurde in this book (Fig. 1 and 3). The use of non-dried agar preparations increased the background fluorescence, but still allowed observation of IFC-positive colonies.

Immuno-enzyme colony staining resulted in a red staining of Eca colonies in the agar, which were clearly visible under the stereo microscope (see Fig 5). However, more than 5% of the colonies of saprophytes from cattle slurry gave a positive reaction in the controls without conjugate, thus indicating that alkaline phosphatase enzymes were also produced by the organisms themselves.

Immunogold colony staining of Ech was only succesful for colonies on the surface of the agar medium. Colonies in the agar were not stained, indicating that the conjugate particles could not penetrate into the agar layer. Enrichment of Ech on potato roots growing on agar showed the potential of this technique for the detection of colonization patterns of target organisms *in situ* on roots, using a stereo microscope at low magnification (see Fig. 4 in Underberg and Van Vuurde, this book).

DISCUSSION AND CONCLUSIONS

Of the investigated immuno colony staining techniques, IFC was most successful. With IFC in combination with pour plating in selective medium, high detectability can be combined with improved specificity. Reisolation from IFC-positive colonies can be used for confirmation and to check for cross-reacting bacteria. When cross-reacting saprophytes are found, they will be used either (a) to improve the specificity of antiserum by absorption or, indirectly, for research for more typical antigens to produce specific antibodies as done for Ech by Van der Wolf (this book) or (b) to develop a more selective medium using inhibitory compounds to eliminate the cross-reacting organism.

IFC is presently evaluated for its characteristics in large scale routine screening of Ech in potato and Xcb in begonia using tissue culture plates to miniaturize the method and to make it cost effective. Clear colony differentiation on the base of IFC makes the use of an image analyser for automated reading of plates for target bacteria possible.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

PRODUCTION OF MONOCLONAL ANTIBODIES SPECIFIC TO XYLOPHILUS AMPELINUS

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INTRODUCTION

Xanthomonas ampelina Panagopoulos 1969, recently reclassified as Xylophilus ampelinus (Willems et al., 1987), is the causal agent of the bacterial blight of grapevine. The disease has been detected in Greece, France, South Africa, Portugal, Spain, Italy and Turkey (Ridé et al., 1983). Isolation, in vitro culture, and biochemical characterization, the serological techniques such as the indirect immunofluorescent staining (Ridé et al., 1977) and ELISA indirect (Aramburu et al., 1984), DNA-DNA and rRNA-DNA hybridization (Willems et al. , 1987) have been utilized for its diagnosis. The serological methods prove the most rapid, cheap, and convenient for large-scale application. The disadvantages of serological methods used with polyclonal antibodies are primarily: moderate specificity due to frequent cross-reactions with saprophytic flora and the variability of antisera from different immunizations.

We considered the possibility of producing and using monoclonal antibodies (MAb) to improve a specific serological diagnosis to assay the discrimination of X. ampelinus isolates and to improve the ELISA sensitivity by new variants using MAb.

MATERIAL AND METHODS

Bacterial isolates. Sixty-three characterized X. ampelinus isolates from different sites were used. The origin of these isolates is shown in Table 1. The isolates were cultured on PYGA medium (yeast extract, 0.5%; bactopeptone, 0.5%; glucose, 1%; agar, 2%). Twenty - three unclassified saprophytic bacteria from grapevine grown in a number of areas of Spain, were isolated on PYGA medium. Twenty-six characterized bacterial isolates of Agrobacterium, Corynebacterium, Erwinia, Pseudomonas, Rhizobium, and Xanthomonas have been used. The references are shown in Table 2. All bacterial isolates are kept in the phytopathogenic bacteria collection of the I.V.I.A.

Antiserum production . Californian x Neozelander rabbits were immunized with somatic antigen (O) of X. ampelinus 64.11. The antigen was obtained by treating a suspension of 3.10^9 cfu/ml phosphate saline buffer (PSB) pH 7.2, for 2 h at 100°C. The immunization procedure consisted of one intravenous injection of 2 ml of the antigen every 5 day . On the 35th day the animal was bled. The antisera was maintained at -70°C.

Production and characterization of hybridomas secreting MAb specific to X. ampelinus. Production of hybrid cells secreting specific MAb was performed by fusion between a non secreting mouse myeloma (P3 x 63-Ag 8653) and spleen cells from X. ampelinus-immunized mice. BALB/$_c$ mouse were immunized by intra-peritoneal injection of 0.1 ml of whole cells antigen preparation (10^9 cfu X. ampelinus 60.7 per ml of PBS) every 10 day , for two months. Hybridization was conducted basically following the procedure described by Vela et al., (1986). Screening for presence of antibodies against X. ampelinus was performed by indirect ELISA (see below). Specific antibody-secreting hybridomas were cloned under conditions of limited dilution using feeder layers (Sanz et al., 1985). Determination of MAb isotype by

immunodifusion, production of ascitic fluids and antibody purification were made following the procedure described by Vela et al., (1986).

Indirect ELISA (ELISA-I). The whole bacterial cells in 100 µl carbonate buffer (0.05M, pH 9.6) were incubated overnight at 4°C. Plates were washed with PBS-Tween 20 (PBS + 0.02% Tween-20) and 100 µl/well of undiluted cell-free culture fluid or 0.1 µg/ml purified MAb or dilutions of different MAbs were pipetted into the microplate (M129A-Dynatech) wells and incubated for 2 h at 37°C. After washing, biotin-labelled anti-mouse immunoglobulins (Sigma) were used as conjugate at 0.1 µg/ml concentration and incubate for 1 h at 37°C. After washing, 100 µl/well streptavidin-peroxidase conjugate (Sigma) was added and incubated for 30 min at room temperature. After washing, 125 µl/well peroxidase substrate (Sanchez-Vizcaíno and Cambra, 1987) was added. The final coloration was obtained after 5-10 m incubation and adding 20 µl/well of 3N sulphuric acid. Reading of results was made with an ELISA-reader at 450 nm. As a control, some wells of the plates were coated with Erwinia carotovora pv atroseptica suspension made in carbonate buffer, and the remaining operations, as indicated above.

For the screening of hybridomes secreting specific MAb, reactions were considered positive when the absorbance readings were three-fold those obtained with the control. In other tests, a reaction was deemed positive when the absorbance readings were twice those obtained with the control.

Sensitivity of ELISA-I B/SA (biotin-streptavidin system) to detect X. ampelinus was assayed using dilutions of X. ampelinus ranging from 10^9 to 10 cfu/ml of carbonate buffer. As negative control, bacteria uncoated wells were use, in which carbonate buffer was added. MAbs utilized were 8c and 5c at a concentration of 0.1 µg/ml.

Table 1. SEROLOGICAL RELATIONSHIP OF XYLOPHILUS AMPELINUS ISOLATES USING SPECIFIC MONOCLONAL ANTIBODIES AND AN ANTISERUM WITH THE ELISA-INDIRECT METHOD

X. AMPELINUS ISOLATES AND ORIGIN		MONOCLONAL ANTIBODIES REACTION 5C MAb	8C MAb	ANTISERA REACTION 8 AS
* 60-7	Zaragoza (Spain)			
G4	Zaragoza (Spain)			
64-11	Zaragoza (Spain)			
67-1b	Zaragoza (Spain)			
67-2a	Zaragoza (Spain)			
69-7b	Zaragoza (Spain)			
70-3a	Zaragoza (Spain)			
71-12	Zaragoza (Spain)			
71-19	Zaragoza (Spain)			
119-2	Zaragoza (Spain)			
119-8	Zaragoza (Spain)			
120-1	Zaragoza (Spain)			
128-3	Zaragoza (Spain)			
190-1	La Rioja (Spain)			
196-1	La Rioja (Spain)			
249-3	Orense (Spain)			
337-1b	Orense (Spain)			
337-2b	Orense (Spain)			
337-3b	Orense (Spain)			
338-10	Orense (Spain)			
338-20	Orense (Spain)			
420-1	Pontevedra (Spain)			
420-2	Pontevedra (Spain)			
420-2.1	Pontevedra (Spain)			
420-5	Pontevedra (Spain)			
420-6	Pontevedra (Spain)			
420-9.1	Pontevedra (Spain)			
420-9.2	Pontevedra (Spain)			
420-10	Pontevedra (Spain)			
530-1	Pontevedra (Spain)			
530-1.2	Pontevedra (Spain)			

530-1.3	Pontevedra (Spain)
530-5	Pontevedra (Spain)
530-5.1	Pontevedra (Spain)
539-5	Pontevedra (Spain)
541-2	Pontevedra (Spain)
541-2.1	Pontevedra (Spain)
541-5	Pontevedra (Spain)
553-1	Pontevedra (Spain)
553-1.1	Pontevedra (Spain)
P5	France
P7	France
P8	France
P13	France
C'14	France
2098	France
806-2	France
48	Greece
55	Greece
59	Greece
86	Greece
88	Greece
95	Greece
96	Greece
120	Greece
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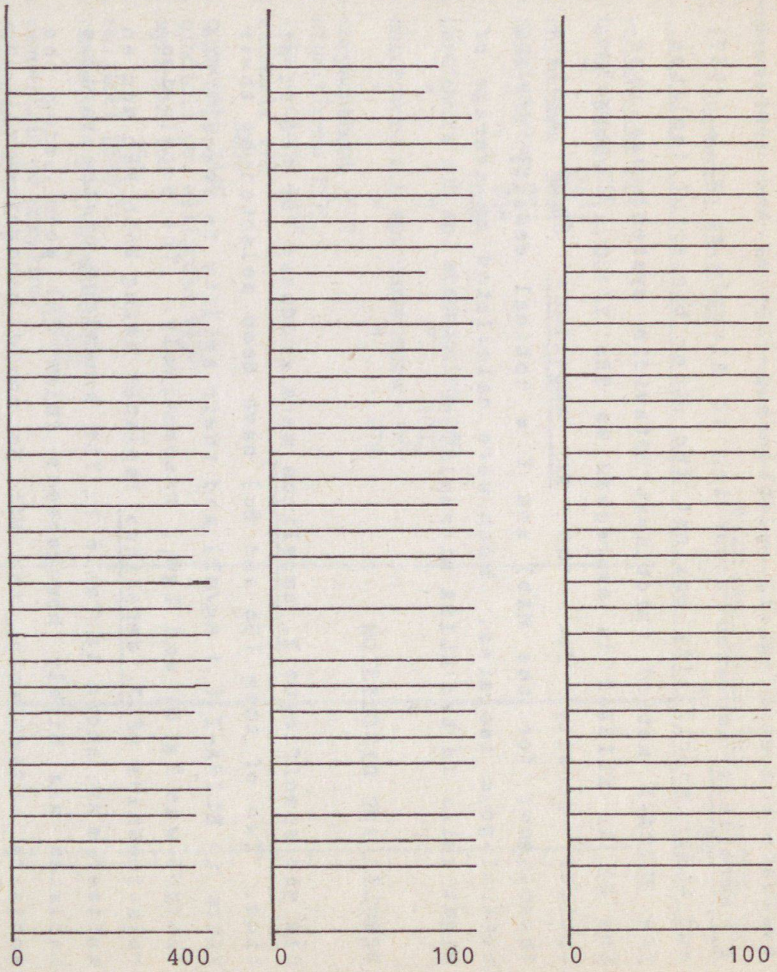
158	Greece
160	Greece
AK15	Greece
AK16	Greece
AK17	Greece
162	Turkey



Serotyping and specificity tests. Two selected MAb (8c and 5c) in comparison with the antiserum (AS.8), were used to evaluate the serological relationship (SR) of the different X. ampelinus isolates according to Alarcón et al., (1987) and their specificity against the other bacterial isolates. The ELISA-I method (biotin-streptavidin system) was used. The SR in ELISA-I is expressed as SR= Y/100.x, where x = optical density for the X. ampelinus 60.7 isolate (homologous for the MAb) and Y = optical density of the heterologous isolates. Both were calculated as average of eight wells in two ELISA microplates.

RESULTS AND DISCUSSION

Six MAb specifics to X. ampelinus were produced for the first time. Two of them (5c and 8c) have been selected by their titre in ELISA-I (1 ng/ml) and their ability to react. The isotype was Ig G_3 and IgG_1, respectively. The serological relationships of X. ampelinus isolates using both MAb and an antiserum are shown in Table 1. The behaviour of the various isolates was highly homogeneous (high SR) when using the antisera. Similarly, the MAb 5c could recognize all the isolates of X. ampelinus, showing high SR. This would imply that MAb 5c represents an epitope, and possibly the majority, common to all the isolates assayed. However, it has been possible to discriminate isolates using MAb 8c that represents an antigenic determinant, not being present in all of them.The reaction of MAb 8c, when it occurred, showed extremely variable SRs. Greek isolates were particularly discriminated. This demonstrates that, although X. ampelinus forms a serologically compact group, it is possible to select specific antigens, and/or selective to some isolates. Both MAb have appeared completely specific to X. ampelinus (see Table 2). There was no reaction; not even against the saprophytic flora of grapevine, nor against other of the bacteria assayed. The antiserum produced frequent cross-reactions, both against the saprophytic flora (SR variation

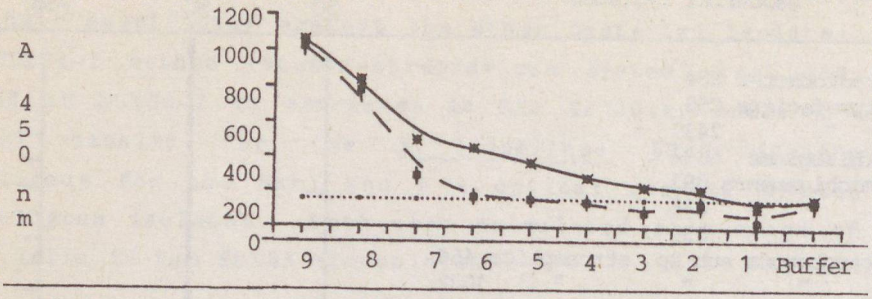
Table 2. CROSS REACTIONS PERCENTAGE OF DIFFERENT BACTERIA WITH MONOCLONALS ANTIBODIES SPECIFIC FOR X. AMPELINUS AND AND ANTISERUM.

Bacterial isolates	Monoclonal antibodies 5C	Monoclonal antibodies 8C	Antiserum AS8
A. radiobacter K84			
A. tumefaciens C58			
" 2437			
A. rhizogenes 5834			
C. michiganense 093			
" 818			
" 030			
E. carotovora sub sp. atroseptica 466			
" " " 1002			
E. carotovora sub sp. carotovora 195			
" " " 878			
E. chrysanthemi 597			
E. nigrifluens 1576			
E. rubrifaciens 1975			
P. cichorii 479			
" 5800			
P. corrugata 2445			
P. gladioli pv. alliicola 1435			
P. syringae 162			
" 164			
R. meliloti L530			
X. campestris pv juglandis 884			
" " " 885			
X. campestris pv corylina 1			
X. fragariae 351-1			
" 351-2			
	0	100	0 100

from 0 to 35%), and against other bacteria representative of different genus (SR variation from 0 to 65%).

ELISA-I B/SA was set up, and appeared highly sensitive to detect the bacterium using the MAb. Table 3 shows the results. Using this method, approximately 100 cfu can be detected utilizing MAb 5c. Use of MAb permits completely specific diagnosis, thereby increasing the sensitivity of the serological detection techniques. The MAb allow availability of homogeneous and well characterized antibodies, and open new ways to antigenic determinants understanding, and for general or discriminating detection of the bacterium.

Table 3. SENSITIVITY OF ELISA-INDIRECT METHOD (BIOTIN/STREPTAVIDINE SYSTEM) TO DETECT XYLOPHILUS AMPELINUS USING SPECIFIC MABS



MAb 5C	1059	840	498	452	367	282	228	218	113	165
MAb 8C	1021	780	292	172	157	142	86	133	35	142
Negative Control	157	157	157	157	157	157	157	157	157	157

LOG(CFU/ml)

—— MAb 5C — MAb 8C ..... Negative Control

—— MAb 5C — MAb 8C ..... Negative Control

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

Plant Pathogenic Bacteria Z. Klement (ed.)

XANTHOMONAS ALBILINEANS SEROVARS AND DIAGNOSIS OF SUGARCANE LEAF SCALD

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ABSTRACT:

We identified three serovars of *Xanthomonas albilineans* using three antisera based on immunofluorescence and agglutination assays : strains of serovar 1 were from South Africa, Australia, Barbados, Brasil, Guadeloupe, India, Mauritius, United States, Réunion and Taïwan ; serovar 2 from tropical Africa (Burkina Faso, Cameroon, Côte d'Ivoire, Kenya and Zaïre) and serovar 3 from the West Indies (St Kitts and Guadeloupe). Each serovar reacted with only one of the three antisera when these were highly diluted. By mixing strains of the three serovars, we prepared a polyvalent antiserum for rapid and accurate diagnosis of sugarcane leaf scald. After verifying that strains of the three known serovars reacted with this antiserum, we then used it for detection of the pathogen in various sugarcane samples.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

DAS ELISA TEST TO DETECT XANTHOMONAS CAMPESTRIS PV. DIEFFENBACHIAE IN ANTHURIUM PROPAGATIVE MATERIAL

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INTRODUCTION

Xanthomonas campestris pv. dieffenbachiae was identified on *Anthurium* in different production areas of the American continent : Hawaï (Hayward 1972), (Nishijima 1985), French Indies (Hostachy 1985), (Rott et Prior 1987), Venezuela (Guevara 1985) and California (Cooksey 1985). In the infected areas, the disease is a limiting factor for flower production which is of great export interest.

The dissemination of the bacteria takes place mainly by uncontrolled propagation. DAS ELISA is proposed for a routine diagnosis in propagative material. This method was compared to the indirect immunofluorescence technique in leaf and stem samples of healthy and inoculated plants, and also of symptomless and naturally infected material. Antibodies against different isolated strains are evaluated for their specificity among different genus, species and pathovar of *Xanthomonas* and against the saprophytic bacterial flora isolated from Anthurium.

MATERIALS AND METHODS

Plant samples

Healthy plants were from *in vitro* culture.
Inoculated plants were supplied by Philippe Prior (INRA Guadeloupe).
Symptomless and naturally infected material were collected in French West Indies.

Bacterial strains.

Strains 110.16 (Prior) and 11066 (CRA) was used for antisera production. Strains used for specificity tests were from CRA and CFBP (Angers). Saprophytic bacterial flora were isolated from plants collected in West Indies.

Antigens preparation.

Strains were grown on YDA medium during 48 th at 30°C, washed, centrifuged at 2500g during 15' and the concentration was calibrated to 10^9 c.f.u. ml^{-1}. Bacteria were heat-killed during 2 days at 100°C and the suspension was inoculated in 6-month old rabbits.

Bacterial suspensions.

To evaluate sensibility of detection bacterial suspensions were prepared by growing on YDA medium. The initial suspension was calibrated to 10^8 c.f.u. ml^{-1}.

Preparation of plant samples.

Samples of different organs (leaf, stem, rhizome) were taken and calibrated to 0,3 g.
To evaluate the sensitivity of detection in plant extracts, calibrated inoculum were introduced in extracts from healthy plants.

Antisera production.

Two protocols for immunisation were used. The short immunisation protocol is described in table1.
For the long immunisation protocol one additional immunisation each month was made during 6 months. Titres of antisera were determined by indirect immunofluorescence method (Faure et al. 1977)

Antibodies preparation.

Antibodies were purified as described by Goumas (1987) and conjugated to alkaline phosphatase adding glutaraldehyde as described by Avrameas (1969).

Methods of diagnosis.

Three methods were compared : isolation on YDA medium, immunofluorescence technique and DAS ELISA. Ouchterlony double diffusion (O.D.D.) was used to control the results.

Table 1. Short immunisation Protocol

Injection nb	Day	ml	Method	
1	0	0.5	IV	blood recovery
2	3	1	IV	
3	6	1.5	IV	
4	8	1.5	IV	
5	10	2	IV	
6	13	2+2	IV+SC	
7	16	2+2	IV+SC	blood recovery
8	20	2+2	SC+SC	
9	22	2+2	SC+SC	
10	24	2+2	SC+S	
-	29	-	-	blood revovery

IV: intra-venous
SC: subcutaneous

RESULTS

Specificity

Polyclonal serum anti *Xanthomonas campestris pv dieffenbachiae* (x.c.pv.d.) was found to be specific for tested strains isolated from *Anthurium* of different geographical origins.

No cross reactions were noticed with :

1) tested strains of x.c.pv.d. isolated from other Araceae (Table 2).
2) most of the other *Xanthomonas campestris pathovars* (table 2).
3) Strains of other phytopathogenic genus (table 3).
4) some saprophytic bacterial strains isolated from Anthurium (table 4).

Nevertheless cross reactions were observed with *Xanthomonas campestris pv oryzicola* and sometimes with *Xanthomonas campestris pv. oryzae, manihotis, campestris.* Results were confirmed by O.D.D. (table 5).

Sensitivity.

The best results were obtained for a concentration of 2 µg ml^{-1} IgG for the coating and a dilution of 1/2000 for the conjugate.

The best sensitivity (2,5 10^4 b ml^{-1}) was obtained in plant juice infected by different levels of inoculum. The addition of urea (2M) (GOUMAS 1987) did not improve the result

Correlation between the results of the different diagnosis tests. The correlation of results among ELISA, IF and O.D.D. is shown below.

Correlation among the 3 tests		No correlation			
+	-	I+Ser-	I-Ser+	I-Ser Var	40: samples I: isolation Ser: serology
13	24	1	1	1	

On tested plants the infection was controlled at several levels from the rhizome to the leaves.

DISCUSSION AND CONCLUSION

The sensitivity obtained in infected plant juice allows exploiting the use of the method in routine control.

The detected level of inoculum on *Anthurium* infected by *X.c. pv.d.* (2 10^4 b ml^{-1}) is higher than the detected level of *X.c.pv. Pelargonii* on *Pelargonium* (JP Narcy personnal communication). Nevertheless, it takes place in the lowest levels mentioned by other authors in studies on *Xanthomonas* by ELISA.

10^4 b ml^{-1} (Nemeth, 1983) *X.c. pv phaseoli*
10^4 b ml^{-1} (Civerolo, 1982) *X.c. citri*
10^5 b ml^{-1} (Lopez, and al., 1987) *X. ampelina*
10^5 b ml^{1} (Alvarez, 1985) *X.c. pv campestris.*

Table 2. Specificity of antisera

host	Antigen	Strains	Short immunization antiserum 1		Long immunization antiserum 2		reference antiserum 3		reference study
			ELISA	IF	ELISA	IF	ELISA	IF	IF
Anthurium	*X.c. dieffenbachiae*	11016	+	+	+	+	+	+	+
	X.c. dieffenbachiae	11066	+	+	+	+	+	+	
	X.c. dieffenbachiae	X1Ven	+	+	+	+	+	+	+
	X.c. dieffenbachiae	X1Haw	+F	+F	+	+	+	+	+
	X.c. dieffenbachiae	X2Haw	+F	+F	+	+	+	+	+
	X.c. dieffenbachiae	X3Haw	+F	+F	+	+	+	+	+
Other Araceae	*X.c. dieffenbachiae*	XD1	-	-	-	-	-	-	-
	X.c. dieffenbachiae	XD2	-	-	-	-	-	-	-
	X.c. dieffenbachiae	XD3	-	-	-	-	-	-	-
	X.c. dieffenbachiae	XD4	-	-	-	-	-	-	-
	X.c. dieffenbachiae	XD7	-	-	-	-	-	-	-
	X.c. dieffenbachiae	XD8	-	-	-	-	-	-	-
	X.c. dieffenbachiae	XD9	-	-	-	-	-	-	-
Plants of other families	*X.c. campestris*	10418	-	-	+	+	-	-	-
	X.c. vesicatoria	10601	-	-	-	-	-	-	-
	X.c. manihotis	10501	-	-	+	+	-	-	-
	X.c. begonise	10150	-	-	-	-	-	-	-
	X.c. begonise	10169	-	-	-	-	-	-	
	X.c. juglandis	1023	-	-	-	-	-	-	-
	X.c. juglandis	1023	-	-	-	-	-	-	-
	X.c. incanae	1438	-	-	-	-	-	-	-
	X.c. citri	1814	-	-	-	-	-	-	-
	X.c. malvacearum	2012	-	-	-	-	-	-	-
	X.c. mangiferae indicae	1716	-	-	-	-	-	-	-
	X.c. mangiferae indicae	1717	-	-	-	-	-	-	-
	X.c. orgeae	1948	-	-	+	+	-	-	-
	X.c. orgeicola	2286	+	+	+	+	+	+	+
	X.c. phaseoli	1816	-	-	-	-	-	-	-
	X.c. vasculorum	1289	-	-	-	-	-	-	-
	X.c. pelargoni	1384	-	-	-	-	-	-	-

ELISA: coating 2 µg conjuguant 1/2000
IF: dilutions of antisera 1-2 : 1/500
3 : 1/1280

F: weak reaction

against strains of Xanthomonas campestris

Code	Pathovar	host	Origin	obtained by
X1VEN	*dieffenbachiae*	*Anthurium andreanum*	Venezuela	Y.M. Guevara
X1HAW	*dieffenbachiae*	*Anthurium andreanum*	Hawai	W. Nishijima
X2HAW	*dieffenbachiae*	*Anthurium andreanum*	Hawai	W. Nishijima
X3HAW	*dieffenbachiae*	*Anthurium andreanum*	Hawai	W. Nishijima
XD1	*dieffenbachiae*	*Philodendron* sp	Floride	J.W. Miller
XD2	*dieffenbachiae*	*Philodendron* sp	Floride	J.W. Miller
XD3	*dieffenbachiae*	*Philodendron* sp	Floride	J.W. Miller
XD4	*dieffenbachiae*	*Philodendron* sp	Floride	J.W. Miller
XD7	*dieffenbachiae*	*Philodendron scandens oxycardium*	Floride	A.R. Chase
XD8	*dieffenbachiae*	*Philodendron scandens oxycardium*	Floride	A.R. Chase
XD9	*dieffenbachiae*	*Philodendron scandens oxycardium*	Floride	A.R. Chase
CNRA 10150	*begonise*	*Begonia* sp	nd	CNRA*
CNRA 10169	*begonise*	*Begonia* sp	nd	CNRA
CNRA 10418	*campestris*	*Brassica* sp	nd	CNRA
CNBP 1814	*citri*	*Citrus* sp	Réunion	CNBP**
CNBP 1438	*incanae*	*Matthiola incana*	Etats-unis	CNBP
CNBP 1023	*juglandis*	*Juglans regia*	France	CNBP
CNBP 1024	*juglandis*	*Juglans regia*	France	CNBP
CNBP 2012	*malvacearum*	*Gossypium hirsutum*	Burkina Faso	CNBP
CNBP 1716	*mangiferae indicae*	*Mangifera indica*	Inde	CNBP
CNBP 1717	*mangiferae indicae*	*Mangifera indica*	Réunion	CNBP
CNRA 10501	*maniholis*	*Manihol osculenta*	nd	CNRA
CNBP 1948	*oryzae*	*Oryza sativa*	Cameroun	CNBP
CNBP 2286	*oryzicola*	*Oryza sativa*	Malaisie	CNBP
CNRA 1384	*pelargonii*	*Pelargonium sp*	nd	CNRA
CNRA 1816	*phaseoli*	*Phaseolus vulgaris*	nd	CNRA
CNBP 1289	*vasculorum*	*Saccarum officinarum*	Réunion	CNBP
CNRA 10601	*vesicatoria*	*Lycopersicon esculentum*	nd	CNRA

Reference of pathovars used in table 2.

nd : origine non déterminée
* CNRA: Centre National de la Recherche Agronomique, Versailles, France
** CNBP: Collection Nationale de Bactéries Phytopathogénes, Institut national de la recherche agronomique, Angers, France

Table 3. Specificity controlled by DAS ELISA against strains of other phytopathogenic genus

Antigen	Reference strains	Short immunisation antiserum	Long immunisation antiserum	Ref antiserum
Pseudomonas syringae phasedicola	CNRA 20501	-	-	-
Pseudomonas syringae pisi	CNRA 11	-	-	-
Pseudomonas cichorii	CNRA 21300	-	-	-
Pseudomonas viridiflava	CNRA 20201	-	-	-
Pseudomonas fluorescens	CNRA 20303	-	-	-
Pseudomonas marginalis	CNRA 21202	-	-	-
Agrobacterium tumefaciens	CNRA 60105	-	-	-
Erwinia chrysanthemi	CNRA 30317	-	-	-
Erwinia carotovora	CNRA 40990	-	-	-
Erwinia atroseptica	CNRA 40884	-	-	-
Corymbacterium fascians	CNRA 50102	-	-	-

Table 4. Specificity against saprophytic bacterial flora of Anthurium

Strains	Gram	Hugh Leifson	Fluorescence on King B medium	Elisa	IF
S1	+	inert	-	-	-
S2	-	F	-	-	-
S3	-	F	-	-	-
S4	-	0	-	-	-
S5	-	0	+	-	-
S6	-	0	+	-	-
S7	-	F	-	-	-
S8	-	F	-	-	-
S9	-	F	-	-	-
S10	+	0	-	-	-
S11	+	0	-	-	-
S12	-	F	-	-	-
S13	-	inert	-	-	-
S14	+	0	-	-	-
S16	-	F	-	-	-
S17	+	0	-	-	-
S18	+	inert	-	-	-
S19	-	0	+	-	-
S20	+	inert	-	-	-
S21	-	alkalinising	-	-	-
S22	+	0	-	-	-

Table 5. O.D.D. Results

Strains			Long immunisation serum	Short immunisation serum
X.c.	*dieffenbachiae*	*anthurium* 11016	+	+
	dieffenbachiae	*anthurium* 11066	+	+
	dieffenbachiae	*anthurium* X1Yen	+	+
	dieffenbachiae	*anthurium* X1Haw	-	-
	dieffenbachiae	*anthurium* X2Haw	-	-
	dieffenbachiae	*anthurium* X3Haw	-	+
X.c.	*dieffenbachiae*	*philodendron* XD1	-	-
	dieffenbachiae	*philodendron* XD2	-	-
	dieffenbachiae	*philodendron* XD3	-	-
X.c.	*campestris*	10418	-	-
X.c.	*manihotis*	10501	-	-
X.c.	*begoniae*	10150	-	-
X.c.	*citri*	1814	-	-
X.c.	*phasaoli*	1816	-	-
X.c.	*oryzae*	1948	-	+

Although the treatment with urea mentioned by Goumas (1987) has not improved the detected level of inoculum for *X.c. pv. d.* on *Anthurium*, a more exhaustive study of the conditions of extraction of antigens in plant samples should allow reducing the background reaction and to improve the detection level of the inoculum. This one might be sufficient for the detection of the disease in the fields for *X.c pv campestris* on cabbage but not sufficient to estimate the rate of contamination in cabbage seeds (Alvarez 1985). This estimation in the culture and in propagative material is actually made on *Anthurium* in collaboration with Plant Protection Service in Guadeloupe. The incidence of the low level of inoculum non detected in propagative material on epidemic evolution must be studied in different agronomical and geographical contexts. This study shows a good specificity of the reactives against the strains of *X. c pv d* of French and American origin and against the saprophytic bacterial flora isolated from *Anthurium* in Guadeloupe. The extension of the study to pathogenic strains from other geographical origins and to saprophytic bacterial flora isolated in others productions areas is necessary before advising the use of ELISA for the sanitary control in the different *Anthurium* production areas. The recognition by the reagents of strains non-pathogenic for *Anthurium* has been established in this study, nevertheless cross-reactions noticed with other *campestris pathovars (XU. c pv oryzicola, oryzae, manihotis, campestris)* might lead to faulty diagnosis if those strains are present in the epiphyll or telluric flora of *Anthurium* cultivated after plants infected by those different pathovars.

This study corroborates a variability already shown by Prior (1987) in IF inside the *dieffenbachia pathovar* infecting different, Araceae plants. The technique described seems to be useful for the routine diagnosis for a sanitary selection of *Anthurium* and for the studies on epidemiology and of host specificity within the genus *xanthomona campestris*.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

CROSS-REACTIONS BETWEEN RHODOCOCCUS FASCIANS AND OTHER BACTERIA IN INDIRECT IMMUNOFLUORESCENCE

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Rhodococcus fascians (Tilford) Goodfellow, the causal agent of "leafy gall" in several plant species, can colonize and survive also on the outer tissue layer of the lily bulbs (Miller *et al.* 1980). Since "leafy gall" pathogen does not occur in Italy, in order to prevent its admittance, it is necessary to rely on quick and accurate techniques for detecting this bacterium in lily bulbs imported from foreign countries.

Digat (1978) obtained positive results in *Pelargonium* and *Begonia* x *Elatior* by means of the immunofluorescence antibody staining (IFAS).

The aim of this work was to single out the best procedure in the antisera production and to evaluate their specificity in IFAS.

Antisera against *R. fascians* NCPPB 1675 were prepared by inoculating bacterial suspensions containing about 4×10^9 cfu/ml of living or heat-killed cells into 'New Zealand' white rabbits. Somatic antigens, at 3 mg/ml of protein concentration, obtained following the thrichloroacetic acid extraction (Sutherland 1978), were injected, too. Two different schedules of immunization were used (Trigalet *et al.* 1978): 1) short-term immunization (STI), carried out by four injections at weekly intervals, and 2) long-term immunization (LTI), consisting of STI steps followed by a week of rest plus another set of four weekly injections. Incomplete adjuvant of Freund (2 ml/injection) was added to the antigens.

Antisera at 1:100 to 1:800 dilutions were tested by indirect IFAS against *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey *et al.*, the other bacterium pathogenic on lily, and against some of the most widespread soilborne and plant decaying bacteria. Consequently, suspensions containing about 1×10^3 cfu/ml of *R. fascians* PD 293 and PD 300, *E. c.* subsp. *carotovora* ATCC 15173 and NCPPB 468, *Erwinia herbicola* (Löhnis) Dye NCPPB 2971, *Pseudomonas fluorescens* (Trevisan) Migula ATCC 13525, *Arthrobacter simplex* (Jensen) Lochhead ATCC 6946 and *Bacillus subtilis* (Ehrenberg) Cohn ATCC 6051 were utilized for the cross-reaction tests.

The results showed that the highest specificity of antiserum/antigen reaction was obtained when antiserum against living cells and LTI was used. At lower values of dilutions cross-reactions occurred with all the tested bacteria, except *P. fluorescens* . On the contrary, at the 1:800 di-

lution the antiserum reacted strongly with the *R. fascians* isolates, and very weakly with *A. simplex*, a closely related species.

From the foregoing it seems that the indirect IFAS technique, at definite conditions, may be useful for a reliable detection of *R. fascians* in naturally infected samples.

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

Plant Pathogenic Bacteria
Z. Klement (ed.)

IN SITU DETECTION OF ERWINIA CHRYSANTHEMI ON POTATO ROOTS USING IMMUNOFLUORESCENCE AND IMMUNOGOLD STAINING

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INTRODUCTION

The *in situ* detection of plant pathogens in complex ecosystems, e.g. soil and rhizosphere, requires highly sensitive and highly specific methods. Methods are also needed to follow the population dynamics of pathogens in and on plant tissue. Serological methods, employing immunoglobulins labelled with fluorochromes like fluorescein isothiocyanate (FITC) have been used in medical and veterinarian research for the detection of bacterial cells *in situ*. In plant bacteriology, direct and indirect immunofluorescence cell staining play a prominent role (Schaad, 1979). Recently, immunofluorescence colony staining (IFC) has shown its potential for plant bacteria detection (Van Vuurde, 1987). Within the last years, gold-labelled immunoglobulins have been applied successfully in plant virology (Patterson & Verduin, 1987). In the beginning, the properties of small electron-dense gold particles (5-15 nm) have been employed in electron microscopy. But recently, methods are developed to make antibodies labelled with larger gold particles (> 15 nm) to be used in light microcopy (Van Laere et al.,1985). This paper describes the adaptation of immunofluorescence and immunogold staining for the detection of *Erwinia chrysanthemi* (Ech) colonizing potato roots. In the discussion, a preliminary evaluation of the immunogold staining is given.

MATERIALS AND METHODS

Potato plants, *Solanum tuberosum* L. cv. 'Bintje', are grown in *in vitro* tube culture on Murashige-Skoog (MS) medium (Flow Laboratories) pH 5.7 complemented with sugar 25 g/l and agar 8 g/l (Agar technical No. 3, Oxoid). Stem-cuttings (1 cm length, 1 leaf) of these *in vitro* plants are made and transferred to Petri dishes (15 cm diameter) containing one of the following 6 media: MS without sugar, with 5 and 25 g sugar per litre with a pH of either 5.7 or 6.7. The plates are incubated at an angle of ca. 75°, in order to stimulate root formation in the agar for 10 days at 20 ^{o}C and 16 h light. Then the plants are inoculated as follows: ca. 3 mm from the root tip a well of ca. 2 mm diameter is cut into the agar and filled with ca. 5×10^{5} cells of Ech (IPO 502=PD 226). After filling the wells with inoculum, the plants are incubated for 3-10 days under the same conditions as described above. The roots, with the agar or prepared free of agar, are transferred to one of the two staining solutions described below.

* This research was partially supported by the Gottlieb Daimler-und-Carl Benz-Stiftung, Ladenburg, West Germany.

1. Immunofluorescence staining (IF staining)

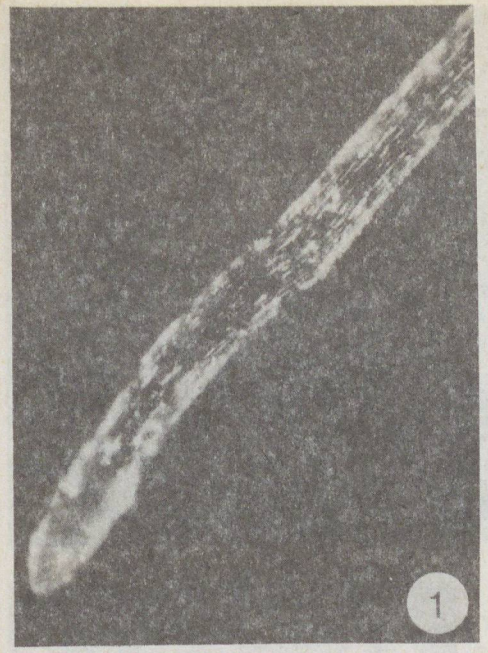
IgG type antibodies (IPO 8276 B) raised in a rabbit against washed Ech cells are coupled to FITC (Sigma) following the method of Allan & Kelman (1977). The working dilution of the conjugate for this technique (diluted in 0.01 M phosphate buffered saline, pH 7.4) is 1:100. The antibody preparation is checked for cross-reactivity in enzyme linked immunosorbent assay (ELISA) and to a limited extend in IFC with bacteria of other genera and with other *Erwinia* spp. and was determined to be highly specific (Van der Wolf, 1989). The infected roots, and not-infected control roots, are incubated for 2-3 h in the diluted FITC-labelled antibody suspension at room temperature. The preparations are observed under UV light at 490 nm with 4x (N.A. 0.12), 25x (water immersion, N.A. 0.60) and 50x (water immersion, N.A. 1.00) objective magnification.

2. Immunogold staining

For immunogold staining the same antibodies are used as for IF staining. They are coupled to 30 nm colloidal gold particles in a suspension (Janssen Life Science Products, Beerse, Belgium) adjusted to pH 9.0 with 0.02 M K_2CO_3 prior to coupling (Roth, 1983). For coupling, 0.05 mg of IgG is added per ml of gold suspension and stirred rapidly for 2 min. The gold-labelled antibodies are stabilized by the addition of polyethylene glycol (MW 20,000) to a final concentration of 0.05% and the addition of bovine serum albumin to a final concentration of 1%. The suspension is centrifuged at 12,000 g for 15 min at 4 °C to remove unlabelled antibodies. Approximately three quarters of the supernatant are discarded and the pellet is resuspended in the remaining supernatant. The working dilution is determined to be 1:8 to 1:20 (in 0.002 M borax buffer, pH 9.0). As the same antibodies were used as for IF staining, no further tests for cross-reactivity were performed. The incubation of roots with the diluted gold labelled antibody suspension takes place as in IF staining. The preparations are observed with darkfield and lightfield illumination under a stereo microscope with a zoom objective (0.6 - 3.1x) or under a compound microscope with transmitted light and/or epipolarized light (employing an epipolarization filterblock, Leitz) with 4x, 25x or 50x objective magnification.

RESULTS

It was possible to adapt immunofluorescence and immunogold staining for the detection of Ech cells and colonies on potato roots. From the experiments done so far, two colonization patterns have been observed. One is the denser colonization along the longitudinal cell walls than on the rest of the root surface demonstrated by IF staining at 4x and at 25x objective magnification (Fig. 1 and 2, respectively). The other is the more dense colonization at points where the side roots are formed, which was shown by IF staining (Fig. 3). Sometimes, a denser colonization can be found close to the root tip; demonstrated by immunogold staining visible as a red stain in darkfield illumination (Fig. 4). When epipolarized light is used, the colour of the gold particles is yellowish golden against a dark background. When the background itself reflects the polarized light, e.g. due to auto-reflection of the root, the gold particles are seen as dark dots against the whitish reflecting background. Our preliminary data show that the autoreflection of the roots is very variable. The comparison of immunogold stained Ech on a reflecting preparation of the root surface illuminated with epipolarized light and with transmitted light is given in Fig. 5 and 6, respectively.





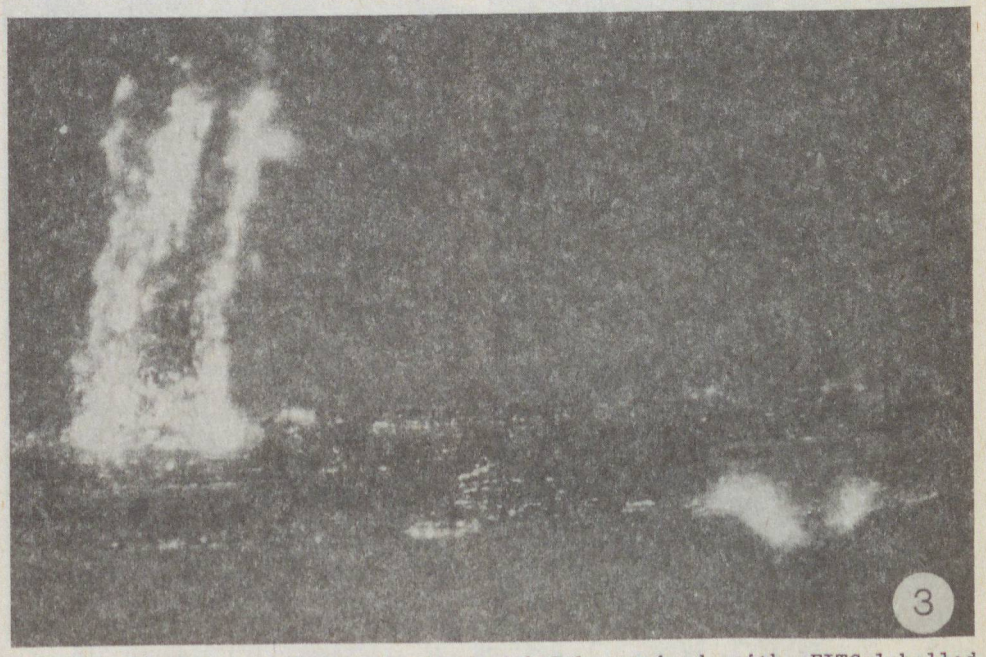


Fig. 1 and 2. Root surface colonized with Ech stained with FITC-labelled antibodies. Note that denser colonization can be observed at the longitudinal cell walls, than on the rest of the root surface.(Fig. 1: 4x obj. magn., Fig. 2: 25x obj. magn.).
Fig. 3. Colonization pattern of Ech at the point of side root formation at 4x obj. magn. Staining as in Fig. 1 and 2.







Fig. 4. Colonization of Ech on the root tip at 3.1x obj.magn. Staining with gold-labelled antibodies, visible as a red stain (arrow).
Fig. 5 and 6. Root surface colonized with Ech stained with gold-labelled antibodies at 25x obj. magn. Fig. 5. Epipolarized light. Gold particles can be recognized as dark dots. Fig. 6. Transmitted light results in the red colour of the gold particles.

DISCUSSION

Our results show that both methods can be useful tools for *in situ* studies. It was possible to detect bacterial colonization patterns in a model system, set up to evaluate the relationship between the population dynamics of Ech and the potato root surface. The usefulness of the methods for *in vivo* detection remains to be investigated. From our experiments, a qualitative and quantitative comparison of the two methods is not yet possible. Our preliminary results indicate a higher sensitivity of detection and a higher intensity of the staining for the IF staining. Further work on the immunogold staining will be necessary to improve and optimize this method for research on bacteria as has been done for the IF staining. Especially the specificity and sensitivity of the immunogold staining need to be evaluated.

Also, the labelling procedure of large gold particles to antibodies needs further investigation and optimization. Unfortunately, there is a lack of knowledge about the efficiency of this procedure. In our experiments, the coupled product was used the same day. After one to two weeks of storage, gold precipitates were visible and the reactivity of the gold-labelled antibodies was strongly decreased. This indicates that the stabilization of the label has to be improved. Methods to reduce autoreflection of the roots in epipolarization microscopy need to be worked out. Reduction of the reflection of a petiole section was demonstrated by Van Lent & Verduin (1987) employing toluidin blue staining.

The immunogold staining in turn has several promising advantages. For example, that the label does not fade during observation and that less expensive standard halogen microscope lamps can also be used to illuminate the preparations. Furthermore, there are various illumination and microscopy systems applicable for an immunogold stained preparation in order to achieve a high contrast picture: For low magnifications, a stereo microscope with dark- and brightfield illumination can be used. For high microscopical magnifications, transmitted light or epipolarized light might be used alone or in combination. Our experiments indicate, that a combination can make it easier to correlate the stained parts to their localization in the tissue (unpublished data). Phase contrast and interference microscopy can be applied, also. Immunogold staining can be combined with silver staining, which results in an enhanced signal. Furthermore, the same preparation can be observed in light and electron microscopy, as gold particles are electron dense.

The outlined potential advantages of the immunogold staining justify further investigations to make this technique suitable for ecological studies.

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SESSION 9

MISCELLANEOUS

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

Plant Pathogenic Bacteria Z. Klement (ed.)

OCCURRENCE AND DISTRIBUTION OF TOMATO STOLBUR IN GREECE

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SUMMARY

The symptoms of tomato stolbur in Greece are described. Pleomorphic mycoplasma-like organisms (MLO's) were seen by thin-section electron microscopy in the sieve tubers of infected plant tissues. MLO's were also detected in the sieve tubes by bright field and fluorescence microscopy after staining of hand and microtome tissue sections with toluidine blue and Hoechst 33258 stain respectively.

Remission of symptoms occurred when infected tomato plants were injected with tetracycline hydrochloride, but not with benzyl-penicillin.

Healthy periwinkle plants showed typical "yellows" symptoms when connected via Cuscuta campestris L. with stolbur diseased tomato plants. MLO's were detected in diseased periwinkles by electron and fluorescence microscopy. Natural transmission studied on 13 tomato cultivars ranged from 1.25% to 10% .

The disease was detected in northern and central Greece, in Attica and Corfu.

INTRODUCTION

Tomato stolbur was first recorded as plant disease in Chechoslovakia in 1932 although it was presumed from early reports that it was present there from 1921. The disease has been reported to occur also in USSR, Hungary, Austria, France, Switzerland, Italy, Yugoslavia, Bulgaria, Romania, United Kingdom, Saudi Arabia and Iraq.

Until 1969 when Ploaie and Maramorosch (1969), first reporte the presence of mycoplasma-like organisms (MLO's) in stolbur infected plants, all early reports on stolbur were totally based on symptoms. Since 1969 some of the reports were based or symptoms but in several others electron microscopy was used to demonstrate the presence of MLO's. No other methods have been reported for the visualization of stolbur MLO's.

We observed tomato stolbur for the first time in Greece in a tomato field in northern Greece in 1985 although it was possibly present there from 1965.

This paper describes the symptoms of tomato stolbur in Greece, confirms the MLO etiology of the disease and further explores its distribution in the country and the natural transmission of MLO's in northern Greece.

MATERIALS AND METHODS

Plant material

Samples of stolbur diseased tomato plants were collected late in the summer from field plantations grown in northern Greece and other places of the country and checked for the presence of MLO's as described below.

Electron microscopy

Plant tissue pieces (2-3 mm) cut from green petals or sepals of malformed or healthy flowers, were prefixed in fixative (3% glutaraldehyde, 2% sucrose, 2mM $CaCl_2$ in Sorensen phosphate buffer 0.1M, pH 7.2), post-fixed in 1% osmium tetroxide, dehydrated, embedded in a resin mixture and further treated and examined as described by Norris and McCoy (1983).

Fluorescence and bright-field microscopy

Ultramicrotome sections, 100-200 nm thick, or hand sections stained with Hoechst 33258 fluorescent stain (H-stain) (1 µg/cm^3 for 1 h) or with 1% solution of toluidine blue (for 30 sec),

were examined under a UV Zeiss microscope systeme (Alivizatos and Markham, 1986) or in a bright field Zeiss photomicroscope respectively.

Antibiotic treatments

Twelve tomato plants with typical stolbur symptoms, selected from each field were transferred in pots in a greenhouse and four of them were injected with tetracycline-HCl (50 mg/plant), four with benzylpenicillin (50 mg/plant) and four with 1% (w/v) solution of citric acid, used as diluent of antibiotics. All plants were sprayed with insecticide (Alivizatos, 1984) and kept in the greenhouse at 30±1°C.

Transmission tests

Each of four tomato plants with stolbur symptoms selected from each field were transferred in pots, connected via Cuscuta campestris L. with each of four healthy periwinkle plants (Catharanthus roseus (L.) G. Don) for 1 month, then sprayed and kept in a greenhouse as above.

To study natural transmission, 80 healthy plants from each of 13 varieties of tomato were planted in rectangular plots early in April in a field in northern Greece, where tomato stolbur was observed the previous year. Plants were checked for symptoms and the presence of MLO's early in September. The year of experimentation was characterized by poor rainfall in the spring and by warm and dry summer.

RESULTS

Symptoms

The first symptoms appeared in June including proliferation of lateral shoots, leaf yellowing and leaf size reduction. Subsequently plants showed slow growth, malformed flowers (green petals, connected or separated giant sepals, lengthened hyperus, underdeveloped stemens) fruit size reduction and undeveloped or

underdeveloped flesh of the fruit (Fig. 1). The shoots often produced terminal malformed flowers. Early in September the young leaves and sepals appeared violet in colour.

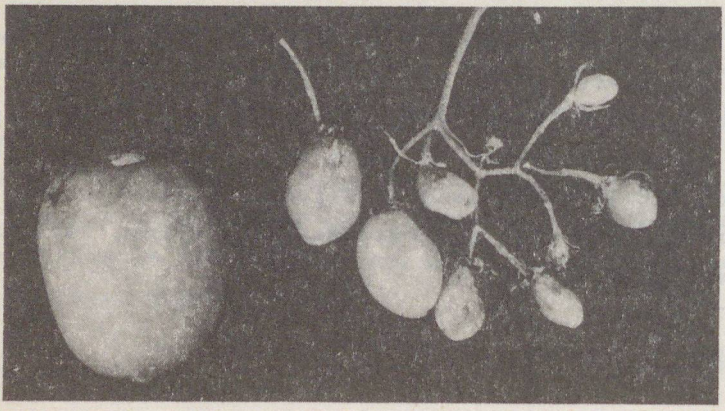

Fig. 1. Symptoms of tomato stolbur on tomato fruits (right: healthy fruit).

Microscopy

Electron microscopy revealed numerous pleomorphic or even filamentous MLO's in a number of sieve tubes of infected flower tissues but not in healthy ones (Fig. 2). No helical cells or virus-like particles were seen to be associated with MLO's.

Bright-field light microscopy revealed many blue bodies in a number of sieve tubes of infected but not of healthy plants. Nuclei of parenchyma cells were also stained blue in sections of both infected and healthy plants (Fig. 3).

Bright fluorescence was seen by fluorescence microscopy from some sieve tubes of infected but not healthy tissues. Fluorescent nuclei were seen in some parenchyma cells.

Antibiotic tests

All diseased plants injected with tetracycline-HCl showed remission of symptoms after 1 month. However all plants injected

either with benzylpenicillin or with citric acid continued to show typical stolbur symptoms even after 5 months.

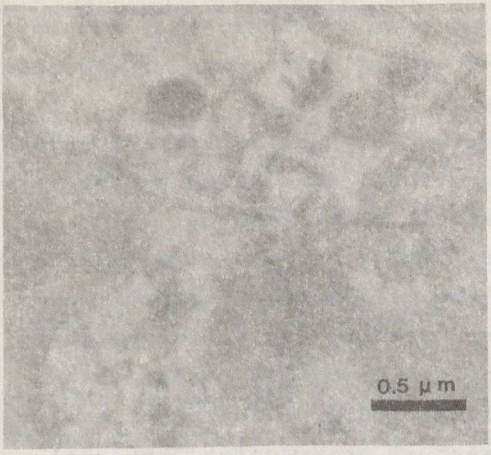

Fig. 2. Section through sieve tubes of infected petals, showing MLO's.

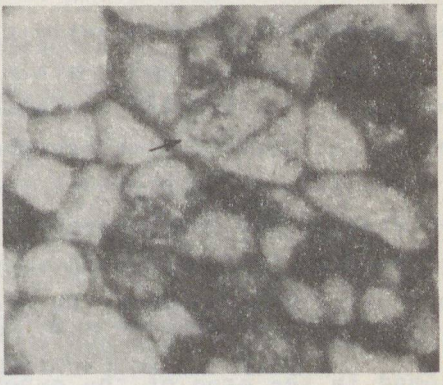

Fig. 3. Stolbur MLO's in the sieve tubes of infected tomato stained with toluidine blue.

Transmission tests

All healthy periwinkle plants connected via C. campestris with stolbur diseased tomato plants 2 months later showed, proliferation of lateral shoots with very short internodes and small yellow-greenish leaves. MLO's were detected by light and electron microscopy in all diseased periwinkles.

All 13 varieties of tomato exposed to natural transmission by leafhoppers, showed plants with stolbur symptoms. In most varieties 2.5-5% of the plants became infected, but among all varieties this ranged from 1.25% to 10%.

Disease distribution

Based on the results of the previous tests, tomato stolbur was detected in northern and central Greece, in Attica and the island of Corfu.

DISCUSSION

This study confirms the MLO etiology of tomato stolbur and consists of the first report of this disease in Greece.

The variation in MLO morphology seen by electron microscopy could be attributed to the varying age of sieve tube and MLO cells and needs further investigation.

Toluidine blue and H-stain proved very useful for the quick staining of microtome and hand sections, thus allowing the easy and quick detection of mycoplasmas in plant tissues.

The present work proved the existence of natural transmission of stolbur MLO and consequently the existence of natural infectivity of leafhoppers in northern Greece. The vector which is most likely to be there is Hyalesthes obsoletus, since this leafhopper which is known to transmit only stolbur MLO, has been found in the area of the experiment (S.Drosopoulos, personal communication), the dry and warm weather in the spring and summer favoured this vector and the percentages of naturally infected plants were similar to those reported for this vector from other countries (Brčák 1979).

Since stolbur under natural conditions occurs in foci and its appearance depends on various factors (vector, conditions etc) its distribution in Greece needs further investigation.

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

Plant Pathogenic Bacteria
Z. Klement (ed.)

COLONIZATION OF PIERCE'S DISEASE RESISTANT AND SUSCEPTIBLE GRAPEVINES BY XYLELLA FASTIDIOSA

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ABSTRACT

Muscadine grapes (Vitis rotundifolia), native to the Southeastern United States, are resistant, or tolerant, to Pierce's disease. Pierce's disease strains of Xylella fastidiosa were used to inoculate petioles or stem internodes of resistant muscadine and susceptible V. vinifera grapevines with 10^5-10^6 bacteria per inoculation site. After inoculation, samples were taken weekly and bacterial populations determined using dilution plating techniques. In inoculated petioles, multiplication rates were higher and final populations were 10-100 times greater in V. vinifera than in V. rotundifolia. In stem inoculations, upward migration rates were higher in V. rotundifolia during the first 2 weeks after inoculation, but no further movement of X. fastidiosa occurred in weeks 3-8. A steady upward migration of bacteria occurred throughout the 8 weeks in V. vinifera. Both bacterial multiplication and migration rates were lower in the resistant than in the susceptible grapevines.

INTRODUCTION

Pierce's disease of grapevine is the major limiting factor in the production of bunch grapes (Vitis vinifera L. and V. labrusca L.) in the southeastern U.S.A. Production of grapes requires the use of resistant, or tolerant, cultivars derived from Vitis species native to this area. Muscadine grapes (V. rotundifolia Michx.) are popular because of their resistance to Pierce's disease.

Xylella fastidiosa Wells et al, a xylem-limited bacterium, is the causal agent of Pierce's disease (Davis et al 1978, Hopkins 1977, Wells et al 1987). In a field study, the bacterium was found to cause vascular occlusions and leaf marginal necrosis symptoms 2 months earlier in susceptible V. labrusca 'Schuyler' than in tolerant V. rotundifolia 'Carlos' and 'Welder' (Hopkins & Thompson 1984). The present study was undertaken to compare the rate of colonization of resistant V. rotundifolia and susceptible V. vinifera by X. fastidiosa.

MATERIALS AND METHODS

Xylella fastidiosa strains were isolated from naturally infected bunch and muscadine grapevines. Isolates were single-colony transferred twice prior to use in colonization experiments. Rooted green cuttings with a minimum of 8 nodes at inoculation were used in the tests. Grape cultivars used in the test were V. vinifera 'Carignane', highly susceptible to X. fastidiosa, and V. rotundifolia 'Carlos', moderately resistant.

Inoculum was prepared by growing X. fastidiosa on PD3 medium (Davis et al 1980) for 4-6 days at 28°C. Bacterial cells were suspended in a phosphate buffer (pH 7.0) and their concentration was adjusted turbidimetrically to 10^7-10^8 colony-forming units per ml. Dilution plating was used to confirm the concentrations. One drop (0.02 ml) of inoculum was placed on the grapevine petiole and a dissecting needle was used to pierce the petiole 3-5 times through the drop, resulting in the inoculum being pulled into the plant. This means that approximately 4×10^5 to 4×10^6 bacteria were inoculated into each petiole. In bacterial migration tests, the third internode from the base was inoculated at 2 points, using the technique described for petioles.

To determine populations of X. fastidiosa in the inoculated petioles, two-cm-long petiole samples centered on the inoculation points were surface sterilized in 1% sodium hypochlorite for 3 min, rinsed in water, and ground in succinate-citrate-phosphate buffer (pH 7.0). The suspension was filtered through cheesecloth, and the bacteria concentrated by centrifugation and resuspension in 2 ml of buffer. Bacterial populations were determined by dilution plating on PD3 media. Bacterial migration was determined by cutting entire plants into

internode and petiole samples and numbering them from the base with the inoculated internode labeled 0. Internodes and petioles were surface sterilized and cut into 0.5-1.0 cm sections. The sections were squeezed with forceps and the sap exuding from each section was blotted onto PD3 medium. X. fastidiosa colonies could be identified by size and appearance after 7 days of growth.

RESULTS

Bacterial multiplication at the inoculation site. In both V. vinifera and V. rotundifolia, populations of X. fastidiosa in the inoculated petiole declined during the first 2-3 days after inoculation (Fig. 1). In both, the most rapid multiplication of bacteria occurred 3-14 days after inoculation and populations stabilized or increased

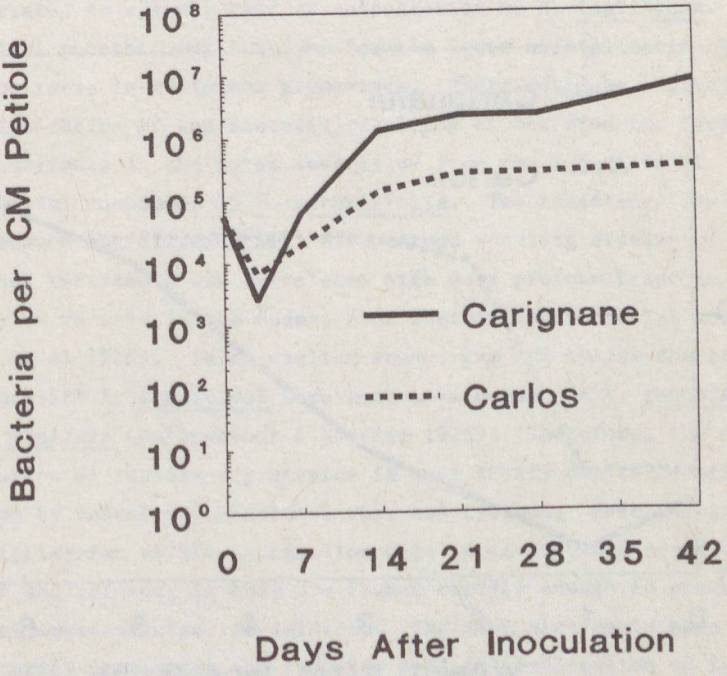

Fig. 1. Growth curves of a Pierce's disease strain of Xylella fastidiosa in the inoculated petiole of susceptible Vitis vinifera 'Carignane' and resistant V. rotundifolia 'Carlos'.

slowly from 14-72 days post-inoculation. During the time of rapid multiplication, the multiplication rate was lower in the resistant V. rotundifolia than in V. vinifera and the maximum population of bacteria was 10-100 fold higher in V. vinifera.

Bacterial migration from the inoculation point. Inoculation of X. fastidiosa into a single internode near the base of the grapevines resulted in bacteria being detected 3 and 4 internodes above the inoculation point in V. vinifera and V. rotundifolia, respectively, 2-3 hrs after inoculation (Fig. 2). Initial upward migration of bacteria in the stem was more rapid in the V. rotundifolia than in the V. vinifera, however there was very little migration after the first 2 wks in the resistant V. rotundifolia. In the susceptible V. vinifera, X. fastidiosa migration rate in the stem was slower but constant throughout the 8-wk test.

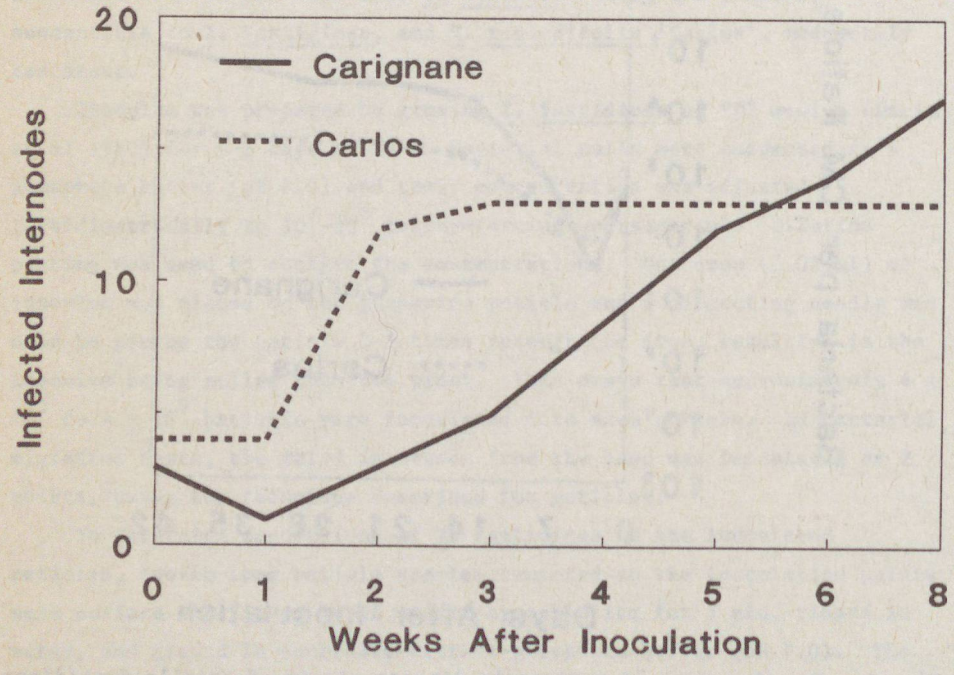

Fig. 2. Upward migration of Xylella fastidiosa in the stems of Vitis vinifera 'Carignane' and V. rotundifolia 'Carlos' after inoculation into a single internode.

In V. vinifera, upward migration of bacteria into the petioles was similar to migration in the internode. However, in the resistant V. rotundifolia, upward migration into the petioles was delayed by 1-2 weeks when compared with movement in the internodes. Also X. fastidiosa could not be detected in the petioles at the uppermost 3-4 internodes that contained detectable bacteria on any sampling date.

In inoculated V. vinifera, leaf marginal necrosis symptoms were first observed 3-4 weeks after inoculation in the lower 2-3 leaves and progressed up the stem throughout the tests. In V. rotundifolia, mild leaf marginal necrosis symptoms were first observed in the lower 2-3 leaves 6-8 wks post-inoculation.

DISCUSSION

Resistance to Pierce's disease in V. rotundifolia 'Carlos' appears to be related to a lower rate of colonization by X. fastidiosa. There are several possible explanations for the lower multiplication and migration rates in resistant grapevines. There could be a direct effect on multiplication of the bacteria resulting either from the lack of proper nutrients in the xylem vessels or from the induction of antibacterial compounds in V. rotundifolia. The resistance could also be due to anatomical differences. With ratoon stunting disease of sugarcane, resistance was correlated with more profuse branching of the large xylem vessels in the nodes, thus restricting bacterial movement (Teakle et al 1978). In an earlier study, gum and tylose formation after infection with X. fastidiosa were much more common in V. rotundifolia than V. vinifera (Mollenhauer & Hopkins 1976). Therefore, the reduced colonization of resistant grapevine is most likely due to localization of infection by vascular occlusions, gums and tyloses. Even the lower rate of multiplication at the inoculation site in the petiole could be due to the gums and tyloses, if they are formed rapidly enough to prevent vessel to vessel movement after inoculation. The gums also could have antibacterial properties and function both in localization of infection and in a chemical resistance mechanism.

ACKNOWLEDGMENT

The technical assistance of C. M. Thompson is gratefully acknowledged.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

A FAST SCREENING METHOD FOR THE DETECTION OF RESISTANCE CLAVIBACTER MICHIGANENSIS IN TOMATO

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INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* (Smith) Davies et al., formerly known as *Corynebacterium michiganense* (Smith) Jensen, is the causal agent of bacterial canker in tomato. This disease occurs in many tomato growing regions all over the world and can cause considerable yield losses. Wilting, usually unilateral, is the first and main symptom of this vascular disease. Since chemical control of the pathogen is difficult, resistant tomato cultivars would be very valuable. Despite extensive investigations, however, no absolute resistance has been found as yet, either in cultivated tomatoes, or in wild relatives (Laterrot et al. 1978; Van Steekelenburg 1985). Only partially resistant material is available now, but the level of resistance is considered to be not high enough and the material is still far from being economically applicable. For this reason, a program which aims at the introduction of resistance to *C. michiganensis* in cultivated tomatoes was started. Besides the screening of wild Lycopersicon species, somaclonal variation, i.e. genetic variation induced by tissue culture (Larkin and Scowcroft 1981), is explored. In a number of cases, somaclonal variation has led to resistance (Evans et al. 1984). For this purpose, somaclonal variants (R1) were regenerated from leaf discs of *in vitro* grown 'Moneymaker' plants. Their progeny (R2) will be screened for enhanced resistance against *C. michiganensis*. To do so, a suitable screening method must be set up (reviewed by Laterrot et al. 1978). So far, we applied a

double inoculation technique (Lindhout and Purimuhua 1987). Five-week old plants were inoculated by excision of the top with an infected knife and injection of 10 µl of the bacterial suspension into the stem. The disease development was assessed from 2 to 5 weeks after inoculation. In this way, reliable differences between susceptible and partially resistant genotypes were found. However, for the screening of thousands of plants, this method is very time-and space-consuming, especially since the screening has to be carried out in a special quarantine greenhouse. This paper describes the development of a fast screening method to assess the resistance of tomato to *C. michiganensis*.

MATERIAL AND METHODS

Pathogen

The bacterial strain of *C. michiganensis* used in this study was Cm 542, obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, UK: NCPPB 1064. In previous experiments this strain was found to be highly aggressive (Van den Bulk et al. 1989). The experiments were carried out with permission of the National Plant Protection Service under restricted conditions.

Plant material

The susceptible genotype Moneymaker (Rijk Zwaan BV, De Lier, the Netherlands) and the partially resistant line Irat L3 (Laterrot et al. 1978) were used.

Cotyledons test

Ten-day-old seedlings of 'Moneymaker' and 'Irat L3' were inoculated by dusting the cotyledons with Carborundum and rubbing them lightly with a cotton plug dipped in a bacterial suspension (10^7 bacteria/ml). Plants were incubated in a greenhouse at 25°C under a plastic tunnel to ensure a high relative humidity. After 5 days, the affected area of the cotyledons was estimated and plants were classified from 1 to 5 (0%, 1-25%, 26-50%, 51-75% and 76-100% affected area respectively). The experiment was carried out in a randomized block design with 10 plants per plot and 4 replications.

In vitro test

Disinfected seeds of 'Moneymaker' and 'Irat L3' were placed on MS-medium with 2% sucrose and 0.75% agar in 330-ml jars (4

seeds/jar) and incubated at 25°C in the light. After 10 days, the tops of the plants were transferred to fresh medium. After another 10 days, plants were inoculated by excision of the first leaf with an infected scalpel (10^7 bacteria/ml). After 6 and 11 days, plants were assessed for disease symptoms. The experiment was carried out in a randomized block design with 4 plants per plot and 3 replications.

Greenhouse tests

Four different inoculation techniques were compared using tomato plants of 'Moneymaker' and 'Irat L3' of five different ages.

1) Plants of 1, 2, 3, 4 or 5 weeks' age (time after sowing) were inoculated by dipping the roots in a suspension of 10^7 bacteria/ml before transplantation.
2) Plants of 1, 2, 3, 4 or 5 weeks' age were inoculated by excision of the first true leave or -if not yet present- of one of the cotyledons with a scalpel dipped in a suspension of 10^7 bacteria/ml. A 20 µl drop of the same bacterial suspension was placed on the wound.
3) Plants of 4 or 5 weeks' age were inoculated by excision of the top with an infected knife (10^7 bacteria/ml).
4) Plants of 4 or 5 weeks' age were inoculated by excision of the top and injection of 20 µl of the bacterial suspension into the stem (double inoculation).

The last two methods could not be applied to younger plants. The disease was assessed three weeks after inoculation. Plants were considered healthy when no wilting or wilting of only one leaflet was observed. This was based on the observation that (healthy) control plants sometimes showed a wilted leaflet, probably due to temporary unfavourable conditions. The experiment was carried out in a randomized block design with 1-2 plants per plot and 5 replications.

The inoculation by excision of a leaf was evaluated further. Two- or three-week-old plants of 'Moneymaker' and 'Irat L3' were inoculated by excision of one of the cotyledons (2-week old plants) or by excision of the first true leaf (3-week-old plants) with an infected knife (dipped in 10^7 bacteria/ml). A 20 µl drop of the same suspension was placed on the wound. The disease was assessed after 9, 11, 19, and 28 days. Plants were considered healthy when not more than one leaflet was wilted. The experiment was carried out in a randomized block design with 10 plants per plot and 8 replications.

In a duplicate experiment, two-week-old plants were inoculated by excision of one of the cotyledons and addition of a 20 µl drop to the wound (10^7 bacteria/ml). The disease was assessed after 12, 16, and 21 days. The experiment was carried out in a randomized block design with 90 replicates and 6 plants per replicate.

RESULTS AND DISCUSSION

Cotyledons test

Symptoms of infected cotyledons of young tomato plants readily appeared, as has been described by Chaldecott and Preece (1983). However, both the susceptible 'Moneymaker' and the partially resistant 'Irat L3' were affected (Fig 1). Probably the resistance of the latter is not expressed in the cotyledons, rendering this method useless as a technique for resistance.

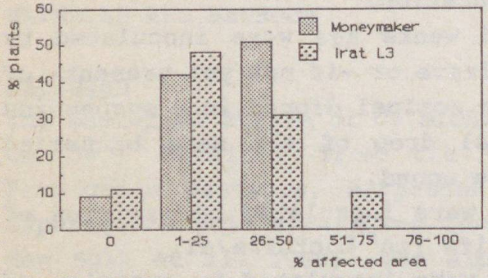

Fig 1: Affected area of cotyledons of 'Moneymaker' and 'Irat L3' inoculated with *C. michiganense*.

In vitro test

Six days after inoculation, all plants of 'Moneymaker' and 'Irat L3' were affected, although the 'Moneymaker' plants were somewhat more wilted than the Irat plants (results not shown). After 11 days, the difference between both genotypes could no longer be seen. These observations show that infection of *in vitro* grown plants is possible very well, but that the partial resistance of 'Irat L3' is not or only slightly expressed. This may be due to the fragile habitus of *in vitro* grown plants, which make them more receptive to the disease. Perhaps a very low inoculation pressure will enhance the contrast between both genotypes.

Greenhouse tests

Dipping the roots in a bacterial suspension before transplantation did not result in a high percentage of infection after three weeks (Table 1). Probably the bacteria need more

Table 1: Percentage of diseased plants of 'Moneymaker' (MM) and 'Irat L3' (IR) three weeks after inoculation. Plants were inoculated with different techniques at 1,2,3,4 or 5 weeks after sowing.

age	dipping		top		double		leave	
	MM	IR	MM	IR	MM	IR	MM	IR
1	30	40	-	-	-	-	70	60
2	10	0	-	-	-	-	70	0
3	20	0	-	-	-	-	100	0
4	30	0	80	0	100	0	100	0
5	0	0	100	0	100	0	80	0

time to penetrate the vascular system of the plant. Inoculation by excision of the top or by double inoculation resulted in a considerable infection of the susceptible 'Moneymaker' plants of both ages tested and no infection of plants of the partially resistant 'Irat L3'. The same holds for inoculation by excision of a leaf for 2- to 5-week-old plants. One-week old plants were too tiny to withstand the excision of a leaf properly. Inoculation by excision of the top and by double inoculation could only be applied on plants of 4 weeks or older. Thus, in order to accelerate the screening method as much as possible, the leaf inoculation method applied on plants of 2- or 3-weeks old seems to be the most promising.

In an extended experiment, the disease development of susceptible and partially resistant plants, inoculated 2 weeks after sowing by excision of one of the cotyledons or after 3 weeks by excision of the first true leaf, was followed in time. Both 2- and 3-week-old plants of 'Moneymaker' became more rapidly diseased than those of 'Irat L3', thus confirming the presence of partial resistance in the latter genotype (Fig 2). The contrast between both genotypes was comparable when 2- or 3-week-old plants were used and was optimal two weeks after inoculations. However, the time on which optimal contrast can be found depends on the environmental conditions

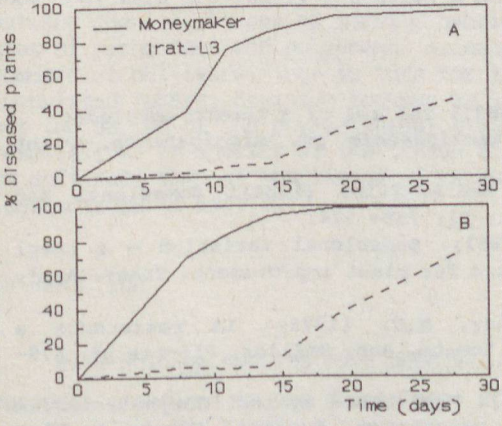

Fig 2: Disease development of 'Moneymaker' and 'Irat L3' plants inoculated 2 (A) or 3 (B) weeks after inoculation by excision of a cotyledon (A) or the first leaf (B) with a scalpel infected with *C. michiganense*.

during the experiment. In a following experiment with higher mean temperatures (30 °C at noon instead of 25 °C) in the greenhouse during the first week after inoculation, the disease developed faster in both 'Moneymaker' and 'Irat L3' (Fig 3).

In conclusion, inoculation of cotyledons by rubbing them with a bacterial suspension or inoculation of *in vitro* grown plants quickly led to symptoms, but did not or only slightly discriminate between a susceptible and a partially resistant genotype. Inoculation of 2-week-old plants by excision of a leaf with an infected knife quickly discriminated between a susceptible and a partially resistant genotype. Care must be taken, however, to control experimental conditions since interaction between those conditions and disease development readily occurs. Experiments are presently carried out to refine the screening method and to screen somaclonal variants for enhanced resistance.

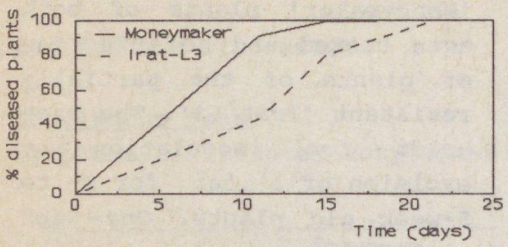

Fig 3: Disease development of 'Moneymaker' and 'Irat-L3' plants inoculated 2 weeks after sowing by excision of a cotyledon with an infected scalpel.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

FACTORS AFFECTING HIGH RESISTANCE TO ERWINIA SOFT ROT OF TUBERS FROM INTERSPECIFIC SOMATIC HYBRIDS OF SOLANUM BREVIDENS AND SOLANUM TUBEROSUM

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ABSTRACT

Tubers from somatic hybrids produced by protoplast fusion between S. brevidens, a diploid, non-tuber-bearing wild species, and a tetraploid S. tuberosum showed resistance to decay caused by soft rot erwinias. Tubers of the S. tuberosum fusion parent, and potato cultivar Russet Burbank are susceptible to bacterial soft rot. Furthermore, some of the sexual progeny from crosses between the somatic hybrids and cultivar Katahdin had the same high level of resistance as the somatic hybrids. Thus, the resistance incorporated from S. brevidens by somatic fusion was sexually transferrred. Interspecific somatic hybridization enable using new sources of disease resistance that were previously unavailable because of sexual incompatibilities between species. Tubers of somatic hybrids indicated higher level of activities of peroxidase and polyphenol oxidase then tubers of parental line and commercial cultivars. This is ture for intact tubers and also for injured or inoculated tubers. Bruising induced an increase of the respiration activity but the period of the physiological disorder (expressed as an activation of respiration) is shorter at the tissue of somatic hybrid tubers (resistant to soft rot) than at the tissue of parental line and commercial cultivars (susceptible to soft rot).

INTRODUCTION

Wild Solanum species have been valuable as sources of desirable agronomic features in the development of potato cultivars. However, at present only a limited number of tuber-bearing Solanum species are involved in breeding programs. Although full utilization of wild Solanum species can be limited, by sexual incompatibilities between gametic cells, agronomically important traits of related wild species can theoretically be incorporated into breeding programs by means of protoplast fusions between sexually incompatible species (1). Somatic hybrids between Solanum species using protoplast fusion have been obtained by several research groups (2, 3).

Inter and intra-specific hybrids have been produced that clearly show phenotypic characteristics intermediate between those of the parental types used in the fusion (4). Some of the hybrids expressed resistance to late blight and leaf roll virus (5) some to soft rot caused by Erwinia (6). Thus, certain desirable characters present in wild Solanum species and incorporated by protoplast fusion into S. tuberosum were transferred in subsequent sexual crosses with a commercial cultivar (6).

Tubers of potato cultivars vary in their relative susceptibility to bacterial soft rot (7, 8); but none of the common cultivars are considered to be highly resistant, although a range in relative susceptibility has been reported (7, 9, 10). The genetic basis of resistance to Erwinia soft rot is not well known, and some investigators describe it to be complex. Immunity has not been found either in potato cultivars (7, 8, 10) or in accessions of wild Solanum species (9, 11).

The somatic hybrid materials are valuable not only for direct potential use in breeding programs (6), but also for the examination of the factors affecting high resistance to Erwinia soft rot of tubers from somatic hybrids of S. brevidens and S. tuberosum.

The possible involvement of phenols system and related oxidative enzymes in disease resistance has been discussed in a number of reviews (12, 13, 14). One of the most widely accepted explanations of how the system operates in diseased plants is that following infections by pathogens the plants' oxidative enzymes catalyzing oxidation of phenolic compounds are induced. Oxidation products such as quinones, are toxic to the invading microorganism (12, 14, 15). Several reports on a wide range of pathogens have described the increased peroxidase (PO) and polyphenol oxidase (PPO) activities following the host-pathogen interaction. However, some of them have suggested that increases in PO activity are not directly involved in induced resistance. The investigation reported here was designed to test whether potato tubers differing in susceptibility to soft rot also show differences in the activities of PO and PPO in intact, mechanically injured and infected tubers.

The tubers of somatic hybrids indicated high level of resistance to mechanical damage (unpublished results). It was not surprising because several investigators showed the correlation between the resistance to soft rot and severity of sustained by tubers during mechanical harvesting and handling (7, 16). Generally the resistance to mechanical damage is influenced by the rate of the wound-healing process (16, 17, 18). If the wound-

healing is very intensive the tissue shows only very weak symptoms of damage and infection usually does not appear (16, 18). One of the effects of mechanical damage of potato tubers tissue is the induction of the post-wounding respiration. An evaluation of the respiration rate for the potato tubers sample could be a tool in predicting the susceptibility of potato tubers to mechanical damage (19, 20). In the present study relationships are sought between the observed differences in potato tubers susceptibility to soft rot and the respiration rate.

MATERIALS AND METHODS

Sources of test material

The materials used in this study were obtained from Dr. John Helgeson and Dr. Sandra Austin, Department of Plant Pathology, University of Wisconsin-Madison, USA. The production and field evaluation of these plants have been described previously (4, 5, 6).

Testing for PO and PPO activities

Ten tubers of each cultivars were used for the determination of enzymes activities. Tubers were injected with sterile polypropylene pipet tips containing sterile water (25 µl) or suspension of Erwinia carotovora spp. carotovora (Ecc-SR 394), 25 µl of 5×10^7 cfu/ml. Tubers were incubated at 22 ^{o}C in a dew chamber with a relative humidity (RH) of 92%. After 12, 24 and 48 h of incubation tubers were sliced vertically to the injection points. For determination PPO and PO activities tissue surrounding injection site was collected. Tissue from intact tubers was used as a control. For enzyme extraction and routine assay of PO and PPO the method described by Basham et al. was used. Standard deviations were calculated on the basis of two independent experiments.

Respiratory measurements

The respiration rate was determined for intact and bruised tubers on the basis of the CO_2 release from the tubers. The conversion of CO_2 to CH_4 was conducted by Methanizer (Shimadzu MTN-1). CH_4 concentration was measured by the Gas-chromatograph (Shimadzu, GC-9AM, Japan) equipped with Flame-Ion-

ization Detector. Gas-chromatograph was equipped with Supelco column (4 x 1/8, MR43854) filled with Poropak Q (80-100 mesh), nitrogen was the carrier gas, operating temperature was 40 ^{o}C, the time of analysis 1 minute. Peaks were integrated with Integrator Chromatopac (Shimadzu, C-R3A).

Ten tubers of each investigated line or cultivar were bruised at three sites. For determination of respiration rate tubers were incubated in tightly closed jars. Samples of gas for injection to Gas-chromatograph have been taken immediately after packing tubers to the jars and after 30 and 60 min of incubation at the jars. After determination of CO_2 concentration, tubers were taken out from the jars and incubated in dew chamber for 24, 48 and 72 h. After this the procedure described earlier was repeated. Respiration rate was expressed in µl CO_2 per 1 g fresh weight per 1 h. Analyses were performed in duplicates, 2 jars with tubers of each line was investigated. Standard deviation was calculated on the basis of two independent experiments.

RESULTS AND DISCUSSION

Determination of PO activity in tissue of intact tubers of Russet Burbank (RB) and hexaploid somatic hybrids of Russet Burbank and S. brevidens (RB + 6A) indicates two times higher enzyme activity at the tissue of somatic hybrids tubers than at the tissue of RB (Table 1).

Table 1 Changes of PO and PPO activities

Solanum line	PO activity (units/g FW/sec^{-1}) Time after injection			PPO activity (units/g FW/sec^{-1}) Time after injection		
	12 h	24 h	48 h	12 h	24 h	48 h
Russet Burbank						
Intact tubers	15.6			5.1		
Injected tubers:						
Sterile water	17.3	18.8	34.3	6.5	5.5	6.4
ECC-SR 394	23.0	27.8	40.4	10.3	2.9	4.5
Somatic hybrids (RB + 6A)						
Intact tubers	32.7			10.1		
Injected tubers:						
Sterile water	35.5	63.7	69.2	11.0	10.3	13.0
ECC-SR 394	39.0	65.8	76.6	11.9	14.3	12.1

Incubation in dew chamber at 22 ^{o}C in RH 92 %. Enzymes activites were determined 12, 24, 48 h after injection. Injection were 25 µl of sterile water or 25 ul of Ecc-SR 394 suspension (5 x 10^{7} cfu/ml) Data give the mean of two independent experiments, SD $\leq$ 10 %.

Tissue of RB injected with sterile water indicates lower activation of PO than tissue injected with bacterial suspension, respectively 20 and 78% after 24 h and 120 and 160% of control after 48 h. It was not true for somatic hybrid tuber tissue where even injection of sterile water induced increase of PO activity about 95% during first 24 h.

The results presented in this study reveal that, as in many other plant-pathogen interactions (12, 13, 15), PO activity increases in potato tuber tissues mechanically injured or infected by Erwinia. The presented results showed that PO is involved in the resistance mechanism. The above assumption is based on the following observation: intact tubers of resistant lines indicated higher level of PO activity than tubers of susceptible ones. Increase of PO activity after mechanical injury of infection if quicker in resistant lines than in susceptible ones.

However, the fact that the PO activity increases to a similar extent in mechanically injured and infected tissue of somatic hybrids indicates no specific reaction. This kind of reaction was observed by other authors (15). Manibhushanrao et al. concluded that increased PO activity is rather a biochemical symptom of tissue destruction and one of the events reflecting the abnormal physiological status of host plants.

The activity of PPO was also higher in the tissue of somatic hybrids than at the tissue of RB (Table 1). However, we could not find significant differences between the PPO activities in the intact, mechanically injured and infected tubers of RB. In case of somatic hybrids only infection with soft rot bactèria induced significant increase of PPO activity during first 24 h after inoculation (about 30%) (Table 2).

The possible involvement of the phenol oxidation system in resistance to bacterial soft rot of potato tubers was shown. Mechanical injury or infection of the tissue of the tubers susceptible to soft rot showed no significant increase in PPO activity during 48 h after infection. However, tissue of tubers resistant to soft rot infected with Erwinia showed an increase of PPO activity in comparison with intact tubers.

The role of phenols and their oxidation products have been studied intensively in bacterial disease (12, 13, 14, 15). Basham et al. showed that tomato cultivars known to possess a high degree of field resistance to

Pseudomonas syringae pv. tomato indicate high level of PPO activity. On the other hand, in apples Erwinia infection increases PPO activity, both in virulent and avirulent infections (21). Reports on the participation of oxidative enzymes are highly contradictory. It indicates that disease resistance in higher plants is mediated by many different mechanism.

Bruising induces activation of post-wounding respiration but the pattern of it varies in investigated lines (Fig. 1). Intensity of respiration in the tissue of parental line S. tuberosum (R4) increased by about 40% in the results of mechanical damage. This phenomenon could be observed 72 h after bruising. In contrast in the tissue of hexaploid somatic hybrids of S. tuberosum and S. brevidens (R4 + 6A) bruising induced an increase of respiration activity by about 30% first 24 h since bruising. Later slow decrease of the post-injury respiration rate was observed. The tissue of somatic hybrids (RB + 6A) indicated high activity of respiration directly

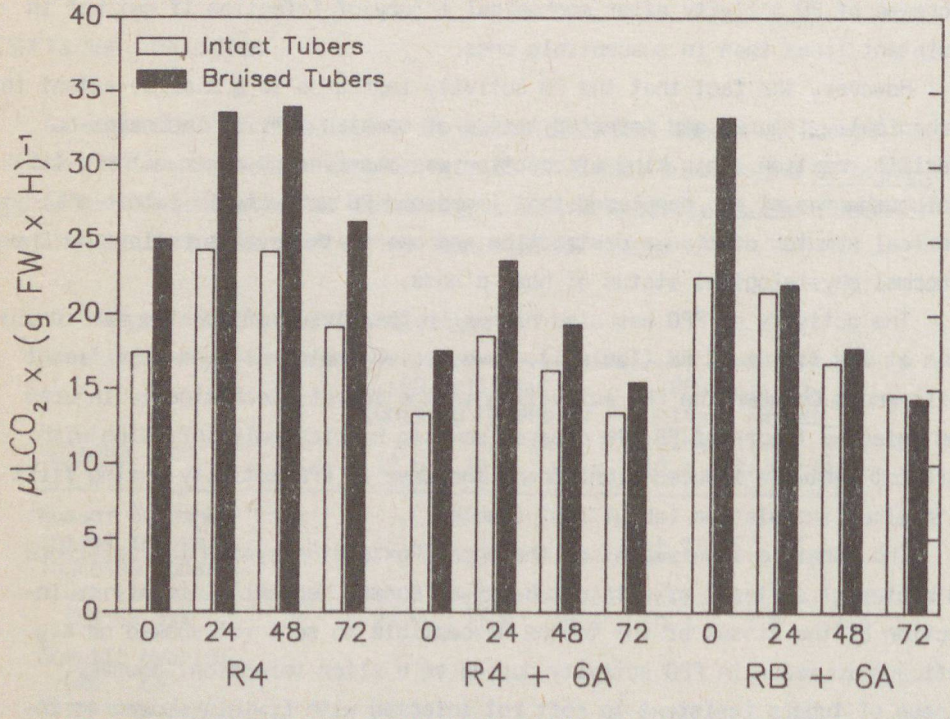

Figure 1 Change in potato tubers respiration after bruising. Incubation was provided for 24, 48 and 72 h in dew chamber at 22 °C in RH 92%. R4 - S. tuberosum, R4 + 6A - somatic hybrids of S. tuberosum and S. brevidens, RB + 6A - somatic hybrid of Russet Burbank and S. brevidens.

after bruising. However, during 24 h the respiration rate decreased and attained the level characteristic for intact tubers (Fig. 1).

These results indicate that although mechanical damage induces physiological disorder in the tissue of all investigated lines, this process is repressed earlier and more effectively in the tissue of somatic hybrids. This observation is in accordance with the fact that evaluation of respiration is a tool in description of quality of potatoes (19, 20).

The objective of this study was to describe biochemical parameters that could affect resistance to soft rot. Presented results indicate that high activities of PO and PPO and the ability of the tissue of somatic hybrids tubers to stop post-wounding respiration play an important role in resistance mechanism.

Acknowledgements

Appreciation is expressed to Dr. Arthur Kelman for his cooperation and assistance in this project and to Dr. Sandra Austin and Dr. John Helgeson for making tubers of somatic hybrids available. Part of this work was supported by Project CPBP 05.02. from the Polish Academy of Science.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

DOSE-RESPONSE OF RICE CULTIVARS IR20 AND PERUM KARUPPAN TO VIRULENT AND AVIRULENT RACES OF XANTHOMONAS CAMPESTRIS PV. ORYZAE

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ABSTRACT

Inheritance studies have shown that Perum Karuppan (PK) is similar to IR20 for resistance to bacterial blight (BB). Pathologically however, IR20 was more resistant than PK at maximum tillering to race 1. Both were susceptible to race 2. The dose-response of PK to incompatible race PX061 and compatible race PX086, was compared with IR20. The dose levels varied from 0.51 to 1.9 x 10^3 cfu/leaf estimated 1 hr after inoculation.

IR20 and PK showed high leaf infection with PX086 at a mean initial dose of 8.7 x 10 and 1.1 x 10^2 cfu/leaf, respectively, while to the incompatible PX061, leaf infection increased when initial inoculum was 1.9 x 10^2 and 1.4 x 10^2 cfu/leaf, respectively at both maximum tillering and booting stages. Lesion areas of the two cultivars differed. IR20 was resistant to PX061 even at the highest initial dose of 7.6 x 10^2 cfu/leaf. PK showed susceptible reaction to PX086 at maximum tillering and booting at 1.1 x 10^2 cfu/leaf and to PX061 at maximum tillering only. At booting, PK was resistant to PX061 even at the highest initial dose of 1.9 x 10^3 cfu/leaf, indicating that the resistance functions against PX061 at booting but not to PX086. The susceptible check IR24 showed high leaf infection and lesion area at an initial mean dose of 3.2 x 10 cfu/leaf for PX061 and PX086, respectively at maximum tillering; at booting, these reactions occurred at 1.3 x 10^2 and 2.5 x 10^2 cfu/leaf.

The relative resistance of PK and IR20 was also estimated by the ratio of their lesion areas. At maximum tillering, IR20 was twice more resistant than PK to PX061, but at booting they were similar. The estimated relative resistance of both cultivars based on ED_{50} showed a similar trend of response to PX061; against PX086, the relative resistance between IR20 and PK at maximum tillering and booting based on lesion area and ED_{50} was not significantly different as they were both susceptible.

INTRODUCTION

Bacterial blight of rice caused by Xanthomonas campestris pv. oryzae (Xco) has been controlled economically by utilizing resistant rice cultivars. There are a large number of resistance sources identified from the vast collection of rice germplasm. However, to be able to utilize these resistances, it is necessary to assess and characterize the relative

resistance of the cultivars to races of the pathogen.

Generally, resistance of cultivars to bacterial blight is affected by crop growth and the differential response to the pathogen (Mew, 1987). The resistance conveyed by cultivar Perum Karuppan (PK) was reported to be similar to that of cultivar IR20 (Sidhu et al. 1978). Pathologically, however, IR20 was more resistant to race 1 (PX061) than PK at maximum tillering. Both cultivars were susceptible to race 2 (PX086) at all growth stages. To quantify the relative resistance of these two cultivars, the dose-response of PK to incompatible race PX061 and compatible race PX086 was compared with that of IR20 at maximum tillering and booting stages. The effect of inoculum dose on the expression of resistance of PK and IR20 was also compared.

MATERIALS AND METHODS

Test plants and bacterial strains

Three cultivars were studied: cultivar IR20 has resistance to PX061 at all growth stages, Perum Karuppan also has resistance to PX086 but functions only at the booting stage. Cultivar IR24 was used as susceptible check. The Xco races PX061 and PX086 were used; PX086 is virulent to all the three cultivars.

Plant growth and experimental layout

Sowing of three rices in plastic trays was staggered to synchronize inoculation at 40-50 days after sowing for maximum tillering (MTS) and 75-85 DAS for booting stages (BS). Fifteen seedlings per cultivar were transplanted individually in 3 x 5 rows to 13 x 9 inch plastic trays at 12 days after sowing. The soil was prepared from paddy field. An additional 3 g of ammonium sulfate was applied to trays one week before inoculation. The trays were arranged following a split-split plot design with cultivars as mainplot, races as subplot and inoculum dilution as sub-subplot in 3 replications.

Challenge/inoculum doses and inoculation

Suspensions of PX061 and PX086 were prepared from 48-hr old cultures and adjusted to an absorbance of 0.05 at 590 nm on a spectrophotometer. Based on preliminary experiments, minimal inoculum dose of 5-7 x 10^7 cfu/ml was necessary to cause 95-100% infection on all inoculated leaves of a susceptible cultivar at MTS. The concentrated bacterial suspensions of each strain were used to prepare a series of four-fold dilutions using sterile distilled water. Six inoculum doses were used to inoculate 100-

130 leaves/rep in IR20 and IR24 and 87-100 leaves/rep in PK at MTS: at BS, 62-86 leaves/rep in IR20 and IR24 and 56-63 leaves/rep in PK were inoculated. All fully expanded leaves were clipped 1-2 cms from leaf tip by a pair of scissors dipped in each inoculum suspension. The different dilutions of inoculum were kept at 10°C while other dilutions were being used for inoculation. The lowest inoculum concentration was inoculated ahead of the higher concentrations.

Estimation of bacterial number at the infection court

The number of cells at the point of inoculation was determined for each cultivar to represent the challenge dose of the cultivar. To estimate the number of cells at the point of inoculation one hour after inoculation, the about 0.5 cm long leaf samples inoculated individually with PX061 and PX086 at approximately 2×10^8 cfu/ml were cut aseptically and placed in a sterile plastic bag. The samples of each cultivar were disinfested with 70% ethanol and rinsed vigorously in sterile distilled water. The leaf specimens were homogenized in 5-ml sterile distilled water with a leaf presser. The leaf extract was diluted ten-fold for the viability count and 0.1 ml of the diluted extract was plated on Suwa's medium. Plate counts were taken four days after incubation.

Disease readings

Host response was based on positive lesion development per leaf of all inoculated leaves estimated at 14 and 21 days after inoculation (dai). From such readings, leaf infections were based on scores $\geq 1\%$ diseased leaf area, thus estimation of the quantal response was based on $\geq 1\%$ for presence and $< 1\%$ for absence of bacterial blight lesion. Lesion area was based on mean of all progressing lesion inoculated with highest concentration at 14 dai for maximum tillering stage and 21 dai for booting stage. The estimate of relative resistance was based on the ratio of lesion areas of the cultivars.

Determination of the ED_{50}

The proportion of diseased ($\geq 1\%$ DLA) and healthy ($< 1\%$ DLA) leaves over the total number of inoculated leaves at each challenge dose estimated one hr after inoculation of each cultivar were used to calculate for the ED_{50} using probit analysis based on Quant 13 program (modified and provided by D.J. Finney).

The estimate of relative resistance was based on the ratio of ED_{50}'s of PX061 and PX086 on the cultivars.

RESULTS

Leaf infections and lesion areas increased gradually with increasing doses of bacterial concentration in the infection site however, some differences on leaf infection between avirulent and virulent isolates at a certain dose were observed. On IR20, percent leaf infections on PX086 at maximum tillering stage (MTS) was greater than on PX061 when initial inoculum was 2.18 x 10 and 1.19 x 10 cfu/leaf, respectively (Fig. 1). At booting stage, a remarkable difference on leaf infections of IR20 caused by PX061 and PX086 was observed at initial concentrations of 4.7 x 10 and 8.7 x 10 cfu/ml, respectively. PK also showed marked difference on leaf infection at MTS between PX061 and PX086 at initial inoculum of 3.5 x 10 and 1.19 x 10^2, respectively (Fig. 2). At booting stage, PK showed 9.3% leaf infections to avirulent PX061 at initial inoculum of 3.5 x 10 cfu/leaf while to virulent PX086, leaf infections was 65.03% at 1.19 x 10^2 cfu/leaf (Fig. 2). On susceptible IR24, leaf infection increased gradually for both isolates at MTS; at BS, the difference was observed at initial conc. of 3.2 x 10 for PX061 and 6.4 x 10 for PX086 (Fig. 3).

Qualitatively, lesion area of IR20 was always resistant to PX061 at MTS and BS (Fig. 1). IR20 was 2.3855 times more resistant than PK at MTS, however PK was as resistant (1.4461X) as IR20 at BS to PX061 (Table 1). The ED_{50} was used to compare the relative resistance of the three cultivars. PK was 3.1433X more resistant than IR20 at MTS and as resistant as IR20 at BS to PX061 (Table 1). No significant difference was noted between these two cultivars at both stages to PX086 (Table 2).

The response of the three cultivars to the challenge doses of PX061 and PX086 was also determined by relating the proportion of diseased leaves transformed to $\ln(1-x)^{-1}$ to the doses applied. All combinations showed a linear relationship between the dose and the transformed proportion of diseased leaves which indicated that the cells are acting independently and are homogeneous in virulence to infect the cultivars (Fig. 4). However, the resistance of IR20 functions at MTS and BS to PX061 while that of PK was at BS to PX061.

DISCUSSION

Physiologic specialization of Xco, the rice bacterial blight pathogen has been reasonably well understood (Mew, 1987). Applying the molecular genetic techniques, scientists were able to elucidate the genotypes of the races based on restriction fragment length polymorphism pattern and

Table 1. Lesion area and median effective dose (ED_{50}) and estimates of relative resistance of cultivars having either Xa-4[a] (IR20) or Xa-4[b] (Perum Karuppan) genes for BB resistance to PX061 at maximum tillering and booting. IRRI, 1987.

Cultivar	Maximum tillering			Booting		
	Lesion area[1]	Relative resistance[2]		Lesion area[1]	Relative resistance	
IR24	53.0	1.0000		30.74	1.0000	
Perum Karuppan (Xa-4[b])	19.8	2.6767	1.0000	10.99	2.7971	1.0000
IR20 (Xa-4[a])	8.3	6.3855	2.3855	7.6	4.0447	1.4461
	ED_{50}	Relative resistance[2]		ED_{50}	Relative resistance	
IR20 (1)	86.99	1.0000		107.15	1.0000	
Perum Karuppan (2)	27.71	2.1433	1.0000	89.83	1.1928	1.0000
IR24 (3)	7.61	11.4278	3.6402	52.66	2.0347	1.7058

Table 2. Lesion area and median effective dose (ED_{50}) and estimates of relative resistance of cultivars having either Xa-4[a] (IR20) or Xa-4[b] (Perum Karuppan) genes for BB resistance to PX086 at maximum tillering and booting. IRRI, 1987.

Cultivar	Maximum tillering			Booting		
	Lesion area[1]	Relative resistance[2]		Lesion area[1]	Relative resistance	
IR24	40.1	1.0000		23.1	1.0000	
Perum Karuppan	43.3	0.9261	1.0000	22.8	1.0132	1.0000
IR20	39.6	1.0126	1.0934	18.9	1.2184	1.2025
	ED_{50}	Relative resistance[2]		ED_{50}	Relative resistance	
Perum Karuppan	34.02	1.0000		132.48	1.0000	
IR20	25.30	1.3448	1.0000	73.32	1.8063	1.0000
IR24	14.79	2.3003	1.7105	40.42	3.2770	1.8141

[1]Estimated on leaves inoculated with highest inoculum dose at 14 days after inoculation (MTS) and 21 dai (BS).
[2]Relative to second named cultivar.

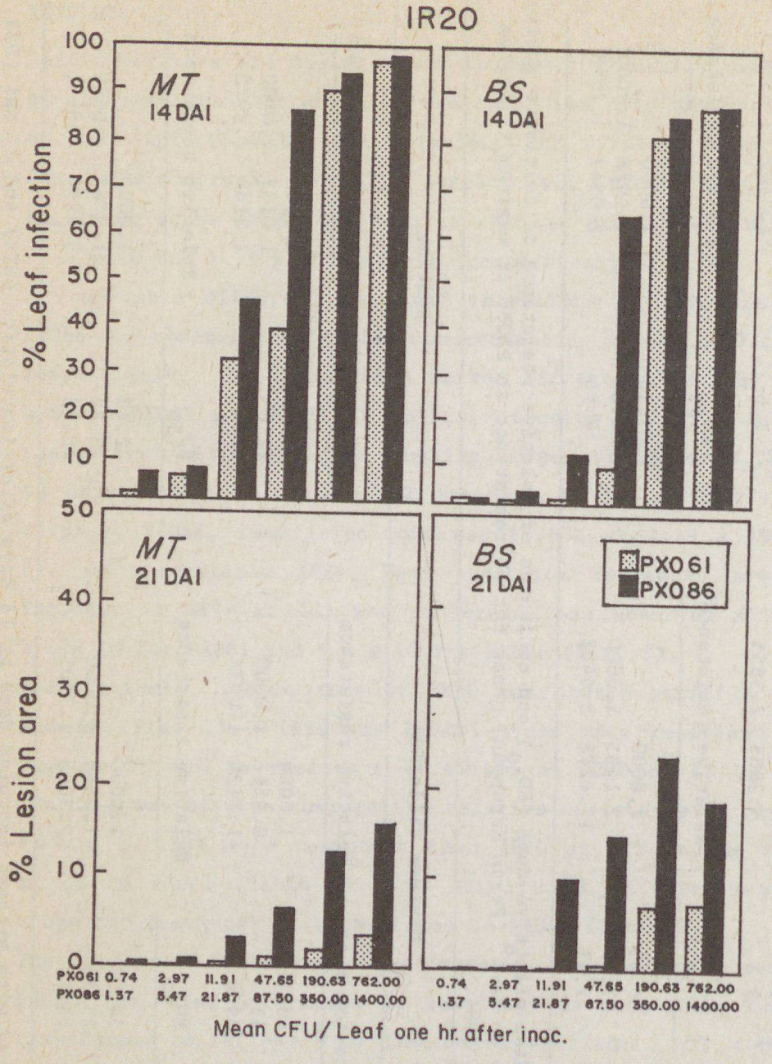

Fig. 1. Leaf infections at 14 days after inoculation (dai) and lesion areas at 21 dai of cultivar IR20 initiated at varying doses of PXO61 (race 1) and PXO86 (race 2) of Xanthomonas campestris pv. oryzae at maximum tillering (MT) and booting (BS) stages.

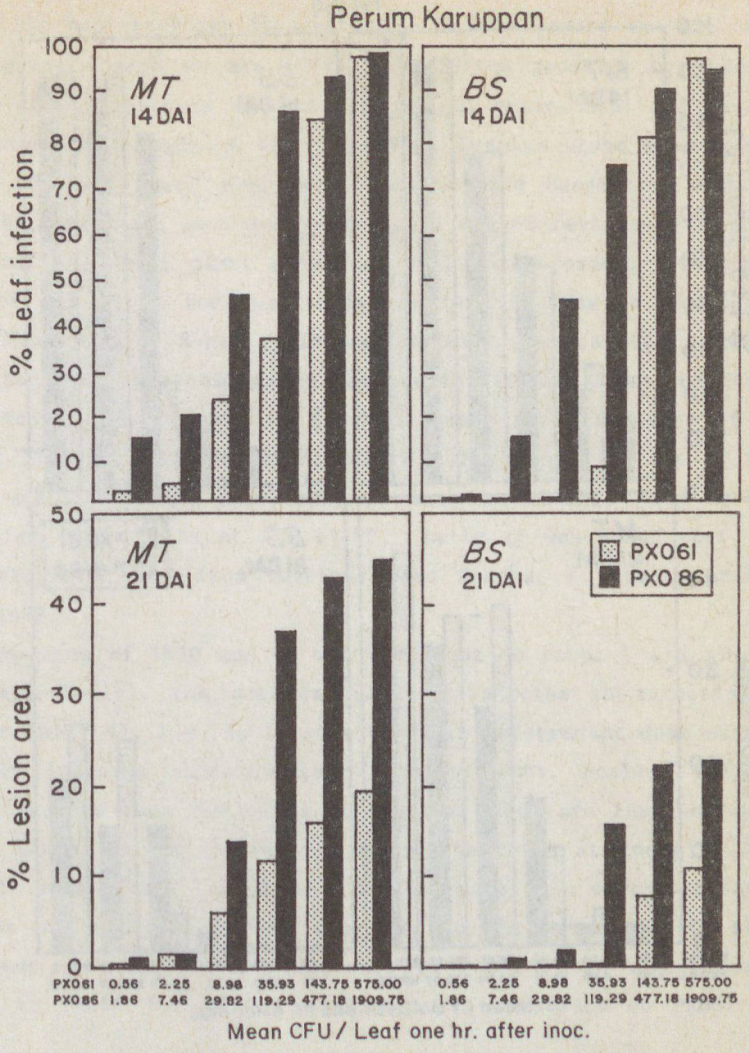

Fig. 2. Leaf infections at 14 days after inoculation (dai) and lesion areas at 21 dai of cultivar Perum Karuppan initiated at varying doses of PX061 (race 1) and PX086 (race 2) of Xanthomonas campestris pv. oryzae at maximum tillering (MT) and booting (BS) stages.

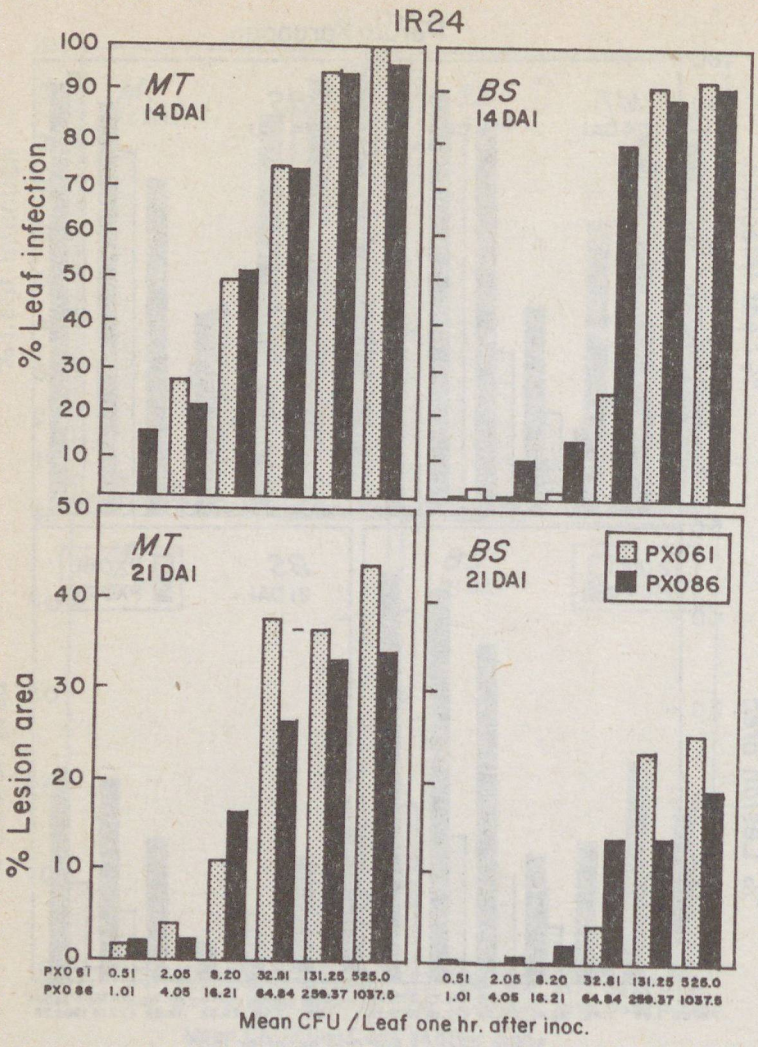

Fig. 3. Leaf infections at 14 days after inoculation (dai) and lesion areas at 21 dai of cultivar Perum Karuppan initiated at varying doses of PX061 (race 1) and PX086 (race 2) of Xanthomonas campestris pv. oryzae at maximum tillering (MT) and booting (BS) stages.

specificity of the host-pathogen interaction. In this regard we have made remarkable progress (Leach et al. 1989). Mendelian genetics has helped to demonstrate the inheritance of bacterial blight resistance, and so far 14 genes are identified (Ogawa & Khush, 1989). Despite these progresses, only few rice cultivars have been well characterized based on their resistance to BB. Earlier, we tried to evaluate the BB resistance in rice cultivars according to crop plant growth as well as differential response to virulence of the races for qualitative resistance (Mew et al. 1981, 1982, Zhang & Mew, 1985). Since resistance of many rice cultivars appear incomplete or partial, we attempted in the present study to use the dose-response relationship as a means to further assess the resistance of PK as compared to IR20, which seems quantitative. PK belongs to one of the rice cultivar groups reported previously to have an allelic gene $Xa\text{-}4^b$ against $Xa\text{-}4^a$ for IR20 (Sidhu et al. 1978). Later it was found that $Xa\text{-}4^b$ is identical to Xa-3 from cultivar Wase Aikoku, a japonica rice (Ogawa et al. 1987).

Although the reaction of IR20 and PK was different to races 1 & 2 (PXO61 and PXO86, respectively), the dose relation in response to infection appears similar (Fig. 4), i.e. followed a distinct independent dose action theory at lower inoculum concentration (Ercolani 1984, Boelema 1977). This indicates perhaps that the cells of the two races are homogenous in virulence, and there was no interaction among the cells at lower concentration in each of the two races. Because rice is a self-pollinated crop, the ratio of outcross is less than 1%, so the tested plants also appear homogenous with regard to resistance to PXO61 at MT and BS and susceptibility to PXO86 for IR20, and likewise, resistance to PXO61 at BS for PK.

The relative resistance estimated on the basis of lesion area (or length) provides a clear distinction of the three rice cultivars at MT and BT to the two races. We also estimated the relative resistance of the cultivars based on the ratio of ED_{50} of the two races. Finney used the term "potency" to refer to the ratio of equally effective doses (Finney, 1971). The relative potency provides a convenient description of the difference between the two series. We feel that it is more relevant to use "relative resistance" as we assessed resistance of the rice cultivars. From these estimates, it was clear that IR20 and PK are distinct in their resistance to race 1 (PXO61) at booting, and no difference to race 2 (PXO86) both at booting and maximum tillering stage.

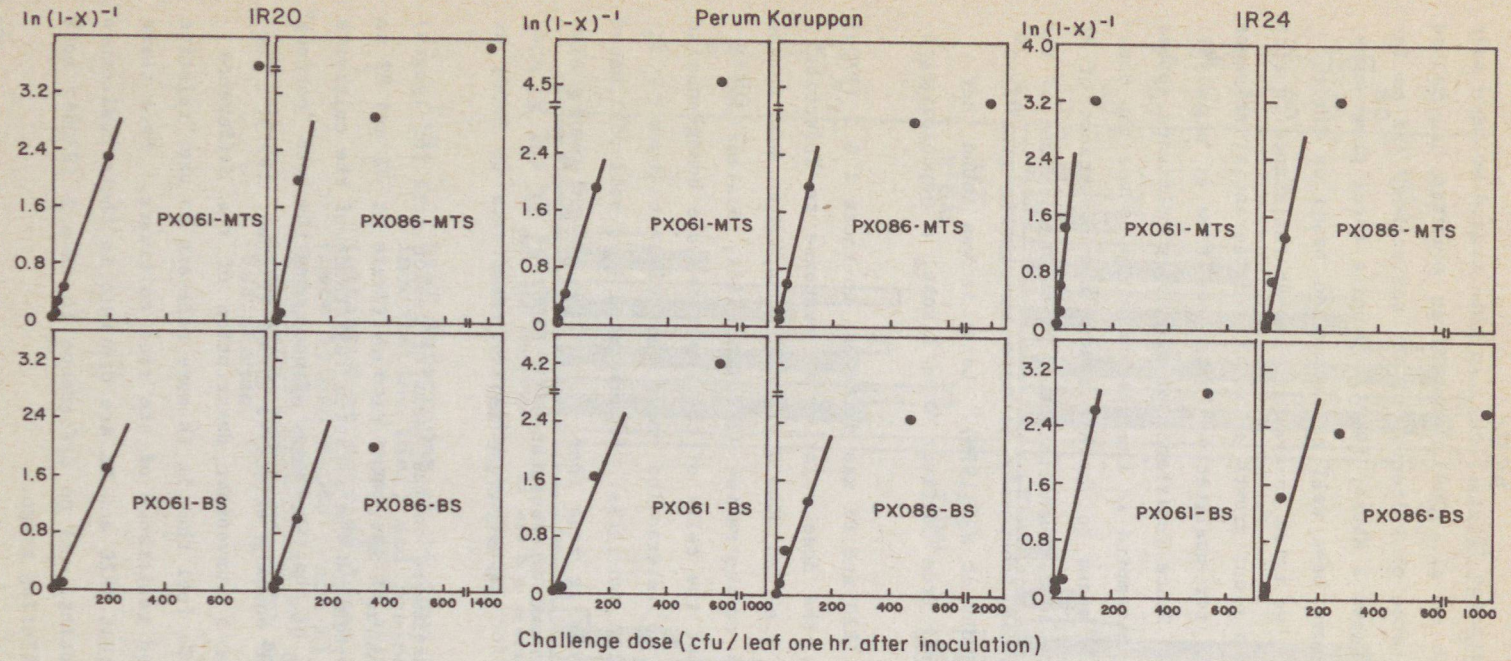

Fig. 4. Relationship between ln $(1-x)^{-1}$ (x= proportion of diseased leaves) and the challenge dose of Xanthomonas campestris pv. oryzae in rice cultivars IR20, Perum Karuppan and IR24.

The ratio for relative resistance thus provides a means of using ED_{50} and lesion area (or length) to assess the quantitative difference of rice cultivars to BB resistance.

ACKNOWLEDGEMENT

We thank Dr. D.J. Finney for his assistance in the data analysis and for providing us the Quant 13 program as modified from the program of Tang Zhung-Ming.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

PLUM SUSCEPTIBILITY TO XANTHOMONAS CAMPESTRIS PV. PRUNI IN THE PO VALLEY

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INTRODUCTION

Xanthomonas campestris pv. *pruni* (Smith) Dye was first reported in Italy in the 50's in Campania on apricot and in Sicily on almond. In the Po Valley, Northern Italy, after a first report in 1970, epidemics of bacterial spot have occurred in plums since the end of the 70's (Bazzi and Mazzucchi, 1980). In 1987 epidemics were also observed in peach orchards with cling peaches and nectarines in North-Eastern Italy (Stefani *et al.*, 1989). The introduction of more susceptible Japanese-type cultivars probably favoured the outbreak of these epidemics in plums. As a result, the resistance to this disease has become one of the most important traits considered in plum breeding in Italy. This paper reports the results of a comparative study set up to assess the susceptibility of 22 plum cultivars in the field.

MATERIALS AND METHODS

Bacterial cultures

The virulent strain *X. campestris* pv. *pruni* (X.c.p.) NCPPB 3155 (=IPV-B01192), isolated from plum in the Po Valley, was used. This was grown in nutrient A broth (Stolp and Starr, 1964) in a rotary shaker at 27°C for 30h, centrifuged at 8,000 g in a LAPX 200 α-Laval continuous centrifuge, and used to prepare the inoculum.

Research supported by Italian Ministry of Agriculture and Forestry Rome under project "Frutticoltura e Agrumicoltura".

Plum cultivars

A total of 22 European and Japanese-type cultivars was tested (Table 1). They were grafted onto myrobalan B, (*Prunus cerasifera*), open-centre trained, for 3 years in an experimental field, set out in randomized blocks with 4 replications, at Ozzano dell'Emilia near Bologna.

Inoculation

The source of inoculum was both experimental and natural. The experimental inoculum consisted of a bacterial suspension sprayed on a single branch of each tree in the last ten days of May 1988. The final pellet of the broth culture was resuspended in water to a concentration of approximately 10^8 C.F.U. ml^{-1}, equal to 0.1 A_{660}. For inoculation, each branch was covered with a large polyethylene bag in the late afternoon and then sprayed with the suspension. This moist chamber was removed the next morning. The May inoculation was made on the leaves and fruitlets.

The natural inoculum consisted of bacteria evaded from primary infections, caused by the experimental inoculation, and secondary infections during the growing season (June-October).

Disease assessment

Two field assessments were made in the periods June 15th - July 15th and September 29th - October 7th. A sample of 100 leaves and 20 fruitlets was collected from each tree. In June the samples were only taken from the inoculated branches; in September they were taken randomly from all the branches not previously sampled. The fruit sampling in June was carried out at the same time for all the cultivars.

Disease intensity was assessed by counting the leaf and fruit spots (Terzano Model LMMP electric colony counter) using as a reference a single polygonal, water-soaked and/or necrotic spot with sides measuring approximately 1.5 mm. Areas of confluent infection were counted as multiples of the reference spot. Disease intensity in the leaves was expressed as the number of spots per leaf and per cm^2 of leaf, and

likewise in the fruits, the number of spots per fruit and per cm^2 of fruit. The leaf surface area was calculated approximately as an ellipse on the basis of measurement of the two axes; that of the fruits as a prolate sphaeroid (Hodgman, 1951). Reisolation of X.c.p. from random samples of typical water-soaked leaf and fruit spots was successful on YDC-agar (Stolp and Starr, 1964).

Statistical analysis

Means were compared according to the cluster procedure of Scott and Knott (1974) and to Spearman's rank correlation coefficient (Boldrini, 1962).

RESULTS AND DISCUSSION

Experimental inoculation gave rise to primary infections which appeared in the first week of June on leaves and fruits. In the majority of cultivars the leaf spots were quite uniform, individual or confluent. In "Frontier" numerous circular spots, 2-3 mm in diameter, with a water-soaked halo, were observed associated with a few X.c.p. spots; within a few days the infected tissue dried up and dropped away, causing a shot-hole appearance. Attempts to reisolate X.c.p. from these spots were not successful. These spots were associated with a fungus of the genus *Helminthosporium* (Ciccarone, pers.com.). The number of spots per leaf varied from 15.3 in "Çalita" to 0.12 in "Valor" (Tab.1). The number of spots per cm^2 of leaf ranged from 0.9 in "Sorriso di Primavera" to 0 in "Valor". Two groups of cultivars were significantly different ($p \leq 0.05$). The fruit spots were not uniform among all the cultivars; in the majority the infected areas were circular, 2-4 mm in diameter, initially water-soaked, becoming gradually brown and cracked at the centre. In "Burmosa", "Fialetovaja Desertnaja", "Morettini 355" and "Obilnaja", necrotic specks were observed with no cracking; in "Valor" these had a reddish hue. Attempts to isolate X.c.p. from these specks were never successful. Four and three groups of cultivars

TABLE 1. Plum leaf susceptibility to *X.c.p.* assessed by late Spring and early Autumn indexes. The Spring indexes refer to an assessment made 5 weeks after primary high dose experimental infection. The Autumn indexes refer to an assessment at the end of September of the overall response of the cvs to natural secondary infections.

CULTIVAR	Late Spring indexes		Early Autumn indexes	
	Spots/leaf	Spots/cm^2	Spots/leaf	Spots/cm^2
Calita	15.13 A	0.58 A	4.07 A	0.14 A
Sorriso di Primavera	13.92 A	0.98 A	0.44 B	0.02 C
Black Gold	12.20 A	0.35 B	0.33 B	0.01 C
Simka	11.79 A	0.59 A	0.24 B	0.01 C
Black Star	9.87 A	0.71 A	0.25 B	0.01 C
Verity	9.71 A	0.32 B	0.05 B	0.00 C
Shiro	9.54 A	0.77 A	1.43 B	0.08 B
Burmosa	8.60 A	0.30 B	0.40 B	0.01 C
Angeleno	5.05 B	0.23 B	0.88 B	0.05 C
Ozark Premier	4.79 B	0.28 B	0.10 B	0.00 C
Fialetovaja Desertnaja	3.70 B	0.15 B	0.74 B	0.02 C
Morettini 355	3.04 B	0.14 B	0.61 B	0.02 C
Stanley	2.22 B	0.11 B	0.26 B	0.01 C
Empress	1.71 B	0.06 B	0.03 B	0.00 C
Black Amber	1.62 B	0.11 B	0.54 B	0.03 C
Frontier	1.55 B	0.09 B	0.36 B	0.02 C
Zemlianichnaja	1.39 B	0.07 B	0.34 B	0.02 C
Black Diamond	1.32 B	0.05 B	0.12 B	0.00 C
Obilnaja	0.74 B	0.05 B	0.32 B	0.01 C
Pobeda	0.69 B	0.07 B	0.17 B	0.01 C
Cinerova Rana	0.51 B	0.02 B	0.01 B	0.00 C
Valor	0.12 B	0.00 B	0.05 B	0.00 C

TABLE 2. Plum fruit susceptibility to *X.c.p.* on the basis of late Spring indexes assessed 5 weeks after primary high dose experimental infection.

CULTIVAR	LATE SPRING INDEXES	
	Spots/fruit	Spots/cm^2
Frontier	35.40 A	0.55 A
Black Star	18.14 B	0.59 A
Calita	17.20 B	0.34 B
Verity	11.79 C	0.36 B
Burmosa	11.25 C	0.18 C
Fialetovaja Desertnaja	10.36 C	0.23 C
Black Gold	7.71 C	0.44 B
Shiro	5.73 D	0.32 B
Obilnaja	5.06 D	0.10 C
Morettini 355	3.62 D	0.07 C
Ozark Premier	2.47 D	0.03 C
Black Amber	2.17 D	0.04 C
Black Diamond	1.87 D	0.07 C
Empress	1.60 D	0.02 C
Sorriso di Primavera	1.09 D	0.04 C
Zemljanichnaja	0.42 D	0.02 C
Stanley	0.40 D	0.00 C
Pobeda	0.28 D	0.01 C
Valor	0.00 D	0.00 C
Angeleno	0.00 D	0.00 C
Cinerova Rana	N.D. (*)	N.D.
Simka	N.D.	N.D.

(*) N.D. = no fruits

Values followed by the same letter are not significantly different at $P \leq 0.05$ (Scott and Knott, 1974).

were shown to be significantly different (Tab.3) on the basis of the number of spots per fruit and per cm^2 of fruit.

At the beginning of October, fewer leaf spots were counted than in the Spring (Tab.1). The number of spots per leaf varied from 4.07 in "Calita" to 0.01 in "Cinerova Rana"; with the exception of "Calita" there was no significant difference between the varieties. The number of spots per cm^2 of leaf ranged from 0.14 in "Calita" to 0 for "Verity", "Ozark Premier", "Empress", "Black Diamond", "Cinerova Rana" and "Valor". "Calita" and "Shiro" were statistically different from one another and significantly different from the remaining twenty cultivars. The susceptibility of "Shiro", "Burmosa", "Ozark Premier" and "Stanley", assessed in decreasing order in Maryland (Keil and Fogle, 1974) fits quite well with our one-season data.

The position of certain cultivars in the rank based on the number of spots per leaf was different from that in the rank based on the number of spots per cm^2 of leaf in both late Spring and early Autumn indexes. Clear examples were the cases of "Empress", "Black Gold", "Calita", "Shiro" for the Spring indexes and "Burmosa" for the early Autumn indexes. Obviously this was an expression of leaf surface-area effect; similarly there was a clear fruit surface-area effect in "Black Gold". A significant interaction between cultivar and inoculation conditions was observed.

The rankings of the 22 cultivars at the two assessment times were compared on the basis of the number of spots per leaf and per cm^2 of leaf (Tab.3). The Spearman coefficients were respectively 0.44 and 0.42. Clearly the cultivar response to high dose infection in the Spring was different from the cumulative response following secondary Summer infections at natural dose levels. The cases of "Black Amber", "Simka" and "Verity" were the most evident. These results indicate that susceptibility assessment was influenced by the inoculation conditions.

"Tough testing" discriminates between high and low resistance, masking partial resistance, whereas "soft testing" also reveals intermediate levels of resistance

(Zadoks and Schein, 1979). The differentiated response of the plum varieties to the Spring infections can be compared to the result of a "tough testing", the wide range of responses to Summer infections to that of "soft testing". Consequently, the Spring ranking can be of greater interest to a breeder whereas the early Autumn one is more useful to the fruit-grower. The Spearman coefficients relative to the comparison of the rankings in the leaves and in the fruits were 0.45 and 0.48 respectively. Evidently leaf and fruit susceptibility were not correlated. The clearest examples being the cases of "Angeleno", "Frontier", "Oblinaja" and "Sorriso di Primavera".

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

Plant Pathogenic Bacteria Z. Klement (ed.)

IN VITRO SCREENING FOR BACTERIAL CANKER RESISTANCE IN CHERRY

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INTRODUCTION

One of the objectives of the U.K. cherry breeding programme is to develop new rootstocks and cultivars resistant to bacterial canker, caused by Pseudomonas syringae pv. morsprunorum. Formerly, material from the breeding programme was screened in the field (Garrett, 1979) but, to obviate the introduction of the disease on to the fruit breeders plots, it was necessary to devise an in vitro method for the early assessment of susceptibility. A procedure based upon the method of Klement et al (1984), for inoculation of apricot shoots, was adopted.

METHODS

Two-year-old dormant shoots of approx. 10 mm diameter are cut into c. 20-25 cm lengths and stood in pots of wet sand. They are inoculated, through a freshly made cut across the upper end of the shoot, with 30 µl of a suspension of the pathogen at a concentration of 10^7 cfu/ml. Once the inoculum has been adsorbed the cut end is sealed with 'parafilm' to prevent drying out. The pots are held at 15°C for 7 days with a 16 hr daylength. To simulate winter chilling the shoots are removed from the sand and placed in polythene bags in a refrigerator held at -5°C for 7 days. The shoots are then thawed out, the basal end is recut and they are replaced in the pots of wet sand and held at 15°C for a further 4 weeks. Necrosis is then recorded. The extent of necrosis varies from 2 mm on control shoots (due to tissue bruising by the secateurs) to 15-20 cm necrosis on a susceptible rootstock or cultivar. A minimum of 15 replicates is normally used.

The validity of the method was checked using a range of cultivars representing different levels of field resistance to bacterial canker. The best correspondence was obtained with cultivars of either high or low resistance. Test reproducibility was also best with these cultivars and less good with those of intermediate resistance.

RESULTS

New cherry rootstock and cultivar selections, together with standards of known susceptibility, were screened against races 1 and 2 of Pseudomonas syringae pv. morsprunorum in winter 1988 (Table 1).

Table 1. Canker length (cm) on dormant 2 year detached shoots of cherry inoculated with Pseudomonas syringae pv. morsprunorum (mean of 15 shoots)

	Race 1	Race 2
Rootstock		
F12/1	0.74	0.56
Colt	1.60	1.56
Charger	0.46	0.61
CR-708.1	0.19	0.21
-268.1	1.19	1.00
-266.2	1.67	1.94
-271.4	7.20	1.03
-596.6	4.38	4.09
Cultivars		
Napoleon	21.51	12.01
Merchant	12.19	7.26
Inga	11.94	2.34
C3-68	7.93	7.19
-39	12.45	13.31
-25	21.29	12.34
-11	17.97	12.81

Several F1/3 rootstock crosses (CR series) showed good resistance to both races of the pathogen; one is showing promise as sweet cherry rootstock and another as a rootstock for *Prunus padus* types. The 4 derivatives of the sweet cherry Inga (C3 series) were generally more susceptible to bacterial canker than Inga. In further tests of 12 more C3 derivatives in 1989 only one was significantly more resistant to infection than Inga.

CONCLUSIONS

This *in vitro* test provides a useful screen for the elimination of highly susceptible rootstocks and cultivars and for indicating those which are of high resistance. Material giving intermediate results and which has desirable growth characteristics needs further testing. The test has also been used successfully to compare the virulence of strains of *P. syringae* pv. *morsprunorum* on cherry. It has not proved as suitable for the testing of plum cultivars for which some modification in procedure is clearly required.

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

Plant Pathogenic Bacteria
Z. Klement (ed.)

SPECIES DIVERSITY AND PATHOLOGICAL SPECIALIZATION OF TOMATO PITH NECROSIS BACTERIA

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Pith necrosis of greenhouse grown tomato plants normally occurs in Portugal in association with other symptoms, like stem patches, leaf spots, fruit specks, vascular discoloration, and general chlorosis of the plants. In each greenhouse, one of these symptoms is usually present with a higher incidence, and some of the others score lower frequencies among affected plants. It has been repeatedly observed that in places where the soils have a high clay and silt content and neutral or slightly alkaline reaction, the dominant symptoms can be found in the internal tissues (pith necrosis, discoloration of the vascular bundles), while in soils with acid reaction and low clay content "external" symptoms are the most frequent.

The bacterial populations isolated from plants affected by this syndrome are not homogeneous. As Martins (1989) has demonstrated, there is a certain degree of uniformity of characteristics among the strains of bacteria isolated from each greenhouse, in spite of the fact that those strains represent species of bacteria often with low systematic affinity. The same analysis has shown that greenhouses can be ordered on the basis of the characteristics of their bacterial populations, and that this ordination parallels the range of the soil types. The present paper reports on a similar investigation of the relationships between the types of lesions, the composition of the bacterial populations present in them, and the characteristics of the soils where they occur.

MATERIAL AND METHODS

Diseased tomato plants (cv. Dombo, Dombito and Carmelo) were collected in several plastic covered greenhouses in Portugal (A to F; see Martins, 1989, for details of their location). Greenhouses are ordered from A to F according to the gradient of pH, humidity and clay and silt content. A to D had sandy soils with acid reaction. Soil in C was consolidated due to the precipitation of mineral salts from the nutrient solution. Soils in E and F had a high clay and silt content, were slightly alkaline and compacted. All but D were very poor in organic matter, overirrigated and overfertilized.

Bacteria were isolated from macerates of pieces of tissue detached from the margins of lesions using nutrient agar plus 5% sucrose. Microbiological tests were performed as described by Schaad (1980). Utilization of carbon compounds was tested in the mineral base medium C of Dye (1968).

RESULTS AND DISCUSSION

Bacterial strains were clustered in 15 groups after a principal components analysis of the data matrix of nearly two hundred strains and the 29 characteristics that were not common to more than 90% of them. The distribution of the bacterial groups by the types of lesions where they were present is summarized in Table 1. An association seems to exist between the fluorescent pseudomonads and the development of lesions in the more superficial tissues of the plant, while other bacterial groups were present in greater numbers in internal lesions.

The distribution of the strains by their place of isolation and lesion types (Table 2) shows a remarkable correlation between the external symptoms (where fluorescent pseudomonads are dominant) and soils with low clay content and low pH, while the remaining bacterial groups prefer the other extreme of the soil range. Fermentative bacteria (*Erwinia* spp.) were present in mixed populations isolated from greenhouses with an intermediate position in the range of the soil characteristics.

Reorganizing the data matrix by grouping the bacterial

TABLE 1

Distribution of isolated bacterial groups by the types of lesions

Bacterial groups(1)	Type of lesion(2) (no. isolated strains) External	Internal
Fluorescent pseudomonads:		
a (*Pseudomonas syringae*)	12	11
b (*P. syringae*)	27	1
c (*P. fluorescens*)	12	1
d (*P. putida*)	5	3
e (*P. viridiflava*)		8
f (*P. cichorii*)		5
Rhizobiaceae (?):		
g		8
h	5	1
i	3	31
j	2	6
k	1	3
l	1	5
m	7	9
Fermentative:		
n		6
o	5	21

(1) Characteristics of bacterial groups in Martins (1989).
(2) Leaf spots, fruit specks, chlorosis of the whole plant and stem patches pooled as "external"; pith necrosis and vessel discoloration pooled as "internal".

TABLE 2

Distribution of types of lesions and major bacterial groups by greenhouses

	Greenhouses (no. isolated strains) A	B	C	D	E	F
External symptoms:						
Fruit specks	5	3	5	3		
Stem patches	5					12
Leaf spots	6	18		8		
Chlorotic plants	15					
Internal symptoms:						
Necrotic vessels			25	39	12	
Pith necrosis		10			5	28
Bacteria:						
Fluorescent pseudomonads	31	16	5	27	6	
Rhizobiaceae (?)			21	10	11	40
Fermentative		15	4	13		

strains on the basis of the type of lesion from which they were isolated, a canonical variate analysis separates the groups of strains corresponding to "external" from those corresponding to "internal" symptoms (Fig. 1), with two exceptions.

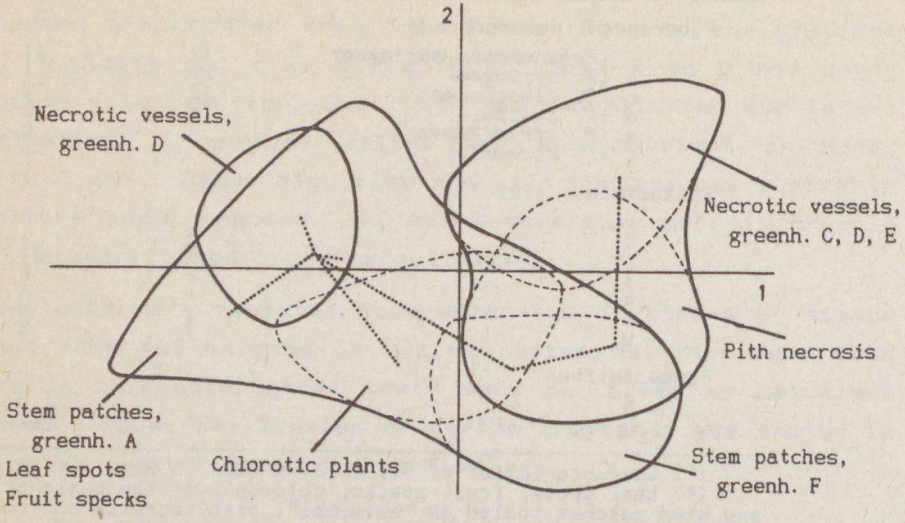

Fig. 1: Outlines of the projections of bacterial strains isolated from different types of lesions on the first two canonical variates. Dotted, minimum spanning tree of Mahalanobis generalized distances between group centroids.

One of the exceptions is a group of *Rhizobiaceae* from stem patches which is projected with the bacteria from pith necrosis, to which they are very similar in their microbiological characteristics. The other is a group of fluorescent pseudomonads isolated from necrotic vessels of a plant grown in greenhouse D, which is projected near the majority of the strains from "external" lesions. Greenhouse D was the one which showed the greatest heterogeneity in all aspects: there could be found "external" as well as "internal" symptoms, and the widest microbiological diversity; it was also the one with the highest organic matter content in the soil, and was irrigated by microjet while the others were drip-irrigated. The presence of fluorescent pseudomonads in necrotic vessels could well be the result from the contamination of pruning wounds by surface bacteria carried there by irrigation water.

It can be concluded from the information in Table 2 and

Fig. 1 that there is a general correlation between fluorescent pseudomonads, "external" lesions, and acid sandy soils (siderophores are certainly very useful when iron is easily washed from the soil), and, on the other hand, between the remaining isolates, "internal" lesions, and neutral or slightly alkaline heavy soils (where low oxygen tensions are common, and nitrate respiration may be advantageous to denitrifying *Rhyzobiaceae*). So, when exceptions occur, the factors determining the composition of the bacterial populations of the phytosphere of plants affected by this syndrome should be searched among the soil characteristics. The correlation found between the composition of epiphytic bacterial populations and soil type mirrors the one described by Sands & Rovira (1971) for bacterial populations from soil and rhizosphere. This fact may not be casual, and the soil properties, through their effects on the physiology of the tomato plant, do have an influence on the space where epiphytic bacteria live, and on the substances available for their nutrition.

High nitrogen levels produce fast and soft vegetative growth, but they can also provoke the relative unavailability of some oligoelements, that can be expected in neutral soils as well as in acid soils after liming, and can be aggravated by low levels of organic matter. Oligoelement deficiencies can be responsible for chlorosis, loss of meristematic activity and of cell wall resistance. In crops affected by the pith necrosis syndrome, apparently healthy plants often show a white, dry and laddered pith, from which all attempts to isolate bacteria have failed.

When fruits are growing, they drain elaborate substances and minerals from the surrounding tissues. Low temperatures at night, which induce high rates of translocation in tomato plants (Went, 1957), can intensify this process, and constitute a great stress on plants otherwise weakened by nutritional imbalances.

These environmental conditions and physiological processes may act as predisposing factors for the invasion of the plants by the "low-grade pathogens" associated with this disease.

Their consideration may contribute to a better understanding of the observations made in Portugal and reported by Scarlett *& al.* (1978), that pith necrosis is favoured by low night temperatures, is most frequent with high nitrogen levels in the soil, starts near the point of attachment of the first truss when its fruits are growing, and is accompanied by chlorosis of the upper leaves (and often of the whole plant).

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

Plant Pathogenic Bacteria Z. Klement (ed.)

STUDIES ON SURVIVAL & LOCALIZATION OF PSEUDOMONAS SOLANACEARUM IN CLAYS EXTRACTED FROM VERTISOLS

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ABSTRACT

A mucoid and rough strains of P. solanacearum survived in vertisol-extracted montmorillonite maintained at pF 2 (-0.1 bar) whereas, at pF 4 (-10 bar) a rapid decline was observed. In situ examinations in SEM and TEM showed bacteria embedded in the deformable 3D-network of montmorillonite. Observations suggest that size reduction of this network when water potential increases, could explain the decline observed. Moreover, embedding properties of montmorillonite are suspected to be involved in suppressiveness of this vertisol.

Although it is known since earlier studies that various types of soils can be infested by P. solanacearum, rather few attempts to characterize relationships between this species and common soil constituents, were made. Among them, particular attention was generally paid to clay minerals (Stotzky, 1980). In Guadeloupe, the suppressiveness of vertisols to P. solanacearum was mainly attributed to physical properties of montmorillonite, their constitutive clay (Béreau & Messiaen, 1975; Rat, 1978). A previous study demonstrated the incidence of pure clays on P. solanacearum when water potentials vary (Schmit & Robert, 1984). The present paper investigates survival and localization of P. solanacearum in clays extracted from suppressive vertisols.

MATERIAL AND METHODS

Clay minerals. Crude clay (< 2 µm) was extracted by sedimentation (Stockes'law), from a Guadeloupe vertisol (Grande-Terre, St. François) having a very high clay content (> 90% of dry weight). A purified clay was also obtained by deferrification and hydrogen peroxide treatment from the

horizon B of the same soil. Suspensions of these clays were autoclaved (115 ^{o}C, 30 mn, 2 times) in diluted suspensions and aseptically concentrated with mild centrifugation (2000 g, 15 mn).

Bacterial strains. Two strains of Pseudomonas solanacearumm 1000, mucoid, virulent and 2000 rough avirulent, were prepared as described earlier (Schmit & Robert, 1984) and finally adjusted to 5.10^{9} bacteria ml^{-1} in sterile distilled water by spectrophotometry.

Preparation of bacteria-clay samples for survival studies and electron microscopy. Suspensions of each strain were mixed with 2g of clay extracted from vertisol and 2g of purified clay to obtain pastes of fluid consistence with a ratio of 10^{11} bacteria.g^{-1} (dried clay) for SEM and $1.5.10^{10}$ bacteria.g^{-1} for TEM and survival studies. These bacteria-clay samples were placed to different water potentials pF 2(-0.1 bar), 4(-10 bar) and 4.4(-25 bar). Control samples with bacteria alone, without any clay, were treated identically as pastes of bacteria.

Low water potential value was accurately maintained in the pneumatic device described by (Tessier & Berrier, 1979). Higher values were obtained with Richards' apparatus, (Richards, 1947). Bacterial populations were periodically evaluated on subsamples dispersed, diluted and plated on TZC Kelman's medium. Water content was determined by wet and dry weighing, at the same time.

Localization of bacteria in clays by electron microscopy. Pieces of bacteria-clay pastes were processed after 12 days for scanning (SEM) or transmission (TEM) electron microscopy.

For SEM a small piece of bacteria-clay paste was immediately frozen in fluorocarbon cooled with liquid nitrogen, and put, under vacuum, into the "Cryoscan" chamber. A fresh undisturbed surface obtained by breaking the top of the sample was gold-metallized and observed.

Processing samples for TEM required some modifications of double fixation procedures. In smectite clays, water has a great incidence on cohesion; any important variations of water activity will result in destruction of sample initial organization. So, before final processing, the relative concentrations of each solution used were experimentally determined with test-pieces of bacteria-clay pastes. We considered that concentration of processing fluids was adequate when no changes in dimensions and shape of test pieces occurred after overnight immersion.

Processing of bacteria mixed with clays extracted from vertisol, horizon A, was as follows:

Fixation of small pieces 1.5 hours with 4 per cent glutaraldehyde in a 25 mM phosphate buffer. Washings in buffer 2 hours. Postfixation with 1 per cent osmium tetraoxide in same buffer. Washings in buffer 40 mn. Dehydration in graded buffered ethanol (1°, 2.5^{o}, 5°, 10°, 20°, 30°, 35°, 42.5^{o}, 50°, 60°, 70°, 82.5^{o}, 96°, 100° four times) with progressive decrease to zero of buffer concentration. Increasing amounts of propylene oxide were added step by step from ethanol 70° to ethanol 100° and followed by four baths of pure propylene oxide. Each dehydration step lasted 20 minutes. Infiltration was performed by spurr resin (Spurr, 1969). Pieces were left in pure resin for 2 days (1 bath/day) before inclusion and curing 15 h at 70 ^{o}C.

Sections obtained with a diamond knife were contrasted on grids with uranyl acetate (1 per cent in 50° ethanol, 7 mn) and lead citrate (0.4 per cent in decarbonated water, 13 mn).

RESULTS

Evolution of bacterial population in clays submitted to water potentials (Figure 1). At constant low water potential, numbers of bacteria of both mucoid (1000) and rough (2000) strains is maintained close to the initial level. Bacterial counts (unreported data) performed on similar samples 26 days after applying pF 2.2 gave results (within 1 log from initial counts). At the opposite, higher water potentials (pH 4 & 4.4) cause rapid decline of both strains.

Comparison between survival curves obtained with bacteria-clay samples and bacteria without clay treated identically (control samples), indicated that mere dessication of bacterial cells could not entirely account for mortality observed at high water potential. No significant difference between clays extracted from vertisols.

Localization of bacteria in clay matrix. (Figure 2 & 3). A 3D-network of tactoids, typical of the structural organization of these clays is visible in both SEM and TEM. At 10 w water potential (Figs 2a, 2d, 3a to 3d) bacteria are visible in the pores of this network which are larger in size than bacterial cells.

When water potential is raised to 4, pore sizes decrease (Figs 2b & c) to a level that exceeds cell mean dimansions. Bacteria are no longer observed.

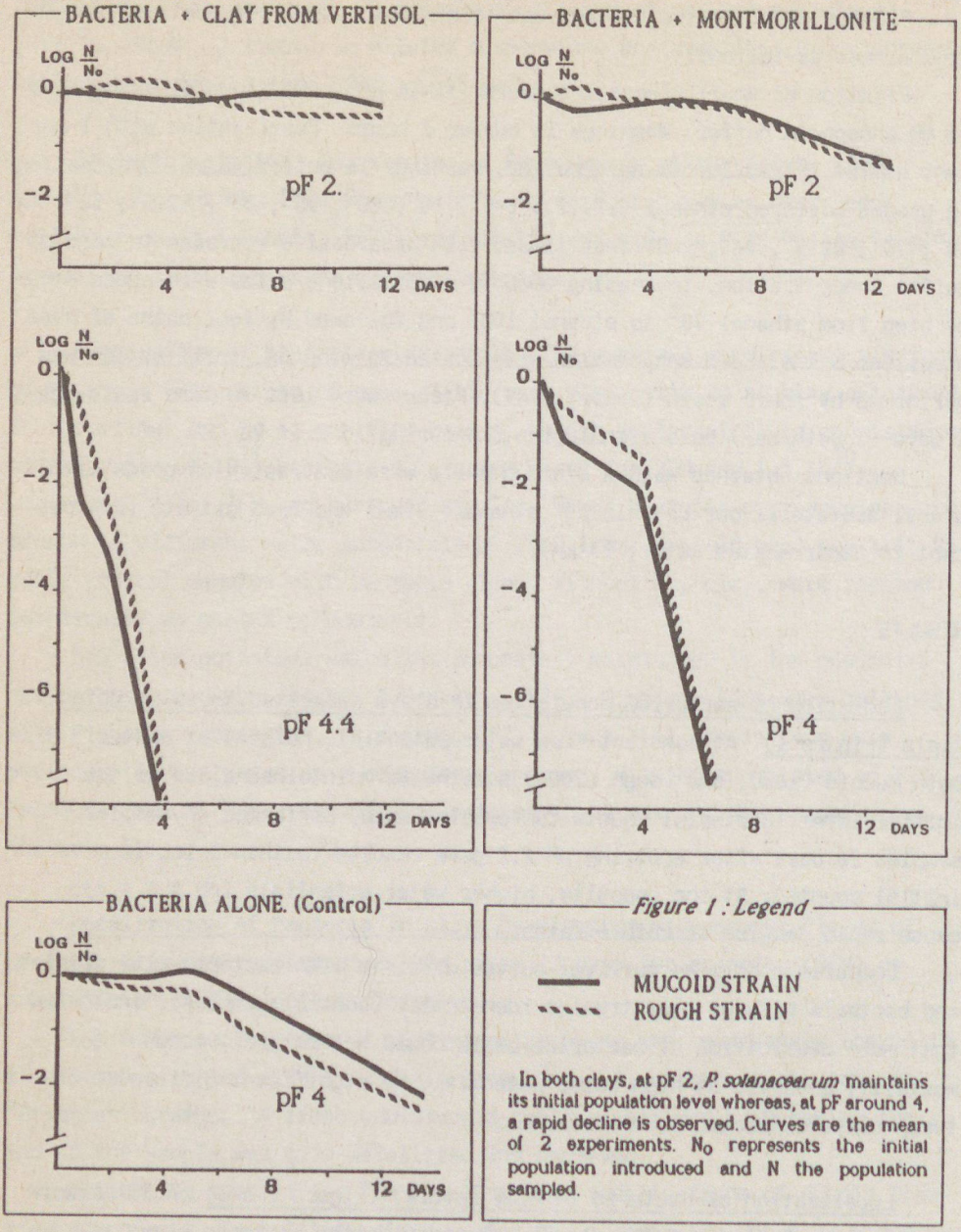
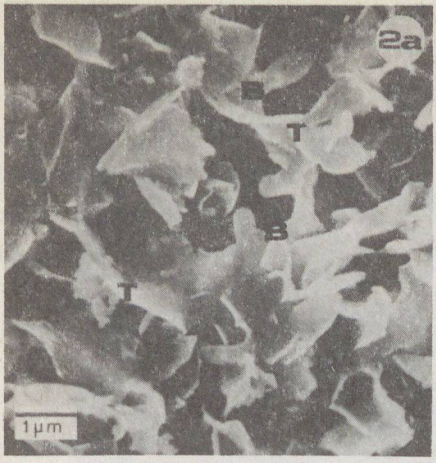
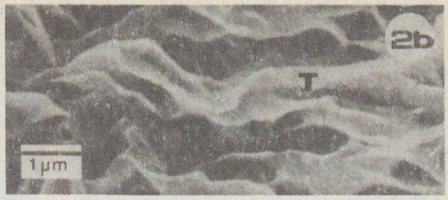


Figure 1 Survival of P. solanacearum in montmorillonite and in clays extracted from a vertisol (Guadeloupe), at 2 water potentials







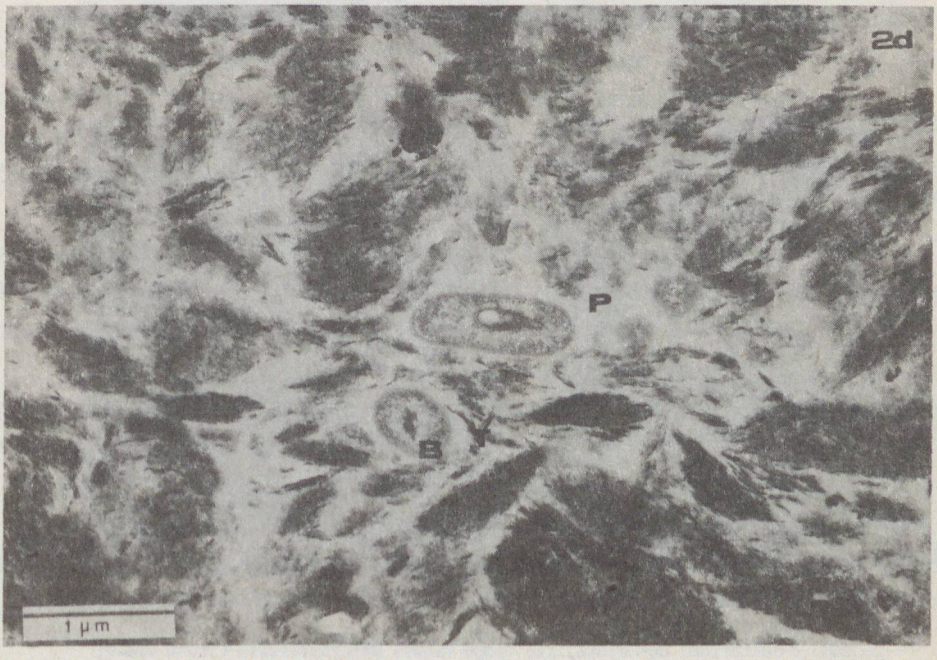


Figure 2 Localization in SEM & TEM of P. solanacearum in purified or soil-extracted montmorillonite

Legend: B=bacterium; P=pore; T=tactoid

Fig. 2a SEM micrograph of bacteria (1000) in porosity (P) of a purified montmorillonite maintained at pF 2(-0.1 bar). Bacterial cells (B) are partly covered by tactoids (T)

Fig. 2b & c Same clay at pF 3(-1 bar) and pF4(-10 bar), respectively. As water potential increases, pore size decreases, 3D-network flatten and tactoids come in contact. Micrograph sizes are proportional to apparent volume of the sample at -0.1, -1, -10 bar

Fig. 2d P. solanacearum in montmorillonite extracted from Guadeloupe vertisol, pF 2(-0.1 bar). Tactoids sectioned under different orientations delimit pores where bacteria are localized. Ultrastructure of cell appears well-preserved

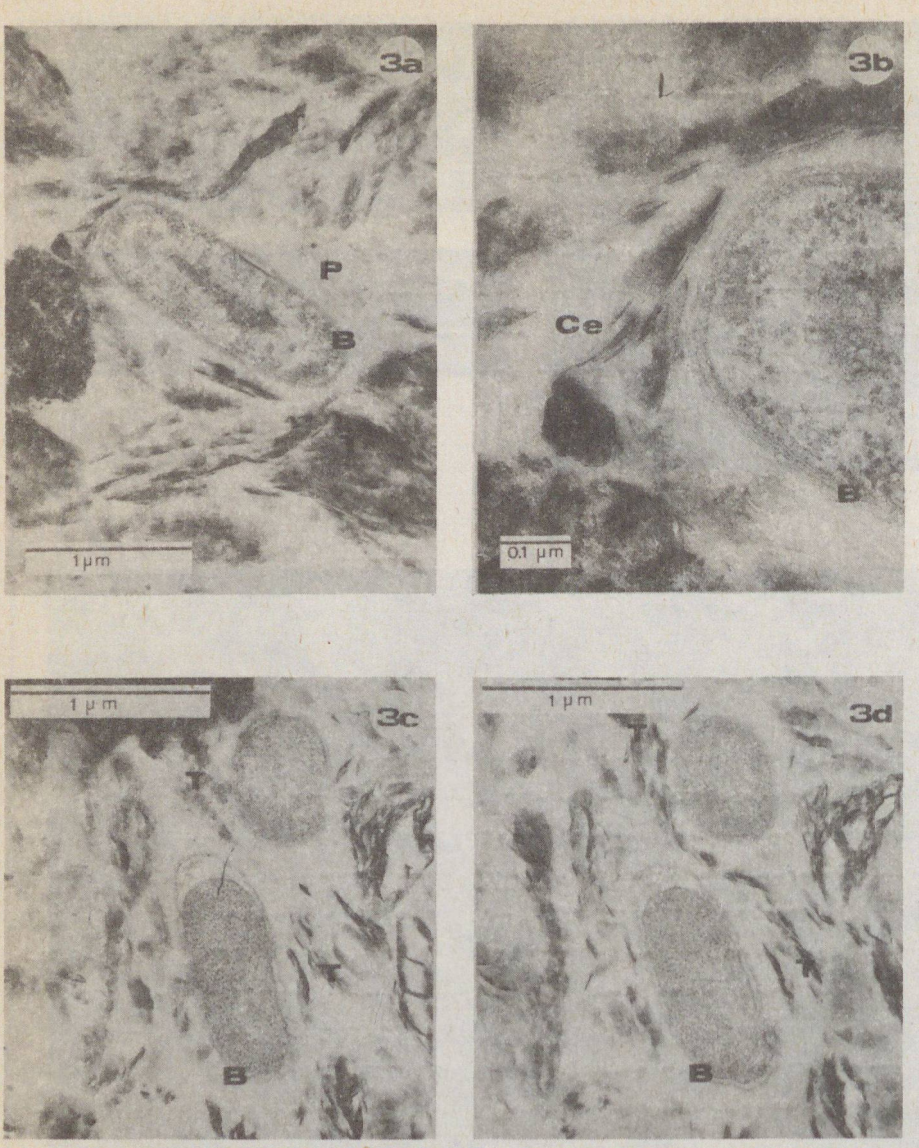


Figure 3 Aspects of bacteria embedded in tactoids of soil-extracted montmorillonite in transmission electron microscopy.
Legend: B=bacterium; Ce=cristallite (edge view); Cf=cristallite (front view); P=pore; T=tactoid

Fig. 3a P. solanacearum (1000) in montmorillonite extracted from vertisol, pF 2(-0.1 bar). Several tactoids wrap and bend around a bacterial cell

Fig. 3b Magnification of 3a. Cristallites of montmorillonite (Ce, Cf) are in close contact with outer membrane

Fig. 3c & d P. solanacearum (1000) in montmorillonite extracted from Guadeloupe vertisol, pF 2(-0.1 bar). Same area seen on 2 near sections. Note orientation and extension in space of tactoids around bacteria (3D-network). These views (see also 3a & b) strongly suggest that movements in clay matrix are impaired as well as availability for infection

Ultrathin sections of bacteria-clay samples examined in TEM (Figs 2d, 3a to 3d) show that tactoids composed of individual cristallites of montmorillonite are deformable and able to wrap around bacterial cells. On sections, tactoids delimit open spaces corresponding to pores already observed in SEM. Close contacts between bacterial cells and cristallites (Figure 3b) suggest an association of bacteria with particles and a subsequent immobilization. Morphological details of bacteria look well preserved.

DISCUSSION

The outstanding feature of methods used is the preservation of initial structure of the delicate samples composed of biological and mineral elements. In the case of SEM 'cryoscan' device allows observations of hydrated specimen. In TEM, processing of sample without macroscopical disturbance, is assumed to preserve the organization SEM and TEM results are complementary. Structural aspects of clays observed here are in agreement with those described by Tessier (1984) for pure minerals. However, if method is adequate for rather hydrated specimens (low pF range), it was unsuccessful for water potential around 4 (unreported assays).

Microscopic studies illustrate how water potential affects the organization of montmorillonite when water contained in porosity is removed. These observations support the hypothesis of a mechanical effect caused by decrease of pore sizes under a lethal limit for cells.

This effect of water potential and clay is not specific and has already been observed with other minerals of different composition and structure like kaolinite (Schmit & Robert, 1984).

Other non spore-forming, Gram-negative bacteria, phytopathogenic or not, may be affected in the same way. In this respect, results contribute to ecology of bacterial populations in soils with high clay content, at the aggregate level.

Obviously, the mechanical properties of tactoid 3D-network reported above cannot account entirely for suppressiveness of Guadeloupe vertisols as other clays are known to give similar results in same conditions.

Apart from mechanical lethal effect observed at high water potential, SEM and mostly TEM, show clearly the embedding abilities of montmorillonite matrix, effective even at low range pF. Such properties probably immobilize bacteria, limit theri passive movements and reduce subsequently their availability for root infection.

Adhesion process described since early studies (Marshall, 1968) should be now investigated in situ in bacteria-clay-root system. Cytochemical methods applied successfully to root-soil interface (Guckert & al., 1975) and those used here could be adapted to further investigations relative to infection by P. solanacearum, or other soil-borne bacteria, in more complex environment like rhizosphere and rhizoplan.

ACKNOWLEDGEMENT

Technical assistance of M. T. Rousseau is gratefully acknowledged.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

SCREENING CRITERIA FOR BACTERIAL LEAF STREAK IN BREAD WHEAT, DURUM WHEAT AND TRITICALE

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ABSTRACT

Forty genotypes of small grains were spray-inoculated with Xanthomonas campestris pv. undulosa at the end of the tillering stage. At the flowering stage, disease severity was assessed on the flag leaf (F) and the leaf below (F-1), using a 0-6 scale. F leaves consistently scored lower than F-1 leaves. The logarithm of colony forming units per 10 g grain and 1000-grain weight did not differentiate resistant from susceptible genotypes.

INTRODUCTION

Xanthomonas campestris pv. undulosa (Smith, Jones and Reddy) Dye 1978, X. c. pv. undulosa, is the causal agent of bacterial leaf streak (BLS) on bread wheat (BW) (Triticum aestivum), durum wheat (DW) (Triticum turgidum var. durum) and triticale (T) (X triticosecale Wittmack). The disease is known as "black chaff" when found on the glumes. Reports of measurable damages due to BLS have been made during this decade, especially in developing countries (Duveiller 1989). A major problem in identifying resistant genotypes is that the disease does not always result in an epidemic, even in countries where BLS is a potential threat. Consequently, selecting for resistance to X. c. pv. undulosa under natural conditions may not be very reliable, and comparison of screening results from different locations becomes doubtful. Screening criteria was analyzed under high artificial inoculum pressure in the highlands of Central Mexico where high rainfall during and immediately after heading favor infection and symptom expression of X. c. pv. undulosa.

MATERIALS AND METHODS

Forty genotypes were kindly supplied by Drs. S. Rajaram (BW), P. Brajcich (DW) and G. Varughese (T):

Bread wheats: 1) Pavon 76, 2) Anahuac 75, 3) Thornbird"S", 4) Alondra, 5) Shanghai 18 7B-0Y, 6) 793-3402, 7) Chuan Mai #18, 8) Shanghai 17 40B-0Y, 9) Wuhan 3 44B-0Y, 10) CMH 82-493 CMH 82-493-3Y-3B-1Y-1B 2Y.1B.0Y, 11) Genaro, 12) Seri 82, 13) Wuhan 1 43B-0Y;

Durum wheats: 1) Ch67/Cando ICD78-0059-5AP-1AP-2AP-0AP, 2) Tito, 3) Altar 84, 4) Eids"S" CD10535-0-1M-1Y-2Y-0M, 5) A63040/Sty//Lds/3/Win/4/Erp/Ruso CD35072-C-5Y-1M-0Y, 6) Fa/Cando ICD78-0001-3AP-1AP-0SH, 7) Fillo"S" CD59105-C-5M-28Y-6M-2Y-0M, 8) Carc"S"/Auk"S" CD56981-4Y-1M-1Y-0M, 9) Carc "S"/Auk"S" CD56981-4Y-3M-3Y-0M, 10) Chen"S"/Altar 84 CD57005-6Y-1M-6Y-0B, 11) Stn"S"//Hui"S"/Somo"S" CD66589-C-1M-3Y-6M-0Y, 12) Sula"S"//Wls/Dws/5023 CD66590-B-1B-1Y-14M-1Y-0M, 13) Altar 84/Aos"S", 14) Chen"S"/Altar 84 CD57005-6Y-1M-8M-0Y, 15) Fillo"S" CD59105-C-5M-13Y-5M-1Y-0M, 16) Fillo"S" CD59105-C-5M-13Y-10M-2Y-0M, 17) Fillo"S" CD59105-C-5M-28Y-3M-2Y-0M, 18) Yavaros;

Triticales: 1) Venus, 2) Siskiyou, 3) Buf 1 CT4161-0M-0Y-0M-1Y-3Y-0Y, 4) Beagle"S"/Adx, 5) Octo NV//Drira/Kgr/3/Mus"S"/Lynx"S", 6) Mus"S"*2/Zebra 79, 7) an unknown line from Brazil, 8) Ate"S"/Df"S"/Df"S", 9) Stier 15-1.

In BW and T, X. c. pv. undulosa strain CB4 (from triticale, Obregon, 1987) was used at about 10^9 colony forming units per milliliter (CFU/ml); in DW, the inoculum was a mixture of strains CB120, CB122, CB131, CB133 and CB134 from durum wheat (Obregon, 1988), at about 10^{10} CFU/ml. Genotypes were planted in three areas, each one corresponding to one crop, in two replicates, at Toluca, Mexico (2649 meters above-sea-level), during the 1988 rainy season (May to October). Individual plots were 2.0 x 0.5 m. Plants were spray-inoculated at stage 6 (Feekes' scale). At the end of flowering (stage 10.5.3) for each replicate, 25 flag (F) and flag minus one leaves (F-1) were sampled at random in durum and 30 of each leaf in the other two crops. Each leaf was scored using a severity scale based on the percentage of leaf area damage observed: 0 = no symptoms; 1 = <10%; 2 = 10-25%; 3 = 26-50%; 4 = 51-75%; 5 = 76-99%; 6 = 100%

Class distribution of F and F-1 leaves was considered.

Confidence intervals (5%) were calculated for average scores of F. At harvest, 1000-grain weight was measured. Three 10 g grain samples per line were washed during 30 min. in sterile saline and assayed (3 rep./subsample)

by dilution plating onto WBC agar (Duveiller 1989). The logarithm (log) of the average CFU/10 g seed was calculated.

RESULTS

F leaves exhibited consistently lower levels of disease than F-1 leaves and a vertical disease gradient was established in the canopy (Table 1).

Table 1. Average percentages of X. c. pv. undulosa damage to flag (F) and flag-minus-one (F-1) leaves scored on a 0-6 severity scale for 13 bread wheat, 18 durum wheat and 9 triticale genotypes subjected to heavy artificial inoculation.

		Disease severity score						
		0	1	2	3	4	5	6
Bread wheat	F	72	11	8	7	2	0	0
	F-1	47	23	9	11	8	1	0
Durum wheat	F	13	25	16	28	14	3	0
	F-1	0	4	12	30	31	21	2
Triticale	F	53	22	15	10	3	0	0
	F-1	17	17	15	34	15	2	0

Confidence intervals allowed differentiation between genotypes (Table 2-4) Thornbird"S" (BW3) (Table 2), Venus (T1) and Siskiyou (T2) (Table 4), considered resistant after previous field trials, were classified as more resistant than susceptible material in their respective crop groups; e. g. Alondra (BW4) (Table 2) and Stier 15-1 (T9) (Table 4). Differences between lines were not so marked in DW (Table 3), due to a more intense disease expression or lack of resistance. In some cases (BW6, T4) (Tables 2,4), F leaf score was low but F-1 leaf score was high. This observation may indicate that resistance to BLS is determined not only by the average disease all over the plant, but by the disease severity principally at the level of flag leaf, or that escape is occurring on some genotypes as a function of the distance between F and F-1 leaves. Values for 1000-grain weight did not permit differentiation between resistant and susceptible lines. All BW, DW, and T lines were found to have seed-borne bacteria, even when disease scores were very low. No varietal differences in the log of

CFU/10 g were observed.

Table 2. Average disease score on a 0-6 severity scale for the flag leaf (F) and the leaf below (F-1) of 13 bread wheat genotypes under heavy artificial X. c. pv. undulosa pressure.

Genotype[1]	Average score F	Confidence intervals F (5%)	Average score F-1	Thousand-grain weight	log CFU[2]/10 g seed
5	0.00	0.00	0.13	45.2	4.2
3	0.02	0.03	0.63	41.0	7.2
8	0.05	0.11	0.60	42.3	6.8
6	0.17	0.15	1.58	30.6	7.1
9	0.17	0.13	0.40	35.1	4.7
12	0.30	0.17	0.63	31.0	6.5
1	0.30	0.23	1.30	33.7	7.6
13	0.40	0.22	0.78	37.9	5.1
11	0.48	0.27	0.62	32.9	6.7
7	0.67	0.26	1.88	36.5	6.4
2	0.75	0.27	1.45	35.8	7.1
4	1.18	0.32	2.40	36.3	7.3
10	2.35	0.24	2.22	29.8	7.5

1 identified by their list number in Material and Methods

2 colony forming units

DISCUSSION

Artificial, high X. c. pv. undulosa inoculum pressure caused some degree of infection in all varieties tested, suggesting that resistance to BLS is only partial. In the crops, a vertical disease gradient was induced by spray-inoculation at an earlier stage of crop development; thus disease scores in upper leaves allowed a statistical separation of genotypes that was consistent with our previous knowledge of their resistance to X. c. pv. undulosa. Spray of concentrated inoculum at the end of tillering and disease scoring at flowering may be recommendable. Breeders need a reliable and rapid index. Results suggest that once inoculum pressure is established at the end of tillering, BLS assessment may follow the prin-

ciples used for other foliar diseases of wheat (Saari and Prescott 1975). To avoid confusion with melanism induced by abiotic factors (Duveiller 1989), symptom assessment should be limited to the leaves. Thousand-grain weight is reduced by X. c. pv. undulosa (Duveiller 1989) but would seem an unreliable resistance indicator, since this yield component also depends on several other factors of greater significance than BLS.

Table 3. Average disease score on a 0-6 severity scale for the flag leaf (F) and the leaf below (F-1) of 18 durum wheat genotypes under heavy artificial X. c. pv. undulosa pressure.

Genotype[1]	Average score F	Confidence intervals F (5%)	Average score F-1	Thousand-grain weight	log CFU[2]/10 g seed
11	0.78	0.94	3.16	22.9	7.7
6	1.16	0.89	3.72	24.9	7.9
15	1.58	1.14	3.66	28.4	7.8
18	1.70	0.99	3.02	27.3	8.1
16	1.82	1.15	3.46	29.8	7.7
4	1.84	1.22	2.80	17.1	7.8
10	1.90	1.23	3.54	19.4	7.5
14	2.10	1.22	4.04	26.0	7.8
3	2.22	1.12	3.64	22.0	7.8
17	2.26	1.14	3.52	27.2	7.9
13	2.34	1.12	3.90	25.3	7.7
12	2.54	0.89	3.58	22.4	8.0
5	2.56	1.28	4.00	27.7	7.5
9	2.58	1.07	3.04	25.1	7.8
7	2.62	1.07	3.42	26.6	8.4
2	2.76	1.23	4.06	24.8	8.2
8	2.88	1.00	3.82	20.1	7.8
1	3.26	1.25	3.80	18.9	7.9

1 identified by their list number in Material and Methods

2 colony forming units

Table 4. Average disease score on a 0-6 severity scale for the flag leaf (F) and the leaf below (F-1) of 9 triticale genotypes under heavy artificial X. c. pv. undulosa pressure.

Genotype[1]	Average score F	Confidence intervals F (5%)	Average score F-1	Thousand-grain weight	log CFU[2]/10 g seed
2	0.35	0.14	1.47	43.0	8.3
4	0.45	0.18	3.10	33.9	8.4
1	0.55	0.20	1.42	33.7	8.8
3	0.72	0.26	2.47	43.4	8.5
7	0.90	0.27	2.23	32.9	8.6
8	1.10	0.34	1.70	45.1	8.4
9	1.12	0.28	2.53	27.1	8.8
6	1.28	0.30	2.20	41.9	8.2
5	1.50	0.40	2.67	39.9	7.6

1 identified by their list number in Material and Methods

2 colony forming units

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ACKNOWLEDGMENTS

The author is indebted to Ing. C. Martinez C. for her technical work. This study is funded by the Belgian Administration for Development Cooperation (BADC).

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

Plant Pathogenic Bacteria Z. Klement (ed.)

POTATO SELECTION FOR RESISTANCE TO BACTERIOSES

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In the Ukraine potato growing is seriously hindered by bacterioses: ring rot, black leg and wet rot. They are especially dangerous in the period of potato storage, losses reaching 30 per cent. The main cause of recognized seed potato varieties in great demand being rejected is that they are bacterial disease agents.

A system of bacteriosis control measures has been developed but it is not highly efficient in the case of susceptible varieties. Developing resistant potato varieties is one of the radical methods of control of these pathogenes.

Since 1981 purposeful selection of potatoes for resistance to bacterioses has been carried out at the Ukrainian Research Institute of Potato Growing jointly with the Zhitomir Agricultural Institute.

In selecting potato for resistance to ring rot we used the field technique consisting in inoculation of the top part of a bulb with bacterial suspension of the Corynebacterium sepedonicus causal agent in 10^9 concentration with the help of a modified syringe needle. Infected bulbs (15 to 30 of each hybrid) were placed into polyethylene sacks and kept under conditions of 20-22 ^{o}C with relative humidity of 95 per cent. The disease development was estimated by stems after anthesis and by bulbs a month after harvesting.

Hybrid resistance was determined by percentage of bulbs with ring rot signs in a total amount analyzed. The relatively ring rot resistant variety Gatchinsky and the susceptible one Nezabudka were used as model varieties.

As a result of estimating 450 various potato hybrids obtained by 65 crossing combinations a number of relatively ring rot resistant genotypes have been selected. The highest efficiency of resistant genotypes was observed in case of the Adretta variety being used as a maternal one in the

crossing combinations Adr. x Zarevo, Adr. x Galina, Adr. x Certa, Adr. x Monica.

In each of them 2 or 3 relatively ring rot resistant hybrids were obtained. In the combination Adr. x 337-4c/71 the new potato variety of Svitanok Kievsky(SK) has been bred and recognized. It is relatively ring rot resistant and also possesses a number of other agronomic characters. This variety accounted for five combinations that are of practical interest for selection by its resistance to the pathogene.

The potato hybrids from each population (SK x Hitte, SK x Genetic, 77.583/13 x Hydra) with bulb affection not exceeding 10 per cent were selected. The 81.669/7 (SK x Omega) hybrid relatively resistant not only to ring rot but also to potato - root eelworm, possessing other agronomic characters was submitted to the State Commission of Variety Testing.

In selecting potato for wet rot resistance the laboratory method of infecting the whole bulb was used. Bacterial suspension (0.2 ml) consisting of two virulent stocks Erwinia carotovora var. atroseptica, Erwinia carotovora var. carotovora in the concentration 10^6 was injected into each bulb in the navel area to the depth of 15 mm. Injected bulbs were placed into polyethylene sacks with wet filter paper inside (for creating humidity nearing 100 per cent), and then were kept in the incubation chamber under 22-25 ^{o}C. Five days later they were evaluated by ninepoint scale where 9 is the minimum bulb infection and 1 is maximum. The relatively bacterial rot resistant Sapphire variety and the susceptible Nezabudka variety were used as models.

The detailed study of 4557 potato control samples has discovered no immune forms resistant to wet rot. The degree of their bulb affection varied greatly ranging from average resistance (12.3 per cent) to high susceptibility (100 per cent). This is due to potato rot resistance being a complex character and determined by numerous characteristics. As a rule, they are polygenically inherited. The largest amount of resistant hybrids was obtained when relatively resistant parental forms were used in hybridization. Moreover, resistance of hybrid progeny to wet rot increases if both parental pairs are resistant to this disease. Thus crossing two relatively wet rot resistant varieties Adretta x Sapphire affection of which did not exceed 7 resulted in 35 per cent of hybrids being relatively resistant to this pathogen.

In crossing relatively resistant Adretta variety and susceptible Perlyna variety only 11 per cent of resistant genotypes were obtained from

the hybrid progeny. And finally crossing two non-resistant varieties Nemeshayevsky Bely x 40-4c/72 did not result in hybrids with bulbs relatively resistant to bacterial rot.

So hybrids obtained from both resistant parents were characterized by positive transgression which was proved by larger amounts of resistant hybrdis. And vice versa crossing susceptible parents in most cases resulted in negative transgression.

Populations from crossing SK x Hydra, Adr. x Galina, Vysheborsky x Zarevo, Sapphire x Amaril is of practical importance in selecting potatoes for wet rot resistance. The above-mentioned populations gave 3 to 8 relatively wet rot resistant genotypes.

Among genotypes obtained on the basis of interspecific hybridization potato hybrid 120/36 pedigreed by three wild species Solanum leptostigma, S. demissum, S. andigenum was selected. Moreover, the sources of wet rot resistant bulbs obtained on the basis of cell technology were also selected.

For this purpose we used regenerated plants which were isolated from protoplasts of somatic cells and pollinia. The study of 71 potato samples obtained on the basis of cell manipulations gives evidence that wet rot affection of genotypes of regenerated plants is significantly less than that of original forms from which they were isolated.

This regularity is especially clearly seen in case of the Zarevo variety. With bulb maceration of 5 or 6 it was possible to obtain 64.5 per cent of regenerated plants with wet rot affection of 6 or 7. In case of Gatchinsky variety only 17.4 per cent of samples with resistance higher than in the parental forms was selected. This peculiarity must be taken into account in cell selection for resistance to pathogens of various origin.

The experiments made it possible to select two regenerated potato samples such as 83.102p and 83.119n obtained on the basis of cell technologies characterized not only by wet rot resistance but also by a complex of other agronomic characters.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

PERPETUATION OF XANTHOMONAS CAMPESTRIS PV. ORYZAE (ISHIYAMA) DYE IN THE RHIZOSPHERE OF SUCCEEDING CROPS

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INTRODUCTION

Bacterial leaf blight (BLB) of rice caused by Xanthomonas campestris pv. oryzae is the most serious disease of this crop in South-East Asia including India. In India, where two or more crops of rice are grown in a year, the bacterium survives on the crop itself. But in areas like North India where only one crop is grown in a year the bacterium must have some mode of perpetuation for the recurrence of the disease. There are reports of transmission of the bacterium through seed (Srivastava and Rao, 1964; Reddy, 1983) and its survival on the wild rice (Kulkarni and Thombre, 1968; Devadath et al., 1974), but no information is available on its survival in the rhizosphere of succeeding crops. The present investigation was undertaken to see the survival of the bacterium in the rhizosphere of succeeding crops under North Indian conditions.

MATERIAL AND METHODS

The investigations were carried out for two successive years i.e. 1986-87 and 1987-88 at the experimental area of Punjab Agricultural University, Ludhiaan. Rice crops artificially inoculated with X. campestris pv. oryzae were harvested at the end of October, 1986 and 1987 and wheat (Triticum aestivum), Brassica campestris, Brassica campestris var. toria, potato (Solanum tuberosum), Egyptian clover (Trifolium alexandrium), tomato (Lycopersicon esculentum) and winter maize (Zea mays) were shown in these fields in both years. The soil samples from the rhizosphere of the mentioned crops were collected at monthly intervals starting from November.

The field was irrigated 2-3 days before the collection of samples. Five plants of each crop were carefully removed with the help of a khurpi, along with the soil adhering to it. The plant tops were removed, the balls of earth were shaken vigorously to remove the soil from the roots. The soil still adhering to the roots was removed mechanically with a sterile scalpel. The roots and rootlets left in the soil samples were hand picked and the soil sample was mixed thoroughly. In 1987-88, the soil samples were also collected from the rhizosphere of the mentioned crops grown in the farmer's fields. These crops, sown in the fields from where rice crop severely infected with BLB was harvested, were selected. The remaining procedure was the same.

Isolation from soil

The isolation of X. campestris pv. oryzae was made using a dilution plate technique (Tuite, 1969). One gram of soil sample was mixed in nine ml of sterile distilled water in a test tube and ten-fold serial dilutions up to 10^{-6} were prepared. A new medium (potato 250 g, peptone 2 g, yeast extract 2 g, sucrose 15 g, potassium dihydrogen phosphate 0.15 g, disodium hydrogen phosphate 0.5 g, calcium nitrate 0.5 g, potassium chloride 0.05 g, ferrous sulphate 0.05 g and agar 15 g) was devised for the isolation of the bacterium. To ward off the fungal contamination, Aureofungin (100 mg commercial/l) was added to the medium before pouring it into petri plates. Twenty ml of the medium was poured in each petri plate as a bottom layer. One ml of 10^{-6} sample dilution mixed with 5 ml of medium was poured as a top layer. After solidification, the plates were incubated at $28\pm1^{o}C$ and 5 days after incubation, the colonies of X. campestris pv. oryzae were counted. The identification of X. campestris pv. oryzae was based on colony morphology, aesculin hydrolysis, gelatine liquefaction, protein digestion, urease production, acid production and pathogenicity. The pathogenicity was proved on TN-1 plants by leaf clipping method.

RESULTS

The plate count of X. campestris pv. oryzae population revealed that newly devised medium proved very effective for its isolation from soil. The population of X. campestris pv. oryzae recovered from soil samples on this medium was significantly more in comparison to Wakimoto's PSPA, YDC and NA media.

Table 1. Survival of Xanthomonas campestris pv. oryzae in the rhizosphere of different crops during 1986-87

Crop	pH of soil	No. of colonies per plate at 10^{-6} dilution*								
		Nov.	Dec.	Jan.	Feb.	March	April	May	June	July
Wheat	5.7	110	112	96	76	81	72	68	57	48
Brassica campestris	6.3	25	10	8	2	0	0	0	0	0
Brassica campestris var. toria	6.2	24	12	10	3	1	0	0	0	0
Potato	6.4	50	33	0	0	0	0	0	0	0
Egyptian Clover	6.0	184	92	80	98	42	48	34	30	16
Tomato	6.1	128	61	30	0	0	0	0	0	0
Winter maize	6.2	105	114	107	118	98	68	48	32	40

*Average of five replications.

Table 2. Survival of Xanthomonas campestris pv. oryzae in rhizosphere of different crops during 1987-88

Crop	pH of soil	No. of colonies per plate at 10^{-6} dilution*								
		Nov.	Dec.	Jan.	Feb.	March	April	May	June	July
Wheat	5.8	132	130	90	95	87	74	78	30	40
Brassica campestris	5.9	62	17	15	12	11	4	0	0	0
Brassica campestris var. toria	6.0	31	8	0	0	0	0	0	0	0
Potato	5.9	78	56	25	6	2	0	0	0	0
Egyptian Clover	6.1	156	178	120	100	93	72	37	40	27
Tomato	6.2	98	75	82	22	8	0	0	0	0
Winter Maize	6.0	97	110	108	88	74	57	48	43	38

*Average of five replications.

Table 3. Survival of Xanthomonas campestris pv. oryzae in the rhizosphere of different crops during 1987-88*

Crop	pH of soil	No. of colonies per plate at 10^{-6} dilution**								
		Nov.	Dec.	Jan.	Feb.	March	April	May	June	July
Wheat	6.1	128	132	104	79	68	60	30	28	22
Brassica campestris	5.9	75	60	16	3	0	0	0	0	0
Brassica campestris var. toria	6.0	96	32	9	3	0	0	0	0	0
Potato	5.8	68	70	40	23	6	0	0	0	0
Egyptian clover	6.0	148	135	120	129	93	58	52	28	21
Tomato	6.1	112	81	60	25	0	0	0	0	0
Winter maize	6.2	121	118	117	112	73	79	58	42	40

* Soil samples collected from farmers' fields.

**Average of five replications.

The population of X. campestris pv. oryzae recovered from the rhizosphere of seven crops at monthly intervals during 1986-87 revealed that the bacterium survived in the rhizosphere of potato and tomato for 2 and 3 months, respectively (Table 1). It survived for 4 months in the rhizosphere of Brassica campestris and for 5 months in the rhizosphere of B. campestris var. toria. The bacterium survived for 9 months in the rhizosphere of wheat, winter maize and Egyptian clover.

During 1987-88 the bacterium survived for 5 months in the rhizosphere of potato and tomato (Table 2). It survived for 2 and 6 months in the rhizosphere of B. campestris var. toria and. B. campestris respectively. It survived for 9 months in the rhizosphere of wheat, winter maize and Egyptian clover.

The isolation of the bacterium from the rhizosphere of there crops grown in the fields during 1987-88 showed that the bacterium survived for 4 months in the rhizosphere of B. campestris, B. campestris var. toria and tomato (Table 3). It survived for 5 months in the rhizosphere of potato. Again it survived for 9 months in the rhizosphere of wheat, winter maize and Egyptian clover.

All the cultures of X. campestris pv. oryzae isolated from the rhizosphere of these crops proved pathogenic on TN-1 plants on inoculation. In addition to X. campestris pv. oryzae, the colonies of Erwinia sp., Bacillus sp., Flavobacterium sp., and Pseudomonas sp. were commonly found during the isolation.

DISCUSSION

Rhizosphere is a metabolically active soil zone and supports the growth of several micro-organisms (Clark, 1949). It has been estimated that root exudates and sloughed cells are sources of as much as 30-40 per cent of organic inputs to below ground portion of terrestrial ecosystems (Coleman et al., 1978).

The saprophytic survival of plant pathogenic bacteria on root surfaces or in rhizosphere of nonhost plants was first revealed by Valleau et al. (1944). They showed that Pseudomonas tabaci was primarily a rhizosphere inhabitant and was also associated with roots of wheat and rye sown after harvesting a tobacco crop infected with wildfire. They also postulated that the disease phase of its life cycle was only casual and not necessary for its survival. Schneider and Grogan (1977) found that P. tomato also survived in the rhizosphere of various plants growing in soils with no known history of tomato culture and soil-borne inoculum was adequate for initiation of epidemics of bacterial speck.

Tsuyama (1962) reported that Erwinia soft rot bacteria lived in soil, especially in the rhizosphere, on root surfaces and on leaf surfaces of susceptible host plants. Later on, Kikumoto and Sakamoto (1969) observed that the growth of soft rot Erwinia was selectively stimulated in the rhizosphere of Chinese cabbage, Brassica chinensis, teosinte, Chinese chives and tomato and of three weeds namely, Sonchus oleraceus, Chenopodium album and Commelina communis. No similar effect was noted with all the seven species of leguminous crops tested.

Okabe (1969) found that the population of P. solanacearum in the rhizosphere of different weeds growingin artificially infested soils varied considerably depending upon the plant species. It was very high in Capsella soil (either fresh or air-dried) very poor in Stellaria and Poa soils and high in Equisetum soil only when it was air-dried before infestation. Smith (1944) also demonstrated that crops grown in rotation having varying degrees of resistance to P. solanacearum affected the population of bac-

terium to different degrees, presumably due to biological effects of the crop on soil microbes.

X. campestris pv. oryzae is an ubiquitous pathogen and has been found to survive in seed (Fang et al., 1956), in diseased straw in outdoor piles or indoors in temperate region (Isaka, 1962; Yu and Cho, 1978), in rice stubbles (Watanabe, 1975) and in the rhizosphere of certain weeds namely Leersia sayanuka (Goto et al., 1953; Inoue et al., 1957; Yoshimura et al., 1959).

The present study also revealed the survival of X. campestris pv. oryzae in the rhizosphere of wheat, Egyptian clover, winter maize, potato, tomato, Brassica campestris, and B. campestris var. toria for different periods. The differences in survival period of the bacterium in the rhizosphere of these crops may be due to the differences in nutrients released by different plant spp. and their direct/indirect effect on the rhizosphere microflora as reported by Okabe (1969) for P. solanacearum. The survival of X. campestris pv, oryzae in rhizosphere of wheat, Egyptian clover and winter maize up to 9 months in North India is of great significance because sowing of next rice orop coincides with the harvesting of these crops and major acreage of rice is transplanted in fields vacated by wheat and Egyptian clover.

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

Plant Pathogenic Bacteria Z. Klement (ed.)

BACTERIOSIS OF OAK (QUERCUS ROBUR L.) AND MAPLE (ACER PLATANOIDES L.) IN ARID ZONE

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INTRODUCTION

The health and life-span of protective plantations in arid zone is affected not only by unfavourable growing conditions but also by negative consequences of bacterial diseases.

Information concerning bacteriosis of various types of woods in different regions of USSR is scarce. It is usually restricted to description of symptoms of diseases without identification of pathogenic agents (Gninenko, 1985; Golgofskaya, 1985; Derevyankin, 1985; Derevyankin, Kryukova, 1985; Shevchenko et al., 1985).

The objective of this study was to investigate the etiology of oak and eagle claw maple diseases and to identify the pathogens.

The studies of bacterial diseases of oak and maple were carried out in artificial plantations in semi-desert wormwood-gramineous steppes of Kalmykiya and in the subzone of dry steppe in Ural area of Kazakhstan.

The detailed information concerning forest growing conditions in these zones may be found in "Stationary Studies of Laboratory of Forest Research"(1984).

MATERIALS AND METHODS

Bacteriological studies involved the samples of infected branches, roots and seeds of oak and maple collected during

1984-1987. Isolation of bacteria and investigation of their properties was carried out according to the methods previously described (Beltyukova et al, 1968; Gvozdyak, Yakovleva, 1980; Smirnov et al, 1983).

Pathogenic properties of obtained isolates were assessed during artificial infection of English oak, eagle claw, Tatarian and Guinnal maple both under natural conditions and in control plantings. The suspension of bacterial cells was introduced by syringe or pipette in cuts made in pretreated trunks or branches of 17-20 year-old oak and maple trees. The cuts were made using sterile drills, awls, needles or scalpel in the shape of T-like insections. The depth of drill induced holes was 25-30 mm, the awl punches were about 10 mm deep, the invasion in other cases affected both the bark and alburnum up to 5-8 mm. The artificial infection of trees was carried out early in May, and symptoms were detected in August and October. Several trees were left to monitor the further development of disease. In order to obtain experimental data the infected trees were felled. Trunks and branches were cut into 50 cm sections and split along the infected area. The length and breadth of zones around initial cuts were measured which were distinctly visible due to different colouring of infected wood.

RESULTS

Oak bacteriosis: The bacterial symtoms of oak diseases in shield forests in Kalmykiya became distinctive in 1984-1987 after pronounced physiological withering due to the repetitive droughts and massive damage of green leaves by oakworm Totrix viridana L.

As a rule, branches of withering trees were damaged by worms Asterodiaspis quercicola Bouche; As. variolosus Ratz; and by bark miner Gracilaria syniploniella S.R. and less frequently by Agrilus viridis L. and Zeuzera pyrina L. There was a marked correlation between quantity of insects and the condition of the forest.

The bacterial symptoms of disease were usually manifested in young branches in areas damaged by insects, most frequently



Fig. 1 Necrosis of the oak branches around worm damage. a) on the bark; b) inside the bark

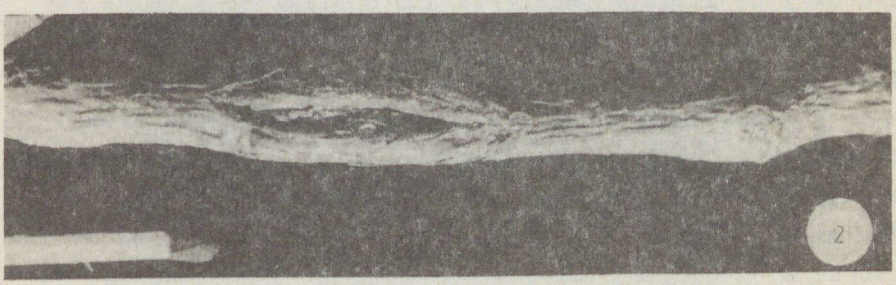

Fig. 2 Cankers on young oak branches as a result of necrotization of the bark and alburnum

by worms which introduced bacterial infection. Due to this fact brown necrotized zones of round, oval or irregular shape and various size appeared around the punched area (Fig. 1). The wood was also subjected to necrosis which resulted in further propagation of infection through vascular bundles of branches and trunk. Decay of bark and necrosis of alburnum originated in open or closed ulcers on buds (Fig. 2) which, depending on the tree state, localized or spread over the vast areas of the branch cambium (and even trunk) causing partial dieback of the crown or the whole tree. The trees damaged from bacteriosis were characterized by the dark-brown colour of the trunk with distinct marks of receptacles of gray fluid formed under the bark of infected trees.

Bacteriological analysis of necrotized sections of bark and wood revealed the presence of pathogenic bacteria belonging

to the Pseudomonas genus. The isolated bacteria of Erwinia genus were less aggressive.

According to biological properties the pathogenic bacteria Pseudomonas genus were divided into two groups and identified as P. syringae pV. syringae and P. Cichorii.

Pathogenic and biological properties of these bacteria were assessed in comparative studies involving collection strains P. syringae pV. syringae 281 and P. Cichorii 9048, obtained in the Institute of Microbiology and Virology, Ukraine Academy of Sciences.

The artificial infection of branches and trunks of 30-35 year old oaks by isolated bacteria and collection strains resulted in grey colouring of the wood around the infected area spreading up and down as red-yellow or gray-brown stripes with length of 3.0-150.0 cm and 1.0-2.5 cm deep inside the trunk. Occasionally, we observed the development of open canker formed at the output of above stripes on the surface of infected wood.

The artificial infection was most effective in case of weak trees, e.g. growing in water-deficient saline soils. We failed to detect the difference in symptoms of diseases during inoculation by isolated bacteria P. syringae pV. syringae and P. cichori.

Bacteriosis of Eagle Claw Maple: A massive dieback of of eagle claw maple was observed. Dieback was detected in more than 80% trees of various ages. The infected maple trees exhibited vast open cankers with grey and occasionally black tracks of flowing liquid. These cankers were detected on trunks and branches at arbitrary height. Sometimes we observed a plentiful gray juice flow through the knots or cuts of a seemingly healthy trunk. The wood of infected trees becomes dark or light brown in colour. However, as a rule the root wood retained its natural colouring. Trunks of most trees were encircled by cankers.

Samples of infected trunks and branches were used to isolate bacteria whose pathogenic properties were checked on maples and indicator plants. Pathogenic and biochemical studies enabled us to classify them as bacteria belonging to Bacillus genus. These bacteria were isolated in pure culture or in

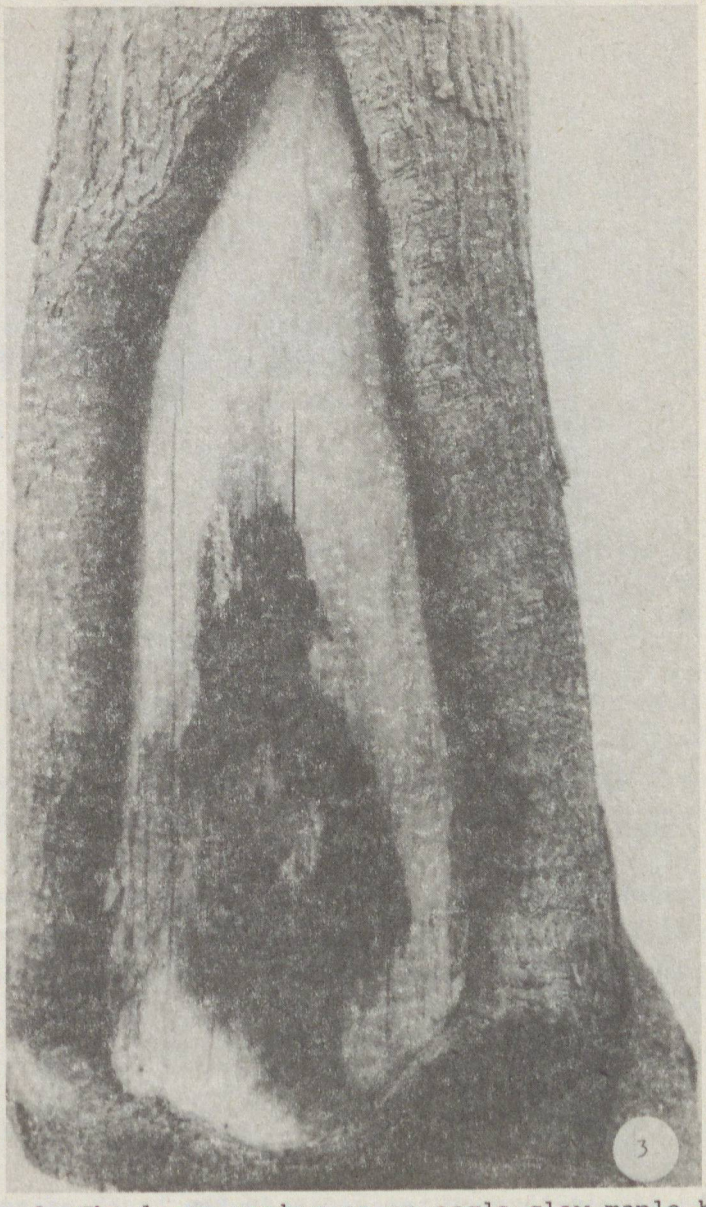

Fig. 3 The large canker on an eagle claw maple bark with traces of sap



Fig. 4 The liquid through the knots in the trunk of seemingly healthy maple

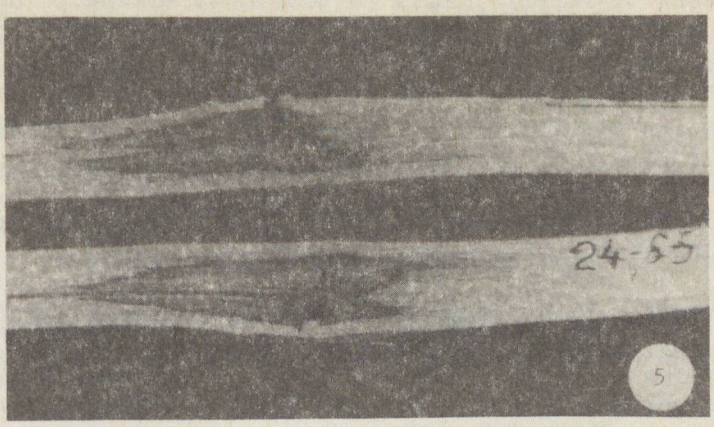

Fig. 5 Inoculation of maple branch by Bacillus subtilis strain

mixture with other bacteria and mycobiota of unidentified origin. The artificial infection of maple branches resulted in intensive darkening of the xylem (Fig. 5) and minor destruction of infected areas.

The obtained experimental data shows that in extreme plantation conditions the English oak (Q. robur. L.) is infected by phytopathogenic bacteria P. syringae pV. syringae and D. cichorii. The weakened trees are most sensitive to the action of these bacteria.

The eagle claw maple (A. platanoides) is subjected to infection by bacteria Bacillus subtilis, which presumably stems from the negative effects of dry steppes and semi-deserts upon forest plantations.

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These volumes may be useful for all those interested in plant pathogenic bacteria and bacterial plant diseases.

ISBN 963 05 5871 8 (A–B)
ISBN 963 05 5873 4 (B)

67942

EDITED BY
Z. KLEMENT

PART B

PLANT PATHOGENIC BACTERIA

1828