Conductimetric detection of *Pseudomonas syringae* pathovar *pisi* in pea seeds and soft rot *Erwinia* spp. on potato tubers

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Cover photograph: immunofluorescence cell staining of *Pseudomonas syringae* pathovar *pisi* in pea seed extract after enrichment in a selective medium.

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STELLINGEN

1. Geleidbaarheidsmetingen kunnen in combinatie met andere technieken worden gebruikt voor detectie en identificatie van plantepathogene bacteriën.

   Dit proefschrift

2. Het is jammer dat de populariteit van archeologie en prehistorie misbruikt wordt om een deel van het Groene Hart om te vormen tot een nieuwbouwwijk.

   (Dreigend) faillissement *Archeon*, 1996

3. De recente ophef omtrent het gebruik van 'paddo' als nieuwe drug bij de turbo-jeugd in Nederland is nogal overdreven, zeker als men in ogenschouw neemt dat de hallucinerende werking van paddestoelen al meer dan 1000 jaar bekend is.

4. De Europese bezorgdheid over overdracht van antibiotica-resistentie-genen vanuit genetisch gemanipuleerd voedsel naar (darm)bacteriën van mens en dier is terecht, gelet op de huidige problemen in de gezondheidszorg bij het ontstaan en verspreiden van pathogene multi-drug resistente bacteriën, zoals de methicilline-resistente *Staphylococcus aureus* (MSRA).

   Wadman, M. (1996) Genetic resistance spreads to consumers. *Nature* **383**: 564.

5. Het is verbazingwekkend dat juist in Nederland niet afdoende rekening gehouden wordt met de combinatie van wind en regen bij het bouwen van een prestigieuze brug.

   Erasmusbrug wappert als vaatdoek bij sterke wind. *De Telegraaf*, 5 november 1996.

6. De overvloed van kleuren en dimensies bij grafieken en tabellen gegenereerd m.b.v. de modernste grafische software gaat vaak ten koste van de duidelijkheid van de boodschap die men wil overbrengen.

7. Gezien de wijze waarop de meeste proefschriften worden doorgelezen verdient het de aanbeveling belangrijke conclusies ook maar in de stellingen, dankbetuigingen en het nawoord op te nemen.

Stellingen behorende bij het proefschrift "Conductimetric detection of *Pseudomonas syringae* pathovar *pisi* in pea seeds and soft rot *Erwinia* spp. on potato tubers"

Bart Fraaije

Wageningen, 9 december 1996

Contents

Chapter 1. Introduction

1.1 Pea bacterial blight

1.1.1 Epidemiology of pea bacterial blight

Bacterial blight caused by *Pseudomonas syringae* pathovar *pisi* (Sackett) Young *et al.* (*Psp*) has become a serious disease for protein pea (*Pisum sativum*) in Europe since 1985 (Stead and Pemberton, 1987; Roberts *et al.*, 1991; Schmit, 1991). *Psp* is a Gram-negative, rod-shaped, chemo-organotrophic bacterium with a strictly respiratory type of metabolism. *Psp* is seed-borne and contaminated seeds provide the primary source of inoculum for infection in the field (Skoric, 1927). Under optimal climatic conditions for transmission, such as high moisture content of the soil (Roberts, 1992), low contamination levels of *Psp* on seed can generate plants carrying epiphytic *Psp* (Grondeau *et al.*, 1994). Consequently, the epiphytic *Psp* can spread rapidly within the crop, mainly by windblown rain-splash, hail, dust, machinery, insects and birds, whereas seed infection can occur in the apparent absence of field symptoms (Stead and Pemberton, 1987).

1.1.2 Pea bacterial blight pathogenesis

So far, seven different races of *Psp* have been distinguished on the basis of their reactions on a series of nine different pea cultivars (Taylor *et al.*, 1989; Bevan *et al.*, 1995). The interactions between races and cultivars were explained in terms of a gene-for-gene relationship, possibly involving six putative resistance (R1-R6)/avirulence (A1-A6) gene pairs. In a compatible interaction between pea and *Psp* disease symptoms develop, showing up as water-soaked lesions around inoculation points, whereas in an incompatible interaction host defence mechanisms are triggered, resulting in a hypersensitive response or absence of disease symptoms. Race 2, and to a lesser extent races 4 and 6, are the most frequently occurring races of *Psp* in Europe, while races 1 and 7 are only sporadically isolated from imported seed lots in the UK (J.D. Taylor, personal communication). The predominance of race 2 can partly be explained by the lack of the matching resistance gene R2 in many cultivars, whereas race 6, lacking avirulence genes, and therefore pathogenic to all pea cultivars, constitutes only a small proportion of the total population of isolates (Taylor *et al.*, 1989).

1.1.3 Identification of *Pseudomonas syringae* pv. *pisi*

Psp did not show a single uniform phenotype when physiological and serological characteristics were studied (Grondeau *et al.*, 1992). Although all *Psp* isolates

were positive in the hyper-sensitive reaction on tobacco, levan positive, oxidase negative, arginine dihydrolase positive and non-pectolytic, various tests showed variation. Most of the *Psp* isolates were positive for fluorescent pigment production (93 %), ice nucleation activity (97 %) and homoserine utilization (75 %), whereas the carbon sources esculin (86%) and DL-lactate (85%) were not utilized. With regard to serology, three different serogroups APT-PIS, HEL2 and RIB, dependent on the structure of the lipopolysaccharide (LPS) O-antigen sidechain (Samson and Saunier, 1987), were observed for *Psp*. Of the isolates tested 88.5, 11.4 and 0.1 % belonged to the serogroups APT-PIS, HEL2 and RIB, respectively. For some *P. s.* pv. *syringae* (*Pss*) isolates showing *Psp*-related phenotypes, pathogenicity tests on pea were required for their identification. Furthermore, the phenotypic diversity of *Psp* was not linked to geographical origin or race structure. However, *Psp* could be subdivided into two separate race-related groups, consisting of races 1, 5 and 7, and races 2, 3, 4 and 6, based on isozyme analysis of esterases (Malandrin *et al.*, 1994) or isoleucine amino-peptidases (B.A. Fraaije, unpublished), DNA probes (Rasmussen and Reeves, 1992) and cross-reactivity in slide agglutination testing with flagellar antigens of *P. s.* pv. *phaseolicola* (race 2) (N.F. Lyons and B.A. Fraaije, unpublished).

1.1.4 Control of pea bacterial blight

The use of resistant cultivars and healthy seeds are the most important ways to control pea bacterial blight.

Pea blight resistance breeding is currently focussed on a combined incorporation of the six specific resistance genes (R1-R6) and a race non-specific resistance gene, which has been found in accessions of *Pisum abyssinicum*, into commercial lines of pea (*Pisum sativum*) (Taylor *et al.*, 1994).

Although *Psp* is not a quarantine organism in the EC anymore, for the production of healthy seeds reliable and sensitive methods are needed, as the role of seed infection is crucial in pea blight epidemiology and uninfected plants guarantee a higher yield (Roberts, 1993). The current bacterial blight test used in The Netherlands for routine indexing of pea seed lots consists of an extraction procedure and an immunofluorescence (IF) test, followed by dilution plating of IF-positive seed extracts in order to isolate and identify *Psp* (Franken and Van den Bovenkamp, 1990). However, to distinguish *Psp* from closely related *Pss*, which are able to give cross-reactions in serology, pathogenicity tests on pea are still needed, making the test laborious and time-consuming. Enzyme-linked immuno-sorbent assay (ELISA) using monoclonal antibodies was not suitable for a direct detection of *Psp* in pea seed extracts because of its relatively low sensitivity of

10^5-10^7 cells ml^{-1} (Candlish *et al.*, 1988). The polymerase chain reaction (PCR) for detection of *Psp* in seed extracts has to be improved with regard to efficiency, as false positive and false negative results were obtained. This was probably due to cross-reacting saprophytes and presence of PCR-inhibitory compounds in the seed extracts, respectively (Reeves *et al.*, 1994). An enrichment procedure for *Psp* in seed extracts before applying ELISA or PCR may solve the problems of sensitivity and specificity, providing that the growth of cross-reacting saprophytes can be inhibited.

1.2 Potato soft rot diseases caused by pectolytic *Erwinia* spp.

1.2.1 Epidemiology of potato soft rot diseases

Pectolytic *Erwinia* spp. are involved in soft rot diseases of various agricultural crops, such as potato, sugar beet and chichory. *Erwinia* spp. are Gram-negative, rod-shaped, chemo-organotrophic, non-sporeforming, facultative anaerobes, and have both a repiratory and a fermentative type of metabolism. They belong to the family of the *Enterobacteriaceae*. *Erwinia* spp. associated with potato tuber soft rot have been studied extensively, because of their economic importance. The different *Erwinia* spp. that are involved in potato diseases are *Erwinia carotovora* subspecies *atroseptica* (Van Hall) Dye (*Eca*), *Erwinia carotovora* subspecies *carotovora* (Jones) Bergey *et al.* (*Ecc*) and *Erwinia chrysanthemi* Burkholder *et al.* (*Ech*). Under cool and moist conditions *Eca* is the causal agent of blackleg, a blackening of the stem base which originates from the mother tuber (Pérombelon and Kelman, 1987). *Ecc* mainly causes aerial stem rot, often due to injury, although under high field temperatures it has been reported to incite blackleg symptoms (Molina and Harrison, 1977). Under hot and humid (tropical) conditions *Ech* can also induce blackleg symptoms, but recently this bacterium has also been found in association with stem wet rot in temperate regions (De Vries, 1990). The symptoms of stem wet rot caused by *Ech* are often difficult to distinguish from typical blackleg symptoms of *Eca* in temperate regions where both pathogens occur (De Boer, 1994). In The Netherlands only *Eca* and *Ech* are regarded to be pathogenic. The major primary source of inoculum of these pathogenic *Erwinia* spp. are seed tubers, where the bacteria can survive for a long time in the lenticels and wounds during storage. In contrast to *Ecc*, both *Eca* and *Ech* cannot survive well in the environment. In the field, rotting mother tubers can contaminate progeny tubers by release of bacteria into the soil followed by transmission by soil water. Daughter tubers can also be contaminated by stolon infection (stolon end rot) of blackleg affected plants. The spread of bacteria from

rotting tubers into progeny tubers can be facilitated by mechanical harvesting and handling (Pérombelon and Hyman, 1992).

1.2.2 Potato soft rot pathogenesis

Pectolytic enzymes of *Erwinia* spp. play a major role in soft rot pathogenesis and elicitation of hypersensitive responses (Collmer and Keen, 1986; Kotoujansky, 1987; Barras *et al.*, 1994). Various isoenzymes of polygalacturonase (PG) and pectate lyase (PL) of *Erwinia* spp. were shown to be able to degrade pectic polymers of middle lamellae and primary cell walls of higher plants, resulting in maceration of plant tissue (Lei *et al.*, 1985; Roberts *et al.* 1986; Barras *et al.*, 1987). Furthermore, less virulent and avirulent strains of *Erwinia* spp. were obtained with transposon mutagenesis, due to a defective motility and impairment in the synthesis and secretion of pectolytic enzymes, respectively (Andro *et al.*, 1984; Hinton *et al.*, 1989; Pirhonen *et al.*, 1991). The regulation of extracellular pectolytic enzyme production of *Erwinia* spp. during interaction with the host plant is very complex, involving both positive and negative regulators responding to various metabolic and environmental stimuli, such as pectic derivatives (Condemine *et al.*, 1986), calcium (Flego, 1994), temperature, nitrogen starvation, oxygen limitation, osmolarity (Hugouvieux-Cotte-Pattat *et al.*, 1992), iron concentration (Sauvage and Expert, 1994) and bacterial cell density (Flego, 1994; Pirhonen *et al.*, 1993). Pectolytic enzymes of *Erwinia* spp. can also elicit plant defense responses, such as production of β-1,3-glucanase (Palva *et al.*, 1993) and phenylalanine ammonialyase (Yang *et al.*, 1989, 1992; Rumeau *et al.*, 1990), by releasing oligogalacturonates with a high degree of polymerization (DP) from the plant cell wall which may function as endogenous elicitors (Davis *et al.*, 1984; Yang *et al.*, 1992). Consequently, the possession of a battery of various inducible pectolytic enzymes with different modes of action is very beneficial for *Erwinia* spp., because it enables the bacteria to attack plant cell walls of different hosts (Beaulieu *et al.*, 1993), to cope better with changing environmental conditions and to overcome plant defence responses by cleavage of oligo-galacturonates with a high DP (Yang *et al.*, 1992).

1.2.3 Identification of soft rot *Erwinia* spp.

E. chrysanthemi differs significantly from *E. carotovora* with regard to biochemical and physiological properties (Graham, 1972; Dickey, 1979; Thomson *et al.*, 1981), and results obtained in DNA reassociation studies support the validity of maintaining the two species as designated (Pérombelon and Kelman, 1980). *Ech* is a highly polymorphic species with a wide range of hosts, and can be divided

into different subgroups on the basis of biochemical and physiological properties (Samson *et al.*, 1987; Ngwira and Samson, 1990), serology (Samson *et al.*, 1990), pectic enzyme profiles (Ried and Collmer, 1986), host specificity (Dickey, 1981), restriction fragment length polymorphism (Boccara *et al.*, 1991) and ribotyping (Nassar *et al.*, 1994). In temperate regions, such as France and The Netherlands, all *Ech* potato strains isolated thus far belonged to serogroup 1 and to flagella type 1 (O1:H1), and to biovars 1, 5 or 7 (Samson *et al.*, 1987; Janse and Ruissen, 1988), whereas in Australia *Ech* isolates from rotting potato tubers were consistent with biovar 3 (Cother and Powell, 1983). Although *Eca* and *Ecc* are closely related subspecies, they can be differentiated from each other on the basis of physiological, biochemical and serological characteristics (Graham, 1972; De Boer *et al.*, 1987), DNA probes (Ward and De Boer, 1994; Darrasse *et al.*, 1994) and cellular fatty acid composition (De Boer and Sasser, 1986). *Eca* has a narrow host range, which is mainly restricted to potato grown in cool climates, and forms a serologically homogeneous group of bacteria (Pérombelon and Kelman, 1980). Most *Eca* strains, depending on geographic origin, between 55 and 96 %, belong to serogroup I, whereas less frequently occurring *Eca* strains were classified into serogroups XVIII, XX and XXII (De Boer *et al.*, 1987). *Ecc* is pathogenic to a wide range of temperate and tropical crops (Pérombelon and Kelman, 1980) and serologically far more heterogeneous than *Eca*, since more than 36 different serogroups have been reported for potato isolates alone (De Boer, 1994).

1.2.4 Control of potato soft rot diseases

Good cultural practices and the use of resistant cultivars and healthy seed tubers are the best strategies to control the disease.

Cultural measures to prevent spread and multiplication of *Erwinia* spp. include the continuous use of high-grade propagation material, crop rotation, removal of diseased plants, disinfection of machinery, early harvesting under dry soil conditions, careful mechanical handling and removal of rotten tubers during grading. Furthermore, tubers have to be stored under dry conditions at a cool temperature in order to prevent growth of soft rot *Erwinia* spp. (Pérombelon and Hyman, 1992).

Various biochemical resistance factors to *Erwinia* spp. have been found in potato tubers, such as degree of esterification of cell wall pectin (McMillan *et al.*, 1993), high dry matter and low reducing sugar content (Tzeng *et al.*, 1990), high Ca^{2+} concentration and oxygen dependent formation of suberins, phytoalexins and other phenolic compounds (Lyon, 1989). However, the genetic resistance

found so far is partial, polygenic (Lyon, 1989) and too difficult to screen, because cultivar resistance is strongly dependent on environmental conditions and of the screening method used (Gans *et al.*, 1991; Koppel, 1993; Lojkowska and Kelman, 1994). An alternative way for resistance breeding is the use of gene technology. Transgenic potato plants resistant to *Erwinia carotovora* were obtained by introduction of foreign genes coding for antibacterial proteins, such as bacteriophage T4 lysozyme (Düring *et al.*, 1993). However, environmental and human health concerns may limit immediate commercial production of genetically modified potato cultivars resistant to *Erwinia* spp. (De Boer, 1994).

In temperate regions much attention has been paid to improve the quality and health of seed potatoes. A clear relationship was found between the number of *Eca* present on seed potatoes and the incidence of blackleg in the field, whereas the incidence of blackleg late in the season was negatively correlated with the tuber yield (Bain *et al.*, 1990). Field inspection for blackleg alone failed to assure the status of this pathogen in seed lots, because post-harvest contamination and latent infections cannot be detected by visual observation. Therefore, laboratory testing in addition to field inspection is currently used in The Netherlands for seed certification in order to control blackleg (De Boer *et al.*, 1996). The aim of laboratory testing is to detect 10^2-10^4 viable *Eca* cells per tuber, which is the amount of inoculum needed to incite blackleg under most field conditions (Bain *et al.*, 1990). Techniques based on dilution plating, serology and DNA technology have been developed to detect *Erwinia* spp. on potato tubers. Dilution plating on crystal violet pectate (CVP) medium (Pérombelon *et al.*, 1987) lacks sensitivity, due to the presence of large numbers of potato tuber-associated saprophytes and *Ecc*, and is both unreliable (Janse and Spit, 1989) and time-consuming. Immunofluorescence cell and colony staining (Allan and Kelman, 1977; Van Vuurde and Roozen, 1990), although laborious, and ELISA, in combination with an enrichment step (Gorris *et al.*, 1994), have good potential for routine application. However, serological techniques sometimes suffer from false positive and false negative reactions, due to cross-reacting saprophytes (Van der Wolf *et al.*, 1994) and variation in serotypes (De Boer *et al.*, 1987; Samson *et al.*, 1990). Polymerase chain reaction (PCR) assays for *Eca* and *Ech* are promising rapid techniques with high specificity and sensitivity (De Boer and Ward, 1995; Smid *et al.*, 1995), but are laborious and expensive to perform. However, the use of a fast and automated screening technique, such as conductivity measurements, prior to PCR may well be suited for routine blackleg indexing of seed potatoes.

1.3 *Conductimetry*

Impedance can be defined simply as the resistance to flow of an alternating current as it passes through a conducting material. The impedance of a system is a function of its resistance, capacitance and the applied frequency (Firstenberg-Eden and Eden, 1984). It is usually the resistance of a solution which is measured and this is most frequently recorded as changes in conductance, the reciprocal of resistance. These changes occur in an electrolyte-containing solution (culture medium) due to metabolism of uncharged or weakly charged substrates, which are converted to highly charged end products, e.g. carbohydrates, proteins and lipids to ionized acids and amines (Owens, 1985). Conductance changes as a result of bacterial metabolism can be measured directly in a growth medium (direct conductimetry) or indirectly, e.g. in a KOH-solution which traps CO_2 evolved from the medium, as described by Owens *et al.* (1989). A conductance system can be considered as measuring net changes in conductance in the culture medium at regular intervals. By reaching a threshold value of conductance change or conductance change rate, which can be set according to the users wishes, the system will detect microbial activity. The time required to reach the point of detection is called the detection time and depends upon the physiological conditions of the system (i.e. temperature, media, electrode type) and the microbial characteristics (i.e. microbial concentration, metabolism and generation time) (Firstenberg-Eden and Eden, 1984). When the growth rate of a test population is more or less constant under experimental conditions, the detection time will correlate with initial bacterial concentrations and conductimetry can be used for automated quantitative estimation of bacteria in samples.

Conductance measurements have been used for many purposes (see review of Silley and Forsythe (1996)), such as rapid measurements of total microbial activity in many food products (Hardy *et al.*, 1977; Martins and Selby, 1980; Firstenberg-Eden and Tricarico, 1983), detection of various pathogenic food-borne bacteria (Bolton, 1990; Pless *et al.*, 1994; Capell *et al.*, 1995), growth monitoring of lactic acid bacteria starter cultures (Lanzanova *et al.*, 1993), antibiotic sensitivity testing (Porter *et al.*, 1983), and for predictive microbial growth modelling (Borch and Wallentin, 1993). Recently, Franken and Van der Zouwen (1993) reported that conductimetry could be used for automated identification and detection of plant pathogenic bacteria, such as *Erwinia*, *Pseudomonas* and *Xanthomonas* spp., but that media and incubation conditions should be improved to diminish the influence of interfering saprophytes and to improve the detection threshold.

1.4 Aim of this study

The aim of the research described in this thesis was to develop suitable enrichment media for detection and quantification of *Psp* and *Erwinia* spp. in seed (tuber) extracts, to be used directly in automated conductance measurements or before detection by PCR or serological techniques. In Chapter 2, the development of a conductimetric assay based on Special Pepton Yeast Extract Broth (SPYE; Malthus Instruments Ltd., Crawley, UK) for detection of *Psp* in pea seeds was studied. The sensitivity of this conductimetric assay for detection of *Psp* in pea seed extracts was compared with serological assays and dilution plating by using two different extraction methods. In Chapter 3, a conductance medium solely based on conversion of L-asparagine (AM) was developed and compared with SPYE with regard to the potential of application in both direct conductimetric detection and enrichment of *Psp* in pea seed extracts prior to serological detection. Chapter 4 describes the development of a conductimetric assay based on polypectate conversion for detection of *Erwinia* spp. in potato peel extracts. In Chapter 5, the mechanism behind the conductance responses of *Erwinia* spp. in pectate media was analysed by determining the production of pectolytic enzymes and the accumulation of products during metabolization of polygalacturonic acid. The potential of application of the polypectate medium for direct conductimetric detection of *Eca* and *Ech* in peel extracts was examined by comparing results of conductimetry with dilution plating, PCR and serological assays with respect to the detection threshold level and the potential for quantification (Chapters 6 and 7). In Chapter 8, the results reported in this thesis are summarized and discussed in view of recent developments.

References

Allan, E. and Kelman, A. (1977) Immunofluorescent stain procedures for detection and identification of *Erwinia carotovora* var. *atroseptica*. *Phytopathology* **67:** 1305-1312.

Andro, T., Chambost, J-P., Kotoujansky, A., Cattaneo, J., Bertheau, Y., Barras, F., Van Gijsegem, F. and Coleno, A. (1984) Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and cellulase. *Journal of Bacteriology* **160:** 1199-1203.

Bain, R.A., Pérombelon, M.C.M., Tsror, L. and Nachmias, A. (1990) Blackleg development and tuber yield in relation to numbers of *Erwinia carotovora* subsp. *atroseptica* on seed potatoes. *Plant Pathology* **39:** 125-133.

Barras, F., Thurn, K.K. and Chatterjee, A.K. (1987) Resolution of four pectate lyase structural genes of *Erwinia chrysanthemi* (EC16) and characterization of the enzymes produced in *Escherichia coli*. *Molecular General Genetics* **209:** 319-325.

Barras, F., Van Gijsegem, F. and Chatterjee, A. K. (1994) Extracellular enzymes and pathogenesis of soft-rot *Erwinia* spp.. *Annual Review of Phytopathology* **32:** 201-234.

Beaulieu, C., Boccara, M. and Van Gijsegem, F. (1993) Pathogenic behavior of pectinase-defective *Erwinia chrysanthemi* mutants on different plants. *Molecular Plant-Microbe Interactions* **6:** 197-202.

Bevan, J.R., Taylor, J.D., Crute, I.R., Hunter, P.J. and Vivian, A. (1995) Genetics of specific resistance in pea (*Pisum sativum*) cultivars to seven races of *Pseudomonas syringae* pv. *pisi*. *Plant Pathology* **44:** 98-108.

Boccara, M., Vedel, R., Lalo, D., Lebrun, M-H. and Lafay, J.F. (1991) Genetic diversity and host range in strains of *Erwinia chrysanthemi*. *Molecular Plant-Microbe Interactions* **4:** 293-299.

Bolton, F.J. (1990) Conductance and impedance methods for detecting pathogens. In *Rapid Methods and Automation in Microbiology and Immunology* eds Vaheri, A., Tilton, R.C. and Balows, A. pp. 176-182. Berlin: Springer Verlag.

Borch, E. and Wallentin, C. (1993) Conductance measurements for data generation in predictive modelling. *Journal of Industrial Microbiology* **12:** 286-290.

Candlish, A.A.G., Taylor, J.D. and Cameron, J. (1988) Immunological methods as applied to bacterial blight. In *Proceedings of the 1988 Brighton Crop Protection Conference-Pests and Diseases* ed. The British Crop Protection Council pp. 787-794. Lavenham, England: The Lavenham Press Ltd..

Capell, C.J., Kirby, R.M. and Moss, M.O. (1995) A method and medium for the electrical detection of *Listeria* spp. from food. *International Journal of Food Microbiology* **25:** 169-177.

Collmer, A. and Keen, N.T. (1986) The role of pectic enzymes in plant pathogenesis. *Annual Review of Phytopathology* **24:** 383-409.

Condemine, G., Hugovieux-Cotte-Pattat, N. and Robert-Baudouy, J. (1986) Isolation of *Erwinia chrysanthemi kduD* mutants altered in pectin degradation. *Journal of Bacteriology* **165:** 937-941.

Cother, E.J. and Powell, V. (1983) Physiological and pathological characteristics of *Erwinia chrysanthemi* isolates from potato tubers. *Journal of Applied Bacteriology* **54:** 37-43.

Darrasse, A., Kotoujansky, A. and Bertheau, Y. (1994) Isolation by genomic subtraction of DNA probes specific for *Erwinia carotovora* subsp. *atroseptica*. *Applied and Environmental Microbiology* **60:** 298-306.

Davis, K.R., Lyon, G.D., Darvill, A.G. and Albersheim, P. (1984) XXV. Endopolygalacturonic acid lyase from *Erwinia carotovora* elicits phytoalexin accumulation by releasing plant cell wall fragments. *Plant Physiology* **74:** 52-60.

De Boer S.H. (1994) Prospects for control of potato diseases caused by pectolytic erwinias. In *Advances in potato pest biology and management* eds Zehnder G.W., Powelson M.L., Jansson, R.K. and Raman K.V. (eds) pp. 136-148. APS press, St. Paul, Minnesota.

De Boer, S.H. and Sasser, M. (1986) Differentiation of *Erwinia carotovora* spp. *carotovora* and *E. carotovora* spp. *atroseptica* on the basis of cellular fatty acid composition. *Canadian Journal of Microbiology* **32:** 796-800.

De Boer, S.H., Slack, S.A., Van den Bovenkamp, G.W. and Mastenbroek, I. (1996) A role for pathogen indexing procedures in potato certification. In *Advances in Botanical Research Vol. 23, Pathogen indexing technologies* eds Andrews, J.H., Tommerup, I.C. and De Boer, S.H. pp. 217-242. London, UK: Academic Press.

De Boer, S.H., Verdonck, L., Vruggink, H., Harju, P., Bång, H.O. and De Ley, J. (1987) Serological and biochemical variation among potato strains of *Erwinia carotovora* subsp. *atroseptica* and their taxonomic relationship to other *E. carotovora* strains. *Journal of Applied Bacteriology* **63:** 487-495.

De Boer, S.H. and Ward, L.J. (1995) PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. *Phytopathology* **85:** 854-858.

De Vries P.M. (1990) Stengelnatrot symptomen in aardappelplanten. *Aardappelwereld* **4:** 15-18.

Dickey, R.S. (1979) *Erwinia chrysanthemi*: a comparative study of phenotypic properties of strains from several hosts and other *Erwinia* species. *Phytopathology* **69:** 324-329.

Dickey, R.S. (1981) *Erwinia chrysanthemi*: reaction of eight plant species to strains from several hosts and to strains of other *Erwinia* species. *Phytopathology* **71:** 23-29.

Düring, K., Porsch, P., Fladung, M. and Lörz, H. (1993) Transgenic potato plants resistant to the phytopathogenic bacterium *Erwinia carotovora*. *The Plant Journal* **3:** 587-598.

Firstenberg-Eden, R. and Eden, G. (1984) Impedance Microbiology 170 pp.. Letchworth, UK: Research Studies Press.

Firstenberg-Eden, R. and Tricarico, M.K. (1983) Impedimetric determination of total, mesophilic and psychrotropic counts in raw milk. *Journal of Food Science* **48:** 1750-1754.

Flego, D. (1994) Virulence in *Erwinia carotovora* subsp. *carotovora*: gene regulation and bacterial cooperation. Doctoral Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Franken, A.A.J.M. and Van den Bovenkamp (1990) The application of the combined use of immunofluorescence microscopy and dilution-plating to detect *Pseudomonas syringae* pv. *pisi* in pea seeds. In *Proceedings of the 7th International Conference on Plant Pathogenic Bacteria* ed. Klement, Z. pp. 871-875. Budapest, Hungary: Akadémiai Kiadó.

Franken, A.A.J.M. and Van der Zouwen, P.S. (1993) Direct and indirect conductimetry for identification and detection of plant pathogenic bacteria. *Journal of Applied Bacteriology* **74:** 234-242.

Gans, P.T., Jellis, G.J., Little, G., Logan, C. and Wastie, R.L. (1991) A comparison of methods to evaluate the susceptibility of potato cultivars to blackleg (caused by *Erwinia carotovora* subsp. *atroseptica*) in the field at different sites. *Plant Pathology* **40:** 238-248.

Gorris, M.T., Alarcon, B., Lopez, M.M. and Cambra, M. (1994) Characterization of monoclonal antibodies specific for *Erwinia carotovora* subsp. *atroseptica* and comparison of serological methods for its sensitive detection on potato tubers. *Applied and Environmental Microbiology* **60:** 2076-2085.

Graham, D.C. (1972) Identification of soft rot coliform bacteria. In *Proceedings of the 3rd International Conference on Plant Pathogenic Bacteria* ed. Maas-Geesteranus, H.P. pp. 273-279. Wageningen, The Netherlands.

Grondeau, C., Poutier, F. and Samson, R. (1994) Pea seed contamination by *Pseudomonas syringae* pv. *pisi*: description and consequences. In *Proceedings of the 8th International Conference of Plant Pathogenic Bacteria* eds Lemaittre, M., Freignoun, S., Rudolph, K. and Swings, J.G. pp. 779-783. Paris, France: INRA editions.

Grondeau, C., Saunier, M., Poutier, F. and Samson, R. (1992) Evaluation of physiological and serological profiles of *Pseudomonas syringae* pv. *pisi* for pea blight identification. *Plant Pathology* **41:** 495-505.

Hardy, D., Kraeger, S.J., Dufour, S.W. and Cady, P. (1977) Rapid detection of microbial contamination in frozen vegetables by automated impedance measurements. *Applied and Environmental Microbiology* **34:** 14-17.

Hinton, J.C.D., Sidebotham, J.M., Hyman, L.J., Pérombelon, M.C.M. and Salmond, G.P.C. (1989) Isolation and characterisation of transposon-induced mutants of *Erwinia carotovora* subsp. *atroseptica* exhibiting reduced virulence. *Molecular General Genetics* **217:** 141-148.

Hugouvieux-Cotte-Pattat, N., Domiminguez, H. and Robert-Baudouy, J. (1992) Environmental conditions affect transcriptions of the pectinase genes of *Erwinia chrysanthemi* 3937. *Journal of Bacteriology* **174:** 7807-7818.

Janse, J.D. and Ruissen, M.A. (1988) Characterization and classification of *Erwinia chrysanthemi* strains from several hosts in the Netherlands. *Phytopathology* **78:** 800-808.

Janse, J.D. and Spit, B.E. (1989) A note on the limitations of identifying soft rot Erwinias by temperature tolerances and sensitivity to erythromycin on a pectate medium. *Journal of Phytopathology* **125:** 265-268.

Koppel, M. (1993) Methods for assessing potato tubers for resistance to bacterial soft rot. *Potato Research* **36:** 183-188.

Kotoujansky, A. (1987) Molecular genetics of pathogenesis by soft-rot Erwinias. *Annual Review of Phytopathology* **25:** 405-430.

Lanzanova, M., Mucchetti, G. and Neviani, E. (1993) Analysis of conductance changes as a growth index of lactic acid bacteria. *Journal of Dairy Science* **76:** 20-28.

Lei, S-P., Lin, H-C, Heffernan, L. and Wilcox, G. (1985) Evidence that polygalacturonase is a virulence determinant in *Erwinia carotovora*. *Journal of Bacteriology* **164:** 831-835.

Lojkowska, E. and Kelman, A. (1994) Comparison of the effectiveness of different methods of screening for bacterial soft rot resistance of potato tubers. *American Potato Journal* **71:** 99-113.

Lyon, G.D. (1989) The biochemical basis of resistance of potatoes to soft rot *Erwinia* spp.-a review. *Plant Pathology* **38:** 313-339.

Malandrin, L., Grondeau, C. and Samson, R. (1994) Contribition of isozym analysis to the identification of *Pseudomonas syringae* pathovar *pisi*. In *Proceedings of the 8th International Conference of Plant Pathogenic Bacteria* eds Lemaittre, M., Freignoun, S., Rudolph, K. and Swings, J.G. pp. 343-347. Paris, France: INRA editions.

Martins, S.B. and Selby, M.J. (1980) Evaluation of a rapid method for the quantitative estimation of coliforms in meat by impedimetric procedures. *Applied and Environmental Microbiology* **39:** 518-524.

McMillan, G.P., Hedley, D., Fyffe, L. and Pérombelon, M.C.M. (1993) Potato resistance to soft-rot erwinias is related to cell wall pectin esterification. *Physiological and Molecular Plant Pathology* **42:** 279-289.

Molina, J.J. and Harrison M.D. (1977) The role of *Erwinia carotovora* in the epidemiology of potato blackleg. I. Relationship of *Erwinia carotovora* var. *carotovora* and *Erwinia carotovora* var. *atroseptica* to potato blackleg in Colorado. *American Potato Journal* **54:** 587-591.

Nassar, A., Bertheau, Y., Dervin, C., Narcy, J-P. and Lemattre, M. (1994) Ribotyping of *Erwinia chrysanthemi* strains in relation to their pathogenic and geographic distribution. *Applied and Environmental Microbiology* **60:** 3781-3789.

Ngwira, N. and Samson, R. (1990) *Erwinia chrysanthemi*: description of two new biovars (bv 8 and 9) isolated from Kalanchoe and maize host plants. *Agronomie* **10:** 341-345.

Owens, J.D. (1985) Formulation of culture media for conductimetric assays: theoretical considerations. *Journal of General Microbiology* **131:** 3055-3076.

Owens, J.D., Thomas, D.S., Thompson, P.S. and Timmerman, J.W. (1989) Indirect conductimetry: a novel approach to the conductimetric enumeration of microbial populations. *Letters in Applied Microbiology* **9:** 245-249.

Palva, T.K., Holmstrom, K-O., Heino, P. and Palva, E.T. (1993) Induction of plant defence response by exoenzymes of *Erwinia carotovora* subsp. *carotovora*. *Molecular Plant-Microbe Interactions* **6:** 190-196.

Pérombelon, M.C.M. and Kelman, A. (1980) Ecology of the soft rot Erwinias. *Annual Review of Phytopathology* **18:** 361-387.

Pérombelon, M.C.M. and Kelman, A. (1987) Blackleg and other potato diseases caused by soft rot *Erwinias*: Proposal for revision of terminology. *Plant Disease* **71:** 283-285.

Pérombelon, M.C.M., Lumb, V.M. and Hyman L.J. (1987) A rapid method to identify and quantify soft rot erwinias on seed potato tubers. *EPPO bulletin* **17:** 25-35.

Pérombelon, M.C.M. and Hyman, L.J. (1992) Control of potato blackleg: production of healthy seed. *Aspects of Applied Biology* **33:** 77-84.

Pirhonen, M., Saarilahti, H., Karlsson, M-B. and Palva, E.T. (1991) Identification of pathogenicity determinants of *Erwinia carotovora* subsp. *carotovora* by transposon mutagenesis. *Molecular Plant-Microbe Interactions* **4:** 276-283.

Pirhonen, M., Flego, D., Heikinheimo, R. and Palva, E.T. (1993) A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *The EMBO Journal* **12:** 2467-2476.

Pless, P., Futschik, K. and Schopf, E. (1994) Rapid detection of Salmonellae by means of a new impedance-splitting method. *Journal of Food Protection* **57:** 369-376.

Porter, I.A., Reid, T.M.S., Wood, W.J., Gibson, D.M. and Hobbs, G. (1983) Conductance measurements for determining antibiotic sensitivity. In *Antibiotics, Assessment of Antimicrobial Activity and Resistance* eds Russel, A.D. and Quesnel, L.B. pp. 49-60. New York: Academic Press.

Rasmussen, O.F. and Reeves, J.C. (1992) DNA probes for the detection of plant pathogenic bacteria *Journal of Biotechnology* **25:** 203-220.

Reeves, J.C., Rasmussen, O.F. and Simpkins, S.A. (1994) The use of a DNA probe and PCR for the detection of *Pseudomonas syringae* pv. *pisi* in pea seed. In *Proceedings of the 8th International Conference of Plant Pathogenic Bacteria* eds Lemaittre, M., Freignoun, S., Rudolph, K. and Swings, J.G. pp. 383-390. Paris, France: INRA editions.

Ried, J.L. and Collmer, A. (1986) Comparison of pectic enzymes produced by *Erwinia chrysanthemi*, *Erwinia carotovora* subsp. *carotovora*, and *Erwinia carotovora* subsp. *atroseptica*. *Applied and Environmental Microbiology* **52:** 305-310.

Roberts, D.P., Berman, P.M., Allen, C., Stromberg, V.K., Lacy, G.H. and Mount, M.S. (1986) Requirement for two or more *Erwinia carotovora* subsp. *carotovora* pectolytic gene products for maceration of potato tuber tissue by *Escherichia coli*. *Journal of Bacteriology* **167:** 279-284.

Roberts, S.J. (1992) Effect of soil moisture on the transmission of pea bacterial blight (*Pseudomonas syringae* pv. *pisi*) from seed to seedling. *Plant Pathology* **41:** 136-140.

Roberts, S.J. (1993) Effect of bacterial blight (*Pseudomonas syringae* pv. *pisi*) on the growth and yield of single pea (*Pisum sativum*) plants under glasshouse conditions. *Plant Pathology* **42:** 568-576.

Roberts, S.J., Reeves, J.C., Biddle, A.J., Taylor, J.D. and Higgins, P. (1991) Prevalence of pea bacterial blight in UK seed stocks, 1986-1990. *Aspects of Applied Biology* **27:** 327-332.

Rumeau, D., Maher, E.A., Kelman, A. and Showalter, A.M. (1990) Extensin and phenylalanine ammonia-lyase gene expression altered in potato tubers in response to wounding, hypoxia, and *Erwinia carotovora* infection. *Plant Physiology* **93:** 1134-1139.

Samson, R., Ngwira, N. and Rivera, N. (1990) Biochemical and serological diversity of *Erwinia chrysanthemi*. In *Proceedings of the 7th International Conference on Plant Pathogenic Bacteria* ed. Klement, Z. pp. 895-900. Budapest, Hungary: Akadémiai Kiadó.

Samson, R., Poutier, F., Sailly, M. and Jouan, B. (1987) Caractérisation des *Erwinia chrysanthemi* isolées de *Solanum tuberosum* et d'autres plantes-hôtes selon les biovars et sérogroupes. *EPPO bulletin* **17:** 11-16.

Samson, R. and Saunier, M. (1987) Désignation de références sérologiques pour six sérogroupes de pathovars de *Pseudomonas syringae* sur la base de leur lipopolyoside. *EPPO bulletin* **17:** 165-171.

Sauvage, C. and Expert, D. (1994) Differential regulation by iron of *Erwinia chrysanthemi* pectate lyases: pathogenicity of iron transport regulatory (*cbr*) mutants. *Molecular Plant-Microbe Interactions* **7:** 71-77.

Schmit, J. (1991) Races of *Pseudomonas syringae* pv. *pisi*. Occurence in France and host specificity towards winter and spring cultivars of protein peas. In *Proceedings of the 4th International Working Group on Pseudomonas syringae pathovars* eds Durbin, R.D., Surico, G. and Mugnai, L. pp. 256-262. Florence, Italy: Stamperia Granducale.

Silley, P. and Forsythe, S. (1996) Impedance microbiology-a rapid change for microbiologists. *Journal of Applied Bacteriology* **80:** 233-243.

Skoric, V. (1927) Bacterial blight of pea: overwintering, dissemination and pathological histology. *Phytopathology* **17:** 611-627.

Smid, E.J., Jansen, A.H.J. and Gorris, L.M.G. (1995) Detection of *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* in potato tubers using polymerase chain reaction. *Plant Pathology* **44:** 1058-1069.

Stead, D.E. and Pemberton, A.W. (1987) Recent problems with *Pseudomonas syringae* pv. *pisi* in UK. *EPPO bulletin* **17:** 291-294.

Taylor, J.D., Bevan, J.R., Crute, I.R. and Reader, S.L. (1989) Genetic relationship between races of *Pseudomonas syringae* pv. *pisi* and cultivars of *Pisum sativum*. *Plant Pathology* **38:** 364-375.

Taylor, J.D., Roberts, S.J. and Schmit, J. (1994) Screening for resistance to pea bacterial blight (*Pseudomonas syringae* pv. *pisi*). In *Proceedings of the 8th International Conference of Plant Pathogenic Bacteria* eds Lemaittre, M., Freignoun, S., Rudolph, K. and Swings, J.G. pp. 1027. Paris, France: INRA editions.

Thomson, S.V., Hildebrand, D.C. and Schroth, M.N. (1981) Identification and nutritional differentiation of the Erwinia sugar beet pathogen from members of *Erwinia carotovora* and *Erwinia chrysanthemi*. *Phytopathology* **71:** 1037-1042.

Tzeng, K-C., McGuire, R.G. and Kelman, A. (1990) Resistance of tubers from different potato cultivars to soft rot caused by *Erwinia carotovora* subsp. *atroseptica*. *American Potato Journal* **67:** 287-305.

Van der Wolf, J.M., Van Beckhoven, J.R.C.M., De Vries, Ph.M. and Van Vuurde, J.W.L. (1994) Verification of ELISA results by immunomagnetic isolation of antigens from extracts and analysis with SDS-PAGE and Western blotting, demonstrated for *Erwinia* spp. in potatoes. *Journal of Applied Bacteriology* **77:** 160-168.

Van Vuurde, J.W.L. and Roozen, N.J.M. (1990) Comparison of immunofluorescence colony-staining in media, selective isolation on pectate medium, ELISA and immunofluorescence cell staining for detection of Erwinia carotovora subsp. *atroseptica* and *E. chrysanthemi* in cattle manure slurry. *Netherlands Journal of Plant Pathology* **96:** 75-89.

Ward, L.J. and De Boer, S.H. (1994) Specific detection of *Erwinia carotovora* subsp. *atroseptica* with a digoxigenin-labeled DNA probe. *Phytopathology* **84:** 180-186.

Yang, Z., Cramer, C.L. and Lacy, G.H. (1989) System for simultaneous study of bacterial and plant gene expression in soft rot of potato. *Molecular Plant-Microbe Interactions* **4:** 195-201.

Yang, Z., Cramer, C.L. and Lacy, G.H. (1992) *Erwinia carotovora* subsp. *carotovora* pectic enzymes: in planta gene activation and roles in soft-rot pathogenesis. *Molecular Plant-Microbe Interactions* **5:** 104-112.

Chapter 2. Serological and conductimetric assays for the detection of *Pseudomonas syringae* pathovar *pisi* in pea seeds

Abstract

Test protocols for detecting *Pseudomonas syringae* pv. *pisi* (*Psp*), the causal agent of bacterial blight, in pea seeds are generally based on dilution plating assays. These assays are usually very specific and reliable, but are time-consuming and laborious. Tests suitable for large-scale screening of seed lots are therefore needed. Conductimetric assays, immunofluorescence microscopy (IF) and an enzyme-linked immunosorbent assay (ELISA) for detecting *Psp* in pea seed extracts were compared with dilution plating by two extraction methods, viz. 6 h soaking of seeds and 2 h soaking of flour of ground pea seeds in water. In general, the detection of *Psp* with conductimetric, IF and dilution plating assays in the extracts of the ground and 2 h-soaked pea samples was less sensitive than detection in the extracts of the 6 h-soaked pea seeds. The detection threshold of these assays varied per seed lot between 0 and 4.08 log cfu ml^{-1} for the 6 h soaking procedure. The detection threshold of ELISA varied for both extraction methods generally between 4.08 and 6.08 log cfu ml^{-1}. The detection times recorded in the conductimetric assays correlated well ($-0.89 < r < -0.97$) with the log colony-forming units of *Psp* added to seed extracts at 27 as well as at 17 °C. However, confirmation of results by isolation on semi-selective media after conductimetry was more successful at 17 °C than at 27 °C, because of the relatively lower activity of saprophytic *Pseudomonas* spp. at this temperature.

Introduction

The seed-borne bacterium *Pseudomonas syringae* pv. *pisi* (*Psp*) causes bacterial blight in pea (*Pisum sativum*) and is a serious threat for all pea-growing areas (Boelema, 1972). The use of disease-free seed is one of the most important ways to control bacterial blight. To determine whether a seed lot is infected or healthy, reliable and sensitive detection methods are needed. Most of the current tests for the detection and identification of plant pathogens in seed are based on serology and plating assays (Franken and Van Vuurde, 1990), but new, recently developed nucleic acid techniques, like PCR (polymerase chain reaction) and RFLP (restriction fragment length polymorphism) analyses, have a high potential for application (Ball and Reeves, 1991).

The current bacterial blight test used in The Netherlands for routine indexing of pea seed lots consists of an extraction procedure, an immunofluorescence (IF)

test and a dilution plating assay of the seed extract followed by confirmation tests of suspected colonies. In this protocol IF is used as a screening step before dilution plating is conducted. Only IF-positive pea seed lots are plated (Franken and Van den Bovenkamp, 1990). In addition to identification tests, such as slide agglutination (Taylor, 1970, 1972; Lyons and Taylor, 1990) and IF, pathogenicity testing is used to identify the seven currently known races of *Psp* (Taylor *et al.*, 1989) and to distinguish *Psp* from *P. s.* pv. *syringae* (*Pss*) and other organisms from seed extracts that are able to give cross-reactions in serology (Grondeau *et al.*, 1992). The use of pathogenicity testing to distinguish *P. syringae* pathovars could possibly be restricted by the improvement of serological techniques and the production of high quality antisera (Samson and Saunier, 1987; Candlish *et al.*, 1988). Mazarei and Kerr (1990) developed an indirect ELISA (enzyme-linked immunosorbent assay) based on polyclonal antibodies to distinguish pathovars of *P. syringae* from pea. However, ELISA cannot generally be used for the direct detection of *Psp* in seed extracts because of its relatively low sensitivity of 10^5 to 10^7 cells ml^{-1} (Candlish *et al.*, 1988). Concentration of seed extracts or development of enrichment procedures for *Psp* in seed extract may solve this problem. Conductimetric assays were found very useful for development of enrichment procedures for detection of food-borne bacteria (Ur and Brown, 1975; Bolton, 1990). In this technique changes in conductance in a culture medium as a result of bacterial metabolism are measured automatically. Recently, Franken and Van der Zouwen (1993) reported the potential of these techniques for detection and identification of some plant pathogenic bacteria.

In this paper, the application of an enzyme-linked immunosorbent assay (ELISA) and IF for the detection of *Psp* in pea seed lots is described. The potential of conductimetric assays for enumeration and detection of *Psp* in seed extracts is also discussed.

Materials and Methods

Media

Nutrient Agar (Oxoid) was used for maintaining of bacterial cultures. Tryptic Soy Broth (TSB; Difco) was used for growing of overnight cultures. Tryptone Soya Agar (TSA; Oxoid) or 5 % (w/v) Sucrose Nutrient Agar (SNA; Taylor and Dye, 1972) plates were used for the determination of the number of cfu added to the seed extracts. King's medium B agar (KB, King *et al.*, 1954) and SNAC, which is SNA containing (g l^{-1}): boric acid, 1.5; cephalexin, 0.08; cycloheximide, 0.2, were used for dilution plating of seed extracts. The addition of these antibiotics to KB

was reported by Mohan and Schaad (1987). Special Peptone Yeast Extract Broth (SPYE; Malthus Instruments Ltd, Crawly, UK) was used as a base medium for the conductimetric assays with pure cultures. SPYEC, which is SPYE containing (g l^{-1}): boric acid, 1.5; cephalexin, 0.08; cycloheximide 0.2; cefuroxime, 0.1 (N.F. Lyons, personal communication), was used to measure conductance responses of spiked and unspiked seed extracts.

Antisera

The rabbit polyclonal antibody (PAb) 104 was used in IF, ELISA and agglutination tests. PAb 104, prepared against whole living cells of *Psp* strain 519 (National Collection of Plant Pathogenic Bacteria, Harpenden, UK, no. 2585, race 2, blue fluorescent), was obtained from the Research Institute for Plant Protection (IPO-DLO), Wageningen, The Netherlands. This antisera was known to react specifically with all strains tested of *Psp* in IF at 1 : 500 dilution (A.A.J.M. Franken and G.W. Van den Bovenkamp, unpublished). PAb 85, a polyclonal antibody raised against *Clavibacter michiganensis* subsp. *michiganensis*, a bacterial pathogen of tomato, was used as control.

Immunofluorescence microscopy (IF)

Indirect IF of seed extracts was performed with 1 : 2000 diluted PAb 104 and 1 : 100 diluted fluorescein-isothiocyanate conjugated secondary goat anti-rabbit antibodies (Sigma) as described by Van Vuurde *et al.* (1983). Rhodamine, 1 : 100 diluted, was used as counter stain. Fifty μl per sample were fixed on multitest slides (diam. 8 mm). At least 50 microscope fields were counted (field coefficient 18, objective magnification 63, internal magnification 1.25, ocular magnification 10) which corresponds to *ca* 2 μl seed extract.

ELISA

Each well of a 96-well microtitre plate was incubated at room temperature for 1 h with 100 μl of poly-L-lysine solution (0.1 mg ml^{-1} in carbonate buffer, pH 9.6). The wells were washed four times in PBST (0.05 % Tween 20 in 0.01 mol l^{-1} phosphate buffered saline, pH 7.2) with an automatic wellwasher and coated with 50 μl antigen solution. After incubation at 4 °C overnight and washing, wells were blocked at 37 °C for 1 h with PBST containing 0.5 % bovine serum albumin (BSA). After washing, 1 : 1000 diluted PAb 104 was added to the wells and the plate was incubated at 4 °C for 3 h. The plate was washed as before and incubated with a 1 : 1000 dilution (in PBST with 0.2 % BSA) of goat anti-rabbit immunoglobulin coupled with alkaline phosphatase at 37 °C for 2 h. After

washing, the substrate, 0.75 mg ml^{-1} *p*-nitrophenyl phosphate in substrate buffer (10 % (v/v) diethanolamine, pH 9.8) was added. Finally, the absorbance was measured with an automatic reader at 405 nm after 45 minutes (A_{405}-values). Results are expressed as corrected A_{405}-values, i.e. A_{405}-values with Pab 104 minus the A_{405}-values read with negative control Pab 85.

Pea seed lots

Pea seed lots 6268, 6330, 7289 and 7292, stored at 4-6 °C, were harvested in 1986 and checked for bacterial blight in 1987 by the method reported by Franken and Van den Bovenkamp (1990). Seed lots 6330 and 6268 were found positive with IF and dilution plating. Seed lot 7289 was found positive with IF and negative with dilution plating in 1987. Seed lot 7292 was found negative with IF, dilution plating was not conducted. Seed lot 0-1991 was first checked in 1991 and found to be positive with IF and dilution plating. All *Psp* colonies isolated from these seed lots were blue fluorescent strains (unpublished results).

Preparation of inoculum

The strain *Psp* 518 (National Collection of Plant Pathogenic Bacteria, Harpenden, UK, no. 2222, race 1, non-fluorescent) was obtained from the Research Institute for Plant Protection (IPO) Wageningen, The Netherlands. This strain was grown overnight at room temperature to give suspensions of *ca* 10^9 cfu ml^{-1}. The numbers of cfu were calculated according to ISO 7218 (Anon., 1985). Inoculum dilutions of pure cultures were made in saline or pea seed extract for testing pure cultures and inoculated (spiked) pea seed extracts, respectively.

Sample treatment

Each pea seed lot was tested in samples of 1000 seeds. Individual samples were either first ground with a coffee grinder and soaked at 4-6 °C for 2 h in tap water (Taylor *et al.*, unpublished) or immediately soaked for 6 h at 4-6 °C in tap water (Franken and Van den Bovenkamp, 1990). Sterile tap water was added to the 6 h soaked samples in a quantity corresponding to 2.0 ml multiplied by the weight (in g) of 1000 seeds. Twice the amount of water was added to the ground samples. After soaking, soaking fluids were spiked by adding dilutions of overnight cultures of *Psp* 518 (dilutions were made with the seed extracts). *Psp* 518 (non-fluorescent) was added to suspension water in order to distinguish it easily from blue fluorescent *Psp* strains which were isolated before from the seed lots. Dilution plating, IF, ELISA and conductimetric assays were done in duplicate with unspiked and spiked samples. Saprophytes and suspected colonies were

identified by an oxidase reaction (Kovacs, 1956), fluorescence production on KB, and levan production on SNAC (Lelliot *et al.*, 1966). *Psp* isolates were confirmed by slide agglutination tests (Lyons and Taylor, 1990), IF and pathogenicity testing on pea cultivar Kelvedon Wonder (Taylor *et al.*, 1989).

Conductance measurements

For the conductance measurements the Malthus 2000 series Analyzer (Malthus Instruments Ltd, Crawley, UK) was used. Malthus conductance tubes with 2 ml of media were inoculated with 1 ml of seed extract or bacterial suspension. The tubes were incubated at 27 °C or 17 °C. The direct conductance responses in the cells were recorded at 18 min intervals and the detection time (Td; defined here as the time (h) needed to a give an average conductance change of 1 μS h^{-1}), maximum rate of conductance change (μS h^{-1}) and the maximum conductance change (μS) were determined. To determine the detection time of the ground, 2 h-soaked samples, the conductance responses were corrected for the base line drift. After detection, samples in the tube, 1 : 10000 diluted, were plated on KB and SNAC in order to isolate and identify *Psp* suspected colonies.

Statistical analysis

Analysis of variance (ANOVA) was done with the statistical program Genstat (Rothamsted Experimental Station). In this, ANOVA effects of seed lots were specified as block effect terms. The extraction method and bacterial concentration effects were defined as treatment effect terms. Sign tests were done as described by Siegel (1956).

Results

Dilution plating

To compare the specificity and sensitivity of techniques for detecting *Psp* in pea seeds, seed lots were spiked with different cell concentrations of *Psp* 518. The number of cfu added to the extracts as well as the number of cfu recovered in dilution plating were determined. Table 1 shows that *Psp* 518 could be recovered in dilution plating in nearly the same numbers as were added to the extracts (correlation coefficient r = 0.96 for 6 h soaking; r = 0.99 for grinding plus 2 h soaking). Soaking for 6 h gave higher recoveries than grinding plus 2 h soaking (P < 0.001, as determined by ANOVA). The occurrence and total amounts of saprophytes were dependent of the seed lot and extraction method used. The total yield of saprophytes on KB varied from 5.12 to 2.34 log cfu ml^{-1}.

Table 1. Detection of *Pseudomonas syringae* pv. *pisi* strain 518 in pea seeds using two extraction methods, viz. 6 h soaking of seeds (soaking) and 2 h soaking of flour of ground seeds (grinding) in water

Cell concentrations of *Psp* added to seed extracts (log cfu ml^{-1})	Plating[1]		IF[2]		ELISA[3]	
	soaking	grinding	soaking	grinding	soaking	grinding
Unspiked	<u>0.32</u>	<u>0.00</u>	3.55	2.17	0.09	0.17
2.08	2.23	2.15	<u>3.56</u>	1.91	0.05	0.20
4.08	4.07	3.86	4.97	<u>2.41</u>	<u>0.09</u>	<u>0.20</u>
6.08	-	-	5.98	5.31	1.01	0.51
8.08	-	-	-	-	1.04	0.95

Underlined figures indicate the detection threshold of the techniques used; - = not tested.
1 Results of dilution plating on SNAC in log cfu ml^{-1}; standard error of difference of means is 0.084 (n = 10).
2 Results of immunofluorescence cell staining (IF) in log cells ml^{-1}; standard error of difference of means is 0.416 (n = 10).
3 Corrected absorbance values in ELISA; standard error of difference of means is 0.098 (n = 10).

On SNAC only seed lot 0-1991 yielded saprophytes, namely 1.60 log cfu ml^{-1} for the 6 h-soaked sample and 2.57 log cfu ml^{-1} for the ground, 2 h-soaked sample. The sensitivity of detecting *Psp* 518 in the seed extracts tested was in the range of 0-10^2 cfu ml^{-1}.

Immunofluorescence microscopy (IF)

IF was used for testing seed lots examining 50 microscope fields (Table 1). For every seed lot tested, fluorescent cells were found in 50 microscope fields when 6 h soaking was used. IF-results corresponded well to those found in 1987, except that one seed lot, negative before, was now found to be positive. In nearly every instance, soaking for 6 h resulted in more fluorescent cells in IF than grinding plus 2 h soaking, although this varied with the concentration of bacterial cells (P = 0.02). In general, grinding plus 2 h soaking yielded cells only at very high cell concentrations added. Controls yielded more cells than the same extracts to which cells were added after grinding plus 2 h soaking. For 6 h soaking a higher correlation was found between the number of cfu added to the seed extracts and the number of cells found in IF (r = 0.52 for 6 h soaking; r = 0.09 for grinding plus 2 h soaking). The threshold for detecting the cells added to the seed extracts was for the 6 h-soaked pea samples often 10- to 100-fold lower than for the ground, 2 h-soaked samples.

Enzyme-linked immunosorbent assay (ELISA)

Table 1 shows that the sensitivity of ELISA for detecting *Psp* 518 in seed extracts was 10^4-10^6 cells ml^{-1}. The effect of the two extraction methods were dependent on the cell concentration (P < 0.001). Mean A_{405}-values with Pab 85 (negative control) were 0.24 ± 0.107 (n = 25) for grinding plus 2 h soaking and 0.13 ± 0.050 (n = 25) for 6 h soaking. The coefficient of correlation (r) between the log number of cfu added and the absorbance values was 0.82 and 0.72 for 6 h soaking and grinding, followed by 2 h soaking, respectively. In general, the difference in A_{405}-values between healthy and infected seed lots was higher for 6 h soaking than grinding plus 2 h soaking.

Conductimetry

At 27 °C and 17 °C a linear relation was found between detection times and log cfu ml^{-1} (Fig. 1). Detection times were higher at 17 °C than at 27 °C for both extraction methods. For detecting *Psp* 518 in seed extracts, grinding followed by 2 h soaking gave much higher detection times than 6 h soaking at 17 °C and 27 °C (P < 0.001 for both temperatures) (Fig. 1).

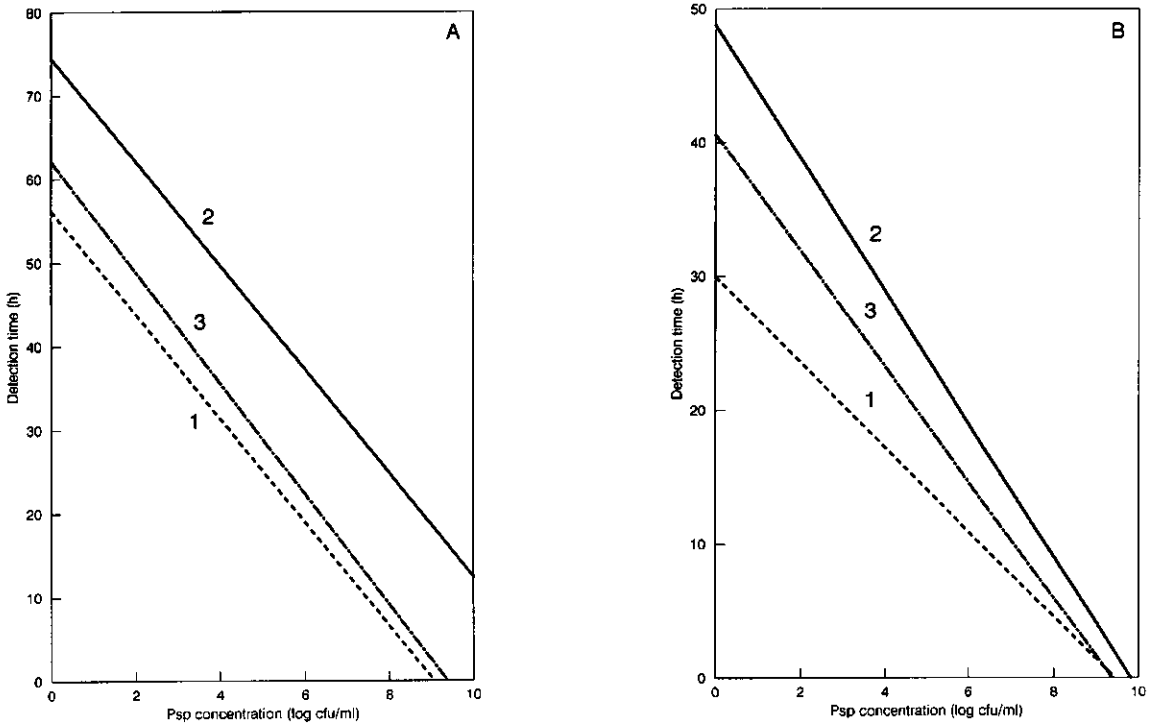

Figure 1. The relationship between detection times in conductimetry and the number of cfu of *Pseudomonas syringae* pv. *pisi* (*Psp*) added to seed extracts in Special Peptone Yeast Extract with the addition of cephalexin, cefuroxime, cycloheximide and boric acid at 17 (**A**) and 27 °C (**B**). 1 = 6 h soaking of pea seeds, 2 = grinding plus 2 h soaking of pea seeds, 3 = pure culture. Equations for the calibration lines at 17 °C are: $y = -6.2x + 56.2$ ($r = -0.97$, n = 20), $y = -6.2x + 74.5$ ($r = -0.89$, n = 20) and $y = -6.6x + 62.0$ ($r = -0.98$, n = 16) for lines 1, 2 and 3, respectively, and at 27 °C: $y = -3.2x + 29.9$ ($r = -0.93$, n = 31), $y = -5.0x + 49.0$ ($r = -0.94$, n = 39) and $y = -4.3x + 40.6$ ($r = -0.94$, n = 15) for lines 1, 2 and 3, respectively, in which y = detection time (h) and x = log cfu of *Psp* ml^{-1}.

Effects of the extraction methods, however, depended on the concentration used (P = 0.002 and P < 0.001 for 27 °C and 17 °C, respectively). For both extraction methods high negative correlations were found between the log cfu ml^{-1} of *Psp* 518 added to seed extracts and the detection time at 27 °C (r = -0.93 for 6 h soaking; r = -0.94 for grinding, followed by 2 h soaking) and at 17 °C (r = -0.97 for 6 h soaking; r = -0.89 for grinding, followed by 2 h soaking). In general, no differences were found between samples (P = 0.275) at 27 °C, although detection times varied with the extraction method used (P = 0.039). At 17 °C, however, the detection times varied with the extraction method and seed sample tested (P < 0.001). After detection in conductimetry the broths from the conductivity cells were plated. The 6 h-soaked, unspiked samples of seed lot 7292, yielded at 27 °C levan producing, oxidase-negative, blue fluorescent *Psp* colonies. These colonies, the concentration of which in the seed extract was in the range of 0-10 cfu ml^{-1} in dilution plating, gave an average Td of 40.2 h (n = 2). The 6 h-soaked and ground plus 2 h-soaked unspiked samples of seed lot O-1991, with average Td of 29.9 and 45.6 h (n = 2), respectively, yielded only oxidase-positive, yellow/green fluorescent, levan producing, or oxidase-positive, blue/green fluorescent, levan non-producing *Pseudomonas* strains at 27 °C. The presence of these strains could also be deduced from the deviating conductance response curves (results not shown), as compared with *Psp* 518. Non-fluorescent *Psp* 518 bacteria, confirmed by agglutination, could be re-isolated after 67 h incubation at 27 °C from all spiked samples, except from seed lot O-1991, where *Psp* 518 bacteria could be isolated only from the 10^8 cells ml^{-1} spiked samples. At 17 °C *Psp* could be isolated from the enrichment broths of all spiked samples and cell concentrations added. *Psp* could also be isolated from the 6 h-soaked unspiked samples of seed lots 0-1991 and 7292, detected within 59.7 h (n = 2) and 85.5 h (n = 1) at 17 °C, respectively.

Correlations between results obtained with dilution plating, IF, ELISA and conductimetric assays

In all cases, correlations between the conductimetric assays and the other tests were negative. Highest negative correlations were obtained for conductimetric assays and dilution plating (r = -0.86 and -0.91 for 6 h soaking at 27 and 17 °C, respectively; r = -0.96 and -0.91 for grinding, followed by 2 h soaking at 27 and 17 °C, respectively). Negative correlations were also found between detection times and absorbance values in ELISA (r = -0.79 and -0.64 for 6 h soaking at 27 and 17 °C, respectively; r = -0.73 and -0.67 for grinding, followed by 2 h soaking at 27 and 17 °C, respectively), and detection times and log cells ml^{-1} in IF (r = -

0.60 and -0.47 for 6 h soaking at 27 and 17 °C, respectively; r = -0.47 and r = -0.57 for grinding, followed by 2 h soaking at 27 and 17 °C, respectively). The correlation coefficient between dilution plating results (expressed as log cfu of *Psp* ml^{-1}) and IF or ELISA values was in all instances smaller than 0.37. Correlation coefficients of IF and ELISA results varied between 0.4 and 0.5 for both extraction methods and temperatures.

Discussion

Rapid and sensitive tests are needed for large-scale routine testing of seed lots. In this study the merits of current tests such as dilution plating, IF and ELISA, and the potential of conductimetric assays for detecting *Psp* in pea seeds, using two different extraction methods, were investigated.

The enumeration of *Psp* 518 added to the seed extracts was according to the obtained detection times in the conductimetric assays more rapid for the 6 h soaking than for the grinding plus 2 h soaking extraction method (Fig. 1). The higher and lower detection times in conductimetry for *Psp* in seed extracts, compared with testing *Psp* as a pure culture, may respectively be explained by the release of toxic and growth-stimulating substances from the seeds, which depends on the extraction method used. These results were confirmed by the slightly lower recovery of *Psp* in dilution plating when grinding plus 2 h soaking was compared with 6 h soaking of pea seeds (Table 1).

In this study no bacteria could be isolated from the long-stored pea seed lots which were found positive in dilution plating on SNAC in 1987. This means that the viability of saprophytes and *Psp* in pea seeds decreased in time during storage, as is also known for *P. s.* pv. *phaseolicola* in bean seeds (Taylor *et al.*, 1979). *Psp*, however, could be isolated from the enrichment broth of sample 7292 in several conductimetric assays.

The poor correlation found between the results of dilution plating and IF/ELISA was probably caused by the presence of dormant, damaged or dead cells in the seed extracts (IF) or variations in (soluble) antigens of *Psp* per seed lot (ELISA). In general, however, high values in IF or ELISA also yielded higher number of colonies, indicating that there was a high rank correlation. The detection threshold of IF was found to be lower for 6 h soaking than for grinding followed by 2 h soaking (Table 1). This may be caused by quenching of the fluorescence and incomplete staining of cells by substances released by the flour of ground seeds, leading to negative results in the tests used.

In most cases higher background absorbance values were obtained for the unspiked 2 h-soaked pea seed flour than for the unspiked 6 h-soaked seeds.

Binding of pea phosphatases or pea proteins, non-specifically reacting with the antiserum, to poly-L-lysine may have caused these higher absorbance values. ELISA was generally less sensitive for detecting *Psp* than IF, dilution plating and conductimetric assays.

Although high correlations were found between the detection times found in conductimetry and results obtained in the other tests, saprophytic micro-organisms may still interfere with the conductimetric assay. In this study the seed lots tested were relatively clean, with the exception of seed lot 0-1991. Recently, we have found more seed lots containing saprophytes which grew very fast in the medium used. Therefore, the selectivity of the medium needs to be improved. Incubation at 17 °C generally increases the probability of re-isolating *Psp* from the conductivity cells. For the time being, the presence of *Psp* in seed lots, suspected of being infected on the basis of detection times, should always be confirmed by other tests.

For a first screening of seed lots, a sensitive technique is needed, while for confirmation generally a more specific test is needed (Sheppard *et al.*, 1986). We feel that the use of an enrichment procedure for *Psp* may improve the detection level of tests as shown by Wong (1991) for *Xanthomonas campestris* pv. *phaseoli* in bean seed. In this respect conductimetric assays, possibly in combination with specific serological techniques or DNA assays, such as immunomagnetic separation (Parmar *et al.*, 1992) or PCR (Rasmussen and Wulff, 1991), may be become highly suited for large-scale routine indexing of seed lots, provided that a good correlation between detection times and contamination level of seed lots has been established. Studies are now in progress to investigate the possibility of combining enrichment procedures or conductimetric assays with serological assays, using polyclonal or monoclonal antibodies.

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References

Anon. (1985) *ISO 7218 Microbiology-General guidance for Microbiological Examinations.* Geneva: International Organization for Standardization.

Ball, S.F.J. and Reeves, J.C. (1991) The application of new techniques in the rapid testing of seed-borne pathogens. *Plant Varieties and Seeds* **4**: 169-176.

Boelema, B.H. (1972) Bacterial blight (*Pseudomonas pisi* Sackett) of peas in South-Africa, with special reference to frost as a predisposing factor (thesis). *Mededelingen Landbouwhogeschool Wageningen* **72:** 77-83.

Bolton, F.J. (1990) An investigation of indirect conductimetry for detection of some food-borne bacteria. *Journal of Applied Bacteriology* **69:** 655-661.

Candlish, A.A.G., Taylor, J.D. and Cameron, J. (1988) Immunological methods as applied to bacterial pea blight. In *Proceedings of the 1988 Brighton Crop Protection Conference-Pests and Diseases* ed. The British Crop Protection Council pp. 787-794. Lavenham, England: The Lavenham Press Limited.

Franken, A.A.J.M. and Van den Bovenkamp, G.W. (1990) The application of the combined use of immunofluorescence microscopy and dilution-plating to detect *Pseudomonas syringae* pv. *pisi* in pea seeds. In *Proceedings of the 7th International Conference on Plant Pathogenic Bacteria* ed. Klement, Z. pp. 871-875. Budapest, Hungary: Akadémiai Kiadó.

Franken, A.A.J.M. and Van der Zouwen, P.S. (1993) Direct and indirect conductimetry for identification and detection of plant pathogenic bacteria. *Journal of Applied Bacteriology* **74:** 234-242.

Franken, A.A.J.M. and Van Vuurde, J.W.L. (1990) Problems and new approaches in the use of serology for seed-borne bacteria. *Seed Science and Technology* **18:** 415-426.

Grondeau, C., Saunier, M., Poutier, F. and Samson, R. (1992) Evaluation of physiological and serological profiles of *Pseudomonas syringae* pv. *pisi* for pea blight identification. *Plant Pathology* **41:** 495-505.

King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* **44:** 301-307.

Kovacs, N. (1956) Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature* **178:** 703.

Lelliot, R.A., Billing, E. and Hayward, A.C. (1966) A determinative scheme for the fluorescent plant pathogenic pseudomonads. *Journal of Applied Bacteriology* **29:** 470-489.

Lyons, N.F. and Taylor, J.D. (1990) Serological detection and identification of bacteria from plants by the conjugated *Staphylococcus aureus* slide agglutination test. *Plant Pathology* **39:** 584-590.

Mazarei, M. and Kerr, A. (1990) Distinguish pathovars of *Pseudomonas syringae* on peas: nutritional, pathogenicity and serological tests. *Plant Pathology* **39:** 278-285.

Mohan, S.K. and Schaad, N.W. (1987) An improved agar plating assay for detecting *Pseudomonas syringae* pv. *syringae* and *P.s.* pv. *phaseolicola* in contaminated bean seed. *Phytopathology* **77:** 1390-1394.

Parmar, N., Easter, M.C. and Forsythe, S.J. (1992) The detection of *Salmonella enteritidis* and *S. typhimurium* using immunomagnetic separation and conductance microbiology. *Letters in Applied Microbiology* **15:** 175-178.

Rasmussen, O.F. and Wulff, B.S. (1991) Detection of *Pseudomonas syringae* pv. *pisi* using PCR. In *Proceedings of the 4th International Working Group on Pseudomonas syringae pathovars* eds Durbin, R.D., Surico, G. and Mugnai, L. pp. 369-376. Florence, Italy: Stamperia Granducale.

Samson, R. and Saunier, M. (1987) Désignation de références sérologiques pour six sérogroupes de pathovars de *Pseudomonas syringae* sur la base de leur lipopolyoside. *EPPO Bulletin* **17:** 165-171.

Sheppard, J.W., Wright, P.F. and Desavigny, D.H. (1986) Methods for the evaluation of EIA tests for use in the detection of seed-borne diseases. *Seed Science and Technology* **14:** 49-59.

Siegel, S. (1956) *Nonparametric Statistics for the Behavioral Sciences* pp. 312. Tokyo: McGraw-Hill Inc..

Taylor, J.D. (1970). Bacteriophage and serological methods for the identification of *Pseudomonas phaseolicola* (Burk.) Dowson. *Annals of Applied Biology* **66:** 387-395.

Taylor, J.D. (1972). Specificity of bacteriophages and antiserum for *Pseudomonas pisi*. *New Zealand Journal of Agricultural Research* **15:** 421-431.

Taylor, J.D., Bevan, J.R., Crute, I.R. and Reader, S.L. (1989) Genetic relationship between races of *Pseudomonas syringae* pv. *pisi* and cultivars of *Pisum sativum*. *Plant pathology* **38:** 364-375.

Taylor, J.D., Dudley, C.L. and Presley, L. (1979) Studies of halo-blight seed infection and disease transmission in dwarf beans. *Annals of Applied Biology* **93:** 167-172.

Taylor, J.D. and Dye, D.W. (1972) A survey of the organisms associated with bacterial blight of peas. *New Zealand Journal of Agricultural Research* **15:** 432-440.

Ur, A. and Brown, D.F.J. (1975) Impedance monitoring of bacterial activity. *Journal of Medical Microbiology* **8**: 19-28.

Van Vuurde, J.W.L., Van den Bovenkamp, G.W. and Birnbaum, Y. (1983) Immunofluorescence microscopy and enzyme-linked immunosorbent assays as potential routine tests for detection of *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* in bean seed. *Seed Science and Technology* **11**: 547-559.

Wong, W.C. (1991) Methods for recovery and immunodetection of *Xanthomonas campestris* pv. *phaseoli* in navy bean seed. *Journal of Applied Bacteriology* **71**: 124-129.

Chapter 3. Development of an enrichment medium to improve the serological detection of *Pseudomonas syringae* pathovar *pisi* extracted from *Pisum sativum* (pea) seeds

Abstract

Pea blight, caused by the seed-borne bacterium *Pseudomonas syringae* pv. *pisi* (*Psp*), can be controlled by the use of disease-free seed. A rapid, reliable and sensitive method for detecting *Psp* in pea seeds could be the use of an enrichment procedure followed by serological confirmation of the presence of the pathogen. *Psp* and growth-interfering pea-associated saprophytes from different seed lots were isolated on semi-selective media and characterized. The sensitivities of the most frequently occurring saprophytes, belonging to *P. fluorescens, P. putida* or to a complex group of *P. syringae* and *P. viridiflava*, were analysed for a range of selective agents by conductimetric assays in Special Peptone Yeast Extract (SPYE), a basic conductance medium. Although the growth of a group of saprophytes could be suppressed, *P. fluorescens* was not affected. In comparison with SPYE, higher and more specific conductance responses for *Pseudomonas* spp., *Psp* included, were obtained in minimal medium supplemented with L-asparagine (AM). AM supplemented with cycloheximide, cephalexin, cefuroxime and boric acid (AMC) was not sufficiently selective for direct automated conductimetric detection of *Psp* in pea seed extracts, due to conductance responses of fluorescent *Pseudomonas* spp.. However, enrichment of seed extracts in AMC in combination with immuno-fluorescence cell staining (IF) was highly suitable, as less than 10^3 cells ml^{-1} could be detected at 27 °C after 48 h enrichment. Pathogenicity testing of positive seed extracts was performed to exclude false positive results in IF due to the presence of cross-reacting *P. s.* pv. *syringae* strains.

Introduction

Pseudomonas syringae pv. *pisi* (*Psp*), a seed-transmitted plant pathogenic bacterium, is the causal agent of pea bacterial blight (Boelema, 1972). The most efficient way to control bacterial blight is the use of resistant varieties and healthy seeds. For determining the health status of pea seeds, reliable, rapid and sensitive detection methods are needed. The traditional test protocol for pea bacterial blight, consisting of extraction, isolation by dilution plating and identification by serology and pathogenicity testing, is laborious and time-consuming (Ball and Reeves, 1991). Additionally, the isolation of *Psp* can be unreliable when stressed *Psp* cells or large numbers of saprophytes are present

in the seeds. Immunofluorescence cell staining (IF) is also unreliable as dead cells can be detected, but can be used as a primary screening technique in combination with the traditional test (Franken and Van den Bovenkamp, 1990). Enzyme-linked immunosorbent assay (ELISA), which can be performed automatically, has a high potential for application, but cannot be used directly due to its low sensitivity of 10^5-10^7 cells ml^{-1} (Candlish *et al.*, 1988). The use of an enrichment procedure prior to serological detection may improve the sensitivity, and, possibly, the specifity when growth of cross-reacting saprophytes can be inhibited. Conductimetry was applied succesfully for the development of enrichment procedures for detection of pathogenic food-borne bacteria (Ur and Brown, 1975; Bolton, 1990) and may be useful for plant pathogenic bacteria as well. Franken and Van der Zouwen (1993) reported some suitable media for conductimetric assays of different plant pathogenic bacteria, *Psp* included. Fraaije *et al.* (1993) used Special Peptone Yeast Extract (SPYE), supplemented with selective agents, to detect *Psp* conductimetrically. However, false positive results due to conductance responses of interfering saprophytes were obtained. Therefore, for conductimetric detection a more selective medium is needed. Selective media can be developed by means of adding antimicrobial agents to a medium which supports growth of the target organism. A rapid and objective way for testing effects of antibiotics is the use of conductimetry, which cannot only show suppression of growth caused by selective agents, but can also give information on the bactericidal rate, duration of suppression and length of exposure required to obtain an effect (Porter *et al.*, 1983; Hogg *et al.*, 1987).

The main goal of our research was to develop an enrichment medium for *Psp* in pea seed extracts, to be used directly in conductimetry or prior to serological detection. Conductance measurements in SPYE were used for determining the effects of selective agents on *Psp* and pea-associated saprophytes. The most suitable agents were used for the development of a selective enrichment medium for *Psp* in pea seed extracts. The final enrichment medium was tested both in conductimetry and in combination with serological and pathogenicity tests. Test results were compared with respect to sensitivity, specificity and speed in order to find the most optimal detection method for *Psp* in pea seeds.

Materials and methods

Bacterial strains

The *Psp* strains 299A, 202 (race 2, NCPPB 3516, National Collection of Plant Pathogenic Bacteria, Harpenden, UK), 870A (race 3, ICMP 10213, International

Collection of Micro-organisms from Plants, Auckland, New Zealand), 895A (race 4), 974B (race 5), 1704B (race 6, ICMP 10222), 2960 (serotype HEL2, Collection of Institute National de la Recherche Agronomique, Angers, France (INRA)) and *P. s.* pv. *syringae* (*Pss*) 1212 (VCRI 112521, Victoria Crop Research Institute, Horsham, Australia), 1480A (serotype APT-PIS) and 2970 (serotype HEL2, INRA) were kindly provided by Dr J.D. Taylor (Horticulture Research International (HRI), Wellesbourne, UK). *Psp* 518 (NCPPB 2222, race 1) and 538 (race 6, serotype RIB, NCPPB 1653) were obtained from the Research Institute for Plant Protection (IPO-DLO), Wageningen, The Netherlands. Serotypes APT-PIS, HEL2 and RIB are described by Grondeau *et al.* (1992).

Media

Nutrient Agar (Oxoid) and Tryptic Soy Broth (TSB; Difco) were used for maintaining bacterial cultures and for growing of overnight cultures, respectively. King's medium B (KB; King *et al.*, 1954) and Sucrose Nutrient Agar (Taylor and Dye, 1972), pH 7.3, containing the selective agents (g l^{-1}): boric acid, 1.5; cephalexine, 0.08 and cycloheximide, 0.2, as reported by Mohan and Schaad (1987) (SNAC) were used for dilution plating of seed extracts. Special Peptone Yeast Extract broth (SPYE; Malthus Instruments Ltd, Crawly, UK) and minimal medium supplemented with L-aspararagine (AM) containing (g l^{-1}): $NH_4H_2PO_4$, 1.5; $MgSO_4.7H_2O$, 0.3; KCl, 0.3; NaCl, 2.3; L-asparagine monohydrate, 7.5; pH 7.2, were used as basic conductance media. SPYEC (Fraaije *et al.*, 1993) and AMC, which are, respectively, SPYE and AM containing the same antibiotics as in SNAC with the extra addition of 0.1 g l^{-1} cefuroxime (N.F. Lyons, personal communication), were used for enrichment of *Psp* in pea seed extracts.

Antibiotics and selective agents

Stock solutions of selective agents were prepared in suitable organic solvents or in filter-sterilized distilled water.

Antisera

The rabbit polyclonal antibody (Pab) 104, prepared against cells of *Psp* strain 519 (race 2, serotype APT-PIS, NCPPB 2585) was obtained from IPO-DLO, Wageningen, The Netherlands. Pab 153C, a *Staphylococcus aureus* conjugated polyclonal antibody prepared against a somatic extract of *Psp* 299A (race 1, serotype APT-PIS, ICMP 10210) was kindly provided by Dr N.F. Lyons (HRI, Wellesbourne, UK). Pab 322, prepared against cells of *P. s.* pv. *helianthi* (serotype HEL2), was kindly provided by Dr R. Samson (INRA, Angers, France).

Sample preparation

Pea seed extract samples were prepared by soaking thousand seeds per seed lot during 6 h at 4 °C in sterile tap water in a volume of 2.0 ml multiplied by the weight of the seeds in grams as described by Franken and Van den Bovenkamp (1990).

Preparation of bacterial inoculum

Bacterial strains were grown overnight in TSB at room temperature on a rotary shaker at 250 rpm, resulting in suspensions of *ca* 10^9 cfu ml^{-1}. Dilutions of overnight cultures were prepared in saline (0.85 % (w/v) NaCl, pH 7) and pea seed extracts for testing pure cultures and inoculated pea seed extracts, respectively.

Isolation and identification of Psp and related pea-associated saprophytes

From different pea seed lots, *Psp* colonies and saprophytes were isolated on SNAC and KB plates by dilution plating of crude seed extracts, directly or after 40 h enrichment in SPYEC at 27 °C. Suspected *Psp* strains, which formed levan colonies on SNAC plates after 72 h incubation at 27 °C, and saprophytes were identified further by slide agglutination tests, IF, pathogenicity tests on pea seedlings, oxidase reaction (Kovacs, 1956), fluorescent pigment production in KB and cellular fatty acid profiling (Janse *et al.*, 1992). Biochemical characteristics were determined by the API 20 NE system (Biomérieux SA, Marcy-l'Etoile, France).

Immunofluorescence cell staining (IF)

Indirect IF of seed extracts, before and after enrichment, was performed with diluted Pab 104 or Pab 322, and 1 : 100 diluted fluorescein-isothiocyanate conjugated secondary goat anti-rabbit antibodies (Sigma) as described by Van Vuurde *et al.* (1983). Rhodamine, 1 : 100 diluted, was used as counter stain. Fifty μl per sample was fixed on multitest slides (diam. 8 mm) and *ca* 2 μl per sample was screened under a UV microscope for the presence of stained cells.

Slide agglutination testing

Staphylococcus aureus conjugated slide agglutination tests were done as described by Lyons and Taylor (1990). Reactions were performed with 5 μl bacterial sample, pure culture of *ca* 10^9 cells ml^{-1} or enriched pea seed extract, and 5 μl Pab 153C, and recorded within 2 minutes after mixing.

Pathogenicity testing

Pathogenicity tests were performed with pea cultivars Kelvedon Wonder or Maxi, which are both susceptible to all known races of *Psp* (Taylor *et al.*, 1989). Suspensions of pure cultures or enriched seed extracts were injected with a syringae under the epidermis of stem and leaves of young, two or three-leaved pea seedlings. Inoculated seedlings were incubated in a greenhouse at 25 °C. Plants showing water-soaked lesions, the typical pea blight reaction caused by *Psp*, hypersensitive host-response reactions, such as brown necrotic spots around inoculation points and stem collapse, or no symptoms were recorded 3-7 days after inoculation.

Conductimetric assays

Conductance responses at 27 °C were measured with the Malthus 2000 series Analyser (Malthus Instruments Ltd, Crawley, UK) using 8 ml cells with 2 ml of conductance medium. The cells were inoculated with 1 ml of seed extract or bacterial suspension in order to detect *Psp* or to determine the sensitivity of bacterial isolates to antimicrobial agents. For determining sensitivities, bacterial concentrations of 10^6-10^7 cfu ml^{-1} were used. Conductance responses were recorded at 18 minutes intervals for 84 h and the detection time (Td; time (h) needed to give an average conductance change of 1 $\mu S\ h^{-1}$), maximum rate of conductance change (ΔG in $\mu S\ h^{-1}$) and the maximum conductance change (G_{max} in μS) were determined.

Testing sensitivity to selective agents

For testing the effect of selective agents conductimetrically, the ratio (in %) of Td or ΔG obtained for the antimicrobial agent containing conductance medium relative to the same medium without selective agent (control) was determined. An isolate was recorded as sensitive (s) to an agent if it did not generate a Td or when ΔG decreased to 0-33 % of the control. An intermediate reaction (i) to an agent was defined when both a Td was generated and the Td was more than 200 % of the control or ΔG was 33-67 % of the control. Resistant reactions (r) were recorded when the Td generated was less than 200 % of the control or ΔG was higher than 67 % of the control. Additionally, a series of antibiotic sensitivities were determined using the API ATB PSE system (API SYSTEM SA, La Balme Les Grottes, France).

Results

Selection and characterization of bacterial strains isolated from pea seeds

Saprophytes and suspected *Psp* colonies, which were isolated from 21 different seed lots, were characterized and identified by physiological, biochemical, serological and pathogenicity tests. On the basis of the physiological and biochemical tests various profiles were obtained (Table 1). All *Psp* and *Pss* reference strains clustered together in profile J. However, *Psp* produced typical water-soaked lesions around inoculution points on stem and leaves of pea seedlings, whereas seedlings inoculated with *Pss* showed brown necrotic hypersensitive responses in pathogenicity testing after five days. The biochemical characteristics of profiles H, I and J were almost identical, but isolates of these profiles could be distinguished from each other by their distinct colony morphology on SNA and KB plates and by their reaction in the pathogenicity tests. Isolates with profile H or I produced no symptoms at all. Saprophytes belonging to the other profiles were biochemically clearly different from *Psp*. Fatty acid analysis designated strains with profiles A, B, C and D to be *P. fluorescens* and strains belonging to profiles E, F and G as *P. putida*. Isolates with profiles H, I and J were clustered together in a complex group of *P. viridiflava* and *P. syringae* pathovars. Isolates with profiles K and L, which were clearly distinct from *Pseudomonas* spp., were presumptively identificated as *Enterobacter* and *Arthobacter* spp., respectively. *Psp* was isolated from 10 of the 21 pea seed samples tested, whereas *P. fluorescens* (profile A), strains with profile H, strains with profile I and *Pss* were the most frequently isolated saprophytes in 8, 11, 5 and 6 out of the 21 seed lots tested, respectively.

With regard to serology, two *Pss* isolates, strains B10 and B12, reacted weakly in slide agglutination with Pab 153C, directed against LPS of *Psp* serogroup APT-PIS, whereas *Pss* B20, and isolate S11 of profile I reacted strongly (Table 2). These isolates were also tested in IF using various dilutions of antisera and several test strains with known serotype. *Pss* B10 and B12 reacted strongly with Pab 322, directed against LPS of *Psp* serogroup HEL2, whereas *Pss* B20 reacted strongly with both Pab 322 and Pab 104, the latter directed against LPS of serogroup APT-PIS. The LPS molecules of serogroups APT-PIS and HEL2 have probably epitopes in common, as representative serogroup APT-PIS isolates reacted strongly with the HEL2 serum, even at the highest dilution, whereas HEL2 isolates were only negative for a reaction with the APT-PIS serum at the lowest concentration tested. The RIB strain *Psp* 538 reacted much stronger with the HEL2 serum than the APT-PIS serum.

Table 1. Biochemical profiling of *Pseudomonas syringae* pv. *pisi* and related pea-associated bacteria isolated from pea seeds

Characteristics	Profiles											
	A	B	C	D	E	F	G	H	I[3]	J	K	L
API 20 NE system[1]:												
Nitrate reduction to nitrite	-	+	-	-	-	-	-	-	-	-	-	v
Indole production	-	-	-	-	-	-	-	-	-	-	-	-
Acid from glucose	-	-	-	-	-	-	-	-	-	-	+	-
Arginine dihydrolase	+	+	+	+	+	+	+	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-
Esculine hydrolysis	-	-	+	-	-	-	-	+	+	v	+	+
Gelatine protease	+	+	+	+	-	-	-	v	v	v	-	+
PNPG β-galactosidase	-	-	-	-	-	-	-	-	-	-	+	+
Assimilation of:												
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	-	+	+	+	+	+
Mannose	+	+	+	+	-	+	-	+	+	+	+	+
Mannitol	+	+	+	+	-	-	-	+	+	+	+	+
N-acetyl-glucosamine	+	+	+	+	-	-	-	-	-	-	+	+
Maltose	-	-	-	-	-	-	-	-	-	-	+	+
Gluconate	+	+	+	+	+	+	+	+	+	+	+	+
Caprate	+	+	+	+	+	+	+	+	+	+	-	-
Adipate	-	-	-	+	+	-	-	-	-	-	-	-
Malate	+	+	+	+	+	+	+	+	+	+	+	-
Citrate	+	+	+	+	+	+	+	+	+	+	+	-
Phenyl-acetate	-	-	-	-	+	-	-	-	-	-	-	-
Other tests[2]:												
Oxidase	+	+	+	+	+	+	+	-[4]	-	-	-	-
Fluorescence on KB	+	+	+	+	+	+	+	-[4]	v	v	-	-
Levan production on SNA	+	+	-	-	-	-	-	-	v	+	-	+

1 The API 20 NE strips were used as described by the manufacturer, readings after 24 and 48 h; + = positive reaction, - = negative reaction, v = variable reaction. Abbreviation PNPG: p-nitrophenyl-β-D-galactopyranoside.
2 *+ = positive reaction, - = negative reaction, v = variable reaction.*
3 Several strains of profile I produced a yellow pigment during the assimilation of glucose, mannose, arabinose and gluconate; the levan production of the strains in this profile was slow compared to the strains of the other profiles.
4 Strains of profile H produced mucous, yellow pigmented kolonies on KB.

Table 2. Serological grouping of *Pseudomonas syringae* strains and cross-reacting isolates by *Staphylococcus aureus* conjugated slide agglutination and immunofluorescence cell staining (IF)

Strains	Serogroup	Agglutination[1]	IF[2]					
			Pab 104 (APT-PIS)			Pab 322 (HEL2)		
			1:100	1:1000	1:5000	1:100	1:1000	1:5000
Test strains:								
Psp[3]	APT-PIS	4	4	4	4	4	4	4
Psp 2960	HEL2	3	3	2	1	4	4	4
Psp 538	RIB	3	3	1	0	3	3	1
Pss 1212	?	0	1	0	0	1	0	0
Pss 1480A	APT-PIS	4	4	4	3	3	3	3
Pss 2970	HEL2	2	3	3	1	4	4	3
Cross-reacting isolates:								
Pss B10		2	1	0	0	4	4	4
Pss B12		3	3	2	1	4	4	4
Pss B20		4	4	4	3	3	3	3
Saprophyte S11		4	1	0	0	1	0	0

1 The slide agglutination test was performed Pab 153C, an APT-PIS serum. Results expressed as: 0 = no reaction within 2 min, 1 = weak reaction within 2 min, 2 = weak reaction within 30 s, 3 = clear agglutionation within 30 s, 4 = strong agglutination within 15 s.

2 IF was performed with dilutions of Pab 104, an APT-PIS serum, and Pab 322, a HEL2 serum. Results expressed as: 0 = no staining, 1 = very weak staining, 2 = weak staining, 3 = clear staining, 4 = strong staining.

3 *Psp* strains 299A, 202, 870A, 895A, 974B and 1704B reacted similarly in the serological tests.

As the IF results of isolate S11 were negative, the positive reaction of this isolate in slide agglutination is probably caused by a non-specific serological reaction.

Testing sensitivity to selective agents

With regard to conductimetry, all isolates belonging to profiles A-K grew rapidly in SPYE at 27°C. To improve the selectivity of SPYE, the sensitivity of *Psp*, *Pss* and isolates with profiles A, H and I, representing the most frequently isolated saprophytes from pea seeds, was tested for a range of selective agents.

Tables 3 summarizes the effects of the antimicrobial agents tested in conductimetry in SPYE and with the API ATB-PSE system.

In comparison with *Psp*, saprophytes of profile I were only slightly more sensitive to cefsulodine, which is a antibiotic with a mode of action related to cefuroxime. Moreover, the rate of growth of saprophytes of profile H and *P. fluorescens* (profile A), the two most interfering saprophytes, was not affected by any of the selective agents tested in comparison with *Psp*. Also the growth of *Pss*, biochemically and serologically closely related to *Psp*, could not be restricted.

Three distinct types of conductance responses were determined in conductimetry, which expressed different modes of action of the selective agents. Firstly, addition of hygromycine B, gramacidine S, gentamicin, hydrogen peroxide and 2,3-butanedione at inhibitory concentrations resulted in an increase of Td, a so called distinct lag phase as reported by Hogg *et al.* (1987), whereas ΔG was not affected. This bactericidal mode of action can be explained by a delay in growth due to resuscitation of injured cells or selection of resistant strains, as reported by *Porter et al.* (1983). Secondly, an equal Td and a decrease of ΔG, reflecting a retarded growth by a more bacteriostatic mode of action, was observed when inhibitory concentrations of moxalactam, sulphamethoxazole and tetracycline were tested. Thirdly, a decrease of Td and increase of ΔG was obtained when low concentrations of agents such as cetrimide, deoxycholic acid, cephalexine and boric acid were tested. This effect of enhanced metabolic activity can be explained by an increased membrane permeability through detergent action in case of cetrimide, deoxycholic acid and boric acid.

Basal medium for conductimetry

In comparison with *Psp*, *Pss* and isolates belonging to profile H or I, the metabolic activities of *P. fluorescens* (profile A, B, C and D) and *P. putida* (profile E, F and G) were much higher in SPYE at 27 °C (Table 4).

Table 3. The sensitivities of *Pseudomonas syringae* pv. *pisi* and various pea-associated isolates to a range of selective agents determined with the API ATB-PSE antibiogram and with conductimetric assays in Special Pepton Yeast Extract broth (SPYE) at 27 °C

		Sensitivities of isolates[2]				
Chemicals	Concentration[1]	A	H	I	*Psp*	*Pss*
API ATB-PSE[3]:						
Piperacilline	16-64	i	s	s	s	s
Imipenen	4-8	i	s	s	s	s
Aztreonam	4-32	r	i	i	i	i
Cefsulodine	8-32	r	i	s	i	i
Ceftazidime	4-32	s	s	s	s	s
Tobramycine	4-8	s	s	s	s	s
Amikacine	8-16	s	s	s	s	s
Gentamicine	4-8	s	s	s	s	s
Netelmicine	8-16	s	s	s	s	s
Colistine	4	r	s	s	s	s
Ciprofloxacine	1-2	s	s	s	s	s
Fosfomycine	32-64	i	s	i	s	s
Cotrimoxazole	2-8	i	i	s	s	s
Conductimetry:						
Gentamicin SO_4	2-10	i	s	s	s	s
Hygromycin B	50-100	i	s	s	s	s
Primaquine $2H_3PO_4$	100-500	r	r	r	r	r
Cephalothin Na	100-500	r	r	r	r	r
Cephalexin hydrate	100-500	r	r	r	r	r
Cefuroxime Na	100-500	r	r	r	r	r
Cefamandole Na	100-500	r	r	r	r	r
Moxalactam Na	100-500	i	i	i	s	s
Monensin Na	100-500	r	r	r	r	r
Nitrofurantoin	100-500	i	i	i	i	i
Carbenicillin Na_2	100-500	r	r	r	i	r
Gramicidin S HCl	50-100	r	r	i	i	r
Sulphamethoxazole	100-500	r	r	r	r	i
Tetracycline HCl	2-10	i	i	i	i	s
Novobiocin Na	100-500	r	r	i	i	i
Nalidixic acid	10-50	r	r	r	r	r
EDTA	100-500	r	i	i	i	i
L(+)-ascorbic acid	1000	r	r	r	s	i
Boric acid	1000-2000	r	r	r	r	r
2,3-butanedione	33-167	r	r	r	r	r
Cetrimide	33-100	r	i	i	i	i
Hydrogen peroxide	1-10	i	s	s	s	s

1 The concentration of a selective agent in the medium tested is given in mg l^{-1}, except for 2,3-butanedione (in ppm), cetrimide (in ppm) and hydrogen peroxide (in mM). Of each selective agents 1 or 2 concentrations were tested, first figure is the lowest concentration.

2 The isolates tested belonged to profiles A, H, I and J, as determined in Table 1. Of each profile at least 10 isolates were tested in the API ATB-PSE antibiogram, while isolates S1 and S22 (A), S13 (H), S10 and S15 (I), *Pss* 1212 and *Psp* B28, 299A and 518 (Psp) were tested conductimetrically. Results expressed as: s = sensitive reaction, no growth, i = intermediate reaction, only growth at the lowest antibiotic concentration, r = resistant reaction, growth not inhibited.

3 The API PSE-ATB antibiogram was used as described by the manufacturer, recording for growth after 48 h was done by visual observation.

Table 4. Conductance responses of *Pseudomonas syringae* pv. *pisi* and other pea-associated *Pseudomonas* spp. at 27 °C.

Isolates[1]		Conductance measurements[2]			
		SPYE		AM	
		ΔG	G_{max}	ΔG	G_{max}
A	(6)	23.4 ± 4.3	335 ± 40	46.4 ± 13.0	928 ± 77
B	(1)	16.2 ± 0.5	314 ± 25	81.8 ± 2.9	980 ± 28
C	(1)	30.9 ± 4.8	327 ± 34	36.5 ± 9.1	956 ± 78
D	(1)	19.8 ± 2.7	303 ± 30	48.4 ± 4.8	831 ± 3
E	(1)	15.7 ± 1.7	320 ± 53	60.5 ± 31.0	946 ± 87
F	(3)	24.9 ± 2.3	270 ± 50	65.7 ± 7.2	918 ± 61
G	(1)	33.2 ± 1.4	371 ± 20	53.1 ± 6.3	998 ± 30
H	(2)	6.6 ± 0.2	310 ± 38	32.3 ± 5.3	865 ± 13
I	(3)	5.4 ± 1.1	217 ± 30	35.3 ± 15.0	939 ± 10
Psp	(10)	5.9 ± 3.7	198 ± 45	24.0 ± 8.0	887 ± 80
Pss	(6)	7.0 ± 3.0	220 ± 50	29.9 ± 7.8	851 ± 80

1 The Pseudomonas spp. strains tested belonged to different profiles (A-I) as determined in Table 1; between brackets number of strains tested.

2 Conductance changes were recorded in Special Peptone Yeast Extract (SPYE) and minimal medium supplemented with 0.75 % (w/v) L-asparagine monohydrate (AM); results expressed as: ΔG = the average maximum rate of conductance change (μS h^{-1}) and G_{max} = the maximum conductance change (μS), recorded within 50 h after detection or after 120 h of incubation in SPYE and AM, respectively.

Therefore, in order to improve the rate of growth of *Psp* relative to other *Pseudomonas* spp. during enrichment, other conductance media based on conversion of charged amino acids were tested. Of the various amino acids tested in minimal medium (data not shown), the conversion of L-asparagine monohydrate by *Psp* was most suited for conductimetry, because extremely high conductance responses up to 3000 μS at a concentration of 3.0 % were recorded (Fig. 1). The maximum conductance change (G_{max}) increased almost linearly with the L-asparagine concentration. However, ΔG decreased at concentrations of L-asparagine monohydrate higher than 2.0 % (w/v). Because the conductance changes in minimal medium were recorded most rapidly with addition of 0.75 % of L-asparagine, this concentration was used in further studies.

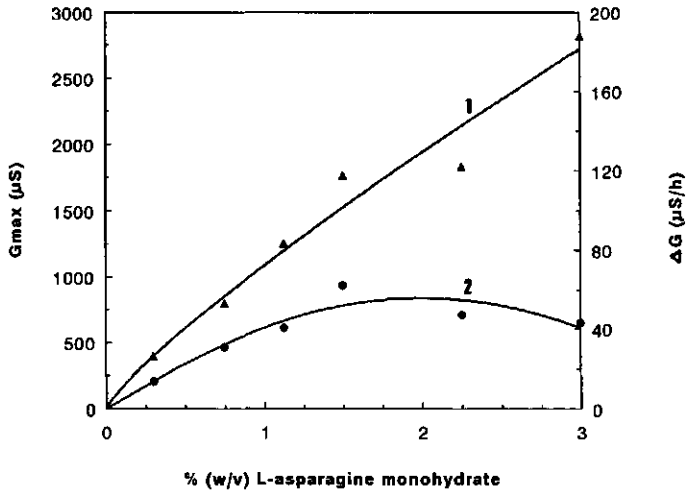

Figure 1. The effect of the L-asparagine concentration on the conductance responses of *Pseudomonas syringae* pv. *pisi* 518 in minimal medium, pH 7.2, at 27 °C. 1 = maximum conductance change (G_{max} in μS), 2 = maximum rate of conductance change (ΔG in $\mu S\ h^{-1}$).

When comparing SPYE with minimal medium supplemented with 0.75 % (w/v) L-asparagine monohydrate (AM), the ΔG of *Psp* was much lower in SPYE than in AM (Table 4). Moreover, the relative differences between *Psp* and the different *Pseudomonas* spp. tested with respect to ΔG were less in AM than in SPYE at 27 °C. Since no additional selective agents were found in this study to improve the medium selectivity significantly, AM was supplemented with the same selective agents as in SPYEC, because these selective agents inhibited the growth of microbes other than *Pseudomonas* spp., incuding *Psp*, and improved the yield of *Psp* after enrichment (data not shown). As higher yields of *Psp* were obtained with seed extracts enriched in AMC rather than in SPYEC (Fig. 2), AMC was used further to test enrichment of *Psp* from pea seeds.

Enrichment of seed extracts in AMC in order to detect Psp; comparison of detection techniques

For 35 different commercial seed lots, the results of dilution plating, serology, conductimetry and pathogenicity testing, performed to detect *Psp* in seed extracts directly or after enrichment in AMC at 27 °C, were compared (Table 5).

For IF performed directly with crude seed extracts, 28 of the 35 seed lots tested were found positive for *Psp* with a concentration of at least 10^3 cells ml^{-1} extract. Of these 28 seed lots, 12 were also found positive by dilution plating and were verified with pathogenicity tests.

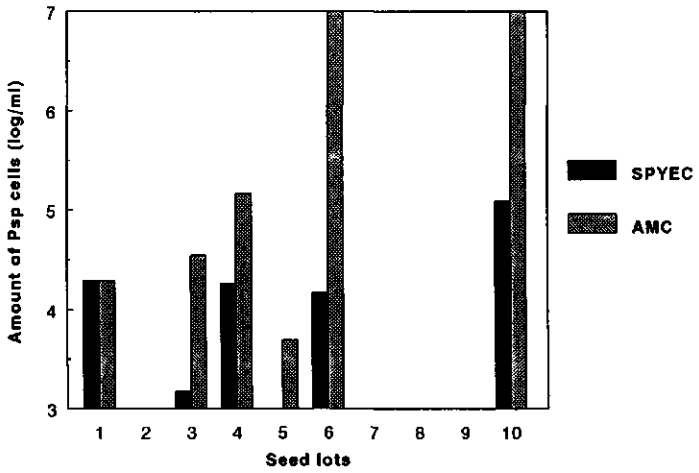

Figure 2. Enrichment of *Pseudomonas syringae* pv. *pisi* in pea seed extracts in selective Special Pepton Yeast Extract Broth (SPYEC) and in a selective L-asparagine based minimal medium (AMC) at 27 °C. After 42 h incubation cell concentrations of *Psp* were determined with immunofluorescence cell staining.

Besides the 12 seed lots positive in dilution plating, seven other lots were found positive for *Psp* with pathogenicity testing of enriched seed extracts. When testing dilutions of pure cultures, the detection threshold of pathogenicity testing was *ca* 10^4 viable cells ml^{-1}, which was determined with dilution plating.

After 48 h of enrichment 33 seed lots were found positive with IF, meaning at least a concentration of 10^3 cells ml^{-1} was detected. After 72 h enrichment only 13 lots were found clearly positive with slide agglutination, due to its low sensitivity of 10^7-10^8 cell ml^{-1}. Furthermore, no correlation was found between contamination levels of *Psp* found in IF or dilution plating of crude seed extracts and Td obtained in conductimetry. Most of the conductance responses of the 21 positive pea seed lots, which were detected within 48 h in AMC at 27 °C, were mainly generated by fast growing fluorescent *P. fluorescens* and *P. putida*, which could be isolated in high numbers by dilution plating after enrichment. However, for inoculated seed extracts of relatively clean seed lots a high correlation ($r = -0.99$) was found between inoculum level of *Psp* and Td in conductimetry (Fig. 3).

Discussion

Psp and related pea-associated saprophytes were isolated from 21 different pea seed lots by dilution plating of seed extracts. The most frequently occurring isolates belonged to *P. fluorescens*, *P. putida* and to a complex group of *P. syringae* and *P. viridiflava*, as shown by fatty acid analysis, and could be distinguished from each other by biochemical and physiological properties (profiles A-L).

Table 5. Results of pea seed lots tested for presence of *Pseudomonas syringae* pv. *pisi* in seed extracts before or after enrichment in L-asparagine minimal medium supplemented with boric acid, cephalexin, cycloheximide and cefuroxime (AMC) at 27 °C.

Seed lots	Plating[1]	IF[2] t = 0 h	IF[2] t = 48 h	Conductimetry[3]	Agglutination[4]	Pathogenicity[5]	
1	4.13 *Psp*	5.59	6.86	21.6	+	+	(48 h)
2	<0.82	<3.00	4.83	>96.0	-	nd	
3	<0.82	<3.00	4.46	>96.0	-	-	(48 h)
4	<0.82	4.87	4.99	77.4	+	+	(48 h)
5	<0.82	3.16	5.47	69.0	-	*	(48 h)
6	<0.82	3.79	5.47	42.6	+	+	(96 h)
7	3.36 *Pss*	<3.00	4.69	18.3	-	-	(48 h)
8	2.34 *Psp*	4.69	5.87	32.1	-	nd	
9	0.82 *Psp*	4.39	4.69	41.7	-	nd	
10	1.67 *Pss*	3.16	3.54	37.5	-	nd	
11	<0.82	<3.00	3.37	68.1	-	nd	
12	<0.82	4.39	5.47	>96.0	+	+	(96 h)
13	2.49 *Psp*	3.33	5.09	20.1	-	nd	
14	1.42 *Psp*	3.97	5.69	39.9	-	-	(96 h)
15	<0.82	<3.00	<3.00	90.9	-	nd	
16	1.52 *Psp*	4.20	4.45	29.7	-	nd	
17	<0.82	<3.00	3.85	63.6	-	-	(48 h)
18	0.82 *Sap*	4.29	4.51	43.8	+/-	+	(96 h)
19	<0.82	<3.00	<3.00	45.3	-	nd	
20	0.82 *Sap*	3.16	3.37	30.9	-	*	(48 h)
21	1.82 *Pss*	4.72	>7.00	25.5	+	-	(96 h)
22	2.12 *Sap*	4.87	6.99	50.7	+	+	(96 h)
23	<0.82	4.32	>7.00	46.8	+	+	(48 h)

Table 5. continued.

Seed lots	Plating[1]	IF[2] t = 0 h	IF[2] t = 48 h	Conductimetry[3]	Agglutination[4]	Pathogenicity[5]
24	1.90 *Psp*	5.17	>7.00	39.3	+	+ (96 h)
25	2.95 *Pss*	3.16	4.43	21.0	-	* (48 h)
26	0.82 *Psp*	4.99	5.87	>96.0	+	+ (96 h)
27	1.87 *Pss*	3.31	4.85	25.8	-	nd
28	0.82 *Sap*	3.90	5.87	29.4	-	nd
29	<0.82	4.33	>7.00	>96.0	+	+ (96 h)
30	2.81 *Psp*	4.99	5.69	25.2	+/-	- (96 h)
31	1.42 *Sap*	3.64	3.77	26.1	-	nd
32	3.10 *Psp*	5.39	6.29	27.9	+	+ (48 h)
33	1.52 *Psp*	4.69	5.39	54.9	+	+ (96 h)
34	<0.82	4.39	4.69	52.2	-	nd
35	0.82 *Psp*	3.70	5.39	50.1	+	+ (96 h)

1 Total number of bacteria in log cells ml^{-1} seed extract on SNAC before enrichment; suspected *P. syringae* colonies were identified with pathogenicity testing as *Pseudomonas syringae* pv. *pisi* (*Psp*), *Pseudomonas syringae* pv. *syringae* (*Pss*) or saprophytic *Pseudomonas* spp. (*Sap*) by causing a water-soaking, host-response or negative reaction, respectively.

2 Concentration of *Psp* in log cells ml^{-1} seed extract, determined with immunofluorescence cell staining (IF), using Pab 104, 1 : 2000 diluted. Seed extracts were screened directly (t = 0 h) or after 48 h enrichment (t = 48 h). Samples with concentrations >log 3.00 cells ml^{-1} were considered to be positive.

3 Detection times (Td) in h of the conductimetric assays in AMC at 27 °C are presented, samples detected within 48 h were considered to be positive.

4 Results of slide agglutination tests performed with seed extracts after 73 h enriched in AMC at 27 °C; Results expressed as: - = no or very weak reaction within 2 minutes, +/- = weak reaction within 30 s and + = clear agglutination within 30 s.

5 Results of pathogenicity tests performed with seed extracts after 48 or 96 h enrichment in AMC at 27 °C; Results expressed as: nd = not determined; + = at least 1 of 3 pea seedlings tested showed typical pea blight symptoms, * = at least 1 of the 3 pea seedlings tested showed hypersensitive host-response reactions caused by *Pss* and - = no reaction observed with 3 pea seedlings.

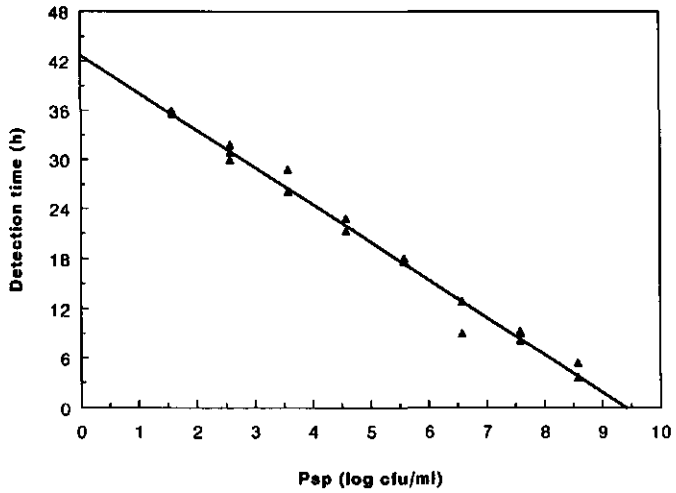

Figure 3. The relationship between detection times in conductimetry and the number of cfu of *Pseudomonas syringae* pv. *pisi* 518 added to pea seed extracts; $y = -4.5x + 42.8$. The conductimetric assays were performed in L-asparagine minimal medium supplemented with boric acid, cephalexin, cycloheximide and cefuroxime (AMC) at 27 °C.

Most *Psp* and *Pss* strains, which clustered together in profile J, could be distinguished by serology. However, some *Pss* isolates which belonged to the APT-PIS and HEL2 serogroups of *Psp* were able to give specific cross-reactions in serology, and for their identification pathogenicity testing was required.

Testing of various antimicrobial agents in conductimetric assays showed that *Psp* was more sensitive to most of the antimicrobial agents tested in comparison with the most frequently isolated saprophytic *Pseudomonas* spp.. Differences in sensitivity to selective agents between *Psp* and *Pss* were not found. Some inhibition of growth was obtained for saprophytes belonging to profile I, but saprophytes of profile H and fast growing *P. fluorescens* isolates of profile A were not affected. Although SPYE was used initially for sensitivity testing and enrichment, AM was shown to be better suited for conductimetry and enrichment. The extremely high conductance changes during conversion of L-asparagine were probably based on excretion of charged ammonium ions, as reported for the yeast *Rhodotorula rubra* (Owens *et al.*, 1992). In AM, the conductance responses for all *Pseudomonas* spp. tested were much higher and more reproducible with respect to G_{max} and ΔG. Moreover, higher yields of *Psp* in seed extracts were obtained after enrichment in AMC in comparison with SPYEC.

Applying AMC for enrichment of *Psp* in seed extracts showed that conductimetry could not be used for direct detection of *Psp*, as detection times were not related to contamination levels of *Psp* in seed extracts. This was mainly

due to competition by fluorescent *Pseudomonas* spp., which also were able to generate conductance responses. However, the use of an enrichment procedure of at least 48 h prior to serological detection has a high potential for application, because of the enhanced sensitivity in the IF method. A high sensitivity is needed to control pea bacterial blight, as seeds carrying a single bacterium can be the primary source of infection in the field (Grondeau *et al.*, 1994).

IF combined with enrichment was the most sensitive technique tested, with a detection threshold of *ca* 10^3 cells ml^{-1}. For routine indexing of seed lots for pea bacterial blight, ELISA might be better suited than IF, because of its higher speed, simplicity and lower cost-price. With regard to the specificity of serological techniques using APT-PIS and HEL2 antisera, false positive reactions were obtained for HEL2 and APT-PIS serotypes of *Pss*, whereas false negative reactions could be caused by RIB isolates of *Psp*. Grondeau *et al.* (1992) reported that, respectively, 11.6 and 10.0 % of *Pss* strains tested belonged to the serogroups APT-PIS and HEL2. Because only 0.1 % of the *Psp* strains belonged to the serogroup RIB, for testing, the risk of false negative reactions will be tolerable. However, the risk of an unacceptable number of false positive reactions, which is dependent on the incidence and contamination levels of seed lots with cross-reacting *Pss*, has to be investigated. To check for cross-reactions in serology after enrichment, samples have to be verified by other specific confirmation tests, such as pathogenicity testing, as described in this study. However, the use of other tests, reducing time and labour, and also able to detect stressed, less virulent cells of *Psp*, such as serology using other specific antisera or PCR (Rasmussen and Wulff, 1991; Reeves *et al.*, 1994) might be more suitable, provided that specific primers are available for the latter technique.

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References

Ball, S.F.L. and Reeves, J.C. (1991) The application of new techniques in the rapid testing for seed-borne pathogens. *Plant Varieties and Seeds* **4**: 169-176.

Boelema, B.H. (1972) Bacterial blight (*Pseudomonas pisi* Sackett) of peas in South-Africa, with special reference to frost as a predisposing factor (thesis). *Mededelingen Landbouwhogeschool Wageningen* **72:** 77-83.

Bolton, F.J. (1990) Conductance and impedance methods for detecting pathogens. In *Rapid Methods and Automation in Microbiology and Immunology* eds Vaheri, A., Tilton, R.C. and Balows, A. pp. 176-182. Berlin: Springer Verlag.

Candlish, A.A.G., Taylor, J.D. and Cameron, J. (1988) Immunological methods as applied to bacterial pea blight. In *Proceedings of the 1988 Brighton Crop Protection Conference-Pests and Diseases* ed. The British Crop Protection Council pp. 787-794. Lavenham, England: The Lavenham Press Ltd.

Fraaije, B.A., Franken, A.A.J.M., Van der Zouwen, P.S., Bino, R.J. and Langerak, C.J. (1993) Serological and conductimetric assays for the detection of *Pseudomonas syringae* pv. *pisi* in pea seeds. *Journal of Applied Bacteriology* **75:** 409-415.

Franken, A.A.J.M. and Van der Zouwen, P.S. (1993) Direct and indirect conductimetry for identification and detection of plant pathogenic bacteria. *Journal of Applied Bacteriology* **74:** 234-242.

Franken, A.A.J.M. and Van den Bovenkamp, G.W. (1990) The application of the combined use of immunofluorescence microscopy and dilution-plating to detect *Pseudomonas syringae* pv. *pisi* in pea seeds. In *Proceedings of the 7th International Conference on Plant Pathogenic Bacteria* ed. Klement, Z. pp. 871-875. Budapest, Hungary: Akadémiai Kaidó.

Grondeau, C., Saunier, M., Poutier, F. and Samson, R. (1992) Evaluation of physiological and serological profiles of *Pseudomonas syringae* pv. *pisi* for pea blight identification. *Plant Pathology* **41:** 495-505.

Grondeau, C., Poutier, F. and Samson, R. (1994) Pea seed contamination by *Pseudomonas syringae* pv. *pisi*: description and consequences. In *Proceedings of the 8th International Conference on Plant Pathogenic Bacteria* eds Lemattre, M., Freigoun, S., Rudolph, K. and Swings, J.G. pp. 779-783. Paris, France: INRA editions (Les Colloques, no66).

Hogg, G.M., Patterson, M.F. and Barr, J.G. (1987) Correlation between antibiotic sensitivity testing by conventional and conductivity measurements. *Journal of Applied Bacteriology* **62:** 189-195.

Janse, J.D., Derks, J.H.J., Spit, B.E. and Van der Tuin (1992) Classification of fluorescent soft rot *Pseudomonas* bacteria, including *P. marginalis* strains, using whole cell fatty acid analysis. *Systematic and Applied Microbiology* **15:** 538-553.

King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicin* **44:** 301-307.

Kovacs, N. (1956) Identification of *Pseudomonas pyocynea* by the oxidase reaction. *Nature* **178:** 703.

Lyons, N.F. and Taylor, J.D. (1990) Serological detection and identification of bacteria from plants by the conjugated *Staphylococcus aureus* slide agglutination test. *Plant Pathology* **39:** 584-590.

Mohan, S.K. and Schaad, N.W. (1987) An improved agar plating assay for detecting *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *phaseolicola* in contaminated bean seed. *Phytopathology* **77:** 1390-1394.

Owens, J.D., Konířová, L. and Thomas, D.S. (1992) Causes of conductance change in yeast cultures. *Journal of Applied Bacteriology* **72:** 32-38.

Porter, I.A., Reid, T.M.S., Wood, W.J., Gibson, D.M. and Hobbs, G. (1983) Conductance measurements for determining antibiotic sensitivity. In *Antibiotics, Assessment of Antimicrobial Activity and Resistance* eds Russell, A.D. and Quesnel, L.P. pp. 49-60. New York: Academic press.

Rasmussen, O.F. and Wulf, B.S. (1991) Detection of *Pseudomonas syringae* pv. *pisi* using PCR. In *Proceedings of the 4th International Working Group on Pseudomonas syringae pathovars* eds Durbin, R.D., Surico, G. and Mugnai, L. pp. 369-376. Florence, Italy: Stamperia Granducale.

Reeves, J.C., Rasmussen, O.F. and Simpkins, S.A. (1994) The use of a DNA probe and PCR for the detection of *Pseudomonas syringae* pv. *pisi* in pea seed. In *Proceedings of the 8th International Conference on Plant Pathogenic Bacteria* eds Lemattre, M., Freigoun, S., Rudolph, K. and Swings, J.G. pp. 383-390. Paris, France: INRA editions (Les Colloques, no66).

Taylor, J.D., Bevan, J.R., Crute, I.R. and Reader, S.L. (1989) Genetic relationship between races of *Pseudomonas syringae* pv. *pisi* and cultivars of *Pisum sativum*. *Plant Pathology* **38:** 364-375.

Taylor, J.D. and Dye, D.W. (1972) A survey of the organisms associated with bacterial blight of peas. *New Zealand Journal of Agricultural Research* **15:** 432-440.

Ur, A. and Brown, D.F.J. (1975) Impedance monotoring of bacterial activity. *Journal of Medical Microbiology* **8**: 19-28.

Van Vuurde, J.W.L., Van den Bovenkamp, G.W. and Bimbaum, Y. (1983) Immunofluorescence microscopy and enzyme-linked immunosorbent assays as potential routine tests for detection of *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* in bean seed. *Seed Science and Technology* **11**: 547-559.

Chapter 4. The development of a conductimetric assay for automated detection of metabolically active soft rot *Erwinia* spp. in potato tuber peel extracts

Abstract

Four media were tested for their ability to detect the soft rot potato pathogens *Erwinia chrysanthemi* (*Ech*) and *Erwinia carotovora* subsp. *atroseptica* (*Eca*) in potato tubers by means of automated conductance measurements. The specificity of the conductimetric assays was determined by testing a set of different *Erwinia* spp. and potato-associated saprophytes, including the genera *Pseudomonas*, *Bacillus*, *Enterobacter* and *Flavobacterium*. All bacteria tested produced conductance responses in Special Peptone Yeast Extract, whereas in minimal medium with L-asparagine only *Erwinia* spp. and *Pseudomonas* spp. were able to generate large conductance responses. In minimal medium supplemented with glucose and trimethylamine N-oxide only Enterobacteriaceae, *Erwinia* spp. included, generated conductance responses, while with pectate as sole carbon source only *Erwinia* spp. produced distinct conductance responses. The pectate medium proved to be particularly useful for specific automated conductimetric detection of *Erwinia* spp. in potato peel extracts. Within 48 h, the detection threshold of the conductrimetric assay for *Eca* varied between 10^2 and 10^3 cfu per ml peel extract at both incubation temperatures of 20 and 26 °C. *Ech* was detected at concentrations of 10^4 to 10^5 or 10^3 to 10^4 cfu ml^{-1} at 20 and 26 °C, respectively. To eliminate false positive reactions in conductimetry caused by *Erwinia carotovora* subsp. *carotovora*, results of the conductance measurements have to be confirmed by other techniques, like serology or DNA assays.

Introduction

Potatoes may suffer from various bacterial diseases. The bacterial genera involved are *Clavibacter*, *Erwinia*, *Pseudomonas* and *Streptomyces* (Salmond, 1992). Because of their great economic impact, much interest is being paid to the detection and identification of the soft rot pathogens *Erwinia chrysanthemi* (*Ech*), *Erwinia carotovora* subsp. *carotovora* (*Ecc*) and *Erwinia carotovora* subsp. *atroseptica* (*Eca*). In The Netherlands only *Ech*, as primary agent of stem wet rot (De Vries, 1990), and *Eca*, as causal agent of blackleg, are considered to be a problem for seed potato production (Anon., 1982). *Eca* and *Ech* are both tuber-transmitted bacteria, and for the control of these soft rot diseases testing of seed potatoes with rapid, reliable and sensitive diagnostic assays is needed. In The Netherlands field inspection and laboratory testing by means of ELISA are

currently used to control blackleg (De Boer *et al.*, 1996).

For automated detection of bacteria, conductance measurements in specific media may be useful. Conductance changes arise as a result of the production or uptake of strongly ionized metabolites, due to bacterial metabolic activity. Direct conductimetric assays based on conversions of uncharged or weakly charged substrates into highly charged products by specific enzymes, like trimethylamine N-oxide (TMAO) reductase, lysine decarboxylase and urease, have shown their potential for rapid screening of food products for food-borne human pathogenic bacteria of the genera *Campylobacter* (Bolton, 1990), *Listeria* (Hancock *et al.*, 1993), *Salmonella* (Gibson, 1987; Ogden, 1988) and *Yersinia* (Walker, 1989). Franken and Van der Zouwen (1993) did demonstrate the feasibility of detecting plant pathogenic bacteria, including the genus *Erwinia*, by means of conductance measurements.

To develop a suitable conductance medium for the detection of soft rot Erwinias there are two major requirements. First, relevant saprophytes isolated from the rhizosphere of potato should not interfere with the conductance responses, and second, detection should occur reasonably fast. In this study several media, based on different substrates, were tested for their ability to generate large and specific conductance changes rapidly during growth of *Erwinia* spp.. Conductance measurements were performed with pure cultures of *Erwinia* spp., potato-associated saprophytes and with inoculated potato peel extracts. The potential of the application of the medium based on pectate for automated conductimetric detection of *Erwinia* spp. in potato peel extracts is demonstrated and discussed.

Materials and methods

Media

Nutrient Agar (NA; Oxoid) and a growth factor medium, containing (g l^{-1}): $NH_4H_2PO_4$, 0.5; $MgSO_4.7H_2O$, 0.05; $FeSO_4.7H_2O$, 0.017; NaCl, 0.1; K_2HPO_4, 0.4; yeast extract (Oxoid), 3.0; glucose, 1.0; bacto-agar (Difco), 15.0; pH 7.2, were used for maintaining bacterial cultures. Tryptic Soy Broth (TSB; Difco) was used for growing of overnight cultures. King's medium B (KB; King *et al.*, 1954) and Double Layer Crystal Violet Pectate medium (DLCVP) of Pérombelon and Burnett (1991), without antibiotics, were used for dilution plating assays of potato peel extracts. Special Peptone Yeast Extract broth (SPYE; Malthus Instruments Ltd, Crawly, UK) and three minimal media containing (g l^{-1}): $NaNO_3$, 1.50; $MgSO_4.7H_2O$, 0.30; NaCl, 2.30; K_2HPO_4, 6.00; KH_2PO_4, 2.25; pH 7.2,

supplemented with either 1.5 % (w/v) sodium polypectate (HP Bulmer, Hereford, England), 0.75 % (w/v) L-asparagine monohydrate or 1.5 % (w/v) glucose + 0.75 % (w/v) trimethylamine N-oxide dihydrate (TMAO) were used in the conductimetric assays.

Bacterial strains

All bacterial strains used in the conductance measurement studies are presented in Table 1.

Isolation of bacterial strains

In addition to the bacterial strains of Table 1, a set of *Erwinia* spp. and saprophytes was isolated from potato peel extracts, directly or after 24 h enrichment at 25 °C in minimal medium supplemented with 0.25 % (w/v) L-asparagine monohydrate, by dilution plating and incubation on DLCVP at 25 °C.

Preparation of samples

Potato peels were extracted by a power-driven roller press (Pollähne, Wennigsen, Germany). Before use, extracts were wringed through cheese cloth and allowed to settle down for 1 h to remove starch and peel debris. Bacterial strains were grown overnight in TSB at room temperature on a rotary shaker at 250 rpm, resulting in suspensions of *ca* 10^9 cfu ml^{-1}. Dilutions of overnight cultures were prepared in saline (0.85 % (w/v) NaCl; pH 7) and potato peel extract for testing pure cultures and inoculated potato peel extracts, respectively.

Identification of bacterial strains

All bacterial strains used in this study were identified or checked by biochemical, serological or fatty acid analysis. Biochemical analysis included acid production from organic compounds, and was used for the characterization of *Erwinia*-type pit-forming bacteria on DLCVP. For this purpose minimal medium of Ayers *et al.* (1919), pH 7.2, was supplemented with 0.3 % (w/v) D-glucose monohydrate, D-salicin, sodium gluconate, raffinose, palatinose, methyl α-D-glucopyranoside, L-asparagine monohydrate or lactose. Bromthymol blue was used as pH-indicator (blue-green), as suggested by Dickey and Kelman (1988). Wells of 96-well microtitre plates were filled with 200 μl of the various media, and 100 μl of an overnight culture diluted to *ca* 10^7 cfu ml^{-1}, was added. The microtitre plates were covered with mineral oil and incubated at 25 °C. Anaerobic acid production was recorded by change in colour of the pH-indicator, blue-green to yellow, for a period of three days.

Table 1. Bacterial strains used for conductrimetric testing of media for specific detection of *Erwinia* spp..

Bacteria	Strain designation	Source[1]
E. c. subsp. *atroseptica* (*Eca*)	1061, 1064, 1068, 1071, P603, P606, P609, P644	1
	A18, A20, A35	2
	707	3
E. c. subsp. *carotovora* (*Ecc*)	139, 257, 921, 972, S94	1
	C12, C18, C21, C27, C28, C29	2
	955	3
E. chrysanthemi (*Ech*)	976, 979, 990, 991, P602, P650, P652, P653	1
	B18, B22	2
	482, 581, 1883	3
Enterobacter spp. (*Ent*)	S89, S113	1
	S14, S15, S16, S17	2
Flavobacterium spp. (*Fla*)	S18	2
	1400	3
Bacillus polymyxa (*Bac*)	S23, S24	2
Klebsiella spp. (*Kle*)	S119	1
Xanthomonas maltophilia (*Xma*)	S11, S21	2
Comamonas spp. (*Com*)	S103	1
Janthinobacterium spp. (*Jan*)	S126	1
Pseudomonas fluorescens (*Pfl*)	S96	1
	S13	2
Pseudomonas putida (*Ppu*)	S110	1
Pseudomonas aerofaciens (*Pae*)	S109	1
Unidentified saprophyte	S12	2

1 Source names and location: 1 = Research Institute for Plant Protection (IPO-DLO, Wageningen, The Netherlands), 2 = Centre for Plant Breeding and Reproduction Research (CPRO-DLO, Wageningen, The Netherlands), 3 = Plant Protection Service (PD, Wageningen, The Netherlands).

Direct immunofluorescence cell staining (IF) was performed according to Van Vuurde *et al.* (1983), using fluorescein-isothiocyanate (FITC) conjugated polyclonal antibodies (Pabs) 8898 and 9024/5C, obtained from IPO-DLO, Wageningen, The Netherlands. The working-dilution of FITC-labeled Pab 8898, prepared against whole cells of *Eca* strain 161 (collection of IPO-DLO) and absorbed with cross-reacting *Ecc* and *Comamonas* strains to reduce cross-reactions, was 1 : 1000. Pab 9024/5C, prepared against a cell extract of *Ech* strain 502 (IPO-DLO collection), was used in a 1 : 500 dilution.

The cellular fatty acid analyses were performed by the Plant Protection Service, Wageningen, The Netherlands, using the Microbial Identification System (MIDI, Newark, DE, USA).

Conductance measurements

All conductance responses were measured with a Malthus 2000 series Analyser (Malthus Instruments Ltd, Crawley, UK). In most experiments reusable 8 ml tubes with 2 ml of culture medium were inoculated with 0 to 1 ml bacterial suspension or peel extract and filled with distilled water till a final volume of 3 ml. Conductance responses of pure cultures were recorded in duplicate at 18 minute intervals for 84 h at 27 °C, and detection time (Td in h), maximum rate of conductance change (ΔG in $\mu S\ h^{-1}$) and the maximum conductance change (G_{max} in μS) were determined. The detection time was defined as the time at which three consecutive conductance changes of 1.0 μS or more were recorded. The conductance responses of *Erwinia*-inoculated peel extracts were recorded at 20 and 26 °C. To determine the detection thresholds of inoculated extracts in PM, detection was achieved when conductance responses exceeded 50 or 75 μS at 20 and 26 °C, respectively.

Results

Identification of bacterial strains isolated from potato tubers

To develop a selective conductance medium for the detection of *Erwinia* spp. on potato tubers, a set of possibly interfering saprophytes was isolated and identified to be tested later in conductimetric assays. Suspected pectolytic *Erwinia* spp. strains (pit-forming colonies on DLCVP) and (non-)pectolytic saprophytes isolated from potato peel extract were identified by biochemical, serological and fatty acid analysis. The results of the biochemical tests of the isolated pectolytic *Erwinia* spp. strains are presented in Table 2.

Table 2. Profiles of *Erwinia* spp. isolated from potato tubers based on biochemical characterization

Carbon source	Profile A	Profile B	Profile C
Acid production from:			
D-glucose	+	+	+
D-salicin	+	+	+
Lactose	+	+	-
Gluconate	+	v	+/-
Raffinose	+	+	v
Palatinose	+	-	-
Methyl α-D-glucopyranoside	+/-	-	-
Alkali production from:			
L-asparagine	+	+	-[1]

+ = strong acid (yellow) or alkali (blue) production, +/- = weak acid production (green-yellow), - = no acid or alkali production (green-blue), v = variable reaction.
1 Most Ech strains tested utilized L-asparagine without alkalinization of the medium.

All fermentative strains belonging to profiles A, B and C were designated by fatty acid analysis as *Eca*, *Ecc* and *Ech*, respectively. Strong fermentative saprophytes, biochemically related to *Erwinia* spp. but not pectolytic (no pit-forming colonies on DLCVP), were designated as *Enterobacter* spp. (e.g. *Ent* S14, S15, S16 and S17 in Table 1). Pectolytic, weak or non-fermentative saprophytes were identified by fatty acid analysis as *Flavobacterium* spp. (*Fla* S18), *Bacillus polymyxa* (*Bac* S23 and S24) or remained unidentified (S12). Other sets of saprophytes, non-pectolytic with a more respiratory type of metabolism, were identified as *Pseudomonas fluorescens* (*Pfl* S13) and *Xanthomonas maltophilia* (*Xma* S11 and S21).

Serological analysis by immunofluorescence showed all *Eca* strains tested to react strongly with the *Eca* antiserum Pab 8898, except for strain *Eca* A20. This strain was recently isolated and showed only a weak reaction. Among the remaining *Erwinia* spp. and saprophytes tested, only *Ent* S89 was able to give a strong reaction with Pab 8898, whereas *Com* S103 and *Jan* 126 reacted weakly. All *Ech* strains tested reacted strongly with the *Ech* antiserum Pab 9024/5C. Among the other bacteria tested, only *Pse* S96 and *Ppu* S110 reacted strongly with Pab 9024/5C, whereas *Pae* S109 and *Ent* S113 reacted weakly.

In addition to bacterial strains isolated from potato tubers some ELISA cross-reacting organisms, such as *Enterobacter*, *Janthinobacterium*, *Comamonas* and *Pseudomonas* spp., were included in the conductivity studies.

Selection of an Erwinia-specific conductance medium

Pure cultures of all bacteria selected (see Table 1), *Erwinia* spp. as well as potato-associated saprophytes, were tested in various media at 27 °C (Table 3). All bacteria tested were able to give a distinct increase in conductance in SPYE. In minimal medium with L-asparagine (AM), all *Pseudomonas* and *Erwinia* spp. tested were able to produce large conductance changes up to *ca* 1100 μS, with the exception of three Ech strains (*Ech* 482, B18 and B22). *Enterobacter*, *Janthinobacterium* and *Comamonas* spp. gave significantly lower responses in AM, while other bacterial species tested did not give a conductance respons at all. In minimal medium with glucose and TMAO (GTM) only *Erwinia*, *Enterobacter* and *Klebsiella* spp. were able to produce positive conductance changes. When TMAO was omitted also positive conductance changes were obtained, however, these responses were much weaker, viz. 10 to 50 % of the conductance signal in GTM (results not shown). Strains of *Pseudomonas* spp. produced negative conductance changes in GTM as a result of a decreasing conductivity. In minimal medium with pectate as the sole carbon source (PM), only *Erwinia* spp. strains were able to produce signals higher than 50 μS within 84 h. An example of the conductance changes in the various media is given for *Ecc* strain 257 (Fig. 1).

Because of the large conductance responses in AM and the high specificity of PM when testing pure cultures, the potential of both media for detection of *Erwinia* spp. in potato peel extracts was further analysed.

Conductimetric assays of inoculated potato peel extracts

When 1 ml peel extract was added to 2 ml saline and directly tested for conductance changes at 20 °C, conductance responses of 200 up to 600 μS were recorded. These signals were generated by bacteria present in the extracts, as no signal was obtained with filter-sterilized extract (Fig. 2). The peel extract signal coincided with the signal of L-asparagine conversion when 1 ml extract was added to minimal medium with 0.375 % (w/v) L-asparagine monohydrate (Fig. 2). As particularly amino acids carrying net charges at pH values between 4 and 8 are able to cause conductance changes during conversion, a range of amino acids, which are known to be present in potato tubers, were tested.

Table 3. Conductance responses of *Erwinia* spp. and potato-associated saprophytes in various media at 27 °C

Bacterial species[1]	Cells added[2]	Medium[3]	Td[4]	ΔG[5]	G_{max}[6]
Erwinia carotovora subsp. *atroseptica* (12)	5.9-6.6	SPYE	4.8-7.2	31.8-76.0	214-233
		AM	7.7-13.2	45.5-82.2	898-1091
		GTM	24.6-37.2	7.5-15.3	114-204
		PM	15.8-21.3	12.0-18.0	132-220
Erwinia carotovora subsp. *carotovora* (12)	6.0-6.7	SPYE	3.6-6.0	48.2-77.9	200-270
		AM	4.2-8.9	77.9-163.5	968-1113
		GTM	14.1-26.4	8.4-14.1	86-167
		PM	10.8-15.6	12.9-21.6	162-255
Erwinia chrysanthemi (13)	5.5-6.7	SPYE	5.4-9.5	19.7-52.2	220-359
		AM	12.9--	--17.3	--1055
		GTM	20.9-48.0	8.1-16.7	64-180
		PM	18.5-29.6	8.0-16.2	216-290
Enterobacter ssp. (6)	5.9-7.0	SPYE	3.0-5.7	23.2-124.4	223-716
		AM	18.3-46.1	5.5-12.5	134-537
		GTM	12.3-25.8	5.9-11.7	0-127
		PM	-	-	-
Flavobacterium ssp. (2)	7.0	SPYE	17.0-26.4	6.7-8.5	326-414
		AM	-	-	-
		GTM	-	-	-
		PM	-	-	-
Bacillus polymyxa (2)	5.3-5.8	SPYE	7.2-9.3	6.3-7.4	54-58
		AM	-	-	-
		GTM	-	-	-
		PM	-	-	-
Klebsiella ssp. (1)	6.7	SPYE	3.0	91.7	606
		AM	46.1	7.2	206
		GTM	12.3	11.7	127
		PM	-	-	-

Table 3. continued.

Bacterial species[1]	Cells added[2]	Medium[3]	Td[4]	ΔG[5]	G_{max}[6]
Xanthomonas maltophilia (2)	7.0-7.1	SPYE	10.4-12.5	11.4-18.4	352-433
		AM	-	-	-
		GTM	-	-	-
		PM	-	-	-
Comamonas ssp. (1)	6.9	SPYE	41.6	8.5	204
		AM	-	3.7	163
		GTM	-	-	-
		PM	-	-	-
Janthinobacterium spp. (1)	7.0	SPYE	39.0	5.5	169
		AM	-	-	169
		GTM	-	-	-
		PM	-	-	-
Pseudomonas ssp. (4)	6.2-6.7	SPYE	8.0-21.3	5.7-41.2	273-338
		AM	6.3-8.3	42.7-68.2	683-846
		GTM	-	-	-82-0
		PM	-	-	-
Unidentified saprophyte (1)	6.6	SPYE	58.2	5.7	200
		AM	-	-	-
		GTM	-	-	-
		PM	-	-	-

- = no detection.
1 Bacterial species; between brackets number of strains tested.
2 Number of cells added in log cfu; when more strains were tested, the range of the numbers added is given.
3 SPYE = Special Peptone Yeast Extract, AM = L-asparagine minimal medium, PM = pectate minimal medium, GTM = glucose-TMAO minimal medium.
4 Td = detection time (h).
5 ΔG = maximum conductance change rate (μS h^{-1}); only maximum conductance change rates higher as 3.5 μS h^{-1} were recorded.
6 G_{max} = maximum conductance change (μS); only maximum conductance changes higher as 50 μS were recorded.

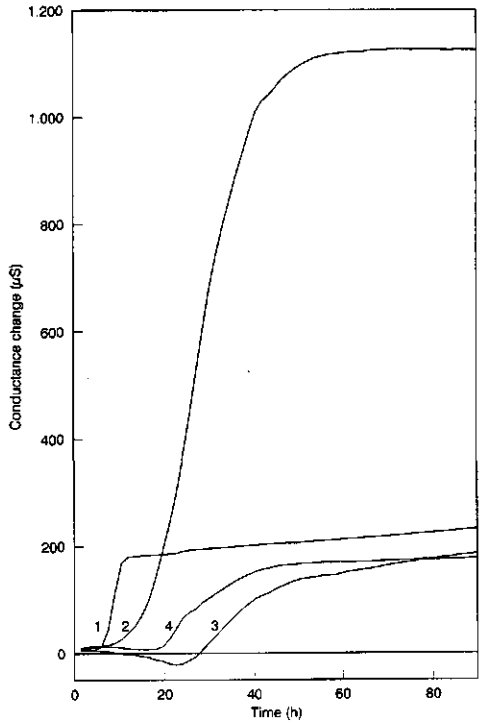

Figure 1. Conductance responses of 10^6 cfu of *Erwinia carotovora* subsp. *carotovora* in different media at 27 °C. 1 = Special Peptone Yeast Extract (SPYE), 2 = L-asparagine minimal medium (AM), 3 = Glucose-TMAO minimal medium (GTM), 4 = Pectate minimal medium (PM).

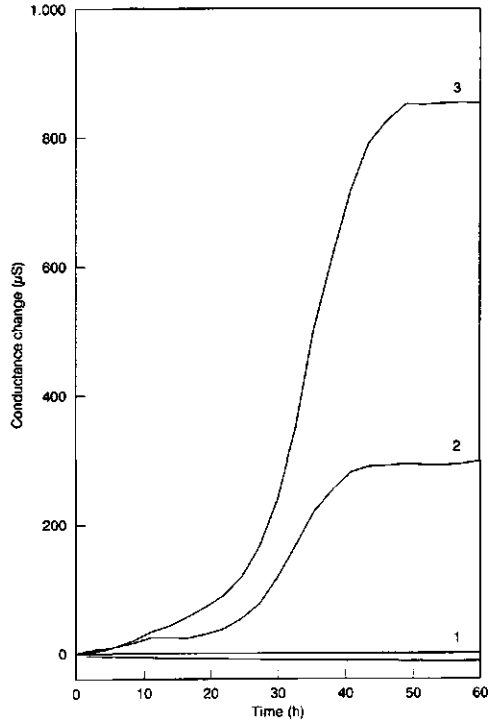

Figure 2. Conductance responses of potato peel extracts at 20 °C. 1 = 1 ml filter-sterilized extract in 2 ml saline, 2 = 1 ml peel extract in 2 ml saline, 3 = 1 ml peel extract in 2 ml minimal medium supplemented with 0.375 % (w/v) L-asparagine.

In minimal medium supplemented with 0.25 % (w/v) L-aspartic acid, L-arginine, L-cysteine, L-glutamic acid, L-glutamine, L-histidine, L-methionine, L-serine, L-threonine or DL-tryptophan, only with aspartic acid conductance responses higher than 50 μS were recorded for *Erwinia* spp., varying from 400 to 700 μS at 27 °C. As *Pseudomonas* spp., which could be isolated in high numbers after enrichment of peel extracts, can also generate conductance responses in minimal media with asparagine or its degradation product aspartic acid, the more selective PM was used in further studies. To reduce the interfering signal of peel extracts itself, extracts were 10 times diluted. By testing various bacterial concentrations, ranging from 10^2 to 10^8 cells ml^{-1}, in potato peel extract, the sensitivity of the conductimetric assays for detecting *Eca* and *Ech* was determined. The detection threshold, i.e. the lowest concentration of bacterial cells in potato peel extract that was found to be detected in PM within 48 h, for *Eca* varied between 10^2 and 10^3 cfu ml^{-1} at both 20 and 26 °C, dependent on the potato peel extract used. *Ech* was detected between 10^4 and 10^5 or 10^3 and 10^4 cfu ml^{-1} at 20 and 26 °C, respectively. Representative examples of conductance measurements of various *Ech* and *Eca* concentrations in potato peel extracts at 20 °C are shown in Figure 3.

Discussion

Because all bacteria tested were able to produce conductance responses in SPYE rapidly, this medium is not suited for specific detection of *Erwinia* spp..

In GTM, only the Enterobacteriaceae tested, *Erwinia* spp. included, were able to give positive conductance responses. As also signals were obtained without TMAO, the signal in GTM is only partly due to TMAO reductase activity, as reported before by Owens *et al.* (1985).

In AM, all *Erwinia* spp. and *Pseudomonas* spp. tested were able to generate large conductance responses, due to the presence of asparaginases, enabling the bacteria to use L-asparagine as a sole carbon and nitrogen source. Asparaginases in *Erwinia carotovora* are present in large amounts, emphasized by the recovery of this enzym at large scale for commercial purposes (Lee *et al.*, 1986). The high preference for asparagine is also demonstrated by a strong positive chemotaxis of *Erwinia* spp. to this compound in root exudates (Stanghellini, 1982). The large conductance changes during microbial conversion of asparagine and aspartic acid are primarily based on excretion of charged ammonium ions and alkalinization of the medium, as reported for the yeast *Rhodotorula rubra* (Owens *et al.*, 1992).

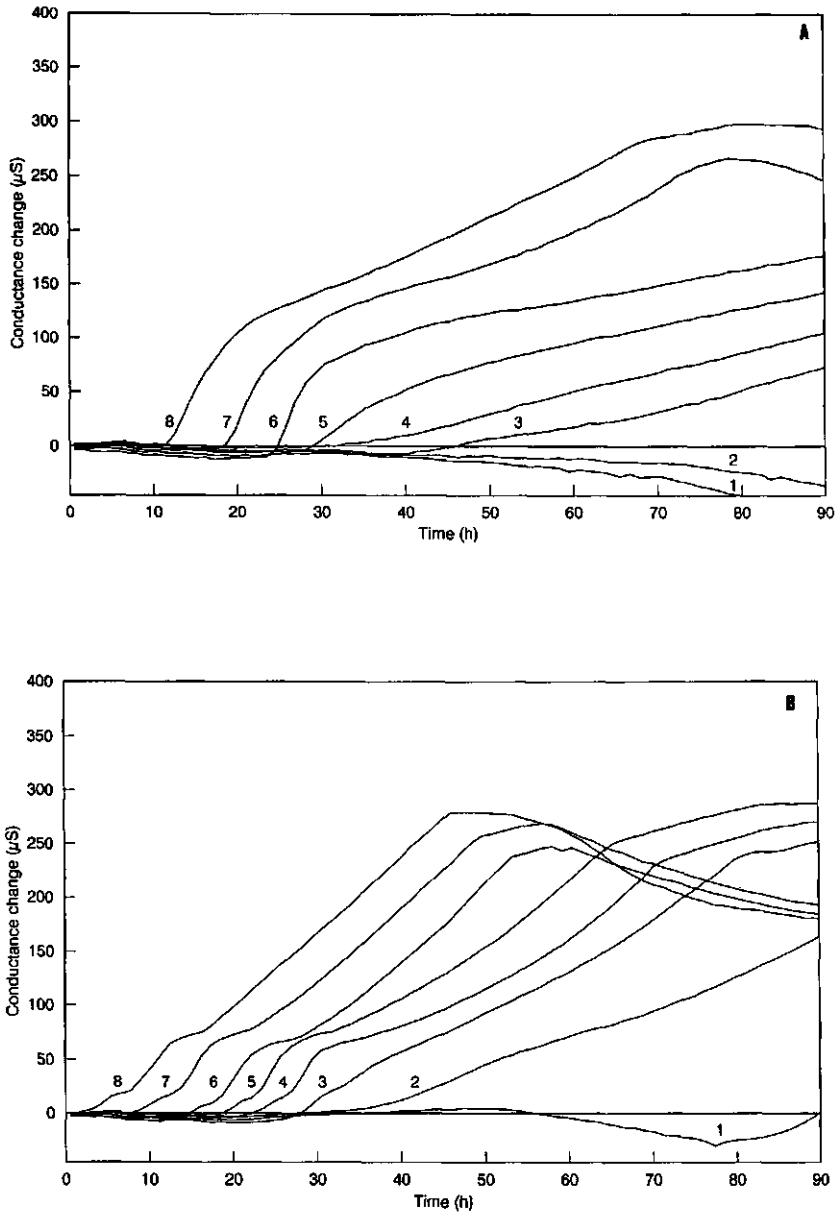

Figure 3. Conductance responses of potato peel extracts inoculated with *Erwinia chrysanthemi* (**A**) and *Erwinia carotovora* subsp. *atroseptica* (**B**) in pectate minimal medium at 20 °C. 1 = unspiked peel extract, 2 = 10^2, 3 = 10^3, 4 = 10^4, 5 = 10^5, 6 = 10^6, 7 = 10^7, 8 = 10^8 cfu ml^{-1} potato peel extract.

In PM, only *Erwinia* spp. were able to generate conductance responses higher than 50 μS. With regard to the pectate conversion, plant pathogenic *Erwinia* spp. produce a range of pectolytic enzymes, which are considered to be a major virulence factor (Collmer and Keen, 1986). The mechanism behind the increase in conductance in PM is associated with extracellular hydrolysis of pectate polymers (Owens, 1985). During the depolymerization of pectate by pectate lyases and polygalacturonases, large amounts of a series of saturated and unsaturated oligogalacturonides and D-galacturonic acid are produced by *E. carotovora* (Stack *et al.*, 1980) and *E. chrysanthemi* (Preston *et al.*, 1992). Among the other bacteria tested, only the *Klebsiella* spp. was able to grow rapidly in pectate minimal medium, however, the conductance signal did not exceed 50 μS. The growth of *Klebsiella* spp. was only associated with pectate lyase activity, resulting in accumulation of large amounts of unsaturated oligo-galacturonides in the medium (results not shown). Therefore, pectate lyase activity, in contrast with polygalacturonase activity of *Erwinia* spp., seems not to be an important conductimetric factor. The lack of rapid growth in PM by the other bacteria may be explained by the absence of pectolytic enzymes, specific growth factors or induction of extracellular pectolytic enzym production.

For conductimetric detection of *Eca* and *Ech*, PM is more suited than AM, because of its higher specificity and the inability of some *Ech* strains to grow in AM. Besides *Ecc*, no false positive conductance responses higher than 50 μS were obtained in PM with the common potato-associated saprophytes tested. After testing peel extracts in conductimetry, an interfering signal of peel extract itself was recorded. This signal is probably due to bacterial conversion of asparagine, which is present in relatively large amounts in potato tubers (Woolfe, 1987), at levels high enough to cause the conductance responses found in this study. To reduce the interfering signal of peel extract itself in PM, extracts were 10 times diluted. Within 48 h, *Eca* was detected with conductimetry at concentrations of 10^2 to 10^3 cfu per ml peel extract at both 20 and 26 °C. *Ech* was detected at concentrations of 10^4 to 10^5 or 10^3 to 10^4 cfu ml^{-1} at 20 and 26 °C, respectively. The temperature-dependent detection thresholds of *Eca* and *Ech* can be explained by differences in growth rate and competition between *Erwinia* spp. and other bacteria present in potato peel extracts. It is known that *Eca* tends to predominate in rotting tubers and diseased stems at temperatures lower than 25 °C, whereas *Ecc* and *Ech* prevail at higher temperatures (Pérombelon and Kelman, 1987).

For blackleg laboratory testing of potato seed lots ELISA is applied in The Netherlands. However, some *Erwinia carotovora* subsp. *carotovora*, *Comamonas*,

Pseudomonas, *Janthinobacterium* and *Enterobacter* spp. strains can cross-react with the LPS-based polyclonal antisera against *Eca* and *Ech*, as reported by Van der Wolf *et al.* (1993, 1994). Furthermore, the sensitivity of ELISA of 10^6 to 10^7 cells per ml peel extract is not high enough to guarantee the development of healthy potato plants in the field next year. With regard to specificity and sensitivity, automated conductance measurements in PM combined with ELISA, using monoclonal antibodies (Gorris *et al.*, 1993), or PCR (De Boer and Ward, 1995; Smid *et al.*, 1995) for verification of positive conductimetric samples may be better suited for routine large-scale blackleg indexing of potato seed lots. This is currently under study.

Acknowledgements

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References

Ayers, S.H., Rupp, P. and Johnson, W.T. (1919) A study of the alkali forming bacteria in milk. *Bulletin of United States Departement of Agriculture* **782:** 1-39.

Anon. (1982) *Zwartbenigheid en stengelnatrot bij aardappelen* pp. 1-12. Wageningen, The Netherlands: Werkgroep Onderzoek Knolziekten.

Bolton, F.J. (1990) Conductance and impedance methods for detecting pathogens. In *Rapid Methods and Automation in Microbiology and Immunology* eds Vaheri, A., Tilton, R.C. and Balows, A. pp. 176-182. Berlin: Springer Verlag.

Collmer, A. and Keen, N.T. (1986) The role of pectic enzymes in plant pathogenesis. *Annual Review of Phytopathology* **24:** 383-409.

De Boer, S.H., Slack, S.A., Van den Bovenkamp, G.W. and Mastenbroek, I. (1996) A role for pathogen indexing procedures in potato certification. In *Advances in Botanical Research Vol. 23, Pathogen Indexing Technologies* eds Andrews, J.W., Tommerup, I.C. and De Boer, S.H. pp. 217-242. London: Academic Press.

De Boer, S.H. and Ward, L.J. (1995) PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. *Phytopathology* **85:** 854-858.

De Vries, P.M. (1990) Stengelnatrot symptomen in aardappelplanten. *Aardappelwereld* **4:** 15-18.

Dickey, R.S. and Kelman A. (1988) B. Erwinia. In *Laboratory guide for identification of plant pathogenic bacteria* ed. Schaad, N.W., pp. 44-59. St. Paul, Minnesota: The American Phytopathological Society.

Franken, A.A.J.M. and Van der Zouwen, P.S. (1993) Direct and indirect conductimetry for identification and detection of plant pathogenic bacteria. *Journal of Applied Bacteriology*, **74:** 234-242.

Gibson, D.M. (1987) Some modification to the media for rapid automated detection of salmonellas by conductance measurement. *Journal of Applied Bacteriology* **63:** 299-304.

Gorris, M.T., Alarcon, B., Lopez, M.M. and Cambra, M. (1994) Characterization of monoclonal antibodies specific for *Erwinia carotovora* subsp. *atroseptica* and comparison of serological methods for its sensitive detection on potato tubers. *Applied and Environmental Microbiology* **60:** 2076-2085.

Hancock, I., Bointon, B.M. and Mc Athey, P. (1993) Rapid detection of *Listeria* species by selective impedimetric assay. *Letters in Applied Microbiology* **16:** 311-314.

King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicin* **44:** 301-307.

Lee, S.M., Ross, J.T., Gustafson, M.E., Wroble, M.H. and Muschik, G.M. (1986) Large-Scale recovery of L-asparaginase from *Erwinia carotovora*. *Applied Biochemistry and Biotechnology* **12:** 229-247.

Ogden, I.D. (1988) A conductance medium to distinguish between *Salmonella* and *Citrobacter* spp.. *Journal of Food Microbiology* **7:** 287-297.

Owens, J.D. (1985) Formulation of culture media for conductimetric assays: theoretical considerations. *Journal of General Microbiology* **131:** 3055-3076.

Owens, J.D., Konířová, L. and Thomas, D.S. (1992) Causes of conductance change in yeast cultures. *Journal of Applied Bacteriology* **72:** 32-38.

Owens, J.D., Miskin, D.R., Wacher-Viveros, M.C. and Benge, L.C.A. (1985) Sources of conductance changes during bacterial reduction of trimethylamine oxide to trimethylammonium in phosphate buffer. *Journal of General Microbiology* **131:** 1357-1361.

Pérombelon, M.C.M. and Burnett, E.M. (1991) Two modified crystal violet pectate (CVP) media for the detection, isolation and enumeration of soft rot erwinias. *Potato Research* **34:** 79-85.

Pérombelon, M.C.M. and Kelman, A. (1987) Blackleg and other potato diseases caused by soft rot erwinias: proposal for revision of terminology. *Plant Disease* **71:** 283-285.

Preston, J.F., Rice, J.D., Ingram, L.O. and Keen, N.T. (1992) Differential depolymerization mechanisms of pectate lyases secreted by *Erwinia chrysanthemi* EC16. *Journal of Bacteriology* **174:** 2039-2042.

Salmond, G.P.C. (1992) Bacterial diseases of potatoes: from classical phytobacteriology to molecular pathogenicity. *Netherlands Journal of Plant Pathology* **98:** 115-126.

Smid, E.J., Jansen, A.H.J. and Gorris, L.M.G. (1995) Detection of *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* in potato tubers using polymerase chain reaction. *Plant Pathology* **44:** 1058-1069.

Stack, J.P., Mount, M.S., Berman, P.M. and Hubbard, J.P. (1980) Pectic enzyme complex from *Erwinia carotovora*: a model for degradation and assimilation of host pectic fractions. *Phytopathology* **70:** 267-272.

Stanghellini, E.M. (1982) Soft-rotting bacteria in the rhizosphere. In *Phytopathogenic prokaryotes* eds Mount, M.S. and Lacy, G.M. Vol. 1, pp. 249-260. New York: Academic Press.

Van der Wolf, J.M., Van Beckhoven, J.R.C.M., De Boef, E. and Roozen, N.J.M. (1993) Serological characterization of fluorescent Pseudomonas strains cross-reacting with antibodies against Erwinia chrysanthemi. *Netherlands Journal of Plant Pathology* **99:** 51-60.

Van der Wolf, J.M., Van Beckhoven, J.R.C.M., De Vries, Ph.M. and Van Vuurde, J.W.L. (1994) Verification of ELISA results by immunomagnetic isolation of antigens from extracts and analysis with SDS-PAGE and Western blotting, demonstrated for *Erwinia* spp. in potatoes. *Journal of Applied Bacteriology* **77:** 160-168.

Van Vuurde, J.W.L., Van den Bovenkamp, G.W. and Birnbaum, Y. (1983) Immunofluorescence microscopy and enzyme-linked immunosorbent assays as potential routine tests for detection of *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* in bean seed. *Seed Science and Technology* **11:** 547-559.

Walker, S.J. (1989) Development of an impedimetric medium for detection of *Yersinia enterolitica* in pasteurized milk. In *Modern Microbiological Methods for Dairy Products, Proceedings of International Seminar in Santander*, Spain, pp.288-291.

Woolfe, J.A. (1987) Proteins and other nitrogenous constituents of the tuber. In *Potato in the human diet*, pp. 58-82. Cambridge: University Press.

Chapter 5. Analysis of conductance responses during depolymerization of pectate by soft rot *Erwinia* spp. and other pectolytic bacteria isolated from potato tubers

Abstract

Different bacteria isolated from potato tubers were screened for their pectolytic properties by examining pitting in polypectate agar, recording conductance responses in polypectate medium and performing potato tuber soft rot tests. For bacteria found positive in conductimetry, the role of polygalacturonase (PG) and pectate lyase (PL) in the generation of conductance changes in a polygalacturonic acid (PGA) medium was further analysed using enzyme activity staining after gel electrophoresis and high-performance anion exchange chromatography. The extent of the conductance changes during depolymerization of PGA was dependent on the amounts of galacturonate monomers and oligomers accumulated in the medium. In comparison with an unidentified saprophyte and a *Klebsiella* strain, both mainly having PL activity, soft rot *Erwinia* spp. rapidly produced larger conductance responses, due to a combined action of multiple isozymic forms of PG and PL. The responses of *Erwinia* spp. were initially associated with the accumulation of large amounts of monomers and saturated dimers to heptamers, due to PG activity. Subsequently, as well as monomers and saturated dimers, large amounts of unsaturated dimers were also detected, due to PL activity. The role of PG as an important conductimetric factor was also demonstrated for a pectinase preparation derived from *Aspergillus niger*. Besides detection, automated conductimetric assays in pectate media may also be useful for monitoring of pectolytic activity in pectinase preparations and for screening of pectolytic activity of micro-organisms under different media and growth conditions.

Introduction

Recently, plant pathogenic *Erwinia carotovora* and *Erwinia chrysanthemi* bacteria have been detected in potato peel extracts by automated conductance measurements in a pectate medium (Fraaije *et al.*, 1996). Because of the specificity of the conductance responses in pectate medium, the ability of *Erwinia* spp. to cause conductance changes seems to be mainly associated with pectolytic activity. However, other pectolytic bacterial strains isolated from potato tubers, such as *Pseudomonas*, *Flavobacterium* and *Xanthomonas* spp., were not able to generate significant conductance responses in pectate medium, whereas *Klebsiella* spp. only produced weak conductance responses.

Soft rot *Erwinia* spp. produce multiple isozymic forms (≥3) of polygalacturonase (PG) and pectate lyase (PL) (Ried and Collmer, 1986; McMillan *et al.*, 1994), enabling the bacteria to split α-1,4-glycosidic linkages in low methoxylated pectins by hydrolysis and β-elimination, respectively. In comparison with *Erwinia* spp., the pectolytic systems of *Pseudomonas*, *Xanthomonas* and *Cytophaga* spp., which have been associated with spoilage of vegetables (Liao, 1989), and those of the enterobacteria *Klebsiella* and *Yersinia* spp. (Bagley and Starr, 1979) were much simpler, as only one or two different forms of PL were detected.

This study was carried out to establish why plant pathogenic *Erwinia* spp. cause considerable conductance responses in pectate medium, while other pectolytic bacteria cause only weak or no responses at all. Various bacterial species isolated from potato, including *Erwinia* spp., were screened for their pectolytic properties by examining pitting in polypectate agar, recording conductance responses in polypectate medium and performing potato tuber soft rot tests. For bacteria found positive in conductimetry, the generation of conductance changes, the production of pectolytic enzymes and the formation of degradation products were simultaneously determined in time during depolymerization of polygalacturonic acid (PGA). The synthesis of pectolytic enzymes and accumulation of degradation products were determined with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) activity staining and high-performance anion exchange chromatography (HPAEC), respectively. By comparing the results of HPAEC, SDS-PAGE activity staining and conductimetry, the role of PG and PL in the generation of conductance responses during depolymerization of PGA was analysed for different pectolytic bacteria and discussed.

Materials and methods

Media

Nutrient Agar (NA; Oxoid) and a growth factor medium, containing (g l^{-1}): $NH_4H_2PO_4$, 0.5; $MgSO_4.7H_2O$, 0.05; $FeSO_4.7H_2O$, 0.017; NaCl, 0.1; K_2HPO_4, 0.4; yeast extract (Oxoid), 3.0; glucose, 1.0; bacto-agar (Difco), 15.0; pH 7.2, were used for maintaining bacterial cultures. Tryptic Soy Broth (TSB; Difco) was used for growing of overnight cultures. King's medium B (KB; King *et al.*, 1954) was used for counting total numbers of bacteria, whereas Double Layer Crystal Violet Pectate agar (DLCVP; Pérombelon and Burnett, 1991), without antibiotics, was used in the polypectate pit-formation tests. Minimal media containing (g l^{-1}):

$NaNO_3$, 1.5; $MgSO_4.7H_2O$, 0.3; NaCl, 2.3; K_2HPO_4, 4.0; KH_2PO_4, 1.5; pH 7.2, supplemented with either 2.0 % sodium polypectate (HP Bulmer, Hereford, England) or 2.0 % PGA sodium salt (Sigma P-1879), were used in conductimetric assays.

Bacterial strains

All bacterial strains tested are listed in Table 1.

Table 1. Bacterial strains tested for pectolytic activity

Bacterial strains	Source[1]
E. carotovora subsp. *atroseptica* (*Eca*) 1061	1
E. carotovora subsp. *carotovora* (*Ecc*) 257	1
E. chrysanthemi (*Ech*) B19	2
Enterobacter spp. (*Ent*) S89	1
Flavobacterium spp. (*Fla*) S18	2
Klebsiella spp. (*Kle*) S119	1
Pseudomonas fluorescens (*Pfl*) S26	2
Xanthomonas maltophilia (*Xma*) S11	2
Unidentified saprophyte (*Sap*) S12	2

1 Source names: 1 = Research Institute for Plant Protection (IPO-DLO, Wageningen, The Netherlands), 2 = Centre for Plant Breeding and Reproduction Research (CPRO-DLO, Wageningen, The Netherlands).

Preparation of bacterial inoculum

Bacteria were grown overnight in TSB at 25 °C on a rotary shaker at 250 rpm, resulting in suspensions of *ca* 10^9 cfu ml^{-1}. Inoculum dilutions were made in distilled water.

Preparation of potato peel extracts

The peelings of five tubers of cultivar Bintje were extracted using a power-driven Pollähne roller press (Wennigsen, Germany). Subsequently, extracts were filtered through cheese cloth, centrifuged at 14,000 × *g* for 5 min in an Eppendorf centrifuge and filter-sterilized through a 0.2 μm syringe filter holder (Sartorius AG, Gottingen, Germany) to remove soil, peel debris, starch and micro-organisms.

Conductance measurements

Conductance changes were monitored with a Malthus 2000 series analyser (Malthus Instruments Ltd., Crawley, UK). Reusable 8 ml tubes containing 2 ml of medium were inoculated with 100 μl bacterial suspension and the volume was

made up to 3 ml with pure distilled water or distilled water in combination with 50 µl potato peel extract. The conductance responses were recorded in duplicate at 6 or 18 minute intervals at 25 or 27 °C. For each sample, the detection time (Td in h) and maximum conductance change (G_{max} in µS) were determined. For samples with distilled water, the Td was defined as the time needed to give an overall conductance change of 25 µS or more. For samples with distilled water and potato peel extract, detection was achieved when the conductance responses exceeded 50 µS, as bacterial conversion of asparagine and aspartic acid in peel extract itself can cause additional conductance responses up to 25 µS (Fraaije *et al.*, 1996).

Potato tuber soft rot testing

Tubers of cultivar Bintje, which were surface sterilized with 96 % alcohol, were inoculated by inserting micro-pipette tips with 0.1 ml bacterial suspension of *ca* 10^8 cfu ml^{-1} in tuber tissue to a depth of 5 to 7 mm. Incubation was carried out in a container at 25 °C and incidence of soft rot was determined five days after inoculation by slicing the tubers and examining the presence of decayed tissue.

SDS-PAGE activity staining of pectolytic enzymes

SDS-PAGE was performed with the Midget-gel system of LKB, according to the procedure described by Laemmli (1970). Resolving gels contained 10 % acrylamide, 0.3 % bisacrylamide, 0.1 % SDS and 0.15 % PGA. After centrifugation at 14,000 × *g* for 10 min, medium samples (15 µl of supernatant) were applied directly onto the gel. After electrophoresis, *ca* 1 h at a constant voltage of 200 V, pectolytic enzym activity was restored in the gel by displacement of SDS with a 1.0 % Triton X-100 solution. Finally, after incubation of the gel for 1 h at 37 °C in 50 mM phosphate buffer, pH 5.2 for PG, and in the same buffer with 1 mM $CaCl_2$ at pH 8.3 for PL, molecular forms of PG and PL were visualized within the gel by ruthenium red staining as described by Lewosz (1993). Phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa), all obtained from Biorad, were used as molecular mass standards.

High-performance anion exchange chromatography (HPAEC)

HPAEC of degradation products of PGA was performed with a Dionex BioLC system (Dionex, Sunnyvale, CA) equipped with a Dionex Carbopack PA-100 column (250×4 mm). After centrifugation at 14,000 * *g* for 10 min, the

supernatant of medium samples (20 μl) was tested in HPAEC. After sample injection, the column, pre-equilibrated with a solution consisting of 0.2 M NaAc in 0.1 M in NaOH, was eluted with two successive linear gradients of NaAc in 0.1 M NaOH (0.2-0.7 M for 40 min and 0.7-1.0 M, for 5 min), washed for 5 min with 1 M NaAc in 0.1 M NaOH and then re-equilibrated for 15 min with a solution of 0.2 M NaAc in 0.1 M NaOH. The flow rate was 1 ml min^{-1}. Detection, performed at 1 μC, was made with a PAD II pulsed-amperometric detector (Dionex, Sunnyvale, CA) equipped with a gold working-electrode and an Ag/AgCl reference electrode. The following pulse potentials and durations were used: E_1 = 0.1 V, t_1 = 400 ms; E_2 = 0.7 V, t_2 = 200 ms; E_3 = -0.1 V, t_3 = 400 ms.

Results

Analysis of pectolytic activity of potato-associated bacteria

Results of the conductance measurements in polypectate minimal medium, pit formation in DLCVP and the potato tuber soft rot tests are presented in Table 2. The *Erwinia* spp. strains, *E. carotovora* subsp. *atroseptica* (*Eca*) 1061, *E. c.* subsp. *carotovora* (*Ecc*) 257 and *E. chrysanthemi* (*Ech*) S19, *Flavobacterium* (*Fla*) S18, *Klebsiella* (*Kle*) S119 and saprophyte S12 were able to generate conductance changes in polypectate minimal medium at 25 °C. Addition of potato peel extract resulted in a shorter Td for *Fla* S18 and *Erwinia* spp., whereas *Kle* S119 and saprophyte S12 were detected later. Of the bacteria tested, only *Ecc* 257 was not able to grow on DLCVP, maybe as a result of sensitivity to crystal violet, as 50 other *Ecc* strains isolated from potato all grew and formed pits on DLCVP (data not shown). Besides *Erwinia* spp., *Fla* S18 and saprophyte S12 also formed pits on DLCVP, whereas all other bacteria, although growing on DLCVP, did not. In potato tuber soft rot testing, only the *Erwinia* spp. strains were able to macerate potato tissue.

Conductance changes during depolymerization of PGA

To analyse the role of pectolytic enzymes in conductimetry, the generation of conductance responses, production of pectolytic enzymes and formation of degradation products in PGA minimal medium were simultaneously monitored in time during depolymerization of PGA at 27 °C. All the bacteria giving a positive response in conductimetry and *P. fluorescens* (*Pfl*) S26, which was negative, were tested.

Table 2. Results of conductimetric assays in polypectate minimal medium, pit formation on polypectate agar and potato tuber soft rot testing at 25 °C for pectolytic potato-associated bacteria

	Conductance measurements [1]					
	- extract		+ extract			
Strains	Td	G_{max}	Td	G_{max}	Pitting[2]	Potato soft rot[3]
Eca 1061	21.3	206	11.6	211	+	+
Ecc 257	17.0	318	9.2	379	*	+
Ech B19	22.8	348	12.6	388	+	+
Ent S89	-	-	-	-	-	-
Fla S18	40.0	72	21.3	77	+	-
Kle S119	63.2	97	81.6	113	-	-
Pfl S26	-	-	-	-	-	-
Xma S11	-	-	-	-	-	-
Sap S12	52.7	109	103.5	86	+	-
Control (water)	-	-	-	-	*	-

- = negative reaction, + = positive reaction.

1 For each strain *ca* 10^7 cfu per tube were tested in conductimetry. Conductance changes were measured in 2.0 % polypectate minimal medium, with or without addition of potato peel extracts. For samples positive in conductimetry, results are given as detection time (Td in h) and maximum conductance change (G_{max}, absolute value in μS).

2 The ability of the bacteria to form pits in DLCVP at 25 °C within 5 days. * = no growth observed.

3 Incidence of potato tuber soft rot determined 5 days after inoculation at 25 °C.

The results of the conductimetric assays in PGA minimal medium at 27 °C are shown in Figure 1A. The *Erwinia* spp. strains grew rapidly in PGA minimal medium, resulting in rapid conductance changes up to 175 μS within 48 h. The size of the conductance responses was dependent on the concentration of PGA in the medium, as shown for *Ecc* 257 in Figure 1B. *Kle* S119 was also able to grow rapidly in PGA minimal medium (visual observation), but generated only a conductance response of *ca* 30 μS within 48 h, whereas subsequently conductance changes up to 75 μS were recorded. Saprophyte S12 grew weakly in PGA minimal medium, resulting in a delayed conductance response of *ca* 75 μS. *Fla* S18 and *Pfl* S26 were not able to grow in PGA minimal medium and conductance changes were not measured. However, for *Fla* S18, a conductance response up to 75 μS was obtained within 24 h when high numbers of bacteria (>10^7 cfu per conductance tube) were added to PGA minimal medium (data not shown).

Besides the bacterial cultures, also a pectinase preparation derived from *Aspergillus niger* (Sigma P-9179) was tested in conductimetry. This preparation, which mainly contained PG activity at pH 5.4, rapidly generated conductance changes up to 100 μS in PGA minimal medium at 25 °C (Fig. 1C).

Accumulation of products during depolymerization of PGA

For the bacteria tested, various patterns of a series of saturated and a series of unsaturated galacturonates with different degrees of polymerization (DP) were detected with HPAEC during conversion of PGA at 27 °C (see Table 3). For *Erwinia* spp., the series of oligogalacturonates accumulated in time were more or less similar. Initially, besides galacturonate monomers, large amounts of saturated dimers to heptamers were formed within 46 h, due to PG activity. Unsaturated oligomers (DP 2-4), products of PL activity, generally accumulated somewhat later in time, as illustrated for *Eca* 1061 in Figure 2. After 140 h, large amounts of monomers together with saturated and unsaturated dimers were detected, whereas saturated and unsaturated trimers were present in smaller amounts. *Kle* S119 generated considerable amounts of unsaturated dimers and trimers, due to PL activity, whereas trace amounts of saturated dimers, due to PG activity, were found towards the end of incubation. Saprophyte S12 showed both PL and PG activity, because small amounts of a series of unsaturated (DP 2-12) oligomers together with monomers and trace amounts of saturated oligomers (DP 4-8) were detected late in time. *Pfl* S26 showed a weak PG activity, because only a trace amount of saturated dimers was detected. For *Fla* S18 no degradation products were detected at all.

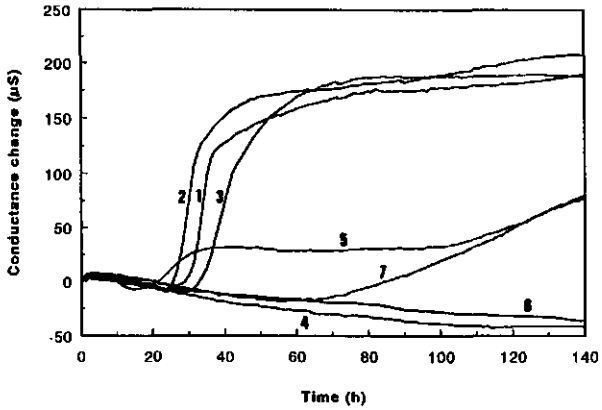

Figure 1A. Conductance responses of bacterial strains in 2.0 % PGA minimal medium, pH 7.2, at 27 °C. 1 = *Eca* 1061, 2 = *Ecc* 257, 3 = *Ech* B19, 4 = *Fla* S18, 5 = *Kle* S119, 6 = *Pfl* S26, 7 = *Sap* S12. For each strain *ca* 10^5 cfu per tube were tested in conductimetry.

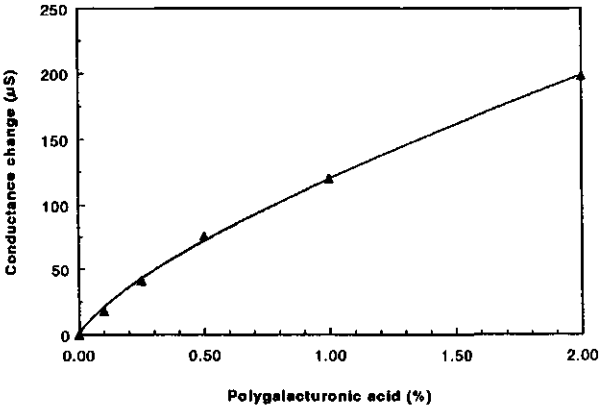

Figure 1B. The effect of the PGA concentration on the conductance responses of *Erwinia carotovora* subsp. *carotovora* 257 in minimal medium, pH 7.2, at 27 °C.

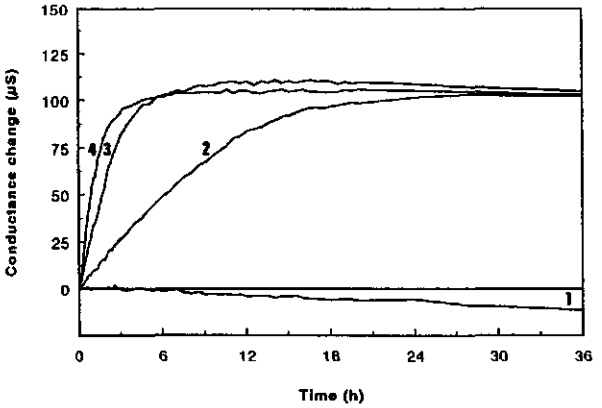

Figure 1C. Conductance responses of a pectinase preparation derived from Aspergillus niger in 2.0 % PGA medium, pH 5.4, at 25 °C. Different amounts of pectinase were tested; 1 = 0 units (U) (control), 2 = 0.37 U, 3 = 1.83 U, 4 = 3.66 U.

Table 3. Oligogalacturonates accumulating during depolymerization of polygalacturonic acid (PGA)

	DP of saturated oligogalacturonates[2]				DP of unsaturated oligogalacturonates[2]			
Strains[1]	28h	46 h	72h	140 h	28 h	46 h	72 h	140 h
Eca 1061	-	1-6	1-4	1-3	-	2	2	2
Ecc 257	2-7	1-4	1-3	1-2	2-4[t]	2,3[t]	2	2
Ech B19	2	1-5	1-5	1-3	2	2,3[t]	2,3[t]	2,3[t]
Fla S18	-	-	-	-	-	-	-	-
Kle S119	-	-	-	2[t]	2[t]	2[t]	2,3[t]	2,3[t]
Pfl S26	-	-	-	2[t]	-	-	-	-
Sap S12	-	-	-	1, 4-8[t]	-	-	-	2-9, 10-12[t]

- = no oligogalacturonates detected, t = trace amounts detected.

1 For each strain 1 ml bacterial suspension (containing *ca* 10^5 cfu) was incubated in 2.0 % PGA minimal medium for 140 h at 27 °C.

2 The formation of oligogalacturonates in time was measured with HPAEC; the figures indicate the degree of polymerization (DP) of the saturated and unsaturated oligogalacturonates accumulated.

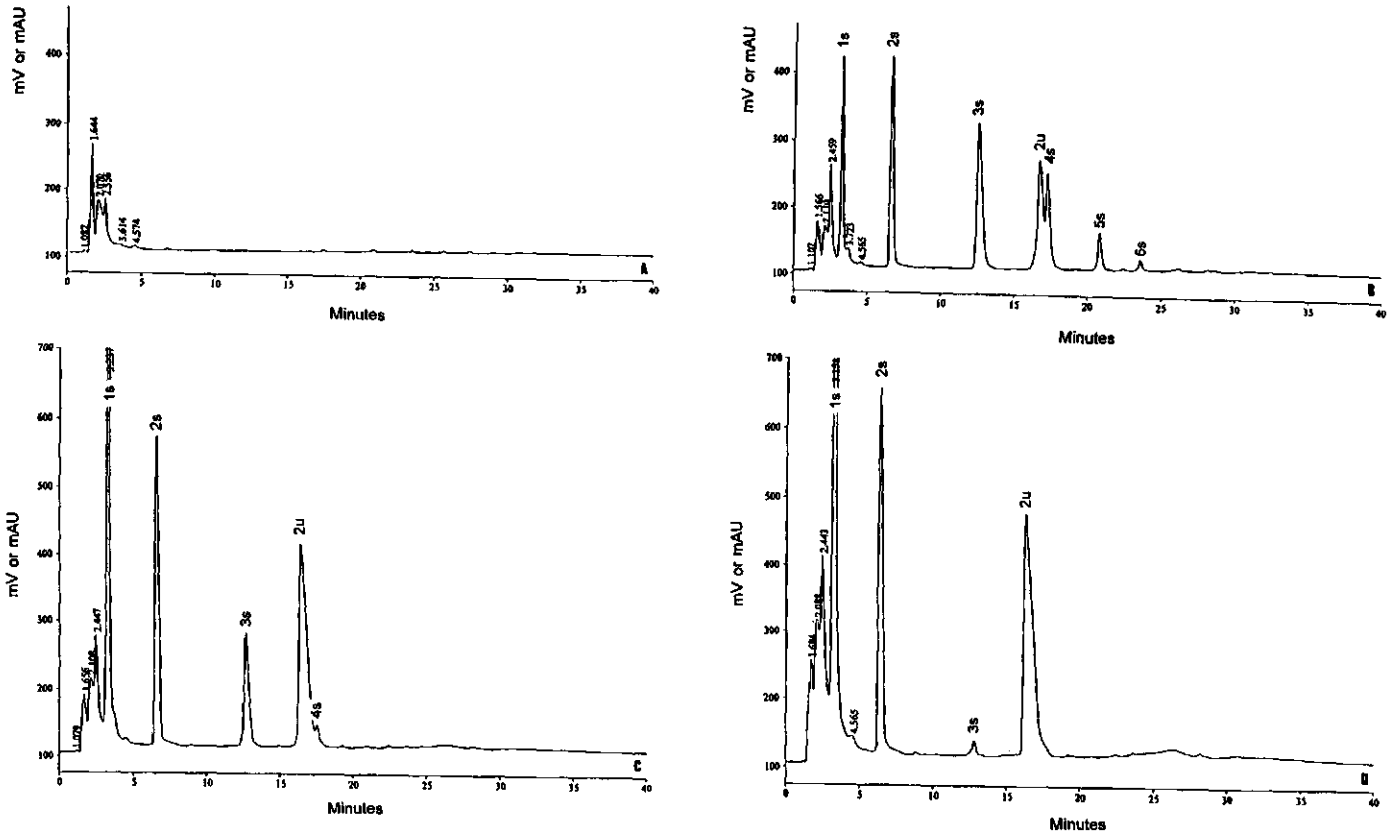

Figure 2. High-performance anion exchange chromatography (HPAEC) profiles of oligogalacturonates accumulated during incubation of *Erwinia carotovora* subsp. *atroseptica* 1061 in 2 % (w/v) polygalacturonic acid (PGA) minimal medium, pH 7.2, at 27 °C. One ml bacterial suspension with *ca* 10^5 cfu was added to 2 ml medium, samples were analyzed after 28 (**A**), 46 (**B**), 72 (**C**) and 140 h (**D**) of incubation. The figures (1-6) indicate the degree of polymerization of the products and whether they are saturated (s) or unsaturated (u).

The production of pectolytic enzymes during depolymerization of PGA

Different forms of PL and PG were detected in SDS-PAGE activity staining during incubation of the bacteria tested in PGA minimal medium at 27 °C (See Fig.3).

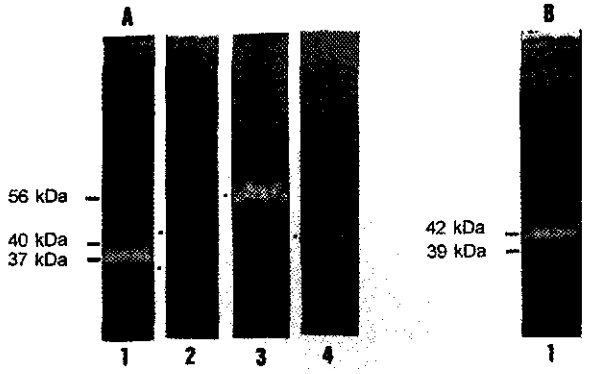

Figure 3. Production of polygalacturonase (PG) and pectate lyase (PL) isozymes determined with SDS-PAGE activity staining. Samples were taken after 72 h incubation of 1 ml bacterial suspension (containing *ca* 10^5 cfu) in 2 ml 2.0 % PGA minimal medium at 27 °C. PG and PL isozymes resolved in the gel were visualized by their activities at pH 5.2 and 8.3, respectively. **A.** PL isozymes: lane 1 = *Ecc* 257, 2 = *Ech* B19, 3 = *Kle* S119, 4 = *Sap* S12. **B.** PG isozymes: lane 1 = *Ecc* 257.

Eca 1061 and *Ecc* 257 produced at least 2 different PGs, having an estimated molecular weight (MW) of 39 and 42 kDa, and 3 PLs of 37, 40 and 56 kDa. For *Ech* B19, 2 PLs of 33 and 42 kDa were detected, whereas at least 1 band of 48 kDa showed a faint PG activity. All the PGs and PLs of *Ecc* 257 were already detected after 28 h of incubation, whereas the PGs and PLs of *Eca* 1061 and *Ech* B19 were detected after 46 h. For *Kle* S119, only a single PL of 58 kDa was detected after 28 h. Saprophyte S12 produced a single PL form of 40 kDa, which was detected after 72 h. Although *Kle* S119, *Pfl* S26 and saprophyte S12 showed PG activity in HPAEC, no molecular forms of PG were found with SDS-PAGE activity staining. For *Fla* S18, no forms of PG or PL were detected during incubation in PGA minimal medium.

Discussion

All the bacteria which produced considerable amounts of extracellular PL and/or PG during depolymerization of PGA, i.e. *Erwinia* spp., *Kle* S119 and saprophyte S12, were also able to generate conductance responses in PGA minimal medium. *Fla* S18 only produced conductance responses in PGA minimal medium when high cell numbers were present. The *Erwinia* spp. strains rapidly generated conductance responses up to 200 μS in PGA minimal medium. The size of the

responses showed a near linear relationship with the PGA concentration (Fig. 1B). The conductance responses of the *Erwinia* spp. strains were initially associated with the accumulation of large amounts of monomers and saturated oligomers (DP 2-7) formed by endoPG activity, whereas later in time, besides monomers and saturated dimers and trimers, also large amounts of unsaturated dimers, formed by PL activity, were detected. The PL production was probably stimulated by PG activity, as saturated oligomers of low DP, particularly dimers which can be taken up easily, can induce the PL synthesis effectively (Collmer *et al.*, 1982). Furthermore, for *Eca* 1061, *Ecc* 257 and *Ech* B19, different forms of PG and PL were detected in SDS-PAGE activity staining. For the *E. carotovora* strains, producing PGs of 39 and 41 kDa, and PLs of 37, 40 and 56 kDa, similar MWs have been reported by other groups; PG of 43 kDa (Lei *et al.*, 1985a), PG and PLs of 39 kDa (McMillan *et al.*, 1992), PLs of 41 and 44 kDa (Lei *et al.*, 1985b) and PL of 56 kDa (Trollinger *et al.*, 1989). For *Ech*, the MWs of 33 and 42 kDa for PL determined in this study were close to the MWs of 33 and 40 kDa, reported by Bagley and Starr (1979) and Keen and Tamaki (1986), respectively, whereas no MW for PG has been reported thus far. For saprophyte S12, producing conductance changes up to 100 μS late in time, both endoPL and endoPG activity are likely to be produced, as monomers and a series of saturated (DP 4-8) and unsaturated oligomers (DP 2-12) were released. The absence of saturated dimers and trimers can be explained by uptake of these oligomers as substrates. *Kle* S119 rapidly generated a conductance response up to 30 μS in PGA minimal medium, which was associated with PL activity splitting PGA in a less random endolytic or exolytic fashion, as only unsaturated dimers and trimers were detected. A delayed conductance response of 75 μS was recorded. This late increase was probably caused by a weak PG activity, since at that time saturated dimers appeared in HPAEC analysis. For both saprophyte S12 and *Kle* S119, only a single form of PL was found with SDS-PAGE activity staining, whereas the PG activity was probably not high enough for detection. The PL of 58 kDa from *Kle* S119 was clearly different from the 2 PLs of 71 kDa reported for *Klebsiella oxytoca* (Bagley and Starr, 1979). The inability of *Kle* S119 to form pits in DLCVP, in contrast with the other pectolytic enzym producing bacteria, may be explained by the absence of PL and/or PG isozymes spitting polypectate in a more endolytic fashion.

In comparison with the saprophytic bacteria, *Erwinia* spp. rapidly produced larger conductance responses, due to a combined action of PG and PL isozymes, enabling the bacteria to depolymerize PGA more efficiently. Particularly, the connection between the rapid formation of oligogalacturonates

with a low DP and the slow uptake and metabolism of these products, resulting in an early accumulation of these products, is apparently responsible for the conductance signal. Indeed, Owens (1985) already reported the increase in conductivity during hydrolysis of PGA to be due to the greater mobility of the monomer residues and that its extent would depend upon the degree to which monomers accumulate or are metabolized by the microbes. Accordingly, a rapid conductance response up to 100 μS at 25 °C during depolymerization of PGA was generated by a pectinase preparation derived from *A. niger*, which was mainly due to PG activity.

The pectolytic activity of the *Erwinia* spp. and the *Flavobacterium* strain tested was even further enhanced in polypectate minimal medium by addition of potato peel extract. For plant pathogenic *Erwinia* spp., an inducible synthesis of large amounts of pectolytic enzymes with different modes of action, far beyond metabolic needs, is very beneficial. Namely, it enables the bacteria to attack plant cell walls of different hosts (Beaulieu *et al.*, 1993), to cope better with changing environmental conditions (Hugouvieux-Cotte-Pattat *et al.*, 1992), and to overcome plant defence responses elicited by oligogalacturonates with a high DP (Yang *et al.*, 1992).

Our data showed that, besides for detection of soft rot *Erwinia* spp., conductimetric assays in pectate media may also have good prospects for screening of pectolytic activity of micro-organism under different media and growth conditions. Additionally, conductimetry may also be applied to monitor the enzymatic activity of pectinase preparations, which are commonly used in the food industry for extraction and clarification of fruit juices and for maceration of fruit and vegetable tissues (Rombouts and Pilnik, 1980).

REFERENCES

Bagley, S.T. and Starr, M.P. (1979) Characterization of intracellular polygalacturonic acid *trans*-eliminase from *Klebsiella oxytoca*, *Yersinia enterolytica*, and *Erwinia chrysanthemi*. *Current Microbiology* **2:** 381-386.

Beaulieu, C., Boccara, M. and Van Gijsegem, F. (1993) Pathogenic behavior of pectinase-defective *Erwinia chrysanthemi* mutants on different plants. *Molecular Plant-Microbe Interactions* **6:** 197-202.

Collmer, A., Berman, P. and Mount, M.S. (1982) Pectate lyase regulation and bacterial soft-rot pathogenesis. In *Phytopathogenic prokaryotes* Vol. 1 eds Mount, M.S. and Lacy, G.H. pp. 395-422. New York: Academic Press.

Fraaije, B.A., Birnbaum, Y., Franken, A.A.J.M. and Van den Bulk, R.W. (1996) The development of a conductimetric assay for automated detection of metabolically active soft rot *Erwinia* spp. in potato tuber peel extracts. *Journal of Applied Bacteriology*, in press.

Hugouvieux-Cotte-Pattat, N., Dominguez, H. and Robert-Baudouy, J. (1992) Environmental conditions affect transcription of the pectinase genes of *Erwinia chrysanthemi* 3937. *Journal of Bacteriology* **174:** 7807-7818.

Keen, N.T. and Tamaki, S. (1986) Structure of two pectate lyase genes from *Erwinia chrysanthemi* EC16 and their high-level expression in *Escherichia coli*. *Journal of Bacteriology* **168:** 595-606.

King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicin* **44:** 301-307.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.

Lei, S-P., Lin, H-C., Heffernan, L. and Wilcox, G. (1985a) Evidence that polygalacturonase is a virulence determinant in *Erwinia carotovora*. Journal of Bacteriology **164:** 831-835.

Lei, S-P., Lin, H-C., Heffernan, L. and Wilcox, G. (1985b) Cloning of the pectate lyase genes from *Erwinia carotovora* and their expression in *Escherichia coli*. *Gene* **35:** 63-70.

Lewosz, J. (1993) Repression of several polygalacturonases and diminution of the virulence of *Erwinia carotovora* ssp. *atroseptica* by potato tuber extracts. In *Abstracts of the 12th Triennial Conference of the European Association for Potato Research* (EAPR) pp. 382-383. Paris: INRA editions.

Liao, C-H. (1989) Analysis of pectate lyases produced by soft rot bacteria associated with spoilage of vegetables. *Applied and Environmental Microbiology* **55:** 1677-1683.

McMillan, G.P., Barrett, A.M. and Pérombelon, M.C.M. (1994) An isoelectric focusing study of the effect of methyl-esterified pectic substances on the production of extracellular pectin isoenzymes by soft rot *Erwinia* spp.. *Journal of Applied Bacteriology* **77:** 175-184.

McMillan, G.P., Johnston, D.J. and Pérombelon, M.C.M. (1992) Purification to homogeneity of extracellular polygalacturonase and isoenzymes of pectate lyase of *Erwinia carotovora* subsp. *atroseptica* by column chromatography. *Journal of Applied Bacteriology* **73:** 83-86.

Owens, J.D. (1985) Formulation of culture media for conductimetric assays: theoretical considerations. *Journal of General Microbiology* **131:** 3055-3076.

Pérombelon, M.C.M. and Burnett, E.M. (1991) Two modified crystal violet pectate (CVP) media for the detection, isolation and enumeration of soft rot erwinias. *Potato Research* **34:** 79-85.

Ried, J.L. and Collmer, A. (1986) Comparison of pectic enzymes produced by *Erwinia chrysanthemi*, *Erwinia carotovora* subsp. *carotovora*, and *Erwinia carotovora* subsp. *atroseptica*. *Applied and Environmental Microbiology* **52:** 305-310.

Rombouts, F. M. and Pilnik., W. (1980) Pectic enzymes. In *Microbial enzymes and bioconversions* Economic Microbiology Vol. 5. ed. Rose, A.H. pp. 227-282. London: Academic Press.

Trollinger, D., Berry, S., Belser, W. and Keen, N.T. (1989) Cloning and characterization of a pectate lyase gene from *Erwinia carotovora* EC153. *Molecular Plant-Microbe Interactions* **2:** 17-25.

Yang, Z., Cramer, C.L. and Lacy, G.H. (1992) *Erwinia carotovora* subsp. *carotovora* pectic enzymes: *in planta* gene activation and roles in soft-rot pathogenesis. *Molecular Plant-Microbe Interactions* **5:** 104-112.

Chapter 6. Detection of soft rot *Erwinia* spp. on seed potatoes: conductimetry in comparison with dilution plating, PCR and serological assays

Abstract

Automated conductance measurements in polypectate medium were used for the detection of pathogenic soft rot *Erwinia* spp. in potato peel extracts. The detection threshold for *Erwinia carotovora* subsp. *atroseptica* (*Eca*) in inoculated peel extracts was *ca* 10^4 colony forming units (cfu) ml^{-1} when samples were considered positive on the basis of a response within 48 h at 20 °C. Detection of *E. chrysanthemi* (*Ech*) was less sensitive, only 10^5 cfu ml^{-1} peel extract were detected within 36 h at 25 °C. The linear correlation between detection times in conductimetry and inoculum levels of *Eca* and *Ech* in peel extracts was used for a quantitative estimation of *Eca* and *Ech* in naturally contaminated peel extracts. Samples giving a positive conductimetric response had to be confirmed with an enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR) for the presence of *Eca* and *Ech*, because *E. carotovora* subsp. *carotovora* (*Ecc*) also generated a conductance response. Conductimetry was sensitive and efficient for detection of contamination levels of *Eca* higher than 10^4 cfu ml^{-1} peel extract. For *Ech*, conductimetric detection was less sensitive and inefficient due to low contamination levels of *Ech* and the presence of high numbers of *Ecc* in many samples after enrichment, which interfered with the test. Immunofluoresence cel staining (IF) combined with enrichment and immuno-fluorescence colony staining (IFC) were suited to detect and quantify low numbers of *Eca* and *Ech* at less than 10^4 cells ml^{-1} in peel extracts. However, since false positive and negative reactions in serology were observed, the use of PCR after enrichment, or in combination with IFC to confirm positive results, was required for accurate detection.

Introduction

Pectolytic erwinias are involved in soft rot diseases of various agricultural crops such as potato, sugar beet and chicory. *Erwinia* spp. associated with soft rot of potato have been studied extensively because of their economic importance. The different *Erwinia* spp. that are involved in potato diseases are *Erwinia carotovora* subsp. *atroseptica* (*Eca*), *Erwinia carotovora* subsp. *carotovora* (*Ecc*) and *Erwinia chrysanthemi* (*Ech*). They all can cause tuber soft rot. Under cool and moist conditions *Eca* is the main causal agent of blackleg, a blackening of the stem base of potato plants which originates from the mother tuber (Pérombelon and

Kelman, 1987). *Ecc* mainly causes aerial stem rot, often due to injury, although under high field temperatures it has been reported to incite blackleg-like symptoms (Molina and Harrison, 1977). Under hot and humid (tropical) conditions *Ech* can also induce blackleg-like symptoms, but recently this bacterium has also been found in association with stem rot in temperate regions (De Vries, 1990). The symptoms of stem wet rot, caused by *Ech*, are often difficult to distinguish from typical blackleg symptoms of *Eca* in temperate regions where both pathogens occur (De Boer, 1994).

Stem rot diseases caused by *Eca* and *Ech* are seed tuber borne. Besides resistance breeding (See Düring *et al.*, 1993), good cultural practices and the use of healthy seeds are the best strategies to control the disease (Pérombelon and Hyman, 1992). Bain *et al.* (1990) found a clear relationship between the number of *Eca* bacteria present on seed potatoes and the incidence of blackleg in the field. Moreover, the incidence of blackleg late in the season was negatively correlated to tuber yield.

In temperate regions much attention has been paid to improve the quality and health of seed potatoes. Field inspection for blackleg failed to assure the status of this pathogen in seed lots, because post-harvest contamination and latent infections cannot be detected by visual observation. Therefore, laboratory testing with ELISA in addition to field inspection is currently used in The Netherlands for seed certification to control blackleg (De Boer *et al.*, 1996).

The aim of laboratory testing is to detect 10^2-10^4 colony forming units (cfu) of *Eca* per tuber, which is the minimum amount of inoculum needed to incite blackleg symptoms under most field conditions (Bain *et al.*, 1990). Techniques based on dilution plating, serology and DNA technology have been developed to detect *Eca* and *Ech* on potato tubers. Dilution plating on crystal violet pectate (CVP) medium (Pérombelon *et al.*, 1987) lacks sensitivity due to the presence of large numbers of potato tuber-associated saprophytes and *Ecc*, and is both unreliable (Janse and Spit, 1989) and time-consuming. Immunofluorescence cell (IF) and colony staining (IFC) (Allan and Kelman, 1977; Van Vuurde and Roozen, 1990), although laborious, and ELISA, in combination with an enrichment step (Gorris *et al.*, 1994), have good potential for routine application. However, serological techniques sometimes show false positive and false negative reactions, due to cross-reacting saprophytes (Van der Wolf *et al.*, 1994) and variation in serotypes (De Boer *et al.*, 1987; Samson *et al.*, 1987). Polymerase chain reaction assays (PCR) for *Eca* and *Ech* are rapid techniques with high specificity and sensitivity (De Boer and Ward, 1995; Smid *et al.*, 1995). Nevertheless, the application of PCR for large-scale routine indexing of potato tuber seed lots is expensive and

laborious.

Automated conductimetric assays have already shown their potential for rapid screening of food products for both pathogenic (Bolton, 1990) and non-pathogenic bacteria (Hardy *et al.*, 1977; Cady *et al.*, 1978) and might also be useful for routine indexing of seed potatoes (Fraaije *et al.*, 1996).

This paper describes the use of conductance measurements as a primary screening technique for quantifying the number of viable soft rot *Erwinia* spp. on potato tubers. Conductimetric assays were combined with ELISA, PCR and dilution plating to verify the presence of *Eca*, *Ech* and *Ecc* in potato peel extract samples after enrichment in polypectate medium. Results obtained with conductimetry were compared with dilution plating, IFC and IF combined with enrichment in order to determine the sensitivity, specificity and efficiency of the test.

Materials and methods

Bacterial strains

Two strains of *Eca*, 1061 and P644, and of *Ech*, P652 and P991, from the culture collection of the Research Institute for Plant Protection (IPO-DLO, Wageningen, The Netherlands) were used for inoculation of peel extracts. Bacteria were grown overnight in TSB at room temperature on a rotary shaker at 250 rpm to *ca* 10^9 cfu ml^{-1}. Dilutions of bacterial broth cultures were made in saline (0.85 % (w/v) NaCl; pH 7) and potato peel extract for preparing pure culture dilutions and to inoculate potato peel extract.

Media

Nutrient Agar (NA; Oxoid) and a growth-factors medium (Van Vuurde and Roozen, 1990) were used for maintaining bacterial cultures of *E. carotovora* and *E. chrysanthemi*, respectively. Tryptic Soy Broth (TSB, Difco) was used for growing overnight cultures. Double Layer Crystal Violet Pectate medium (DLCVP; Pérombelon and Burnett, 1991) without antibiotics was used for dilution plating. Minimal medium containing (g l^{-1}): $NaNO_3$, 1.50; $MgSO_4.7H_2O$, 0.30; sodium taurocholate (Difco), 0.2; NaCl, 2.30; K_2HPO_4, 4.0 and KH_2PO_4, 1.5, was supplemented with either 0.5 % (w/v) L-asparagine monohydrate (AM) or 2.0 % (w/v) sodium polypectate (HP Bulmer, Hereford, UK) (PM) for enrichment of *Erwinia* spp. in potato peel extracts. During enrichment of potato peel extracts in PM, the conductance change responses were recorded simultaneously.

Potato seed lots

In 1994, 122 potato tuber seed lots were tested within four weeks after harvest. The General Netherlands Inspection Service for Agricultural Seeds and Seed Potatoes (NAK) kindly provided 53 commercial seed lots, of which four (seed lots 1-4) were derived from fields without disease symptoms (category A) and 49 (seed lots 5-53) from fields where blackleg symptoms were observed sporadically (cat. B). We also harvested potatoes from healthy plants (cat. C; seed lots 54-61) and naturally *Erwinia*-infested plants (cat. D; seed lots 62-122) during the growing season from control field plots at the experimental farm of the NAK, Tollebeek, The Netherlands.

Preparation of samples

The peel from ten potatoes of every seed lot was extracted by a power-driven roller press (Pollähne, Wennigsen, Germany). Extracts were filtered through cheese cloth and left standing for at least 1 h to settle soil, starch and peel debris, before sampling from the top layer.

Enrichment in AM and immunofluorescence cell staining (IF)

Tubes with 4.5 ml of AM were inoculated with 0.5 ml extract, and incubated without shaking at 25 °C for 22 h. Bacterial cells in enriched peel extracts (20 µl) were stained as described by Van Vuurde *et al.* (1983), using FITC-conjugated polyclonal antibodies (Pab) 9024/5C and 8898. Bacto-FA rhodamine (code 2340, Difco), 1 : 100 diluted, was used as counter stain. The working dilutions of Pab 9024/5C, directed against lipopolysaccharide (LPS) of *Ech*, and Pab 8898, directed against LPS of *Eca*, were 1 : 600 and 1 : 1000, respectively. Stained cells fixed on multitest slides (diam. 8 mm) were counted in at least 25 fields under a UV microscope (field coefficient 18, objective magnification 63, internal magnification 1.25, ocular magnification 10), which corresponds to ca 1 µl extract.

Enrichment in PM and conductance measurements

A Malthus 2000 series analyser (Malthus Instruments Ltd, Crawley, UK) was used for recording conductance change responses of potato peel extracts during enrichment in PM. Reusable 8 ml tubes with 2 ml of PM were inoculated with peel extract and filled with distilled water to a final volume of 3 ml. Only small volumes of 20 and 50 µl peel extract were tested at 25 and 20 °C, respectively, in order to reduce the temperature-dependent background conductance signal of peel extract itself (Fraaije *et al.*, 1996) to a value below 15 µS. Conductance

changes were recorded in duplicate at 18 min intervals for at least 48 h. To determine the detection times in conductimetry for *Eca* and *Ech* in peel extracts, the response was considered to be positive when the conductance change of peel extracts in PM exceeded 25 μS. After 48 h enrichment in PM, samples were used directly for dilution plating or transferred to Eppendorf tubes and stored at -20 °C until ELISA and PCR were performed.

Dilution plating

Dilutions of potato peel extract, before and after enrichment in AM or PM, were plated on DLCVP plates. Suspected *Erwinia* spp. colonies, which formed typical pits on DLCVP after 48 to 96 h incubation at 25 °C, were isolated and identified by biochemical, serological and fatty acid analyses as described previously (Fraaije *et al.*, 1996).

Immunofluorescence colony staining (IFC)

IFC of 20 μl samples of diluted potato peel extracts was performed in 24-well tissue culture plates, with each well containing 300 μl molten PT medium (Burr and Schroth, 1977) with polygalacturonate, according to the procedure of Van Vuurde and Van der Wolf (1995). After 48 h of incubation at 27 °C, target colonies were stained with the fluorescein-isothiocyanate (FITC) conjugated polyclonal antibodies 8898 and 8174. The working dilutions of Pab 8898, directed against LPS of *Eca*, and Pab 8174, directed against LPS of *Ech*, were both 1 : 100. Stained colonies in the agar were counted under a UV microscope at a magnification of 20 times. Fluorescent colonies suspected of being *Eca* were further tested by PCR.

ELISA

ELISA was done according to an indirect antigen coated plate procedure. Polystyrene plates were coated with 100 μl sample and incubated overnight at 4 °C. After two washings with tap water, wells were blocked for 1 h at 37 °C with PBST (0.1 % Tween-20 in 0.01 mol l^{-1} phosphate buffered saline, pH 7.2) containing 5 % (w/v) skim milk powder. After washing, 1 : 2000 diluted Pab 8898 (in PBST with 0.1 % bovine serum albumin (BSA)) or 1 : 0.25 similarly diluted Mab 27D10 exudate, a monoclonal antibody directed against LPS of *Ech*, was added to the wells and the plate was incubated for 2 h at 4 °C. After washing, the plate was incubated for 1 h at 4 °C with a 1 : 1000 dilution (in PBST with 0.1 % BSA) of goat anti-rabbit or goat anti-mouse immunoglobulins, conjugated with alkaline phosphatase. After four washings, the substrate, 0.75 mg ml^{-1} of *p*-nitrophenyl

phosphate in substrate buffer (10 % (v/v) diethanolamine, pH 9.8), was added. The absorbance was measured with an automatic reader at 405 nm after 1 h (A_{405}-values). A_{405}-values greater than twice the mean of negative control values (clean seed extract) were considered to be positive.

PCR

PCR was used to verify positive results for *Eca* in IFC and conductimetric assays. Positive colonies in IFC were punched from the agar, suspended in 20 µl purified water, boiled for 10 min, and tested directly in PCR. DNA was extracted from 40 µl of conductimetric samples enriched in PM. Five µl 14 % (w/v) SDS and 5 µl 100 mM EDTA solution was added to each sample and heated for 1 h at 55 °C. Twenty-five µl ammonium acetate (7.5 M) was mixed with heat-treated samples which were then centrifuged at 14,000 × *g* for 10 min. DNA was precipitated by adding 1 volume of isopropanol and washed with 70 % (v/v) ethanol. Final pellets were dried at 55 °C for 15 min, dissolved in 50 µl purified water, and heated to 55 °C prior to PCR. The PCR assays, using *Taq* DNA polymerase (BioCan Scientific, Missisauga, Canada) and the ECA1f and ECA1r primers, were performed with 1 µl sample in 20 µl reaction mixture as described previously by De Boer and Ward (1995), except that 0.2 % (w/v) skim milk powder was added to the reaction mixture (De Boer *et al.*, 1995). The PCR conditions with an Ericomp Easy Cycler (San Diego, US) were 95 °C for 4 min, followed by 40 cycles at 94 °C for 45 s, 62 °C for 45 s and 72 °C for 1 min. The PCR was terminated with a DNA extension at 72 °C for 5 min. PCR products were analyzed on 2 % (w/v) agarose gels, containing ethidium bromide, by electrophoresis of 5 µl sample at 5 V cm^{-1} for 1 h. The presence of a 690 bp DNA fragment confirmed the presence of *Eca*.

Results

Conductimetric detection of Eca and Ech

A clear positive relationship occurred between detection time (Td) in conductimetry and the concentration of *Erwinia* spp. inoculated into potato peel extracts (Figs 1A, C). Within 48 h, detection time in PM at 20 °C (Td_{20}) was linearly correlated (r = -0.94) with *Eca* concentrations >log 4.0 cfu ml^{-1} potato peel extract (Fig. 1B). *Eca* detection was less sensitive at 25 °C in comparison with 20 °C, probably due to poor competition of *Eca* with other micro-organisms present in potato peel extracts, which could be isolated in high numbers after enrichment at 25 °C. *Ech* was detected somewhat earlier at 25 than 20 °C,

probably because the positive effect of the higher growth rate of *Ech* at 25 °C (results not shown) was almost counteracted by the higher competitive ability at 20 °C. The detection threshold for *Ech*, i.e. the lowest concentration of bacterial cells that gave a positive response in the assay, was 10 times higher than that for *Eca* after 48 h incubation. For *Ech*, a high linear correlation coefficient (r = -0.96) between detection time in PM at 25 °C and inoculum concentrations >log 5.0 cfu ml^{-1} was obtained (Fig. 1D).

Comparison of conductimetry with ELISA and PCR

Results of conductance measurements were verified with PCR and ELISA. After 48 h enrichment in PM at 20 °C, initial inoculum concentrations of 10^2, 10^3-10^4 and 10^4 *Eca* cfu ml^{-1} potato peel extract were detected in PCR, ELISA and conductimetry, respectively (Table 1). The detection thresholds for *Ech* were 10^5 cfu ml^{-1} for both ELISA and conductimetry after 36 h enrichment in PM at 25 °C, whereas a reliable PCR assay for *Ech* was not available at the time (Table 1). Conductimetric detection of both *Eca* and *Ech* occurred when cell concentrations reached 10^7-10^8 cells ml^{-1} during enrichment. As the detection threshold of PCR for pure cultures of *Eca* was 10^4 cells ml^{-1} and 10^6-10^7 *Eca* or *Ech* cells ml^{-1} were detected in ELISA (data not shown), both techniques could be used for confirmation. Indeed, all samples positive in conductimetry were confirmed by both techniques (Table 1).

Screening of peel extracts naturally infected with Erwiniae

To determine the efficiency of the conductance measurements, all 122 seed lots were also tested with ELISA for the presence of *Eca* and *Ech*. All samples that were positive in ELISA or conductimetry were further tested by dilution plating and PCR (Table 2).

Nineteen and 32 seed lots were positive for *Eca* in ELISA and conductimetry, respectively. For 15 of the 32 positive conductimetric samples the presence of *Eca* was confirmed by both ELISA and PCR. For three of the four samples (seed lots 70, 95 and 111) which were positive in ELISA and negative in conductimetry, the inconsistency between ELISA and conductimetry results could be explained by the higher sensitivity of ELISA, as these samples were also found positive in PCR and dilution plating. The positive ELISA result of seed lot 90, which was negative in all other tests, was probably caused by dead cells or a false positive reaction in serology. The negative ELISA, and positive PCR and conductimetric results of seed lots 21 and 31 can be explained by mixed *Eca* and *Ecc* populations, which were present in these samples.

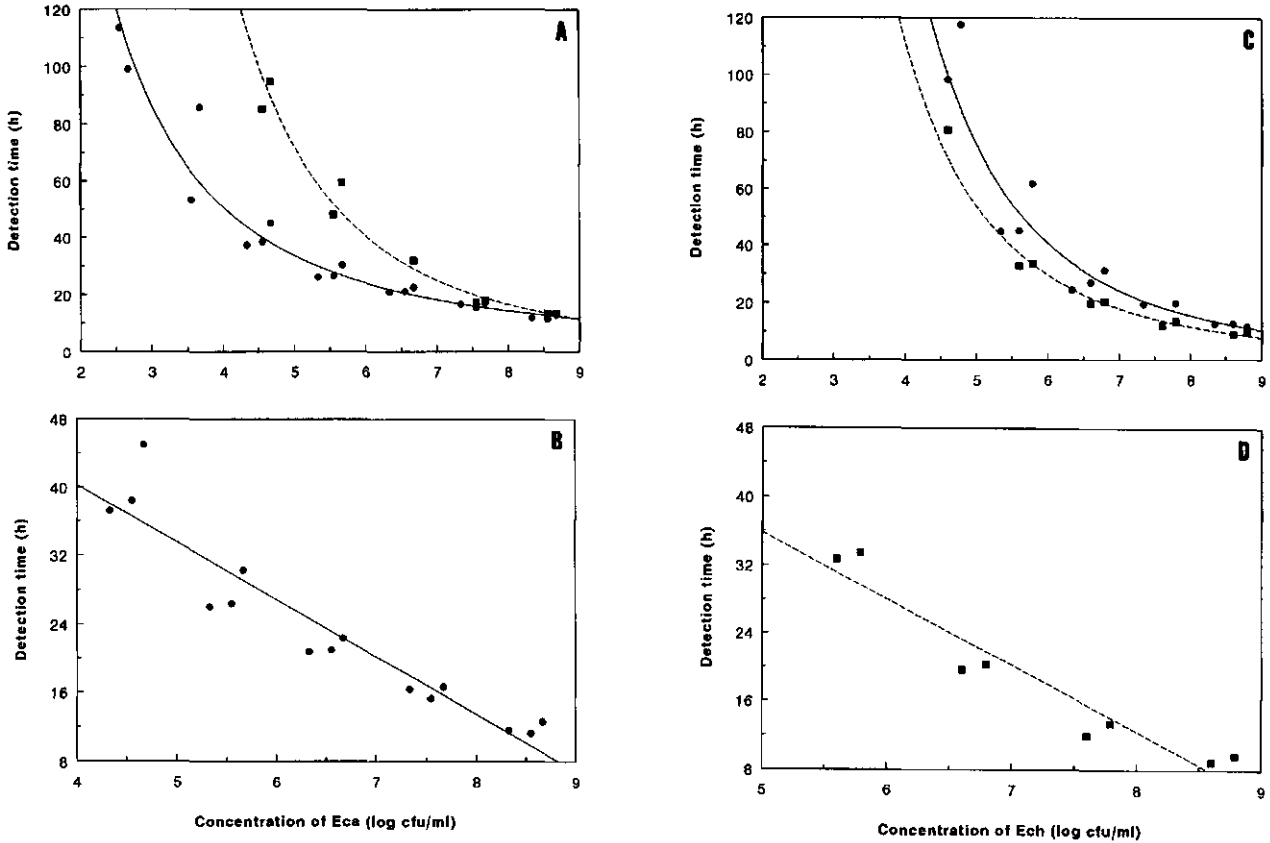

Figure 1. The relationship between detection times in conductimetry and concentration of *Erwinia* spp. in inoculated potato peel extracts at 20 °C (—●-) and 25 °C (-■-). **A.** Conductimetric detection of *Eca* in inoculated peel extracts during enrichment in polypectate medium (PM) at 20 and 25 °C. **B.** Linear relationship between detection time and *Eca* concentrations >log 4.0 cfu ml^{-1}; $y = -6.9x + 66.9$. **C.** Conductimetric detection of *Ech* in inoculated peel extracts during enrichment in PM at 20 and 25 °C. **D.** Linear relationship between detection time and *Ech* concentrations >log 5.0 cfu ml^{-1}; $y = -7.8x + 74.9$.

Table 1. Results of conductimetry, ELISA and PCR for detection of *Eca* and *Ech* in inoculated potato peel extracts after enrichment in polypectate medium (PM) at 20 and 25 °C

Concentration of cells added to peel extract (cfu ml^{-1})	Detection of *Eca*			Detection of *Ech*	
	Td20[1]	ELISA	PCR	Td25[2]	ELISA
0	-	-	-	-	-
10	-	-	-	-	-
10^2	-	-	+	-	-
10^3	-	+/-	+	-	-
10^4	+	+	+	-	-
10^5	+	+	+	+	+
10^6	+	+	+	+	+
10^7	+	+	+	+	+
10^8	+	+	+	+	+

+ = positive reaction, - = negative reaction.
1 Td20 = detection time (h) of peel extract in PM at 20 °C; all samples detected within 48 h were considered to be positive for presence of *Eca*.
2 Td25 = detection time (h) of peel extract in PM at 25 °C; all samples detected within 36 h were considered to be positive for presence of *Ech*.

Table 2. Results of naturally infected potato seed lots positive for *Eca* or *Ech* with conductimetry or ELISA after enrichment in polypectate medium (PM), using PCR and dilution plating to verify results of enriched samples

	Detection of *Eca*			Detection of *Ech*		Isolation[3]	
Seed lots	Td20[1]	ELISA	PCR	Td25[2]	ELISA	Direct	After enrichment
1	28.8	-	-	15.9	-	-	*Ecc*
2	-	-	-	30.6	-	-	nd
4	-	-	-	26.9	-	-	nd
6	-	-	-	34.5	-	-	nd
7	-	-	-	34.5	-	-	*Ecc*
8	-	-	-	30.5	-	-	nd
12	25.1	-	-	14.1	-	*Ecc*	*Ecc*
13	42.2	-	-	23.1	-	*Ecc*	*Ecc*
14	-	-	-	34.4	-	-	nd
15	20.4	-	-	11.7	-	*Ecc*	*Ecc*
16	-	-	-	29.3	-	-	nd
17	-	-	-	31.7	-	-	nd
18	45.0	-	-	27.8	-	-	nd
19	-	-	-	17.7	-	*Ecc*	nd
20	12.0	-	-	6.9	-	-	nd
21	35.1	-	+	22.4	-	*Eca*	*Ecc*
22	-	-	-	30.8	-	-	nd
25	-	-	-	33.9	-	-	nd
27	-	-	-	17.1	+	-	nd
28	46.2	-	-	-	-	*Ecc*	nd
30	-	-	-	34.5	-	-	nd
31	36.3	-	+	16.5	-	*Eca*/*Ecc*	*Ecc*
33	47.1	-	-	29.1	-	-	nd
38	24.9	-	-	11.9	-	*Ech*	*Ecc*
45	13.4	-	-	8.4	-	*Ecc*	*Ecc*
46	42.7	-	-	29.3	-	-	*Ecc*
48	-	-	-	30.8	-	-	*Ecc*
49	39.3	-	-	23.9	-	-	*Ecc*
50	34.1	-	-	25.8	-	*Ecc*	*Ecc*
51	-	-	-	27.0	-	*Ecc*	*Ecc*
52	34.5	-	-	22.2	-	*Ecc*	*Ecc*
53	44.4	+	+	-	-	*Eca*	*Eca*
66	38.6	-	-	24.9	-	-	*Ecc*
69	-	-	-	31.2	-	-	*Ecc*
70	-	+	+	-	-	-	*Ecc*/*Eca*
88	-	-	-	21.6	-	-	*Ecc*
89	37.8	+	+	-	-	-	*Eca*
90	-	+	-	-	-	-	-
91	25.8	+	+	26.6	-	*Eca*	*Eca*
93	37.5	+	+	36.0	+	-	*Eca*
94	44.1	+	+	-	-	-	*Eca*
95	-	+	+	29.4	-	-	*Eca*
96	33.8	+	+	-	-	-	*Eca*
97	45.0	+	+	-	-	-	*Eca*
98	14.6	+	+	13.8	-	*Eca*	*Eca*

Table 2. Continued

Seed lots	Detection of *Eca*			Detection of *Ech*		Isolation[3]	
	Td20[1]	ELISA	PCR	Td25[2]	ELISA	Direct	After enrichment
99	39.9	+	+	-	-	-	*Eca*
100	41.4	+	+	-	-	-	*Eca*
101	39.9	+	+	-	-	-	*Eca*
102	32.6	+	+	33.8	-	*Eca*	*Eca*
103	31.8	+	+	30.8	-	*Eca*	*Eca*
104	36.9	+	+	35.4	-	-	*Eca*
105	41.9	+	+	-	-	-	*Eca*
111	-	+	+	-	-	*Eca*	*Eca*

+ = positive reaction in ELISA and PCR, - = negative reaction in ELISA and PCR.
1 Td20 = detection time (h) of peel extract in PM at 20 °C; all samples with Td20 <48 h were considered to be positive in conductimetry.
2 Td25 = detection time (h) of peel extract in PM at 25 °C; all samples with Td25 <36 h were considered to be positive in conductimetry.
3 Isolation of *Erwinia* spp. after dilution plating; the detection thresholds of dilution plating, performed directly with peel extracts and after enrichment were higher than 10^4 and 10^6 cfu ml^{-1}, respectively.
- = *Erwinia* spp. were not isolated, nd = dilution plating was not done.

The conductance signals were probably caused by high numbers of *Ecc* (>10^7 cells ml^{-1}) during enrichment, but low numbers of *Eca* (<10^6 cells ml^{-1}), not detectable by ELISA and conductimetry, could still be detected by PCR. The positive conductance signals of the 15 remaining seed lots, which were negative in both ELISA and PCR, were probably due to high numbers of *Ecc*, which were isolated before or after enrichment from all these samples (Table 2).

Respectively, two and 40 of the 122 seed lots tested were found positive for *Ech* in ELISA and conductimetry. The positive ELISA results of seed lots 27 and 93 were probably due to dead cells or false positive reactions since *Ech* could not be isolated and both samples were negative in IFC (Tables 2 and 3). Furthermore, the conductance response of most of the 40 positive conductimetric samples were probably generated by *Ecc* and *Eca*, which could be isolated in high numbers after enrichment (Table 2).

The results of the conductimetric assays at 20 and 25 °C indicated that the higher temperature favoured the growth of *Ecc* in the peel extracts, while *Eca* was enriched better at the lower temperature. *Eca* was generally detected slightly faster at 20 °C, whereas *Ecc* was detected about twice as fast at 25 °C (Table 2 and Fig. 2).

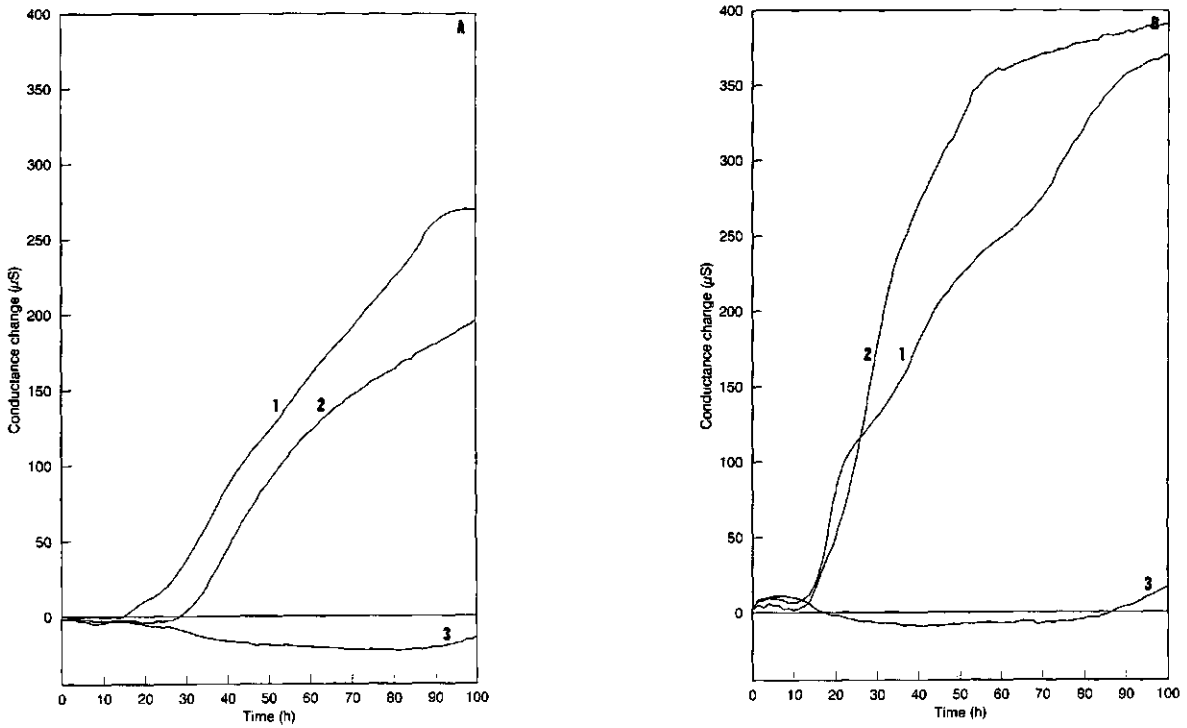

Figure 2. Conductance responses of potato peel extracts in polypectate medium. **A.** Peel extracts at 20 °C. **B.** Peel extracts at 25 °C. 1 = seed lot 1, contaminated with *Ecc*, 2 = seed lot 31, contaminated with *Eca* and *Ecc*, 3 = seed lot 37, no *Erwinia* spp. isolated.

Quantitative estimation of Eca and Ech in potato peel extracts

To evaluate the value of conductimetry, a quantitative comparison with immunofluorescence techniques and dilution plating was made. For all positive seed lots, the contamination level with *Eca* and *Ech* in peel extracts before or after enrichment was estimated by conductimetry, dilution plating and immunofluorescence staining techniques (Table 3).

IFC-PCR for *Eca* was positive for 23 of 122 potato seed lots tested, of which 18 were positive after enrichment in PCR (Table 2) and 15 in conductimetry combined with PCR (Table 3). IFC-PCR was more sensitive than dilution plating and conductimetry, although for some seed lots (98 and 111) the contamination level of *Eca* was underestimated. False negative reactions in IFC-PCR were found for two seed lots (31 and 97), which were positive with at least three other techniques and from which *Eca* was isolated. The negative IFC result for seed lot 31 could be ascribed to the presence of an unusual *Eca* serotype, which reacted only weakly with Pab 8898, directed against LPS of serogroup I. All *Eca* strains tested in The Netherlands thus far, have been placed in serogroup I (M. Appels, personal communication). Twenty-five peel extracts enriched in AM had more than 10^4 *Eca* cells ml^{-1} in IF. Of these, 18 lots were also positive in IFC-PCR, 8 in dilution plating, 20 in PCR after enrichment, 18 in both ELISA and PCR after enrichment (Tables 2 and 3), and 17 in conductimetry combined with PCR. Apparent false positive IF-reactions were found for seed lots 16, 17, 19 and 22.

For *Ech*, 16 potato seed lots were found positive by one of the five techniques tested, whereas only 10 seed lots were positive with two techniques and no seed lots were positive with all techniques tested. Seven seed lots were positive in IFC or dilution plating and contained at least 10^4 cells ml^{-1}, but in ELISA none of these seven lots were positive, while only five were positive in conductimetry. The conductimetric response of these five lots was probably caused by *Ecc* or *Eca*, because they were negative in ELISA for *Ech*. Of the 10 seed lots found positive with two techniques, eight were positive in IFC and seven in IF. Unfortunately, since *Ech* was only isolated from two seed lots and serological tests were inconsistent, another confirmation test, like PCR, was not available to verify results obtained in conductimetry and serology.

Table 3. Quantitative estimation of *Eca* and *Ech* in peel extracts of naturally infected potato seed lots. Comparison of conductimetry with IFC and dilution plating, performed directly, and with IF, PCR and ELISA, performed after enrichment

Concentration of *Eca* (log cells ml^{-1})	Positive potato seed lots				
	Without enrichment[1]			After enrichment[2]	
	IFC-PCR[3]	Plating	Conductimetry +PCR[4]	IF	PCR-ELISA[5]
<4.0	7,13,14,24 62,70,89,93 94,95,96,99 100,104,105 111				
4.0-6.0	21,53,91,98 101,102,103	21,31,53,91 102,103,111	21,31,53,89 93,94,96,97 99,100,101 102,103,104 105	16,17,19,22 31,33	21,31
>6.0		98	91,98	21,53,70,89 91,93,94,95 96,97,98,99 100,101,102 103,104,105 111	53,70,89,91 93,94,95,96 97,98,99 100,101,102 103,104,105 111

Table 3. continued.

Concentration of *Ech* (log cells ml^{-1})	Without enrichment[1]		Conductimetry +ELISA[6]	After enrichment[2]	
	IFC	Plating		IF	ELISA
<4.0	6,8,12,14 16,20,23,30 33,34,35,47 65,86,119				
4.0-6.0	21,25,38,50 51,82	38,42	93	8,23,25,26 30,33,36,65 119	
>6.0			27		27,93

1 IFC and plating on DLCVP were done directly with dilutions of peel extracts.
2 For doing IF, peel extracts were enriched in AM for 22 h at 25 °C; ELISA and PCR were done after enrichment of peel extracts in PM for 48 h at 20 °C or 36 h at 25 °C for the detection of *Eca* and *Ech*, respectively.
3 IFC-PCR for detection of *Eca*; PCR was used for confirmation of positive colonies in IFC.
4 Conductimetry-PCR; all samples with Td20 <30 h were suspected to be contaminated with >10^6 cfu ml^{-1}, while samples with a Td20 between 36 and 48 h were suspected to be contaminated with 10^4 to 10^6 cfu ml^{-1}, PCR was used for confirmation of the presence of *Eca* in the suspected samples.
5 PCR-ELISA, samples positive in PCR were considered to contain at least 10^4 cells ml^{-1}, ELISA was performed to determine which samples yielded more than 10^5 cell ml^{-1}.
6 Conductimetry-ELISA, all samples with Td25 <30 h were suspected to be contaminated with >10^6 cells ml^{-1}, while samples with a Td25 between 30 and 36 h were suspected to yield at least >10^5 cells ml^{-1}, ELISA was used for confirmation of the presence of *Ech* in the suspected samples.

Discussion

The detection thresholds of the conductimetric assays for *Eca* and *Ech* in potato peel extract were temperature-dependent. High linear correlations between detection times in conductimetry and cell concentrations in inoculated peel extract were obtained for *Eca* and *Ech*. By using calibration curves, it was possible to estimate the tuber-borne contamination levels of *Eca* and *Ech* in potato tuber seed lots. *Eca* in inoculated peel extracts was detected in 48 h at 20 °C with a detection threshold of 10^4 cfu ml^{-1}. *Ech* was detected in 36 h at 25 °C with a detection threshold of 10^5 cfu ml^{-1}. However, when seed lots naturally contaminated with *Erwinia* spp. were tested, *Ecc* was also able to generate conductance responses in PM, particularly at 25 °C, making the calibration curves less reliable. Therefore, the presence of *Eca* and *Ech* in positive conductimetric samples had to be confirmed with ELISA or PCR.

It was difficult to determine the sensitivity and efficiency of conductimetry for *Ech*, as *Ech* was only isolated from two of the 122 seed lots tested and there were discrepancies among the serological test results. The contradictory results in the different serological assays may partly be caused by non-specific binding or cross-reactions of *Pseudomonas* spp. with the *Ech* antisera used, as reported by Van der Wolf *et al.* (1993). High numbers of *Pseudomonas* spp. were especially present after enrichment in AM at 25 °C (results not shown). Furthermore, many samples were detected in conductimetry due to the presence of high numbers of *E. carotovora* after enrichment, and a PCR assay with high specificity was not available to confirm the results obtained in serology and conductimetry. However, in a later test series with other samples, a nested PCR procedure to confirm IFC-positive colonies showed promising results, as the presence of *Ech* could be confirmed in *ca* 95 % of the samples tested (J.M. Van der Wolf, personal communication).

About 20 % of the 122 seed lots was found positive for *Eca* by direct IFC combined with PCR or by PCR after enrichment. In conductimetry 26 % of the seed lots tested was found suspected to be contaminated with *Eca*. After verification of the conductimetric-positive samples, 53 % of these samples was found positive for *Eca*. Most of the conductance responses of the other samples, if not all, was caused by *Ecc*. Of the seed lots positive in IFC-PCR or PCR after enrichment, 68 % was also found positive in conductimetry combined with PCR. The remaining samples were probably not detected due to the low contamination levels of *Eca* less than 10^4 cfu ml^{-1} peel extract. Conductimetry was less laborious and more sensitive than dilution plating for the detection of *Eca* in naturally contaminated peel extracts, because more samples were found positive

(Table 3). Direct IFC, performed with crude peel extracts, and IF and PCR, performed after enrichment, were adequately sensitive for quantifying and detecting low numbers ($<10^4$ cells ml^{-1}) of *Eca* in peel extracts.

Our results suggest that automated conductance measurements can already be used for an efficient screening of seed potatoes for *Eca*. Samples negative in a conductimetric assay can be certified without further testing, because the contamination level of *Eca* will be less than 10^4 cfu ml^{-1}. The conductimetric detection of *Ech* was less sensitive and inefficient, because too many samples needed further testing.

Different approaches are possible to improve the sensitivity and specificity of the conductimetric assays for both *Eca* and *Ech*. Other procedures to prepare samples, such as immunomagnetic separation (Parmar *et al.*, 1992), may be used to reduce the number of *Ecc* and other non-target organisms. Furthermore, to restrict the growth of non-target organisms, including *Ecc*, the use of a selective pre-enrichment medium, commonly used for conductimetric detection of food-borne bacteria such as *Salmonella* (Pless *et al.*, 1994) and *Listeria* spp. (Capell *et al.*, 1995), might be considered.

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References

Allan, E. and Kelman, A. (1977) Immunofluorescent stain procedures for detection and identification of *Erwinia carotovora* var. *atroseptica*. *Phytopatology* **67**: 1305-1312.

Bain, R.A., Pérombelon, M.C.M., Tsror, L. and Nachmias, A. (1990) Blackleg development and tuber yield in relation to numbers of *Erwinia carotovora* subsp. *atroseptica* on seed potatoes. *Plant Pathology* **39**: 125-133.

Bolton, F.J. (1990) Conductance and impedance methods for detecting pathogens. In *Rapid methods and automation in microbiology and immunology* eds Vaheri, A., Tilton, R.C. and Balows, A. pp. 176-182. Berlin: Springer Verlag.

Burr, T.J. and Schroth, M.N. (1977) Occurrence of soft rot *Erwinia* spp. in soil and plant material. *Phytopathology* **67**: 1382-1387.

Cady, P., Hardy, D., Martins, S., Dufour, S.W. and Kraeger, S.J. (1978) Automated impedance measurements for rapid screening of milk microbial content. *Journal of Food Protection* **41**: 277-283.

Capell, C.J., Kirby, R.M. and Moss, M.O. (1995) A method and medium for the electrical detection of *Listeria* spp. from food. *International Journal of Food Microbiology* **25:** 169-177.
De Boer, S.H., Verdonck, L., Vruggink, H., Harju, P., Bång, H.O. and De Ley, J. (1987) Serological and biochemical variation among potato strains of *Erwinia carotovora* subsp. *atroseptica* and their taxonomic relationship to other *E.carotovora* strains. *Journal of Applied Bacteriology* **63:** 487-495.
De Boer, S.H. (1994) Prospects for control of potato diseases caused by pectolytic erwinias. In *Advances in potato pest biology and management* eds Zehnder, G.W., Powelson, M.L., Jansson, R.K. and Raman, K.V. pp. 136-148. St. Paul, Minnesota: APS Press.
De Boer, S.H. and Ward, L.J. (1995) PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. *Phytopathology* **85:** 854-858.
De Boer, S.H., Ward, L.J., Li, X. and Chittaranjan, S. (1995) Attenuation of PCR inhibition in the presence of plant compounds by addition of BLOTTO. *Nucleic Acids Research* **23:** 2567-2568.
De Boer, S.H., Slack, S.A., Van den Bovenkamp, G.W. and Mastenbroek, I. (1996) A role for pathogen indexing procedures in potato certification. In *Advances in Botanical Research Vol. 23, Pathogen indexing technologies* eds Andrews, J.H., Tommerup, I.C. and De Boer, S.H. pp. 217-242. London, UK: Academic press.
De Vries, P.M. (1990) Stengelnatrot symptomen in aardappelplanten. *Aardappelwereld* **4:** 15-18.
Düring, K., Porsch, P., Fladung, M. and Lörz, H. (1993) Transgenic potato plants to the phytopathogenic bacterium *Erwinia carotovora*. *The Plant Journal* **3:** 587-598.
Fraaije, B.A., Birnbaum, Y., Franken, A.A.J.M. and Van den Bulk, R.W. (1996) The development of a conductimetric assay for automated detection of metabolically active soft rot *Erwinia* spp. in potato tuber peel extracts. *Journal of Applied Bacteriology*, in press.
Gorris, M.T., Alarcon, B., Lopez, M.M. and Cambra, M. (1994) Characterization of monoclonal antibodies specific for *Erwinia carotovora* subsp. *atroseptica* and comparison of serological methods for its sensitive detection on potato tubers. *Applied and Environmental Microbiology* **60:** 2076-2085.
Hardy, D., Kraeger, S.J., Dufour, S.W. and Cady, P. (1977) Rapid detection of microbial contamination in frozen vegetables by automated conductance measurements. *Applied and Environmental Microbiology* **34:** 14-17.
Janse, J.D. and Spit, B.E. (1989) A note on the limitations of identifying soft rot Erwinias by temperature tolerances and sensitivity to erythromycin on a pectate medium. *Journal of Phythopathology* **125:** 265-268.
Molina, J.J. and Harrison, M.D. (1977) The role of *Erwinia carotovora* in the epidemiology of potato blackleg. I. Relationship of *Erwinia carotovora* var. *carotovora* and *Erwinia carotovora* var. *atroseptica* to potato blackleg in Colorado. *American Potato Journal* **54:** 587-591.
Parmar, N., Easter, M.C. and Forsythe, S.J. (1992) The detection of *Salmonella enteritidis* and *S. typhimurium* using immunomagnetic separation and conductance microbiology. *Letters in Applied Microbiology* **15:** 175-178.
Pérombelon, M.C.M., Lumb, V.M. and Hyman, L.J. (1987) A rapid method to identify and quantify soft rot erwinias on seed potato tubers. *EPPO bulletin* **17:** 25-35.
Pérombelon, M.C.M. and Kelman, A. (1987) Blackleg and other potato diseases caused by soft rot *Erwinias*: Proposal for revision of terminology. *Plant Disease* **71:** 283-285.
Pérombelon, M.C.M. and Burnett, E.M. (1991) Two modified crystal violet pectate (CVP) media for the detection, isolation and enumeration of soft rot erwinias. *Potato Research* **34:** 79-85.
Pérombelon, M.C.M. and Hyman, L.J. (1992) Control of potato blackleg: production of healthy seed. *Aspects of Applied Biology* **33:** 77-84.
Pless, P., Futschik, K. and Schopf, E. (1994) Rapid detection of Salmonellae by means of a new impedance-splitting method. *Journal of Food Protection* **57:** 369-376.
Samson, R., Poutier, F., Sailly, M. and Jouan, B. (1987) Caractérisation des *Erwinia chrysanthemi* isolées de *Solanum tuberosum* et d'autres plantes-hôtes selon les biovars et sérogroupes. *EPPO Bulletin* **17:** 11-16.
Smid, E.J., Jansen, A.H.J. and Gorris, L.M.G. (1995) Detection of *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* in potato tubers using polymerase chain reaction. *Plant Pathology* **44:** 1058-1069.
Van der Wolf, J.M., Van Beckhoven, J.R.C.M., De Boef, E. and Roozen, N.J.M. (1993) Serological characterization of fluorescent *Pseudomonas* strains cross-reacting with antibodies against *Erwinia chrysanthemi*. *Netherlands Journal of Plant Pathology* **99:** 51-60.

Van der Wolf, J.M., Van Beckhoven, J.R.C.M., De Vries, Ph.M. and Van Vuurde, J.W.L. (1994) Verification of ELISA results by immunomagnetic isolation of antigens from extracts and analysis with SDS-PAGE and Western blotting, demonstrated for *Erwinia* spp. in potatoes. *Journal of Applied Bacteriology* **77**: 160-168.

Van Vuurde, J.W.L. and Roozen, N.J.M. (1990) Comparison of immunofluorescence colony-staining in media, selective isolation on pectate medium, ELISA and immunofluorescence cell staining for detection of *Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi* in cattle manure slurry. *Netherlands Journal of Plant Pathology* **96**: 75-89.

Van Vuurde, J.W.L., Van den Bovenkamp, G.W. and Birnbaum, Y. (1983) Immunofluorescence microscopy and enzyme-linked immunosorbent assays as potential routine tests for detection of *Pseudomonas syringae* pv. *phaeseolicola* and *Xanthomonas campestris* pv. *phaeseoli* in bean seed. *Seed Sciece and Technology* **11**: 547-559.

Van Vuurde, J.W.L. and Van der Wolf, J.M. (1995) Immunofluorescence colony-staining (IFC). In *Molecular Microbial Ecology Manual* eds Akkermans, A.D.L., Van Elsas, J-D. and De Bruijn, F.J. pp. 1-19. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Chapter 7. Comparison of methods for detection of *Erwinia carotovora* subsp. *atroseptica* in progeny tubers derived from inoculated tubers of *Solanum tuberosum* (potato)

Abstract

Several techniques for detection of *Erwinia carotovora* subsp. *atroseptica* (*Eca*) on potato tubers were used to study the transmission of *Eca* from inoculated mother tubers into progeny tubers for 12 different potato cultivars. The sensitivity of the polymerase chain reaction (PCR) for a direct detection of *Eca* in crude peel extracts was only 10^7-10^8 cells ml^{-1}, due to inhibition of PCR amplification by potato tuber-derived compounds. With dilution plating and ELISA, 10^5 and 10^7 cells ml^{-1} were detected, respectively. Immunofluorescence cell staining (IF) with a detection threshold of 10^4-10^5 cells ml^{-1} was shown to be most sensitive for direct detection of *Eca* in peel extracts and transmission of *Eca* was found for six out of the 12 potato cultivars tested, whereas with the other techniques tested no transmission was found. After 48 h enrichment of peel extracts in polypectate medium at 20 °C, IF and PCR detected an initial inoculum concentration of 10 *Eca* cells ml^{-1}, whereas 10^2, 10^4 and 10^4 cells ml^{-1} were detected in dilution plating, ELISA and conductimetry, respectively. With dilution plating, PCR and IF, performed after enrichment, transmission of *Eca* was found for, respectively, four, six and seven out of 12 potato cultivars tested. No transmission was found using ELISA and conductimetry, due to their lower sensitivity. *Erwinia carotovora* subsp. *carotovora* (*Ecc*) was only detected in the progeny tubers of control plants. No relation was found between the transmission of *Eca* to progeny tubers and blackleg incidence in the field.

Introduction

Blackleg, mainly caused by *Erwinia carotovora* subsp. *atroseptica* (Van Hall) Dye (*Eca*), is an important bacterial potato disease for seed producers in temperate regions. *Eca* is a seed-borne bacterium and cannot survive well in the environment, in contrast to the closely related *E. carotovora* subsp. *carotovora* (Jones) Bergey *et al.* (*Ecc*), which is in The Netherlands not regarded to be pathogenic. The disease control of blackleg relies primarily on the production of healthy potato seeds (Pérombelon and Hyman, 1992). To guarantee the health status of the seed stocks, seed certification was traditionally based on a visual crop inspection for blackleg affected plants. Currently, in countries like The Netherlands, certification is also based on laboratory tests in order to estimate tuber contamination and verify field inspection results. Blackleg incidence in the

field is related to tuber contamination, but can be affected by environmental and cultural factors (De Boer *et al.*, 1996). The aim of laboratory testing is to detect the presence of *ca* 10^3 viable *Eca* cells per tuber, which is generally considered as a threshold level for seed contamination below which, under most conditions, no blackleg development is expected (Bain *et al.*, 1990). So far, none of the laboratory tests developed were completely satisfactory when used on a routine basis. Dilution plating is laborious and time-consuming, while the sensitivity of the assay depends on the amount of saprophytes growing on the media. With regard to sensitivity, immunofluorescense cell and colony staining (Allan and Kelman, 1977; Van Vuurde and Roozen, 1990; Jones *et al.*, 1994) and ELISA after enrichment (Gorris *et al.*, 1994) were the most suitable serological techniques applied. However, despite the development of monoclonal antibodies for *Eca*, false positive and negative reactions in serology can still occur (Hyman *et al.*, 1995). Recently, highly specific and sensitive detection methods based on DNA-technology, like PCR (De Boer and Ward, 1995; Smid *et al.*, 1995), have been developed for detection of *Eca* on seed potatoes. Fraaije *et al.* (1996) used conductimetry for detection of *Eca* on potato tubers. For seed lots found positive in conductimetry, the presence of *Eca* has to be confirmed by PCR or ELISA, as *Ecc* and *E. chrysanthemi* Burkholder *et al.* (*Ech*) also can cause conductance change responses.

In this study, the potential of applying ELISA, immunofluorescence cell staining (IF), PCR and conductimetry for indexing transmission of *Eca* from inoculated mother tubers into progeny tubers was investigated. The sensitivity and the specificity of these tests for detection of *Eca* in peel extracts, directly or after enrichment in polypectate medium, were determined and compared. Furthermore, for 12 different potato cultivars the relation between blackleg incidence in the field and transmission of *Eca* to progeny tubers was examined.

Materials and methods

Bacterial strains and inoculation of tubers

Eca strains A21, A33 and A36, from the culture collection of the DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO, Wageningen, The Netherlands), were used for vacuum infiltration of tubers. These *Eca* strains were expected to be highly pathogenic, as they were recently isolated from progeny tubers derived from plants with typical blackleg symptoms. The *Eca* strains P644 and 1061, obtained from the culture collection of the DLO-Research Institute for Plant Protection (IPO-DLO, Wageningen, The Netherlands) were used for

inoculation of peel extracts. Bacteria were grown overnight in Tryptic Soy Broth (TSB, Difco) at 25 °C on a rotary shaker at 250 rpm, resulting in suspensions of *ca* 10^9 colony forming units (cfu) per ml. Bacterial cultures were mixed and inoculum dilutions were made in tap water and potato peel extracts for vacuum infiltration of tubers and preparation of inoculated peel extracts.

Media

Nutrient Agar (NA; Oxoid) was used for maintaining bacterial cultures. Double Layer Crystal Violet Pectate medium (DLCVP; Pérombelon and Burnett, 1991), without antibiotics, was used for dilution plating of bacterial cultures and potato peel extracts. Polypectate minimal medium (PM; Fraaije *et al.*, 1996), containing 20 g l^{-1} sodium polypectate (HP Bulmer, Hereford, England), was used for the enrichment of peel extracts and simultaneous monitoring of conductance changes.

Antisera and DNA primers

Polyclonal antiserum (Pab) 8898, prepared against whole cells of *Eca* strain 161 (culture collection IPO-DLO), a serogroup I isolate, and absorbed with cross-reacting *Ecc* and *Comamonas* spp. strains, was obtained from IPO-DLO, Wageningen, The Netherlands. The primers ECA1f and ECA1r (De Boer and Ward, 1995) were kindly provided by Dr. De Boer (Agriculture Canada Research Station, Vancouver, Canada) for performing PCR.

Field experiment

Seed tubers of potato cultivars Agria, Alcmaria, Amazone, Arinda, Bintje, Désirée, Hertha, Karnico, Kondor, Morene, Producent and Venouska were supplied by Dr. Allefs (Agrico Research, Emmeloord, The Netherlands). The relationship between tuber resistance and blackleg development in the field for all these cultivars is known (Allefs *et al.*, 1995). After storage at 4 °C until use, 25 tubers of each cultivar were vacuum infiltrated during 15 min at a pressure of *ca* 13 kPa with tap water (control) or with tap water containing *ca* 10^6 cfu of *Eca* per ml. Tubers were air dried for four days at room temperature, stored for nine days at 15 °C and planted on 26 April 1995 at the experimental farm of the General Netherlands Inspection Service for Agricultural Seeds and Seed Potatoes in Tollebeek, The Netherlands. Non-emergence and typical blackleg-associated symptoms were scored seven weeks after planting. Disease incidence was expressed as the total percentage of non-emerged plants and plants with at least one stem with blackleg-associated symptoms. Progeny tubers were harvested by hand on

August 25 and stored at 4 °C until tested for the presence of *Eca* in the last week of September.

Sample preparation

The peelings from ten progeny tubers of every cultivar were extracted using a power driven Pollähne roller press (Wennigsen, Germany). Extracts were filtered through cheese cloth and left standing for 1 h to settle soil, starch and peel debris, before sampling from the the top layer. Samples were used directly for enrichment, dilution plating and IF, or stored at -20 °C until performing ELISA and PCR.

Enrichment and conductance measurements

For the enrichment of peel extracts reusable 8 ml tubes containing 2 ml of PM were inoculated with 100 µl peel extract and filled with distilled water till a final volume of 3 ml. During enrichment at 20 °C, conductance changes were monitored automatically with a Malthus 2000 series analyser (Malthus Instruments Ltd, Crawley, UK). Conductance changes were recorded in duplicate at 18 min intervals for 72 h. To determine the detection treshold, conductimetric assays were performed with potato peel extracts inoculated with concentrations of 10-10^6 *Eca* cells ml^{-1}. The detection time (Td) was defined as the time at which the conductance responses of a given sample exceeded 50 µS. Samples which were detected within 48 h in PM at 20 °C were considered to be positive in conductimetry. After 48 h enrichment of peel extracts in PM at 20 °C, samples for dilution plating and IF were taken and tested directly, while samples for ELISA and PCR were stored at -20 °C prior to testing.

Immunofluorescence cell staining (IF)

For IF, 50 µl reaction mix, consisting of 1 : 500 diluted fluorescein-isothiocyanate (FITC) conjugated Pab 8898 and 1 : 50 diluted rhodamine, were added to crude peel extract samples of 50 µl. Samples of enriched peel extracts were tested undiluted, while samples of crude peel extracts were 10 times diluted to improve the fixation and to reduce the background staining of soil, starch and plant debris. After 30 min incubation at room temperature, the mixtures were centrifuged at 14,000 × *g* for 10 min. The stained bacterial cells in the pellets were resuspended in 50 µl saline (0.85 % (w/v) NaCl; pH 7), and 10 µl suspension was fixed directly on multitest slides (diam. 4 mm), as described by Van Vuurde *et al.* (1983). Finally, ca 2 µl per sample was screened under a UV microscope for the presence of *Eca* cells.

Dilution plating

For dilution plating, 20 µl sample, 1 : 10^4 diluted for direct plating and 1 : 10^6 diluted for plating after 48 h enrichment in PM, was plated on DLVCP. Suspected *Erwinia* spp. strains, which formed typical pit-forming colonies on DLCVP plates within four days incubation at 25 °C, were identified by IF and biochemical tests, as described previously by Fraaije *et al.* (1996).

ELISA

For ELISA, the wells of microtitre plates were filled with 50 µl of sample and 50 µl of sample buffer (2.0 % (w/v) polyvinylpyrrolidone (PVP), 0.2 % bovine serum albumin (BSA), 0.1 % (v/v) Tween-20 in 0.01 M phosphate buffered saline (PBS), pH 7.2), and incubated overnight at 4 °C. After two washings with tap water, wells were blocked for 1 h at 37 °C with PBST (0.1 % Tween-20 in PBS), containing 0.5 % (w/v) BSA. After washing, 1:1000 diluted (in PBST with 0.2 % (w/v) BSA) Pab 8898 was added to the wells and the plate was incubated for 2 h at 37 °C. Subsequently, the plate was washed and incubated for 2 h at 37 °C with a 1 : 1000 dilution (in PBST with 0.2 % (w/v) BSA) of goat anti-rabbit immunoglobulin coupled with alkaline phosphatase. After washing, 100 µl of 0.75 mg ml^{-1} *p*-nitrophenyl phosphate in substrate buffer (10 % (v/v) diethanolamine, pH 9.8) was added for the colour reaction. The absorbance was measured with an automatic reader at 405 nm after 1 h. A_{405}-values exceeding twice the mean of the negative control values (clean extracts) were considered to be positive.

PCR

For performing PCR, DNA was extracted from 40 µl of conductimetric samples enriched in PM at 20 °C. Five µl 14 % (w/v) SDS and 5 µl 100 mM EDTA solution was added to each sample and heated for 1 h at 55 °C. Twenty-five µl ammonium acetate (7.5 M) was mixed with heat-treated samples which were then centrifuged at 14,000 * *g* for 10 min. DNA from supernatant fractions was precipitated by adding 1 volume of isopropanol and washed with 70 % (v/v) ethanol. Final pellets were dried at 55 °C for 15 min, dissolved in 50 µl purified water, and heated to 55 °C prior to PCR. Individual PCR reactions (20 µl) were performed with 1.5 µl DNA sample and 0.375 U *Taq* polymerase (Super Taq, Sphaero Q) in PCR buffer provided by the manufacturer, containing 0.5 µM of each of the primers ECA1f and ECA1r, 125 µM of each of the desoxynucleoside triphosphates (Pharmacia) and 0.2 % (w/v) skim milk powder. PCR was performed in a Hybaid thermocycler (OmniGene). After denaturing the template DNA at 95 °C for 4 min, 40 cycles were done at 94 °C for 45 s, 62 °C for 45 s

and 72 °C for 1 min. PCR was terminated with a DNA extension at 72 °C for 5 min. PCR products were analysed on 2 % (w/v) agarose gels, containing ethidium bromide, by electrophoresis of 5 µl sample in Tris-borate buffer as described by *Maniatis et al.* (1982). The presence of a 690 bp DNA fragment confirmed the presence of *Eca*.

Results

Blackleg development in the field

None of the tubers that were vacuum infiltrated with tap water showed non-emergence or blackleg-associated symptoms. For each cultivar vacuum infiltrated with *Eca*, the percentage of tubers not emerged and plants with blackleg symptoms are shown in Figure 1.

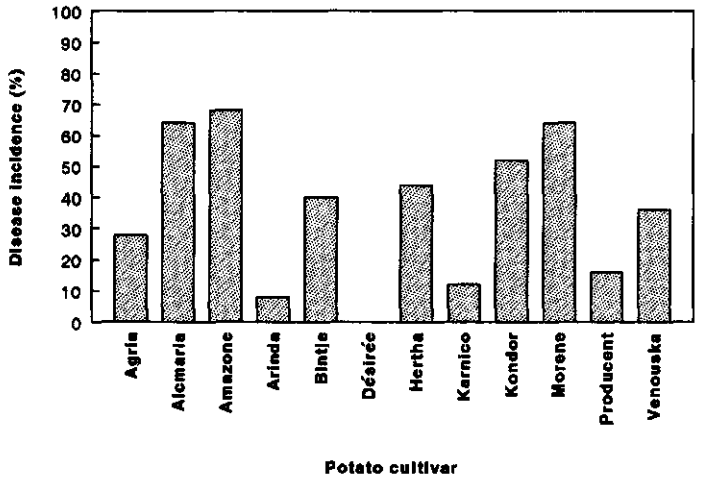

Figure 1. Percentage of tubers of 12 different potato cultivars vacuum infiltrated with *Eca* which did not emerge or developed blackleg-associated symptoms, seven weeks after planting

The incidence of non-emergence was low, as only one tuber of Agria and Alcmaria did not sprout. The disease incidence varied from 0 % for cultivar Désirée to 68 % for cultivar Amazone, whereas the average disease incidence was 36 %. Most of the cultivars showed symptoms of dessication, wilting and leaf-green discoloration, while especially Alcmaria, Kondor and Morene showed dark basal stem rot symptoms which are typical for blackleg.

Table 1. Determination of detection thresholds of dilution plating, ELISA, IF, PCR and conductimetry for *Eca* in inoculated potato peel extracts of cultivars Désirée and Amazone, before and after 48 h enrichment in polypectate medium (PM) at 20 °C

Concentration of *Eca* (cfu ml^{-1})	Direct detection					Detection after enrichment			
	Plating	ELISA	IF	PCR[1]	Conductimetry[2]	Plating	ELISA	IF	PCR
Uninoculated	-	-	-	-	>72.0	-	-	-	-
10	-	-	-	-	>72.0	-	-	+	+
10^2	-	-	-	-	>72.0	+	-	+	+
10^3	-	-	-	-	62.9	+	-	+	+
10^4	-	-	+/-	-	41.6	+	+	+	+
10^5	+	-	+	-	29.2	+	+	+	+
10^6	+	-	+	-	20.7	+	+	+	+
10^7	+	+	+	+/-	16.7	+	+	+	+
10^8	+	+	+	+	10.7	+	+	+	+

+ = positive reaction, - = negative reaction, +/- = variable reaction.

1 When potato peel extracts were inoculated after filtration through a 0.2 μm filter, all *Eca* concentrations >10^3 cells ml^{-1} were detected in PCR.

2 Detection times (h) of the conductimetric assays of peel extract in PM at 20 °C are presented; only samples with a detection time <48 h were considered to be positive in conductimetry.

Detection of Eca in inoculated peel extracts

The results of dilution plating, IF, ELISA, PCR and conductimetry for detection of *Eca* in inoculated peel extracts are presented in Table 1. The detection thresholds for *Eca* in inoculated peel extracts were 10^4-10^5, 10^5, 10^7 and 10^7-10^8 cells ml^{-1} for IF, dilution plating, ELISA and PCR, respectively. After 48 h enrichment of peel extracts in PM at 20 °C, an initial inoculum concentration of *Eca* of 10 cells ml^{-1} peel extract was detected with PCR and IF, whereas an initial concentration of 10^2 cells ml^{-1} was detected by dilution plating and 10^4 cells ml^{-1} in ELISA and conductimetry. By comparing the sensitivity of the techniques, before and after enrichment, it is clear that the sensitivity of most techniques increased a factor 10^3, due to the multiplication of *Eca* in PM. However, the sensitivity of PCR increased a factor 10^6, indicating that inhibition took place by performing PCR directly in peel extracts. The inhibitory effect of crude peel extracts could be reduced by dilution of the extract or be eliminated by filtration through a 0.2 μm syringe filter holder (Sartorius AG, Göttingen, Germany) after centrifuging at 14,000 × *g* for 5 min. After filtration, 10^3 *Eca* cells ml^{-1} peel extract could be detected by PCR (Fig. 2).

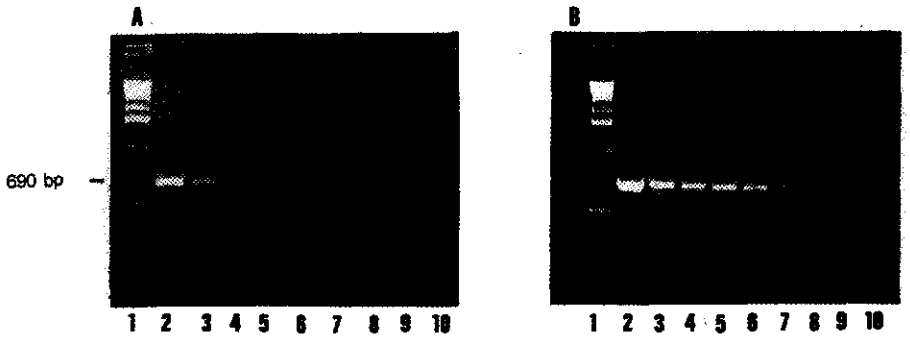

Figure 2. Detection of *Eca* in inoculated potato peel extracts by PCR, pure peel extracts (**A**) compared with filter-sterilized peel extracts (**B**). Lanes: 1 = DNA marker, 2 = 10^8, 3 = 10^7, 4 = 10^6, 5 = 10^5, 6 = 10^4, 7 = 10^3, 8 = 10^2 and 9 = 10 *Eca* cells/ml potato peel extract; 10 = uninoculated potato peel extract.

Detection of Eca in progeny tubers

The results of dilution plating, IF, ELISA, PCR and conductimetry for detection of *Eca* in the progeny tubers of vacuum infiltrated mother tubers of 12 different potato cultivars are presented in Table 2.

Eca was not detected by IF, dilution plating and PCR in the progeny tubers of the water vacuum infiltrated mother tubers of the 12 cultivars tested.

Table 2. Detection of *Eca* in potato peel extracts by dilution plating, conductimetry, IF, ELISA and PCR, before and after 48 h enrichment in polypectate medium (PM) at 20 °C

Samples of Progeny tubers	Direct detection					Detection after enrichment			
	Plating[1]	ELISA	IF[2]	PCR	Conductimetry[3]	Plating[1]	ELISA	IF[2]	PCR
Control mother tubers:									
Agria	-	-	-	-	-	Ecc	-	-	-
Alcmaria	-	-	-	-	-	-	-	-	-
Amazone	-	-	-	-	-	-	-	-	-
Arinda	Ecc	-	-	-	21.2	Ecc	+	-	-
Bintje	-	-	-	-	40.3	Ecc	-	-	-
Désirée	-	-	-	-	-	-	-	-	-
Hertha	-	-	-	-	-	Ecc	-	-	-
Karnico	-	-	-	-	-	-	-	-	-
Kondor	-	-	-	-	-	-	-	-	-
Morene	-	-	-	-	-	Ecc	-	-	-
Producent	-	-	-	-	-	-	-	-	-
Venouska	-	-	-	-	-	-	-	-	-
Inoculated mother tubers:									
Agria	-	-	-	-	-	-	-	-	-
Alcmaria	-	-	-	-	-	-	-	-	-
Amazone	-	-	5.12	-	-	Eca	-	6.69	+
Arinda	-	-	-	-	-	-	-	-	-
Bintje	-	-	4.54	-	-	-	-	4.47	+
Désirée	-	-	-	-	-	-	-	3.53	+
Hertha	-	-	4.47	-	-	-	-	2.99	-
Karnico	-	-	6.39	-	-	Eca	-	6.29	+
Kondor	-	-	4.17	-	-	Eca	-	6.17	+
Morene	-	-	-	-	-	-	-	-	-
Producent	-	-	5.69	-	-	Eca	-	6.69	+
Venouska	-	-	-	-	-	-	-	-	-

+ = positive reaction, - = negative reaction.
1 The detection thresholds of dilution plating were $>10^5$ and $>10^7$ *Erwinia* spp. cells ml^{-1} peel extract before and after enrichment, respectively.
2 Results of IF are expressed in log cells ml^{-1}; the detection thresholds of IF were 3.69 and 2.69 log cells ml^{-1} peel extract before and after enrichment, respectively.
3 Only for samples with detection times <48 h, considered to be positive in conductimetry, the average detection times ($n \approx 2$) are given.

The positive ELISA result of cultivar Arinda after enrichment is probably due to serologically cross-reacting *Ecc*, as *Eca* was not detected by other techniques applied and *Ecc* was only isolated in high numbers (>10^8 cells ml^{-1}) after enrichment.Ecc was also isolated after enrichment from the progeny tubers of cultivars Agria, Bintje, Hertha and Morene. Only two samples, Arinda and Bintje, with detection times of 21.2 and 40.3 h, respectively, were detected in conductimetry. These responses were produced by *Ecc* bacteria, which were isolated in numbers higher than 10^8 cells ml^{-1} after 48 h enrichment. As the growth rate of *Ecc* and *Eca* in peel extracts in PM at 20 °C were about equal (data not shown), the initial concentrations of *Ecc* in peel extracts of cultivars Bintje and Arinda were, *ca* 10^4 and 10^6 cells ml^{-1}, respectively, estimated from a calibration curve relating Td in conductimetry to numbers of *Eca* present in peel extracts. For cultivar Arinda, the presence of 10^6 *Ecc* cells ml^{-1} before enrichment could be confirmed by dilution plating.

With IF performed directly in peel extracts, *Eca* was detected in the progeny tubers of six out of the 12 cultivars, of which the mother tubers had been vacuum infiltrated with *Eca*. However, the cells stained were small and variable in size, and cell debris and lipopolysaccharide (LPS) particles were observed frequently. With ELISA, dilution plating and PCR performed directly in peel extracts, no samples were found positive for the presence of *Eca*. After enrichment of peel extracts, seven samples were found positive in IF, including the six samples which were IF-positive before enrichment. Compared to the cells stained in IF in peel extracts directly, the cells stained in IF after enrichment were larger and more equally sized, whereas LPS particles were present in large amounts and cell debris was observed less frequently. No samples were positive in ELISA, while, respectively, four and six out of the seven samples positive in IF were also found positive in dilution plating and PCR after enrichment. The differences in the number of positive samples for IF, PCR, dilution plating and ELISA can be explained by an decreasing sensitivity of these techniques in this order of succession. One IF-positive sample, which had the lowest number of stained cells, could not be detected by PCR, because the concentration of *Eca* was probably below the detection level for PCR. Only four IF-positive samples, those with the highest numbers of cells stained, were detected by dilution plating. Apparently, the detection levels of ELISA and conductimetry for detecting *Eca* were just not reached.

Discussion

Relation between blackleg incidence and transmission of Eca

The results of blackleg development in the field of the cultivars tested were similar to those found by Allefs *et al.* (1995). Cultivars Désirée, Arinda, Karnico and Producent were the most resistant, i.e. showed the lowest disease incidence, while Amazone, Alcmaria and Morene were highly susceptible. Transmission of *Eca* was not related to blackleg susceptibility. Progeny tubers of the most resistant cultivar Désirée, for which no blackleg development was observed, were infected with *Eca*, whereas *Eca* was not detected in progeny tubers of the highly susceptible cultivar Alcmaria. For the cultivars tested, differences in transmission of *Eca* from mother tubers into the stem base can be ascribed to cultivar differences in the rate of mother tuber decay (Allefs *et al.*, 1995), and the degree of lignification of the stem base (Weber, 1990). The low contamination levels of *Eca* in the progeny tubers in comparison with previous years (unpublished results) were probably due to the extreme hot and dry potato growing season of 1995. Under these conditions, which are unfavourable for bacterial growth, there is no or little transmission of *Eca* from rotting mother tubers to progeny tubers by soil water, but stolon end rot and vascular infection may still take place (Pérombelon and Hyman, 1992). In this study, contamination of progeny tubers with *Eca* may have occurred late in the growing season due to a slow built up of *Eca* populations in the mother tubers to levels high enough for invading stolons and, consequently, infection of progeny tubers. This is in agreement with the absence of *Eca* in progeny tubers of early potato cultivars, which show an rapid average mother tuber decay in the field, such as Alcmaria, Arinda and Venouska (Allefs *et al.*, 1995). The contrary was found for the late potato cultivars Hertha, Karnico and Producent. Contamination could also have occurred during harvest, as rotten mother tubers were present among the progeny tubers. The presence of large numbers of *Ecc* in progeny tubers of control plants may also be due to spread of soil-borne *Ecc* from colonized mother tubers to progeny tubers during harvest.

Comparison of methods for laboratory testing

For a direct sensitive detection of *Eca* in peel extracts on a routine base, IF with a detection threshold of 10^4-10^5 cells ml^{-1} peel extract was most suited. However, as also non-viable cells not able to incite blackleg will be counted in IF, seed lots can be declassified incorrectly. PCR was not suited for a direct sensitive detection of *Eca* in crude peel extracts, as the detection threshold for two

cultivars tested (Désirée and Amazone) was 10^7-10^8 cells ml^{-1}, in contrast to a detection threshold of 10^3-10^4 cells ml^{-1} when testing pure cultures. This effect was due to the presence of inhibitory compounds in the potato peel extracts prepared. The inhibition of PCR was reduced or even eliminated by dilution or filtration of crude potato peel extracts through a 0.2 μm filter, indicating that large particles, such as soil and starch, or matrix-bound compounds are involved. Demeke and Adams (1992) reported that some plant polysaccharides, such as dextrane sulfate, can inhibit PCR amplification, while in their studies starch and pectin showed no inhibitory effects. When using a different sample preparation, based on a less severe bacterium extraction step, De Boer and Ward (1995) found only for two out of 84 PCR-negative samples inhibition of PCR amplification, due to potato tuber-derived compounds. Smid *et al.* (1995) were able to overcome the problem of PCR-inhibitory potato-derived compounds by using a two-step DNA extraction procedure, which included a low-speed centrifugation of potato extracts to get rid of interfering particles.

After 48 h enrichment of peel extracts in PM at 20 °C, no inhibition of PCR was observed, and both IF and PCR were able to detect an initial concentration of 10 *Eca* cells ml^{-1} peel extract. Furthermore, a high correlation was found between numbers of *Eca* cells found in IF, PCR and dilution plating (Table 2), indicating that most of the cells counted in IF were viable and intact after enrichment. With regard to sensitivity, specificity, speed and labour, IF and PCR are higly suited to detect initial contamination levels of *Eca* of less than 10^4 cells ml^{-1} peel extract after enrichment. For laboratory testing on a routine base, IF can be used first to screen seed lots for presence of *Eca* after enrichment of potato peel extracts, while PCR can be applied to verify the positive samples, provided that the number of false negative reactions in serology is acceptable. For the detection of contamination levels of *Eca* higher than 10^4 cells ml^{-1} peel extract, automated conductance measurements may well be suited for a primary screening, after which PCR or ELISA can be used to verify the positive samples.

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References

Allan, E. and Kelman, A. (1977) Immunofluorescent stain procedures for detection and identification of *Erwinia carotovora* var. *atroseptica*. *Phytopathology* **67:** 1305-1312.

Allefs, S.J.H.M., Van Dooijeweert, W., De Jong, E.R., Prummel, W. and Hoogendoorn, C. (1995) The role of the seed tuber in determining partial resistance to potato blackleg caused by *Erwinia* spp.. *European Journal of Plant Pathology* **101:** 189-199.

Bain, R.A., Pérombelon, M.C.M., Tsror, L. and Nachmias, A. (1990) Blackleg development and tuber yield in relation to numbers of *Erwinia carotovora* subsp. *atroseptica* on seed potatoes. *Plant Pathology* **39:** 125-133.

De Boer, S.H., Slack, S.A., Van den Bovenkamp, G.W. and Mastenbroek, I. (1996) A role for pathogen indexing procedures in potato certification. In *Advances in Botanical Research Vol. 23, Pathogen indexing technologies* eds Andrews, J.H., Tommerup, I.C. and De Boer, S.H. pp. 217-242. London, UK: Academic press.

De Boer, S.H. and Ward, L.J. (1995) PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. *Phytopathology* **85:** 854-858.

Demeke, T. and Adams, R.P. (1992) The effects of plant polysaccharides and buffer additives on PCR. *BioTechniques* **12:** 332-334.

Fraaije, B.A., Birnbaum, Y., Franken, A.A.J.M. and Van den Bulk, R.W. (1996) The development of a conductimetric assay for automated detection of metabolically active soft rot *Erwinia* spp. in potato tuber peel extracts. *Journal of Applied Bacteriology*, in press.

Gorris, M.T., Alarcon, B., Lopez, M.M. and Cambra, M. (1994) Characterization of monoclonal antibodies specific for *Erwinia carotovora* subsp. *atroseptica* and comparison of serological methods for its sensitive detection on potato tubers. *Applied and Environmental Microbiology* **60:** 2076-2085.

Hyman, J.L., Wallace, A., Lopez, M.M., Cambra, M., Gorris, M.T. and Pérombelon, M.C.M. (1995) Chararacterization of monoclonal antibodies against *Erwinia carotovora* subsp. *atroseptica* serogroup I: specificity and epitope analysis. *Journal of Applied Bacteriology* **78:** 437-444.

Jones, D.A.C., Hyman, L.J., Tumeseit, M., Smith, P. and Pérombelon, M.C.M. (1994) Blackleg potential of potato seed: determination of tuber contamination by *Erwinia carotovora* subsp. *atroseptica* by immunofluorescence colony staining and stock and tuber sampling. *Annals of Applied Biology* **124:** 557-568.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y..

Pérombelon, M.C.M. and Burnett, E.M. (1991) Two modified crystal violet pectate (CVP) media for the detection, isolation and enumeration of soft rot erwinias. *Potato Research* **34:** 79-85.

Pérombelon, M.C.M. and Hyman, L.J. (1992) Control of potato blackleg: production of healthy seed. *Aspects of Applied Biology* **33:** 77-84.

Smid, E.J., Jansen, A.H.J. and Gorris, L.M.G. (1995) Detection of *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* in potato tubers using polymerase chain reaction. *Plant Pathology* **44:** 1058-1069.

Van Vuurde, J.W.L and Roozen, N.J.M. (1990) Comparison of immunofluorescence colony-staining in media, selective isolation on pectate medium, ELISA and immunofluorescence cell staining for detection of *Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi* in cattle manure slurry. *Netherlands Journal of Plant Pathology* **96:** 75-89.

Van Vuurde, J.W.L., Van den Bovenkamp, G.W. and Birnbaum, Y. (1983) Immunofluorescence microscopy and enzyme-linked immunosorbent assays as potential routine tests for detection of *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* in bean seed. *Seed Science and Technology* **11:** 547-559.

Weber, J. (1990) *Erwinia*: a review of recent research. In *Proceedings 11th Triennial Conference of the European Association for Potato Research*, Edinburgh, UK, pp. 112-121.

Chapter 8. General Discussion

8.1 Detection of *Pseudomonas syringae* pv. *pisi* in pea seeds

The traditional test protocol for pea bacterial blight, which is based on extraction, isolation by dilution plating and identification by host inoculation, is labour intensive and time consuming (Ball and Reeves, 1991). To improve the test efficiency without the need for isolation and identification, rapid techniques with high specificity and sensitivity are needed.

For conductimetric detection of *Psp* in pea seeds, Special Peptone Yeast Extract broth supplemented with cephalexin, cefuroxime, cycloheximide and boric acid (SPYEC) was used first as conductance medium. When seed extracts with low contamination levels of bacteria were tested, the detection times recorded in conductimetric assays in SPYEC correlated well with the numbers of *Psp* added to seed extracts at 17 as well as at 27 °C (Chapter 2). However, the relation between detection times and inoculation levels of *Psp* in seed extracts was dependent on the extraction method used. In comparison with *Psp* tested as pure culture, the detection of *Psp* in seed extracts prepared by 6 h soaking of seeds was faster, probably due to the release of growth-stimulating substances from the seeds. Additionally, the slower detection of *Psp* in the ground and 2 h-soaked pea samples could be explained by the release of toxic compounds affecting bacterial growth.

When seed lots with high contamination levels of bacteria were tested, the correlation between detection times and numbers of *Psp* present in seed extracts was poor. The poor correlation was due to the presence of saprophytic *Pseudomonas* spp. in seed extracts, which were able to interfere with the conductimetric assay by overgrowing *Psp* and generating conductance responses as well. Therefore, the presence of *Psp* in positive seed lots, suspected of being infected on the basis of detection times, had to be confirmed by other techniques. To improve the medium selectivity, a range of different antimicrobial agents was tested to restrict the growth of frequently occurring interfering saprophytes, which all were identified as *Pseudomonas* spp. strains (Chapter 3). However, *Psp* was more sensitive to the selective agents tested in comparison with most of the saprophytes tested. Furthermore, a minimal medium based on L-asparagine as sole carbon source (AM) was tested for conductimetric detection of *Psp* in pea seeds. To detect *Psp* in seed extracts conductimetrically, AM was supplemented with the same selective agents as in SPYEC (AMC) in order to restrict the growth of microbes other than *Pseudomonas* spp.. Although, AMC was not sufficiently selective to be used for a direct automated conductimetric detection of *Psp* in

pea seed extracts, in comparison with SPYEC, higher yields of *Psp* were obtained after enrichment of seed extracts in AMC. With immunofluorescence cell staining (IF) an initial concentration of less than 10^3 cells ml^{-1} could be detected in naturally contaminated seed extracts after 48 h enrichment at 27 °C. Recent studies showed that an indirect ELISA, detecting bacterial LPS released into AMC following the addition of EDTA and a heat treatment, was applied succesfully for a sensitive and accurate indexing of seed lots for pea blight (Lyons *et al.*, 1995). With this test procedure, an initial concentration of *Psp* of 10 cells ml^{-1} could be detected in inoculated pea seed extracts after 72 h enrichment in AMC at 27 °C. A high sensitivity is needed to control pea bacterial blight, as seeds carrying a single bacterium can be the primary source of infection in the field (Grondeau *et al.*, 1994). However, although serological detection of *Psp* in seed extracts after enrichment is sensitive, false negative and false positive reactions can be obtained in serology (Chapter 3). With regard to the specificity of the serological techniques, three different LPS O-chain based serogroups have been found for *Psp* (Grondeau *et al.*, 1992). Of the *Psp* isolates tested, 88.5, 11.4 and 0.1 % belonged to the serogroups APT-PIS, HEL2 and RIB, respectively. When using specific antisera directed against LPS of these three serogroups, cross-reactions were obtained for pea-associated *P. s.* pv. *syringae* (*Pss*) and *P. viridiflava* isolates, due to shared epitopes on the LPS O-chain (Grondeau *et al.*, 1992). Consequently, for an accurate detection, the presence of *Psp* in enriched seed extracts, found positive with serology, has to be confirmed with other specific tests, because *Pss* is able to grow in AMC. In this study, pathogenicity testing was used for verification (Chapter 3), but the polymerase chain reaction (PCR) using specific primers (Reeves *et al.*, 1994) might be more suitable, because it is a rapid and work-saving procedure which also allows detection of stressed, less virulent *Psp* cells in enriched seed extracts.

8.2 Detection of soft rot *Erwinia* spp. on potato tubers

Under weather conditions prevailing in The Netherlands only *Erwinia chrysanthemi* (*Ech*), as primary agent of stem wet rot (De Vries, 1990), and *Erwinia carotovora* subsp. *atroseptica* (*Eca*), as causal agent of blackleg, are considered to be a problem for seed potato production (Anon., 1982). Field inspection and laboratory testing with ELISA, the latter used additionally to detect latent infected seed lots that escaped field inspection, are currently used to control blackleg in The Netherlands. However, only the severest infected seed lots are detected, because of the low sensitivity of ELISA of 10^5-10^6 cells ml^{-1} peel extract (De Boer *et al.*, 1996). The aim of the laboratory test is to detect an

amount of 10^2-10^4 viable cells per tuber, which is generally the minimum size of inoculum needed to incite blackleg under most field conditions (Bain *et al.*, 1990).

To develop a specific conductimetric assay for detection of potato pathogenic *Erwinia* spp., *E. c.* subsp. *carotovora* (*Ecc*), *Eca*, *Ech* and a set of potato-associated saprophytes were tested for their ability to generate conductance responses in different media (Chapter 4). In minimal medium supplemented with L-asparagine, only *Pseudomonas* and *Erwinia* spp. were able to generate large conductance responses rapidly, whereas with polypectate as sole carbon source only *Erwinia* spp. produced distinct conductance responses. The high conductance responses of *Erwinia* spp. in pectate media were due to the release of large amounts of a series of oligogalacturonates during depolymerization of pectate by a combined action of extracellular polygalacturonases (PGs) and pectate lyases (PLs) (Chapter 5). Other highly pectolytic bacteria, such as *Klebsiella* and an unidentified saprophyte, could only generate weak conductance responses in pectate media, due to PL activity.

Because the growth of interfering *Pseudomonas* spp. could not be restricted by adding selective agents to L-asparagine minimal medium (B.A. Fraaije, unpublished), minimal medium with polypectate as sole carbon source was most suitable to be used for conductimetric detection of *Erwinia* spp. in potato peel extracts. Only small samples of potato peel extracts could be tested conductimetrically, because interfering conductance responses were recorded, due to bacterial conversion of asparagine/aspartic acid present in potato peel extract itself (Chapter 4). The detection threshold for *Eca* in inoculated potato peel extracts was generally *ca* 10^4 cells ml^{-1}, when samples were considered positive on the basis of a response within 48 h at 20 °C. About 10^5 cells of *Ech* ml^{-1} were detected within 36 h at 25 °C (Chapter 6). When naturally contaminated potato peel extracts were tested, samples with a positive conductance response had to be confirmed with other techniques, such as ELISA or PCR, for presence of *Eca* and *Ech*, since *Ecc* was also able to generate a conductance response. The conductimetric detection of contamination levels of *Eca* higher than 10^4 cells ml^{-1} peel extract was specific and efficient, because most of the seed lots tested were negative in conductimetry, meaning that an additional test to check the presence of *Eca* was superfluous. Consequently, large-scale certification of seed lots for contamination levels of *Eca* higher than 10^4 cells ml^{-1} peel extract to control blackleg can be done with automated conductance measurements as a primary screening, after which PCR can be used to verify the positive samples. For *Ech*, the conductimetric detection was less specific and sensitive, and unefficient, due to the presence of low contamination levels of *Ech* and high

numbers of *Ecc* after enrichment, which interfered with the test.

To improve the sensitivity of conductimetry, larger samples can be tested after removal of asparagine/aspartic acid in peel extracts by centrifugation. Additionally, the use of an immunomagnetic separation procedure (Van der Wolf *et al.*, 1996) or a selective pre-enrichment step, performed prior to conductimetry to increase the ratio of target bacteria to non-target bacteria, may also improve the sensitivity and specificity. Furthermore, the selectivity of the polypectate conductance medium for *Ech* might be increased by adding the selective agent Brilliant Green, because the growth of both *Eca* and *Ecc* was restricted when pure cultures were tested (B.A. Fraaije, unpublished).

Immunofluorescence colony staining (IFC), for both *Eca* and *Ech* (Chapter 6) and enrichment combined with IF or PCR, for *Eca* (Chapters 6 and 7), were suitable to detect and quantify low numbers of bacteria, viz. $10\text{-}10^4$ cells ml^{-1} in potato peel extracts. With regard to serology, false positive and false negative reactions were observed (Chapters 6 and 7). However, since the chance of false negative reactions caused by unusual serotypes of *Ech* and *Eca* is negligible in The Netherlands (Anon., 1982), only false positive reactions in serology are considered as a major problem for laboratory testing. To exclude false positive reactions in IFC or IF using enrichment, PCR was applied succesfully (Chapters 6 and 7). If desirable, false negative serological reactions of enriched peel extracts can be excluded by testing all samples with PCR. The latter test protocol, although being laborious and expensive, might be very useful in small-scale blackleg indexing of valuable young clonal material from the top of the selection system in order to eradicate the disease, because of the specificity and the extremely low detection limit of 10 cells ml^{-1} potato peel extract.

References

Anon. (1982) Zwartbenigheid en stengelnatrot bij aardappelen. pp. 1-12. Wageningen,The Netherlands: Werkgroep Onderzoek Knolziekten.

Bain, R.A., Pérombelon, M.C.M., Tsror, L. and Nachmias, A. (1990) Blackleg development and tuber yield in relation to numbers of *Erwinia carotovora* subsp. *atroseptica* on seed potatoes. *Plant Pathology* **39:** 125-133.

Ball, S.F.L. and Reeves, J.C. (1991) The application of new techniques in the rapid testing for seed-borne pathogens. *Plant Varieties and Seeds* **4:** 169-176.

De Boer, S.H., Slack, S.A, Van den Bovenkamp, G.W. and Mastenbroek, I. (1996) A role for pathogen indexing procedures in potato certification. In *Advances in Botanical Research Vol. 23, Pathogen indexing technologies* eds Andrews, J.H., Tommerup, I.C. and De Boer, S.H. pp. 217-242. London, UK: Academic Press.

De Vries P.M. (1990) Stengelnatrot symptomen in aardappelplanten. *Aardappelwereld* **4:** 15-18.

Grondeau, C., Saunier, M., Poutier, F. and Samson, R. (1992) Evaluation of physiological and serological profiles of *Pseudomonas syringae* pv. *pisi* for pea blight identification. *Plant Pathology* **41:** 495-505.

Grondeau, C., Poutier, F. and Samson, R. (1994) Pea seed contamination by *Pseudomonas syringae* pv. *pisi*: description and consequences. In *Proceedings of the 8th International Conference of Plant Pathogenic Bacteria* eds Lemaittre, M., Freignoun, S., Rudolph, K. and Swings, J.G. pp. 779-783. Paris, France: INRA editions.

Lyons, N.F., Taylor, J.D., Roberts, S.J., Maury, Y., Duby, C., Masmoudi, K., Faris-Mokaiesh, S., Corbière, R., Mendes-Pereira, E., Spire, D., Samson, R., Malandrin, L., Grondeau, C., Franken, A.A.J.M. and Fraaije, B.A. (1995) International programme on the serological detection of bacterial, fungal and viral pathogens of protein pea seeds. *EPPO Bulletin* **25**: 393-401.

Reeves, J.C., Rasmussen, O.F. and Simpkins, S.A. (1994) The use of a DNA probe and PCR for the detection of *Pseudomonas syringae* pv. *pisi* in pea seed. In *Proceedings of the 8th International Conference of Plant Pathogenic Bacteria* eds Lemaittre, M., Freignoun, S., Rudolph, K. and Swings, J.G. pp. 383-390. Paris, France: INRA editions.

Smid, E.J., Jansen, A.H.J. and Gorris, L.G.M. (1995) Detection of *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* in potato tubers using polymerase chain reaction. *Plant Pathology* **44**: 1058-1069.

Van der Wolf, J.M., Hyman, L.J., Jones, D.A.C., Grevesse, C., Van Beckhoven, J.M.C.M., Van Vuurde, J.W.L. and Pérombelon, M.C.M. (1996) Immunomagnetic separation of *Erwinia carotovora* subsp. *atroseptica* from potato peel extracts to improve detection sensitivity on a crystal violet pectate medium or by PCR. *Journal of Applied Bacteriology* **80**: 487-495.

Summary

Pea bacterial blight and potato blackleg are diseases caused by *Pseudomonas syringae* pv. *pisi* (*Psp*) and soft rot *Erwinia* spp., respectively. The primary source of inoculum for these bacteria is contaminated plant propagation material, i.e. pea seeds and potato tubers. One of the best ways to control the diseases is the use of healthy planting material. To check the health status of this material, sensitive and specific methods are needed to detect the bacteria.

In Chapter 2 the use of a conductimetric assay to detect *Psp* in pea seed extracts is described. The conductance medium used was based on Special Peptone Yeast Extract broth (SPYE) with the addition of the selective agents boric acid, cycloheximide, cephalexin and cefuroxime to restrict the growth of other micro-organisms. Conductimetric assays, immunofluorescence cell staining (IF) and an enzyme-linked immunosorbent assay (ELISA) for detecting *Psp* in pea seed extracts were compared with dilution plating by two extraction methods, viz. 6 h soaking of pea seeds and 2 h soaking of flour of ground pea seeds in water. In general, the detection of *Psp* with conductimetric, IF and dilution plating assays in the extracts of the ground and 2 h-soaked pea samples was less sensitive than the detection in the extracts of the 6 h-soaked pea samples. The detection thresholds of these assays varied per seed lot between 0 and 10^4 cfu ml^{-1} for the 6 h soaking procedure. The detection threshold of ELISA varied for both extraction methods generally between 10^4 and 10^6 cfu ml^{-1}. Detection times recorded in conductimetric assays correlated well with the number of *Psp* added to seed extracts at 27 as well as at 17 °C. Due to the presence of saprophytic *Pseudomonas* spp., which were able to overgrow *Psp* and to generate conductance responses, conductimetric detection in SPYEC was not useful for routine testing.

In Chapter 3 a medium based on L-asparagine conversion (AM) was found more suitable for conductimetric detection of *Psp* than SPYE, because higher and more specific conductance responses were obtained for *Pseudomonas* spp., *Psp* included. However, AM supplemented with the same selective agents as in SPYEC (AMC) was still not sufficiently selective for a direct conductimetric detection of *Psp* in pea seed extracts, mainly due to the presence of interfering conductance responses caused by *Pseudomonas fluorescens* and *Pseudomonas putida*. Although the medium selectivity could not be improved further by addition of other selective agents, AMC was shown to be useful in an enrichment procedure. In comparison with SPYEC, higher yields of *Psp* were obtained after enrichment of seed extracts in AMC. With IF an initial concentration of less than

10^3 *Psp* cells ml^{-1} could be detected in naturally contaminated seed extracts after 48 h enrichment in AMC at 27 °C. However, although serological detection of *Psp* in seed extracts after enrichment was sensitive, false negative and false positive reactions, due to the presence of unusual serotypes of *Psp* and cross reacting *Pseudomonas syringae* pv. *syringae* (*Pss*), respectively, can still be obtained in serology. Consequently, for an accurate detection the presence of *Psp* in enriched seed extracts, found positive with serology, has to be confirmed with other specific tests. Suitable techniques are pathogenicity testing and the polymerase chain reaction (PCR), provided that specific primers are available for the latter technique to exclude false positive reactions in serology due to the presence of cross-reacting *Pss*.

To develop a specific conductimetric assay for detection of potato pathogenic *Erwinia* spp., *Erwinia carotovora* subsp. *atroseptica* (*Eca*), *Erwinia carotovora* subsp. *carotovora* (*Ecc*), *Erwinia chrysanthemi* (*Ech*) and a set of potato-associated saprophytes were tested for their ability to generate conductance responses in various media (Chapter 4). In SPYE, all bacteria tested, including the genera *Bacillus*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Pseudomonas* and *Xanthomonas*, generated conductance responses, while in minimal medium supplemented with glucose and trimethylamine N-oxide only Enterobacteriaceae, *Erwinia* spp. included, generated conductance responses. Additionally, in minimal medium supplemented with L-asparagine, only *Pseudomonas* and *Erwinia* spp. were able to generate large conductance responses rapidly, whereas with polypectate as sole carbon source only *Erwinia* spp. produced distinct conductance responses.

The high conductance responses of *Erwinia* spp. in pectate media were due to the release of large amounts of saturated and unsaturated oligogalacturonates during depolymerization of pectate by a combined action of extracellular poly-galacturonases (PGs) and pectate lyases (PLs) (Chapter 5). Other highly pectolytic bacteria, such as *Klebsiella* and an unidentified saprophyte, could only generate weak conductance responses in pectate media, due to PL activity.

Because of its specificity, minimal medium with polypectate as sole carbon source (PM) was most suitable for conductimetric detection of *Erwinia* spp. in potato peel extracts. Due to bacterial conversion of asparagine/aspartic acid present in potato peel extract itself, generating an interfering conductance response in PM, only small samples or 10-fold dilutions of potato peel extracts could be tested conductimetrically (Chapter 4). The detection threshold for *Eca* in inoculated potato peel extracts was *ca* 10^4 cells ml^{-1}, when samples were considered positive on the basis of a response at 20 °C within 48 h, while *ca* 10^5

cells of *Ech* ml^{-1} were detected at 25 °C within 36 h (Chapters 4 and 6). Samples with a positive conductance response had to be confirmed with other techniques, such as ELISA or PCR, for presence of *Eca* and *Ech*, since *Ecc* was also able to generate a conductance response. The conductimetric detection of contamination levels of *Eca* higher than 10^4 cells ml^{-1} peel extract was specific and efficient, because most of the seed lots tested were negative in conductimetry, meaning that an additional test to check the presence of *Eca* was superfluous (Chapter 6). Consequently, large-scale certification of seed lots for contamination levels of *Eca* higher than 10^4 cells ml^{-1} peel extract to control blackleg can be done with automated conductance measurements as a primary screening, after which PCR can be used to verify the positive samples. For *Ech*, the conductimetric detection was less specific and sensitive, and unefficient, due to the presence of low contamination levels of *Ech* and high numbers of *Ecc* after enrichment, which interfered with the test (Chapter 6). Further research is needed to improve the sensitivity and specificity of the conductimetric assays for *Erwinia* spp., which for example might be achieved by the use of an immunomagnetic separation procedure or a selective pre-enrichment step before applying conductimetry.

Immunofluorescence colony staining (IFC), for both *Eca* and *Ech* (Chapter 6) and enrichment combined with IF or PCR, for *Eca* (Chapters 6 and 7), were suitable to detect and quantify lower numbers of bacteria, viz. 10-10^4 cells ml^{-1} in potato peel extracts. With regard to serology, false positive and false negative reactions were observed (Chapters 6 and 7). However, since the chance of false negative reactions caused by unusual serotypes of *Ech* and *Eca* is negligible, only false positive reactions in serology are considered as a major problem for laboratory testing. To exclude false positive reactions in IFC or in IF using enrichment, verification with PCR was applied succesfully (Chapters 6 and 7). If required, false negative serological reactions of enriched peel extracts can be excluded by testing all samples with PCR. The latter test protocol, although being laborious and expensive, might be very useful in small-scale blackleg indexing of valuable young clonal material from the top of the selection system in order to eradicate the disease, because of the specificity and the extremely low detection threshold of 10 cells ml^{-1} potato peel extract.

Samenvatting

Bacteriebrand bij de erwt en zwartbenigheid en stengelnatrot bij de aardappel zijn ziekten die respectievelijk veroorzaakt worden door *Pseudomonas syringae* pathovar *pisi* (*Psp*) en natrot *Erwinia* bacteriën. De belangrijkste verspreidingsbron voor deze bacteriën is besmet plantaardig uitgangsmateriaal, hier dus erwtezaden en aardappelpootgoed. Eén van de beste manieren om de ziekten te beheersen is het gebruik van gezond uitgangsmateriaal. Om de gezondheid van het uitgangsmateriaal vast te stellen zijn gevoelige en specifieke methoden nodig die deze plantepathogene bacteriën kunnen aantonen.

Het gebruik van een geleidbaarheidstest om *Psp* aan te tonen in erwtezaadextracten staat beschreven in Hoofdstuk 2. Het geleidbaarheidsmedium dat gebruikt werd was gebaseerd op 'Special Peptone Yeast Extract' bouillon (SPYE) met toevoeging van de selectieve stoffen boorzuur, cycloheximide, cephalexin en cefuroxime om de groei van andere micro-organismen te onderdrukken. Om *Psp* in erwtezaadextracten aan te tonen werden geleidbaarheidsmetingen, immunofluorescentie celkleuring (IF) en een 'enzyme-linked immunosorbent assay' (ELISA) met twee extractiemethoden (het 6 uur weken van erwtezaden versus 2 uur weken van meel van vermalen erwtezaden) vergeleken met de traditionele uitplaattest. De detectie van *Psp* d.m.v. de geleidbaarheidsmetingen, IF en uitplaten in de extracten van de vermalen, 2 uur geweekte erwtezaden was in het algemeen minder gevoelig dan de detectie in de extracten van de 6 uur geweekte erwtezaden. De detectiedrempels van deze technieken varieerde voor de 6 uur weekmethode per zaadpartij tussen 0 en 10^4 kolonie-vormende eenheden (cfu) per ml. De detectiedrempel van ELISA varieerde voor beide extractiemethoden tussen 10^4 en 10^6 cfu per ml. De in de geleidbaarheidsmetingen geregistreerde detectietijden vertoonden bij zowel 27 als 17 °C een lineaire correlatie met het aantal aan de zaadextracten toegevoegde *Psp* bacteriën. Echter door de aanwezigheid van saprofytische *Pseudomonas* spp., die in staat waren *Psp* te overgroeien en geleidbaarheidssignalen te produceren, bleek de conductimetrische detectie in SPYEC niet geschikt voor een routinematige toetsing van erwtezaden op de aanwezigheid van *Psp*.

In Hoofdstuk 3 werd een medium gebaseerd op de omzetting van L-asparagine (AM) beter geschikt bevonden dan SPYE voor de conductimetrische detectie van *Psp*, omdat voor alle geteste *Pseudomonas* spp., inclusief *Psp*, hogere en specifiekere geleidbaarheidssignalen werden verkregen. Helaas bleek de selectiviteit van AM met de toevoeging van dezelfde selectieve stoffen als in SPYE (AMC) nog steeds niet voldoende selectief voor een directe

conductimetrische detectie van *Psp* in zaadextracten, omdat met name *Pseudomonas fluorescens* en *Pseudomonas putida* storende geleidbaarheidssignalen kunnen veroorzaken. Alhoewel de mediumselectiviteit niet verder verbeterd kon worden door toevoeging van andere selectieve stoffen, was AMC goed bruikbaar in een ophopingsprocedure. In vergelijking met SPYEC was de opbrengst aan *Psp* na ophoping vanuit extracten in AMC aanzienlijk hoger. Na 48 uur ophopen van erwtezaadextracten in AMC bij 27 °C kon met IF een initiële concentratie van minder dan 10^3 *Psp* cellen per ml gedetecteerd worden in besmette zaadextracten. Alhoewel de serologische detectie van *Psp* in zaadextracten na ophoping gevoelig genoeg was, kunnen er wel vals negatieve en vals positieve resultaten verkregen worden door de aanwezigheid van respectievelijk ongewone serotypen van *Psp* en kruisreagerende *Pseudomonas syringae* pv. *syringae* cellen. Om uiteindelijk een nauwkeurige detectie van *Psp* in opgehoopte zaadextracten te krijgen zullen extracten die positief zijn bevonden met serologische technieken ook nog eens bevestigd moeten worden met andere specifieke technieken. Hiervoor kunnen pathogeniteitstesten en de polymerase kettingreactie (PCR) gebruikt worden, mits er voor de laatstgenoemde techniek specifieke primers voor *Psp* beschikbaar zijn om vals positieve resultaten veroorzaakt door serologisch kruisreagerende *Pss* cellen uit te sluiten.

In Hoofdstuk 4 werden *Erwinia carotovora* subsp. *atroseptica* (*Eca*), *Erwinia carotovora* subsp. *carotovora* (*Ecc*), *Erwinia chrysanthemi* (*Ech*) en een reeks saprofytische isolaten uit aardappelschilextracten getest op hun vermogen om geleidbaarheidssignalen te produceren in verschillende media. Het doel van dit onderzoek was om een specifieke geleidbaarheidstest voor detectie van plantepathogene natrot *Erwinia* bacteriën (*Eca* en *Ech*) te ontwikkelen. Alle geteste bacterie-isolaten waaronder de genera *Bacillus*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Pseudomonas* en *Xanthomonas* produceerden een geleidbaarheidssignaal in SPYE. In minimaal medium met glucose en trimethylamine N-oxide vormden alleen de Enterobacteriaceae, inclusief *Erwinia* spp., een signaal. Verder waren alleen de geteste *Pseudomonas* en *Erwinia* isolaten in staat om in minimaal medium met L-asparagine een signaal te produceren, terwijl met polypectaat als enige koolstofbron alleen *Erwinia* bacteriën een duidelijk signaal afgaven.

De geleidbaarheidssignalen van de *Erwinia* bacteriën in pectaat media werden veroorzaakt door het vrijkomen van grote hoeveelheden galacturonaat-oligomeren gedurende de depolymerisatie van pectaat door een gecombineerde werking van polygalacturonases (PGs) en pectaatlyases (PLs), extracellulair geproduceerde enzymen van *Erwinia* bacteriën (Hoofdstuk 5). Andere sterk

pectolytische bacteriën, zoals *Klebsiella* en een ongeïdentificeerde saprofyt, produceerden slechts een zwak signaal, wat veroorzaakt werd door PL activiteit.

Vanwege de specificiteit werd minimaal medium met polypectaat (PM) verder gebruikt voor de conductimetrische detectie van *Erwinia* spp. in aardappelschil-extracten. Omdat door bacteriële omzetting van L-asparagine/aspartaat in het aardappelschilextract zelf een storend geleidbaarheidssignaal werd verkregen, konden slechts kleine monstervolumes of 10-voudige verdunningen van aardappelschilextracten conductimetrisch getest worden (Hoofdstuk 4). De detectiedrempel voor *Eca* in geïnoculeerde schilextracten was ca 10^4 cellen per ml, wanneer monsters positief werden beschouwd op basis van een geleidbaarheidssignaal binnen 48 uur bij 20 °C. Ongeveer 10^5 *Ech* cellen konden worden aangetoond binnen 36 uur bij 25 °C (Hoofdstukken 4 en 6). Omdat *Ecc* ook een geleidbaarheidssignaal kan produceren in PM moest voor de monsters die positief waren bevonden in de geleidbaarheidstesten de aanwezigheid van *Eca* en *Ech* bepaald worden met andere technieken, zoals ELISA en PCR. De conductimetrische detectie van besmettingsniveaus van *Eca* van meer dan 10^4 cellen per ml schilextract was specifiek en efficiënt, omdat de meeste monsters negatief waren in de geleidbaarheidstesten en dus niet verder geverifieerd hoefden te worden. Voor certificatie van basispootgoed om op zwartbenigheid te controleren kunnen geautomatiseerde geleidbaarheidsmetingen als voorscreeningsmethodiek gebruikt worden, gevolgd door PCR om positieve monsters te verifieren. Hierbij konden ca 10^4 *Eca* cellen per ml schilextract worden aangetoond.

De conductimetrische detectie van *Ech* was behalve minder specifiek en minder gevoelig ook onefficiënt, omdat de geteste pootgoedpartijen besmet waren met lage aantallen *Ech* bacteriën en er tijdens detectie veel geleidbaarheidssignalen veroorzaakt werden door *Ecc* bacteriën (Hoofdstuk 6). Meer onderzoek is nodig om de gevoeligheid en specificiteit van de geleidbaarheidstesten voor *Erwinia* spp. verder te verbeteren. Hierbij kan worden gedacht aan het toepassen van een immunomagnetische isolatie-procedure of het gebruik van een selectieve voorophopingsstap, om het aantal doelbacteriën in de monsters te verhogen alvorens een geleidbaarheidstest uit te voeren.

De immunofluorescentie koloniekleuring techniek (IFC), toegepast voor detectie van zowel *Eca* als *Ech* (Hoofdstuk 6), en immunofluorescentie celkleuring en PCR, toegepast na ophoping van *Eca* (Hoofdstukken 6 en 7), waren in staat om zeer lage aantallen van deze doelbacteriën in schilextracten te quantificeren, namelijk 10 tot 10^4 cellen per ml. Wat betreft de serologische technieken werden er vals positieve en vals negatieve resultaten verkregen

(Hoofdstukken 6 en 7). Omdat de kans op vals negatieve serologische reacties veroorzaakt door afwijkende serotypen van *Eca* verwaarloosbaar is, worden alleen de vals positieve serologische reacties als een probleem beschouwd voor routinematige laboratoriumtesten. PCR, toegepast ter verificatie van positieve monsters, bleek goed bruikbaar om de vals positieve reacties in IFC en in IF na verrijking uit te sluiten (Hoofdstukken 6 en 7). Wanneer het wenselijk is om ook vals negatieve serologische reacties na ophoping uit te sluiten, zullen alle monsters met PCR getest moeten worden. De laatst genoemde methodiek, alhoewel arbeidsintensief en duur, kan vanwege de hoge specificiteit en een extreem lage detectiedrempel van 10 cellen per ml aardappelschilextract zeer nuttig zijn voor een kleinschalige keuring van hoogwaardig basispootgoed of stammenmateriaal om de toename van zwartbenigheid tegen te gaan.

List of publications

Some Chapters described in this thesis are based on the following papers:

Fraaije, B.A., Franken, A.A.J.M., Van der Zouwen, P.S., Bino, R.J. and Langerak, C.J. (1993). Serological and conductimetric assays for the detection of *Pseudomonas syringae* pathovar *pisi* in pea seeds. *Journal of Applied Bacteriology* **75:** 409-415. (Chapter 2).

Fraaije, B.A., Birnbaum, Y., Franken, A.A.J.M. and Van den Bulk, R.W. (1996) The development of a conductimetric assay for automated detection of metabolically active soft rot *Erwinia* spp. in potato tuber peel extracts. *Journal of Applied Bacteriology* **81:** 375-382. (Chapter 4).

Fraaije, B.A., Bosveld, M., Van den Bulk, R.W. and Rombouts, F.M. Analysis of conductance responses during depolymerization of pectate by soft rot *Erwinia* spp. and other pectolytic bacteria isolated from potato tubers. *Journal of Applied Bacteriology*, submitted. (Chapter 5).

Fraaije, B.A., Appels, M., De Boer, S.H., Van Vuurde, J.W.L. and Van den Bulk, R.W. Detection of soft rot *Erwinia* spp. on seed potatoes: conductimetry in comparison with dilution plating, PCR and serological assays. *European Journal of Plant Pathology*, submitted. (Chapter 6).

Fraaije, B.A., Birnbaum, Y. and Van den Bulk, R.W. Comparison of methods for detection of *Erwinia carotovora* subsp. *atroseptica* in progeny tubers derived from inoculated tubers of *Solanum tuberosum L. Journal of Phytopathology*, in press. (Chapter 7).

Other publications:

Bussink, H.J.D., Buxton, F.P., Fraaije, B.A., De Graaff, L.H. and Visser, J. (1992) The polygalacturonases of *Aspergillus niger* are encoded by a family of diverged genes. *European Journal of Biochemistry* **208:** 83-90.

Fraaije, B.A., Franken, A.A.J.M. and Van der Zouwen, P.S. (1994) The use of conductimetric assays for the detection of *Pseudomonas syringae* pv. *pisi* in pea seeds. In *Proceedings of the 8th International Conference Plant Pathogenic Bacteria*, Versailles (France), June 9-12, 1992 eds Lemattre, M., Freigoun, S., Rudolph, K. and Swings, J.G. pp. 349-354. Paris, France: INRA Editions (Les Colloques no66).

Franken, A.A.J.M., Fraaije, B.A. and Van der Zouwen, P.S. (1994) The application of conductimetric assays in phytopathology. In *Proceedings of the 8th International Conference Plant Pathogenic Bacteria*, Versailles (France), June 9-12, 1992 eds Lemattre, M., Freigoun, S., Rudolph, K. and Swings, J.G. pp. 735-740. Paris, France: INRA Editions (Les Colloques no66).

Lyons, N.F., Taylor, J.D., Roberts, S.J., Maury, Y., Duby, C., Masmoudi, K., Faris-Mokaiesh, S., Corbière, R., Mendes-Pereira, E., Spire, D., Samson, R., Malandrin, L., Grondeau, C., Franken, A.A.J.M. and Fraaije, B.A. (1995) International programme on the serological detection of bacterial, fungal and viral pathogens of protein pea seeds. *EPPO Bulletin* **25:** 393-401.

Curriculum vitae

Bartholomeus Antonius (Bart) Fraaije werd op 10 januari 1966 geboren in 's-Hertogenbosch. Na het behalen van het Atheneum diploma aan het Jacob Roelands Lyceum te Boxtel in 1985, werd in augustus 1985 begonnen met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen. In 1991 werd het doctoraal examen Moleculaire Wetenschappen, oriëntatie's Chemisch-biologisch en Biotechnologisch, met als hoofdvakken Moleculaire Genetica en Industriële Microbiologie, behaald. Vervolgens werkte hij 4 jaar in de functie van wetenschappelijk onderzoeker microbioloog/ fytobacterioloog bij het Centrum voor Plantenveredelings- en Reproductie-onderzoek (CPRO-DLO). Het onderzoek dat gedurende de periode juni 1991 tot januari 1996 werd uitgevoerd op de afdeling Reproductie-technologie, sectie Gezondheid, van het CPRO-DLO resulteerde in dit proefschrift.

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