Resistance to *Erwinia* spp. in potato (*Solanum tuberosum* L.)

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Resistance to *Erwinia* spp. in potato (*Solanum tuberosum* L.)

Resistentie tegen *Erwinia* spp. in aardappel
(*Solanum tuberosum* L.)

Sjefke Allefs

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 30 mei 1995
des namiddags te half twee in de Aula
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STELLINGEN

1. *De resistentie van aardappelgenotypen tegen zwartbenigheid kan niet betrouwbaar worden vastgesteld in het zaailingstadium, noch direct aan knolmateriaal uit bewaring. Dit proefschrift.*

2. Bij het zoeken naar bronnen van resistentie tegen zwartbenigheid in het gewas aardappel dient rekening te worden gehouden met het bestaan van componenten van resistentie. *Dit proefschrift.*

3. Het verdient aanbeveling om de termen zwartbenigheid en stengelnatrot slechts te gebruiken voor de beschrijving van symptomen zonder direct een relatie te leggen met de fytopathogene bacteriën die deze veroorzaken. *M.C.M. Pérombelon & A. Kelman, 1987, Plant Disease 71: 283-285.*

4. Deklassering van pootaardappelen op basis van het percentage waargenomen planten met zwartbenigheid is weinig effectief als maatregel ter verlaging van de besmettingsgraad van het te oogsten pootgoed. *M.C.M. Pérombelon, 1992, Netherlands Journal of Plant Pathology 98 Supplement 2: 135-146.*

5. Door de voortdurende afname van het beschikbare landbouwareaal per capita, die wordt veroorzaakt door bodemerosie, verstedelijking en toename van de wereldbevolking, zal verbetering van het opbrengend vermogen van landbouwgewassen nog lange tijd één van de belangrijkste veredelingsdoelen blijven. *D. Pimentel et al., 1995, Science 267: 1117-1121.*

6. De neiging van veel moleculair biologen om langs biotechnologische weg verkregen resistentie tegen fytopathogenen te presenteren als absoluut, ook in die gevallen waarin een dosis-respons relatie verwacht mag worden, is veelal terug te voeren op hun onbestemde gevoelens jegens kwantitatieve data.

7. Zij die overwegen om de relatie tussen aardappel en pectolytische *Erwinia* spp. te gaan bestuderen moeten zich realiseren dat daar een hoop rottigheid van komt.

8. Hedendaagse natuurontwikkelingsprojecten luiden een nieuw hoofdstuk in in de geschiedenis van de Nederlandse tuinarchitectuur.

9. Pijprokers dienen nadrukkelijker te worden gewezen op het gevaar dat zij lopen tijdens het rijden in een auto met airbag(s).

10. Als de uitstoot van kooldioxide als vervuilend moet worden beschouwd, is het voor het milieu beter om de fiets te laten staan indien de af te leggen afstand ook met het openbaar vervoer kan worden overbrugd.

11. Het verdwijnen van de smalfilmindustrie zal toekomstige cultuurhistorici nog lelijk opbreken.

12. Het bestaan van door beursgoeroes geëxploiteerde 06-lijnen doet vermoeden dat zulks lucratiever is dan het effectueren van de verstrekte tips en adviezen.

13. Van integratie van de Europese bevolking zal nauwelijks sprake zijn zolang een Nederlandse regenjas waterdicht is en een Italiaanse mooi.

Stellingen behorend bij het proefschrift van Sjefke Allefs, getiteld 'Resistance to *Erwinia spp.* in potato (*Solanum tuberosum* L.)', te verdedigen op 30 mei 1995 in de Aula van de Landbouwuniversiteit te Wageningen.

Abstract

Blackleg is a disease of potato, *Solanum tuberosum*, which is caused by the bacteria *Erwinia carotovora* subsp. *carotovora* (*Ecc*), *E.c.* subsp. *atroseptica* (*Eca*) or *E. chrysanthemi* (*Ech*). Incidence of blackleg negatively affects the quality of seed potatoes. Disease control relies on phytosanitary measures aiming to reduce the spread of the pathogen. Partial resistance has been identified but exploitation of genotypic variation has been hindered by the lack of efficient and accurate methods for measuring resistance.

The partial resistance of 12 cultivars was studied in terms of components of resistance. Tuber tissue resistance was considered as a putative component and studied under aerobic and anaerobic conditions. Significant and reproducible differences for resistance were found, but these differences had no explanatory value in relation to resistance to blackleg in the field. However, it was found that the mother tubers of plants in the field play a role in determining field resistance but that this role for a given cultivar can not be predicted from resistance screening under laboratory conditions. Another important component of resistance was found to be located in the stem base. When stem base resistance was measured by inoculation with *Ech*, this component accounted for 58% and 65% of the variance in field experiments for resistance to *Eca* and *Ech*, respectively. Resistance of the above ground stem tissue was of minor importance in determining blackleg resistance.

A study on one first, and 11 second backcross populations which were derived from somatic hybrids between *S. tuberosum* and *S. brevidens*, revealed that relatively high levels of tuber tissue resistance could be found in this material. As for cultivars, no correlation was found between tuber tissue resistance and resistance to blackleg. It is doubtful whether the material is useful as a source of resistance to blackleg. In addition, attempts were undertaken to introduce resistance in potato cultivars by means of genetic transformation with constructs encoding the antibacterial peptides cecropin B, α-hordothionin and tachyplesin I. Transcription of the cecropin B gene was found in transgenic plants but no expression of this peptide. This was probably a result of instability of cecropin B in potato tissue. Expression of α-hordothionin was found but the transgenic plants did not show enhanced resistance. Expression of tachyplesin I was detected in transgenic plants of cvs Bintje, Karnico and Kondor. Small tubers of these transgenic plants showed a slight improvement of tuber tissue resistance in a repeated experiment, especially under aerobic conditions.

Voorwoord

Dit proefschrift doet verslag van een onderzoek dat gedurende viereneenhalf jaar is uitgevoerd bij het Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO) te Wageningen in opdracht van Agrico Research B.V., te Bant.

Het is niet zo moeilijk om de personen te noemen die een essentiële bijdrage leverden aan de totstandkoming en uitvoering van het onderzoeksproject en dit proefschrift. Het zogenoemde *Erwinia*-project kwam tot stand door initiatief van ir. W. Prummel van Agrico Research. Van zijn enthousiasme voor het onderzoek en van de ongedwongen wijze waarop hij zijn kennis van en ervaring in de aardappelveredeling ten dienste stelde, ging gedurende de uitvoering van het project een grote stimulans uit. Coosje Hoogendoorn, hoofd van de afdeling Akkerbouw- en Voedergewassen, heeft het project zeer kundig begeleid en er mede zorg voor gedragen dat de resultaten van het onderzoek niet alleen voor Agrico Research bruikbaar waren maar ook vanuit wetenschappelijk oogpunt interessant. Op een paar maanden na is Willem van Dooijeweert gedurende het gehele *Erwinia*-project werkzaam geweest als onderzoeksassistent. Willem heeft het grootste deel van de onderzoekswerkzaamheden verricht en hij heeft daarbij een enorme inzet aan de dag gelegd. Eliza de Jong was gedurende ruim twee jaar als onderzoeksassistent werkzaam binnen het *Erwinia*-project en heeft in die periode zelfstandig en nauwgezet de transformaties van aardappelrassen uitgevoerd. Vervolgens heeft zij de expressie van de ingebrachte genen in de verkregen transformanten geanalyseerd. Marien Winters, kweker bij Agrico Research, heeft vanuit het bedrijf voor de nodige technische ondersteuning gezorgd en tevens zitting gehad in de begeleidingscommissie. Daarin hebben naast voornoemde personen ook Willem Stiekema, Leontine Colon, Tini Colijn en Pim Lindhout zitting gehad.

Zonder de ongecompliceerde en collegiale instelling van Dion Florack zouden de moleculair biologisch getinte hoofdstukken niet in hun huidige vorm tot stand zijn gekomen. Paul Keizer, die als statisticus al belangrijke bijdragen leverde bij de afronding van menig promotie-onderzoek, heeft ook mij veelvuldig en geduldig geholpen bij de analyses van de onderzoeksgegevens. Ir. E.H. Woltjer, directeur van Agrico Research, heeft bij aanvang en gedurende de uitvoering van het *Erwinia*-project een aantal malen zijn goedkeuring aan verschillende zaken verleend waardoor het *Erwinia*-project zijn uiteindelijke omvang en kwaliteit heeft kunnen krijgen. Alle genoemde personen ben ik erg dankbaar voor hun bijdragen die uiteindelijk hebben geleid tot de totstandkoming van dit proefschrift.

Tevens ben ik de professoren dr. ir. J.E. Parlevliet en dr. B. Schippers erkentelijk voor hun bereidheid om als promotor op te treden en de diverse hoofdstukken te becommentariëren.

Collega's van de afdelingen Akkerbouw- en Voedergewassen, Moleculaire Biologie, diverse ondersteunde afdelingen en Agrico Research ben ik dankbaar voor hun kleine en grotere inspanningen tijdens de uitvoering van het onderzoek.

Mijn ouders ben ik dankbaar omdat ze mij altijd hebben gestimuleerd om mijn capaciteiten aan te wenden en kansen te benutten. Wendy, mijn echtgenote, dank ik voor haar steun en voor de dispensatie van huishoudelijke taken.

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Note

Some chapters of this thesis are based on the following papers:

Allefs JJHM, W van Dooijeweert, ER de Jong, W Prummel & J Hoogendoorn. Factors affecting potato tuber tissue resistance to pectolytic *Erwinia* spp. in a tuber slice assay. Submitted. (Chapter 2).

Allefs JJHM, W van Dooijeweert, ER de Jong, W Prummel & J Hoogendoorn, 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. (Chapter 3).

Allefs JJHM, W van Dooijweert, LCP Keizer & J Hoogendoorn. Components of partial resistance to potato blackleg caused by pectolytic *Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi*. Submitted. (Chapter 4).

Allefs JJHM, W van Dooijeweert, ER de Jong, W Prummel & J Hoogendoorn. Resistance to *Erwinia carotovora* subsp. *atroseptica* in second backcross populations of somatic hybrids between *Solanum tuberosum* and *S. brevidens*. Potato Research, in press. (Chapter 5).

Allefs JJHM, ER de Jong, DEA Florack, J Hoogendoorn & WJ Stiekema. *Erwinia* soft rot resistance of potato cultivars expressing antimicrobial peptide tachyplesin I. Submitted. (Chapter 6).

Allefs JJHM, DEA Florack, J Hoogendoorn & WJ Stiekema. *Erwinia* soft rot resistance of potato cultivars transformed with a gene construct coding for antimicrobial peptide cecropin B is not altered. American Potato Journal, in press. (Chapter 8).

Chapter 1

Introduction

Pectolytic *Erwinia* spp.

Erwinia carotovora subsp. *carotovora* (Jones) Bergey *et al.*, *E.c.* subsp. *atroseptica* van Hall (Dye) and *E. chrysanthemi* (Burkholder *et al.*) are plant pathogenic bacteria belonging to the *Enterobacteriaceae* family. They are rod-shaped, Gram-negative, non spore-forming, facultative anaerobes and characterized by their ability to produce large amounts of pectolytic enzymes which enable them to macerate various plant tissues (Pérombelon & Kelman, 1980). The classification of these so called soft rotting *Erwinia* (sub-)spp. is based on biochemical and physiological characteristics rather than on host range. *E. carotovora* subsp. *atroseptica* (*Eca*) has a lower optimal growth temperature than *E. carotovora* subsp. *carotovora* (*Ecc*) and *E. chrysanthemi* (*Ech*). This seems to be reflected in the relatively small host range of *Eca* which is mainly restricted to potato in temperate regions (Pérombelon & Kelman, 1980). Most strains of *Eca* belong to one serogroup (De Boer *et al.*, 1979). In comparison with *Eca*, *Ecc* has a slightly higher optimum growth temperature and the capacity to grow at 37 °C (Pérombelon & Kelman, 1980). *Ecc* has a wide distribution in temperate and tropical regions and is pathogenic to a wide range of plants (Pérombelon 1988). Over 50 serogroups of *Ecc* were identified on the basis of immunodiffusion tests (De Boer *et al.*, 1987). *Ech* has the highest optimal growth temperature, most strains are able to grow at 39 °C (Pérombelon & Kelman, 1980). *Ech* strains can be subdivided into serogroups on the basis of at least three somatic (O) and three flagellar (F) antigens (Samson *et al.*, 1990) and into nine biovars (Ngwira & Samson, 1990), a classification which is mainly based on sugar catabolizing ability. *Ech* is pathogenic to a wide range of ornamental and food crops in tropical and subtropical regions as well as to crops that are grown in glasshouses (Pérombelon & Kelman, 1980). *Ech* is also pathogenic to potato and a few other crops grown in temperate regions (Pérombelon & Kelman, 1987; Janse & Ruissen, 1988). Dutch potato strains of *Ech* belong to serogroup 1 and to biovars 5 and 7. With the strains from kalanchoe and chicory, they apparently form a separate group which is characterized by non-pathogenicity on *Philodendron*, inability to grow at 39 °C and a weaker pectolytic activity at 37 °C (Janse & Ruissen, 1988).

Potato soft rot and blackleg.

When potato plants in the field or tubers in storage are infected with pectolytic *Erwinia* spp., various symptoms may develop depending on environmental factors of which temperature and moisture are the most important (Elphinstone, 1987). In the field, non-emergence occurs when the seed potato decays before development of above ground parts. Rot of the seed potato by *Erwinia* after emergence may be followed by colonization of the vascular stem tissue. Especially under wet conditions, the bacteria may spread to the parenchymatous tissue and cause a basal rot in one or more stems. This symptom is called blackleg. Under dry conditions, however, the bacteria might remain restricted to the vascular system, often resulting in discolorization of the stem base and in wilting symptoms (Pérombelon & Kelman, 1987). In areas were potatoes are overhead irrigated, *Erwinia* spp., especially *Ecc*, may cause so called aerial stem rot in above ground parts of the stem. This usually originates from a source of infection other than the rotting seed tuber (Powelson, 1980; Pérombelon & Kelman, 1987). Infection of tubers in storage, under certain conditions, results in decay. This is called potato soft rot. The symptoms caused by the different *Erwinia* spp. are indistinguishable and can also be caused by other pathogens (Pérombelon & Kelman, 1987).

Accurate data about the economical impact of potato diseases caused by *Erwinia* spp. are not available. Pérombelon & Kelman (1980) estimated annual losses of $ 50 to 100 million on a worldwide basis but the damage varies from country to country. In some countries, the main recognized problem is soft rot in storage, while blackleg in the field is the main problem in others. Losses by soft rot in Poland in the years 1976-1979 were estimated to be on average 6.7% (Pietkiewicz, 1981). Blackleg in temperate regions seldom exceeds 2% of the plants but even this low incidence may have a serous impact on yield (Elphinstone, 1987, Bain *et al.*, 1990). In the Netherlands, were storage of potatoes in well conditioned facilities is common, soft rot only occasionally causes damage. However, blackleg is a serious problem during production of seed potatoes of which about 75% are exported (*Anonymous*, 1994), partly to countries were environmental conditions may be more favourable for the pathogenic development of the bacteria. In spite of the poor relation between the occurrence of blackleg diseased plants and the extent to which the harvested seed tubers are latently infected (Hidalgo, 1988, Pérombelon 1992), blackleg incidence is thought to affect the phytosanitary quality of the seed potatoes and therefore, zero and low tolerances for blackleg during basic and certified seed production, respectively, are applied in the Dutch Seed Certification Programme (Hidalgo, 1988).

Low incidence of blackleg thus leads to declassification which implies an important financial loss for the seed potato grower.

Epidemiology.

In spite of the low incidence of blackleg in temperate regions, high proportions of commercial seed potatoes are latently infected with *Erwinia* bacteria (Pérombelon & Kelman, 1980, Tsror (Lahkim) *et al.*, 1993). The major source of contamination is formed by the presence of rotting mother tubers during harvest and post harvest mechanical handling (Elphinstone & Pérombelon, 1986; Pérombelon, 1992). Bacteria enter the tuber surface through lenticels and deep wounds where they survive until planted in the following season. However, already after one multiplication cycle in the field, tubers harvested from plants which were grown from true potato seed or stem cuttings may also become infected, indicating that other infection routes, for example contaminated irrigation water, aerosols, insects and soil water are also important (Pérombelon, 1992). In storage, especially when tuber tissue is exposed to anaerobis, soft rot may develop out of latent infections (Pérombelon & Kelman, 1980).

The plant-pathogen interaction.

Pectolytic *Erwinia* (sub-)spp. secrete several cell wall degrading enzymes such as pectinases, cellulases and proteases in the extracelluar environment. The group of pectinases secreted *in vitro* comprises of polygalacturonase, pectin lyase, pectin methylesterase and pectate lyase (Collmer & Keen, 1986; Kotoujansky, 1987). The latter enzyme is produced in four to five isozyme forms depending on (sub-)species and strain (Salmond, 1992). There is considerable evidence that the pectinases, especially pectate lyases, play an important role in the pathogenicity of *Erwinia* spp. while the role of cellulases and proteases is not clear (Salmond, 1992). Other, as yet unidentified gene products that are induced by plant extracts also play a role in pathogenesis (Beaulieu & Van Gijsegem, 1990). In contrast to host-specialized phytopathogenic bacteria such as *Xanthomonas* and *Pseudomonas* spp., of which several also secrete cell wall degrading enzymes, pectolytic *Erwinia* spp. do not show a narrow host specificity and are therefore described as unsophisticated opportunistic or general pathogens that simply macerate host tissue if it is suitable as a substrate (Salmond, 1992). The host-pathogen interaction, however, is more complex than this

description suggests. *In planta* studies revealed that in potato slices, *Ecc* pectate lyase reaction products stimulate pectinase production but also elicit defence related phenylalanine ammonia-lyase (PAL) mRNA synthesis (Yang *et al.*, 1992). Other enzymes such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), which is involved in isoprenoid synthesis and pathogenesis related protein β-1,3-glucanase, are also induced by *Ecc* (Yang *et al.*, 1991; Palva *et al.*, 1993). Within different host species, the isozyme forms of the *Ech* pectinases seem to play a differential role in pathogenicity which probably contributes to its wide host range (Beaulieu *et al.*, 1993).

Numerous studies have been performed to elucidate the biochemical basis of differences in resistance of potato tuber tissue to *Erwinia* whilst that of potato stem tissue has had relatively little attention (Lyon, 1989). Several resistance factors have been identified which can be divided into oxygen dependent and oxygen independent factors. Production of phytoalexins and phenolics which inhibit the growth of *Erwinia*, as well as the response of tuber tissue to wounding, are considered as important oxygen dependent mechanisms of resistance (Lyon, 1989; Lyon, 1992). The response of tuber tissue to wounding involves the formation of lignin and suberin, substances that can not be degraded by the *Erwinia* secreted enzymes. High degrees of esterified pectin and high Ca^{2+} concentrations in the cell wall, which negatively affect the suitability of pectin as a substrate for certain pectinases, are considered as important oxygen independent resistance mechanisms. Results from different studies concerning the role of esterified pectin, however, are somewhat conflicting (Pagel & Heitefuss, 1989; McMillan *et al.*, 1993). High dry matter and low reducing sugar content of tuber tissue may also be considered as oxygen independent resistance factors although for the latter, again, results are conflicting (Lyon, 1989; Tzeng *et al.*, 1990). The division into oxygen dependent and oxygen independent mechanisms is meaningful on the basis of the observation that resistance to soft rot under low oxygen or anaerobic conditions is strongly reduced compared with resistance under aerobic conditions (De Boer & Kelman, 1978; Pérombelon & Kelman, 1980). Oxygen dependent responses require appropriate mRNA and protein synthesis which is inhibited by anaerobic stress (Davis *et al.*, 1990). A recent study indicates that the reduced resistance under anaerobic conditions is not solely caused by changes in host tissue metabolism but may also partly be explained by changes in *Eca* energy metabolism resulting in a stimulation of bacterial growth (Smid *et al.*, 1993). None of the mechanisms of resistance that are identified so far, show a tight correlation with differences for resistance found in sets of potato clones. As a result, indirect selection on the basis of one biochemical characteristic, or a combination thereof, is not

suitable for identification of resistant clones. It is likely that the resistance of a given clone is determined by several biochemical mechanisms that may partly be in interaction with each other and that are strongly affected by environmental conditions. It may be due to this complex situation, that there are as yet no indications for the existence of specific genes for resistance to *Erwinia* (Lyon, 1989).

Disease control.

Erwinia bacteria in deep wounds and lenticels are well protected against liquid chemicals and therefore, in spite of several attempts, disease control by treating seed potatoes with bactericides has not been successful (Pérombelon, 1988; Weber, 1990).

Decontamination of seed potatoes by dipping in water of 55 °C for 5 min and subsequent drying has been successful under experimental conditions in Scotland but the treatment is expensive to perform and may not be suitable for decontamination of *Ech* which has a several degrees C higher thermal tolerance than *Eca* and *Ecc* (Robinson & Foster, 1987; Pérombelon, 1992). Hot water treatment is not applied in the Netherlands.

Disease control is still partly based on certification schemes in which declassification follows upon blackleg incidence during seed tuber production in the field. This is done in spite of the knowledge that the rotting mother tubers and not the blackleg plants form the main source of contamination (Pérombelon, 1988). The amount of blackleg that can be expected in the following season is clearly related to the level of seed tuber contamination (Bain *et al.*, 1990). As a consequence, certification schemes are expected to be more effective when based on accurate assessments of the seed tuber contamination level. Attempts are being made to develop a serological or DNA hybridisation technique with which this can be done (Pérombelon, 1992). As the epidemiology of the disease became better understood, phytosanitary measures were defined to reduce the risk of contamination. Avoiding extensive wounding during harvest and grading are examples of such measures on which disease control nowadays partly relies (Pérombelon, 1992).

Breeding for resistance.

In addition to the measures described above, the use of resistant cultivars has been suggested to be an effective way of controlling blackleg (Wastie & Mackay, 1985; Weber 1990; Pérombelon, 1992). Breeding for resistance to blackleg, as is the case for any desirable trait, requires heritable genetic variation and methods with which this variation can be measured. Immunity of potato genotypes to *Erwinia* has not yet been found. However, differences for resistance in modern and primitive cultivars were reported in many studies. Most of these concern resistance of tubers to soft rot (Brierley, 1928; Nielsen, 1954; Zadina & Dobiáš, 1976; Pietkiewicz, 1980; Bourne *et al.*, 1981; Hidalgo & Echandi, 1982; Krause *et al.*, 1982; Ciampi-Panno & Andrade-Soto, 1984; Corsini & Pavek, 1986; Huaman *et al.*, 1989). Cultivar resistance is strongly dependent on environmental conditions and of the screening method used (Wastie & Mackay, 1985; Bain & Pérombelon, 1988; Koppel, 1993). Attempts to use the variation that is found in cultivars for breeding purposes have been very limited. Lellbach (1978) screened the progenies of a three cultivar diallel cross for resistance to soft rot and concluded that resistance was primarily controlled by genes with additive effects. Dobiáš (1977a) found more resistant clones and a higher average level of soft rot resistance in progenies of which either one parent or both were relatively resistant compared with clones of progenies from two susceptible parents.

Genetic variation was not only detected in cultivars but also in wild species of the section *petota* and in interspecific hybrids. Such sources were either screened for soft rot resistance (French & de Lindo, 1979; Corsini & Pavek, 1986; Pawlak *et al.*, 1987; Austin *et al.*, 1988; Zimnoch-Guzowska & Lojkowska, 1993), blackleg (Lojkowska & Kelman, 1989) or for a combination of both (van Soest, 1983; Huaman *et al.*, 1988; Hoekstra, 1990). The frequency and level of resistance in wild species and derived material is usually higher than those found in cultivars (Corsini & Pavek, 1986, Huaman *et al.*, 1988). Helgeson *et al.* (1993) reported that resistance to soft rot in somatic hybrids between *S. tuberosum* and the non tuber-bearing species *S. brevidens* was transmitted to the progenies after backcrossing with modern cultivars.

Numerous methods for screening for resistance have been described. They can be grouped into (1) laboratory methods for screening of tuber material for resistance to soft rot (Henniger, 1965; Kiel, 1967; Hahn, 1974; Dobiáš, 1976; Munzert & Hunnius, 1980; Bourne *et al.*, 1981; Hidalgo & Echandi, 1983; Lapwood *et al.*, 1984; Bain & Pérombelon, 1988) or for screening of detached stems for resistance to blackleg (Lapwood & Read, 1986a), (2) glasshouse methods for screening of potted plants for resistance to blackleg (Dobiáš, 1977b, Hidalgo & Echandi, 1982; Wastie, 1984; Bisht

et al., 1993) or a combination of soft rot and blackleg (Munzert, 1975) and (3) field methods for screening for resistance to blackleg and yield reduction after inoculation of the seed before planting (Hossain & Logan, 1983; Lapwood & Legg, 1983; Lapwood & Gans; 1984; Lapwood & Read, 1986b, Gans *et al.*, 1991).

Although it is generally accepted that there is genetic variation for resistance available and in spite of the numerous methods described with which this variation can be identified, it has not been claimed so far that breeding cultivars with an appropriate level of resistance to soft rot or blackleg has been successful. As far as resistance to blackleg is concerned, this may be due to a number of reasons (Wastie & Mackay, 1985): (1) It is known that environmental conditions such as soil temperature and moisture as well as oxygen concentration affect resistance levels of clones, but the precise conditions under which these should be screened have not clearly been defined. (2) The complex relationship between the tuber and stem phases of the disease is not well understood. (3) High repeatability of results of a screening method *per se* does not make it suitable for screening purposes. Results should be due to resistance factors that are well expressed under field conditions. (4) Is has been shown that results of cultivar screenings in the field are consistent with resistance levels under practical conditions (Gans *et al.*, 1991). However, field screenings alone are not suitable for large scale application in breeding programmes as they require considerable quantities of tuber material per genotype which is not available in early stages of the selection procedure. The suitability of screening methods for early selection has had little attention. (5) The resistance found so far is partial and possibly polygenic and needs to be combined with several other agronomic traits which is difficult to achieve and time consuming. Finally, (6) the cultivated potato is a tetraploid species. The genetics behind breeding goals are usually more complicated when compared with diploid crops which negatively affects the speed of the selection process.

Resistance through genetic engineering.

Nowadays, *Agrobacterium tumefaciens* mediated transformation is a well developed technique to introduce single genes in potato and other crops thus enlarging the available genetic variation with desirable agronomic traits (Vayda & Belknap, 1992). Several peptide families with strong *in vitro* antibacterial activity have been isolated from a number of organisms and phages (Boman, 1991). This suggests that it may be feasible to transform potato with the genes that are coding for these peptides,

aiming to enhance resistance to plant pathogenic bacteria (Casteels *et al.*, 1989; Destéfano-Beltrán *et al.*, 1990). Thus genes coding for cecropin like peptides, that originally were isolated from the hemolymph of giant silk moth (*Hyalophora cecropia*) pupae, were transferred to tobacco and potato. Transgenic plants showed a delayed symptom expression after inoculation with *Pseudomonas solanacearum*, the causal agent of bacterial wilt disease (Jaynes *et al.*, 1993; Jia *et al.*, 1993). Düring *et al.* (1993) transformed potato with a gene coding for bacteriophage T4 lysozyme, a peptide which degrades the peptidoglycan bacterial cell wall, and found both a reduction of transgenic tuber tissue maceration by *Eca* as well as a better emergence of inoculated seed pieces after planting in soil. Genetic engineered potato cultivars with resistance to *Erwinia* have not yet been introduced on the market.

Outline of the research.

This thesis reports about studies on the identification of components of resistance and sources of resistance to blackleg. Since blackleg of stems of field-grown plants is preceded by rot of the mother tuber (Pérombelon & Kelman, 1980), resistance of both tuber and above ground stem tissue were considered as putative components of field resistance. The stem base, which may function as a barrier against invading *Erwinia* bacteria from the rotting mother tuber (Munzert & Hunnius, 1980; Weber, 1990), was considered as another component of resistance. Chapters 2, 3 and 4 describe the experiments to characterize these components of resistance and their relation to resistance under field conditions. Chapter 5 describes *S. brevidens* as a source of resistance to blackleg. Chapters 6 to 8 describe the possibilities to obtain resistance to *Erwinia* by means of introducing genes into potato cultivars that code for antibacterial peptides such as tachyplesin I, α-hordothionin and cecropin B. Throughout this thesis, the implications of the results for practical breeding programmes are being evaluated.

Chapter 2

Factors affecting potato tuber tissue resistance to pectolytic *Erwinia* spp. in a tuber slice assay

Abstract

Potato tuber slices and small tubers of 12 cultivars were inoculated with isolates of *Erwinia carotovora* subsp. *atroseptica* (*Eca*), *E. carotovora* subsp. *carotovora* (*Ecc*) and *E. chrysanthemi* (*Ech*). Cultivar resistance was expressed as the mean diameter of the rotted tissue area. Cultivar ranking after incubation in air was different when compared with cultivar ranking under anaerobic conditions, and depended on the (sub-)species of bacteria used. These interaction effects were reproducible in repeated experiments. When compared with the effect of inoculation with different (sub-)species, the rank order of the cultivars was less affected by isolates within (sub-)species. Cultivar ranking was hardly affected by site of inoculation being either in the medullary tissue or in the cortex. In general, results of experiments with either seed tubers or small tubers from two years were reproducible, except when tuber tissue was inoculated with *Ech* and incubated aerobically. Then, cultivars only showed relatively small differences for resistance. Correlation coefficients between the results from slice and small tuber screenings were 0.71 and 0.64 for *Eca* and *Ecc* respectively.

The results of this study show that tuber tissue resistance of clones to different pectolytic *Erwinia* spp. can be determined efficiently and reproducibly if the oxygen concentration during incubation is standardised.

Introduction

Breeding of potato cultivars with high levels of resistance to pectolytic *Erwinia* spp., the causal agents of blackleg and tuber soft rot (Pérombelon & Kelman 1987), requires screening. Screening methods should meet a number of requirements. They should be easy to perform and suitable for screening large numbers of clones in early vegetative generations when there is only a limited amount of plant material available. Results must be reproducible and in agreement with the level of resistance that the screened clones will show under field or storage conditions.

Several methods have been described for the screening for tuber soft rot resistance either by inoculation of whole tubers (Henniger 1965; Kiel 1967; Hahn 1974; De Boer & Kelman 1978; Munzert & Hunnius 1980; Bourne *et al.* 1981; Lapwood *et al.* 1984; Bain & Pérombelon 1988) or slices (Dobiáš 1976; Lapwood *et al.* 1984). Of these, the method for slices as described by Lapwood *et al.* (1984) has been tested most extensively and appears suitable for use as a screening method in breeding programmes. It has already been shown that factors such as bacterial species (*Erwinia carotovora* subsp. *atroseptica* (*Eca*) and *E. carotovora* subsp. *carotovora* (*Ecc*)), wound healing period, year of testing (Lapwood *et al.* 1984), incubation temperature, incubation time (Lapwood & Read 1985), time of testing, source of tubers, number of replications per clone, the institution where the test is performed (Wastie *et al.* 1988) and inoculum concentration (Lapwood *et al.* 1984; Lapwood & Read 1985; Wastie *et al.* 1988) have only minor effects on the ranking order of the clones tested. However, other important factors with potential effects on tuber tissue resistance of potato clones remained to be studied. Here we present results of experiments in which a set of 12 cultivars was inoculated with different isolates of each of the pectolytic *Erwinia* (sub-)spp. in order to determine the effect on cultivar resistance of *Erwinia* isolates relative to the effect of *Erwinia* (sub-)spp. Studies on this specific aspect of *Erwinia* resistance screening are scarce (Wolters & Collins 1994). The effect of inoculation near the centre of a slice versus inoculation in the cortex was compared. We also determined the suitability of a screening method for application to early vegetative generations by testing small tubers harvested from glasshouse-grown plants. Experiments were not only carried out with isolates of *Eca* and *Ecc*, but also with so called 'cold strains' of *E. chrysanthemi* (*Ech*) which had been isolated from blackleg diseased potato plants in the Netherlands (Janse & Ruissen 1988). Since tuber tissue resistance is strongly affected by the oxygen concentration during incubation (De Boer & Kelman 1978; Bain & Pérombelon 1988), a modification of the incubation conditions was introduced which enabled us to perform the experiments at different oxygen concentrations. Finally, for a subset of the cultivars used, results of the method studied were compared with results of screening according to the "pipette tip method" as originally described by Maher and Kelman (1983). This "pipette tip method" recently has been recognized for its potential as a standard method for soft rot resistance screening (Allefs *et al.* 1993).

Materials and methods

In 1991 and 1992, seed tubers, 40-50 mm in diameter, of the cvs Agria, Alcmaria, Amazone, Arinda, Bintje, Désirée, Hertha, Karnico, Kondor, Morene, Producent and Venouska were harvested from the same field and stored at 4 °C until tested. These cultivars were chosen since they had shown differences for susceptibility to blackleg under field conditions (Prummel, unpublished results). Small tubers, approximately 20 mm in diameter, were harvested from a multiplication of the cultivars in the glasshouse during the summers of 1991 and 1992. Plants were grown to maturity in 1 ℓ pots containing potting compost.

Bacterial strains and preparation of inoculum. *Eca* IPO 161 (=PD 230), *Ecc* IPO 163 (=PD 1407) and *Ech* IPO 502 (=PD 226) were obtained from the DLO-Research Institute for Plant Protection (IPO-DLO), Wageningen. *Eca* PD 755, *Ecc* PD 452, *Ecc* PD 1006, *Ech* PD 482 and *Ech* PD 581 were obtained from the Plant Protection Service (PD), Wageningen, the Netherlands. *Eca* SCRI 1039 was kindly provided by Dr Pérombelon, Scottish Crop Research Institute, Dundee, UK. All isolates were isolated from potato plants in the Netherlands except *Eca* SCRI 1039 which was isolated from a blackleg plant in Scotland (Bain *et al.* 1990). Dutch isolates within (sub-)species were isolated in different years. *Ech* isolates IPO 502 and PD 581 were identified as biovar 7 and *Ech* PD 482 as biovar 5 and are so called 'cold strains' which show a lower optimum temperature for pectolytic activity than *Ech* strains from (sub-)tropical regions (Janse & Ruissen 1988).

Inoculum of the bacteria was prepared as follows. *Eca* and *Ecc* were grown on Bouillon Agar medium containing 8.0 g 'Lab-Lemco' Broth (Oxoid), 5.0 g $ℓ^{-1}$ NaCl and 15.0 g $ℓ^{-1}$ agar, *Ech* was grown on Growth Factor medium containing 0.4 g $ℓ^{-1}$ K_2HPO_4, 0.05 g $ℓ^{-1}$ $MgSO_4.7H_2O$, 0.1 g $ℓ^{-1}$ NaCl, 0.5 g $ℓ^{-1}$ NH_2PO_4, 1.0 g $ℓ^{-1}$ glucose, 3.0 g $ℓ^{-1}$ Yeast Extract (Oxoid) and 15.0 g $ℓ^{-1}$ agar for 48 h at 27 °C. Bacteria were suspended in sterile water and centrifuged for 5 min at 4500 rpm. The bacterial pellet was re-suspended and the concentration determined using a standard curve for each of the species relating colony-forming units (cfu) to optical density at 500 nm. Bacteria were diluted to 1.0 x 10^9 cfu ml^{-1}

Inoculation and incubation. Inoculation of tuber slices was performed essentially as described by Wastie *et al.* (1988). From each seed tuber were cut two or three slices, 1.0 cm thick, one for each treatment concerning site of inoculation, or *Erwinia* isolate, respectively. In contrast to field-grown seed tubers, small tubers were cut

twice tangentially on opposite sides. One cut prevented the small tubers from rolling during handling, the other created a surface for inoculation. After cutting, mean weights of the small tubers were 10.6 and 11.6 g in 1991 and 1992, respectively. Slices and small tubers were placed in plastic trays (40 x 60 x 6 cm) with a perforated bottom. Trays formed experimental units and were divided into two or three randomized blocks, one for each treatment concerning site of inoculation or *Erwinia* isolate. One slice or small tuber per cultivar was placed per block in a randomized position. Ten trays were used per oxygen concentration treatment during incubation (Table 2.1) thus creating ten replicates per combination of cultivar, *Erwinia* isolate and incubation treatment. The trays with cut slices or small tubers were stacked in random order in a temperature-controlled room and incubated overnight at 15 °C. Inoculations were carried out by pipetting 20 µl of bacterial suspension into a well (4.0 mm deep and 5.0 mm diameter) made in each slice or small tuber by pressing with a steel rod in the medullary tissue just near the pith.

Preliminary experiments showed much variation between replicated trays when trays were incubated in plastic bags as described by Lapwood *et al.* (1984). This variation was almost eliminated when trays were incubated in air-tight stainless steel containers, measuring 45 x 65 x 65 cm. The two containers, each containing ten trays, were placed in a growth chamber for three or five days at 20 °C. For aerobic incubation, the containers were flushed with 500 ml min^{-1} air. For anaerobic and low-oxygen incubations, the containers were flushed for 2 min with nitrogen and mixtures of nitrogen and oxygen, respectively. Oxygen concentration of the outlet flow, measured with a paramagnetic sensor (type Servomix 570A, UK), was found to deviate by at most 2% from the desired oxygen concentration, which was reached in about 8 h. Flushing was continued with 500 ml min^{-1} of nitrogen or the appropriate mixture. All gases were bubbled through water, which resulted in 100% relative humidity in the containers.

After incubation, rotted tissue was carefully scraped from the slices and small tubers and the diameter of the resulting cavity was measured in two directions at right angles. The calculated mean diameter was considered as a value for tuber tissue resistance (Wastie *et al.* 1988). Analysis of variance (ANOVA) was carried out using GENSTAT (Payne *et al.* 1987).

Eleven experiments were carried out. Experimental details are summarized in Table 2.1. Seed tubers and small tubers were screened during the period November to July, referred to as the storage period. Except for experiments 6 to 8, one isolate of each of the *Erwinia* (sub-)spp. was used as inoculum (Table 2.1).

Table 2.1. Summary of the 11 experiments carried out with tuber slices. For each of the experiments the year of harvest of tuber material is given, the month of inoculation of slices or small tubers, the *Erwinia* isolates used for inoculation, the duration of incubation in air or nitrogen and the incubation conditions.

Expt	Year of harvest	Month of inoculation	Slices/ small tubers	Inoculum	Duration of incubation (days)	Incubation conditions
1	1991	Dec '91	slices	*Eca* IPO 161, *Ecc* IPO 163, *Ech* IPO 502	5	air vs nitrogen
2	1991	Mar '92	slices	*Eca* IPO 161, *Ecc* IPO 163, *Ech* IPO 502	5	air vs 2% oxygen
3	1991	Mar '92	slices	*Eca* IPO 161, *Ecc* IPO 163, *Ech* IPO 502	5	air vs 5% oxygen
4	1991	Mar '92	slices	*Eca* IPO 161, *Ecc* IPO 163, *Ech* IPO 502	5	air vs nitrogen
5	1991	Jul '92	slices	*Eca* IPO 161, *Ecc* IPO 163, *Ech* IPO 502	5	air vs nitrogen
6	1992	Nov '92	slices	*Ecc* IPO 163, *Ecc* PD 452, *Ecc* PD 1006	5 (air), 3 (nitrogen)	air vs nitrogen
7	1992	Nov '92	slices	*Eca* IPO 161, *Eca* PD 755, *Eca* SCRI 1039	5 (air), 3 (nitrogen)	air vs nitrogen
8	1992	Dec '92	slices	*Ech* IPO 502, *Ech* PD 482, *Ech* PD 581	5 (air), 3 (nitrogen)	air vs nitrogen
9	1991	Dec '91	small tubers	*Eca* IPO 161, *Ecc* IPO 163, *Ech* IPO 502	5 (air), 3 (nitrogen)	air vs nitrogen
10	1992	Dec '92	small tubers	*Eca* IPO 161, *Ecc* IPO 163, *Ech* IPO 502	5 (air), 3 (nitrogen)	air vs nitrogen
11	1991	Nov '91	slices	*Eca* IPO 161[1]	5	air vs nitrogen

[1] Slices were either inoculated in the centre just near the pith or between the vascular ring and peel in the cortex.

Results of an experiment in which whole tubers of cvs Agria, Alcmaria, Bintje, Désirée, Karnico and Producent were screened once according to the "pipette tip method" have been presented earlier (Allefs *et al.* 1993). Because results of this experiment with whole tubers are compared with results of experiments with tuber slices, the experiment with whole tubers is described here briefly. Each of ten tubers per cultivars was inoculated by inserting a yellow polypropylene pipette tip containing 25 µl 1 x 10^9 cfu ml^{-1} of *Eca* SCRI 1039 at a depth of 5.0 mm between rose en heel ends. Tubers were incubated for five days at 20 °C in a mist chamber. After incubation, tubers were sliced and the diameter of the rot was measured at its widest point at 90° to the tip. The tubers used had been harvested in 1992 from the same field as tubers used for experiments with slices (Table 2.1).

Results

The high concentration of bacteria used (1 x 10^9 cfu ml^{-1}), resulted in rot at the site of inoculation of all slices and tubers tested. Control slices that were treated with sterilized water showed no rot after three or five days of incubation.

Incubation of slices in nitrogen was initially carried out for five days. Experiments with tuber slices incubated for two to five days in nitrogen revealed, however, that incubation for three days resulted in a better discrimination of the 12 cultivars (data not shown). Therefore, incubation in nitrogen was restricted to three days in experiments 6 to 8.

Specific measures were taken to decrease the variability between replicates as observed with incubation of trays in plastic bags as described by Lapwood *et al.*, (1984). These improvements, i.e. the construction of air-tight containers that can be placed in temperature controlled chambers and constantly flushed with gases, were very effective and resulted in only small experimental errors. The variance, therefore, could almost completely be explained by the treatment factors and their interaction effects. Thus, effects of the incubation condition (oxygen concentration), inoculum, site of inoculation, cultivar and all possible relevant interactions between these factors were found to be significant ($p<0.05$) in ten of the 11 experiments carried out with slices or small tubers. As will be shown, the most pronounced effects of these factors on cultivar ranking were often found in more than one but not always in all of the repeated experiments. In order to estimate the repeatability and to identify the important interactions, correlation coefficients were calculated for comparisons of cultivar means between treatments and between experiments. Since only a range of

12 cultivars was screened, such coefficients might be strongly affected by one or more cultivars with extreme values. However, no cultivars were found that showed exceptional small or large rots and therefore, it is thought that the calculated correlation coefficients are suitable for an estimation of the importance of the interaction effects found.

The effect of Erwinia (sub-)spp. and isolates on cultivar resistance. In experiments 1, 4 and 5, tuber slices of the 12 cultivars were inoculated with one isolate of *Eca*, *Ecc* or *Ech* and incubated in air or nitrogen (Table 2.1). Typical results for these experiments, as found in experiment 4, are presented in Table 2.2. Within combinations of incubation condition and inoculum, cultivars differed significantly ($P<0.01$) for mean diameter of rotted tuber slice tissue. The most resistant cultivars showed up to two times less rot than the most susceptible cultivars except when slices were incubated aerobically after inoculation with *Ech*. Under these conditions, no or only very small significant differences were found which was typical for all experiments carried out.

Within incubation condition treatments, the resistance of cultivars relative to the resistance of the other cultivars tested and, as a consequence, also the rank order of the cultivars, was affected by the *Erwinia* (sub-)spp. which was used for inoculation, as can be seen in Table 2.2. Under aerobic conditions for example, cv Karnico showed significantly less rot ($P<0.01$) than cvs Amazone and Morene when inoculated with *Eca* but not when inoculated with *Ecc*. The latter cultivars showed significantly less rot than cvs Arinda and Venouska when inoculated with *Ecc* but not with *Eca*. Under anaerobic conditions, cv Karnico showed significantly less rot ($P<0.01$) than cv Morene when inoculated with *Ecc* but not when inoculated with *Eca* or *Ech*. A similar interaction is shown by cvs Morene and Kondor. Many, but not all of these inoculum x cultivar interaction effects were also found in one or both of the experiments 1 and 5 (not shown). Correlation coefficients for comparisons of mean cultivar rots caused by the different *Erwinia* (sub-)spp. are shown in Table 2.3 for experiments 1, 4 and 5. Under aerobic conditions, correlation coefficients for comparisons between (sub-)species varied largely from experiment to experiment. However, as far as comparisons with results from *Ech* inoculated slices are concerned, this is likely due to the absence of clear genotypic variation for resistance to *Ech* in air, which makes calculation of correlation coefficients less appropriate. Under anaerobic conditions, all coefficients were positive and significant ($P<0.05$) for seven out of nine comparisons.

In experiments 6, 7 and 8, tuber slices were inoculated with three isolates of *Ecc*, *Eca* and *Ech*, respectively (Table 2.1). As an example, results of experiment 6 are

Table 2.2. Mean diameter of rotted tuber slice tissue (mm) of 12 cultivars, after inoculation with 3 *Erwinia* (sub-)species and subsequent incubation in air or nitrogen for 5 days at 20 °C (experiment 4, Table 2.1). The rank order is given in parentheses.

	Incubation conditions					
	Air			Nitrogen		
Cultivar	*Eca*	*Ecc*	*Ech*	*Eca*	*Ecc*	*Ech*
Agria	17.1 (1)	20.1 (1)	11.7 (3)	23.4 (2)	24.1 (5)	24.2 (2)
Alcmaria	15.9 (2)	18.6 (3)	12.1 (1)	20.8 (4)	26.4 (3)	21.8 (5)
Amazone	12.1 (8)	12.7 (11)	11.2 (6)	13.5 (12)	16.0 (12)	17.1 (11)
Arinda	12.6 (6)	17.1 (6½)	11.5 (4½)	20.9 (3)	23.4 (6)	22.6 (3)
Bintje	15.7 (3)	19.6 (2)	11.1 (7½)	20.7 (5)	25.9 (4)	22.2 (4)
Désirée	8.6 (11)	14.6 (8½)	10.5 (9)	18.3 (8)	22.9 (9)	20.8 (6)
Hertha	14.3 (4)	17.2 (5)	11.9 (2)	15.5 (11)	21.0 (11)	15.0 (12)
Karnico	8.4 (12)	12.0 (12)	10.2 (10½)	18.6 (7)	22.4 (10)	19.4 (9)
Kondor	13.5 (5)	17.8 (4)	11.5 (4½)	25.1 (1)	29.4 (2)	25.6 (1)
Morene	12.4 (7)	12.8 (10)	10.2 (10½)	18.9 (6)	29.8 (1)	19.7 (8)
Producent	8.9 (10)	14.6 (8½)	9.6 (12)	17.3 (10)	23.2 (7)	17.9 (10)
Venouska	12.0 (9)	17.1 (6½)	11.1 (7½)	17.9 (9)	23.1 (8)	20.5 (7)
Mean	12.6	16.2	11.1	19.2	24.0	20.6

LSD ($P<0.01$) = 2.3 for cultivar x incubation condition x inoculum means; 0.8 for incubation condition x inoculum means.

Table 2.3. Correlation coefficients for comparisons of mean cultivar rot, between *Erwinia* (sub-)species and -isolates. Correlation coefficients for comparisons between *Erwinia* (sub-)species are given in sequence for experiments 1, 4 and 5.

Inoculum	Incubation conditions					
	Air			Nitrogen		
Between Erwinia *(sub-)species*	expt 1	expt 4	expt 5	expt 1	expt 4	expt 5
Eca IPO 161 vs *Ecc* IPO 163	0.42	0.81	0.68	0.88	0.75	0.75
Eca IPO 161 vs *Ech* IPO 502	-0.52	0.79	0.19	0.55	0.94	0.73
Ecc IPO 163 vs *Ech* IPO 502	0.27	0.70	-0.17	0.20	0.63	0.77
Between Erwinia *isolates, within (sub-)species*						
Eca IPO 161 vs PD 755	0.96			0.95		
Eca IPO 161 vs SCRI 1039	0.96			0.98		
Ecc IPO 163 vs PD 452	0.96			0.89		
Ecc IPO 163 vs PD 1006	0.92			0.87		
Ech IPO 502 vs PD 482	0.81			0.52		
Ech IPO 502 vs PD 581	0.79			0.75		

Significance levels: r = 0.58 (P<0.05) and r = 0.71 (P<0.01).

presented in Table 2.4. Within incubation condition treatments, the rank order of cultivars was affected by *Ecc* isolate but the inoculum x cultivar and incubation condition x inoculum x cultivar interaction effects were not significant. In addition, highly significant correlation coefficients (P<0.01) were found for comparisons of mean cultivar rots between the *Ecc* isolate IPO 163 and PD 1006 or PD 452 (Table 2.3). Similar results were obtained in experiments 7 and 8 for isolates of *Eca* and *Ech* respectively. Although the relevant two- and three factor interaction effects were found to be significant (P<0.01) in experiments 7 and 8, correlation coefficients for comparisons between isolates of *Eca* were high and significant (P<0.01). To a lesser extent, this was also found for isolates of *Ech* (Table 2.3). These results indicate that the differences between (sub-)species are greater than the differences between isolates within (sub-)species.

The effect of oxygen concentration during incubation. The effect of the oxygen concentration during incubation on cultivar resistance was studied in experiments 2, 3 and 4 in each of which incubation in air was compared with incubation in 2, 5 and

Table 2.4. Mean diameter of rotted tuber slice tissue (mm) of 12 cultivars, after inoculation with 3 isolates of *Erwinia carotovora* subsp. *carotovora* and subsequent incubation in air or nitrogen for five and three days, respectively, at 20 °C (experiment 6, Table 2.1). The rank order is given in parentheses.

	Incubation conditions											
	Air						Nitrogen					
Cultivar	IPO 163		PD 1006		PD 452		IPO 163		PD 1006		PD 452	
Agria	19.3	(2)	17.3	(2)	15.7	(2)	15.6	(6)	16.7	(5)	15.1	(4)
Alcmaria	20.5	(1)	17.4	(1)	17.4	(1)	16.1	(4)	17.0	(3½)	15.0	(5)
Amazone	14.7	(12)	11.7	(10½)	11.8	(10)	12.6	(12)	13.3	(12)	12.3	(12)
Arinda	17.4	(4½)	14.5	(5½)	14.0	(3½)	15.3	(8)	14.9	(9)	13.3	(8½)
Bintje	17.0	(7)	15.0	(4)	13.6	(6)	14.3	(10)	15.8	(7)	13.3	(8½)
Désirée	16.5	(8½)	13.9	(7)	13.2	(8)	14.2	(11)	13.8	(11)	13.1	(10)
Hertha	17.6	(3)	13.5	(8)	13.7	(5)	15.5	(7)	17.0	(3½)	15.8	(3)
Karnico	14.8	(11)	10.4	(12)	10.7	(12)	16.3	(3)	15.3	(8)	14.1	(6)
Kondor	17.4	(4½)	14.5	(5½)	13.5	(7)	19.0	(2)	19.6	(2)	16.1	(2)
Morene	15.8	(10)	11.7	(10½)	10.8	(11)	21.1	(1)	19.7	(1)	21.2	(1)
Producent	17.3	(6)	15.7	(3)	14.0	(3½)	16.0	(5)	14.8	(10)	13.0	(11)
Venouska	16.5	(8½)	12.4	(9)	13.0	(9)	14.8	(9)	16.0	(6)	13.9	(7)
Mean	17.1		14.0		13.5		15.9		16.2		14.7	

LSD ($P<0.01$) = 2.1 for cultivar x incubation condition x inoculum means; 0.9 for incubation condition x inoculum means.

Table 2.5. Correlation coefficients for comparisons of mean cultivar rot between incubation conditions of experiments 6, 7 and 8.

Incubation condition	Inoculum		
	Eca	*Ecc*	*Ech*
air[1] vs 5% oxygen	0.09	0.48	0.08
air[1] vs 2% oxygen	0.42	0.29	0.56
air[1] vs nitrogen	0.53	0.34	0.40
5% oxygen vs 2% nitrogen	0.63	0.65	0.34
5% oxygen vs nitrogen	0.44	0.69	0.29
2% oxygen vs nitrogen	0.87	0.90	0.83

[1] Mean cultivar rots across experiments 6, 7 and 8 were used for calculations.
Significance levels: $r = 0.58$ ($P<0.05$) and $r = 0.71$ ($P<0.01$).

0% oxygen respectively (Table 2.1). The rank order of the cultivars was strongly affected by oxygen concentration as can be seen in Table 2.2. Cv Hertha for example, when inoculated with *Ecc* showed less rot than cv Kondor under anaerobic but not under aerobic conditions. This was also found in experiments 6 (Table 2.4), 1 and 5. Another example is shown by cv Karnico and the group of cvs Agria, Arinda and Venouska; Karnico showed less rot than Agria, Arinda and Venouska under aerobic but not under anaerobic conditions (Table 2.2). This was also found in experiment 6 (Table 2.4) but not in experiments 1 and 5.

Also for *Eca* and *Ech*, effects of oxygen concentration on cultivar ranking can be seen in Table 2.2. Cvs Amazone and Hertha for example, showed significantly less rot ($P<0.01$) than cv Kondor under anaerobic but not under aerobic conditions. This interaction effect was also found in experiments 1, 5 and 8 for *Ech* and in experiment 1 and 11 (Table 2.6) for *Eca*.

For each of the *Erwinia* (sub-)spp, correlation coefficients for comparisons of mean cultivar rots between the oxygen concentrations during incubation are shown in Table 2.5. All correlation coefficients were positive, but low and not significant ($P<0.01$) except for comparisons between incubation in 2 and 0% oxygen which were high for each of the (sub-)species.

The results presented here indicate that the oxygen concentration during incubation should be accurately controlled and standardised when screening potato clones for tuber tissue resistance to *Erwinia*.

Table 2.6. Mean diameter of rotted tuber slice tissue (mm) of 12 cultivars, after inoculation with *Erwinia carotovora* subsp. *atroseptica* IPO 161 either near the centre or in the cortical tissue and subsequent incubation in air (experiment 11, Table 2.1). The rank order is given in parentheses.

	Incubation conditions							
	Air				Nitrogen			
Cultivar	Centre		Cortex		Centre		Cortex	
Agria	16.9	(3)	14.1	(3)	20.6	(9)	16.8	(10)
Alcmaria	16.4	(4)	15.3	(1)	23.0	(6)	18.8	(6)
Amazone	17.6	(1)	13.9	(4)	18.4	(12)	16.4	(11)
Arinda	15.6	(5½)	12.7	(6)	22.6	(7)	19.2	(5)
Bintje	17.0	(2)	14.2	(2)	24.9	(3)	20.4	(3)
Désirée	9.9	(11)	9.7	(10)	19.5	(10)	16.1	(12)
Hertha	15.1	(7)	11.7	(8)	20.7	(8)	17.3	(9)
Karnico	10.6	(10)	9.3	(11)	24.1	(4)	18.5	(7)
Kondor	15.6	(5½)	11.8	(7)	29.9	(1)	22.9	(1)
Morene	11.4	(9)	10.3	(9)	26.5	(2)	22.3	(2)
Producent	9.8	(12)	8.7	(12)	18.5	(11)	17.7	(8)
Venouska	14.5	(8)	13.0	(5)	23.6	(5)	19.3	(4)
Mean	14.2		12.0		22.7		18.8	

LSD ($P<0.01$) = 2.4 for cultivar x incubation condition x site of inoculation means; 0.9 for incubation condition x site of inoculation means.

Effect of site of inoculation. In experiment 11, slices of the 12 cultivars were inoculated with *Eca* either near the pith in the medullary tissue or between the peel and vascular ring which is in the cortex and outer storage parenchyma. Incubation was in air or nitrogen (Table 2.1). Although, within incubation condition treatments, the rank order of the cultivars was slightly affected by site of inoculation (shown for example by cvs Alcmaria versus Kondor or Hertha under aerobic conditions; Table 2.6), correlation coefficients for comparisons of mean cultivar rots between sites of inoculation were high (0.93) and significant ($P<0.01$), both in air and nitrogen.

Reproducibility of results within and between storage periods. Reproducibility of results was studied by calculation of correlation coefficients for comparisons of mean cultivar rot between different experiments (Table 2.7). Correlations between mean cultivar rots of experiments 1, 4 and 5 that were carried out at different moments

Table 2.7. Correlation coefficients for comparisons of mean cultivar rot between different experiments, within and between storage periods.

Inoculum		Experiments compared	Incubation conditions	
			Air	Nitrogen
Within storage periods				
1991/1992	*Eca*	1 vs 4	0.88	0.82
		1 vs 5	0.82	0.73
		4 vs 5	0.86	0.76
	Ecc	1 vs 4	0.70	0.84
		1 vs 5	0.51	0.67
		4 vs 5	0.75	0.82
	Ech	1 vs 4	-0.17	0.46
		1 vs 5	0.30	0.44
		4 vs 5	0.43	0.79
Between storage periods				
1991 vs 1992	*Eca*	1 vs 7	0.95	0.42
	Ecc	1 vs 6	0.82	0.82
	Ech	1 vs 8	0.08	0.84

Significance levels: r = 0.58 (P<0.05) and r = 0.71 (P<0.01).

during a eight months period within one storage period were highly significant (P<0.01) for *Eca*, mostly significant (P<0.05) for *Ecc* but generally low and not significant for *Ech* especially under aerobic conditions. As stated before, the latter is probably due to the limited genotypic variation for resistance to *Ech* in air. Nevertheless, highly significant (P<0.01) correlation coefficients (0.94, 0.85 and 0.84) were found when results were compared of experiments which were completed within a one month period (experiments 2, 3 and 4).

Correlations between mean cultivar rots of experiments that were carried out in two different storage periods, i.e. with different sources of tubers, either in November or December (experiments 1 and 6 to 8), were high and significant (P<0.01) for *Ecc* and *Eca* only when slices were incubated in air, and for *Ecc* and *Ech* only when slices were incubated in nitrogen (Table 2.7). In spite of the high correlations found, occasionally, the rank order of a cultivar varied markedly from one storage period to another, which can been seen in Tables 2.2 and 2.4 for inoculations with *Ecc* IPO 163 of cv Bintje.

The tuber slice method versus the "pipette tip method". Whole tubers of relatively resistant cvs Désirée, Karnico and Producent and relatively susceptible cvs Agria, Alcmaria and Bintje, were screened for resistance to *Eca* SCRI 1039 according to the "pipette tip method" (Allefs *et al.* 1993). All inoculated sites on the tubers showed rot after five days of incubation in a mist chamber. Mean diameters of rot were 6.2, 5.8, 4.5, 7.3, 6.8 and 8.5 respectively (LSD=2.0, $P<0.01$). When compared with the results of experiment 7 (inoculum *Eca* SCRI 1039 and incubation in air), cvs Agria, Alcmaria and Bintje were found to be the most susceptible and Désirée, Karnico and Producent the most resistant with both methods. With both methods, no significant differences ($P<0.01$) were found within these two groups and group means differed significantly ($P<0.01$).

The use of small tubers. In two successive years, small tubers of the 12 cultivars were harvested from glasshouse-grown plants and screened for resistance in experiments 9 and 10 (Table 2.1). Results for inoculation with *Eca* and incubation under aerobic conditions are presented in Table 2.8. The data illustrate the significant differences found between cultivars for small tuber tissue resistance to *Erwinia* (sub-)spp. ($P<0.01$). Although the rank order of the cultivars in Table 2.8 was slightly affected by the year of testing, correlation coefficients for comparison between the two experiments with small tubers were fairly high after inoculation with *Eca* or *Ecc* but not with *Ech* when incubation was in air (Table 2.9). When incubation was carried out in nitrogen, significant correlations ($P<0.05$) were found for each of the three (sub-)species. Correlation coefficients for comparisons between incubation in air and nitrogen were not significant after inoculation with either *Erwinia* (sub-)spp. (not shown).

Mean cultivar rots of the experiments with small tubers, carried out in December, were compared with results from experiments with slices that were carried out around the same time of the year (Tables 2.8 and 2.9). These Tables show that correlation was found between results of experiments with slices and small tubers for *Eca* and *Ecc* when incubations were carried out in air, but not for *Ech* or when incubation was carried out in nitrogen.

Discussion

Knowledge of the specificity of the relation between potato and *Erwinia* (sub-)spp. is of importance when determining how many and which (sub-)species and isolates of

Table 2.8. Mean diameter of rotted tissue (mm) of slices and small tubers of 12 cultivars, after inoculation with *Erwinia carotovora* subsp. *atroseptica* IPO 161 and subsequent incubation in air (experiments 1, 7, 9 and 10, Table 2.1). The rank order is given in parentheses.

Cultivar	Tuber slices				Small tubers			
	Expt 1 Dec 1991		Expt 7 Nov 1992		Expt 9 Dec 1991		Expt 10 Dec 1992	
Agria	16.2	(1)	18.4	(1)	12.0	(4)	14.9	(6)
Alcmaria	14.2	(3)	15.7	(5)	12.5	(2)	15.9	(2)
Amazone	13.6	(4)	16.3	(3)	14.8	(1)	16.9	(1)
Arinda	13.5	(5)	14.7	(8)	10.2	(8)	12.4	(8)
Bintje	16.0	(2)	17.6	(2)	11.9	(5)	15.3	(5)
Désirée	10.6	(12)	12.2	(11)	8.4	(12)	9.5	(11)
Hertha	13.2	(6)	16.0	(4)	10.9	(6)	15.4	(4)
Karnico	10.9	(10)	11.6	(12)	10.4	(7)	11.0	(10)
Kondor	12.5	(7½)	15.5	(6)	9.3	(10)	13.5	(7)
Morene	10.8	(11)	12.5	(10)	9.8	(9)	11.5	(9)
Producent	11.1	(9)	12.7	(9)	9.1	(11)	9.3	(12)
Venouska	12.5	(7½)	14.9	(7)	12.3	(3)	15.5	(3)
Mean	12.9		14.9		11.0		13.4	
LSD (P<0.01)	2.3		1.6		1.7		1.9	

Table 2.9. Correlation coefficients for comparisons of mean cultivar rot between experiments with small tubers and slices.

Inoculum	Experiment	Incubation conditions	
		Air	Nitrogen
Small tubers 1991 vs 1992[1]			
Eca	9 vs 10	0.87	0.83
Ecc	9 vs 10	0.84	0.69
Ech	9 vs 10	0.28	0.75
Small tubers vs slices[2]			
Eca	9,10 vs 1,7	0.71 (± 0.13)	0.37 (± 0.29)
Ecc	9,10 vs 1,6	0.64 (± 0.10)	0.39 (± 0.23)
Ech	9,10 vs 1,8	0.01 (± 0.13)	0.52 (± 0.14)

[1] Significance levels: r = 0.58 (P<0.05) and r = 0.71 (P<0.01)

[2] Values are means across four comparisons, the range is given in parentheses.

the pathogen should be used for screening purposes. In an attempt to generate such knowledge, most of the inoculations in this study were carried out with isolates of each of the three pectolytic *Erwinia* (sub-)spp. which are pathogenic to potato (Table 2.1). A low level of specificity of the relation between potato and *Erwinia* is expected as a consequence of the broad host range of the pathogen, especially of *Ecc* and *Ech* (Pérombelon 1992). Nevertheless, tuber tissue resistance of the cultivars was clearly and reproducibly affected by *Erwinia* (sub-)spp. Such interactions between cultivars and *Erwinia* (sub-)spp. were also present in data presented by Lapwood *et al.* (1984) for aerobic incubated slices and by Ciampi-Panno and Andrate-Soto (1984), Hidalgo and Echandi (1982) and Lapwood *et al.* (1984) for anaerobic incubated tubers. It is concluded therefore, that screening for resistance can not be carried out with only one (sub-)species, although this has been suggested by Lapwood *et al.* (1984) and more recently by Wolters and Collins (1994). When compared with results from experiments with different (sub-)species, the rank order of the cultivars was less affected by isolates within (sub-)species in experiments 6 to 8 which indicates that screening can be reliably carried out using only one isolate within a (sub-)species.

The diameter of rot, averaged across all cultivars was larger after incubation for five days in nitrogen than after incubation in air (Table 2.2). This is in agreement with earlier reports (Lund & Wyatt 1972; Pérombelon & Lowe 1975; De Boer & Kelman 1978; Maher & Kelman 1983; Vayda *et al.* 1992). Molecular and biochemical mechanisms for this have been discussed by Davis *et al.* (1990). The results of experiments 2 to 4 in which slices were incubated at four differed oxygen concentrations indicate that the effect of oxygen concentration on the relative resistance of cultivars is complex (Table 2.5). This is also illustrated by the results of Vayda *et* al. (1992) who showed that resistance at low oxygen concentrations was comparable with resistance in air when tubers were preincubated at low oxygen concentrations for two days prior to inoculation. It is thought that resistance of cultivars at low oxygen concentrations, which reflects the preformed oxygen independent resistance mechanisms in tuber tissue, is more important under practical storage conditions than resistance shown in air (De Boer & Kelman 1978; Vayda *et al.* 1992). Although this remains to be studied, for practical screening purposes it can be concluded that the oxygen concentration must be standardized.

Lapwood *et al.* (1984) made the well for inoculation of a tuber slice just within the vascular ring. In our preliminary experiments, rot of slices of most of the cultivars which were inoculated that way, was impeded by the tuber skin at a certain time during incubation. As a consequence, the rot did not proceed concentrically any more. We decided therefore to inoculate closer to the middle of a slice, in the

medullary tissue. Results of experiment 11, using only *Eca*, show that the site of inoculation had only minor effects on cultivars ranking and therefore, should not be considered as an important factor. This is in agreement with comparable work on whole tubers by Bain and Pérombelon (1988). The finding that the cortex tissue of all cultivars was significantly more susceptible ($P<0.01$) than the medullary tissue is also in agreement with the work by Bain and Pérombelon (1988) as well as with the recent work of Lojkowska and Kelman (1994). However, the suggestion of these workers that the difference in resistance of medullary and cortical tissue is an important source of variation which affects cultivar ranking in tuber slice inoculation tests, is not supported by our results.

Correlation coefficients for comparisons within storage periods concerning inoculations with *Eca* followed by aerobic incubation (Table 2.7) were slightly higher than those reported by Wastie *et al.* (1988) who also used the slice inoculation method according to Lapwood *et al.* (1984). The same was found for comparisons between storage periods after inoculation with either *Eca* or *Ecc*, as calculated from data presented by Lapwood *et al.* (1984). In general, research teams that used other screening methods also found comparable levels of agreement between results within and between storage periods whether tuber material was incubated either aerobically (Hahn, 1974; Dobiáš, 1976; Workman & Holm, 1984) or anaerobically (Bourne *et al.*, 1981; Bain & Pérombelon, 1988).

Including this study, the slice inoculation method as originally developed by Lapwood *et al.* (1984) is the most extensively studied method for screening for potato tuber tissue resistance to pectolytic *Erwinia* spp. A second method, however, originally described by Maher and Kelman (1983) and indicated here as the "pipette tip method" for whole tubers, recently has been used by several research groups. This method has also potential as a standard method for soft rot resistance testing (Austin *et al.* 1988; Priou *et al.* 1992; Allefs *et al.* 1993; Lojkowska & Kelman 1994). Screening of six cultivars with the "pipette tip method" and with tuber slices using the same isolate (*Eca* SCRI 1039), gave comparable results. Also Priou *et al.* (1992) reported a good correlation between both methods. However, in disagreement with Priou et *al.* (1992) we have the experience that the slice method as used in our experiments is less time consuming than the "pipette tip method".

Breeding for resistance to *Erwinia* spp. involves the selection of clones from populations obtained after crossing. Seedlings and the few small tubers they produce are the earliest material available for selection. Since vegetative multiplication of entire populations is costly, it is desirable to select already among the early generations. In order to determine to what extent resistance of small tubers is comparable with

resistance of field-grown seed potatoes, we simulated the first generation growth of the 12 cultivars in pots in the glasshouse. The small tubers harvested, were screened according to a slightly modified procedure. Reproducibility of results of experiments with small tubers was similar to that obtained with slices (Tables 2.8 and 2.9). For results of experiments with small tubers compared with those with slices, mean correlations were generally lower and only significant ($P<0.05$) after inoculation with *Eca* or *Ecc* and incubation in air (Table 2.9). An explanation for this rather low level of agreement could be that the small tubers were harvested after maturation of the plants, whereas the seed potatoes were harvested in an immature stage after common practised halm destruction.

The inoculation methods described here generate data concerning the speed of the process of rotting of tuber tissue within a fixed time interval. Most of the published methods for screening for resistance to *Erwinia* spp. provide this type of data. However, the speed of rotting is not necessarily correlated with the number of bacteria required for initiation of a progressive rot as can be measured with point titration tests such as the "pipette tip method". Bain and Pérombelon (1988) and, more recently, Lojkowska and Kelman (1994) showed that the resistance of cultivars was dependent on which of the two methods was used, one measuring the weight or diameter of rotted tissue after inoculation at one site of a tuber and the other measuring the number of bacteria that caused initiation of rotting after inoculation with a range of bacterial concentrations. In preliminary experiments, we did not found much variation among the group of 12 cultivars in a point titration type test and, therefore, we decided to focus in this study on tuber tissue resistance to progressive rotting.

Conclusions

The ranking of potato cultivars for resistance to pectolytic *Erwinia* spp., based on the tuber slice inoculation method, is strongly affected by the oxygen concentration during incubation and to a lesser extent by bacterial (sub-)species. Cultivar ranking is hardly affected by the site of inoculation or the isolate used within (sub-)species. The tuber slice method meets most of the requirements of a screening method for breeding programmes, with regard to reproducibility and to screening of large numbers of clones. The predictive value of the method in relation to resistance of clones under field and storage conditions, remains to be studied.

Chapter 3

The role of the seed tuber in determining partial resistance to potato blackleg caused by *Erwinia* spp.

Abstract

In 1991 and 1992, 12 potato cultivars were screened at two locations for resistance to blackleg, after vacuum infiltration of the seed with *Erwinia carotovora* subsp. *atroseptica* or *E. chrysanthemi*. In 1992, cultivars were also screened for resistance to *E. carotovora* subsp. *carotovora*, but disease incidence with *Ecc* was low and consisted largely of non-emergence. Cultivar differences for resistance to *E.c.* subsp. *atroseptica* and *E. chrysanthemi* were found which were consistent over locations and years. Seed tubers of the same cultivars were also screened for resistance to *Erwinia* by using a tuber slice inoculation method. Correlation coefficients for comparisons between resistance to blackleg in the field and tuber tissue resistance under aerobic or anaerobic conditions were not significant. This could partly be explained by drastic changes in relative tuber tissue resistance of the cultivars within a five weeks period after planting in the field. Presprouting of seed tubers in diffuse daylight had a less pronounced effect on relative tuber tissue resistance than planting in the field. Monitoring the process of mother tuber decay during the growing season of 1993 after vacuum infiltration with *E.c.* subsp. *atroseptica* and *E. chrysanthemi* revealed that cultivars differed in the extent to which these bacteria enhanced the process of mother tuber decay. These differences partly explained the cultivar differences for resistance to blackleg in the field.

Introduction

The use of seed potatoes that are latently infected with pectolytic *Erwinia* spp. can result in non-emergence of plants, stunting or wilting, and predominantly in blackleg of stems (Pérombelon & Kelman, 1987), especially under conditions that favour the multiplication of these bacteria (Pérombelon, 1992). Although the bacteria do not seem to reduce yield significantly in temperate regions, infected tubers can under sub-optimal conditions cause severe losses due to rot in storage or due to blackleg associated symptoms when exported to areas were growing conditions are more

favourable for the bacteria. National seed inspection services consider the occurrence of blackleg during seed production as an indication of contamination of the seed. Even low incidence of blackleg leads to declassification of the seed produced (Young, 1990; *Anonymous*, 1994b) and as a consequence to significant economical damage to the seed tuber producers. Breeding for resistance to blackleg may contribute both to a reduction of the problem for seed producers and ware potato growers. Genetic variation for resistance has been identified both in cultivated *Solanum tuberosum* (Zadina & Dobiáš, 1976; Hidalgo & Echandi, 1982) and in species of the *Solanum* section *petota* (Van Soest, 1983; Lojkowska & Kelman, 1989).

Attempts to breed for blackleg resistance will involve the screening of large numbers of clones. Methods are only suitable if they are easy to perform, applicable to early vegetative generations and if they yield reproducible and quantitative data that show good agreement with the resistance of the clones in farmers' fields. Numerous methods for screening of plants in the field (Hossain & Logan, 1983; Lapwood & Legg, 1983; Lapwood & Gans, 1984; Lapwood & Read, 1986b; Gans *et al.*, 1991), or in the glasshouse (Munzert, 1975; Hidalgo & Echandi, 1982; Lapwood & Read, 1986a; Lojkowska & Kelman, 1989) have been described. Field tests correspond most to field conditions but they are expensive to perform and not feasible when only a small amount of seed is available. Since rotting of the mother tuber is an essential step during blackleg development (Pérombelon & Kelman, 1980), it was thought that selection for resistance to blackleg might be carried out by screening for tuber tissue resistance in the laboratory. Of the many methods published (see Chapter 2), the tuber slice inoculation method for screening for tuber tissue resistance as originally described by Lapwood *et al.* (1984) is one of the most extensively studied screening methods. It has been shown that for *Erwinia carotovora* subsp. *atroseptica* (*Eca*), *E.c.* subsp. *carotovora* (*Ecc*) and to a lesser extent also for *E. chrysanthemi* (*Ech*), this method generates reproducible data under different experimental conditions (Lapwood *et al.*, 1984; Lapwood & Read, 1985; Wastie *et al.*, 1988; Chapter 2). The tests are much easier to perform than field tests, yield quantitative data and to some extent seem suitable for screening first generation tubers (Chapter 2), but as far as known from literature, the relation between the results obtained in the tuber slice test and blackleg resistance in the field has not been studied. We studied this relation by screening a range of 12 cultivars for blackleg resistance in the field on two locations in two years and by comparing the results with tuber tissue resistance of the same cultivars as determined with the tuber slice inoculation method. The latter was also used for studying the effect on tuber tissue resistance of presprouting of seed potatoes and their growth in the field.

Finally, the process of mother tuber decay after inoculation with *Eca* and *Ech* was followed under field conditions and the results related to both blackleg and tuber tissue resistance.

Materials and methods

Cvs Agria, Alcmaria, Amazone, Arinda, Bintje, Désirée, Hertha, Karnico, Kondor, Morene, Producent and Venouska were chosen for this study because, over range of years, they differ markedly for the percentage of seed potato fields that is declassified upon blackleg incidence. Furthermore, they represent the early, main and late maturity classes. The seed tubers used in the experiments were 40-50 mm in diameter and harvested from the same field in each of the years 1990 to 1992 with two exceptions. For the field experiments of 1991, tubers of Venouska and Morene were from one different source. For the main field experiment of 1993, tubers of Karnico and Producent were each from a different source. After harvest, tubers were stored at 4 °C until tested.

Bacterial strains and preparation of inoculum. *Eca* IPO 161, *Ecc* IPO 163 and *Ech* IPO 502 were obtained from the DLO-Research Institute for Plant Protection (IPO-DLO), Wageningen, the Netherlands. *Eca* and *Ecc* were grown on Bouillon Agar medium containing 8.0 g ℓ^{-1} 'Lab-Lemco' Broth (Oxoid), 5.0 g ℓ^{-1} NaCl and 15.0 g ℓ^{-1} agar. *Ech* was grown on Growth Factor medium containing 0.4 g ℓ^{-1} K_2HPO_4, 0.05 g ℓ^{-1} $MgSO_4.7H_2O$, 0.1 g ℓ^{-1} NaCl, 0.5 g ℓ^{-1} NH_2PO_4, 1.0 g ℓ^{-1} glucose, 3.0 g ℓ^{-1} Yeast Extract (Oxoid) and 15.0 g ℓ^{-1} agar. Bacteria were cultured for 48 h at 27 °C, subsequently suspended in sterile water and centrifuged for 5 min at 4500 rpm. The bacterial pellet was re-suspended and the concentration determined using a standard curve for each of the species relating colony-forming units (cfu) to optical density at 500 nm. Bacteria were diluted to 1.0×10^9 cfu ml^{-1} in sterile water for tuber slice inoculation and to 3.0×10^7 cfu ml^{-1} (*Eca* and *Ech* in 1991), 2.0×10^7 cfu ml^{-1} (*Ech* in 1992 and 1993) or 5.0×10^7 cfu ml^{-1} (*Ecc*) in tap water for vacuum infiltration of tubers. Concentrations of inoculum for vacuum infiltrations were checked by dilution plating.

Screening for resistance to blackleg in the field. Seed tubers of the 12 cultivars were vacuum-infiltrated with either tap water, *Eca*, or *Ech* in 1991 and also with *Ecc* in 1992 and planted at two locations in the Netherlands. At Wageningen (Wag)

experiments were carried out on a river type of heavy clay soil whereas in the Noordoostpolder (Nop) experiments were planted on a very light sandy soil. At both locations, a split plot design was used with four replicates which consisted of three (1991) or four (1992) randomized plots, one for each of the infiltration treatments. Plots consisted of 12 randomized subplots, one for each cultivar. Subplots consisted of two rows of eight plants.

Vacuum infiltration of the tubers was carried out by submergence into the inoculum at a pressure of 5 kPa for 10 min, followed by a period of recovering for 10 min at atmospheric pressure. In both years, one bacterial suspension of each of the bacteria was used eight times for inoculation of the tubers of one replicate which, per location, were treated in random order. Tubers were air dried overnight at room temperature and planted (Wag, 1991) or stored at 4 °C during one (Nop, 1991), two (Wag, 1992) or three weeks (Nop, 1992) until planted. Planting dates were May 3 and 13 at Wag and May 8 and 19 at Nop in 1991 and 1992 respectively. Trials at Wag were overhead irrigated with approximately 280 mm between the 12th of July and 3rd of August in 1991 and between the 18th of June and 5th of August in 1992. No irrigation was applied to the trials at Nop.

Non-emergence of tubers was scored once during the season and plants showing blackleg associated symptoms three and four times in 1991 and 1992, respectively. Subplots were harvested 22 and 24 weeks after planting at Wag and 19 and 21 weeks after planting at Nop in 1991 and 1992, respectively. Yield was calculated per planted seed tuber and expressed as a percentage of the controls of the same cultivar and replication. Analysis of variance (ANOVA) was carried out for the percentage of diseased plants (non-emergence + blackleg) and for yield using the GENSTAT programme (Payne *et al.*, 1987).

Screening for tuber tissue resistance. Tuber tissue resistance was determined with the slice inoculation method as originally described by Lapwood *et al.* (1984). Tuber material from cold storage was either left untreated, presprouted or planted in the field for one to five weeks in order to study the effect of sprouting and development under field conditions. Presprouting was carried out by placing tubers in diffuse daylight for eight weeks during March to May 1992 at an ambient temperature of approximately 15 °C, according to seed producers' practice. In 1993, tubers were planted in a field with sandy soil on May 26, without prior presprouting treatment. Three plots were planted, each consisting of one row of 25 tubers per cultivar in randomized positions. Plots were harvested in random order, and one at a time, 7, 21 and 35 days after planting. Mother tubers were separated from the sprouts, surface

Table 3.1. Summary of the 9 experiments that were carried out for studying tuber tissue resistance. For each of the experiments the year of harvest of tuber material is given, the month of inoculation and the treatments carried out. In all experiments *Eca*, *Ecc* and *Ech* were used for inoculation. The duration of incubation was 5 days except for the nitrogen treatment in experiments 6 to 9 which was 3 days.

Expt	Year of harvest	Date of inoculation	Treatments
1	1990	June 1991	incubation in air at 15 or 20 °C
2	1991	March 1992	incubation at 20 °C in air or 2% oxygen
3	1991	March 1992	incubation at 20 °C in air or 5% oxygen
4	1991	March 1992	incubation at 20 °C in air or nitrogen
5	1991	May 1992	as 4, but tubers presprouted
6	1992	May 1993	incubation at 20 °C in air or nitrogen
7	1992	June 1993	as 4, but tubers harvested 1 week after planting
8	1992	June 1993	as 4, but tubers harvested 3 weeks after planting
9	1992	July 1993	as 4, but tubers harvested 5 weeks after planting

sterilized for 10 min in 1% hypochlorite, rinsed with tap water, dried overnight at room temperature and then used for inoculation. Tubers that were not planted and used as controls when studying the effect of development under field conditions were also surface sterilized.

For inoculation and incubation of tuber slices, three slices, 1.0 cm thick, one for each *Erwinia* (sub-)spp, were cut per seed tuber and incubated overnight at 15 °C. One fresh wound per slice was made by pressing a steel rod 4.0 mm deep and 5.0 mm diameter in the medullary tissue just near the pith, in which 20 µl of bacterial suspension was pipetted. Slices were placed at randomized positions in trays measuring 60 x 40 x 6 cm with a perforated bottom that were stacked in airtight containers of 65 x 45 x 65 cm. These containers were placed at 15 or 20 °C in a growth chamber and flushed with 500 ml min^{-1} air or nitrogen, or mixtures of nitrogen and oxygen, for five or three days to create different incubation conditions. Ten slices were used for each combination of cultivar, *Erwinia* (sub-)spp. and incubation condition. After incubation, rotted tissue was carefully removed and the diameter of the lesion measured in two directions at right angles. Experimental layout and analysis of mean lesion diameters were as described in Chapter 2.

The results of nine experiments were used for studying tuber tissue resistance in relation to field performance (Table 3.1). Results from experiments 2 to 4 have been described previously in Chapter 2.

Mother tuber decay in the field after vacuum infiltration. In 1993, tubers of the 12 cultivars were vacuum-infiltrated with water, *Eca* or *Ech*, planted at the Wag location and harvested at nine dates during the growing season in order to follow the process of mother tuber decay. A split plot design was used with three replicates which consisted of three randomized plots, one for each infiltration treatment. Plots consisted of nine randomized subplots, one for each harvest date. Subplots consisted of 12 randomized rows of nine plants, one for each cultivar.

Vacuum infiltration was carried out as described, except that in this case all tubers to be used for one infiltration treatment were divided between four randomized portions which were treated one by one in the same bacterial suspension or aliquot of water (controls). Tubers were stored at 4 °C for five (*Ech*, controls) or six (*Eca*) days and planted on the 27th of April. No overhead irrigation was applied. Subplots were harvested 4, 5, 6, 7, 9, 11, 13, 17 and 19 weeks after planting. Plants were carefully lifted and the condition of the mother tuber examined. Rotting mother tubers were bisected and the amount of rot estimated. The number of healthy stems and stems showing blackleg lesions of plants with a rotting or vanished mother tuber was counted at harvest dates 1 to 7. Mother tuber decay was calculated as the mean percentage of rot per cultivar within subplots. For each combination of replicate and cultivar, a three parameter ordinary logistic curve was fitted, relating increase of mother tuber decay with time after planting. Eighteen out of 972 data points, with an exceptional effect on the percentage of fit were omitted. The time after planting in days at which, on average, 50% of the mother tuber was rotted, further referred to as R50-value, was calculated and its variance analyzed by ANOVA.

Meteorological data. Meteorological data for Wag were obtained from the Department of Meteorology, Agricultural University Wageningen and recorded approximately 1.6 km from the trial sites. Meteorological data for Nop were obtained from the Royal Netherlands Meteorological Institute, de Bilt, which were recorded approximately 2 km (rainfall) and 5.2 km (temperature) from the trial sites.

Results

Meteorological data are presented in Table 3.2. Rainfall plus irrigation were approximately twice as high as the expected rainfall on the basis of long term means in 1991 and 1992 at Wag. At Nop rainfall was normal in 1991 but below average in 1992. Mean air temperatures in 1991 and 1993 were normal whereas the season of

Table 3.2. Total rainfall and daily mean temperature between planting and latest date of symptom scoring. Thirty year averages are based on monthly means. Soil temperature was measured at 5 cm below grassland. ND; not determined.

	1991 Wag	1991 Nop	1992 Wag	1992 Nop	1993 Wag
rainfall (mm)	211	212	149	135	243
irrigation (mm)	280	--	280	--	--
average rainfall (mm)	257	192	214	195	202
air temp. (°C)	14.7	13.4[1]	18.8	16.8[1]	15.5
average air temp. (°C)	15.3	ND	15.5	ND	14.6
soil temp. (°C)	16.1	ND	19.2	ND	16.7

[1] Values are based on monthly means.

1992 was relatively hot. Soil temperature is thought to be an important factor for *Erwinia* development (Molina & Harrison, 1980) and it was possible to collect such data from the Wageningen University Meteorological Station.

Resistance to blackleg. Incidence of non-emergence in control treatments was low except at Nop in 1992 (Table 3.3). As the experiments proceeded, on average only 0.3% of the control plants developed blackleg. Non-emergence in the controls, especially at Nop in 1992, is therefore thought not to be caused by *Erwinia* but by other unidentified biotic or abiotic factors. Consequently, disease incidence, which includes non-emergence, was corrected for occurrence of non-emergence in the appropriate controls (Table 3.3). A clear effect of infiltration with *Erwinia* on non-emergence was only found for *Eca* in 1991 when percentages of some cultivars were much higher than in the controls (Table 3.3).

Analysis of data from either combination of year and location concerning disease incidence and yield after infiltration with *Eca* or *Ech*, resulted in comparable experimental errors (not shown) indicating that combined analysis of these data was allowed. Thus, significant year x location x cultivar and treatment x cultivar interactions were found for both disease incidence and yield ($P<0.001$). For disease incidence, also a significant year x treatment x cultivar interaction was found ($P<0.05$) but its effect was less pronounced. In spite of these interactions, disease incidence of 1991 and 1992, when averaged across locations, was well correlated both after infiltration with *Eca* ($r=0.85$, $n=12$) and *Ech* ($r=0.88$). To a lesser extent, this was also the case for yield (*Eca*; $r=0.69$ and *Ech*; $r=0.72$). Disease incidence and yield after

Table 3.3. Percentage of non-emergence, averaged across cultivars and maximum values. Minimum values were 0 for all combinations of location, year and treatment. ND; not determined.

Treatm.	Wag 1991		Nop 1991		Wag 1992		Nop 1992	
	Mean	Max.	Mean	Max.	Mean	Max.	Mean	Max.
Water	0.5	1.6	0.4	1.6	0.7	3.1	7.4	25.0
Eca	6.4	32.8	6.5	18.7	1.1	4.7	7.2	18.7
Ech	0.4	1.6	1.2	6.4	1.6	9.4	8.6	29.7
Ecc	ND	ND	ND	ND	1.0	6.3	10.7	32.8

Table 3.4. Disease incidence (percentage non-emergence + percentage of plants with at least one stem with blackleg associated symptoms). Values for *Eca* and *Ech* are means across 1991 and 1992. *Ecc* data were not statistically analyzed.

Cultivar	Wag			Nop		
	Eca	*Ech*	*Ecc*	*Eca*	*Ech*	*Ecc*
Agria	25.8	39.8	0.0	44.5	43.7	3.1
Alcmaria	62.5	38.1	0.0	51.6	28.9	15.6
Amazone	50.0	48.4	4.7	43.0	44.5	12.5
Arinda	4.7	7.1	0.0	12.5	32.0	1.6
Bintje	15.6	26.6	1.6	17.2	29.7	0.0
Désirée	4.7	25.0	0.0	14.1	19.5	0.0
Hertha	8.9	16.4	0.0	18.0	18.9	6.3
Karnico	8.6	0.8	0.0	8.6	0.0	0.0
Kondor	19.5	41.4	3.1	40.6	78.1	3.1
Morene	19.5	34.4	3.1	18.7	26.6	3.1
Producent	1.6	1.6	1.6	3.1	3.9	1.6
Venouska	45.3	33.6	3.1	50.0	50.0	23.4
mean	22.2	26.1	1.2	26.8	31.3	5.5

LSD (P<0.01) = 15.3 for cultivar x location x inoculum means; 4.7 for location x inoculum means

Table 3.5. Yield as percentage of the controls. Values for *Eca* and *Ech* are means across 1991 and 1992. *Ecc* data were analyzed separately; cultivar yields were not significantly different.

Cultivar	Wag			Nop		
	Eca	*Ech*	*Ecc*	*Eca*	*Ech*	*Ecc*
Agria	85	87	98	90	90	96
Alcmaria	60	88	106	70	76	85
Amazone	85	85	103	88	76	79
Arinda	94	98	100	94	89	94
Bintje	96	89	95	80	80	100
Désirée	95	85	97	93	94	106
Hertha	101	85	96	89	79	77
Karnico	91	94	99	98	101	93
Kondor	93	76	97	81	73	100
Morene	89	80	94	92	79	100
Producent	97	98	97	104	95	99
Venouska	78	85	84	67	76	87
mean	89	88	97	87	84	93

LSD ($P<0.01$) = 23 for cultivar x location x inoculum means; 7 for location x inoculum means

infiltration with *Eca* and *Ech* are therefore presented as means across both years (Tables 3.4 and 3.5 respectively). Significant differences between cultivars were found ($P<0.01$) both for disease incidence and yield after infiltration with *Eca* or *Ech*. Disease incidence caused by *Ech*, averaged across cultivars, was equal to the incidence caused by *Eca* at both locations (Table 3.4) albeit that symptom expression was slightly different: *Ech* showed less non-emergence in 1991 (Table 3.3), less stunting and more wilting and vascular browning (not shown). Infiltration with *Ecc* was only carried out in 1992 and resulted in low disease incidence which consisted of non-emergence for approximately 33% at Wag and 60% at Nop. Yield reduction by *Ecc* was found (Table 3.5) but differences between cultivars were not significant.

The correlation coefficient between disease incidence and yield of subplots was -0.45 (n=480). Within combinations of year, location and *Erwinia* (sub-)spp., correlation coefficients varied from -0.09 to -0.67 (n=48).

Table 3.6. Correlation coefficients for comparisons between blackleg resistance (% of plants with blackleg associated symptoms including non-emergence) and tuber tissue resistance (mean diameter of rot) of stored tubers incubated at 20 °C and several oxygen concentrations. Comparisons refer to experiments of corresponding years and inoculum.

Blackleg resistance		Tuber tissue resistance				
Inoculum	Location	1991	1992			
		Air	Air[1]	5% Oxygen	2% Oxygen	Nitrogen
Eca	Wag	0.37	0.25	-0.40	-0.35	-0.19
Eca	Nop	0.35	0.59	-0.19	0.07	0.29
Ech	Wag	0.19	0.31	-0.64	0.06	0.01
Ech	Nop	0.20	0.56	-0.22	0.65	0.66

[1] Mean cultivar rot across experiments 2, 3 and 4 were used for calculations.
Significance levels: r = 0.58 (P<0.05) and r = 0.71 (P<0.01).

Tissue resistance of stored tubers in relation to blackleg. Tuber tissue resistance of the cultivars, expressed as the diameter of rotted tissue, was determined in 1991 and 1992 in experiments 1 to 4 (Table 3.1). Experiments 2 to 4 have been described previously in Chapter 2. In experiment 1, slices were incubated at 15 and 20 °C. Diameters of rot, averaged across cultivars, were 18.2, 11.9 and 9.6 mm for *Eca*, *Ecc* and *Ech* respectively at 15 °C and 17.4, 15.7 and 11.7 mm (LSD=0.9, P<0.01) at 20 °C.

Results of experiments 1 to 4, which were all carried out in spring with seed tubers from cold storage, were related to disease incidence (blackleg associated symptoms including non-emergence) as found after vacuum infiltration and planting in the field (Table 3.6). Although comparisons were made between results of corresponding years i.e. for tubers from the same source, correlation coefficients were low and if found significant (P<0.05), only for one location. No coefficients are presented for *Ecc*, since disease incidence was very low for most cultivars (Table 3.4).

Correlations between tuber tissue resistance as found in experiments 1 to 4 and yield as percentage of the controls (Table 3.5) were comparable with those found for the correlation between tuber tissue resistance and disease incidence; correlation coefficients were low and in general not significant (data not shown).

Table 3.7. Correlation coefficients for comparisons of mean cultivar rot between stored tubers and tubers that were harvested 1, 3 or 5 weeks after planting in the field.

Time after planting (weeks)	Incubation conditions					
	Air			Nitrogen		
	Eca	*Ech*	*Ecc*	*Eca*	*Ech*	*Ecc*
1	0.89	0.81	0.83	0.92	0.86	0.86
3	0.73	0.76	0.70	0.74	0.34	0.48
5	0.46	0.67	0.48	-0.16	-0.22	-0.13

Significance levels: r = 0.58 (P<0.05) and r = 0.71 (P<0.01).

Effect of presprouting and growth in the field on tuber tissue resistance. For the experiment carried out to study the effect of presprouting of seed potatoes on tuber tissue resistance (experiment 5, Table 3.1), it was found that mean diameters of rot across cultivars were 12.3, 15.7 and 12.6 mm for *Eca*, *Ecc* and *Ech* respectively after incubation in air and 21.5, 23.8 and 24.2 mm after incubation in nitrogen (LSD=1.2, P<0.01) which were comparable with mean diameters of rot in experiment 4 (Table 2.2, Chapter 2). Correlation coefficients for comparisons of tuber tissue resistance between both experiments were 0.82 (*Eca*, r=12), 0.88 (*Ecc*), 0.54 (*Ech*) and 0.71 (*Eca*), 0.88 (*Ecc*), 0.81 (*Ech*) after incubation in air and nitrogen respectively.

Four experiments were carried out in sequence to study the effect of sprout development and growth after planting in the field on tuber tissue resistance (experiments 6 to 9, Table 3.1). Correlation coefficients for comparisons of tuber tissue resistance between seed tubers from storage and seed tubers that were harvested one, three or five weeks after planting are presented in Table 3.7. Correlation coefficients between resistance of stored and planted tubers decreased rapidly with time after planting, especially after incubation in nitrogen. Five weeks after planting, a significant correlation was only found for the combination of *Ech* and incubation in air (P<0.05).

Mother tuber decay in the field after vacuum infiltration. Mother tuber decay of seed tubers infiltrated with water, *Eca* or *Ech* was followed during the growing season of 1993. The mean percentage rot of seed tubers that were infiltrated with water increased during the experiment and varied from 59% (cv Karnico) to 100% (7 cultivars) at the last harvest date. None of these plants showed stems with blackleg

Table 3.8. R50 values; mean time after planting (days) at which average mother tuber decay was 50%. R50 values relative to the controls are given in parenthesis.

Cultivar	Infiltration treatment		
	Water	*Eca*	*Ech*
Agria	85	43 (0.50)	62 (0.74)
Alcmaria	75	45 (0.60)	57 (0.76)
Amazone	93	47 (0.50)	74 (0.80)
Arinda	66	48 (0.73)	69 (1.04)
Bintje	62	43 (0.71)	57 (0.92)
Désirée	94	56 (0.60)	76 (0.81)
Hertha	86	79 (0.92)	76 (0.88)
Karnico	103	92 (0.91)	96 (0.95)
Kondor	72	45 (0.62)	57 (0.79)
Morene	91	50 (0.55)	90 (0.99)
Producent	97	89 (0.91)	96 (0.98)
Venouska	84	53 (0.63)	70 (0.84)
mean	84	58 (0.68)	73 (0.87)

LSD ($P<0.01$) = 12 for cultivar x treatment means; 6 for treatment means.

or associated symptoms, indicating that rotting of water-infiltrated mother tubers was caused by other microorganisms than *Erwinia*.

Cultivars differed significantly for the time after planting at which, on average, 50% of the mother tuber was rotted after infiltration with either water, *Eca* or *Ech* ($P<0.01$). These so called R50 values are presented in Table 3.8. Mean R50 values across cultivars were significantly lower ($P<0.01$) after infiltration with *Erwinia* than after infiltration with water, indicating that the *Erwinia* treatment enhanced mother tuber decay. The rate of this enhancement was not equally distributed across cultivars and the cultivar x treatment interaction was found to be highly significant ($P<0.001$). For example, the cvs Hertha, Karnico and Producent after *Eca* infiltration, and also Arinda, Bintje and Morene after *Ech* infiltration, showed no significant decrease of R50 values ($P<0.01$) when compared with the controls, in contrast to the other cultivars.

The different behaviour of the cultivars is illustrated in Figure 3.1 which shows the relation between mean percentage rot of mother tubers and time after planting for four contrasting cultivars. The fitted logistic curves from Agria and Hertha show that

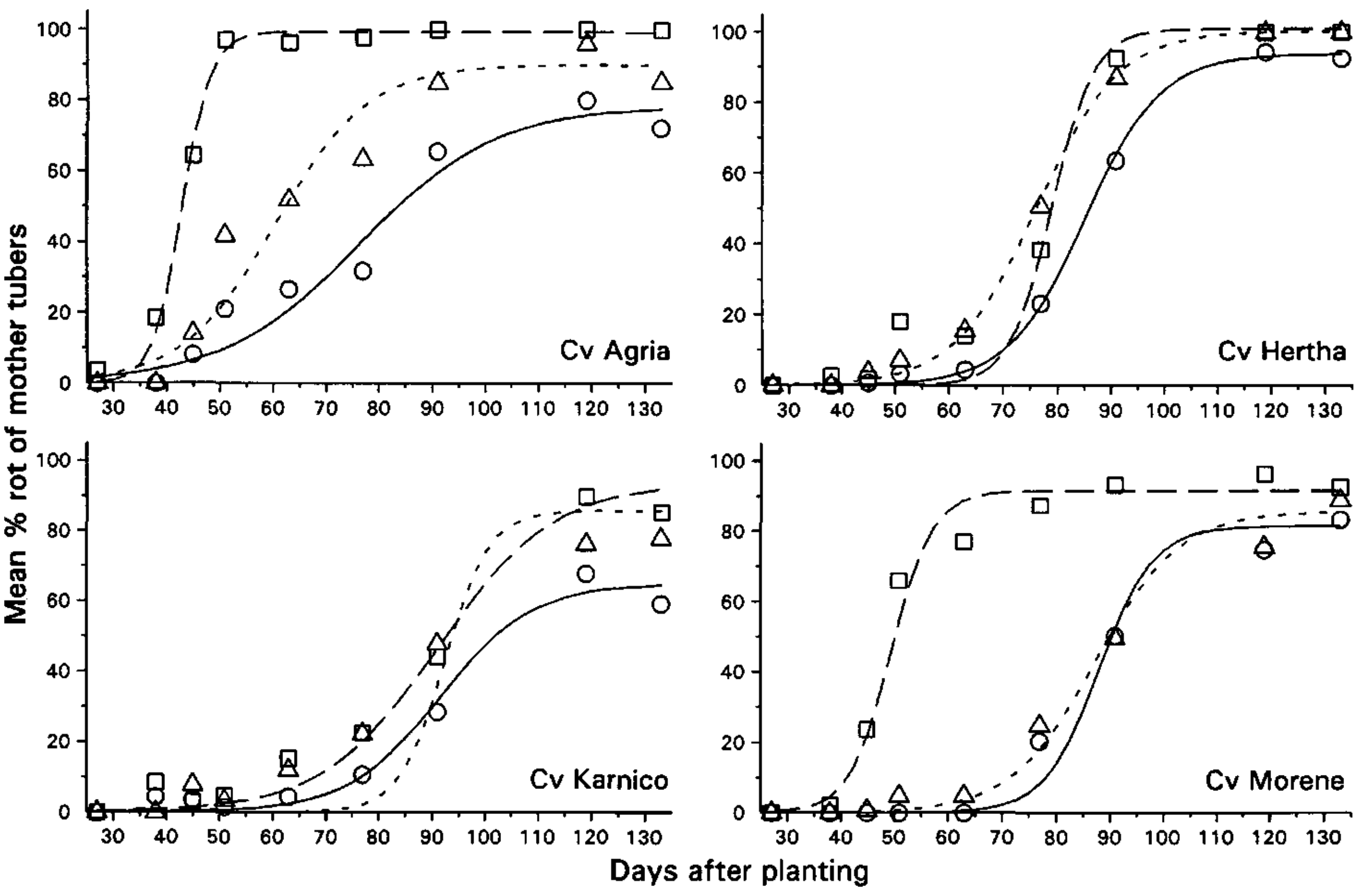

Figure. 3.1. Mean percentage rot of mother tubers of four contrasting cultivars at nine dates after planting of vacuum-infiltrated seed and fitted three parameter ordinary logistic curves. Infiltration treatments were water (open circles, ——), *Eca* (squares,---) and *Ech* (triangles,···).

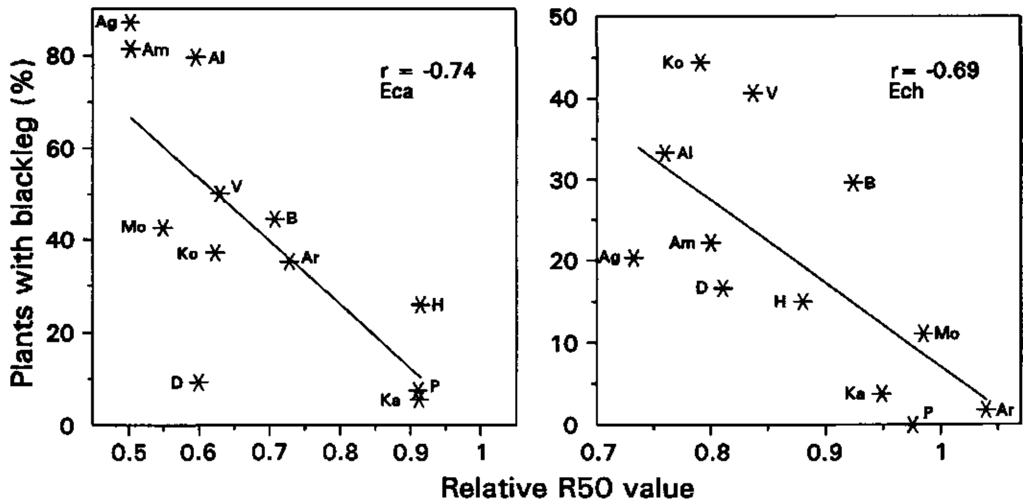

Figure. 3.2. The relationship between the relative R50 values and percentage of plants with blackleg associated symptoms in the 1993 experiment at Wag for Agria (Ag), Alcmaria (Al), Amazone (Am), Arinda (Ar), Bintje (B), Désirée (D), Hertha (H), Karnico (Ka), Kondor (Ko), Morene (M), Producent (P) and Venouska (V) after infiltration of the seed with *Eca* (left panel) or *Ech* (right panel).

these cultivars had comparable R50 values after water infiltration while only Agria showed a clear effect of *Erwinia* infiltration. The curves from Karnico illustrate the large R50 values found for this cultivar (Table 3.8). Another clear cultivar x treatment interaction is seen when curves of Hertha and Morene are compared with only Morene showing an effect of *Eca*.

Relation between mother tuber decay and blackleg. For each cultivar, the enhancing effect of *Erwinia* on the process of mother tuber decay was expressed as a R50 value relative to the controls (Table 3.8) and related to the percentage of plants with blackleg associated symptoms averaged across the 6th and 7th harvest dates (Figure 3.2). Moderate but significant ($P<0.01$; *Eca* and $P<0.05$; *Ech*) negative correlations were found, suggesting that the rate of blackleg development was only partially explained by the effect of *Erwinia* on mother tuber decay. Cvs Alcmaria and

Désirée for example, showed equal relative R50 values for *Eca* but differed strongly for the percentage of blackleg found.

Percentages of plants with blackleg associated symptoms (excluding non-emergence) were 42.1 and 19.9 for *Eca* and *Ech* respectively. Results of the 1993 experiment were in agreement with disease incidence at Wag averaged across 1991 and 1992 ($r=0.81$, $n=12$; *Eca* and $r=0.75$; *Ech*).

Relation between mother tuber decay and tuber tissue resistance after planting. Tuber tissue resistance of the cultivars after planting as studied in experiments 6 and 9 was compared with the appropriate relative R50 values of Table 3.8. For cold stored tubers (experiment 6) only a significant negative correlation ($r=-0.65$, $P<0.05$) was found for *Ech* after incubation in air. Tissue resistance of tubers harvested five weeks after planting (experiment 9) showed no significant correlation ($P<0.05$) with relative R50 values at all (data not shown). This was not caused by the results of Karnico and Producent, the only cultivars of which different sources of tubers were used.

Discussion

Resistance to blackleg. Resistance of the 12 cultivars was studied after planting of vacuum-infiltrated seed at two locations in two successive years. Vacuum infiltration is thought to resemble the natural infection via lenticels and small injuries prior to harvest in soil or during post harvest handling more than other methods that have been used for field tests (Gans *et al.*, 1991). Furthermore, Bain and Pérombelon (1988) showed for a set of cultivars that the number of bacteria infiltrated per tuber is comparable.

Total disease incidence after infiltration with *Eca* was much higher than after infiltration with *Ecc* (Table 3.4). This has been reported earlier (Zink & Secor, 1982; Pérombelon *et al.*, 1987; Pérombelon *et al.*, 1988) and explained by the fact that the soil temperature conditions were more favourable for *Eca* which has a relatively low temperature optimum for growth (Molina & Harrison, 1980). Temperature alone however can not explain the observed differences since it has also been shown that both the *Eca*, *Ecc* and also the *Ech* isolate used are able to macerate tuber tissue at 15 and 20 °C (experiment 1) while mean soil temperatures, at least at Wag, were within that range (Table 3.2). Pérombelon (1992) suggested that low disease incidence by *Ecc* might be explained by its failure to compete with saprophytic bacteria in the mother tuber.

Correlation of disease incidence data between years was high for both *Eca* and *Ech*, in spite of the significant year x location x cultivar interaction found. Also, results concerning groups of the five most resistant (Arinda, Désirée, Hertha, Karnico and Producent) and most susceptible (Agria, Alcmaria, Amazone, Kondor and Venouska) cultivars were consistent for both locations, except for Arinda and Alcmaria which were relatively susceptible and resistant respectively to *Ech* at Nop. Although the importance of *Ech* as causal pathogen of blackleg in temperate regions relative to the importance of *Eca* is not known, results indicate that screening for blackleg resistance in the field should preferably be carried out at different locations with both *Eca* and *Ech*.

Yield, as percentage of the controls, was also affected by year, location and *Erwinia* spp. and, although significant, the correlation between yield and disease incidence was lower than reported by other groups (Bain *et al.*, 1990; Gans *et al.*, 1991).

Tissue resistance of stored tubers in relation to blackleg. It has been shown in a number of studies that the slice inoculation method as originally described by Lapwood *et al.* (1984) is suitable for determination of tuber tissue resistance and that the results obtained, in terms of resistance of clones relative to others, are hardly affected by variation in experimental conditions (Lapwood & Read, 1985; Wastie *et al.*, 1988), although considerable interaction effects of *Erwinia* (sub-)spp. and oxygen concentration during incubation have been found (Chapter 2). However, results from screening for tuber tissue resistance had no predictive value when correlated to results from screening for resistance to blackleg in the field, regardless of the oxygen level during incubation and in spite of the fact that results were compared for experiments in which the same source of tubers had been used (Table 3.6). Results from correlation studies between tuber resistance and resistance to blackleg in the field have not been described before, although some groups have reported on the relation between tuber resistance and blackleg of glasshouse-grown plants. Thus Zadina and Dobiáš (1976) found a correlation coefficient of 0.65 after screening 198 cultivars for resistance to *Eca*, in contrast to the work by Munzert and Hunnius (1980) and Hidalgo and Echandi (1982) who found no correlation for *Eca* and *Ech*, respectively. The absence of a correlation can partly be explained by changes in relative tuber tissue resistance after planting in the field (Table 3.7) which was in contrast to the good reproducibility found for tuber tissue resistance when using tubers stored for various lengths of time, especially after inoculation with *Eca* (Chapter 2). Bain and Pérombelon (1988) tested mother tubers for resistance to *Eca*

after a one month period of growth in the glasshouse in pots containing field soil, and they too did not find a correlation with results from tests for which stored tubers had been used. Results from experiment 5 show that presprouting of tubers did not markedly affect the ranking of the cultivars for resistance.

Mother tuber decay in the field. Infiltration of seed potatoes with *Erwinia* enhanced the natural process of mother tuber decay. The rate at which this occurred was dependent on the cultivar, *Erwinia* spp. and their interaction effects (Table 3.8, Figure 3.1).

A significant but relatively small correlation was found between the percentage of blackleg and mother tuber decay expressed as a R50 value relative to the controls (Figure 3.2). This indicates that there is a role of the mother tuber in blackleg development, but that other factors such as the rate at which the rotting process of the mother tuber proceeds into the stem (Munzert & Hunnius, 1980) and resistance of the stem tissue itself (Pérombelon, 1988), might also be important. Since no correlation was found between relative R50 values of mother tubers in the field and tuber tissue resistance, even five weeks after planting, it will be very difficult, if not impossible, to select for clones with high mother tuber resistance by using the slice inoculation method. Tuber tissue resistance of mother tubers which had been grown in the field for more than five weeks could not be determined due to the start of pathogen independent rotting and extensive cavities forming in the medullar tissue. However, as can be seen in Figure 3.1, the effect of *Erwinia* on mother tuber decay took place in the period beyond five weeks after planting.

Tuber tissue resistance has been determined in air and nitrogen and partly also at intermediate oxygen concentrations. In a well structured and drained soil, oxygen diffuses sufficiently through pores and concentrations in the soil do not differ from the above ground situation. After heavy rainfall or excessive irrigation however, pores become filled with water and dissolved oxygen is soon depleted by plant and microbial respiration (see Drew, 1990). Disease development is often associated with such conditions (Pérombelon & Kelman, 1980; Weber, 1990; Pérombelon, 1992) and therefore, resistance of seed potatoes under anaerobic conditions is expected to be of importance, probably in interaction with moist conditions as such, since resistance of stored tubers is reduced by high water content (Pérombelon & Lowe, 1975; Weber & Jansen, 1984; Weber, 1988). In this study, no indication was found that resistance of seed tubers as expressed under anaerobic conditions is more effective for the control of blackleg than resistance as expressed under aerobic condtions.

Conclusions

Selection for resistance to blackleg in the field by screening stored seed tubers for tuber tissue resistance with the slice inoculation method as originally described by Lapwood *et al.* (1984), either under aerobic or anaerobic conditions, seems not possible. It is not likely that the method can be adapted for this purpose since it was shown that tuber tissue resistance changes soon after planting in field. Also, tuber tissue resistance determined on field planted and subsequently lifted tubers did not show any correlation with the process of mother tuber decay in the field. Mother tuber decay seems to play a significant role in partial resistance to blackleg but other, as yet unidentified factors, are thought to be as important.

Chapter 4

Components of partial resistance to potato blackleg caused by pectolytic *Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi*

Abstract

The various phases of the infection process by pectolytic *Erwinia* spp., the causal pathogens of potato blackleg, were interpreted as components of partial resistance and studied in detail. Glasshouse-grown plants of two potato cultivars were inoculated with antibiotic resistant marker strains of *Erwinia carotovora* subsp. *atroseptica* (*Eca*) or *E. chrysanthemi* (*Ech*). As soon as three days after inoculation of the mother tuber, *Erwinia* bacteria were detected in the stem tissue of the plants. However, blackleg developed only when the rotting mother tuber tissue reached the sites where the stems were attached to the mother tuber. A method was developed for determining differences between cultivars in the extent to which rot in the mother tuber proceeds to the stem. A set of 12 cultivars was screened for this putative component of resistance in the stem base. Significant differences for stem base resistance were found among the cultivars. These differences were more clear and more reproducible when inoculation was carried out with *Ech* in contrast to *Eca*. Glasshouse-grown plants of the same set of cultivars were also screened for above ground stem tissue resistance. Cultivars differed significantly for stem tissue resistance, but results of some cultivars were not consistent among years of testing. Multiple regression analysis revealed that stem base resistance, stem tissue resistance, tuber tissue resistance and resistance of mother tubers in the field account for 63% (*Eca*) and 75% (*Ech*) of the variance found after screening for resistance to blackleg in the field. It was concluded that if results of this study are extrapolated to any set of potato clones, selection for resistance in breeding programmes under laboratory or glasshouse conditions, would be most efficient when directed to clonal differences for stem base resistance.

Introduction

Blackleg is a disease of potato that is caused by the pectolytic *Erwinia* spp. *E. carotovora* subsp. *atroseptica* (*Eca*), *E. chrysanthemi* (*Ech*) and to a lesser extent also by *E.c.* subsp. *carotovora* (*Ecc*) (Pérombelon & Kelman, 1980, Pérombelon *et al.*, 1987). Since genotypic variation for resistance has been found among commercial and primitive cultivars as well as in *Solanum* spp. of the section *petota* (Hidalgo & Echandi, 1982; Huaman *et al.*, 1988; Lojkowska & Kelman, 1989; Gans *et al.*, 1991), breeding for resistance could contribute to disease control. However, only partial resistance has been found within the genus *Solanum* and blackleg resistance of genotypes is difficult to measure, partly because of the lack of knowledge about the mechanisms in the tuber and stem tissue which determine partial resistance. Furthermore, resistance has been found to be strongly affected by environmental conditions (Pérombelon & Kelman, 1980). As a result, no resistance genes have been identified so far (Lyon, 1989) and breeding for resistance to blackleg has not been successful.

If the complexity of partial resistance could be simplified by identifying different components of resistance, breeding for resistance could be carried out more efficiently by selecting clones that are superior for those components that are most important for blackleg resistance under field conditions. Screening for blackleg resistance of potato clones in the field can be carried out by using artificially inoculated seed tubers. Such tests are considered to yield resistance data which show good agreement with resistance in farmer's fields (Gans *et al.*, 1991).

Blackleg of potato stems i.e. the discolorization of the stem tissue accompanying the bacterial rotting process, invariably originates from a rotting mother tuber (Pérombelon & Kelman, 1980) and therefore, the speed of the rotting process of the tuber tissue, as well as that of the stem tissue, can be regarded as putative components of resistance. Several methods have been described for the screening for tuber tissue resistance (see Chapter 2) or stem tissue resistance (Hidalgo & Echandi, 1982; Hossain & Logan, 1983; Wastie, 1984; Lapwood & Read, 1986a). In a previous study (Chapter 3), it was shown that tuber tissue resistance of stored tubers of a set of 12 cultivars was not very well correlated with resistance to blackleg. Resistance of mother tuber tissue as expressed during development and growth of plants in the field, however, seemed to be of importance.

Another component of resistance may be located in the stem base as has been suggested by Munzert (1975) and Munzert and Hunnius (1980), who screened fully grown plants in the glasshouse by inoculating the seed piece from which they were

grown. After incubation, the amount of seed piece decay varied among genotypes. However, between genotypes with completely rotten seed pieces, differences were observed for the percentage of plants with blackleg diseased stems. Here we present further evidence for the existence of a mechanism of resistance which is located in the stem base. Furthermore, we screened the same set of 12 cultivars for above ground stem tissue resistance to *Eca* and *Ech* and studied the role of stem base resistance, stem tissue resistance, tuber tissue resistance (Chapter 2) and mother tuber decay in the field (Chapter 3) as components in determining partial resistance to blackleg in the field. The consequences of the results for resistance breeding are discussed.

Materials and methods

Plant material and inoculum. Seed tubers of 12 cultivars (Agria, Alcmaria, Amazone, Arinda, Bintje, Désirée, Hertha, Karnico, Kondor, Morene, Producent and Venouska) were harvested from one field in each of the years 1990 to 1993 with two exceptions (see Chapter 3). Tubers were stored at 4 °C until tested.

Bacterial isolates *Eca* IPO 161, and *Ech* IPO 502 were obtained from the Research Institute for Plant Protection (IPO-DLO), Wageningen, the Netherlands. Spontaneous mutants of *Eca* IPO 161 and *Ech* IPO 502 were selected on agar medium containing 100 µg ml^{-1} streptomycin or naldinic acid respectively. These antibiotic resistant strains, referred to as Eca^{str} and Ech^{nal} showed no reduced virulence when used for inoculation of tuber slices (data not shown).

Preparation of inoculum was as described in Chapter 2. In short, *Eca* was grown on Bouillon Agar medium containing 8.0 g ℓ^{-1} 'Lab-Lemco' Broth (Oxoid), 5.0 g ℓ^{-1} NaCl and 15.0 g ℓ^{-1} agar. *Ech* was grown on Growth Factor medium containing 0.4 g ℓ^{-1} K_2HPO_4, 0.05 g ℓ^{-1} $MgSO_4.7H_2O$, 0.1 g ℓ^{-1} NaCl, 0.5 g ℓ^{-1} NH_2PO_4, 1.0 g ℓ^{-1} glucose, 3.0 g ℓ^{-1} Yeast Extract (Oxoid) and 15.0 g ℓ^{-1} agar. Bacteria were cultured for 48 h at 27 °C, subsequently suspended in sterile water and centrifuged for 5 min at 4500 rpm. The bacterial pellet was re-suspended and the concentration determined using a standard curve for each of the species relating colony-forming units (cfu) to optical density at 500 nm. In experiments for screening stem base and stem tissue resistance, the pectolytic enzymes which are secreted by the bacterium, were not removed by centrifugation. The initial suspension was directly used for inoculation in order to ensure progressive rotting of the inoculated plant tissues.

Study of the movement of bacteria from the mother tuber into the stem. Seed tubers of cvs Alcmaria and Producent, 28-35 mm diameter and harvested in 1990, were planted in sand in square plastic pots in a glasshouse at 22 °C., the heel end directed to a hole of 18 mm diameter, made in the middle of one of the sides of each pot. Emerged plants were fertilized once a week with 1 x Steiner nutrient solution (Steiner, 1984). After 25 days of growth, plants were inoculated with 20 µl of bacterial suspension containing 1.0 x 10^9 cfu ml^{-1} of either Eca^{str} or Ech^{nal}. Bacteria were inserted by stabbing in the stolon end part of the mother tuber, which could be reached through the hole in the pot. The experiment was carried out in a randomized block design. Two rows of five plants per cultivar per bacterial species were harvested immediately and 3, 7, 14 and 21 days after inoculation. Three types of data were collected. (1) Mother tubers were bisected and the amount of rot was estimated. (2) Stems were scored for presence of blackleg and (3) the main stem of each plant was analyzed for presence of the pathogen. This was carried out as follows. The stems with a small piece of the mother tuber attached, were superficially sterilized in 1% NaOCl for 10 min and then rinsed thoroughly with tap water. A piece of tissue, 1.0 cm long, was cut from the main stem, thereby leaving 1.0 cm of the stem attached to the tuber tissue. The piece of stem tissue was ground in 1.0 ml 0.1 M phosphate buffered saline pH 7.3. Decimal dilutions of 100 µl aliquots of these suspensions were analyzed for presence of *Erwinia* using the immunofluorence colony-staining technique described by Van Vuurde (1987). In short, in wells of 24 wells microtiter plates (Greiner), dilutions of the samples were mixed with agar medium containing the appropriate antibiotic, and incubated for 48 h at 24 °C. The agar layers were dried into a thin film and stained with FITC conjugated antiserum raised against *Eca* or *Ech* (IPO-DLO). The number of positive colonies per well was counted by ultraviolet light microscopy. The concentration of bacteria in the stem tissue was calculated and expressed as the number of cfu stem $sample^{-1}$. Plants which were treated with water served as controls.

Screening for stem base resistance. Seed tubers of the 12 cultivars, 40-50 mm diameter and harvested in 1993, were placed, sprout end upwards, in trays with moist sand at 20 °C in the dark. After two to three weeks, when etiolated sprouts were 1 to 3 cm long, one cylinder of tuber tissue was cut from each tuber. Cylinders were 2.5 cm in diameter and 1.0 cm high and contained one or more sprouts at the centre. Inoculation was carried out by placing the tuber tissue cylinders on a piece of irrigation matting, measuring 25 x 40 cm, that was previously soaked in 600 ml of bacterial suspension. Bacterial concentrations of *Eca* were spectrofotometrically

adjusted to 4.0 x 10^8 and 3.0 x 10^8 cfu ml^{-1} in two successive experiments. For *Ech*, the concentration was 2.0 x 10^8 cfu ml^{-1} in both experiments. Tuber cylinders that were treated with sterilized water served as controls. Experiments were carried out in trays with lids to ensure high relative humidity. Each tray contained 12 randomized rows, one for each cultivar. Rows consisted of eight cylinders. In each of two experiments, five trays were used per bacterial species. After inoculation, the trays were placed at 20 °C in the dark. Sprouts were scored for presence of blackleg symptoms within a 19 days period on seven and eight dates after inoculation in the first and second experiment respectively. The percentage of sprouts per cultivar that showed blackleg at the final scoring date was calculated (referred to as sprout infection frequency), as well as the mean time after inoculation (in days) at which the blackleg symptoms of sprouts were visible (referred to as sprout latency period). Analysis of variance (ANOVA) was carried out using the programme GENSTAT (Payne *et al.*, 1987).

Screening for stem tissue resistance. Seed tubers of the 12 cultivars, 35-40 mm diameter and harvested in the years 1992 and 1993, were planted in 1 ℓ pots containing potting compost and grown for five or six weeks during spring 1993 and 1994 respectively, in a conditioned glasshouse at a 20/18 °C day/night regime before inoculation. The main stem of the plants was kept and the rest removed. Experiments were carried out in a randomized complete block design with ten replicates. Inoculation was carried out by insertion of a cotton thread, approximately 6 cm long and previously soaked in a bacterial suspension. Insertion was carried out with a needle in the middle of the stem at approximately 2 cm from the soil level. One end of the thread was hung in a 1.5 ml Eppendorf tube containing 0.5 ml of the same bacterial suspension. The tube was pressed in the soil near the stem base. Bacterial concentrations in 1993 were 3.0 x 10^9 and 1.5 x 10^9 cfu ml^{-1} for *Eca* and *Ech* respectively and 3.0 x 10^9 cfu ml^{-1} in 1994. Plants that were treated with sterilized water served as controls.

After inoculation, plants were kept in a plastic tunnel to create high relative humidity. Seven days after inoculation, the length of the blackleg lesion was measured on opposite sides along the stem. Analysis of variance (ANOVA) was carried out using the GENSTAT programme (Payne *et al.*, 1987) after square root transformation of the mean blackleg lesion length per stem to obtain equal variances.

Tuber tissue resistance and field data. Two experiments were carried out for determination of tuber tissue resistance of the 12 cultivars. Because results of these

experiments have been presented *in extenso* previously (Chapters 2 and 3), they are described here briefly. Seed tubers, harvested in 1992, were directly tested from 4 °C storage in November 1992 and May 1993. From the tubers, 1.0 cm thick slices were cut, inoculated at the centre with 20 µl 1.0 x 10^9 cfu ml^{-1} *Eca* or *Ech* and incubated in air or nitrogen for five and three days, respectively. Ten slices per cultivar per bacterium were tested in a completely randomized block design. After incubation, the rotted tissue was removed from the slices and the diameter of the resulting cavity was measured in two directions at right angles. Tuber tissue resistance was expressed as the mean diameter of the cavity, averaged across the ten slices.

In 1991 to 1993, field experiments were carried out for assessing the blackleg resistance and the extent to which *Eca* and *Ech* enhanced the process of mother tuber decay. Because results of these field experiments have also been presented previously (Chapter 3), they too are described here briefly. Seed tubers of the 12 cultivars were vacuum-infiltrated in bacterial suspensions of *Eca*, *Ech* or tap water (controls). In 1991 and 1992, vacuum-infiltrated tubers were planted in a split plot design with four replicates at two locations in the Netherlands. Non-emergence of planted tubers was scored once. The number of emerged plants which showed blackleg associated symptoms was scored three or four times.

In 1993, vacuum-infiltrated seed tubers were planted in a split plot design with three replicates at one location. Plants from subplots were lifted at nine dates during the growing season and the amount of rot of the mother tuber was estimated. By fitting ordinary logistic curves that related increase of mother tuber decay with time after planting, the time after planting was determined (in days) at which, on average, 50% of the tuber tissue of individual mother tubers of a given cultivar was decayed. The values from the treatments with *Eca* and *Ech* infiltrated tubers were divided by the values from the appropriate water treated controls. These are referred to as "relative R50 values" for mother tuber decay.

The role of (1) tuber tissue resistance of tubers from storage, (2) mother tuber decay in the field, (3) stem base resistance and (4) stem tissue resistance in determining blackleg resistance of the 12 cultivars in the field experiments of 1991 and 1992 was studied by stepwise multiple regression analysis. Except for mother tuber decay, which was determined once, values used for calculation were means across repeated experiments.

All components of resistance were measured by using tubers from sources other than the sources from which seed tubers for the field tests were derived.

Results

Study of the movement of bacteria from the mother tuber into the stem. The estimated percentage of rot in mother tubers from inoculated glasshouse-grown plants of cvs Alcmaria and Producent increased with time after inoculation (Table 4.1). Mother tubers of the relatively susceptible cv Alcmaria rotted faster than mother tubers of the relatively resistant cv Producent. Already three days after inoculation, when no symptoms could be seen as yet, antibiotic resistant *Erwinia* bacteria could be detected in some stems of plants from both cvs Alcmaria and Producent. The number of bacteria however, was less then 1.0 x 10^3 cfu stem sample^{-1}. In the course of the experiment, no increase was found of the numbers of stems in which *Erwinia* bacteria could be detected nor did the concentration of bacteria per stem sample rise. Blackleg only developed in stems of plants with completely rotten mother tubers. The bacterial concentration in tissue of stems with blackleg was estimated to be equal to or higher than 1.0 x 10^6 cfu stem sample^{-1}. These results indicate that *Erwinia* bacteria are able to reach the stems soon after establishment of rot in the mother tuber but that blackleg will only develop if the mass of *Erwinia* bacteria in the rotting mother tuber tissue contacts the stem base.

Table 4.1. Mean percentage of rot in mother tubers of plants from cvs Alcmaria and Producent 0, 3, 7, 14 and 21 days after inoculation (DAI). The number of plants showing at least one blacklegged stem (BL) and the number of stems in which *Eca*^{str} or *Ech*^{nal} was detected (*E.*) is also given. Ten plants were inoculated per DAI per cultivar per *Erwinia* spp. Only the main stem of each plant was analyzed for presence of *Erwinia*.

	Inoculum											
	Eca^{str}						*Ech*^{nal}					
	Alcmaria			Producent			Alcmaria			Producent		
DAI	% Rot	BL	*E.*	% Rot	BL	*E.*	% Rot	BL	*E.*	% Rot	BL	*E.*
0	0	0	0	0	0	0	0	0	0	0	0	0
3	1	0	5	0	0	6	0	0	0	0	0	0
7	24	0	9	19	0	6	58	0	6	4	0	4
14	65	0	3	35	0	4	100	5	6	67	2	7
21	92	1	2	67	1	1	100	7	5	75	1	6

Stem base resistance. The screening method with etiolated sprouts grown on tuber cylinders was used to determine whether a mechanism of resistance is located in the stem base, i.e the point were the sprout is attached to the tuber. By using unpurified, high concentration bacterial suspensions for inoculation and by inoculating freshly cut cylinders, it was attempted to create conditions that were highly favourable for fast and complete rotting of the tuber tissue cylinders. It was thought that this would allow for the screening of cultivars for differences in the extent to which the rot from the tuber tissue proceeds into the stem tissue. For experiments with *Eca*, this approach was not fully successful. Rotting was initiated in all cylinders tested, but at a certain moment during incubation, rotting stopped in a significant percentage of the cylinders. In experiments with *Ech*, the rotting process of tuber cylinders was completed within about five days after inoculation. This difference between the bacterial species resulted in much lower sprout infection frequencies for *Eca* in contrast to *Ech* (Table 4.2). Despite the low sprout infection frequencies found for *Eca*, some correlation was found for cultivar means between both experiments ($r=0.73$, $P<0.01$).

In experiments with *Eca*, cultivars with positive sprout infection frequency values showed no differences for sprout latency period ($P>0.01$), except for cv Bintje which showed a relatively long sprout latency period in the second experiment.

When inoculation was carried out with *Ech*, cultivars differed for sprout infection frequency (Table 4.2). Cv Producent showed the lowest sprout infection frequency in both experiments. Sprout infection frequencies of cvs Agria and Karnico were not consistent among experiments. The correlation coefficient for comparison of mean sprout infection frequencies between experiments was 0.76 ($P<0.01$). Cultivars differed also significantly for sprout latency period ($P<0.01$, Table 4.2). The correlation coefficient for comparison of mean sprout latency period between experiments was high (0.91; $P<0.01$), even though the sprout latency period of cvs Producent and Karnico were based on only a few diseased sprouts in experiment 2.

In the first experiment with *Ech*, a high negative correlation was found between sprout infection frequency and sprout latency period (-0.84; $P<0.01$), but this was not at a similar level in the second experiment (-0.61; $P<0.05$). This indicates that sprout infection frequency and sprout latency period as factors in determining stem base resistance are not independent. Control tuber cylinders showed no rot at all. These results, especially those from experiments with *Ech*, indicate that the stem base can form a barrier preventing the mass of bacteria in the rotten tuber tissue to spread to the stem, and that there exists genotypic variation for the extent to which bacteria from rotten tuber tissue succeed in passing this barrier.

Table 4.2. Stem base resistance of 12 cultivars as indicated by mean sprout latency period (SLP; days after inoculation at which blackleg of sprouts was observed) and mean sprout infection frequency (SIF; % of sprouts with blackleg 17 (expt 1) or 19 (expt 2) days after inoculation). SLP values are means across the number of tuber tissue cylinders with blacklegged sprouts.

	Inoculum							
	Eca				*Ech*			
	SIF		SLP		SIF		SLP	
Cultivar	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Agria	17.5	12.5	13.0	12.2	97.5	47.5	8.3	10.0
Alcmaria	2.5	7.5	17.0	13.7	87.5	77.5	6.9	11.0
Amazone	12.5	17.5	12.2	12.4	67.5	70.0	9.2	10.4
Arinda	0.0	5.0		10.5	72.5	67.5	13.2	14.3
Bintje	0.0	20.0		16.3	70.0	52.5	10.5	13.0
Désirée	0.0	12.5		13.6	80.0	95.0	11.4	12.8
Hertha	27.5	20.0	12.2	12.3	95.0	92.5	8.7	11.6
Karnico	25.0	35.0	12.2	12.1	62.5	7.5	11.2	12.0
Kondor	25.0	20.0	13.2	12.9	100.0	100.0	6.3	7.9
Morene	15.0	25.0	12.5	12.9	85.0	92.5	8.9	11.3
Producent	0.0	0.0			37.5	5.0	14.4	19.0
Venouska	25.0	40.0	11.7	10.8	97.5	82.5	7.6	9.8
Mean[1]	12.5	17.9	12.5	12.6	79.4	65.8	9.3	11.2
S.E.	9.9	15.4	3.0	2.9	12.6	13.1	2.9	3.6

[1] SLP-values are means across cultivars which showed at least one infected sprout.

Stem tissue resistance. In the spring of 1993 and 1994, experiments were carried out for measuring resistance of the above ground stem tissue of the 12 cultivars to *Eca* and *Ech*. The high concentration of bacteria used for inoculation resulted in an expanding blackleg lesion in almost all stems tested. Only in the experiment of 1993, two plants developed no lesions after inoculation with *Eca*. In 1994, plants of cv Amazone showed a deviating phenotype and were therefore not used for inoculation. Control plants that were treated with water did not develop blackleg lesions. Cultivars differed significantly for stem tissue resistance ($P<0.01$), which was expressed as the length of the blackleg lesion, seven days after inoculation (Table 4.3). Within the group of cvs Alcmaria, Arinda, Bintje, Karnico, Kondor and Producent however, no significant differences were found for *Eca* ($P<0.01$), while differences between some of these cultivars and cvs Désirée, Hertha and Venouska were not consistent among years of testing. When *Ech* was used for inoculation, cv Arinda was found to be significantly more resistant ($P<0.01$) than the group of cvs Agria, Alcmaria, Amazone Désirée, Kondor and Morene. The reaction of the other cultivars was inconsistent among years. For experiments with *Eca*, the linear correlation coefficients for comparison of results between years was high (0.88; $P<0.01$) but this was mainly due to the relatively susceptible reaction of cvs Morene and Agria. For *Ech*, a correlation coefficient of 0.73 was found ($P<0.05$).

Some correlation was found in 1993 for cultivar means between the experiments with *Eca* and *Ech* ($r=0.71$, $n=11$) but not in 1994 ($r=0.28$, $n=11$).

Tuber tissue resistance and field data. Tuber tissue resistance of the 12 cultivars was determined twice in November (early storage) and May (late storage) of the 1992/1993 season. In a previous study (Chapter 2), it was shown that tuber tissue resistance data for *Eca* were only reproducible across different storage periods if incubation of the tuber slices was carried out in air, i.e. under aerobic conditions, whereas for *Ech*, reproducible data were only found when incubation was carried out in nitrogen, i.e. under anaerobic conditions. Therefore, for the study in which the various reliably determinable putative components of resistance are investigated in relation to resistance to blackleg in the field, tuber tissue resistance data of the 12 cultivars concerning resistance to *Eca* are presented which were obtained after aerobic incubation (Table 4.4), while for *Ech* only data concerning resistance under anaerobic conditions are presented in Table 4.4. Correlation coefficients for comparison of tuber tissue resistance between November and May were 0.87 ($P<0.01$) and 0.67 ($P<0.05$) for *Eca* and *Ech* respectively.

Table 4.3. Mean length (√mm) of blackleg lesions in stems of potted potato plants in the glasshouse, 7 days after inoculation with *Eca* or *Ech* in two successive years. ND; not determined

	Inoculum			
	Eca		*Ech*	
Cultivar	1993	1994	1993	1994
Agria	5.76	6.01	7.35	7.66
Alcmaria	3.85	4.95	7.33	8.10
Amazone	4.68	ND	6.04	ND
Arinda	3.34	4.46	4.14	4.85
Bintje	4.06	4.81	5.49	5.57
Désirée	4.20	4.59	6.17	7.19
Hertha	4.06	5.21	6.04	6.49
Karnico	3.57	4.57	5.82	7.51
Kondor	3.92	4.36	6.17	7.64
Morene	5.43	5.63	7.77	7.30
Producent	3.64	4.81	4.25	6.75
Venouska	4.54	4.94	5.49	6.38
Mean	4.25	4.94	6.01	6.86
LSD ($P<0.01$)	0.77	0.82	1.88	1.66

In Table 4.4, relative R50 values for mother tuber decay as determined in the field test of 1993 are reproduced from Chapter 3.

Blackleg resistance of the 12 cultivars in the field (expressed as the mean percentage of plants which showed at least one stem with blackleg or blackleg associated symptoms, including non-emergence), averaged across testing years and locations, is also presented in Table 4.4.

Relation between blackleg resistance and components of resistance. Mother tuber decay in the field was the most important component in determining blackleg resistance of the 12 cultivars to *Eca* while for resistance to *Ech*, the most important component was stem base resistance (Table 4.5). Stepwise multiple regression analysis revealed that mother tuber decay and tuber tissue resistance together accounted for 56% of the variance in the field experiments with *Eca* inoculated seed. Stem base and stem tissue resistance did not add up substantially to the percentage already

Table 4.4. Tuber tissue resistance (mean diameter of rot of tuber slices in mm), relative R50 values for mother tuber decay in the field (see text) and blackleg resistance in the field (mean % plants with blackleg associated symptoms including non-emergence) of the 12 cultivars tested. Values for blackleg resistance are means across experiments at two locations in each of two years. Tuber tissue resistance values are means across two experiments. Relative R50 values are reproduced from Chapter 3. Low relative R50 values indicate that the process of mother tuber decay proceeded faster as result of the inoculation treatment than in the mother tubers of the appropriate control plants.

	Inoculum					
	Eca			*Ech*		
Cultivar	Tuber tissue resistance	Relative R50 value	Blackleg resistance	Tuber tissue resistance	Relative R50 value	Blackleg resistance
Agria	19.9	0.50	35.2	17.2	0.74	41.8
Alcmaria	18.9	0.60	57.0	16.6	0.76	33.5
Amazone	19.5	0.50	46.5	14.9	0.80	46.5
Arinda	17.0	0.73	8.6	14.7	1.04	19.6
Bintje	19.5	0.71	16.4	15.7	0.92	28.1
Désirée	13.3	0.60	9.4	14.7	0.81	22.3
Hertha	18.6	0.92	13.4	17.1	0.88	17.7
Karnico	13.4	0.91	8.6	15.2	0.95	0.4
Kondor	18.6	0.62	30.1	18.2	0.79	59.8
Morene	15.1	0.55	19.1	14.6	0.99	30.5
Producent	13.1	0.91	2.3	14.6	0.98	2.7
Venouska	16.5	0.63	47.7	16.3	0.84	41.8
Mean	17.0	0.68	24.5	15.8	0.87	28.7
LSD ($P<0.01$)	3.2	0.10	10.8	2.1	0.20	10.8

Table 4.5. Blackleg resistance in the field in relation to four components of resistance measured at a set of 12 cultivars. SIF; sprout infection frequency, SLP; sprout latency period (see also Table 4.2), ND; not determined.

Component of resistance	Percentage variance accounted for		
	Eca	*Eca*[1]	*Ech*
Tuber tissue resistance	12.5	2.8	1.5
Mother tuber decay	43.4 *	9.0	5.0
Stem base resistance (SIF)	5.1	5.2	1.0
Stem base resistance (SLP)	ND	52.7 *	63.8 **
Stem tissue resistance	2.4	1.7	3.3
Total	63.4	71.4	74.6

1) With stem base resistance data for *Eca* replaced by those from *Ech*.

* Significant at the $P<0.05$ level; ** Significant at the $P<0.01$ level

accounted for by mother tuber decay and tuber tissue resistance. In experiments with *Ech* inoculated seed, the components of resistance accounted for 75% of the variance but tuber tissue resistance, mother tuber decay and stem tissue resistance did not add to the variance already accounted for by stem base resistance (Table 4.5).

Because measurement of stem base resistance of the 12 cultivars to *Eca* was less accurate due to incomplete rotting of the tuber cylinders in comparison to experiments with *Ech*, multiple regression analysis was also carried out with stem base resistance data for *Eca* replaced by those from experiments with *Ech*. Then, 71% of variance in field experiments with *Eca* inoculated seed was accounted for by the components of resistance studied. The most important components were stem base resistance, that accounted for 58% of the variance and mother tuber decay, that accounted for 9% of the variance not already accounted for by stem base resistance.

Discussion

Erwinia bacteria are able to reach the stem tissue of potato plants from soft rot lesions in the mother tuber. However, the number of bacteria in the stem remains relatively low as long as no blackleg develops (Table 4.1). Bacteria are probably capable of moving through the vascular system from the rotting tuber tissue to the stem. Notwithstanding this ability, rot of stem tissue and hence, development of

blackleg, apparently originates from the rotting mother tuber tissue reaching the sites were the stem is attached to the tuber and not from the relatively few bacteria that migrated previously into the stem through the vascular system. This is in agreement with the observation that the mother tuber of diseased plants in the field is always fully rotting or has completely decayed (Pérombelon & Kelman, 1980).

The observation that usually only one or a few stems of a diseased plant show blackleg (Pérombelon, 1992) point in the direction of a resistance mechanism in the stem base that functions as a barrier against the mass of bacteria in the rotten mother tuber tissue, which is overcome in some, but not all the stems of a plant. The results presented here support the hypothesis that such a mechanism exists and that genotypic variation for this can be found in cultivated potato. This has also been concluded by Munzert (1975) and Munzert and Hunnius (1980). Weber (1990) ascribed differences between cultivars in this component of resistance to differences in the amount and type of lignification in the stem base.

The method which was used here for studying the genotypic variation for stem base resistance was more successful when inoculation was carried out with *Ech* in comparison to *Eca*. In many tuber cylinders, the rotting process by *Eca* did not result in fully rotten seed pieces. This could have been a result of a successful wound healing reaction which impairs the rotting process (Lyon, 1989), or due to the fact that the cortex tissue in the top of the cylinders is less susceptible than the medullary tissue (Chapter 2). Therefore, it can not be excluded that differences between cultivars in tuber tissue resistance could have hindered the accurate determination of stem base resistance to *Eca*.

Results of screening for stem tissue resistance in different years, i.e. with different sources of plant material, have also been presented by Lapwood and Read (1986a). For resistance to *Eca*, they also found significant differences between cultivars which, to some extent, were reproducible across years of testing. Hidalgo and Echandi (1982) found significant differences for resistance to *Ech* between clones of *S. tuberosum* subsp. *andigena*. In the experiments reported here, only a small percentage of the variance found in field tests for resistance to blackleg was accounted for by stem tissue resistance, which may be due to the relatively small amount of genotypic variation found for this component.

Of the components studied, mother tuber decay in the field is the most important in determining resistance of the 12 cultivars to blackleg caused by *Eca* (Table 4.5). Screening of cultivars for this component of resistance however, is even more expensive and time consuming than screening for blackleg resistance in the field. Furthermore, both methods are not applicable to clones from which only a limited

amount of seed is available. The method described here for measuring stem base resistance and, to a lesser extent, also the method for measuring stem tissue resistance, do not have these drawbacks. Regression analysis with stem base resistance data for *Eca* replaced by those from experiments with *Ech* indicated that these data can be used to determine resistance to *Eca* in the field more accurately than when using mother tuber decay. Stem base resistance was found to be also the most important component in determining resistance to *Ech* (Table 4.5). Thus, although these results need to be confirmed using a larger number of cultivars, it appears that stem base resistance to *Ech* is the most reliable component to select for when breeding for blackleg resistance, more so than selection for tuber tissue resistance, mother tuber decay in the field or above ground stem tissue resistance.

Chapter 5

Resistance to *Erwinia carotovora* subsp. *atroseptica* in second backcross populations of somatic hybrids between *Solanum tuberosum* and *S. brevidens*

Abstract

Eleven clones obtained from a cross between cv Katahdin and fusion products between *Solanum tuberosum* and the non tuber-bearing species *S. brevidens*, were backcrossed again with *S. tuberosum* (clone AR80-127-5). Small tubers harvested from 583 seedlings of these second backcross (BC2) populations were screened for tuber tissue resistance to *Erwinia carotovora* subsp. *atroseptica* under aerobic conditions. After multiplication in the field, BC2 clones again were screened for tuber tissue resistance under aerobic as well as anaerobic conditions. In general, the resistance of the BC2 populations was reduced in comparison with the BC1. Variation for resistance was found within the BC2 populations but there was no correlation between the results of the three tests performed. Four BC2 populations were also screened for resistance to blackleg in the field. Significant differences were found between populations for mean percentage of diseased plants, but these differences could not be explained by the resistance of the parental clones.

Introduction

Solanum brevidens Phil. is a non tuber-bearing wild relative of the cultivated potato. The species is known for some characteristics which might be useful in potato breeding, such as resistance to viruses (Jones, 1979; Pehu *et al.*, 1990; Valkonen *et al.*, 1992) and frost tolerance (Ross & Rowe, 1969). Several attempts have been made to integrate these desirable traits into a *S. tuberosum* background, either by direct crossing (Hermsen & Taylor, 1979), which was only successful when combined with embryo rescue techniques (Watanabe *et al.*, 1992), by the use of bridge species (Hermsen, 1983; Ehlenfeldt & Hanneman, 1984) or by somatic hybridization using either intact protoplasts (Barsby *et al.*, 1984; Austin *et al.*, 1986; Fish *et al.*, 1988a; Preiszner *et al.*, 1991) or irradiated protoplasts of the *S. brevidens* parent (Fehér *et al.*, 1992; Puite & Schaart, 1993). After somatic hybridization, hybrid plants could be identified which had a level of resistance to potato leaf roll virus and potato virus Y

comparable with that of the *S. brevidens* parent (Helgeson *et al.*, 1986, Gibson *et al.*, 1988) or which showed intermediate levels of frost tolerance (Preiszner *et al.*, 1991).

Surprisingly, somatic hybrids of diploid *S. brevidens* clone PI 218228 and tetraploid *S. tuberosum* clone PI 203900 showed high levels of resistance to soft rot caused by pectolytic *Erwinia* spp. *E. carotovora* subsp. *atroseptica* (van Hall) Dye (*Eca*), *E.c.* subsp. *carotovora* (*Ecc*) and *E. chrysanthemi* (*Ech*), both under aerobic and anaerobic conditions (Austin *et al.*, 1988). This resistance to soft rot appeared to segregate in populations obtained after backcrossing with the tetraploid cv Katahdin (Helgeson *et al.*, 1993).

McMillan *et al.* (1993) not only confirmed the high level of resistance to soft rot in first backcross generation plants but also found a considerable level of resistance of stem tissue to blackleg after inoculation with *Eca*. We therefore thought that the material might be valuable for breeding for resistance to both soft rot and blackleg. To this end, 583 clones of 11 second backcross populations were screened for resistance of tuber tissue to soft rot. Four populations were selected for screening for resistance to blackleg in the field. The value of the material as a source of resistance to *Eca* and the possibility of early selection are discussed.

Materials and methods

Plant material. True seed of the somatic hybrids of 2x *S. brevidens* PI 218228 and 4x *S. tuberosum* PI 203900 backcrossed once with 4x *S. tuberosum* cv Katahdin (Austin *et al.*, 1988), further referred to as first backcross population (BC1), was kindly supplied by Prof J.P. Helgeson, University of Wisconsin, USA. This seed yielded 83 clones in total, from which 20 were used as the female parent in crosses with 4x *S. tuberosum* clone AR80-127-5. The 20 BC1 parental clones were screened for resistance to a mixture of *Eca*, *Ecc* and *Ech* in a tuber slice assay (Lapwood & Read, 1985). In 1991, seeds of 11 BC2 populations were sown. Seedlings were transplanted to 1 ℓ pots containing potting compost and grown in the glasshouse until maturity. Tubers harvested from seedlings (and plants grown from seed tubers or cuttings in 1 or 2 ℓ pots in the glasshouse) were approximately 15-25 mm in diameter and relatively small in comparison with tubers from field-grown plants. They are referred to as "small tubers". Small tubers were stored at 4 °C until resistance screening or field planting.

In the Netherlands, until spring 1993, plant materials derived from somatic hybrids of *S. tuberosum* and *S. brevidens* were classified as Genetically Modified

Organisms, so seed potato production in the field in 1992 had to take place under restrictions similar to those described by Fish *et al.* (1988b), the most rigorous being the picking of flower buds to prevent pollen dispersal and seed set. From BC1 clones, two to ten plants were grown and from BC2 clones, one plant was grown at the same location on a sandy soil. The male parental clone AR80-127-5 and cvs Katahdin, Agria, Alcmaria, Karnico and Producent, which served as standards in resistance tests, were also grown at this location. Harvested tubers were stored at 4 °C until tested.

Screening for resistance to soft rot. The small tubers, from glasshouse-grown BC2 seedlings and tuber slices of field-grown potatoes were screened for soft rot resistance to *Eca* strain IPO 161, which was obtained from the DLO-Research Institute for Plant Protection (IPO-DLO), Wageningen, the Netherlands. Inoculum was prepared by growing the bacterium for 48 h at 27 °C on Bouillon Agar medium containing 8.0 g ℓ^{-1} 'Lab-Lemco' Broth (Oxoid), 5.0 g ℓ^{-1} NaCl and 15.0 g ℓ^{-1} agar. Bacterial cells were suspended in sterile water, centrifuged for 5 min at 4500 rpm and re-suspended in sterile water. The concentration was determined using a standard curve relating colony-forming units (cfu) to optical density at 500 nm.

For resistance tests, small tubers (cut tangentially on opposite sides to prevent them from rolling during handling and to create a surface for inoculation), or slices cut from larger field-grown tubers, were placed at randomized positions in trays with a perforated bottom measuring 60 x 40 x 6 cm and incubated overnight at 15 °C. Small tubers and slices were inoculated by pressing a well 4.0 mm deep and 5.0 mm diameter into the medullary tissue, into which was pipetted 20 µl of inoculum containing 1.0×10^9 cfu ml^{-1}. Trays were stacked in two airtight containers 65 x 45 x 65 cm deep which were placed in a growth chamber at 20 °C. In the experiment with small tubers, containers were flushed with 500 ml min^{-1} air to create aerobic conditions. In the experiments with slices, one container was flushed with 500 ml min^{-1} air and the other with 500 ml min^{-1} nitrogen to create anaerobic conditions. After incubation, rotted tissue was removed and the diameter of the cavity measured in two directions at right angles. Analysis of mean lesion diameters was carried out by ANOVA using the GENSTAT programme (Payne *et al.*, 1987).

One small tuber per BC2 clone was inoculated and subsequently incubated aerobically for five days. Ten small tubers of the BC1 parental clones, the male parental clone AR80-127-5 and cvs Katahdin, Agria and Producent, (all grown from rooted cuttings in 2 ℓ pots in the glasshouse) were included, except for clone BC1-65 of which only four tubers were used, and clone BC1-72, which was lost during multiplication in 1991. For the tuber slice assay, two 10 mm thick slices were cut

from one tuber per BC2 clone and, after inoculation, incubated either aerobically or anaerobically for five and three days respectively. The screening of tuber slices was carried out in two experiments in February 1993. In the first experiment, populations BC2 nos. 5 - 68 (Table 5.1) were inoculated; populations BC2 nos. 72 - 83 were inoculated one week later. In both experiments, ten slices of the appropriate BC1 parental clones, the male parental clone AR80-127-5 and cvs Katahdin, Agria and Producent were included (except for clone BC1-65 of which only five slices were screened). All the available BC1 clones, except the 11 clones that had been used as parents for BC2 production, were screened in the second experiment. As for the BC2 populations, only one tuber of these remaining BC1 clones was used for inoculation. Tubers for the slice assay were all from the 1992 field multiplication.

Screening for blackleg resistance. Clones of BC2 nos. 5, 23, 65 and 75, of which a least 12 tubers remained from the multiplication in 1992, were screened for resistance to blackleg in the field. Tubers were vacuum-infiltrated with *Eca* strain IPO 161 and planted in three replicates at a location with a very light sandy soil. Replicates consisted of 287 four-plant randomized plots, one for each BC2 clone and parental clone BC1-65, and four for the other parental clones BC1-5, 23 and 75, the male parental clone AR80-127-5 and cvs Katahdin, Agria, Alcmaria, Karnico and Producent.

Vacuum infiltration of tubers was carried out as follows. Tubers from the 1992 field multiplication were submerged in a suspension of 6.5×10^7 cfu ml^{-1} *Eca*, kept for 10 min at 5 kPa and 10 min at atmospheric pressure, dried overnight at room temperature and planted the next day (May 7th 1993). Tubers of the five commercial cultivars, infiltrated with water and planted at a separate site in the same field, served as controls. Non-emergence was scored once, and plants showing blackleg lesions were scored three times during the experiment. The percentage of plants per clone with blackleg symptoms, including non-emergence, was calculated and analyzed by ANOVA using the GENSTAT programme (Payne *et al.*, 1987). About 16 weeks after planting, the plants of all the cultivars, the parental clones and clones of BC2-75 were lifted and the amount of rot of the mother tuber was estimated on a scale from 0 - 4 (0, no rot; 1, approximately one quarter (volume) of the mother tuber rotten; 2, half rotten; 3, three quarters rotten; 4, completely rotten). Only one replicate of BC2 clones nos. 5, 23 and 65, was examined.

Results

Resistance to soft rot. In a preliminary experiment, tuber slices of BC1 clones were screened for resistance to a mixture of *Eca*, *Ecc* and *Ech*. The 11 BC2 populations used for further investigation were chosen because the parental BC1 clones covered the whole range of resistance found in that experiment (data not shown). However, in two further tests, these differences could not be repeated. Glasshouse-grown small tubers of the 11 parental BC1 clones, inoculated with *Eca* alone, did not show significant differences ($P<0.05$) in the mean diameter of rot after aerobic incubation (Table 5.1). The amount of rot of the parental BC1 clones, and also of parental clone AR80-127-5, was comparable with that of the relatively resistant cv Producent. When slices were cut from field-grown potatoes and incubated aerobically, the parental BC1 clones showed small differences that were within the range found for the cultivars. Except for clones BC1-23, 65 and 68, the parental BC1 clones showed significantly smaller ($P<0.05$) rots than cv Katahdin in the appropriate experiments. Katahdin was used as the parent for BC1 production (Austin *et al.*, 1988). Only the parental clones BC1-51 and 75 rotted significantly less ($P<0.05$) than AR80-127-5, the parental clone for BC2 production. Under anaerobic conditions, parental BC1 clones showed significantly smaller rots than the cultivars and clone AR80-127-5, except for clone BC1-51 which did not differ significantly ($P<0.05$) from the relatively resistant cv Karnico in the first experiment with tuber slices. This was also the only clone which, after pair-wise comparisons, showed a significant difference ($P<0.05$) with one (BC1-5) of the other parental BC1 clones tested in the first experiment.

Significant differences ($P<0.05$) were found for the BC2 population means in the experiment with small tubers (Table 5.1) which, as far as they were tested, were higher than that of the corresponding parental BC1 clones. The BC2 means were not significantly different ($P<0.05$) from the AR80-127-5 clone. It should be noted, however, that although grown in the same period of the year, small tubers of BC2 clones were harvested from seedlings whereas small tubers of BC1 clones, AR80-127-5 and cultivars were harvested from rooted cuttings. These may be considered as two physiologically different sources. Nevertheless, in the experiment with aerobically incubated slices from field-grown tubers, the BC2 population means were again higher than those of the corresponding parental BC1 clones, except for BC2-65. The mean rot of the BC1 population, consisting of clones which were not used as parents for BC2 production, was significantly lower ($P<0.05$) when compared with the means of the BC2 populations that were screened in the same experiment. Under anaerobic conditions, BC2 population means differed significantly ($P<0.05$)

Table 5.1. Mean diameter of rot (mm) of the first backcross (BC1) population and 11 BC2 populations, their parents and five cultivars after inoculation of small tubers and tuber slices with 1 x 10^8 cfu ml^{-1} *Erwinia carotovora* subsp. *atroseptica*. Small tubers were incubated in air for 5 days. Slices were either inoculated in air or nitrogen for 5 and 3 days respectively. ND, not determined.

Genotype	No. of clones tested	Diameter of rot (mm)				
		Small tubers, aerobic incubation	Slices, aerobic incubation		Slices, anaerobic incubation	
			Expt 1	Expt 2	Expt 1	Expt 2
Parental clones						
BC1-5 (♀)		8.4	8.3		10.3	
BC1-18 (♀)		7.5	8.0		10.8	
BC1-23 (♀)		7.9	11.7		11.7	
BC1-51 (♀)		7.8	7.3		12.3	
BC1-65 (♀)		8.5	12.8		11.5	
BC1-68 (♀)		8.6	9.7		11.1	
BC1-74 (♀)		9.1		9.8		11.9
BC1-75 (♀)		8.7		7.8		12.6
BC1-82 (♀)		8.8		8.5		11.8
BC1-83 (♀)		7.9		9.3		12.2
AR80-127-5 (♂)		9.1	9.9	10.5	15.4	18.0
Populations						
BC1	43	ND		8.6		12.4
BC2 nr 5	61	10.8	10.2		12.8	
BC2 nr 18	31	10.8	10.2		13.5	
BC2 nr 23	65	11.7	12.1		13.3	
BC2 nr 51	32	10.5	10.2		14.0	
BC2 nr 65	69	10.9	11.0		15.0	
BC2 nr 68	85	11.7	11.1		13.7	
BC2 nr 72	31	14.2		11.9		14.1
BC2 nr 74	71	11.5		11.8		15.4
BC2 nr 75	53	12.1		10.3		15.9
BC2 nr 82	58	11.9		10.9		14.2
BC2 nr 83	27	12.6		10.6		14.6
Cultivars						
Agria		15.9	12.1	11.7	15.6	15.3
Alcmaria		ND	11.5	12.4	17.0	19.8
Karnico		ND	8.2	8.5	14.3	15.1
Katahdin		ND	11.8	12.6	16.5	15.4
Producent		8.1	9.8	8.5	16.0	14.6
s.e.		2.9	2.9	2.9	2.2	2.2
df		677	440	367	440	367

among each other and were all significantly higher (P<0.05) than the means of the corresponding parental BC1 clones. None of the BC2 populations showed significantly smaller (P<0.05) rots than cv Karnico, but all the populations except BC2 nos. 51 and 65 showed significantly smaller (P<0.05) rots than AR80-127-5 in the appropriate experiments.

Figure 5.1 illustrates the variation found in the BC2 populations for resistance of small tubers and tuber slices. Small tubers of the parental BC1 clones 5 and 65 did not differ in soft rot resistance, and no significant difference (P<0.05) was found between means for soft rot resistance of the corresponding BC2 populations. However, the amount of rot of individual BC2 clones varied widely around the parental means (Figure 5.1A). When slices of tubers from these two populations and their parents were screened aerobically, parental clone BC1-65 was less resistant than BC1-5. This difference is also reflected in the corresponding BC2 offspring (Figure 5.1B), but again the population means did not differ significantly (P<0.05). In the experiment with anaerobically incubated slices, parental BC1 clones were clearly more resistant than the AR80-127-5 parent. Clone BC1-5 was slightly (but not significantly) more resistant than BC1-65 and the mean of the corresponding population was significantly (P<0.05) lower (Figure 5.1C).

Correlation coefficients were calculated for comparing the mean diameter of rot between small tubers and aerobically incubated slices for each of the BC2 populations. A significant but low correlation (r=0.47, P<0.01) was found only for BC2-72. Correlation coefficients for comparisons between the amount of rot in aerobically and anaerobically incubated slices were only significant for BC2-5 (r=0.33, P<0.01) and 23 (r=0.31, P<0.05).

Resistance to blackleg. The mean percentage non-emergence in cultivars and BC2 clones varied from 0-10.5% (Table 5.2). Parental clones BC1-23, 65 and 75 showed a relatively high percentage of non-emergence. Due to the limited amount of tuber material available, no water-infiltrated control tubers of BC1 parents and BC2 clones could be planted and, as a consequence, it may be that non-emergence was caused by other factors than *Eca*. However, seven weeks after planting, when non-emergence was scored, 85% of the tubers of non- emerged plants were partially or completely rotten. Only cv Karnico (33%) and parent BC1-23 (26%) showed a relatively high percentage of non-emerged plants from mother tubers that had not rotted. Non-emergence was therefore considered to be mainly a result of the infiltration with *Eca*.

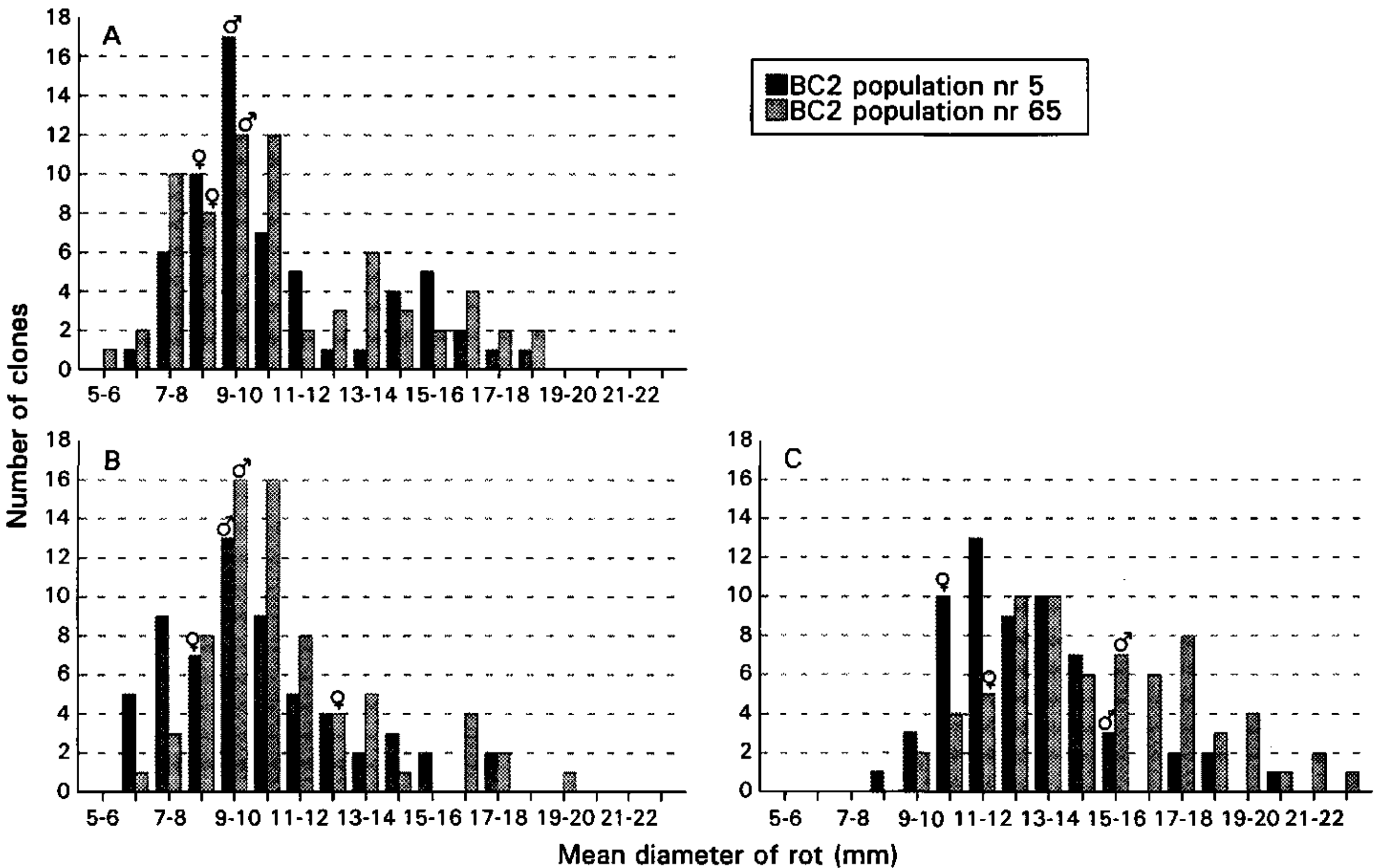

Figure 5.1. Mean diameter of rot for second backcross (BC2) populations nos. 5 and 65 after screening small tubers (A) or tuber slices under aerobic (B) or anaerobic (C) conditions for resistance to 1 x 10^9 cfu ml^{-1} *Eca*. Classes in which parental clones were scored are indicated (♂, ♀).

Table 5.2. Resistance of four second backcross (BC2) populations, their parents and five cultivars to *Eca* in the field. The mean percentage of non-emergence (NE) and blackleg (including NE) per clone, the number of emerged plants examined 16 weeks after planting and the mean percentage of plants per clone with mother tubers (mt) showing less than 50% rot is given per genotype.

	No. of clones tested	Mean % NE per clone	Mean % blackleg per clone	No. of plants examined	Mean % of mt with < 50% rot
Parental clones					
BC1-5 (♀)		0.0	16.7	48	0.0
BC1-23 (♀)		39.6	39.6	29	47.2
BC1-65 (♀)		50.0	50.0	6	66.7
BC1-75 (♀)		45.8	45.8	26	29.2
AR80-127-5 (♂)		0.0	16.7	48	2.1
Populations					
BC2 nr 5	55	10.5	29.1	200	0.6
BC2 nr 23	65	9.1	22.1	237	2.3
BC2 nr 65	67	8.0	26.7	243	2.0
BC2 nr 75	63	4.8	20.5	719	4.8
Cultivars					
Agria		6.3	37.5	45	0.0
Alcmaria		0.0	54.2	48	0.0
Karnico		6.3	25.0	45	0.0
Katahdin		0.0	10.4	48	2.1
Producent		2.1	29.2	47	0.0
s.e. (df=845			26.2		

Water-infiltrated control tubers of the cultivars and AR80-127-5 showed no blackleg. The mean percentage of blackleg, including non-emergence of the parental BC1 clones, varied from 16.7-50% (Table 5.2). However, symptoms on BC1-23, 65 and 75 consisted solely of non-emergence, whereas BC1-5 showed only blackleg. There is no relationship between these results and the soft rot resistance of the BC1 clones presented in Table 5.1. Differences in the mean percentage of blackleg of BC1-5, AR80-127-5 and the cultivars were not significant ($P<0.05$), except for cv Alcmaria, which showed significantly more blackleg. Mean percentages of blackleg per BC2 clone were significantly higher ($P<0.05$) when BC2-5 was compared with BC2 nos. 23 and 75. Clones of BC2-65 also showed significantly more ($P<0.05$) blackleg than BC2-75. The mean percentage of blackleg (including non-emergence) of

individual BC2 clones varied widely within BC2 populations (Figure 5.2). Correlation coefficients for comparisons between the percentage of blackleg of individual BC2 clones (including or excluding non-emergence) and their soft rot resistance were not significant.

A remarkably high percentage of mother tubers of emerged plants of parental clones BC1 23, 65 and 75 showed <50% decay at the end of the growing season (Table 5.2). No or only a small percentage of mother tubers of BC1-5, AR80-127-5 and the cultivars showed <50% decay, whereas these percentages for non-inoculated mother tubers of cvs Agria, Alcmaria, Karnico, Katahdin and Producent were 36, 0, 15, 13 and 31 respectively, indicating that *Eca* had enhanced the decay of the mother tuber. The mean percentage of emerged plants per BC2 clone with mother tubers showing <50% decay was comparable with that of the cultivars, or slightly higher (Table 5.2). For individual clones, maximum percentages were 33, 50, 50 and 100% for BC2 nos. 5, 23, 65 and 75 respectively.

Discussion

Resistance to soft rot. Helgeson *et al.* (1993) tested nine BC1 clones for tuber tissue resistance to *Erwinia carotovora* and classified three of them as resistant. By using a small tuber assay, we were not able to make such a classification for another ten clones of the same BC1 population (Table 5.1). However, when slices were screened under aerobic conditions, seven of the BC1 clones were more resistant than Katahdin, which had been used as a parent for BC1 production. The level of resistance of these clones was comparable with that of the relatively resistant starch cvs Karnico and Producent. Under anaerobic conditions, no segregation among BC1 clones was again apparent, but resistance was clearly higher than that of the cultivars and the AR80-127-5 parent. Austin *et al.* (1988) also found higher levels of resistance of BC1 clones under anaerobic conditions when compared with cultivars.

The individual clones of the BC2 populations which we used were multiplied without any selection for agricultural traits. Nevertheless, populations were slightly biased since only clones which produced enough tubers for resistance screening and further propagation were screened. The results of the resistance tests indicated that the mean level of resistance of the BC2 populations was decreased in comparison with BC1.

On the basis of these results it is not possible to draw conclusions about the genetic basis of the resistance found in BC1 populations. The parental BC1 clones are

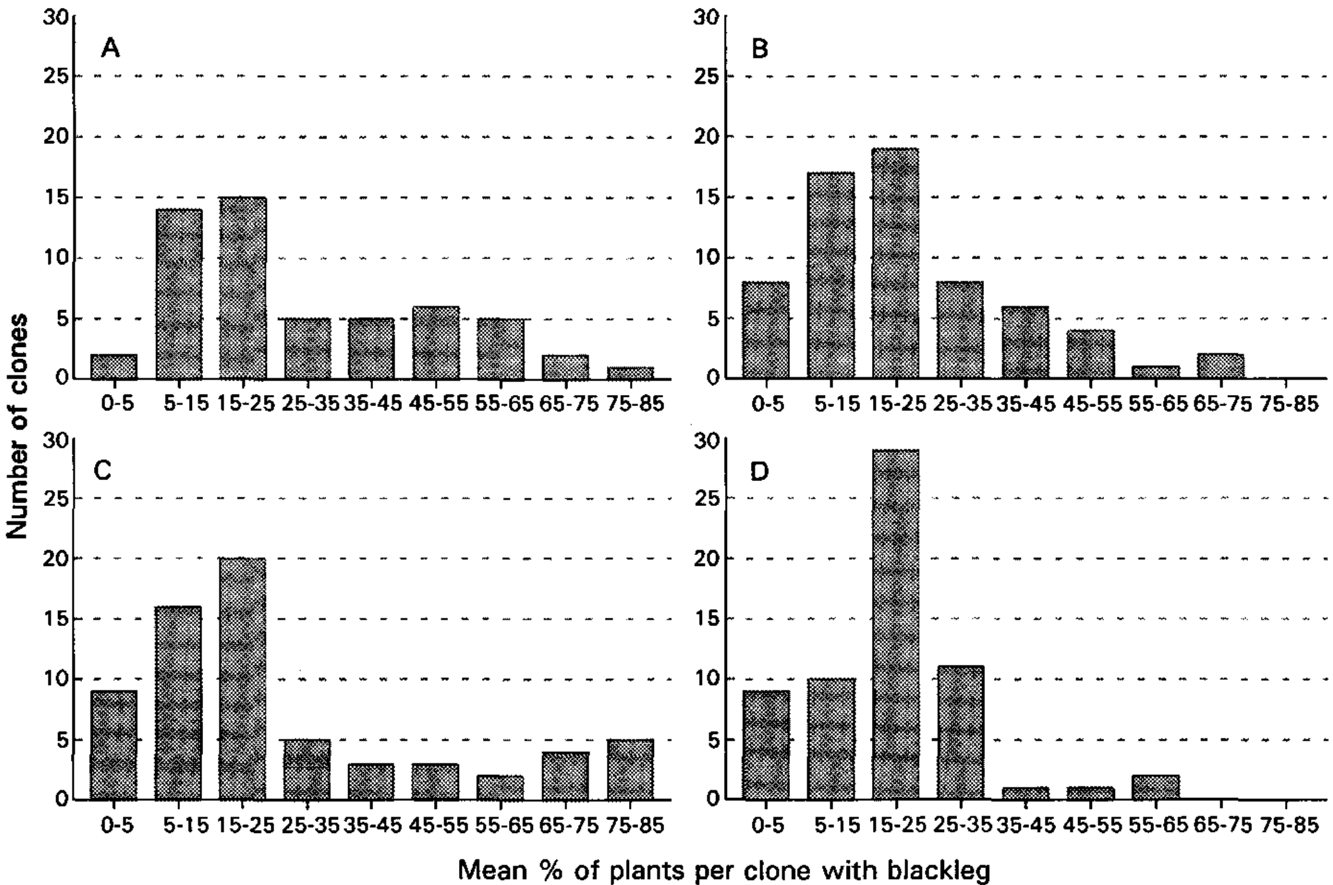

Figure 5.2. Mean percentage of plants per clone of second backcross (BC2) populations nos. 5 (A), 23 (B), 65 (C) and 75 (D) after inoculation of mother tubers with *Eca*. Percentage includes non-emergence.

most likely pentaploids containing an almost complete set of the very homozygous *S. brevidens* donor clone (Williams *et al.*, 1990). After producing a BC2 with a 4x *S. tuberosum* clone, evidence for complex distribution and substantial disruption of chromosomes during meiosis, as well as introgression of the *S. brevidens* genome, has been obtained (McGrath *et al.*, 1994). The expected aneuploid nature of the BC2 populations and the unpredictable segregation ratio of traits make it impossible to determine whether the high resistance of some of the BC2 clones can be ascribed to the effect of one or more genes (if any) from *S. brevidens*.

Except for BC2-72, no correlation was found between the extent of soft rot of small tubers and aerobically incubated slices, but this might be related to the fact that only one small tuber and one slice were screened per BC2 clone. When the same methods were applied to a set of 12 cultivars in ten replicates, a mean correlation coefficient of 0.71 was found (Chapter 2). In order to be able to make a comparison with the BC2 data presented here, subsets were abstracted from the available data of the experiment with 12 cultivars and correlations calculated on a one replicate basis. A mean correlation coefficient of 0.46 was thereby obtained (data not shown).

The absence of a correlation between the results of aerobic and anaerobic incubation has also been reported by Bain and Pérombelon (1988), who screened a set of cultivars under both conditions in a whole tuber assay. A high correlation would have supported the work of McMillan *et al.* (1993), who ascribed the resistance of a particular BC1 clone to the high percentage of esterification of pectin in its cell wall extracts, a mechanism of resistance which is considered to be independent of oxygen. The lack of correlation indicates that other resistance factors, not necessarily derived from *S. brevidens*, might be involved in the BC2 populations.

Resistance to blackleg. A high incidence of non-emergence was found for three of the four parental BC1 clones tested in the field, but surprisingly, the plants that emerged showed no blackleg (Table 5.2). Since rotting of the mother tuber is an essential step for the development of blackleg (Pérombelon & Kelman, 1980), the observation that a relatively high percentage of the mother tubers of these plants was <50% rotted late in the growing season, indicates that non-decay of the mother tuber is an important mechanism of blackleg resistance in this material. Austin *et al.* (1988) also observed that decay of planting material of *S. brevidens* hybrids at harvest was less when compared with that of the parental clone and cultivars. However, this trait appears to be only poorly transmitted to the BC2 populations (Table 5.2).

The significant differences between BC2 populations in the mean percentage of diseased plants cannot be explained by the resistance of the parental BC1 clones, cv Katahdin or AR80-127-5 which, apart from the high incidence of non-emergence of BC1-23, 65 and 75, were all relatively resistant compared with the other cultivars. Testing populations derived from crosses between the three *S. tuberosum* parents is probably necessary to get an indication of the role of *S. brevidens* as a donor of resistance genes in the best BC2 clones (Figure 5.2).

The lack of correlation between resistance to tuber soft rot and resistance to blackleg in the field, as well as the absence of a correlation between soft rot resistance of small tuber and slices, demonstrate the difficulties that potato breeders are likely to encounter when breeding for blackleg resistance.

Chapter 6

Erwinia soft rot resistance of potato cultivars expressing antimicrobial peptide tachyplesin I

Abstract

Tachyplesin I is a 2.3 kDa antimicrobial peptide isolated form Southeast Asian horseshoe crabs. Bacterial suspensions containing 1.0 x 10^6 colony-forming units ml^{-1} of six isolates of pectolytic *Erwinia* spp., the causal pathogens of potato soft rot and blackleg, were killed *in vitro* by 1.4 to 11.1 µg ml^{-1} of tachyplesin I. In an attempt to enhance resistance to *Erwinia* spp., each of the cvs Bintje, Karnico and Kondor were transformed with four gene constructs encoding different precursor tachyplesin I proteins under the control of a cauliflower mosaic virus 35S promotor. Western blot analysis of 25 to 30 independent transgenic plants per construct per cultivar showed that tachyplesin I was found in plants transformed with gene constructs which contained, in addition to the sequence for mature tachyplesin I, an amino terminal sequence of the signal peptide from barley α-hordothionin. No tachyplesin I specific signals were found in plants transformed with gene constructs in which the hordothionin signal peptide was replaced by the signal peptide from tobacco pathogenesis related protein S. Lack of expression in these plants is probably due to the relatively low or non detectable level of transgene transcription or mRNA instability. Small tubers of 17 transgenic clones were screened twice for soft rot resistance to *Erwinia carotovora* subsp. *atroseptica*. Under aerobic conditions, transgenic clones showed less rot in comparison to control tubers. Under anaerobic conditions transgenic clones were only slightly better than the controls.

Introduction

Blackleg of potato plants in the field and soft rot of tubers in storage are important diseases of potato that are caused by the phytopathogenic bacteria *Erwinia carotovora* subsp *carotovora* (*Ecc*), *E.c.* subsp. *atroseptica*, (*Eca*) and *E. chrysanthemi* (*Ech*). Pathogenicity of these Gram-negative bacteria is mainly based on secretion of pectolytic enzymes which enable them to macerate various plant tissues (Pérombelon

& Kelman, 1980). Blackleg and soft rot are seed tuber borne diseases. Tubers may become latently infected in the field or during harvest as well as during post harvest mechanical handling. A progressive rot can develop under certain environmental conditions such as hypoxia which impairs the expression of host defence mechanisms (Pérombelon & Kelman, 1980; Davis *et al.*, 1990). Since the bacteria of latently infected tubers are present in lenticels and deep wounds, where they are well protected against disinfecting liquids, chemical control of the diseases is not possible (Pérombelon, 1988). Disease control mainly relies on both cultural techniques aiming to reduce the risk of contamination and on certification schemes where, for instance, infected seed lots are declassified (Hidalgo, 1988; Pérombelon, 1992).

Breeding for resistance may contribute to a solution (Pérombelon, 1992). Although complete resistance for pectolytic *Erwinia* spp. has not been found amongst potato germplasm, numerous primitive cultivars and wild relatives have been identified that showed higher levels of partial resistance than commonly found in commercial cultivars (Huaman *et al.*, 1988). However, the importance of these sources of resistance in classical breeding programmes has been limited due to the lack of efficient screening methods, the variation in disease assessments due to environmental influences and the tetraploid nature of the cultivated potato which makes breeding for partial resistance very time consuming (Wastie & Mackay, 1985).

The development of efficient *Agrobacterium* mediated transformation protocols for plants (Fraley, 1989) has created novel ways to obtain disease resistance. One such a novel way is the introduction of genes encoding peptides that exhibit antimicrobial properties. Several peptide families that are toxic to bacteria have been identified in different organisms and bacteriophages (Tsugita, 1971; García-Olmedo *et al.*, 1989; Boman, 1991). A limited number of attempts have been undertaken to enhance bacterial disease resistance by transformation with antibacterial peptide encoding gene constructs. It has been claimed that expression of cecropins, small lytic peptides from the giant silk moth (Hofsten *et al.*, 1985), in tobacco and potato resulted in delayed symptom development after inoculation with *Pseudomonas solanacearum*, the causal pathogen of bacterial wilt (Jaynes *et al.*, 1993; Jia *et al.*, 1993). However, Florack *et al.* (1995) did not found enhanced resistance after inoculation with pathogenic *Pseudomonas* spp. of tobacco plants transformed with gene constructs encoding cecropin B, probably as a result of rapid degradation of this peptide in tobacco tissue. Tobacco plants expressing antibacterial thionin from barley showed enhanced resistance to *Pseudomonas syringae* pathovars (Carmona *et al.*, 1993). Transgenic potato plants that expressed bacteriophage T4 lysozyme, a peptide which degrades the cell wall of bacteria, showed less maceration of tuber tissue by *Eca*

compared with controls and showed a higher percentage of emergence of inoculated seed pieces planted in soil (Düring *et al.*, 1993). These results indicate that molecular breeding for resistance may form an alternative for classical breeding.

Tachyplesin I (TPNI) is a member of an antimicrobial peptide family isolated from Southeast Asian horseshoe crabs (*Tachypleus tridentatus*) and related species (Nakamura *et al.*, 1988; Miyata *et al.*, 1989). It is a strongly basic 2.3 kDa peptide containing two intramolecular disulphide bridges and an amidated carboxyl terminal Arg residue (Nakamura *et al.*, 1988). It shows strong antimicrobial activity against both Gram-negative and Gram-positive bacteria and some fungi and antiviral activity against certain enveloped viruses. TPNI is therefore believed to play an important role in horseshoe crab defence mechanisms against invading microorganisms (Nakamura *et al.*, 1988; Miyata *et al.*, 1989; Tamamura *et al.*, 1993). In horseshoe crab hemocytes, TPNI is processed from a prepropeptide consisting of an amino terminal signal peptide (SP) of 23 amino acid residues, the mature antimicrobial peptide of 17 amino acid residues, followed by an amidation signal Gly-Lys-Arg and an additional 34 residual acidic carboxyl terminal polypeptide (AP). The function of the latter is not known but it is thought to play a role in the stabilization of the prepropeptide or in the transport of the peptide to the smaller granules of the hemocytes (Shigenaga *et al.*, 1990).

This chapter reports about the antimicrobial activity of TPNI against several pectolytic *Erwinia* spp., the transformation of three potato cultivars with four gene constructs coding for TPNI and the evaluation of resistance to soft rot by *Eca*. The gene constructs used differed for the origin of the SP and the presence of the AP.

Materials and methods

In vitro *toxicity assays.* Purified TPNI was kindly supplied by Prof S. Iwanaga, Kyushu University, Fukuoka, Japan. Bacterial isolates IPO 161 (*Eca*), 163 (*Ecc*) and 502 (*Ech*) were obtained from the DLO-Research Institute for Plant Protection (IPO-DLO), Wageningen, the Netherlands. Isolates PD 482 (*Ech*), 755 (*Eca*) and 1005 (*Ecc*) were obtained from the Plant Protection Service (PD), Wageningen. *Escherichia coli* K12, collection number PC 1101, was obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands.

Crude extracts of potato were prepared by grounding various tissues from cv Agria under liquid nitrogen and centrifugation for 10 min at 4 °C. Supernatant was mixed with an equal volume of 0.1 M Tris-HCl pH 7.5 and filter sterilized. The protein

concentration was determined using a colorimetric assay (Bio-Rad, USA) according to Bradford (1976).

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of TPNI was essentially performed as described by Nakamura *et al.* (1988). In separate wells of a 96 wells flat bottom tissue culture microplate (Greiner, FRG) 50 µl of a twofold dilution series of TPNI in 0.1 M Tris-HCl pH 7.5 was mixed with 100 µl 2x nutrient broth (NB) and 50 µl of bacterial suspension turbidimetrically adjusted to 4.0 x 10^6 colony-forming units (cfu) ml^{-1}. Mock inoculations were performed with 50 µl of sterile water. Wells in which TPNI was replaced by sterile water served as controls. After incubation at 37 °C for 24 h (*E. coli*) or at 27 °C for 42-48 h (*Erwinia* spp.), absorbance was read at 620 nm on a microplate reader and 100 µl aliquots of 4-7 wells of each strain were used for decimal dilution series plating on Trypticase Soy Agar (TSA). After incubation at 27 °C or 37 °C for 24-72 h, plates were scored for presence of growing colonies.

Activity of TPNI in the presence of crude plant extracts was determined by incubating 50 µl TPNI, 60 µg ml^{-1} in 0.1 M Tris-HCl pH 7.5, with 25 µl crude extract of leaf tissue (0.28 µg protein $µl^{-1}$), tuber tissue (0.38 µg $µl^{-1}$) or stem tissue (0.23 µg $µl^{-1}$) in 0.1 M Tris-HCl pH 7.5 for 1 h at room temperature in separate wells of a 96 wells tissue culture microplate. After incubation, 100 µl 2x NB and 25 µl of 8.0 x 10^6 cfu ml^{-1} *E. coli* K12 were added. Mock inoculations were performed with 25 µl of sterile water. Wells in which TPNI and crude extracts were replaced by 50 and 25 µl 0.1 M Tris-HCl pH 7.5 respectively, served as controls. Plates were incubated at 37 °C for 24 h and 100 µl aliquots used for scoring of growth after dilution plating on TSA.

TPNI encoding gene constructs and transformation of potato. The cDNA clone λTPNI-C1 (Shigenaga *et al.*, 1990) was kindly supplied by Prof S. Iwanaga from the Kyushu University, Fukuoka, Japan. Bluescribe pSK+ (Stratagene, USA) was used for construction of pT-1 to pT-4 (Figure 6.1). Sequences were verified by automated sequencing (Applied Biosystems 370A, USA). *Hpa I*/*Bam* HI fragments as indicated in Figure 6.1 were cloned in the binary vector pCPO5 between a double cauliflower mosaic virus 35 S promotor and a nopaline synthetase terminator (Florack *et al.*, 1994) and conjugated to *Agrobacterium tumefaciens* GV3101 (pMP90RK) by parental mating. After conjugation, the recombinant binary vectors were isolated, transformed to *E. coli* and their composition verified by restriction analysis.

Potato cultivars were transformed essentially according to the procedures described by Stiekema *et al.* (1988). Discs, 2-3 mm thick and 1.0 cm diameter, cut

from field-grown seed potatoes were soaked for 15 min in an overnight culture of *A. tumefaciens* and placed at 24 °C and 16 h light regime on a shoot induction medium containing MS salts plus vitamins (Duchefa, NL), 3% sucrose, 0.8% agar, 0.9 mg ℓ^{-1} indole acetic acid (IAA) and 3.6 mg ℓ^{-1} zeatin riboside (ZR) (Hoekema *et al.*, 1989). After three days, discs were transferred to the same medium containing in addition 250 mg ℓ^{-1} cefotaxim, 200 mg ℓ^{-1} vancomycin and 75 mg ℓ^{-1} kanamycin. Every four weeks, discs were transferred to fresh medium. Shoots were cut from the slices and rooted on the same medium without IAA and ZR. Kanamycin resistant plants were maintained and multiplied *in vitro*. *In vitro* plants were transferred to the glasshouse and grown in 3 ℓ pots until maturity. Harvested tubers were stored at 4 °C. Untransformed *in vitro* plants of the three cultivars served as controls.

TPNI expression analysis. Northern blot analysis was carried out with total RNA fractions of young leaves from glasshouse-grown plants as described (Florack *et al.*, 1994). A ^{32}P-labelled *Sty I/Bam* HI fragment of pT-1, containing ten codons of the carboxyl terminal part of the hordothionin SP and the coding sequence of mature TPNI (Figure 6.1), was used as probe.

Anti TPNI antibodies were raised in a rabbit. A conjugate of Keyhole Limpet Hemocyanin (KLH) and TPNI was used for immunization in order to enhance the immuno-response. Approximately 1.0 mg of purified TPNI was coupled to KLH (Calbiochem, USA) in a molar ratio of 100:1 by 1-ethyl-3-(dimethylaminopropyl) carbodiimide coupling (Deen *et al.*, 1990) and dialysed against 0.01 M phosphate buffered saline. Quarter portions of the conjugate, each containing approximately 4.5 mg of protein, were used for immunisation. The IgG fraction of the polyclonal serum was purified by FPLC on a protein G Superose HR 10/2 column (Pharmacia, USA).

Total potato tissue protein extraction procedures were as described by Florack *et al.* (1994). In short, approximately 50 mg plant tissue was grounded under liquid nitrogen in a 1.5 ml microtube and extracted in 1.0 ml 50 mM H_2SO_4 at 40 °C for 1 h. The suspension was centrifuged twice and the proteins in 730 µl of the supernatant precipitated overnight at 4 °C with 24% (v v^{-1}) trichloroacetic acid. After centrifugation, the pellet was washed once with absolute ethanol, lyophilized and dissolved in 200 µl of water. The concentration of soluble proteins was determined using a colorimetric assay (Bio-Rad) according to Bradford (1976).

Intercellular Fluids (IF's) were isolated by vacuum infiltration of young leaves in water and subsequent centrifugation (De Wit & Spikman, 1982).

Expression of TPNI in transgenic plants was studied by Western blot analysis using procedures described by Florack *et al.* (1994): 30 µg aliquots of total protein extracts or 100 µl samples of IF were separated in 16% SDS-tricine polyacrylamide gels using rainbow-coloured low molecular-weight proteins (Amersham, UK) as markers. After electrophoresis, proteins were blotted onto nitrocellulose BA 85 membranes (Schleicher & Schuell, FRG). TPNI was detected with the chemiluminescent ECL Western blotting detection system (Amersham) using anti TPNI IgG as first and horseradish peroxidase-linked donkey anti rabbit Ig (Amersham) as second antibody.

Tuber tissue resistance tests. Transgenic plants were multiplied *in vitro* and then grown in the glasshouse until maturity. Tubers harvested from these plants were screened for tuber tissue resistance to *Eca* IPO 161 in a randomized block design under aerobic and anaerobic conditions according to the method for small tubers described in Chapter 2. In short, ten small tubers per transgenic clone or non transformed cultivar were cut twice tangentially at opposite sides and incubated overnight at 15 °C. A well, 4.0 mm deep and 5.0 mm diameter was pressed into the medullar tissue in which 20 µl 1.0 x 10^9 cfu ml^{-1} *Eca* was pipetted. Tubers were incubated at 20 °C for three days in nitrogen or five days in air as described (Chapter 2). After incubation, rotted tissue was scraped from the tubers and resistance expressed as the calculated mean diameter of the resulting cavity, measured at right angles. Analysis of variance was carried out by ANOVA.

Results

In vitro *toxicity of TPNI to* Erwinia *spp.* Toxicity of TPNI for two isolates of each of three *Erwinia* spp. that are able to macerate potato tissue was determined *in vitro* (Table 6.1). An *E. coli* K12 isolate was included in the assay in order to allow comparison of results with published TPNI toxicity data (Nakamura *et al.*, 1988; Miyata *et al.*, 1989). Among *E. carotovora* isolates, the MIC's and MBC's of TPNI were almost identical and comparable with the MIC and MBC found for *E. coli*. MIC's and MBC's found for the isolates of *Ech* tested, were slightly lower in comparison to the MIC and MBC found for *E. coli*.

Antibacterial activity of TPNI in the presence of crude potato extracts was studied by incubating a mixture of TPNI and crude extracts for 1 h at room temperature prior to the addition of *E. coli* cells and subsequent incubation at 37 °C (Table 6.2). The final concentration of TPNI in this assay was approximately three times higher than

Table 6.1. Toxicity of TPNI for *Escherichia coli* and pectolytic *Erwinia* spp.

Bacterial species	Isolate	Remarks	MIC[1] ($\mu g\ ml^{-1}$)	MBC[2] ($\mu g\ ml^{-1}$)
E. coli	K12 PC 1011		5.6	5.6
Eca	IPO 161		11.1	11.1
Eca	PD 775		5.6	11.1
Ecc	IPO 163		5.6	11.1
Ecc	PD 1005	indol+	5.6	11.1
Ech	IPO 502	biovar 7	0.7	1.4
Ech	PD 482	biovar 5	1.4	2.8

[1] MIC = minimal inhibitory concentration: lowest concentration of TPNI at which the A_{620} was equal to the mock inoculated wells.

[2] MBC = minimal bactericidal concentration: lowest concentration of TPNI at which no growth was observed after dilution plating.

Table 6.2. Activity of TPNI in the presence of plants extracts. Antibacterial activity of 15 $\mu g\ ml^{-1}$ TPNI against *Escherichia coli* K12 after incubation for 1 h in the presence (+) or absence (-) of crude extracts of potato tuber, leaf or stem tissue. Bacterial growth was scored after dilution plating of 100 µl aliquots of the incubation mixtures.

Conditions		Bacterial growth	
Crude extract	TPNI	Tuber / leaf	Stem
-	+	no	no
+	+	no	yes
boiled[1]	+	no	yes
+	-	yes	yes
boiled[1]	-	yes	yes

[1] Extract boiled for 3 min in a water bath

the MBC (Table 6.1). Incubation of TPNI in the presence of potato leaf or tuber extracts did not affect the antibacterial activity of TPNI. However, antibacterial activity was lost after incubation with a potato stem extract. Loss of activity was also observed when the stem extract was boiled prior to incubation with TPNI.

Expression of TPNI encoding gene constructs in transgenic potato cultivars. Figure 6.1 shows the composition of the four gene constructs used in this study for transformation of potato cultivars. The design was based on the structural organisation of the TPNI precursor from *T. tridentatus* (Shigenaga *et al.*, 1990). In the gene constructs pT-1 and pT-2, the SP of the *tpnI* gene was replaced by the SP of the barley α-hordothionin gene pC1α (Florack *et al.*, 1994). In pT-3 and pT-4, the SP of TPNI was replaced by the SP of tobacco pathogenesis related protein S gene (PR-S; Cornelissen *et al.*, 1986). Nucleotide sequences of both SP's and mature TPNI, except for the carboxyl terminal Arg residue of pT-2 and pT-4, were optimized for codon usage in *Solanaceae* (Wada *et al.*, 1990) and chemically synthesised. A *Taq I/Pvu* II fragment corresponding to nucleotides 170 to 297 of cDNA clone λTPNI-C1 (Shigenaga *et al.*, 1990) encoding the AP of the precursor was ligated to pT-1 and pT-3 to create constructs pT-2 and pT-4 respectively.

Susceptible potato cvs Bintje, Karnico and Kondor were transformed with constructs pT-1 to pT-4. Transgenic plants were grown in the glasshouse and 25 to 30 independent transformants per cultivar per construct analyzed for TPNI expression by Western blot analysis using a polyclonal antiserum. TPNI could be detected in about 10% (cvs Bintje and Kondor) or 30% (cv Karnico) of the plants that were transformed with pT-1 and pT-2 (Table 6.3). These constructs had, in addition to the sequence for mature TPNI, the SP of the α-hordothionin gene in common (Figure 6.1). The highest concentration of TPNI found in extracts of these plants was about 0.003% (0.10 μmol kg^{-1} fresh weight) of the total acid extractable protein of young leaves (ca. 6.6 μg g^{-1}). No TPNI could be detected in plants transformed with constructs pT-3 or pT-4 which both contained the SP of the PR-S gene. Results of an analysis of leaf protein extracts from cv Kondor plants transformed with pT-1 and pT-2 are shown in Figure 6.2 (lanes 1 to 4). TPNI of both the pT-1 and pT-2 transformed plants comigrated with purified TPNI of horseshoe crab. This indicates that both the hordothionin SP and the AP of the TPNI precursor in pT-2 transformed plants were processed. No TPNI was detected in protein extracts of control plants (Figure 6.2, lane 5).

IF, containing the soluble protein fraction from the leaf apoplast, was isolated from TPNI expressing pT-1 and pT-2 transformed clones of the three cultivars and subjected to Western blot analysis. No TPNI specific signals were detected in these IF's (data not shown).

Four pT-1 and pT-2 transformed cv Kondor plants in which TPNI could be detected (Ko-126, 119 and Ko-422, 437 respectively), another four randomly chosen pT-1 and pT-2 transformed plants in which TPNI could not be detected as well as

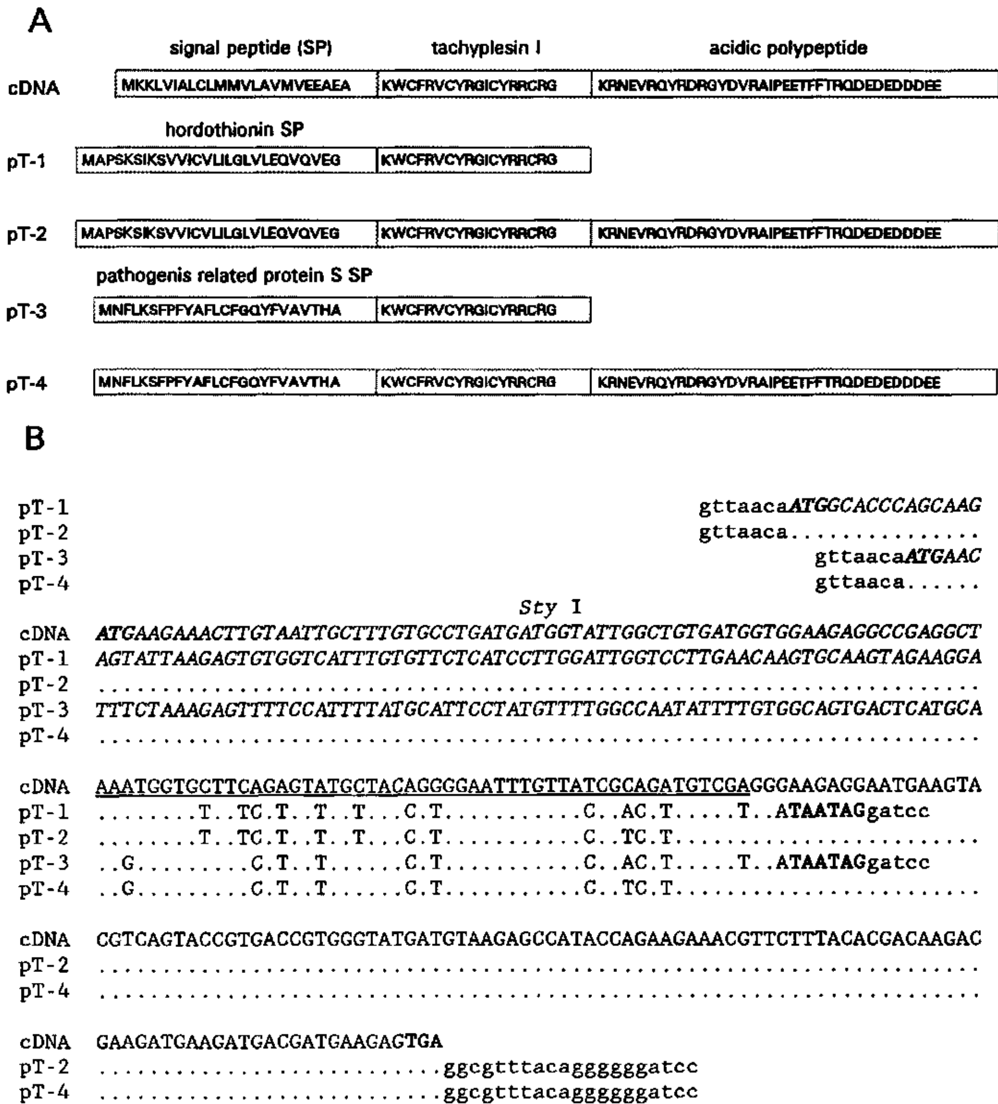

Figure 6.1. Structure and sequences of TPNI cDNA and gene constructs. A. Single letter code amino acid sequences and gene structure of the coding region of cDNA clone λTPNI-C1 (Shigenaga *et al.*, 1990) and gene constructs pT-1 to pT-4. Vertical bars represent proteolytic cleavage sides. B. Nucleotide sequences of the coding region of the cDNA clone λTPNI-C1 and pT-1 to pT-4. SP sequences are in italics. The sequence of the 17 amino acid residues mature TPNI is underlined. Translation start and stop codons are in bold face. Flanking sequences with restriction sites *Hpa* I (gttaac) and *Bam* HI (ggatcc) that were used for cloning in pCPO5 (see text) and optimized translation initiation regions (aaca) are in lower case. Nucleotides common with the cDNA clone (mature TPNI and acidic polypeptide) or with the corresponding construct (SP) are indicated by dots. The position of the restriction site *Sty* I (ccttgg) in pT-1 and pT-2 is indicated.

eight randomly chosen pT-3 and pT-4 transformed plants, were analyzed for *tpnI* gene expression by Northern blot analysis (Figure 6.3). Expression of the genes was detected in all pT-1 and pT-2 transformed plants analyzed. Only minor differences in transcription levels were found. Transcription levels of the *tpnI* gene in pT-3 transformed plants were markedly reduced in comparison to pT-1 and pT-2 transformed plants whereas no expression was detected in pT-4 transformed plants. A kanamycin resistant and non-transformed control plant also showed no transcription signals.

Tuber soft rot resistance of transgenic clones. For each of the six combinations of cultivar and constructs pT-1 or pT-2, the three transgenic clones with the highest expression of TPNI, as determined by Western blot analysis, were multiplied in vitro and then grown in the glasshouse. One of the three selected clones of cv Karnico that was transformed with pT-2 produced only very small tubers and was therefore not included in resistance tests. Under glasshouse conditions, the phenotype of the other transgenic clones did not differ from that of the corresponding non-transformed cultivars.

Small tubers harvested from glasshouse-grown plants were screened for resistance to tuber soft rot in two separate experiments. Mean tuber weight across all tested clones was 14.7 g and 15.1 g in experiments 1 and 2 respectively. Tubers were inoculated with 1.0×10^9 cfu ml^{-1} of *Eca* isolate IPO 161 and incubated either aerobically in air for five days or anaerobically in nitrogen for three days. Under these conditions, the high concentration of inoculum used resulted in rot of all tested tubers. Resistance of the clones was expressed as the mean diameter of rotted tissue and is presented in Table 6.4.

Under aerobic conditions, transgenic clones showed less rot in comparison to the controls which was significant ($P<0.05$) for transgenic clones of cv Bintje (Bi1-234, 256, 260 and Bi2-515 in both experiments and Bi2-520 in the second experiment). The mean diameter of rot of only three transgenic clones (Bi2-520 in experiment 1 and Bi2-516 and Ka2-471 in experiment 2) was the same as the corresponding non-transformed controls. The linear correlation coefficient for comparison of the mean diameter of rot of clones between experiments was high ($r=0.84$, $n=20$) and significant ($P<0.01$).

Under anaerobic conditions 12 out of 17 transgenic clones showed less rot than the appropriate controls in both experiments while the behaviour of four clones was not consistent. Clone Bi1-234 for example showed significantly less rot ($P<0.05$) than the non-transformed control in the first but not in the second experiment when its

Table 6.3. Expression of TPNI in transgenic plants. Number of transgenic plants, out of 30 per combination of gene construct and cultivar, in which expression of TPNI was detected by Western blot analysis.

Cultivar	Gene construct			
	pT-1	pT-2	pT-3	pT-4
Bintje	3	4	0	0[2]
Karnico	9	7[1]	0	0
Kondor	4	4	0	0

[1,2] Only 25 and 28 plants analyzed respectively.

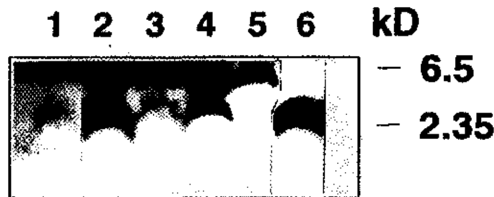

Figure 6.2. Western blot analysis of TPNI expressing potato plants. TPNI specific signals in 30 µg aliquots of leaf protein extracts from kanamycin resistant plants of cv Kondor transformed with the *tpnI* gene construct pT-1 (Ko-125 in lane 1) and pT-2 (Ko-410, 422 and 437 in lanes 2 to 4 respectively). Lane 5 contains protein extract of a non-transformed cv Kondor plant. Lane 6 contains 5 ng of purified TPNI.

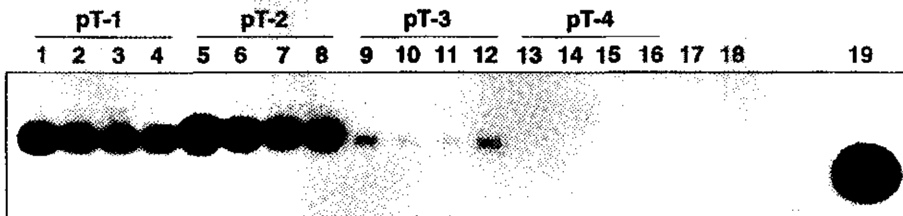

Figure 6.3. Northern blot analysis of TPNI expressing potato plants. Lanes contain 15 µg aliquots of total RNA of kanamycin resistant plants of cv Kondor transformed with the *tpnI* gene constructs pT-1 to pT-4 (Ko-126, 119, 111, 116, 422, 437, 426, 439, 551, 552, 553, 554, 293, 297, 300 and 312 in lanes 1 to 16 respectively), one non-transformed control plant (lane 17) and one kanamycin resistant control plant transformed with binary vector pCPO5 without pT gene insertion (lane 18). A reconstruction was made by adding 1 ng of the *Sty I/Bam* HI fragment of pT-1 DNA to 15 µg aliquots RNA of a control plant (lane 19). This fragment was labelled with ^{32}P and used as probe.

mean diameter of rot was slightly larger. Of the 17 transgenic clones screened, two clones (Ka1-199 and Ko1-119) were significantly more resistant ($P<0.05$) than the controls in both experiments while another nine clones in the first, and two clones in the second experiment were also significantly more resistant ($P<0.05$) than the controls.

Discussion

Toxicity of TPNI for *E. coli* and several pectolytic *Erwinia* spp. which all belong to the *Enterobacteriaceae* family was evaluated *in vitro*. A MIC of 5.6 μg ml^{-1} was found for *E. coli* K12 which was in between the ranges published for this bacteria by Nakamura *et al.* (1988) and Miyata *et al.* (1989), who found MIC values between 6.3-12.5 μg ml^{-1} and 1.6-3.1 μg ml^{-1} respectively. The sensitivity of *Eca* and *Ecc* isolates was comparable with that of *E. coli* whereas *Ech* isolates were slightly more sensitive. The bactericidal activity of TPNI was also observed after incubation with crude potato leaf and tuber extracts but was lost after incubation with crude stem extract, even when this extract was boiled prior to incubation. This finding suggests that the loss of antibacterial activity of TPNI is not caused by proteolytic degradation.

The gene constructs used in this study all encode SP's which are considered to be essential for transition of the precursor to the endoplasmatic reticulum and consequently, intramolecular disulphide bridge forming (Chrispeels; 1991). The hordothionin-SP sequence of constructs pT-1 and pT-2 was chosen because of its plant origin and because of the high levels of expression found in transgenic tobacco plants transformed with gene constructs consisting of the coding sequences of this SP and mature α-hordothionin, a protein of which the precursor shows structural similarities to that of TPNI (Florack *et al.*, 1994). The PR-S-SP sequence of constructs pT-3 and pT-4 was chosen also for its plant origin and its potential role in mediating secretion of proteins to the intercellular spaces (Cornelissen *et al.*, 1986). Secretion of antibacterial peptides in the intercellular spaces might be essential for engineering resistance to soft rotting *Erwinia* since they survive extracelluarly at low densities before causing progressive rots by macerating parenchymatous cells in stored or planted potato tubers (Fox *et al.*, 1971). Gene constructs pT-2 and pT-4 were made in addition to pT-1 and pT-3 to study the role of the AP-sequence found in the cDNA of TPNI (Shigenaga *et al.*, 1990).

After Western blot analysis of the 353 transgenic clones obtained, TPNI was only detected in a fraction of the leaf protein extracts from pT-1 and pT-2 transformed

Table 6.4. Soft rot resistance of TPNI expressing potato plants. Mean diameter of rotted tissue (mm) after inoculation with *Eca* and subsequent incubation in air or nitrogen of non-transformed (NT) cvs Bintje (Bi), Karnico (Ka) and Kondor (Ko) and transgenic clones of these cultivars transformed with pT-1 (Bi1, Ka1 and Ko1 respectively) or pT-2 (Bi2, Ka2 and Ko2 respectively). All transgenic clones showed specific signals after Western blot analysis.

	Incubation conditions			
	Air		Nitrogen	
Clone	Expt 1	Expt 2	Expt 1	Expt 2
Bi-NT	10.1	11.5	17.6	14.4
Bi1-234	7.8 **	8.8 **	15.9 *	14.7
Bi1-256	8.3 *	9.2 **	17.7	14.8
Bi1-260	7.9 **	8.6 **	17.4	14.0
Bi2-515	8.5 *	9.0 **	15.9 *	15.1
Bi2-516	9.2	11.5	16.6	12.6 *
Bi2-520	10.1	9.6 **	15.7 *	13.7
Ka-NT	8.1	8.9	17.3	15.1
Ka1-187	7.0	8.0	16.5	13.1 **
Ka1-191	7.7	8.0	18.3	14.5
Ka1-199	7.4	8.0	15.3 *	13.4 *
Ka2-471	8.0	8.9	17.5	14.5
Ka2-481	7.6	7.9	14.1 **	13.7
Ko-NT	8.1	8.8	20.3	13.5
Ko1-119	7.4	8.7	17.8 **	11.8 *
Ko1-125	7.5	7.8	16.4 **	12.6
Ko1-126	7.5	8.5	16.7 **	12.2
Ko2-410	7.9	8.5	15.0 **	12.8
Ko2-422	7.7	8.4	16.2 **	13.2
Ko2-437	7.6	8.2	15.7 **	13.0
mean	8.1	8.8	16.7	13.6
SED (df=342)	0.8	0.7	0.8	0.7

*, ** rots significantly smaller when compared to the appropriate non-transformed controls at the $P<0.05$ and $P<0.01$ level respectively

clones. The concentration of TPNI in these extracts was equal to or slightly higher than the lowest concentration of about 1 ng horseshoe crab purified TPNI that routinely could be detected by the techniques used. No expression of TPNI was detected in pT-3 and pT-4 transformed clones which was probably due to their low or non-detectable level of transcription (Figure 6.3). Results of the Northern blot analysis suggest that the transcribed mRNA of the PR-S-SP containing gene construct pT-3 is less stable than the mRNA of hordothionin SP containing gene constructs or that the transgene in pT-3 transformed plants is less efficiently transcribed. This might also be the case in pT-4 transformed plants, although, since no transgene transcription was detected at all, it can not be excluded that this is due to incorrect or failure of insertion of T-DNA during the transformation process.

The observation that TPNI specific bands of both pT-1 and pT-2 transformed clones comigrate on SDS-tricine polyacrylamide gels with TPNI purified from horseshoe crab indicate that the hordothionin-SP in these clones and the AP of the precursor in pT-2 transformed clones was processed. In horseshoe crab hemocytes, release of the AP probably requires three steps (Muta *et al.*, 1990; Shigenaga *et al.*, 1990). First, cleavage between Arg and Asn residues at AP positions 3 and 4 (Figure 6.1), which results in a TPNI processing intermediate containing in addition to the mature peptide a carboxyl terminal Gly-Lys sequence that is found in the smaller granules of hemocytes, enzymatic removal of the dibasic sequence (Lys-Arg) and finally, amidation of the carboxyl terminal glycine residue. It is not possible to conclude from our Western blot analysis data whether the processing of the AP containing precursor takes place identically in pT-2 transformed clones or whether the carboxyl terminal Gly residue of TPNI in pT-1 transformed clones is also amidated. Furthermore it is not known from literature whether the removal of the (Lys-Arg) sequence or the amidation of the Gly residue are essential for biological activity of TPNI. Study of the precise processing events and biological activity of TPNI from transgenic potato clones requires the purification of a significant amount of TPNI from transgenic tissue which unfortunately could not be achieved due to the very low levels of expression in our plant material.

The number of TPNI expressing pT-2 transformed clones and their level of expression was almost equal to those of pT-1 transformed clones (Table 6.3, Figure 6.2) indicating that the AP sequence did not markedly affect expression levels of the transgene. This is in contrast to the work of Florack *et al.* (1994) who found strongly increased expression of hordothionin in transgenic tobacco plants when a thionin precursor-derived AP sequence was present in the gene constructs used for transformation.

No TPNI was detected in IF's of transgenic pT-1 and pT-2 transformed clones that did show a TPNI specific signal after Western blot analysis of leaf protein extracts. It is likely therefore that the hordothionin SP of the pT-1 and pT-2 gene constructs did not facilitate secretion of TPNI to the intercellular spaces. Florack *et al.* (1994), who used the hordothionin SP in gene constructs coding for hordothionin, also found no hordothionin in IF's of transgenic tobacco plants that strongly expressed this peptide in young leaves. Unfortunately, due to the lack of expression of TPNI in pT-3 and pT-4 transformed clones, we were not able to analyze whether the PR-S-SP facilitated secretion of TPNI in these clones.

Tubers from TPNI expressing pT-1 and pT-2 transformed clones tended to rot slightly less than the appropriate non-transformed controls. However, results from screenings under anaerobic conditions were less consistent than results from screenings under aerobic conditions. Since the introduced *tpnI* gene constructs function under the control of a constitutive promotor, it is likely that the peptide is expressed during tuber formation. However, its stability and expression after maturing and storage of tubers, or during resistance screening remains to be studied. Since drastic changes in tuber mRNA and protein synthesis occur under anaerobic conditions (Davis *et al.*, 1990), the less clear effect of transformation under anaerobic screening conditions could possibly be explained by anaerobically induced inhibition of 35S mediated TPNI expression.

The level of resistance obtained by the introduction into plants of genes that code for antimicrobial agents such as TPNI, is likely to be related to several factors. Within combinations of cultivar and gene construct a relation is expected between the level of expression of the transgene and resistance expressed as the diameter of rotted tuber tissue. Due to the low expression levels in our transgenic clones and the small size of the groups screened, existence of such relation could not be verified. Another factor of importance might be the concentration of the inoculum used for soft rot resistance testing. Düring *et al.* (1993), who transformed potato plants with a gene construct coding for antibacterial T4 lysozyme, found that soft rot resistance of transgenic tubers, relative to the resistance of control tubers, decreased with increasing inoculum concentrations. As Western blot analysis of IF's indicated that TPNI was probably not secreted, we performed resistance tests with high, rot initiating inocula, expecting that maceration of the plant cells and, as a consequence, contact between *Erwinia* bacteria and TPNI containing cell contents, would be essential for expression of resistance. In a previous study (Chapter 3) it was shown that tuber tissue resistance of stored tubers of a range of 12 cultivars did not correlate with field resistance to blackleg. As a consequence, field tests have to be performed in order to

investigate whether the observed effect of transformation on tuber tissue resistance is of any significance for the level of resistance to blackleg under field conditions. The value of the transgenic approach described here as a potential solution for the losses that are caused by soft rotting *Erwinia* during storage of field-grown potatoes, needs also further investigation.

Chapter 7

Erwinia soft rot resistance of potato cultivars expressing antimicrobial peptide α-hordothionin

Abstract

α-Hordothionin is a 5 kDa peptide which shows antimicrobial activity *in vitro*. Potato cvs Bintje and Kondor were transformed with a gene construct coding for the α-hordothionin precursor from barley. Leaf protein extracts of 32 transgenic clones were analyzed for α-hordothionin expression by a dot blot procedure. Western blot analysis of the clones with the highest level of expression revealed that up to about 1.7% of the acid soluble protein fraction consisted of α-hordothionin. In two experiments, small tubers of five to eight transgenic clones that had been harvested from glasshouse-grown plants were screened for resistance to *Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi* under aerobic and anaerobic conditions. Soft rot resistance of α-hordothionin expressing clones was not clearly improved when compared with kanamycin resistant and non-transformed control clones.

Introduction

Thionins are small (ca 5 kDa) peptides that possess antimicrobial properties *in vitro* (see García-Olmedo *et al.*, 1989; Bohlmann & Apel, 1991). Thionins are divided into five types on the basis of their origin and their molecular and biochemical characteristics (García-Olmedo *et al.*, 1989). Type I thionins are found in the endosperm of monocots. The thionins from wheat endosperm are called purothionins and are subdivided into an α1-, α2- and β-form on the basis of amino acid sequences. The thionins from barley endosperm are called hordothionins and are subdivided in an α- and β-form. Mature type I thionins consist of 45 amino acid residues, are strongly basic and contain four intramolecular disulphide bonds. They are processed from a larger precursor polypeptide consisting of a 28 amino acid residual signal peptide, the mature thionin and an acidic carboxyl terminal polypeptide of 64 amino acid residues.

Many plant pathogenic fungi and bacteria show growth inhibition *in vitro* in the presence of type I thionins (De Bolle *et al.*, 1993; Florack *et al.*, 1993; Molina *et al*,

1993). *Clavibacter* spp. and *Xanthomonas campestris* pv. *vesicatoria* showed inhibition in the presence of 8.0 to 64.0 µg ml^{-1} thionin whereas pectolytic *Erwinia* spp. and *E. salicis* showed no inhibition at concentrations as high as 256 µg ml^{-1} (Florack *et al.*, 1993). Transgenic tobacco plants expressing a genomic α-hordothionin gene construct showed enhanced resistance to *Pseudomonas syringae* pv. *tabaci* and *P.s.* pv. *syringae* (Carmona *et al.*, 1993).

This chapter describes the introduction into potato of a semi-synthetic gene construct coding for the α-hordothionin precursor peptide. The transgenic clones obtained were screened for expression of the gene and for tuber tissue resistance to pectolytic *Erwinia* spp.

Materials and methods

Transformation of potato with a α-hordothionin gene construct. Cvs Kondor and Bintje, both relatively susceptible to potato soft rot (Chapter 2), were transformed as described in Chapter 6. The plant expression vector pC1*α* (Florack *et al.*, 1994), containing the coding sequence for an α-hordothionin precursor under the control of a double cauliflower mosaic virus 35S promotor and nopaline synthase terminator, was used for transformation. This precursor consists of a signal peptide of 28 amino acid residues, the mature α-hordothionin peptide of 45 amino acid residues and a carboxyl terminal acidic polypeptide of 64 amino acid residues. Expression of this gene construct in tobacco resulted in α-hordothionin levels up to 0.7% of total leaf protein (Florack *et al.*, 1994). Plants that were kanamycin resistant (kan^R) were maintained and multiplied *in vitro* and grown in the glasshouse until maturity. Non-transformed plants that had also been multiplied *in vitro* as well as kan^R plants obtained after transformation with binary vector pCPO5 without the α-hordothionin gene insertion, served as controls.

α-Hordothionin expression analysis. Protein extracts from leaf and tuber tissue that was incubated in 50 mM H_2SO_4 and intercellular washing fluid (IF) from young leaves were isolated as described in Chapter 6. IF's were collected by centrifugation after vacuum infiltration in water or 0.5 M NaCl. All transgenic plants were first screened for α-hordothionin expression in a dot blot procedure as described (Florack *et al.*, 1994). In short, 5.0 µg aliquots of leaf protein extracts were blotted onto nitrocellulose BA 85 membranes (Schleicher & Schuell) using a dot blot apparatus (type SRC 96 D, Schleicher & Schuell). A polyclonal antiserum from a rabbit

immunized with a conjugate of bovine serum albumin and α-hordothionin (Florack *et al.*, 1994) was used as primary antibody.

From transgenic plants with strong signals on dot blots, aliquots of 10 to 15 μg leaf or tuber protein extracts or 100 μl samples of IF were analyzed by SDS-tricine polyacrylamide gel electrophoresis and Western blot analysis as described (Florack *et al.*, 1994; Chapter 6).

Tuber tissue resistance tests. Tubers from transgenic and control plants, all harvested from the same multiplication in 1 ℓ pots, were screened for tissue resistance to *Eca* IPO 161 and *Ech* IPO 502 using the method for small tubers as described in Chapter 2. Bacterial isolates were obtained from the DLO-Research Institute for Plant Protection (IPO-DLO), Wageningen. Preparation of inoculum and inoculation procedures were as described in Chapter 2. The concentration of the inoculum used was spectrofotometrically adjusted to 1.0×10^9 colony-forming units ml^{-1}. After inoculation, the tubers were either incubated aerobically in air for five days or anaerobically in nitrogen for three days at 20 °C. Ten tubers per combination of clone, bacterial species and incubation condition were used. After incubation, the rotted tissue was removed and the resistance expressed as the mean diameter of the resulting cavity. Analysis of variance was carried out by ANOVA.

Results

α-Hordothionin expression in transgenic clones. A transformation experiment with the α-hordothionin gene construct yielded 31 transgenic clones of cv Kondor and only one transgenic clone of cv Bintje. This Bintje clone as well as the kan^R control clone of cv Bintje showed a slightly deviating leaf shape and colour during cultivation in the glasshouse whereas transgenic clones of cv Kondor showed no deviating phenotypes.

Transgenic clones were analyzed for expression of α-hordothionin by a dot blot immuno assay. Leaf protein extracts of transgenic clones that showed the highest expression were subjected to Western blot analysis (Figure 7.1, panel A, lanes 1 to 10 and panel B, lanes 4 to 6). These transgenic clones all showed clear bands on Western blots which comigrated with purified α-hordothionin from barley. Non-transformed and kan^R control clones did not show such α-hordothionin specific signals (Figure 7.1, panel A, lanes 11 and 12). The concentration of α-hordothionin was approximately 1.7% (22.3 μmol kg^{-1} fresh weight) of the acid soluble protein fraction of young leaves as determined from a comparison with signal intensity of 50 ng purified thionin

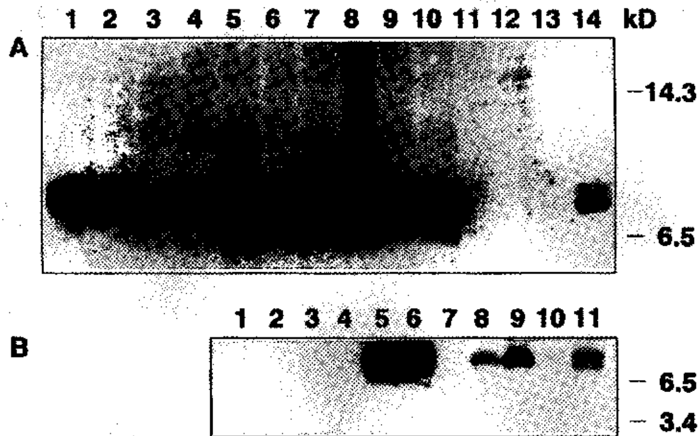

Figure 7.1. Western blot analysis of plants transformed with a gene construct coding for α-hordothionin. A. Aliquots of 10 µg of leaf protein extracts of 10 plants, Ko-142, 146, 149, 150, 151, 155, 156, 159, 164, 169 were separated in lanes 1 to 10 respectively. Lanes 11 and 12 contain leaf protein extract of a kanamycin resistant control plant transformed with binary vector pCPO5 without α-hordothionin gene insertion and of a non transformed control plant respectively. Lanes 13 and 14 contain 10 and 50 ng aliquots of purified α-hordothionin respectively. B. Aliquots of approximately 7 µg of total protein present in intercellular fluid after infiltration of leaves in 0.5 M NaCl of a non-transformed control plant, Ko-151 and 159 were separated in lanes 1 to 3 respectively. 15 µg aliquots of leaf and young tuber protein extracts from these three plants were separated in lanes 4 to 6 and 7 to 9 respectively. Lanes 10 and 11 contain 10 and 50 ng aliquots of purified α-hordothionin respectively.

on the Western blot of Figure 7.1 (panel A). No α-hordothionin specific signals were found in IF's whether isolated from NaCl infiltrated leaves (Figure 7.1, panel B) or water infiltrated leaves (not shown) of the two transgenic clones with the highest level of α-hordothionin expression. However, α-hordothionin was detected in protein extracts of developing tubers (Figure 7.1, panel B). Western blots of protein extracts from matured control tubers showed non-specific bands that comigrated with purified α-hordothionin (data not shown).

Table 7.1. Mean diameter of rotted tissue (mm) of transgenic clones of cvs Kondor (Ko-) and Bintje (Bi-) expressing α-hordothionin, after inoculation with bacterial suspensions of *Eca* and *Ech*. Control clones were either non-transformed (NT) or kanamycin resistant (kan[R]). ND; not determined.

	Incubation conditions					
	Air				Nitrogen	
	Eca		*Ech*		*Eca*	*Ech*
Clone	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 1
Bi-NT	10.3	11.8	9.4	8.2	15.9	15.3
Bi-kan[R]	10.1	11.8	9.8	9.1	13.5	13.8
Bi-271	8.8	8.8	7.9	7.5	12.4	14.4
Ko-NT	10.7	9.2	11.8	9.5	17.1	17.4
Ko-kan[R]	10.5	8.9	11.2	9.4	15.9	15.6
Ko-144	ND	11.0	ND	8.8	ND	ND
Ko-146	9.3	8.9	11.7	9.6	15.7	15.8
Ko-149	8.9	9.4	11.6	8.8	17.0	16.6
Ko-151	8.7	8.5	11.9	9.9	15.9	16.2
Ko-159	8.6	10.8	11.2	9.2	17.1	16.9
Ko-165	ND	9.7	ND	9.2	ND	ND
Ko-166	ND	8.3	ND	9.1	ND	ND

LSD ($P<0.01$) = 1.8 and 2.0 for cultivar x inoculum means in experiments 1 and 2 respectively

Tuber tissue resistance of α-hordothionin expressing clones. Small tubers of cv Kondor clones and the only available transgenic clone of cv Bintje were screened for resistance to *Eca* and *Ech* (Table 7.1). In experiment 1, cv Kondor clones 151 and 159 with the highest level of expression and cv Kondor clones 146 and 149 with the lowest level of expression on Western blots were incubated either aerobically in air or anaerobically in nitrogen after inoculation. Under aerobic conditions, three out of four transgenic clones of cv Kondor (numbers 149, 151 and 159) showed significantly less rot ($P<0.05$) in comparison to either type of control clones when inoculated with *Eca* but not when inoculated with *Ech*. The transgenic clone of cv Bintje (Bi-271) showed less rot than either type of control clones but only significantly ($P<0.05$) after inoculation with *Ech*. Under anaerobic conditions, none of the transgenic clones showed significantly less rot ($P<0.05$) when compared with either type of controls. However, after inoculation with both *Eca* and *Ech*, tubers of the kan[R] control of

cv Bintje showed significantly less rot (P<0.01) in comparison to tubers of the non-transformed control.

A second experiment was carried out with small tubers from plants that had been grown approximately six months later than those from which the tubers of the first experiment had been harvested. In the second experiment, small tubers were only incubated aerobically. After inoculation with *Eca*, only one transgenic clone (Bintje 271) showed significantly less rot (P<0.01) when compared with the appropriate controls. Two transgenic clones of cultivar cv Kondor (numbers 144 and 159) showed significantly larger rots (P<0.01) than either type of control clone. After inoculation with *Ech*, none of the transgenic clones showed significantly less rot (P<0.05) in comparison to either type of control clone (Table 7.1).

Discussion

α-Hordothionin was found to be expressed up to high levels in both leaves and developing tuber tissue of transgenic potato clones and was easy to detect in a dot-blot procedure or by Western blot analysis. In addition, the *α*-hordothionin precursor is properly processed into the mature 5 kDa protein. No *α*-hordothionin could be detected in IF's from young leaves of highly expressing clones which indicates that the *α*-hordothionin is not secreted to the apoplast. These results are in agreement with the work of Florack *et al.* (1994), who transformed tobacco with the same gene construct.

Results of the first experiment in which resistance of small tubers from highly expressing clones of cv Kondor was studied, indicated that tuber tissue resistance to *Eca*, when tested under aerobic conditions, appeared to be only slightly improved. However, the results of the second experiment showed that tuber tissue resistance of transgenic cv Kondor clones was not improved and that there existed no relation between the level of resistance and the level of *α*-hordothionin expression, since the resistance of the group of three clones with low expression levels (Ko-144, 165 and 166) and the resistance of the four highly expressing clones that had been tested in the first experiment did not differ. The only available clone of cv Bintje showed less rot than either type of controls under aerobic conditions. However, it can not be concluded that this is an effect of *α*-hordothionin expression because the clone showed a deviating phenotype. The genotypical changes that apparently had taken place during the transformation process might well have involved the tuber tissue

resistance of this clone. Such changes might also explain why the kan^R control of cv Bintje showed significantly less rot ($P<0.01$) than the non-transformed control under anaerobic conditions (Table 7.1). Cv Bintje has been described earlier as a cultivar that shows a lot of somaclonal variation after transformation (Jongedijk *et al.*, 1992).

Erwinia spp. are among the least sensitive of the plant pathogenic bacteria that were tested *in vitro* for sensitivity to α-hordothionin (Florack *et al.*, 1993). However, *in vitro* toxicity assays are often affected by experimental conditions such as the method used, the ionic strength or the Ca^{2+} concentration (Nordeen *et al.*, 1992; De Bolle *et al.*, 1993; Mills & Hammerschlag, 1993). Therefore it is thought that the *in vitro* toxicity of an antibacterial peptide only weakly predicts its activity *in planta*. Because α-hordothionin has shown a general anti-bacterial activity, and because it was expected on the basis of results from a study on tobacco plants transformed with the α-hordothionin gene construct used (Florack *et al.*, 1994), that high expression levels could be obtained in potato, it was considered worthwhile to test transformed cultivars for resistance to *Erwinia*. Indeed, acid soluble protein extracts of transgenic potato clones contained up to an estimated 1.7% α-hordothionin. However, expression of antibacterial α-hordothionin to such high concentrations in potato tissue could not been shown to result in enhanced resistance to soft rotting *Erwinia* spp. The finding that resistance of the tuber tissue was not improved might also be explained by degradation of the α-hordothionin produced during tuber development combined with a strongly reduced expression in matured tubers or by inactivation due to binding to membranes or other plant cell components.

It remains worthwhile to screen the α-hordothionin expressing potato clones for resistance to pathogens that are much more sensitive to α-hordothionin *in vitro*, for example to *Clavibacter michiganensis* subsp. *sepedonicus* (Florack *et al.*, 1993), the causal agent of bacterial ring rot.

Chapter 8

Erwinia soft rot resistance of potato cultivars transformed with a gene construct coding for antimicrobial peptide cecropin B

Abstract

Cecropin B is a peptide of approximately 4 kDA which shows antimicrobial activity *in vitro* against Gram positive and Gram negative bacteria. Potato cvs Agria, Bintje, Karnico, Kondor and Producent were transformed with a gene construct encoding a cecropin B precursor polypeptide. In total, 49 independent transgenic potato clones were obtained. Northern blot analysis of these plants revealed that the introduced gene was transcribed to detectable levels in almost all plants, the highest transcription level being approximately 0.6% of total mRNA. No cecropin B peptide could be detected in transgenic plants, probably as a result of rapid proteolytic degradation of newly synthesized cecropin B by potato endogenous proteases. Neither small tubers of a group of 11 clones with moderate to high transcription levels nor slices from field-grown tubers of any of the transgenic clones obtained, showed significantly less rot ($P<0.01$) after inoculation with *Erwinia carotovora* subsp *atroseptica* or *E. chrysanthemi*, the plant pathogenic bacteria that cause potato soft rot.

Introduction

Cecropins form a family of small peptides (ca 4 kDa) that originally were isolated from pupae of the giant silkmoth (*Hyalophora cecropia*). Peptides with strong homology to the cecropins of *Hyalophora* were found in several other insects (reviewed in Boman & Hultmark, 1987) as well as in small intestine of pigs (Lee *et al.*, 1989). Cecropins show strong antibacterial activity *in vitro* against a broad range of Gram positive and Gram negative bacteria. In insects, synthesis of cecropins is induced after injection of bacteria in the hemolymph. Cecropins are therefore thought to play an important role in insect immunity (Boman & Hultmark, 1987).

The best described cecropins of *Hyalophora* were designated A, B and D and differ slightly in their 35 to 37 residual amino acid sequence (Boman & Hultmark,

1987). The mature peptides are processed from a precursor protein containing an amino terminal signal peptide followed by two Pro containing dipeptides.

Strong antibacterial activity of cecropins *in vitro* against plant pathogenic bacteria including pectolytic *Erwinia* spp. has been reported in several studies (Destéfano-Beltrán *et al.*, 1990; Nordeen *et al.*, 1992; Mills & Hammerschlag, 1993; Hightower *et al.*, 1994). Attempts have been undertaken to enhance resistance of tobacco and potato to bacterial diseases by *Agrobacterium* mediated transformation with gene constructs that code for cecropins. Results of these studies are conflicting. Jaynes *et al.* (1993) and Jia *et al.* (1993) found delayed symptom development after inoculation of transgenic tobacco and potato plants with *Pseudomonas solanacearum*, whereas Florack *et al.* (1995) found that resistance of transgenic tobacco plants was not altered. In the latter study and another independent one (Hightower *et al.*, 1994), it was found that resistance of transgenic tobacco plants to *P. syringae* pv. *tobacco* was also not improved. In fact both research groups found that the cecropin peptide is degraded rapidly in the presence of various plant extracts.

We describe here the transformation of potato cultivars with a gene construct coding for cecropin B, the analysis of transgene expression and the screening of the transgenic clones for tuber tissue resistance to soft rotting *Erwinia* spp. The cecropin B gene construct used was composed of the cecropin B coding sequence, preceded by a plant-derived signal peptide. It was shown in a previous study (Florack *et al.*, 1995) that this gene construct resulted in stronger cecropin B mRNA expression in tobacco plants than if gene constructs were used that contained a *Hyalophora*-derived signal peptide or no signal peptide at all.

Materials and Methods

Transformation of potato with a cecropin B gene construct. Agrobacterium tumefaciens GV3101 (pMP90RK) mediated transformation of potato cvs Agria, Bintje, Karnico, Kondor and Producent was carried out with the plant expression vector pCPOC4 (Florack *et al.*, 1995), containing a gene construct encoding for the 28 amino acid residual signal peptide of α-hordothionin (Florack *et al.*, 1994) and the mature cecropin B peptide of 36 amino acid residues under the control of a double cauliflower mosaic virus 35S promoter and nopaline synthase terminator. Tuber disc transformation was carried out essentially as described by Stiekema *et al.* (1988). Discs, 1.0 cm diameter, were cut from surface sterilized field-grown seed potatoes and cocultivated for three days with *A. tumefaciens* on a shoot induction medium

containing MS salts plus vitamins (Duchefa, NL), 3% sucrose, 0.8% agar, 0.01 mg ℓ^{-1} 2,4-D and 3.0 mg ℓ^{-1} zeatin. Discs were then transferred to the same medium containing in addition 250 mg ℓ^{-1} cefotaxim, 200 mg ℓ^{-1} vancomycin and 75 mg ℓ^{-1} kanamycin. After two weeks, discs were transferred to a shoot elongation medium that contained the same components as the shoot induction medium including antibiotics but 0.25 mg ℓ^{-1} N^6-benzylaminopurine (BAP) and 0.1 mg ℓ^{-1} gibberellic acid (GA_3) instead of 2,4-D and zeatin. In addition to this procedure, two alternative shoot elongation media were used that contained plant hormones indole acetic acid (0.9 mg ℓ^{-1}) and zeatin riboside (3.6 mg ℓ^{-1}; Hoekema *et al.*, 1989) or GA_3 (0.3 mg ℓ^{-1}), α-naphthaleneacetic acid (0.03 mg ℓ^{-1}) and BAP (3 mg ℓ^{-1}; Jarret *et al*, 1980). In these cases, cocultivation and selection in the presence of antibiotics was directly carried out on shoot elongation medium. Kanamycin resistant (kan^R) plants were maintained *in vitro*. Non-transformed plants and kan^R plants obtained after transformation with the binary vector pCPO5 (Florack *et al.*, 1995) without the cecropin B gene insertion, served as controls.

Transgenic plants were multiplied *in vitro* and then grown in the glasshouse until maturity. Small tubers harvested from such plants were used either for resistance testing or as seed potatoes in a multiplication in the field, which was covered by licence DGM/SVS number BGGO 92/17 of the minister of Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer (VROM). Cultivation in the field was carried out according to the procedures and guidelines of this licence which included the picking of flower buds to prevent seed set and pollen dispersal.

Northern and Western blot analysis. Northern blot analysis was carried out with 12 µg aliquots of total RNA of young leaves from glasshouse-grown plants, as described by Florack *et al.* (1994). The *Hpa* I/*Bam* HI fragment of pCPOC4, containing the cecropin B coding sequence labelled with ^{32}P, was used as probe.

Proteins were extracted by grinding young leaves (ca. 25 mg fresh weight) under liquid nitrogen and incubation of the ground material for 20 min at room temperature with buffer containing 80 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol (Jaynes *et al.*, 1993) and 100 µM chymostatin. Intercellular washing fluids (IF's) were isolated according to the method of De Wit and Spikman (1982). Protein extracts and IF's were subjected to Western analysis using SDS-tricine polyacrylamide gel electrophoresis and immunological detection techniques as described (Florack *et al.*, 1994; Florack *et al.*, 1995). A synthetic cecropin B (American Peptide Co., Santa Clara, USA) was used as a positive control and for reconstructions.

Tuber tissue resistance tests. Small tubers and tuber slices were screened for resistance to *Erwinia carotovora* subsp *atroseptica* (*Eca*) collection number IPO 161 and *E. chrysanthemi* (*Ech*) IPO 502, both obtained from the DLO-Research Institute for Plant Protection (IPO-DLO), Wageningen, the Netherlands, according to the procedures described in Chapter 2 which were based on the method of Lapwood and Read (1985). Small tubers were harvested from glasshouse-grown plants within a three weeks period and stored at 4 °C for three to 21 days. Slices were cut from tubers that were harvested from field-grown plants and stored at 4 °C for eight to 14 weeks. The concentrations of the inocula were 1.0 x 10^9 colony-forming units ml^{-1}. After inoculation, small tubers and slices were incubated aerobically for five days at 20 °C using ten tubers per clone per bacterial species. After incubation, rotted tissue was scraped from the tubers and resistance expressed as the calculated mean diameter of the resulting cavity, measured at right angles. Analysis of variance was carried out by ANOVA.

Results and discussion

Analysis of transgene expression and stability of cecropin B. The transformation experiments that were carried out with the cecropin B coding gene construct yielded 49 independent kan^R clones. Figure 8.1 shows the results of a Northern blot analysis of these clones. Cecropin B gene specific mRNA levels in young leaves varied per transgenic clone from a non-detectable level (Agria 32 and Kondor 72) to about 0.6% of total mRNA (Producent 34 and Kondor 43) when estimating that the polyadenylated RNA fraction is about 0.1% of total RNA. As expected, no cecropin B specific mRNA was detected in control plants (Figure 8.1, lanes marked with C1 and C2). The level of transgene transcription was comparable with that found in tobacco plants transformed with the same gene construct (Florack *et al.*, 1995). Florack *et al.*, (1995) ascribed the considerable transcription levels found to two characteristics of the chemically synthesized hordothionin signal peptide sequence being the optimized translation initiation region and adaptation of codon usage to solanaceous crops.

No cecropin B peptide could be detected by Western blot analysis although approximately 50 µg aliquots of leaf protein extracts from transgenic Kondor plants, with moderate and high transcription levels on Northern blots, were analyzed (data not shown). The detection level was routinely 1-10 ng. Stability of cecropin B was studied by mixing 100 ng aliquots of the peptide with IF or 5.0 µg of total leaf protein, extracted in buffer containing 80 mM Tris-HCl pH 6.8 and 10% glycerol, and

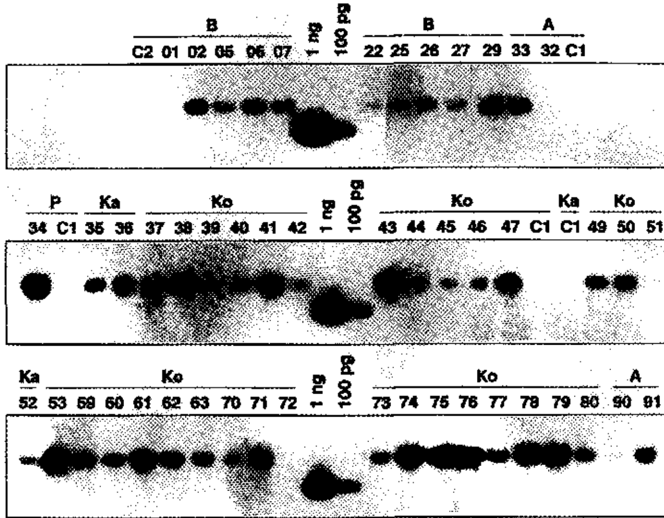

Figure 8.1. Northern blot analysis 48 transgenic clones. Transformed cultivars were Agria (A), Bintje (B), Karnico (Ka), Kondor (Ko) and Producent (P). C1, non-transformed control; C2, kan^R control. Reconstructions were made by adding 1 ng and 100 pg of *Hpa I/Bam*H I fragment of pCPOC4 DNA to RNA of control plants. The ^{32}P labelled fragment was used as probe.

incubation for 1 h at room temperature. Western blot analysis of these mixtures revealed that roughly 90% of cecropin B was degraded unless IF and protein extracts were boiled for 5 min prior to mixing and incubation. Stability of cecropin B in IF and protein extracts slightly increased by the addition of chymostatin, an inhibitor of chymotrypsin-like proteases (data not shown). These results were also in agreement with a previous study on pCPOC4 transformed tobacco plants (Florack *et al.*, 1995). No cecropin B was detected in such plants and the peptide was rapidly degraded when incubated with IF or the protein extract of the tobacco line that was used for transformation.

Soft rot resistance. In a preliminary experiment, small tubers of one transgenic clone of each of the cvs Agria, Bintje, Karnico and Producent with a high transcription level, and seven transgenic clones of cv Kondor with varying transcription levels (Figure 8.1) were screened for resistance to *Eca* and *Ech* (Table 8.1). None of these 11 clones showed a significantly smaller diameter of rotted tissue ($P<0.01$) when compared with the appropriate controls after inoculation with *Eca* or *Ech*, except for one clone, cv Kondor number 79, which showed significantly less rot ($P<0.01$) after inoculation with *Ech*. However, this clone produced no tubers under field conditions which indicates that its cultivar characteristics had been altered during the transformation process. Three transgenic clones (cv Bintje number 29 and cv Kondor numbers 50 and 51) showed significantly more rot ($P<0.01$) than the controls after inoculation with *Eca*.

In a second experiment, tuber slices of field-grown plants of all available transgenic clones, except for cv Kondor number 79, were screened for resistance to *Eca* and *Ech* (Table 8.2). Mean diameters of rotted tissue, averaged across all transgenic clones that were derived from one cultivar, were not markedly smaller in comparison to either non transformed or kan^R controls. Even the most resistant transgenic clones within such groups did not show significantly less rot ($P<0.01$) than

Table 8.1. Diameter of rotted small tuber tissue (mm) after inoculation with *Eca* or *Ech*. Values are means across ten tubers.

	Controls[1]		Transgenic clones		
Cultivar	*Eca*	*Ech*	Clone	*Eca*	*Ech*
Agria	9.0	12.8	91	8.7	11.6
Bintje	8.7	10.6	29	10.3	11.1
Karnico	8.7	12.1	35	7.9	11.1
Producent	7.8	11.2	34	7.7	10.7
Kondor	8.3	11.4	43	7.5	10.0
"			50	11.9	11.5
"			51	10.6	10.5
"			61	8.5	10.4
"			70	8.2	11.0
"			74	8.5	11.3
"			79	8.0	9.8

LSD ($P<0.01$) = 1.5 for cultivar x inoculum means

[1] Agria, Karnico, Kondor, Producent non-transformed; Bintje kan^R

Table 8.2. Mean diameter of rotted tuber slice tissue (mm) after inoculation with *Eca* or *Ech*. NT, non-transformed; ND, not determined.

Cultivar	Controls[1]		Transgenic clones[2]			
	NT	Kan[R]	No tested	Minimum[1]	Mean	Maximum[1]
Eca						
Agria	16.9	ND	4	16.1	17.0	17.7
Bintje	12.2	13.4	10	11.7	13.5	15.6
Karnico	11.1	8.8	3	9.7	10.0	10.2
Producent	12.5	ND	1		10.3	
Kondor	12.4	12.5	30	10.2	11.9	17.5
Ech						
Agria	12.0	ND	4	10.6	12.6	13.6
Bintje	10.7	11.3	10	10.7	11.8	14.3
Karnico	11.4	10.4	3	10.6	11.0	11.3
Producent	11.3	ND	1		11.0	
Kondor	11.4	12.4	30	10.6	12.6	17.7

LSD ($P<0.01$) = 2.4 for cultivar x inoculum means

[1] Values are means across ten slices

[2] Presented are: the number of clones tested, the mean diameter of rot of the most resistant clone, the mean diameter of rot across all tested clones and the mean diameter of rot of the most susceptible clone.

either type of controls. After inoculation with *Eca*, two transgenic clones (cv Kondor number 43 and 47) showed significantly more rot ($P<0.01$) than the controls. The same was found for cv Bintje number 05 and again cv Kondor 43 after inoculation with *Ech*.

Apparently, introduction of the cecropin B gene construct into the five cultivars did not improve their level of soft rot resistance. Since Northern blot analysis revealed that, on average, the cecropin B gene in transgenic clones was well transcribed, the reason for this is probably post transcriptional. Since no cecropin B peptide could be detected in protein extracts of transgenic clones, the concentration of the peptide in tuber tissue might be simply to low for a significant inhibition of the bacterial multiplication. Results of the experiments carried out to study the stability of commercially available cecropin B in the presence of potato extracts indicate that proteolytic activity of potato tissue probably contributes to rapid degradation of *de novo* synthesized cecropin B in transgenic plants and, as a consequence, to the

non detectable level of cecropin B. Protein engineering by altering the primary structure of cecropin B *in such a way that proteolytic degradation of cecropin* B in potato tissue is minimized, but antibacterial activity fully retained, seems necessary for improvement of soft rot resistance by the transgenic approach described here.

Chapter 9

General discussion

The aim of the work described here was to identify components and sources of resistance to pectolytic *Erwinia* spp., the causal pathogens of potato blackleg. Components of resistance were studied in order to obtain a better understanding of the plant-pathogen interaction and to determine how selection for resistance in early stages of a breeding programme can be carried out efficiently. The sources of resistance studied were either cultivars, somatic hybrids between *Solanum tuberosum* and the wild species *S. brevidens* or single genes encoding peptides with antibacterial properties *in vitro*.

Components of resistance

This thesis describes components of resistance in a different way than those described for the so called "compound interest diseases" such as rusts and powerdy mildews that have several to many reproductive cycles per growing season (Parlevliet, 1979). Components of resistance to blackleg in the tuber tissue, the stem base and the stem tissue simply correspond to the stages of the infection process that lead to development of above ground blackleg lesions and associated symptoms caused by pectolytic *Erwinia* spp. In diseased plants, the pathogen goes through these stages only once per growing season. Multiplication of the pathogen is restricted to individual plants and does hardly interfere with the onset of disease development in adjacent plants (Pérombelon, 1992).

Since blackleg in the field invariably originates from a rotting mother tuber (Pérombelon & Kelman, 1980) initially, tuber tissue resistance was considered as an important putative component of resistance and studied in detail (Chapter 2). Tuber tissue resistance of cultivars was strongly affected by the oxygen concentration during incubation and to a lesser extent, also by the *Erwinia* (sub-)spp. used for inoculation. Cultivars differed significantly for tuber tissue resistance to *Erwinia carotovora* subsp. *atroseptica* (*Eca*) and *E.c.* subsp. *carotovora* (*Ecc*) either under aerobic or anaerobic conditions and also for resistance to *E. chrysanthemi* (*Ech*) under anaerobic conditions. Cultivar differences were reproducibly found in repeated experiments. In the course of the work however, it became clear that tuber tissue resistance of seed tubers in

storage did not correlate with resistance to blackleg in the field. The lack of correlation was surprising. It could be explained by the observed rapid changes in tuber tissue resistance after the planting of seed potatoes in the field (Chapter 3). Thus, tuber tissue resistance (of stored tubers) can not be considered as an important component of resistance to blackleg. It can not be excluded however that tuber tissue resistance plays a role in determining the resistance of genotypes to non-emergence in the field, since non-emergence is a result of rot of the seed tuber by *Erwinia* before or during the early phase of germination and sprout development but this was not investigated.

Results of a field experiment in one year showed that cultivars differed for the extent to which the process of mother tuber decay after planting was enhanced by inoculation with *Eca* or *Ech* (Chapter 3). The observed differences between cultivars in disease incidence in the field experiment of 1993 could partly be explained by differences in the extent of mother tuber decay after inoculation. Mother tuber decay by *Erwinia* therefore, can be considered as a component of resistance to blackleg (Chapters 3 and 4). Further studies on the genotypic differences for the process of mother tuber decay may elucidate more important aspects of the potato-*Erwinia* interaction; it would be interesting to see whether similar differences between cultivars in the process of mother tuber decay (Figure 3.1 in Chapter 3) can be observed in different environments. Furthermore, the biochemical factors which trigger the start of the rotting process deserve further study. For example, it could be argued that the relatively high level of resistance of cvs Karnico and Producent is related to their lateness or high starch content. Another important aspect that remains to be studied is the relation between genotypic dependent mother tuber decay and the risk for daughter tuber contamination by bacteria from the rotting mother tuber before or during seed potato harvest and subsequent post harvest handling, since if the mother tubers of a given genotype start to rot relatively late during the growing season they could form an important source of contamination for the daughter tubers.

The mother tubers of water treated control plants also decayed during the growing season. This is in agreement with what is seen in commercial potato cultivation. Mother tubers have usually completely vanished at harvest of a mature crop. Apparently, pectolytic *Erwinia* bacteria have to compete with saprophytic microorganisms and possibly also with other pathogenic microorganisms that can use the mother tuber tissue as a substrate for multiplication. Although competition among pectolytic *Erwinia* has had some attention in literature (Pérombelon *et al.*, 1987; Pérombelon *et al.* 1988), the competition with other microorganisms is poorly

studied. More research on this aspect of blackleg development not only can lead to the identification of microorganisms that can be used for biological control but may also elucidate why isolates of *Ecc* show only weak pathogenicity in field experiments (Pérombelon *et al.*, 1987; Pérombelon *et al.* 1988, Zink & Secor, 1982; Chapter 3), while under laboratory conditions, at 20 °C, *Ecc* IPO 163 is as capable as *Eca* IPO 161 and *Ech* IPO 502 in causing decay of tuber slices and small tubers under aerobic and anaerobic conditions (Chapter 2). In the stem base resistance tests and the tests for stem tissue resistance of glasshouse-grown plants, *Ecc* IPO 163 was found to be even more pathogenic than *Eca* IPO 161 (data not shown). However, in comparison to *Eca* IPO 161 and *Ech* IPO 502, *Ecc* IPO 163 caused far less blackleg in the field experiments of 1992 (Chapter 3). It could be argued therefore, that the weak pathogenicity of *Ecc* is due to a low competitive ability at low cell densities. Because of its low pathogenicity in field experiments, low priority can be given to screening for resistance to *Ecc*.

In addition to tuber tissue resistance of seed tubers in storage (Chapter 2) and mother tuber decay in the field (Chapter 3), two other putative components of resistance were studied; stem base resistance and above ground stem tissue resistance (Chapter 4). It was shown that stem base resistance was the most important of the components studied in determining resistance of the 12 cultivars to blackleg in field experiments. Although the existence of this component was already postulated in the seventies by Munzert (1975), it was surprising and intriguing to find the component to be so important for blackleg resistance. Yet, the mechanism of stem base resistance is not understood and needs further research. Genotypic differences can possibly be explained by differences in the amount and type of lignification (Weber, 1990). In addition, the specificity of stem base resistance to different isolates of the *Erwinia* spp. needs further research.

Some genotypic variation for above ground stem tissue resistance was found among the cultivars. Regression analysis, however, revealed that stem tissue resistance was of minor importance.

Resistance is often defined as the ability of the host to hinder the growth and/or development of the pathogen (Parlevliet, 1979). The most important components of resistance identified, are a reduced mother tuber decay in the field and a reduced chance that the mass of *Erwinia* bacteria in the rotted mother tuber tissue succeed in passing the stem base and subsequently initiate rot of the stem tissue. The differences in the amount of mother tuber decay observed may simply be a result of differences in suitability of the mother tuber tissue as a substrate for bacterial multiplication at certain moments during plant development. The observation that blackleg lesions

develop in some, but often not all stems of a plant (Pérombelon, 1992), also does not point in the direction of a well orchestrated genetic response against the invading bacteria. The stem base could simply act as a physical barrier for the mass of bacteria in the rotting mother tuber that is more often passed in some cultivars than in others. These considerations are not necessarily in conflict with studies by Yang *et al.* (1991) and Yang *et al.* (1992) who found that *Erwinia* bacteria elicit transcription of plant genes involved in the general defence response to invading pathogens. These groups worked with tuber slices under aerobic conditions. Also, genotypic differences for disease severity in stem inoculation assays, to some extent could be ascribed to the expression of genes which products hinder the growth of the bacteria (Abenthum *et al.*, 1993). As was shown in Chapters 3 and 4 however, tuber tissue resistance and stem tissue resistance are of minor importance as components of field resistance. Thus, the host reaction observed in these tissues by the research groups mentioned may take place but, at the same time, may not account for a large percentage of the genotypic variation found for blackleg resistance under field conditions.

Screening methods

The results obtained with the different screening methods as described in Chapters 2 to 5 clearly illustrate the correctness of the statement formulated by Wastie and Mackay (1985) that reproducibility of results of a screening method *per se* does not guarantee a good correlation with resistance under practical conditions. It is amazing that in spite of a large number of studies (reviewed in Chapter 1), so little attention has been paid to this aspect of screening for *Erwinia* resistance. The tuber slice inoculation method as described in Chapter 2, although of little use for screening to blackleg resistance, could be expected to be better suited to screening of potato clones for soft rot resistance. But also for this purpose, the correlation with resistance under practical storage conditions still remains to be determined. In the course of this research, only the usefulness of a method for screening for resistance to blackleg has been studied. It is concluded from the work described in Chapters 2 to 5 that, as yet, no accurate screening methods are available, suitable for selection of large numbers of clones in early stages of the selection process. The screening method for stem base resistance as described in Chapter 4, however, could be considered as a promising candidate as long as it is realized that the results of this method only explain about 60% of the variation for resistance to blackleg in the field. Furthermore, it needs to be realized that the experiments have been done with only 12 cultivars, and the

method should first be validated on a larger group of cultivars that are well characterized for blackleg resistance in the field, before conclusions can be extrapolated and reliably applied in large scale breeding programmes.

The screening for resistance in the field by using vacuum-infiltrated seed tubers can be considered as a reliable method as long as conclusions are drawn from experiments involving more than one year and more than one location. It is the most reliable, but also the most expensive method and only suitable for application in the later stages of a breeding programme.

Now that immunological and molecular techniques for detection of the different *Erwinia* (sub-)spp. in large numbers of samples become available, it is highly recommended to start a survey aiming to determine which of the *Erwinia* (sub-)spp. (and possibly, pathotypes thereof) cause most of the blackleg during seed production in the Netherlands, or, are most threatening for the potato cultivation in areas to which seed potatoes are exported. Results of such a survey would allow breeders to choose the most relevant isolates to be used when screening for resistance of clones.

Sources of resistance

High levels of soft rot resistance have been observed for interspecific somatic hybrids between specific clones of *S. tuberosum* and *S. brevidens*. This resistance seemed transferable to backcross progenies (Austin *et al.*, 1988). As was described in Chapter 5, a small first backcross population of this material was screened for tuber tissue resistance. Clones of this population were scored as relatively resistant, especially under anaerobic conditions. However, similar to the finding that only a poor correlation exists between tuber tissue resistance of cultivars and their resistance to blackleg in field tests (Chapter 3), it was also shown for clones of four second backcross populations from the *S. brevidens*-derived material, that there was no correlation between tuber tissue and blackleg resistance (Chapter 5). Within backcross populations, however, some variation for blackleg resistance was observed and three out of four first backcross generation parental clones showed very little mother tuber decay in the field and, probably in relation to that, no single blackleg stem. Nevertheless, the question arises whether, as a source of resistance to blackleg, it is necessary to use *Solanum* spp. that are taxonomically so distinct from cultivated potato. The time and effort necessary to assess the blackleg resistance of individual clones, the probability that partial resistance to blackleg is most likely of polygenic nature as well as the limited recombination between the *S. tuberosum* and

S. brevidens genomes (McGrath *et al.*, 1994) make introgression of putative resistance genes from *S. brevidens* accessions as a source of resistance likely to be extremely time consuming. A further drawback for the work in Chapter 5 was that the progenies of crosses with the original somatic hybrids, until 1993, were considered as genetically modified organisms and subject to environmental legislation. The guidelines of the licence which covered the work with the somatic hybrids-derived material strongly impaired the breeding process. As far as breeding for resistance to blackleg is concerned, such guidelines make somatic hybrids between two non-crossable parental species as source of resistance very unattractive for use in commercial breeding programmes.

While the genetic variation for resistance to blackleg among the progeny of the somatic hybrids was less than expected, the genetic variation for blackleg resistance within commercial cultivars (Chapter 3) was found to be encouraging and larger than initially expected on the basis of data presented in the literature (Hossain & Logan, 1983; Lapwood & Gans, 1984; Lapwood & Read, 1986b). It should be pointed out that the variation among cultivars was found in spite of the fact that, in general, breeders tended to pay little attention to blackleg resistance during clonal selection. Although every year a certain percentage of the seed potato acreage of even the most resistant cultivars that were tested as part of the work described in Chapters 2 to 4, is still being declassified due to blackleg incidence during seed production in the field, it seems worthwhile to explore the variation found within *S. tuberosum*, since it would contribute much to a solution of the blackleg problem, if newly introduced cultivars would have a level of resistance that would be comparable with the level of the cvs Producent and Karnico.

Transformation with gene constructs encoding antibacterial peptides

Apart from existing resistance in cultivars and wild species, also the effect of genetically engineered resistance was investigated. In spite of the strong expression of α-hordothionin in transgenic clones of cvs Kondor and Bintje, tuber tissue resistance of these clones had not increased in comparison with control clones (Chapter 7). This was most likely the result of the low toxicity of α-hordothionin to *Erwinia* (Florack *et al.*, 1993). Although cecropin B shows a much higher *in vitro* toxicity to *Erwinia* than α-hordothionin (Destéfano-Beltrán *et al.*, 1990; Nordeen *et al.*, 1992; Hightower *et al.*, 1994), improvement of tuber tissue resistance by transformation with a cecropin B encoding gene could also not be achieved (Chapter 8). This is probably

due to the rapid degradation of cecropin B by plant proteolytic enzymes (Chapter 8; Florack *et al.*, 1995). A small but reproducible and significant increase of tuber tissue resistance was obtained after transformation with gene constructs encoding tachyplesin I, a peptide which seems to combine the biochemical stability of α-hordothionin with an *in vitro* toxicity which approximates that of cecropin B. It is concluded from the work described in Chapters 6, 7 and 8, that of the three variants of the biotechnological approach conducted, the transformation with tachyplesin I encoding gene constructs was most successful with respect of enhancement of tuber tissue resistance to *Erwinia*.

The value of this application for practical potato cultivation remains to be studied. Damage to ware potatoes by *Erwinia* soft rot is believed to be associated with anaerobic conditions (Pérombelon & Kelman, 1980; Davis *et al.*, 1990). Results of screening of tachyplesin I expressing clones under anaerobic conditions indicate that resistance to *Eca* is only marginally improved (Chapter 6), and transformation with tachyplesin I encoding genes may not be of any use when aiming to obtain cultivars with good resistance under practical storage conditions. Due to shortage of time and licence regulation, the transformants were not tested in the field. Whether the observed improvement of tuber tissue resistance to *Eca* under aerobic conditions is meaningful with regard to blackleg resistance in the field, can, in view of the existence of components of resistance as discussed above, be only a subject of speculation. As far as the role of the mother tuber is concerned in determining resistance to blackleg, it is likely that due to the constitutive action of the cauliflower mosaic virus (CaMV) 35 S promotor, tachyplesin I is expressed during development of the seed potato similarly as was found for α-hordothionin expressing clones (Chapter 7). It is unknown whether the CaMV 35S promotor is active after maturation and subsequent storage of the seed or even during plant development after planting in the field. It is also unknown whether tachyplesin I is degraded during these phases of vegetative multiplication and, if so, to which extent. If, however, a small amount of tachyplesin I is present in the developing seed tuber, it may well inhibit the growth of *Erwinia* bacteria and cause a shift in favour of saprophytic microorganisms that compete with *Erwinia* for the mother tuber tissue. If these saprophytes are less or not affected by tachyplesin I, than the extent to which *Erwinia* bacteria are able to initiate rot of the mother tuber and the stem tissue will decrease and hence, reduce blackleg. Expression of tachyplesin I in stem tissue might be expected as a result of the use of the CaMV 35S constitutive promotor. However, it is not clear whether the finding that tachyplesin I loses its bactericidal activity in the

presence of crude stem extract (Chapter 6), has any consequences for the expected effect of transgene expression on stem tissue resistance.

Unfortunately, the use of a construct with a signal peptide from the pathogenesis related protein S (PR-S-SP), which can be detected in the apoplast of tobacco leaves (Cornelissen *et al.*, 1986), did not give any transgenic plants that secreted tachyplesin I to detectable amounts in the intercellular fluids (Chapter 6). Several questions about the function of signal peptides arise when taking the results of the work described in Chapters 6 and 7 into account; the remarkable higher level of α-hordothionin expression compared with expression of the tachyplesin I encoding genes may be ascribed to the fact that both the signal peptide and the mature peptide belong to same precursor and that both are from plant origin. Furthermore, it is not clear what causes the transcription level of PR-S-SP-tachyplesin I-encoding constructs to be lower than α-hordothionin signal peptide (HT-SP)-tachyplesin I-encoding constructs (Chapter 6). It would also be interesting to investigate whether secretion of tachyplesin I to detectable levels in the apoplast of potato can be obtained by using other signal peptides. Further research is needed about the function of signal peptides and the possibilities of using signal peptides for the targeting of proteins in transgenic plants.

The results of the work described in Chapters 6, 7 and 8 and the earlier work on transformation to obtain resistance to bacterial diseases that has been reported, make it clear that the introduction of genes encoding antibacterial peptides *per se* does not guarantee enhancement of resistance to bacterial pathogens that are susceptible to such peptides *in vitro*. Successful applications of the transformation approach at laboratory scale has now been described for gene constructs encoding T4 lysozyme (Düring *et al.* 1993) and tachyplesin I (Chapter 6), both resulting in improved resistance of potato to *Eca*. In both cases the positive effect on resistance was found although the levels of transgene expression were relatively low. Carmona *et al.* (1993) reported improved resistance of α-hordothionin expressing tobacco plants when inoculated with *Pseudomonas syringae* pv *tabaci* and *P.s.* pv *syringae*. Recently, Norelli *et al.* (1994) who used gene constructs encoding the antibacterial peptide attacin E, found improved resistance of an apple clone to *E. amylovora*. In contrast, transgenic tomato plants that were transformed with a gene construct encoding α-hordothionin did not show enhanced resistance to *Clavibacter michiganensis* subsp. *michiganensis* or *Xanthomonas campestris* pv *vesicatoria* in spite of the high levels of transgene expression found (Florack, 1994). Also, transformation of potato and tobacco with gene constructs encoding cecropin B did not result in enhanced resistance (Chapter 8; Jaynes *et al.*, 1993; Florack, *et al.*, 1995) unless the primary structure of the peptide

was modified (Jaynes *et al.*, 1993). Apparently, *in vitro* toxicity of an antibacterial peptide and the level of its expression in transgenic plants are not the only factors that determine the success of transformation as a means to acquire resistance. In spite of the broad antibacterial spectrum of most antibacterial peptides, the outcome seems rather unpredictable and to depend on the combination of plant species, peptide and pathogen used. Furthermore, the agricultural value of the enhanced resistances reported still remains to be studied. If, however, a level of resistance to blackleg could be obtained which is equal to or better than the level of resistance of cv Producent (Chapter 3), it is beyond doubt that, from a technical point of view, potato breeders will prefer the biotechnological approach which enables them to add this single trait to commercial cultivars rather than starting a time consuming classical breeding programme.

Conclusions

An attempt was undertaken to analyze the complex interaction between potato cultivars and pectolytic *Erwinia* spp. in terms of components of resistance, aiming to identify the components that determine resistance to blackleg under field conditions. The result of this work indicate that selection for resistance under laboratory or glasshouse conditions can best be carried out by selection for high levels of stem base resistance. Selection for stem base resistance is more efficient than selection for high levels of tuber tissue resistance or stem tissue resistance. The most accurate testing for blackleg resistance can only take place under field conditions. Thus, in spite of the increased knowledge about components of resistance obtained in this study, it is clear that accurate determination of blackleg resistance of a given clone remains laborious and time consuming.

Further research must be focused on the inheritance of important components of resistance before conclusions can be drawn about the existence of resistance genes and the probability that crosses between resistant parents may give a more resistant progeny than when susceptible parents are crossed. Due to the lack of screening methods that can be applied in early phases of the selection process and the uncertainty concerning the existence and quality of resistance genes in wild species or about bactericidal protein constructs, it seems advisable for breeders first to try to explore the genetic variation within cultivated potato. It is obvious that the use of wild relatives such as *S. brevidens* as a source of resistance will be extremely time consuming. The addition of resistance to modern cultivars by means of genetic

transformation with bactericidal protein encoding genes is highly attractive, but as yet, extensive screening of transgenic plants expressing antibacterial peptides under field conditions must elucidate the usefulness of this method to obtain potato cultivars with a high level of resistance to blackleg.

Summary

"Blackleg" is a disease of the potato crop which, in the Netherlands, negatively affects the quality of seed potatoes, thereby causing financial damage to the seed potato grower. The disease is caused by the bacteria *Erwinia carotovora* subsp. *atroseptica* (*Eca*), *E. chrysanthemi* (*Ech*) or, to a lesser extent by *E.c.* subsp. *carotovora* (*Ecc*). These bacteria also can cause "soft rot" of potatoes in storage. Soft rot develops easily when the tuber tissue is subjected to anaerobic conditions, probably as a result of the oxygen dependent resistance mechanisms being hindered. Soft rot is a less important disease in the Netherlands in comparison to blackleg. When the research project started, it was known from literature as well as from practical potato production, that genotypic variation for resistance to blackleg exists among commercially grown cultivars, primitive cultivars and wild relatives of the cultivated potato. The observed resistance, however, always was partial. Several environmental factors affect the expression of resistance and a clear and reliable method for selection of the existing genotypic variation was not available. Attempts to explain the differences for resistance to blackleg in terms of components of resistance are described in Chapters 2 to 4.

Twelve commercial cultivars were screened for resistance to blackleg in the field at two locations (one on sandy soil and the other on clay) in two successive years. Inoculation was carried out by vacuum infiltration of the seed potatoes in bacterial suspensions of *Eca*, *Ech* or *Ecc* before planting. *Ecc* was used in one year only (Chapter 3). Clear differences between the cultivars were found which could be reproduced across years and locations.

Since blackleg in the field invariably originates from a rotting mother tuber, the tuber tissue resistance of 12 cultivars was studied in detail (Chapter 2). Tuber slices cut from seed potatoes from storage and small tubers harvested from glasshouse-grown plants were inoculated with bacterial suspensions and incubated for three or five days at different oxygen concentrations. Differences between cultivars in tuber tissue resistance to *Eca*, *Ech* and *Ecc* were found. The resistance of the cultivars was strongly affected by the oxygen concentration during incubation and the bacterial species that was used for inoculation. The rank order of the cultivars was more affected by the *Erwinia* spp. used for inoculation than by the chosen isolate within an *Erwinia* spp. The rank order of the cultivars in an experiment with *Eca* was hardly affected by the site of inoculation being either in the centre of a slice or at the edge. Cultivars showed hardly differences for resistance to *Ech* when tuber slices or small

tubers were incubated aerobically (in air). In general, cultivar differences were reproducibly found within and between years, except for this last mentioned combination of *Erwinia* spp. and incubation condition. For *Eca* and *Ecc*, a moderate correlation was found between tuber tissue resistance of small tubers and slices when incubation was in air. Unfortunately, hardly any correlation was found between tuber tissue resistance and resistance to blackleg in the field. Subsequently, in a series of four experiments, tuber tissue resistance of the 12 cultivars was studied by inoculation of tuber slices cut from tubers directly from storage or after one, three or five weeks of growth in the field. It appeared that the relative tuber tissue resistance of the cultivars changed drastically after planting. The lack of correlation between tuber tissue resistance and field resistance can be ascribed to this effect. In a field experiment which was carried out with vacuum-infiltrated seed of the 12 cultivars, it was found that the inoculation with *Eca* or *Ech* enhanced the natural process of mother tuber decay and that the differences for resistance to blackleg in the field partly could be ascribed to the rate at which this occurred.

Since the movement of bacteria from the mother tuber into the stem is necessary for development of blackleg, it was then studied whether cultivars differed for the extent to which the bacteria passed the site of attachment between the tuber and sprout. Under laboratory conditions, cultivar differences were found (particularly when inoculation was with *Ech*) for the extent to which the rotting process of tuber tissue proceeds in the stem tissue (Chapter 4). This mechanism of resistance was called stem base resistance. In addition, glasshouse-grown plants of the 12 cultivars showed some differences for above ground stem tissue resistance to blackleg. To some extent, the differences for stem tissue resistance were reproducible among years. Tuber tissue resistance, mother tuber decay in the field, stem base resistance and stem tissue resistance can be considered as components of resistance. Therefore, their importance in relation to field resistance was studied by stepwise multiple regression analysis. For *Eca*, this analysis indicated that the components of resistance together accounted for 63% of the variation in the field. For *Ech*, 65% of the variance was accounted for by stem base resistance. The screening method for this component of resistance, which can be carried out in the laboratory, can therefore be considered as a good candidate for routine application in breeding programmes when aiming to select for high levels of resistance to blackleg.

In the eighties, somatic fusion products were made between *Solanum tuberosum* and non tuber-bearing *S. brevidens* by Prof Helgeson and co-workers. These hybrids, and the offspring obtained by back crossing with cv Katahdin (the BC1), showed a high level of resistance to soft rot. Seed of the BC1 was provided to us by

Prof Helgeson. Eleven BC1-clones were used as mothers in crosses with progenitor AR80-127-5 (Chapter 5). In total 583 clones grown from these second backcross populations (BC2's) were screened for tuber tissue resistance in the seedling stage and after multiplication in the field. Clones from four of these eleven BC2 populations were screened in the field for resistance to blackleg. No clear segregation for resistance was found. The BC2 population means were more or less equal to the mid parent values in those cases were parental clones showed clear differences for tuber tissue resistance. No correlation was found between tuber tissue resistance in the seedling stage, tuber tissue resistance after multiplication in the field or resistance to blackleg. It was not possible to draw conclusions about the existence of resistance genes in *S. brevidens*.

Possibly, resistance to bacterial diseases in plants can also be obtained by transformation with gene constructs encoding peptides with antibacterial properties. Four gene constructs were made encoding tachyplesin I, a peptide which shows antibacterial activity against *Erwinia in vitro* (Chapter 6). The gene constructs encoded precursors consisting of the hordothionin signal peptide (HT-SP) or the signal peptide of pathogenesis related protein "S" (PR-S-SP) and mature tachyplesin I. Two constructs encoded in addition, a precursor with a carboxyl terminal acidic polypeptide as found in the cDNA of tachyplesin I. The cvs Bintje (moderately susceptible), Karnico (partially resistant) and Kondor (susceptible) were transformed with these constructs. Twenty five to 30 transgenic clones per cultivar per construct were analyzed for expression of the tachyplesin I peptide. About 10% of the transgenic clones of cvs Bintje and Kondor and about 30% of the transgenic clones of cv Karnico which were transformed with the constructs containing the HT-SP encoding sequence showed expression of tachyplesin I. Transgenic clones which were transformed with the two other constructs containing the PR-S-SP encoding sequence showed no expression of tachyplesin I. This was possibly a result of the low level of transcription of the introduced genes. In a repeated experiment, small tubers of the three transgenic clones of each cultivar with the highest level of expression of tachyplesin I (only clones transformed with constructs containing the HT-SP encoding sequence were tested), showed a higher level of tuber tissue resistance under aerobic conditions in comparison with non-transformed control clones. The effect of transformation was less clear under anaerobic conditions.

A transformation experiment with a gene construct encoding a precursor which consisted of the HT-SP, mature α-hordothionin and a carboxyl terminal acidic polypeptide, yielded 31 transgenic clones of cv Kondor and one of cv Bintje (Chapter 7). α-Hordothionin is an antibacterial peptide from barley. The introduced

gene was strongly expressed in most of the transgenic clones. Small tubers of clones with varying levels of expression of this gene showed no enhanced resistance to tuber tissue resistance. This could partly be explained by the low toxicity of α-hordothionin to *Erwinia in vitro*.

Cvs Agria (susceptible), Bintje, Karnico, Kondor and Producent (resistant) were transformed with a gene construct encoding a precursor consisting of HT-SP and cecropin B (Chapter 8). Cecropin B is an antibacterial peptide from the silk moth which is toxic for *Erwinia in vitro*. Similar transformations have been carried out by other research groups. Transcription of the introduced gene was found in almost all of the 49 transgenic clones obtained but cecropin B could not be detected. Small tubers en tuber slices cut from tubers which had been harvested from plants in the field showed no enhanced resistance to *Erwinia*. This is probably due to instability of cecropin B in plant tissue.

In conclusion, resistance to blackleg is difficult to assess. Tuber tissue resistance of tubers from storage shows no correlation with resistance to blackleg in the field. Possibly, the cultivar differences found for tuber tissue resistance under aerobic or anaerobic conditions do show a good correlation with resistance to soft rot under practical storage conditions but this remains to be studied. Resistance to blackleg can be determined by screening in the field. Selection for reduced mother tuber decay can not be carried out routinely. There are however good possibilities for selection for stem base resistance. The possibilities for selection in early vegetative generations, however, are still poor. No indication was found of high levels of resistance in *S. brevidens*, whereas the differences between commercial cultivars were larger than expected on the basis of data presented in literature. Since also the usefulness of the resistance obtained by genetic modification needs further studies, plant breeders can best try to explore the genetic variation which is found among cultivated potato. The plant-pathogen interaction between potato and *Erwinia* spp. is worth further studies, especially concerning the heritability of the most important components of resistance and the anatomic and biochemical factors which play a role in the process of mother tuber decay in the field as well as in the transition of this rotting process to the stem tissue.

Samenvatting

"Zwartbenigheid" is een ziekte van het gewas aardappel die in Nederland tot kwaliteitsverlies bij de teelt van pootgoed leidt en daarmee tot financiële schade voor de pootgoedteler. De ziekte wordt veroorzaakt door de bacteriën *Erwinia carotovora* subsp. *atroseptica* (*Eca*), *E. chrysanthemi* (*Ech*) en, in mindere mate, door *E.c.* subsp. *carotovora* (*Ecc*). Deze bacteriën kunnen ook de ziekte "natrot" veroorzaken tijdens bewaring van aardappelen. Natrot treedt vooral op wanneer het knolweefsel wordt blootgesteld aan anaërobe condities, vermoedelijk omdat zuurstof-afhankelijke resistentiemechanismen dan geblokkeerd zijn. In vergelijking met zwartbenigheid is natrot in Nederland van geringer belang. Bij aanvang van het onderzoek was uit de literatuur en vanuit de praktijk bekend dat genetische variatie voor resistentie tegen zwartbenigheid bestaat, zowel binnen commercieel geteelde rassen als in primitieve rassen en wilde verwanten van de cultuuraardappel. De gevonden resistentie was in alle gevallen partieel. Echter, diverse milieufactoren beïnvloeden het niveau van resistentie en een eenduidige manier om de beschikbare genetische variatie te beselecteren, was niet voorhanden. In de hoofdstukken 2 tot en met 4 worden pogingen beschreven om de verschillen voor veldresistentie binnen een groep van 12 rassen te verklaren uit componenten van resistentie.

De 12 commerciële rassen werden getoetst voor veldresistentie tegen zwartbenigheid op een zandgrond- en kleigrondlocatie in twee opeenvolgende seizoenen. Inoculatie vond plaats door middel van vacuüminfiltratie met bacteriesuspensies van *Eca*, *Ech* of *Ecc* van het pootgoed vóór poten. *Ecc* werd in slechts één seizoen gebruikt (Hoofdstuk 3). Er bleken duidelijke rasverschillen te zijn die herhaalbaar waren tussen lokaties en jaren. Omdat zwartbenigheid in het veld altijd ontstaat vanuit een rottende moederknol, werd in eerste instantie de knolweefselresistentie van de 12 rassen in detail bestudeerd (Hoofdstuk 2). Knolschijven die uit pootaardappelen uit de bewaring waren gesneden, en kleine knolletjes die van kasplanten waren geoogst, werden geïnoculeerd met bacteriesuspensies en vervolgens geïncubeerd gedurende drie of vijf dagen bij verschillende zuurstofconcentraties. Er werden rasverschillen gevonden voor knolweefselresistentie tegen *Eca*, *Ech* en *Ecc*. De resistentie van de rassen was sterk afhankelijk van de zuurstofconcentratie tijdens incubatie en van de bacterie waarmee werd getoetst. De rangvolgorde van de rassen werd sterker beïnvloed door de gebruikte *Erwinia* spp. dan door het gekozen isolaat binnen een *Erwinia* soort. De rangvolgorde van de rassen in een experiment met *Eca* werd weinig beïnvloed door de plaats van inoculatie, die middenin een knolschijf of aan de aan de rand ervan

plaatsvond. Wanneer schijven aëroob (in lucht) werden geïncubeerd, vertoonden de rassen nauwelijks verschillen voor resistentie tegen *Ech*. Behalve voor deze laatste combinatie van *Erwinia* spp. en incubatieconditie, waren de rasverschillen over het algemeen reproduceerbaar binnen en tussen jaren. Voor *Eca* en *Ecc* in combinatie met aërobe incubatie, werd een redelijke correlatie gevonden tussen de resultaten met kleine knolletjes en knolschijven. Helaas werd er nagenoeg geen correlatie gevonden tussen knolweefselresistentie en veldresistentie tegen zwartbenigheid. Vervolgens werd in een serie van vier experimenten de knolweefselresistentie van de 12 rassen onderzocht aan knolmateriaal dat direct uit de bewaring werd getoetst, of één, drie dan wel vijf weken na poten in het veld. Daaruit bleek dat de relatieve knolweefselresistentie van de rassen drastisch verandert na poten. Dit kan de geringe correlatie tussen knolweefselresistentie en veldresistentie verklaren. In een veldexperiment met vacuüm geïnfiltreerd pootgoed van de 12 rassen werd gevonden dat inoculatie met *Eca* of *Ech* het natuurlijke proces van moederknolrot versnelt en dat de mate waarin dit gebeurt gedeeltelijk de rasverschillen voor veldresistentie tegen zwartbenigheid verklaart.

Omdat voor het ontstaan van zwartbeen, de bacteriën zich van de moederknol naar de stengel moeten verplaatsen, werd vervolgens onderzocht of er rasverschillen zijn voor de mate waarin de bacterie de overgang tussen knol- en stengelweefsel kan passeren. Onder laboratoriumomstandigheden konden inderdaad rasverschillen worden gevonden (met name voor *Ech*), voor de mate waarin het rottingsproces van het knolweefsel overgaat in het stengelweefsel (Hoofdstuk 4). Deze vorm van resistentie werd stengelbasisresistentie genoemd. Kasplanten van de 12 rassen vertoonden bovendien enige verschillen voor zwartbeenresistentie van de bovengrondse stengel. De gevonden verschillen voor stengelweefselresistentie waren tot op zekere hoogte reproduceerbaar over meerdere jaren. Knolweefselresistentie, moederknolrot in het veld, stengelbasisresistentie en stengelweefselresistentie kunnen als componenten van resistentie worden beschouwd en daarom werd het belang ervan bestudeerd in relatie tot veldresistentie. Dit gebeurde met behulp van stapsgewijze multipele regressie analyse. Voor *Eca* gaf de analyse aan dat de componenten van resistentie gezamenlijk 63% van de variatie in het veld verklaren. Voor *Ech* werd 65% van de variatie verklaard uit stengelbasisresistentie. Daarmee is voor selectie van klonen met een goede resistentie tegen zwartbenigheid, de toetsmethode voor deze component van resistentie, die kan worden uitgevoerd in het laboratorium, een goede kandidaat om routinematig in veredelingsprogramma's te worden toegepast.

Door professor Helgeson en zijn medewerkers van de Universiteit van Wisconsin in de Verenigde Staten werden in de jaren tachtig fusieprodukten gemaakt tussen

Solanum tuberosum en de niet-knoldragende soort *S. brevidens*. Deze hybriden, en de nakomelingschap die ontstond na terugkruising met het ras Katahdin (de BC1), vertoonden een hoog niveau van resistentie tegen natrot. Zaad van de BC1 werd door professor Helgeson aan ons ter beschikking gesteld. Elf BC1-klonen werden als moeder gebruikt in kruisingen met geniteur AR80-127-5 (Hoofdstuk 5). Uit deze tweede terugkruisingsgeneraties (BC2's) werden 583 klonen getoetst voor knolweefselresistentie in het zaailingstadium en na vermeerdering in het veld. Klonen van vier van de elf BC2-populaties werden in het veld getoetst voor resistentie tegen zwartbenigheid. Er werd geen duidelijke uitsplitsing voor resistentie gevonden. De BC2-populatiegemiddelden kwamen grosso modo overeen met de mid parent values in die gevallen waar de ouderklonen duidelijk verschilden voor knolweefselresistentie. Tussen knolweefselresistentie van knolletjes van zaailingen, van knollen na vermeerdering in het veld en zwartbeenresistentie in het veld werd geen correlatie gevonden. Het was niet mogelijk om conclusies te trekken over het voorkomen van resistentiegenen in *S. brevidens*.

Resistentie tegen bacterieziekten in planten kan mogelijk ook verkregen worden door transformatie met genconstructen die coderen voor peptiden met antibacteriële eigenschappen. Er werd een viertal genconstructen gemaakt met de coderende sequentie voor tachyplesine I, een peptide dat *in vitro* antibacteriële activiteit vertoont tegen *Erwinia* (Hoofdstuk 6). De genconstructen codeerden voor precursoreiwitten bestaande uit het signaalpeptide van hordothionine (HT-SP) of het pathogenese gerelateerde eiwit "S" (PR-S-SP), het rijpe tachyplesine I, al dan niet in combinatie met het van nature in de precursor voorkomende carboxylterminale zure polypeptide. Met deze constructen werden de rassen Bintje (matig vatbaar), Karnico (partieel resistent) en Kondor (vatbaar) getransformeerd. Per combinatie van ras en construct werden 25 tot 30 transgene klonen onderzocht op expressie van tachyplesine I. Ongeveer 10% van de transgene klonen van de rassen Bintje en Kondor en 30% van de transgene klonen van het ras Karnico die waren getransformeerd met de twee constructen welke de HT-SP sequentie bevatten, vertoonden expressie van tachyplesine I. Transgene klonen die waren getransformeerd met de overige twee constructen (met PR-S-SP) vertoonden geen expressie, vermoedelijk als gevolg van een geringe transcriptie van de ingebrachte genen. Kleine knolletjes van de drie transgene klonen van elk ras met de hoogste expressie van tachyplesine I (alleen klonen die waren getransformeerd met HT-SP coderende constructen werden getoetst), vertoonden in een herhaald experiment een hoger niveau van knolweefselresistentie onder aërobe omstandigheden in vergelijking met niet getransformeerde controles. Onder anaërobe omstandigheden was het effect van transformatie minder duidelijk.

Een transformatie-experiment met een genconstruct coderend voor een precursor-eiwit, dat bestond uit het HT-SP, rijp α-hordothionine en een carboxylterminaal zuur polypeptide, leverde 31 transgene klonen van het ras Kondor en één transgene kloon van het ras Bintje op (Hoofdstuk 7). α-Hordothionine is een antibacterieel peptide uit gerst. In de meeste transgene klonen kwam het ingebrachte gen in hoge mate tot expressie. Knolletjes van klonen met een matig tot hoog expressieniveau vertoonden geen verhoogde knolweefselresistentie. Dit kan gedeeltelijk verklaard worden op grond van de geringe gevoeligheid *in vitro* van *Erwinia* voor α-hordothionine.

De rassen Agria (vatbaar), Bintje, Karnico, Kondor en Producent (resistent) werden getransformeerd met een genconstruct coderend voor een precursoreiwit bestaande uit het HT-SP en cecropine B (Hoofdstuk 8). Cecropine B is een antibacterieel peptide uit de zijderups dat *in vitro* toxisch is voor *Erwinia*. Soortgelijke transformaties zijn eerder door andere onderzoeksgroepen uitgevoerd. In bijna alle van de 49 verkregen transgene klonen kon transcriptie van het ingebrachte gen worden aangetoond maar niet het peptide. Kleine knolletjes en knolschijven die uit veldvermeerderd materiaal waren gesneden, vertoonden geen hogere niveaus van resistentie tegen *Erwinia*. Dit is vermoedelijk mede een gevolg van instabiliteit van cecropine B in planteweefsel.

Concluderend kan worden gesteld dat resistentie tegen zwartbenigheid moeilijk is vast te stellen. Knolweefselresistentie van knollen uit de bewaring vertoont geen correlatie met resistentie tegen zwartbenigheid in het veld. De gevonden rasverschillen voor knolweefselresistentie onder aërobe dan wel anaërobe condities vertonen mogelijk wel een goede relatie tot resistentie tegen natrot onder praktijkcondities, maar dit moet nog onderzocht worden. Resistentie tegen zwartbenigheid kan worden vastgesteld door middel van toetsing in het veld. Routinematige selectie voor geringe moederknolrot is niet haalbaar. Wel zijn er goede mogelijkheden voor selectie voor stengelbasisresistentie. De mogelijkheden voor selectie in vroege, vegetatieve generaties blijven echter beperkt. Omdat geen aanwijzingen werden gevonden voor hoge niveaus van resistentie in *S. brevidens* terwijl de verschillen tussen commerciële rassen groter waren dan werd verwacht op basis van gegevens uit de literatuur en omdat de bruikbaarheid van resistentie die door middel van genetische modificatie kan worden verkregen nog nader moet worden geëvalueerd, kunnen plantenveredelaars vooralsnog het beste proberen om de genetische variatie binnen de cultuuraardappel te benutten. De plant-pathogeen relatie tussen aardappel en pectolytische *Erwinia* soorten verdient nog verdere studie. Met name wat de erfelijkheidsgraad van de belangrijkste componenten van resistentie betreft en de anatomische en biochemische factoren die een rol spelen bij het proces van moederknolrot in het veld en de overgang van dit rottingsproces naar het stengelweefsel.

References

Abenthum K, S Hildenbrand, L Schilde-Rentschler & H Ninnemann, 1993. Occurrence of the phytoalexins phytuberin, solavetivone and rishitin in stem tissue of potato plants showing blackleg symptoms. Abstracts of the 12th triennial conference of the European Association for Potato Research, Paris, July 18th-23th, 1993: 376-377

Allefs J, W van Dooijeweert, PH Delfosse, E French, L Gutarra De Lindo & M. Pérombelon, 1993. A standardised method for screening of potato tubers for resistance to *Erwinia carotovora* subsp. *atroseptica*. Abstracts of the 12th triennial conference of the European Association for Potato Research, Paris, July 18th-23th, 1993: 311-312

Anonymous, 1994a. 69e Beschrijvende rassenlijst voor landbouwgewassen 1994. CPRO-DLO, Wageningen, 342 p

Anonymous, 1994b. Keuringsreglement NAK, 127 p

Austin S, MK Ehlenfeldt, MA Baer & JP Helgeson, 1986. Somatic hybrids produced by protoplast fusion between *S. tuberosum* and *S. brevidens*: phenotypic variation under field conditions. Theoretical and Applied Genetics 71:682-690

Austin S, E Lojkowska, MK Ehlenfeldt, A Kelman & JP Helgeson, 1988. Fertile interspecific somatic hybrids of *Solanum*: A novel source of resistance to *Erwinia* soft rot. Phytopathology 78:1216-1220

Bain RA & MCM Pérombelon, 1988. Methods of testing potato cultivars for resistance to soft rot of tubers caused by *Erwinia carotovora* subsp. *atroseptica*. Plant Pathology 37:431-437

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

Barsby TL, JF Shepard, RJ Kemble & R Wong, 1984. Somatic hybridization in the genus *Solanum*: *S. tuberosum* and *S. brevidens*. Plant Cell Reports 3:165-167

Beaulieu C & F van Gijsegem, 1990. Identification of plant-inducible genes in *Erwinia chrysanthemi* 3937. Journal of Bacteriology 172:1569-1575

Beaulieu C, M Boccara & F van Gijsegem, 1993. Pathogenic behaviour of pectinase-defective *Erwinia chrysanthemi* mutants on different plants. Molecular Plant-Microbe Interactions 6:197-202

Bisht VS, PS Bains & JR Letal, 1993. A simple and efficient method to assess susceptibility of potato to stem rot by *Erwinia carotovora* subspecies. American Potato Journal 70:611-616

Boer SH de & A Kelmam, 1978. Influence of oxygen concentration and storage factors on susceptibility of potato tubers to bacterial soft rot (*Erwinia carotovora*). Potato Research 21:65-80

Boer SH de, RJ Copeman & H Vruggink, 1979. Serogroups of *Erwinia carotovora* potato strains determined with diffusible somatic antigens. Phytopathology 69:316-319

Boer SH de, L Verdonck, H Vruggink, P Harju, HO Bang & J de Ley, 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

Bohlmann H & K Apel, 1991. Thionins. Annual Review of Plant Physiology and Plant Molecular Biology 42:227-240

Bolle MFC de, FRG Terras, BPA Cammue, SB Rees & WF Broekaert, 1993. *Mirabilis jalapa* antibacterial peptides and *Raphanus sativus* antifungal proteins: a comparative study of their structure and biological activities. In: B Fritig & M Legrand (Eds). Mechanisms of Plant Defense Responses. Kluwer Academic Publishers, The Netherlands: 433-436

Boman HG & D Hultmark, 1987. Cell-free immunity in insects. Annual Review of Microbiology 41:103-126

Boman HG, 1991. Antibacterial peptides: Key components needed in immunity. Cell 65:205-207

Bourne WF, DC McCalmont & RL Wastie, 1981. Assessing potato tubers for susceptibility to bacterial soft rot (*Erwinia carotovora* subsp. *atroseptica*). Potato Research 24:409-415

Bradford MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254

Brierley P, 1928. Pathogenicity of *Bacillus mesentericus, B. aroideae, B. carotovorus*, and *B. phytophthorus* to potato tubers. Phytopathology 18:819-838

Carmona MJ, A Molina, JA Fernández, JJ López-Fando & F García-Olmedo, 1993. Expression of the α-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. The Plant Journal 3:457-462

Casteels P, C Ampe, F Jacobs, M Vaeck & P Tempst, 1989. Apidaecins: antibacterial peptides from honeybees. EMBO Journal 8:2387-2391

Chrispeels MJ, 1991. Sorting of proteins in the secretory system. Annual Review of Plant Physiological and Plant Molecular Biology 42:21-53

Ciampi-Panno L & N Andrade-Soto, 1984. Preliminary evaluation of bacterial soft rot resistance in native Chilean potato clones. American Potato Journal 61:109-112

Collmer A & NT Keen, 1986. The role of pectic enzymes in plant pathogenesis. Annual Review of Phytopathology 24:383-409

Cornelissen BJC, RAM Hooft van Huijsduijnen & JF Bol, 1986. A tobacco mosaic virus-induced tobacco protein is homologous to the sweet-tasting protein thaumatin. Nature 321:531-532

Corsini D & J Pavek, 1986. Bacterial soft-rot resistant potato germplasm. American Potato Journal 63:417-418 (Abstract)

Davis MC, W Butler & ME Vayda, 1990. Molecular responses to environmental stress and their relationship to soft rot. In: ME Vayda & WD Park (Eds). The molecular and cellular biology of the potato. CAB International, Wallingford, UK: 71-87

Deen C, E Claassen, K Gerritse, ND Zegers & WJA Boersma, 1990. A novel carbodiimide coupling method for synthetic peptides. Enhanced anti-peptide antibody responses. Journal of Immunological Methods 129:119-125

Destéfano-Beltrán L, PG Nagpala, MS Cetiner, JH Dodds & JM Jaynes, 1990. Enhancing bacterial and fungal disease resistance in plants: Application to potato. In: ME Vayda & WD Park (Eds). The molecular and cellular biology of the potato. CAB International, Wallingford, UK: 205-221

Dobiáš K, 1976. Methoden zur Prüfung der Resistenz von Kartoffeln gegen den Erreger der Knollennaßfäule. Tagungsbericht, Akademie der Landwirtschaftswissenschaften der Deutschen Demokratischen Republik 140:221-230

Dobiáš K, 1977a. Possibilities of breeding for resistance to bacterial soft rot (*Erwinia carotovora* (Jones) Holland). Rostlinná Vyroba 23:255-260

Dobiáš K, 1977b. Provocation tests for resistance to bacterial soft rot. Ochrana Rostlin 13:101-106

Drew MC, 1990. Sensing soil oxygen. Plant, Cell and Environment 13:681-693

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

Ehlenfeldt MK & RE Hanneman, 1984. The use of Endosperm Balance Number and 2n gametes to transfer exotic germplasm in potato. Theoretical and Applied Genetics 68:155-161

Elphinstone JG & MCM Pérombelon, 1986. Contamination of potatoes by *Erwinia carotovora* during grading. Plant Pathology 35:25-33

Elphinstone JG, 1987. Soft rot and blackleg of potato; *Erwinia* spp. Technical Information Bulletin 21. International Potato Center, Lima, Peru. 18 p

Fehér A, J Preiszner, Z Litkey, Gy Csanádi & D Dudits, 1992. Characterization of chromosome instability in interspecific somatic hybrids obtained by X-ray fusion between potato (*Solanum tuberosum* L.) and *S. brevidens* Phil. Theoretical and Applied Genetics 84:880-890

Fish N, A Karp & MGK Jones, 1988a. Production of somatic hybrids by electrofusion in *Solanum*. Theoretical and Applied Genetics 76:260-266

Fish N, SH Steele & MGK Jones, 1988b. Field assessment of dihaploid *Solanum tuberosum* and *S. brevidens* somatic hybrids. Theoretical and Applied Genetics 76:880-886

Florack DEA, B Visser, PhM de Vries, JWL van Vuurde & WJ Stiekema, 1993. Analysis of the toxicity of purothionins and hordothionins for plant pathogenic bacteria. Netherlands Journal of Plant Pathology 99:259-268

Florack DEA, 1994. Application of hordothionins and cecropin B for engineering bacterial disease resistance into plants. PhD Thesis Wageningen Agricultural University. 120 p

Florack DEA, WG Dirkse, B Visser, F Heidekamp & WJ Stiekema, 1994 Expression of biologically active hordothionins in tobacco. Effects of pre-and pro-sequences at the amino and carboxyl termini of the hordothionin precursor on mature protein expression and sorting. Plant Molecular Biology 24:83-96

Florack D, S Allefs, R Bollen, D Bosch, B Visser & W Stiekema, 1995. Expression of giant silkmoth cecropin B encoding genes in transgenic tobacco. Transgenic Research 4:132-141

Fox RTV, JG Manners, & A Myers, 1971. Ultrastructure of entry and spread of *Erwinia carotovora* var. *atroseptica* into potato tubers. Potato Research 14:61-73

Fraley RT, 1989. Genetic engineering for crop improvement. In: S Kung (Ed). Plant biotechnology. Butterworths, Boston: 395-407

French ER & L de Lindo, 1979. The *Erwinias* of potatoes in Peru. Developments in control of potato bacterial diseases. Report of a planning conference. International Potato Center, Lima, Peru: 88-93

Gans PT, GJ Jellis, G Little, C Logan & RL Wastie, 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

García-Olmedo F, P Rodriguez-Palenzuela, C Hernández-Lucas, F Ponz, C Maraña, MJ Carmona, J Lopez-Fando, JA Fernandez & P Carbonero, 1989. The thionins: a protein family that includes purothionins, viscotoxins and crambins. Oxford Surveys of Plant Molecular and Cell Biology 6:31-60

Gibson RW, MGK Jones & N Fish, 1988. Resistance to potato leaf roll virus and potato virus Y in somatic hybrids between dihaploid *Solanum tuberosum* and *S. brevidens*. Theoretical and Applied Genetics 76:113-117

Hahn W, 1974. Resistenzprüfung der Kartoffelknolle gegen den Erreger der Naßfäule, *Pectobacterium carotovorum* Jones (Waldee) 1. Mitt. Zur Methodiek der Resistenzprüfung. Archiv für Züchtungsforschung 4:133-140

Helgeson JP, GJ Hunt, GT Haberlach & S Austin, 1986. Somatic hybrids between *Solanum brevidens* and *Solanum tuberosum*: Expression of a late blight resistance gene and potato leaf roll resistance. Plant Cell Reports 3:212-214

Helgeson JP, GT Haberlach, MK Ehlenfeldt, G Hunt, JD Pohlman & S Austin, 1993. Sexual progeny of somatic hybrids between potato and *Solanum brevidens*: Potential for use in breeding programs. American Potato Journal 70:437-452

Henniger H, 1965. Untersuchungen über Knollen- und Lagerfäulen der Kartoffel I. Zur Methodik der Resistenzprüfung mit dem Erreger der bakteriellen Knollennaßfäule (*Pectobacterium carotovorum*) var. *atrosepticum* (van Hall) Dowson. Der Züchter 35:174-180

Hermsen JGTh, 1983. Utilization of wide crosses in potato breeding. Report of the XXVI Planning Conference. Dec. 12-14, 1983. International Potato Center, Lima, Peru: 115-132

Hermsen JGTh & LM Taylor, 1979. Successful hybridization of non-tuberous *Solanum etuberosum* Lind. and tuber-bearing *S. pinnatisectum* Dun. Euphytica 28:1-7

Hidalgo OA & E Echandi, 1982. Evaluation of potato clones for resistance to tuber and stem rot induced by *Erwinia chrysanthemi*. American Potato Journal 59:585-592

Hidalgo OA & E Echandi, 1983. Influence of temperature and length of storage on resistance of potato to tuber rot induced by *Erwinia chrysanthemi*. American Potato Journal 60:1-15

Hidalgo O, 1988. Soft rot and blackleg (*Erwinia* spp.) in warm climates. Report of the Planning Conference on Bacterial diseases of the Potato, 1987, Lima, Peru. International Potato Center, Lima: 179-186

Hightower R, C Baden, E Penzes & P Dunsmuir, 1994. The expression of cecropin peptide in transgenic tobacco does not confer resistance to *Pseudomonas syringae* pv *tabaci*. Plant Cell Reports 13:295-299

Hoekema A, MJ Huisman, L Molendijk, PJM van den Elzen & BJC Cornelissen, 1989. The genetic engineering of two commercial potato cultivars for resistance to potato virus X. Bio/Technology 7:273-278

Hoekstra R, 1990. Resistenzen gegen Erwinia in Solanum spp. Tagungsbericht Wintertagung 1990 der DLG-Arbeitsgemeinschaft für Kartoffelzüchtung und Pflanzguterzeugung, 13/14-11-1990, Fulda: 16-26

Hofsten P van, I Faye, K Kockum, Y-L Lee, KG Xanthopoulos, IA Boman, HG Boman, A Angstrom, D Andreu & RB Merrifield, 1985. Molecular cloning, cDNA sequencing, and chemical synthesis of cecropin B from *Hyalophora cecropia*. Proceedings of the National Academy of Sciences of the United States of America 82:2240-2244

Hossain M & C Logan, 1983. A comparison of inoculation methods for determining potato cultivar reaction to black leg. Annals of Applied Biology 103:63-70

Huaman Z, L de Lindo & J Elphinstone, 1988. Resistance to blackleg and soft rot and its potential use in breeding. Report of the planning conference on bacterial diseases of the potato 1987, Lima, Peru. International Potato Center, Lima, Peru: 215-228

Huaman Z, B Tivoli & L de Lindo, 1989. Screening for resistance to *Fusarium* dry rot in progenies of cultivars of *S. tuberosum* spp. *andigena* with resistance to *Erwinia chrysanthemi*. American Potato Journal 66:357-364

Janse JD & MA Ruissen, 1988. Characterization and classification of *Erwinia chrysanthemi* strains from several hosts in The Netherlands. Phytopathology 78:800-808

Jarret RL, PM Hasegawa & HT Erickson, 1980. Factors affecting shoot initiation from tuber discs of potato (Solanum tuberosum). Physiologia Plantarum 49:177-184

Jaynes JM, P Nagpala, L Destéfano-Beltrán, JH Huang, J Kim, T Denny & S Cetiner, 1993. Expression of a Cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas solanacearum*. Plant Science 89:43-53

Jia S-R, Y Xie, T Tang, L-X Feng, D-S Cao, Y-L Zhao, J Yuan, Y-Y Bai, C-X Jiang, JM Jaynes & JD Dodds, 1993. Genetic engineering of Chinese potato cultivars by introducing antibacterial polypeptide gene. In: C You, Z Chen & Y Ding (Eds). Biotechnology in agriculture. Kluwer Academic Publishers, The Netherlands: 208-212

Jones RAC, 1979. Resistance to potato leaf roll virus in *Solanum brevidens*. Potato Research 22:149-152

Jongedijk E, AAJM de Schutter, T Stolte, PJM van den Elzen, BJC Cornelissen, 1992. Increased resistance to potato virus X and preservation of cultivar properties in transgenic potato under field conditions. Bio/Technology 10:422-429

Kiel W, 1967. Entwicklung einer Labormethode zur Resistenzprüfung der Kartoffeln gegen den erreger der Knollennaβfäule (*Pectobacterium carotovorum* var. *atrosepticum* (van Hall) Dowson syn. *Erwinia carotovora* Jones). Nachrichtenblatt für den Pflanzenschutz in der Deutschen Demokratischen Republik 12:237-240

Koppel M, 1993. Methods of 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

Krause B, T Koczy, J Komorowska-Jedrys & E Ratuszniak, 1982. Laboratory assessment of tuber resistance of world potato cultivar collection to main causes of storage rots. Biuletyn Instytutu Ziemniaka 27:111-134

Lapwood DH & PR Legg, 1983. The effect of *Erwinia carotovora* subsp. *atroseptica* (blackleg) on potato plants. I. Growth and yield of different cultivars. Annals of Applied Biology 103:71-78

Lapwood DH & PT Gans, 1984. A method for assessing the field susceptibility of potato cultivars to blackleg (*Erwinia carotovora* subsp. *atroseptica*) Annals of Applied Biology 104:315-320

Lapwood DH, PJ Read & J Spokes, 1984. Methods for assessing the susceptibility of potato tubers of different cultivars to rotting by *Erwinia carotovora* subspecies *atroseptica* and *carotovora*. Plant Pathology 33:13-20

Lapwood DH & PJ Read, 1985. A simplified slice method for assessing tuber susceptibility of potato cultivars to *Erwinia carotovora* subsp. *atroseptica*. Plant Pathology 34:284-286

Lapwood DH & PJ Read, 1986a. The susceptibility of stems of different potato cultivars to blackleg caused by *Erwinia carotovora* subsp. *atroseptica*. Annals of Applied Biology 109:555-560

Lapwood DH & PJ Read, 1986b. A comparison of methods of seed tuber inoculation for assessing the susceptibility of potato cultivars to blackleg (*Erwinia carotovora* subsp. *atroseptica*) in the field. Annals of Applied Biology 109:287-297

Lee J-Y, A Boman, S Chuanxin, M Andersson, H Jörnvall, V Mutt & HG Boman, 1989. Antibacterial peptides from pig intestine: Isolation of a mammalian cecropin. Proceedings of the National Academy of Sciences of the United States of America 86:9159-9162

Lellbach H, 1978. Schätzung genetischer Parameter aus diallelen Kreuzungen bei der Naßfäuleanfälligkeit der Kartoffel. Archiv für Züchtungsforschung 8:193-199

Lojkowska E & A Kelman, 1989. Screening of seedlings of wild *Solanum* species for resistance to bacterial stem rot caused by soft rot *Erwinias*. American Potato Journal 66:379-390

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

Lund BM & GM Wyatt, 1972. The effect of oxygen and carbon dioxide concentrations on bacterial soft rot of potatoes. I. King Edward potatoes inoculated with *Erwinia carotovora* var. *atroseptica*. Potato Research 15:174-179

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

Lyon GD, 1992. The biochemical basis of resistance of potato soft rot bacteria. Netherlands Journal of Plant Pathology 98 Supplement 2:127-133

Maher EA & A Kelman, 1983. Oxygen status of potato tuber tissue in relation to maceration by pectic enzymes of *Erwinia carotovora*. Phytopathology 73:536-539

McGrath JM, SM Wielgus, TF Uchytil, H Kim-Lee, GT Haberlach, CE Williams & JP Helgeson, 1994. Recombination of *Solanum brevidens* chromosomes in the second generation from a somatic hybrid with *S. tuberosum*. Theoretical and Applied Genetics 88:917-924

McMillan GP, D Hedley, L Fyffe & MCM Pérombelon, 1993. Potato resistance to soft-rot erwinias is related to cell wall pectin esterification. Physiological and Molecular Plant Pathology 42:279-289

Mills D & FA Hammerschlag, 1993. Effect of cecropin B on pathogens, protoplasts, and cells. Plant Science 93:143-150

Miyata T, F Tokunaga, T Yoneya, K Yoshikawa, S Iwanaga, M Niwa, T Takao & Y Shimonishi, 1989. Antimicrobial peptides, isolated from horseshoe crab hemocytes, tachyplesin II and polyphemusins I and II: Chemical structures and biological activity. Journal of Biochemistry 106:663-668

Molina JJ & Harrison MD, 1980. The role of *Erwinia carotovora* in the epidemiology of potato blackleg. II. The effect of soil temperature on disease severity. American Potato Journal 57:351-363

Molina A, PA Goy, A Fraile, R Sánchez-Monge & F García-Olmedo, 1993. Inhibition of bacterial and fungal plant pathogens by thionins of types I and II. Plant Science 92:169-177

Munzert M, 1975. Eine Methode zur Prüfung der Resistenz der Kartoffelpflanze gegenüber dem Erreger der Schwarzbeinigkeit (*Erwinia carotovora* var. *atroseptica* (van Hall) Dye). Potato Research 18:308-313

Munzert M & W Hunnius, 1980. Beziehungen zwischen den Resistenzen gegen Schwarzbeinigkeit, Naß- und Trockenfäule der Kartoffel (*Solanum tuberosum* L.). Zeitschrift für Pflanzenzüchtung 85:59-70

Muta T, T Fujimoto, H Nakajima & S Iwanaga, 1990. Tachyplesins isolated from hemocytes of Southeast Asian horseshoe crabs (*Carcinoscorpius rotundicauda* and *Tachypleus gigas*): Identification of a new tachyplesin, tachyplesin III, and a processing intermediate of its precursor. Journal of Biochemistry 108:261-266

Nakamura T, H Furunaka, T Miyata, F Tokunaga, T Muta, S Iwanaga, M Niwa, T Takao & Y Shimonishi, 1988. Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*). The Journal of Biological Chemistry 263:16709-16713

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

Nielsen LW, 1954. The susceptibility of seven potato varieties to bruising and bacterial soft rot. Phytopathology 44:30-35

Nordeen RO, SL Sinden, JM Jaynes & LD Owens, 1992. Activity of cecropin SB37 against protoplasts from several plant species and their bacterial pathogens. Plant Science 82:101-107

Norelli JL, HS Aldwinckle, L Destéfano-Beltrán & JM Jaynes, 1994. Transgenic 'Malling 26' apple expressing the attacin E gene has increased resistance to *Erwinia amylovora*. Euphytica 77:123-128

Pagel W & R Heitefuss, 1989. Calcium content and cell wall polygalacturonans in potato tubers of cultivars with different susceptibilities to *Erwinia carotovora* subsp. *atroseptica*. Physiological and Molecular Plant Pathology 35:11-21

Palva TK, K Holmström, P Heino & ET Palva, 1993. Induction of plant defence response by exoenzymes of *Erwinia carotovora* subsp. *carotovora*. Molecular Plant-Microbe Interactions 6:190-196

Parlevliet JE, 1979. Components of resistance that reduce the rate of epidemic development. Annual Review of Phytopathology 17:203-222

Pawlak A, JJ Pavek & DL Corsini, 1987. Resistance to storage diseases in breeding stocks. In: GJ Jellis & DE Richardson (Eds). The production of new potato varieties: technological advances. Cambridge University Press: 96-98

Payne RW, PW Lane, AE Ainsley, KE Bicknell, PGN Digby, PK Leech, HR Simpson, AD Todd, PJ Verrier, RP White, JC Gower, G Tunnicliffe Wilson & LJ Paterson, 1987. Genstat 5 reference manual, Clarendon Press, Oxford, 749 p

Pehu E, RW Gibson, MGK Jones & A Karp, 1990. Studies on the genetic basis of resistance to potato leaf roll virus, potato virus Y and potato virus X in *Solanum brevidens* using somatic hybrids of *Solanum brevidens* and *Solanum tuberosum*. Plant Science 69:95-101

Pérombelon, MCM & R Lowe, 1975. Studies on the initiation of bacterial soft rot in potato tubers. Potato Research 18:64-82

Pérombelon MCM & A Kelman, 1980. Ecology of the soft rot Erwinias. Annual Review of Phytopathology 18:361-387

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

Pérombelon MCM, VM Lumb & D Zutra, 1987. Pathogenicity of soft rot erwinias to potato plants in Scotland and Israel. Journal of Applied Bacteriology 63:73-84

Pérombelon MCM, 1988. Ecology of Erwinias causing stem and tuber diseases. Report of the Planning Conference on Bacterial diseases of the Potato, 1987, Lima, Peru. International Potato Center, Lima: 143-177

Pérombelon MCM, MM Lopez, J Carbonell & LJ Hyman, 1988. Effects of contamination by *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* of potato seed tubers and of cultivar resistance on blanking or non-emergence and blackleg development in Valencia, Spain. Potato Research 31:591-599

Pérombelon MCM, 1992. Potato blackleg: Epidemiology, host-pathogen interaction and control. Netherlands Journal of Plant Pathology 98 Supplement 2:135-146

Pietkiewicz JB, 1980. Variation in the reaction of potato tubers to diseases. Potato Research 23:473

Pietkiewicz J, 1981. Incidence and noxiousness of bacterial and fungal potato diseases in the years 1976-1979. Biuletyn Institutu Ziemniaka 26:103-126

Powelson M, 1980. Seasonal incidence and cause of blackleg and stem soft rot of potatoes in Oregon. American Potato Journal 57:301-306

Preiszner J, A Fehér, O Veisz, J Sutka & D Dudits, 1991. Characterization of morphological variation and cold resistance in interspecific somatic hybrids between potato (*Solanum tuberosum* L.) and *S. brevidens* Phil. Euphytica 57:37-49

Priou S, K Al Ani & B Jouan, 1992. Comparison of the effectiveness of two methods of screening potato to soft rot induced by *Erwinia carotovora* subsp. *atroseptica* (van Hall, 1902). Proceedings of the Joint Conference of the EAPR Breeding & Varietal Assessment Section and the Eucarpia Potato Section, Landerneau, France: 193-140

Puite KJ & JG Schaart, 1993. Nuclear genomic composition of asymmetric fusion products between irradiated transgenic *Solanum brevidens* and *S. tuberosum*: limited elimination of donor chromosomes and polyploidization of the recipient genome. Theoretical and Applied Genetics 86:237-244

Robinson K & G Foster, 1987. Control of potato blackleg by tuber pasteurisation: the determination of time-temperature combinations for the inactivation of pectolytic erwinia. Potato Research 30:121-125

Ross RW & PR Rowe, 1969. Utilizing the frost resistance of diploid *Solanum* species. American Potato Journal 46:5-13

Salmond GPC, 1992. Bacterial diseases of potatoes: from classical phytobacteriology to molecular pathogenicity. Netherlands Journal of Plant Pathology 98 Supplement 2:115-126

Samson R, N Ngwira & N Rivera, 1990. Biochemical and serological diversity of *Erwinia chrysanthemi*. In: Z Klement (Ed). Proceedings 7th International Conference on Plant Pathogenic Bacteria, 1989. Academiai Kiado, Budapest: 895-900

Shigenaga T, T Muta, Y Toh, F Tokunaga & S Iwanaga, 1990. Antimicrobial tachyplesin peptide precursor. The Journal of Biological Chemistry 265:21350-21354

Smid EJ, AHJ Jansen & CJ Tuijn, 1993. Anaerobic nitrate respiration by *Erwinia carotovora* subsp. *atroseptica* during potato tuber invasion. Applied and Environmental Microbiology 59:3648-3653

Soest LJM van, 1983. Evaluation and distribution of important properties in the German-Netherlands potato collection. Potato Research 26:109-121

Steiner AA, 1984. The universal nutrient solution. Proceedings of the Sixth International Congress on Soilless Culture, Lunteren. International Society for Soilless Culture. Pudoc, Wageningen: 633-650

Stiekema WJ, F Heidekamp, D Louwerse, HA Verhoeven & P Dijkhuis, 1988. Introduction of foreign genes into potato cultivars Bintje and Désirée using an *Agrobacterium tumefaciens* binary vector. Plant Cell Reports 7:47-50

Tamamura H, M Kuroda, M Masuda, A Otaka, S Funakoshi, H Nakashima, N Yamamoto, M Waki, A Matsumoto, JM Lancelin, D Kohda, S Tate, F Inagaki & N Fujii, 1993. A comparative study of the solution structures of tachyplesin I and a novel anti-HIV synthetic peptide, T22 ([$Tyr^{5,12}$,Lys^{7}]-polyphemusin II), determined by nuclear magnetic resonance. Biochemica et Biophysica Acta 1163:209-216

Tsror (Lahkim) L, A Nachmias, O Erlich, M Aharon & MCM Pérombelon, 1993. A 9-year monitoring study of diseases on potato seed tubers imported to Israel. Phytoparasitica 21:321-328

Tsugita A, 1971. Phage lysozyme and other lytic enzymes. In: PD Boyer (Ed). The enzymes, volume 5. Academic Press, New York: 344-411

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

Valkonen JPT, G Brigneti, LF Salazar, E Pehu & RW Gibson, 1992. Interactions of the *Solanum* spp. of the Etuberosa group and nine potato-infecting viruses and a viroid. Annals of Applied Biology 120:301-313

Vayda ME & WR Belknap, 1992. The emergence of transgenic potatoes as commercial products and tools for basic science. Transgenic Research 1:149-163

Vayda ME, LS Antonov, Z Yang, WO Butler & GH Lacy, 1992. Hypoxic stress inhibits aerobic wound-induced resistance and activates hypoxic resistance to bacterial soft rot. American Potato Journal 69:239-253

Vuurde WL van, 1987. New approach in detecting phytopathogenic bacteria by combined immunoisolation and immunoidentification assays. EPPO bulletin 17:139-148

Wada K-N, S-I Aota, R Tsuchiya, R Ishibashi, T Gojobori & T Ikemura, 1990. Codon usages tabulated from the Gen-Bank genetic sequence data. Nucleic Acids Research 18:2367-2411

Wastie RL, 1984. Inoculating plant material by jet injection. Plant Pathology 33:61-63

Wastie RL & GR Mackay, 1985. Breeding for resistance to blackleg - the present and future. In: DC Graham & MD Harrison (Eds). Report of the International Conference on Potato Blackleg Disease. Potato Marketing Board, Oxford: 75-76

Wastie, RL, GJ Jellis, DH Lapwood, C Logan, G Little & MS Phillips, 1988. Assessing potato cultivars for resistance to tuber soft rot (*Erwinia carotovora* subsp. *atroseptica*) at four test centres in the UK. Potato Research 31:67-72

Watanabe K, M Orrillo & S Vega, 1992. Sexual hybrids between 2x potato breeding lines and non-tuber-bearing *Solanum* species. American Potato Journal 69:614 (Abstract)

Weber J & G Jansen, 1984. Resistenzphysiologische Untersuchungen zur Induction und Ausbreitung bakterieller Knollennaβfäule bei Kartoffeln. Archiv für Phytopathologie und Pflanzenschutz 20:297-306

Weber J, 1988. Die Effizienz der Nassfäuleabwehr bei der Wundheilung von Kartoffelknollen. 1. Bestimmung der befallsauslösenden Erregerdichte und ihrer Umweltbeeinflussung. Potato Research 31:3-10

Weber J, 1990. *Erwinia* - a review of recent research. Proceedings of the 11th Triennial Conference of the European Association for Potato Research, Edinburgh, United Kingdom, 8-13th July 1990: 112-121

Williams CE, GJ Hunt & JP Helgeson, 1990. Fertile somatic hybrids of *Solanum* species: RFLP analysis of a hybrid and its sexual progeny from crosses with potato. Theoretical and Applied Genetics 80:545-551

Wit PJGM de & G Spikman, 1982. Evidence for the occurrence of race and cultivar-specific elicitors of necrosis in intercellular fluids of compatible interactions of *Cladosporium fulvum* and tomato. Physiological Plant Pathology 21:1-11

Wolters PJ & WW Collins, 1994. Evaluation of diploid potato clones for resistance to tuber soft rot induced by strains of *Erwinia carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi*. Potato Research 37:143-149

Workman M & DG Holm, 1984. Potato clone variation in blackspot and soft rot susceptibility, redox potential, ascorbic acid, dry matter and potassium. American Potato Journal 61:723-733

Yang Z, H Park, GH Lacy & CL Cramer, 1991. Differential activation of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes by wounding and pathogen challenge. The Plant Cell 3:397-405

Yang Z, CL Cramer & GH Lacy, 1992. *Erwinia carotovora* subsp. *carotovora* pectic enzymes: *in planta* gene activation and roles in soft-rot pathogenesis. Molecular Plant-Microbe Interactions 5:104-112

Young N, 1990. Seed potato systems in developed countries: Canada, The Netherlands and Great Britain. International Potato Center, Lima.

Zadina J & K Dobiáš, 1976. Möglichkeiten der Resistenzzüchtung gegen die Knollennaβfäule bei Kartoffeln. Tagungsbericht, Akademie der Landwirtschaftswissenschaften der Deutschen Demokratischen Republik 140:207-219

Zimnoch-Guzowska E & E Lojkowska, 1993. Resistance to *Erwinia* spp. in diploid potato with a high starch content. Potato Research 36:177-182

Zink RT & GA Secor, 1982. Interaction of fungal wilt pathogens and potato blackleg. Plant Disease 66:1053-1056

Curriculum vitae

Josephus Jacobus Hendricus Maria (Sjefke) Allefs werd geboren op 21 april 1964 te Arnhem. Aldaar volgde hij basis- en voortgezet onderwijs. In 1982 behaalde hij het Gymnasium-β diploma aan het Katholiek Gelders Lyceum. In datzelfde jaar begon hij zijn studie Plantenveredeling aan de toenmalige Landbouwhogeschool te Wageningen. Praktijkperioden werden doorgebracht bij Nunhems Zaden B.V., te Haelen en Sementi Nunhems S.R.L., te S. Agata Bolognese, Italië. Het diploma werd in 1988 behaald met als afstudeervakken Plantenveredeling, Erfelijkheidsleer en Virologie. Van oktober 1988 tot en met december 1989 vervulde hij zijn vervangende dienstplicht bij de Stichting voor Plantenveredeling (SVP), later opgegaan in het DLO-Centrum voor Plantenveredelings- en Reproductieonderzoek (CPRO-DLO), te Wageningen. Vanaf januari 1990 tot en met juni 1994 was hij door Kweek- en Researchbedrijf Agrico B.V., te Emmeloord, gedetacheerd bij CPRO-DLO, alwaar het onderzoek werd verricht dat in dit proefschrift wordt beschreven. Sinds juli 1994 is hij bij Kweek- en Researchbedrijf Agrico B.V. werkzaam in de functie van onderzoeker.