Studies on the basal kernel blight disease of barley: pathogenesis and phylogeny of the causal agent Pseudomonas Syringae pv. syringae and its biological control by antagonistic Pantoea agglomerans
by Andrea Braun

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology
Montana State University

Abstract:
The pathogenesis and phylogeny of Pseudomonas syringae pv. syringae (Pss), the cause of basal kernel blight of barley, and its biological control by antagonistic Pantoea agglomerans Pa, (syn. Erwinia herbicola, Eh) was investigated. Scanning electron micrographs located bacteria close to stomata and bases of kernel trichomes. Light micrographs revealed Pss in intercellular spaces of aleurone and amyloplast cells. Electron micrographs demonstrated Pss in xylem vessels and cell wall degradation in association with capsule protected bacteria.

Fifty-five Pss strains, isolated from infected kernels of 12 barley cultivars were tested for tobacco hypersensitivity, pathogenicity, toxin production and carbon source utilization. Toxin production correlated low ($r=0.31$; $p=0.046$) with pathogenicity, but 96% of toxin-plus strains were pathogenic, supporting the importance of toxins for virulence. Nutritional analyses revealed a phenotypic variability of Pss strains with similarity coefficients between 76-100%. The intrapathovar variation of Pss populations was analyzed by RFLPs of digested DNA using XbaI, SpeI and pulsed field gel electrophoresis. Macrorestriction fingerprinting supported a heterogeneity of Pss populations (43-77% similarity).

Field experiments (1994, 1995) revealed 45-70% disease control, when Pa was applied to barley heads prior to the Pss infection window. Pa provided 80-100% disease reduction in greenhouse studies. Efficacy of Pa was affected by time and rate of application, with a single application providing control. Survival of formulated Pa was better when stored at 4 C than 22 C. Biocontrol activity was not affected by storage.

Multiple mechanisms including antibiosis, preemptive exclusion, and induced systemic resistance were involved in the Pa-Pss interaction. Antibiotics were polar, thermostable, protease insensitive, but base labile ($pH \geq 6.0$). Antibiotic production correlated weakly ($r = 0.43$) with disease suppression. Nutritional niche overlapping indices revealed a high possibility of preemptive exclusion of Pss. Pss populations on barley kernels were reduced 100-fold in the presence of Pa. $FeCl_3$ amendment to the phyllosphere reduced the biocontrol efficacy of Pa, and 2 out of 3 siderophore-minus mutants were less efficient in disease reduction than the parental strain. Eh 460 and 239 reduced kernel blight through induced systemic resistance, similar to the plant activator benzothiadiazole. This is the first report on the induction of systemic resistance in barley by Pantoea agglomerans.

STUDIES ON THE BASAL KERNEL BLIGHT DISEASE OF BARLEY: PATHOGENESIS AND PHYLOGENY OF THE CAUSAL AGENT *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* AND ITS BIOLOGICAL CONTROL BY ANTAGONISTIC *PANTOEA AGGLOMERANS*

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APPROVAL

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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... DAS EINZIGE, WAS WIR BRAUCHEN,

UM GUTE PHILOSOPHEN ZU WERDEN, IST DIE FÄHIGKEIT,

UNS ZU WUNDERN ...

(Jostein Gaarder aus: Sofies Welt -
Ein Roman über die Geschichte der Philosophie)

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ABSTRACT

The pathogenesis and phylogeny of *Pseudomonas syringae* pv. *syringae* (Pss), the cause of basal kernel blight of barley, and its biological control by antagonistic *Pantoea agglomerans* Pa, (syn. *Erwinia herbicola*, Eh) was investigated. Scanning electron micrographs located bacteria close to stomata and bases of kernel trichomes. Light micrographs revealed Pss in intercellular spaces of aleurone and amyloplast cells. Electron micrographs demonstrated Pss in xylem vessels and cell wall degradation in association with capsule protected bacteria.

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CHAPTER 1

LITERATURE REVIEW

Thesis Preface

Barley is the second largest small grain crop produced in the United States of America and in Montana behind wheat production. In 1997, 13.2 million tons of barley were produced in the US with an average yield of 50.7 dt/ha. North Dakota continued to rank as the number one barley producing state, followed by Montana, Idaho, Washington, and Minnesota; with these five states producing more than three-fourths of the US production in 1997. While yields in North Dakota dropped by 8.7 dt/ha to 39.2 dt/ha in 1997 compared to 1996 due to dry weather conditions in the west and the scab disease in the eastern portion of the state, yields in Montana increased by 8.7 dt/ha to 46.1 dt/ha as a result of timely rains (Anonymous, 1997 a). Montana farmers planted 526315 ha of barley in 1997, unchanged from the last four years. Malting and brewing varieties recommended by the American Malting Barley Association accounted for almost 44 percent of the total barley planted (Anonymous, 1997 b). Harrington remained the top malting variety compared to 1996 followed by B 1202, B 2601, and Morex. Baronesse remained in first place among feed-type varieties followed by Hector, Gallatin, and Bowman (Anonymous, 1997, b).

Bacterial Diseases of Small Grains

Bacterial diseases of small grains are generally considered of minor importance compared to other diseases, however, they can cause significant damage when environment favors disease development (Paul & Smith, 1989; Mathre, 1997). Examples of important bacterial diseases on wheat and barley consist of bacterial leaf and kernel blight caused by *Pseudomonas syringae* pv. *syringae* (Peters et al., 1983; Martinez-Miller, 1994; Martinez-Miller & Braun, 1997), bacterial blight of barley caused by *Xanthomonas translucens* pv. *translucens* (Forster, 1997) and basal glume rot caused by *Pseudomonas syringae* pv. *atrofaciens* (von Kietzell, 1995). Bacteria are almost always present on plant surfaces and may live as epiphytes without causing any host damage (Hirano & Upper, 1983, 1990; Lindow, 1985). However, in some years epiphytic populations can become pathogenic. The mechanisms and stimuli for this change are not fully understood, but recent research indicated that population size, including quorum-sensing autoinduction processes, and plant signal molecules can increase virulence gene expression (Pirhonen et al., 1993; Quigley & Gross, 1994; Beck von Bodman & Farrand, 1995; Fuqua et al., 1996; Farrand et al., 1996). Alternatively, new virulent races which may overcome deployed host plant's resistance can occur as a result of genetic changes in pathogen populations through mutation and/or recombination, in addition to selection and migration (Vera Cruz et al., 1996; Vivian & Gibbon, 1997). Understanding these events is critical to the apprehension and needed for control or management of pathogens, such as *P. s.* pv. *syringae*.

The Kernel Blight Disease of Barley

Kernel blight diseases of barley have been reported to be associated with fungi, such as *Bipolaris sorokiniana*, *Fusarium graminearum*, and *Alternaria* spp. (Miles et al., 1987; Johnston, 1997) and with bacteria in the Northern Great Plains (Peters et al., 1983; Martinez-Miller, 1994). *Pseudomonas syringae* pv. *syringae* was first described as causing bacterial kernel spot of Klages barley in Idaho (Peters et al., 1983). The disease has also been reported in Montana and was characterized as a well defined discolored area with distinct margins on the lemma of the kernel (Martinez-Miller, 1994). Another disease caused by *P. s.* pv. *syringae* in Montana is basal kernel blight, which consists of a dark brown discoloration at the embryo end of the kernel (Martinez-Miller & Braun, 1997). Similar symptoms have been associated with *Bacillus* sp. in South Africa by Basson et al. (1990), who found counts of these bacteria on black-end barley kernels significantly higher than on healthy kernels. In the temperate climates of Europe, *P. s.* pv. *atrofaciens* was regularly isolated from basal glume rot symptoms on wheat, barley (Poschenrieder, 1986; Toben et al., 1989) and rye (Gvozdyak & Pasichnik, 1991). Toben et al. (1989,1991) confirmed its pathogenicity in wheat and barley seedling assays, which correlated with typical disease symptoms obtained when adult wheat plants were inoculated at the milk stage (EC 75). However, wheat spikes also developed basal glume rot symptoms when *P. s.* pv. *syringae* isolates from lilac, bean, or *Pennisetum* sp. or *P. s.* pv. *aptata* isolates from sugar beets were inoculated (von Kietzell, 1995).

These diseases are primarily of concern to malting and brewing industries, who discount or even reject barley lots when more than 4 % of kernels are discolored in a lot.

Kernel blight reduces grain yield and quality (Anderson & Banttari, 1976) and Gebhardt et al. (1992) reported, that protein, malt extract, wort color, beer taste and aroma were affected by kernel discoloration. Furthermore, the suppression of germination seemed to be correlated with the formation of black-end kernels (Basson et al.,1990).

We have reported previously on the etiology and significance of basal kernel blight of barley (Martinez-Miller et al., 1998). Kernel discoloration caused by *P. s.* pv. *syringae* was found to vary in severity with cultivars, environmental conditions, and virulence of the pathogen. The critical period for infection increased from late milk to soft dough stages of kernel development (EC 85), with free moisture being necessary for infection and disease development (Martinez-Miller et al., 1998). A similar sensitivity to *P. s.* pv. *atrofaciens* infection was observed by von Kietzell & Rudolph (1991) on wheat plants. While the use of overhead irrigation at soft dough should be avoided by growers during this time, it was useful in screening experiments, in which the susceptibility of cultivars was tested under field conditions (Martinez-Miller et al., 1998).

The Pathogen *Pseudomonas syringae* pv. *syringae*

The bacterium *Pseudomonas syringae* pv. *syringae* [van Hall 1902](Pss) originally isolated from lilac (*Syringa vulgaris* L.), is a common plant pathogen found throughout the world on widely diverse hosts (Bradbury, 1986; Young, 1992; Georgakopoulos & Sands, 1992; Martinez-Miller & Braun, 1997). The disease symptoms associated with the pathogen are of two types. One symptom is known as bacterial canker or dieback on stone fruits

(cherry, apricot, plum) and citrus trees and the other symptom distinguishes foliar or blossom blights (brown spots) on legumes (snap beans, peas, etc.), tomato, wheat, apple and pear (Klement et al., 1990; Goto, 1992; Sigee, 1993). The one-celled bacteria are Gram-negative, straight or curved rods, 0.5-0.7 x 1.5-3.0 μm in size. Cells are motile with one to seven polar flagella and the mol% G+C of the DNA ranges from 58-71. They are catalase-positive and strict aerobes (Palleroni, 1984). A characteristic feature of all *P. syringae* is the production of fluorescent pigments on media devoid of iron (Hildebrand et al., 1988). Species identification is mainly based on levan production on 5 % sucrose medium, oxidase reaction, pectolytic activity on potato slices or pectate gel, arginine dihydrolase activity, and hypersensitivity reaction (HR) on tobacco leaves (LOPAT) (Klement et al., 1990). While most members of the species are variable in the levan reaction, they are oxidase-negative, arginine dihydrolase-negative, non-pectolytic and HR-positive. The species comprises many pathovars on the infrasubspecific level based on distinctive pathogenicity to one or more plant host species (Dye et al., 1980). Pathovar identification is difficult and relies on host specificity as well as nutritional, serological, plasmid, phage, protein, and fatty acid profiles (Klement et al., 1990) and toxin analysis (Gross, 1991; Quigley & Gross, 1994). Currently, the *P. syringae* species consists of 51 pathovars (Young et al., 1996). Hildebrand et al. (1988) identified strains belonging to the pathovar *syringae* based on biochemical characters, since members are positive in trigonelline, quinate and L-lactate utilization, while strains of other pathovars are variable (*pisi*) or negative in some of these traits (*coronafaciens*, *striafaciens*).

Furthermore, identification can be based on commercially available kits, such as the Biolog GN MicroPlate™ system (Biolog, Inc., Hayward, CA). The system provides a standardized method using biochemical tests to identify a broad range of enteric, non-fermenting, and fastidious Gram-negative bacteria. Biolog's MicroLog™ computer software identifies the bacterium from its metabolic pattern in the GN MicroPlate, which is based on the utilization (oxidation) of 95 different carbon sources. Another commercially available source is the Microbial Identification System (MIDI, Microbial ID Inc., Newark, Delaware) which identifies microbes based on their cellular fatty acid compositions, using fatty acid methyl esters of whole cells and high resolution gas chromatography.

Pathogenesis and Population Dynamics of *P. s.* pv. *syringae* (Pss)

In bacterial pathogenesis the infection process can be divided into three phases: (1) migration or attachment to the host (=contact), (2) recognition, and (3) invasion and establishment. These phases often occur in continuity and are difficult to separate (Goto, 1992; Sigee, 1993). For *P. syringae* motility is accomplished by flagella and motile bacteria frequently have an invasive advantage over nonmotile strains, increasing leaf infection (Panopoulos & Schroth, 1974). However, loss in motility did not result in loss of virulence, as inside the leaf inherent differences between motile and nonmotile isolates in virulence were less apparent and bacterial cells in diseased plant tissue were generally nonmotile (Goto, 1992). Furthermore, Chet et al. (1973) observed that *P. syringae* pv. *lachrymans* was capable of following a non host-specific chemical gradient presented by solutions of sugars, amino acids, guttation fluids, etc. However, there was no conclusive evidence that

chemotaxis was required for plant pathogenic bacteria to enter into stomata or wounds. Instead, the process of invasion was substantially affected by the mode of dispersal and sources of inoculum (Goto, 1992), which are for *P. syringae* pathovars aerosols, wind, dew, fog, rain, as well as seeds, plant debris, dormant tissues, and nonhost plants (Hirano & Upper, 1983). Contact (attachment) between host and pathogen was believed to be established when components of bacterial cell walls, such as lipopolysaccharides (LPS - O-antigens) bind to receptor sites on host cell surfaces, such as lectins or other glycoproteins with carbohydrate-binding sites (Sequeira, 1978; Sigee, 1993). Romantschuk et al. (1993) and Suoniemi et al. (1995) studied the attachment of *P. syringae* pathovars to host and non-host plant surfaces as an epiphytic fitness factor. The attachment of phage-resistant non-piliated mutants to leaf surfaces was lowered by a factor between two and ten compared to the piliated parental strains, but pilus-mediated adhesion was not host-specific. Piliated strains adsorbed well to both host and non-host plants (Romantschuk et al., 1993). The ability of the piliation-mutants to cause symptoms when infiltrated into bean leaf tissue was unaffected, but in spray-inoculation experiments the presence of pili correlated positively with frequency of infection (Romantschuk & Bamford, 1986). However, when piliated and non-piliated bacteria were inoculated together as mixtures, the initial epiphytic cell density of each bacterium was similar to that of the separately inoculated plants and the wild-type strain did not have an advantage over the mutant during undisturbed epiphytic growth, indicating that epiphytic growth per se was not dependent on piliation (Suoniemi, et al., 1995). Furthermore, adherence of *Pseudomonas* species to plant cell surfaces inside and outside the plant or other surfaces was mediated by capsular extracellular polysaccharides

(EPS) and biofilms (Smith & Mansfield, 1982; Costerton, 1987; Sigee, 1993; Suoniemi et al., 1995). In addition, EPS caused water-soaking in susceptible cultivars resulting in bacterial multiplication in compatible interactions (El-Banoby & Rudolph, 1979). Many studies have linked free water and high relative humidity to increased epiphytic *P. syringae* populations on plant surfaces and to a subsequent successful plant invasion by improving migratory pathways through natural openings (stomata, hydathodes), through surface wounds or breaks in fragile trichomes (Hirano & Upper, 1983; Goto, 1992; Sigee, 1993). Recognition in the plant-pathogen interaction either resulted in compatibility (disease) or incompatibility (resistance = HR reaction), which will be discussed in more detail below.

With respect to *P. syringae* population dynamics, ecology and epidemiology the tremendous work by Hirano & Upper (1983, 1990) should be acknowledged. They recognized the significance of *P. syringae* as an epiphyte living on plant surfaces of host and nonhost plants as potential inoculum sources for several diseases. In addition, they reported on host and cultivar preference for some genotypes of *P. s.* pv. *syringae* (Hirano & Upper, 1990) and emphasized that its population size at any given time on any given leaf was a function of growth, death, immigration and emigration (Hirano & Upper, 1983, 1990; Kinkel, 1997). Furthermore, they agreed that the amount of disease in a field was directly related to pathogen population sizes on individual leaves within a canopy (Hirano & Upper, 1990) based on earlier studies in which disease incidence could be predicted by the frequency with which pathogen populations exceeded approx. 10^4 cfu per gram of symptomless leaflets (Lindemann et al., 1984).The authors also speculated that the phenotypic and genotypic variability within the species probably allows many populations

to persist under changing environmental conditions, growth and death cycles, immigration and emigration processes in an open system, finally leading to ecosystem stability and evolution based on fitness (Hirano & Upper, 1990).

Another important mechanism of plant pathogenesis in *P. s.* pv. *syringae* is the production of necrosis inducing-toxins, such as syringomycin, its amino acid derivatives, syringotoxin, syringostatin (Gross & Cody, 1985; Gross, 1991), syringopeptin (Ballio et al., 1991; Iacobellis et al., 1992), and pseudomycin (Ballio et al., 1994). Necrosis-toxin production appears limited in *P. syringae* pathovars and has only been described in pathovar *syringae* (Gross, 1991; Quigley & Gross, 1994) and recently in pathovar *atrofaciens* (Braun & Sands, 1995; Vassilev et al., 1996). Most pathogenic strains produced one or more of these low molecular weight lipopeptide antibiotics, which were not host specific but biocidal to a wide spectrum of microbes (Gross, 1991; Iacobellis et al., 1992). Although not exclusively necessary for pathogenicity, syringomycin contributed to virulence in several diseases caused by different ecotypes of *P. s.* pv. *syringae* (Xu & Gross, 1988; Mo & Gross, 1991 a; Gross, 1991). The primary mode of action of syringomycin was determined to be membrane disruption by forming ion channels, releasing monovalent and divalent cations, leading to uncontrolled ion transport across the plasmalemma and finally cell death (Gross & Cody, 1985; Hutchison et al., 1995). Furthermore, syringomycin was found to have biosurfactant activity, lowering the surface tension of water (Hutchison et al., 1995). It also stimulated ATPase activity and uncoupled the oxidative phosphorylation in maize mitochondria of resistant and susceptible lines (Gross & Cody, 1985). Toxin production was reported to be regulated by nutritional and environmental factors, similar to other secondary

metabolites (Gross & Cody, 1985; Gross, 1991). Low phosphate concentrations (<1 mM), moderate temperatures (< 28 C), a pH near neutrality and high iron concentrations were conducive for toxin production (Gross, 1985). It was calculated that between 4-40 ng of iron were needed to produce 50 units of syringomycin, which was the threshold concentration at which symptoms became visible in maize (Gross, 1985). The magnitude of iron required for syringomycin synthesis (>2μM of Fe^{3+} for max. expression; Mo & Gross, 1991 a) and the unavailability of iron to microbes in their environments suggested that *P. s.* pv. *syringae* acquired iron efficiently by the production of its fluorescent siderophore $pyoverdine_{Pss}$ (Gross & Cody, 1984). Research also demonstrated that plant signal molecules, such as arbutin, salicin and other phenolic glucosides activated toxin gene expression, resulting in an activation of phytotoxin production (Mo & Gross, 1991 b; Quigley & Gross, 1994). Moreover, the analysis of *syrB* and *syrC* genes indicated that the gene products function as peptide synthetases in a thiotemplate multienzyme mechanism of syringomycin biosynthesis, which involves non-ribosomal post-translational peptide chain modifications (Zhang et al., 1995).

In relation to recognition, pathogenicity and host range, the discovery of hypersensitivity reaction and pathogenicity genes (*hrp*), their regulation, function, and interaction with other genes, for instance avirulence (*avr*) genes or gene products, may be one of the most important recent findings in phytopathogenic bacteria (Lindgren, 1997). While *avr* genes limit the host range (race/cultivar specificity), they have been found to improve fitness and therefore confer a selective advantage on their host bacteria (Vivian & Gibbon, 1997). *Hrp* genes control the ability of phytopathogenic bacteria to cause disease

on susceptible cultivars and to elicit HR-reactions on resistant cultivars or nonhost plants (Vivian & Gibbon, 1997). Recent studies have demonstrated that Hrp proteins are components of Type III secretion systems, regulatory proteins, proteinaceous elicitors of the HR, and enzymes needed for synthesis of periplasmic β-1,2-glucans (Alfano & Collmer, 1996; Lindgren, 1997). The Type III secretion system is involved in the secretion of pathogenicity proteins across the inner and outer membranes in Gram-negative bacteria and the delivery of such proteins directly into host cells (Alfano & Collmer, 1996; Vivian & Gibbon, 1997). Furthermore, the transcriptional activation of a number of bacterial avirulence (*avr*) genes is controlled by Hrp regulatory proteins, and data suggest that Avr proteins may be transported by Hrp secretion systems directly into plant cells. Thus, avirulence genes may after all be virulence factors, and plants may have evolved to recognize the virulence factors of the pathogen and to localize the damage inflicted by way of a programmed cell-death (HR) (Taylor, 1996; Vivian & Gibbon, 1997). Interesting was also the discovery of the conservation of Type III secretion systems in animal pathogens (*Yersinia, Shigella, Salmonella*) and plant pathogens (*P. syringae*, *P. solanacearum*, *E. amylovora*, *X. c. vesicatoria*), indicating that fundamental determinants of pathogenicity may be linked or have evolved from a common ancestor in completely different pathogens (Alfano & Collmer, 1996; Vivian & Gibbon, 1997; Finlay & Falkow, 1997)

In addition to *hrp*- and *avr*-genes, *tox*-genes (Gross, 1991) and the *lemA/gacA* regulon (= a two-component sensory signal transduction system) (Rich et al., 1992; Barta et al., 1992; Rich et al., 1994) also represent genes encoding proteins that transcriptionally regulate the expression of pathogenicity/virulence factors which contribute to disease and

symptom development in *P. syringae*. LemA serves as a membrane-bound sensor kinase that, in response to external environmental signals, activates the cytoplasmic GacA by phosphorylation. Activated GacA in turn stimulates the expression of several genes including toxin genes, thus leading to the production of syringomycin, in *P. s.* pv. *syringae* (Rich et al., 1994) or tabtoxin in *P. s.* pv. *coronafaciens* (Barta et al., 1992). Although *hrp*- and *avr*-gene regulation seemed to be linked, it is not clear whether virulence factors may be controlled simultaneously by several regulators and/or several regulatory systems can regulate a single virulence factor (Finlay & Falkow, 1997). However, multiple mechanisms have evidently evolved within phytopathogenic bacteria for the secretion of pathogenicity/ virulence factors (Lindgren, 1997). The multiplicity of such factors may allow the bacterium to encounter host resistance as well as to tolerate mutations in one or more virulence genes without severe loss of pathogenicity. Thus, mutations in only one of these genes generally reduces rather than abolishes pathogenicity (Vivian & Gibbon, 1997).

In conclusion, despite their interkingdom distinctness (e.g. plant and animal pathogens, prokaryotes and eukaryotes), the systems seem to have common/conserved themes with regard to microbial pathogenicity (Finlay & Falkow, 1997). These include virulence factors and their regulation (Rahme et al., 1995), resistance mechanisms, such as programmed cell-death systems and signal transduction pathways (Baker et al., 1997), and protein secretion systems (Alfano & Collmer, 1996; Baker et al., 1997). These findings may provide new areas of research, since new classes of broad-spectrum compounds (e.g. inhibitors of conserved bacterial regulatory systems) may be developed or transferred from one system to the other with respect to disease control, once the interconnections between

regulatory systems have been elucidated. Accordingly, resistance to common virulence factors might be transferred from plant to animal pathogens, or vice versa.

Phenotypic and Genetic Diversity of *P. s.* pv. *syringae*

Over the last decade, nucleic acids research has become of increasing importance for the taxonomic identification and classification of bacteria, especially at the pathovar and strain level. In addition, DNA analyses provided information on the assessment of genetic diversity and phylogenetic relationships among bacterial populations and individuals within a population. The heterogeneity of *P. syringae* strains within and between pathovars has been described using both phenotypic (Sands et al., 1970; Endert & Ritchie, 1984; Hirano & Upper, 1985; Roos & Hattingh, 1987; Denny, 1988) or genetic characters (Denny et al., 1988; Cooksey & Graham, 1989; Louws et al., 1994) or a combination of both (Hendson et al., 1992; Liang et al., 1994). Most authors agreed that strains within the pathovar *syringae* represented very diverse populations while strains of pathovars *tomato* (Denny et al., 1988) or *morsprunorum* (Endert & Ritchie, 1984; Liang et al., 1994) were more host-specific and more similar in their genetic structure. The level of genetic diversity in *P. s.* pv. *syringae* supported the hypothesis that variation was higher among strains from diverse habitats (e.g. wide host range Pss) than among those from a uniform habitat (e.g. *P. s.* pv. *tomato* from tomato) (Denny et al., 1988; Cooksey & Graham, 1989). However, Hendson et al. (1992) found no distinctive profiles that clearly differentiated strains of different pathovars (*tomato*, *maculicola*, *antirrhini*), which were isolated from different hosts but exhibited similar nutritional properties and overlapping host ranges. Genetically they all belonged to a single

group with several subgroups, suggesting a common ancestry for these strains. They concluded that nutritional characterization was a more reliable indicator of genetic relationships among strains than the host of origin. Furthermore, the nutritional versatility of Pss was thought to be responsible for the fast adaptation and survival on a multiplicity of plants, including hosts and nonhosts (Hildebrand et al., 1988; Hendson et al., 1992). The recovery of bean strains of Pss from different locations with an identical haplotype, and different haplotypes from within one region, was reported to be attributed to the introduction of the pathogen on bean seed into the areas, since the pathogen is seed-borne and companies distribute seeds throughout the US (Legard et al., 1993). Due to rapid generation times of approximately 3.5 to 4.5 hours after rain events, Pss populations on snap beans had the potential to evolve quickly (Hirano & Upper, 1985). The authors hypothesized that if selection among genotypes (i. e., strains) occurs in each generation, the possibility of several generations per day provides opportunity for amplification of small changes per generation, resulting in large population differences in a relatively short period. The ecosystem in which Pss was an integral component was variable and dynamic and so was the number of Pss genotypes present (Hirano & Upper, 1985).

Although, most studies employed restriction fragment-length polymorphism (RFLP) and DNA-hybridization for the differentiation of *Pseudomonas syringae* between and within pathovars (Denny et al., 1988; Hendson et al., 1992; Legard et al., 1993; Quigley & Gross, 1994; Martinez-Miller, 1994), similar results were obtained with genomic fingerprinting. Rare-cutting restriction endonucleases generated distinctive patterns of large DNA fragments which could be separated by field inversion (Cooksey & Graham, 1989) or pulsed-

field gel electrophoresis (Grothues & Tümmler, 1991). Such macrorestriction fingerprints of the entire bacterial genome were shown to be more sensitive to mutations, including deletions and insertions at a specific restriction site than RFLP analysis with only one DNA-hybridization probe, thus changing the genome profile and exposing polymorphism. The methods separated *Pseudomonas* species (Grotheus & Tümmler,1991) and classified *P. s.* pv. *tomato* to be distinct from other pathovars of *P. syringae* (Cooksey & Graham, 1989). Recently, PCR-RFLP analysis of rRNA operons and rep-PCR-based amplification of variable DNA regions between conserved bacterial repetitive elements have resulted in the rapid and reproducible identification of plant pathogenic bacteria at the species, pathovar and even strain level (Manceau & Horvais, 1997; Louws et al., 1994). Alternatively, Bragard et al. (1997) employed AFLP (Amplified Fragment Length Polymorphism) DNA fingerprinting to characterize 68 xanthomonad strains from small grains and found the results to be consistent with pathogenicity tests and RFLP analysis, recommending AFLP as a method of choice not only for taxonomy, but also for epidemiological studies. Although each of the above mentioned DNA-techniques alone provided some information on whether different isolates represented a single evolutionary line or were composed of several lines that had converged to a similar pathogenic phenotype, the combination of biochemical, genetic and pathological analyses was recommended for the assessment of phylogeny and evolution of strains in relation to the host plant (Denny, 1988; Hendson et al., 1992). In addition, strains of the same geographical origin and host cultivar should be investigated to study genetic diversity and evolutionary relationships within a field population. Accordingly, the variation of the bacterial rice blight pathogen *Xanthomonas oryzae* pv. *oryzae* was

investigated in only one field, on only one cultivar in the Philippines (Vera Cruz et al., 1996). This analysis, done at the micro geographic scale, provided valuable information on the effects of host resistance on the emergence of new virulent races in the population structure of this pathogen.

The Control of Basal Kernel Blight

Due to the apparent ubiquitous distribution and dissemination of *P. s.* pv. *syringae* on nonhost plants in barley growing areas, which resembled the distribution of Pss in bean growing areas in Wisconsin (Hirano & Upper, 1983), the elimination of the pathogen from seeds and foliage of the host may not be sufficient to control the basal kernel blight disease. In addition, the use of bactericides and antibiotics on cereal grains has not been considered practical. Hence, the only control for barley kernel blight suggested to date has been the avoidance of irrigation during the most susceptible kernel developmental stage at soft dough and the use of less susceptible cultivars (Martinez-Miller et al., 1998). Over the last three years we have evaluated a biological control system based on the application of indigenous antagonistic *Pantoea agglomerans* bacteria prior to the *P. s. syringae* infection window to reduce the kernel blight disease incidence (Braun et al., 1996; Braun et al., 1997). Furthermore, the use of biological control agents to control plant diseases, especially with respect to integrated pest management strategies, has become more practical and acceptable in the United States in the last decade, despite its lack of funding (Cook, 1993; Jacobsen, 1997). IPM strategies, including the commercial use of biological control agents, is of particular significance in minor crops or major crops in which no chemicals are available

to control certain pests or diseases; an example being the IR-4 program, a national project promoting pest control products for minor crops. Market studies indicated a growth rate of 10 % per annum for biocontrol-based crop protection. It is predicted that bio-control products will capture a $2-3 billion market share of the $20-30 billion pesticide market at the producer's level worldwide by the turn of the century (Menn et al., 1993).

The Antagonist *Pantoea agglomerans*

Taxonomically, *Pantoea agglomerans* belongs to the family Enterobacteriaceae. The genus *Pantoea* was created in 1989 by Gavini et al. (1989) based on DNA-DNA hybridization and protein electrophoresis. It included two species, *Pantoea agglomerans* [syn. *Enterobacter agglomerans* (Beijerinck 1888) Ewing & Fife 1972; syn. *Erwinia herbicola* (Löhnis 1911) Dye 1964; syn. *Erwinia milletiae* (Kawakami & Yoshida 1920) Magrou 1937] and *Pantoea dispersa*. *P. agglomerans* is frequently referred to by its synonyms, *Enterobacter agglomerans* (Ewing & Fife, 1972) by human pathologists when isolated as clinical isolates from animal or human sources or *Erwinia herbicola* by phytopathologists when isolated from plant sources (Holt et al., 1994). *E. herbicola* exists as an epiphyte on plant surfaces and as a saprophytic secondary organism in lesions caused by many plant pathogens (Dye, 1983; Lelliot & Dickey, 1984). Some strains were reported to cause galls on table beets (Burr et al., 1991), black spot necrosis on beach peas in Canada (Khetmalas et al., 1996), or damage to garlic crops (Koch et al., 1996). *Erwinia herbicola* pv. *gypsophilae* induced gall formation on *Gypsophila paniculata* (Young et al., 1991).

Because the name *Pantoea agglomerans* has finally entered Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and has been approved by the International Society for Plant Pathology (Young et al., 1996), it will be used throughout this report.

The Use of *P. agglomerans* as Biological Control Agent (BCA)

Pantoea agglomerans has been used in several studies to control fungal as well as bacterial plant pathogens. For instance, Kempf (1988) employed *E. herbicola* bacteria to control *Fusarium culmorum* and *Puccinia recondita* f. sp. *tritici* on wheat. He found about 50-60% *F. culmorum* disease suppression on wheat seedlings in the greenhouse after seed coating with *E. herbicola* isolate B247 based on shoot and root dry weight determinations. Furthermore, 78 % disease suppression of *P. recondita* f. sp. *tritici* was revealed when strain B247 was sprayed on wheat leaves two hours prior to uredospore application. Adetuyi (1992), also working on the antagonistic potential of *E. herbicola* against soilborne pathogens on wheat, discovered that antagonists coated onto seeds protected them from attack of *Fusarium nivale* and *Pythium ultimum*. Rate of emergence, stand counts, average root and shoot height were significantly higher in bacterial treated seedlings than in untreated controls, suggesting protection as well as plant growth promoting effects. A plant growth promoting effect of *P. agglomerans* strain D5/23 isolated from the phyllosphere of winter wheat was also noted by Ruppel et al. (1989). The authors detected nitrogenase- and phytohormone activity in these plant associative bacteria in addition to wheat and barley yield increases of up to 500 kg/ha in field experiments (Ruppel et al., 1989; Scholz-Seidel & Ruppel, 1992). Rosales et al. (1993) isolated 23 bacterial strains including one *E.*

herbicola isolate from rice fields in the tropics and tested their effect on germination, seedling development and inhibition of mycelial growth of *Rhizoctonia solani*. All strains inhibited *Rhizoctonia solani in vitro*, however *Bacillus subtilis* isolates performed the best. Seed bacterization provided a sheath blight protection of 4.5 to 73% in a glasshouse trial depending on the isolate screened. Yuen et al. (1994), who studied the inhibition of *Sclerotinia sclerotiorum* on dry edible beans by *E. herbicola*, noticed that strains applied to beans at fully expanded, mature blossom stage prior to inoculation with ascospores of the pathogen, inhibited ascospore germination and subsequent development of white mold lesions in a bioassay, while field applications were less effective due to unfavorable weather conditions for the antagonists. Furthermore, *E. herbicola* was reported to control *Alternaria solani* on tomato (Sujkowski et al., 1994), *Xanthomonas campestris* on onions (Paulraj et al., 1993) and *Verticillium dahliae* in an *in vitro* assay (Berg & Ballin, 1994).

Another often cited example of excellent biocontrol performance of *Erwinia herbicola* is the fire blight system in apples and pears. In research apple orchards, *E. herbicola* has been demonstrated to be an effective antagonist of *Erwinia amylovora* (Beer et al., 1984 a). The protection from fire blight achieved by spraying apple blossoms with *E. herbicola* one day prior to *E. amylovora* was often equivalent (42-51%) to the protection achieved by spraying streptomycin (61%), the most commonly used control agent in North America. Isenbeck & Schulz (1985) isolated several *E. herbicola* strains from blighted ornamentals in Germany that showed antagonistic activity against *E. amylovora in vitro*. Eh 112 y, an isolate obtained from Cornell University demonstrated effective disease reduction in an immature pear fruit assay and under controlled conditions using *Cotoneaster bullatus*

as a test plant if the antagonist was injected 24 h ahead of the pathogen. The antagonist treatment in fact was as effective as the 200 ppm streptomycin control. The authors reported 20-40% fire blight disease reductions under field conditions following natural infection. Vanneste (1990) studied the control of fire blight by *E. herbicola* strain Eh252 in an experimental orchard in France and got 40% disease control compared to the untreated control. Furthermore, Wilson et al. (1990) demonstrated fire blight protection of hawthorn with *E. herbicola* under controlled conditions in the United Kingdom. In New Zealand an *E. herbicola* strain was sprayed on apple blossoms in the orchard and prevented fire blight disease in apple blossom in glasshouse trials (Kearns & Hale, 1993). Field trials conducted in 1991 and 1992 in Oregon and 1992 in Washington revealed a 87.5% fire blight disease reduction in pear blossoms in Oregon 1991 compared to water controls. In 1992 the disease was reduced by 50% and 56% in Washington. However, the streptomycin control resulted in the best disease prevention over all years and locations with 98.8%, 95.5%, and 72.2% disease reduction, respectively (Johnson et al., 1993).

Another example of the engagement of *E. herbicola* in biocontrol was presented by Lindow (1983), when he successfully employed an antagonistic *E. herbicola* isolate (INA-minus) to control frost injury, caused by ice nucleation active (INA-plus) *P. s.* pv. *syringae* bacteria on corn in the field.

The Use of *P. agglomerans* in Systems other than Plant Pathology

In addition to the use of *P. agglomerans* as biocontrol agents in plant pathology, the bacterium and the compounds it produces have been exploited commercially. For instance,

its tyrosine phenol-lyase activity catalyzed the production of L-tyrosine and dihydroxyphenyl-L-alanine (L-dopa), two essential amino acids that can be synthesized from ammonia, pyruvate, and phenol or catechol, respectively (Lloyd-George & Chang, 1995). Furthermore, *P. agglomerans*' massive and fast gluconic acid production by glucose oxidation enhances mineral-phosphate solubility due to acid dissolution of hydroxyapatite (Liu et al., 1992). This is important, since the world's phosphate resources are limited and phosphate is present but unavailable in most soils, due to its structural insolubility, similar to iron.

Herbicolins were described as new acylpeptide antibiotics with antimycetic activity against dermatophytes in human medicine (Winkelmann & Adam, 1980). Furthermore, outer membrane lipopolysaccharides from *P. agglomerans* (LPSp) have been found to inhibit morphine (Okutomi et al., 1992) and cocaine dependent withdrawal signs (Suzuki et al., 1994), revealing their potential use in pharmacotherapy for the prevention of drug abuse. The administration of LPSp showed a marked inhibition of the jumping of mice on naloxone-precipitate withdrawal, however endogenous β-endorphin and tumor necrosis factor (TNF) levels were induced (Okutomi et al., 1992). In a second study, the treatment with LPSp abolished cocaine- induced locomotor enhancement in mice and the cocaine-induced place preference in rats, suggesting that while LPSp itself may possess neither reinforcing nor locomotor enhancing effects, it blocks both the reinforcing and the locomotor enhancing effects of cocaine (Suzuki et al., 1994). The authors claimed that immediately after LPSp administration, a cytokine cascade begins which produces TNF, followed by ACTH/β-LPH gene product, which generates β-endorphin as an endogenous

opioid over a period of 6 h after the injection. As a result, LPSp induced an antinociceptive effect. In 1994, Kamei and coworkers reported that subcutaneous injection of LPSp suppressed hyperalgesia in streptozotocin-induced diabetic mice and also exerted a therapeutic effect on hyperalgesia in these animals. The data indicated that LPSp may be effective in relieving the pain associated with diabetic neuropathy. Recently, a low molecular weight (5 kDa) LPSp from *P. agglomerans* has been recognized as potent antitumor agent in animal tumor models and its use in human cancer therapy is currently under investigation (Goto et al., 1996). Clinical application of LPSp administered intradermally in combination with chemotherapeutics such as cyclophosphamide appeared even more promising in terms of its antitumor effects, whereas pretreatment by anti-tumor necrosis factor antibody reduced the effect exerted by LPSp, indicating that induced TNF might play a crucial role in antitumor activity and the induction of cell mediated immunity (Iwamoto et al., 1996; Inagawa et al., 1997).

In contrast to these studies, a negative effect of endotoxins, associated with LPS from *P. agglomerans* and *Enterobacter spp.*, was found relevant to pathogenesis of alveolitis in organic dust-induced lung diseases (Milanowski et al., 1995). Wood dust from American basswood contained numerous Gram-negative bacteria, including *P. agglomerans*, that were shedding large amounts of the endotoxin-containing membrane vesicles, suggesting that these environmental "dust-borne" endotoxins represented an increased risk for people exposed to various organic dusts (Dutkiewicz et al., 1992).

Biological Control Mechanisms Associated with *P. agglomerans*

Nitrogen Depletion and Acidification

Nitrogen depletion and acidification of the environment have been reported in the interaction between *E. herbicola* and pathogenic bacteria such as *E. amylovora* or *Xanthomonas oryzae* in vitro (Riggle & Klos, 1972; Hsieh & Buddenhagen, 1974; Beer et al., 1984 b). However, organic acid production had never been demonstrated as a mechanism *in planta* (Gibbins, 1978; Wodzinski et al., 1994) and results of the N-depletion hypothesis were inconsistent (Wodzinski et al., 1987; Wodzinski et al., 1994).

Antibiosis

Since *P. agglomerans* are successful inhibitors of plant pathogens, much attention has been paid to the diversity and role of antimicrobial compounds produced by several strains. Herbicolins A and B were described as lipodepsinonapeptide antibiotics that inhibited sterol-requiring fungi (Winkelmann et al., 1980; Aydin et al., 1985). Antibiotic genes were cloned and transferred from *E. herbicola* to *E. coli*, resulting in expression of antifungal activity in *E. coli* (Tenning et al., 1993). Proteinaceous antibiotics that only kill strains of the same or closely related species to *E. herbicola* had been called bacteriocins (Beer & Rundle, 1980; Vidaver, 1983) or bacteriocin-like if they were non-proteinaceous in nature (Beer & Vidaver, 1978; Stein & Beer, 1980). All other compounds with a wider spectrum of activity, including more distantly related bacteria, were designated simply antibiotics. Their chemical structures, if known, mostly contained monocyclic and bicyclic

β-lactam molecules (Parker et al., 1982; Ishimaru et al., 1988; Bainton et al., 1992; Kearns & Hale, 1996), and as such interfered with the transpeptidation step in the cross-linking of amino acids that connect the glycan chains to form the peptidoglycan of bacterial cell walls. This inhibition of the final stage of murein synthesis somehow triggers the autolytic activities of murein hydrolases, resulting in cell lysis (Jacobs, 1997). Biosynthesis of β-lactam antibiotics in *E. carotovora* was shown to be regulated by cell density-dependent gene regulation processes and autoinducer signal molecules (Bainton et al., 1992; Chhabra, et al., 1993). Furthermore, pyrrolnitrin production by *E. herbicola* strain IC1270, usually produced by *Pseudomonas* species, was reported by Chernin et al. (1996), which generated broad spectrum antagonistic activity towards fungal and bacterial phytopathogens, including *P. s.* pv. *syringae*. Another *E. herbicola* antibiotic from strain Eh 252 shared DNA sequence homologies with microcin C7 from *E. coli*, which is a modified heptapeptide and inhibits translation in protein synthesis of *Enterobacteriaceae* (Vanneste & Yu, 1996). Finally, strain Eh 318 was reported to produce two antibiotics termed pantocin A and B, of which pantocin A's structure and function resembled microcin C7 and pantocin B remains to be sequenced and its mode of action determined (Wright, 1997; Wright & Beer, 1997). Studies on the role of *E. herbicola* antibiotics in biocontrol have demonstrated that non-antibiotic producing strains reduced fire blight as efficiently as antibiotic producing strains, indicating no *in-vivo* role for antibiotics (Beer et al., 1984; Wodzinski & Paulin, 1994). Similarly, Lindow (1988) found no correlation of *in vitro* antibiosis with antagonism of ice nucleation active bacteria on leaf surfaces by non-ice nucleation active bacteria. However, for strains Eh 252 (Vanneste et al., 1992) and Eh 318 (Wodzinski et al., 1994; Wright, 1997), effective

antagonists of *E. amylovora* in field tests, there was evidence that the antibiotics may be an important, though not exclusive, mechanism by which *E. herbicola* inhibits *E. amylovora*.

Competition for Space and Nutrients

Hattingh et al. (1986) observed that the most important site of interaction between *E. herbicola* and *E. amylovora* was on the stigmata of apple blossoms. The two species appeared to colonize the stigmatic surface in an identical manner, and were thought to be competing for the same sites. However, Lindow (1987) claimed that while two strains may occupy the same leaf surface, antagonism between those two strains may only occur if they utilize the same limited phylloplane resource. This hypothesis was confirmed by Wilson et al. (1992), who suggested that *E. herbicola* and *E. amylovora* did not compete for physical space, but for a limiting resource, presumably a nutrient, and *E. herbicola* was more efficient than *E. amylovora* in acquiring the limited resource. A microscopic study of the colonization sites indicated that space was not a limiting factor. The conclusion of competition for a limited resource was based on the observation that *E. amylovora* and *E. herbicola* inoculated alone would reach populations of 10^6 cfu/blossom, the apparent carrying capacity of the stigmatic surface of a hawthorn blossom. When coinoculated at the same concentration, *E. amylovora* never reached a population level greater than 10^3 - 10^4 cfu/blossom, while *E. herbicola* still approached 10^6 cfu/blossom. Later studies, which were based on *in vitro* carbon source utilization patterns of biocontrol agents and pathogens, indicated that the more closely related the two competitors are, the greater is their similarity in nutritional requirements based on niche overlap indices (Wilson & Lindow, 1994 a,b). Their studies

resulted in the conclusion that the Ice-minus mutant of *P. syringae* TLP2del1 would be a better biocontrol agent of Ice-plus *P. syringae* than *P. agglomerans*, which competed with *P. syringae* mainly for amino acids and organic acids; but few carbohydrates. However, recent studies revealed no significant correlation between biocontrol effectiveness and nutritional similarity (Ji et al., 1996; Dianese & Wilson, 1996). A lack of correlation was thought to reflect the difficulty to transfer *in vitro* data to *in vivo* events, since for example a differential abundance of carbon sources in the plant environment was not taken into account when establishing the niche overlap indices (NOIs). Hence, current studies are directed toward the development of "weighted NOIs" based on the relative abundance of phyllosphere carbon sources in the particular plant-microbe system (Ji et al., 1996).

Since iron availability is usually limited in most environmental systems (Leong, 1986; Loper & Lindow, 1994), competition for iron as a limited resource has been suggested to operate in many biological control agent-pathogen interactions (Kloepper et al., 1980; Weller & Cook, 1986; Yuen & Schroth, 1986; Leong, 1986; Loper & Buyer, 1991), if their iron uptake systems are unique and a pathogen can not use ferric siderophores from beneficial strains (Leong, 1986; Loper & Buyer, 1991) or the beneficial strains have stronger siderophore-chelators (greater iron(III) affinity, relative kinetics of the iron transport system) than the pathogen (Leong, 1986; Loper & Buyer, 1991; Bull et al., 1994). Studies in the past have concentrated on the cloning of genes involved in siderophore production and the structure identification of siderophores produced by biocontrol agents (Kloepper et al., 1980; Teintze et al., 1981; Moores et al., 1984; Leong, 1986; Costa & Loper, 1994; Feistner & Ishimaru, 1997) or pathogens (Loper et al., 1984; Expert & Toussaint, 1985; Cody & Gross,

1987; Bull et al., 1994) as well as on the importance of siderophore-deficient mutants of pathogens (Cody & Gross, 1987; Loper & Lindow, 1987; Bull et al., 1996). Future research should focus on the importance and ecological fitness of siderophore-deficient biocontrol mutants and the interaction between different siderophores present in the same environment. Cross feeding studies as mentioned by Bull et al. (1994, 1996) could shed some light on whether ferric siderophores produced by the pathogen can be taken up and transported across the membranes by biocontrol agents, and vice versa. This would help elucidate the significance of iron(III)-competition in microbe-plant interactions.

Induction of Systemic Resistance in Monocots

While the induction of systemic resistance (ISR) or systemic acquired resistance (SAR) mechanisms, respectively, in dicots by plant associated bacteria have been demonstrated on several occasions (Kuc & Richmond, 1977; Wei et al., 1991,1996; Oostendorp et al., 1996; Press et al., 1997), ISR/SAR in monocots is less well studied. The most intriguing difference between those two mechanisms is the occurrence of a localized necrosis (HR) on induced leaves in SAR, similar to the gene-for-gene system, which is however, more specific and does not result in cross-resistance to several pathogens, and the dependence on salicylic acid or another systemic signal (jasmonate, systemin, ethylene, etc.) that induces SAR gene expression (Sticher et al., 1997).

Localized acquired resistance (LAR) after induction with some races of *Erysiphe graminis* has been described in barley leaves (Ouchi et al., 1974), in rice by incompatible

strains of *Xanthomonas oryzae* (Horino, 1976), and in wheat against powdery mildew with the nonhost pathogen *Erysiphe graminis* f. sp. *hordei* (Schweizer et al., 1989; Schaffrath et al., 1997). ISR in barley was demonstrated against powdery mildew by the use of extracts from *Bacillus subtilis* (Steiner et al., 1988). SAR was reported in rice after preinoculation with *P. s.* pv. *syringae* (Smith & Metraux, 1991) and in barley after preinoculation with *Erysiphe graminis* f. sp. *hordei* conidia (Hwang & Heitefuss, 1992). According to Smedegaard-Peterson et al. (1991) both virulent and avirulent races of *Erysiphe graminis* f. sp. *hordei* and a race of *Erysiphe graminis* f. sp. *tritici* could induce resistance in barley towards powdery mildew. In wheat infected with barley and wheat strains of *E. graminis*, several resistance-specific cDNAs were induced by pathogen infection (Kessmann et al., 1994). In addition to biological inducers, synthetic chemicals can also trigger acquired resistance, for instance, probenazole, 2,2-dichloro-3,3-dimethylcyclopropane carboxylic acid, 2,6-dichloroisonicotinic acid (INA) and benzo-(1,2,3)-thiodiazole-7-carbothioic acid S-methyl ester (BTH) in rice, INA in barley, and salicylic acid, INA, BTH and CGA245704 (= Actigard®) in wheat (Kogel, et al., 1994; Oostendorp et al., 1996, Sticher et al., 1997; Schaffrath et al., 1997). However, the induction of systemic resistance in barley by *Pantoea agglomerans* has never been reported.

Concluding Remarks and Objectives

Because *P. s.* pv. *syringae* is one of the most important bacterial pathogens on a multitude of plants worldwide, the pathogen has been studied since the start of this century

(Hirano & Upper, 1983). However, studies on its pathogenesis on monocots are not as complete as those for dicot hosts. Its epiphytic lifestyle on barley cultivars has been reported (Georgakopoulos & Sands, 1992) and its pathogenicity was described (Peters et al., 1983; Martinez-Miller, 1994). Since its impact on barley increased in the early 1990s in the barley growing areas in the Northern Prairies of the US, its epidemiology and cultivar response to bacterial attack has been examined (Martinez-Miller, 1994; Martinez-Miller et al., 1998). However, insights into its pathogenesis, phylogeny, and disease control management are still missing.

The present study on the pathogenesis and phylogeny of *Pseudomonas syringae* pv. *syringae* causing basal kernel blight of barley and its biological control by antagonistic *Pantoea agglomerans* had the following objectives:

1. To study the localization and pathogenesis of *P. s.* pv. *syringae* in barley kernels, using light- as well as scanning electron-, and transmission electron microscopy.
2. To determine the phylogenetic and possible evolutionary relationships of *P. s.* pv. *syringae* populations existing in a barley field composed of several different cultivars in one geographical area; and correlations between syringomycin production, pathogenicity tests on tobacco and barley cultivar B 2601, respectively, nutritional profiles and DNA-fingerprinting.
3. To develop an effective and practical biological control strategy using indigenous *Pantoea agglomerans* in glasshouse and field experiments.
4. To investigate mechanisms in the interaction between biocontrol agents, the pathogen, and the host plant barley.

These studies should lead to a better understanding of *P. s.* pv. *syringae* as a causal agent of basal kernel blight of barley and the mechanisms involved in its suppression by *Pantoea agglomerans*. Knowing about the causal agent, their relationship to the host plant, in addition to elucidating the mechanisms by which the pathogen can be controlled by other naturally occurring phyllosphere microorganisms could be used in other plant-pathogen systems for the successful prevention of plant diseases.

Literature Cited

Adetuyi, F. C. 1992. Antagonistic potential of *Pseudomonas syringae* and *Erwinia herbicola* on wheat seeds. Indian Journal of Mycology and Plant Pathology 22(3): 250-254.

Alfano, J. R. and Collmer, A. 1996. Bacterial Pathogens in Plants: Life up against the wall. The Plant Cell 8 (10): 1683-1698.

Anderson, W. H. and Banttari, E. E. 1976. The effect of *Bipolaris sorokiniana* on yield, kernel weight and kernel discoloration in six-row spring barleys. Plant Dis. Rep. 60: 754-758.

Anonymous. 1997 a. National Agricultural Statistics Service. Small grains 1997 summary. Washington D. C.

Anonymous. 1997 b. Montana Agricultural Statistics Service. Barley varieties 1997. 301 S. Park, Drawer 10033. Helena, MT, 59626.

Aydin, M, Lucht, N., König, W. A., Lupp, R., Jung, G., and Winkelmann, G. 1985. Structure elucidation of the peptide antibiotics Herbicolin A and B. Liebigs Ann. Chem. 1985: 2285-2300.

Bainton, N. J., Bycroft, B. W., Chhabra, S. R., Stead, P., Gledhill, L., Hill, P. J., Rees, C. E. D., Winson, M. K., Salmond, G. P. C., Stewart, G. S.A.B., and Williams, P. 1992. A general role for the *lux* autoinducer in bacterial cell signaling: control of antibiotic biosynthesis in *Erwinia*. Gene 116: 87-91.

Baker, B., Zambryski, P., Staskawicz, B., and Dinesh-Kumar, S. P. 1997. Signaling in plant-microbe interactions. Science 276: 726-733.

Ballio, A., Barra, D., Bossa, F., Collina, A., Grgurina, I., Marino, G., Moneti, G., Paci, M., Pucci, P., Segre, A., and Simmaco, M. 1991. Syringopeptins, new phytotoxic lipodepsipeptides of *Pseudomonas syringae* pv. *syringae*. FEBS Letters 291(1): 109-112.

Ballio, A., Bossa, F., Di Giorgio, D., Ferranti, P., Paci, M., Pucci, P., Scaloni, A., Segre, A., Strobel, G. A. 1994. Novel bioactive lipodepsipeptides from *Pseudomonas syringae*: the pseudomycins. FEBS Letters 355: 96-100.

Barta, T. M., Kinscherf, T. G., and Willis, D. K. 1992. Regulation of tabtoxin production by the lemA gene in *Pseudomonas syringae*. J. Bacteriol. 174(9): 3021-3029.

Basson, A.B.K., de Villiers, O. T., and Rabie, C. J. 1990. Effect of black ends on quality characteristics of clipper barley and malt. J. Am. Soc. Brew. Chem. St. Paul, MN: The Society. Winter 1990. 48(1):8-13.

Beck von Bodman, S. and Farrand, S. K. 1995. Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an N-acylhomoserine lactone autoinducer. J. Bacteriol. 177(17): 5000-5008.

Beer, S. V. and Vidaver, A. K. 1978. Bacteriocins produced by *Erwinia herbicola* inhibit *Erwinia amylovora*. In: Proc. of the 3rd international congress of plant pathology. W. Laux (ed.). Munich, Germany, 16-23 August, 1978, pp. 75 (Abstract).

Beer, S. V. and Rundle, J. R. 1980. Inhibition of *Erwinia amylovora* by bacteriocin-like substances. Phytopathology. 70(5): 459 (Abstract).

Beer, S. V., Rundle, J. R., and Norelli, J. L. 1984 a. Recent progress in the development of biological control of fire blight - a review. Acta Hortic. 151: 195-201.

Beer, S. V., Rundle, J. R., and Wodzinski, R. S. 1984 b. Interaction between *Erwinia amylovora* and *Erwinia herbicola* in vitro, in immature pear fruits and in apple blossoms. Acta Hortic. 151: 203-204.

Berg, G. and Ballin, G. 1994. Bacterial antagonists to *Verticillium dahliae* Kleb. J. Phytopathol. (Berl.). 141: 99-110.

Bradbury, J. F. 1986. *Pseudomonas*. In: Guide to plant pathogenic bacteria. CAB International Mycological Institute, Kew, United Kingdom. pp. 154-187.

Bragard, C., Singer, E., Alizadeh, A., Vauterin, L., Maraite, H., and Swings, J. 1997. *Xanthomonas translucens* from small grains: diversity and phytopathological relevance. Phytopathology 87: 1111-1117.

Braun, A. and Sands, D. C. 1995. Identification of two bacterial blights and their suppression by *Pantoea agglomerans*. Phytopathology 85(10): 1163 (Abstract).

Braun, A., Rose, A., Kaliyeva, G.T., Martinez-Miller, C., and Sands, D. C. 1996. Biocontrol of bacterial kernel blight of barley by beneficial *P. agglomerans*. Phytopathology 86 (11): S79 (Abstract).

Braun, A., Sands, D. C., and Jacobsen, B. J. 1997. Suppression of bacterial kernel blight of barley by antagonistic *Pantoea agglomerans*. Biol. Cult. Tests Control Plant Dis. 12: 105.

Bull, C. T., Carnegie, S. R., and Loper, J. E. 1996. Pathogenicity of mutants of *Erwinia carotovora* subsp. *carotovora* deficient in aerobactin and catecholate siderophore production. Phytopathology 86: 260-266.

Bull, C. T., Ishimaru, C. A., and Loper, J. E. 1994. Two genomic regions involved in catechol siderophore production by Erwinia carotovora. Appl. Environ. Microbiol. 60(2): 662-669.

Burr, T. J., Katz, B. H., Abawi, G. S., and Crosier, D. C. 1991. Comparison of tumorigenic strains of *Erwinia herbicola* isolated from table beet with *E. h. gypsophilae*. Plant Dis. 75: 855-858.

Chernin, L., Brandis, A., Ismailov, Z., and Chet, I. 1996. Pyrrolnitrin production by an *Enterobacter agglomerans* strain with broad spectrum antagonistic activity towards fungal and bacterial phytopathogens. Current Microbiology 32: 208-212.

Chet, I., Zilberstein, Y., and Henis, Y. 1973. Chemotaxis of *Pseudomonas lachrymans* to plant extracts and to water droplets collected from the leaf surfaces of resistant and susceptible plants. Physiol. Plant Pathol. 3: 473-479.

Chhabra, S. R., Stead, P., Bainton, N. J., Salmond, G. P. C., Stewart, G. S.A.B., Williams, P., and Bycroft, B. W. 1993. Autoregulation of carbapenem biosynthesis in *E. carotovora* by analogues of N-(3-oxohexanoyl)-L-homoserine lactone. The Journal of Antibiotics 46(3): 441-454.

Cody, Y. S. and Gross, D. C. 1987. Characterization of pyoverdine$_{Pss}$, the fluorescent siderophore produced by *Pseudomonas syringae* pv. *syringae*. Appl. Environ. Microbiol. 53(5): 928-934.

Cook, R. J. 1993. The role of biological control in pest management in the 21st century. In: Proceedings of Beltsville Symposium XVIII, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland, May 2-6, 1993. R. D. Lumsden and J. L. Vaughn (eds.). American Chemical Society, Washington, D.C., pp. 10-21

Cooksey, D. A. and Graham, J. H. 1989. Genomic fingerprinting of two pathovars of phytopathogenic bacteria by rare-cutting restriction enzymes and field inversion gel electrophoresis. Phytopathology 79: 745-750.

Costa, J. M. and Loper, J. E. 1994. Characterization of siderophore production by the biological control agent *Enterobacter cloacae*. Molecular Plant-Microbe Interactions 7(4): 440-448.

Costerton, J. W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C. 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. 41: 435-464.

Denny, T. P., Gilmour, M. N. and Selander, R. K. 1988. Genetic diversity and relationships of two pathovars of *Pseudomonas syringae*. Journal of General Microbiology. 134: 1949-1960.

Denny, T. P. 1988. Phenotypic Diversity in *Pseudomonas syringae* pv. *tomato*. Journal of General Microbiology. 134: 1939-1948.

Dianese, A. C. and Wilson, M. 1996. Evaluation of the importance of nutritional similarity between biocontrol agents and the target pathogen in biocontrol of *Xanthomonas campestris* pv. *vesicatoria* on tomato. Phytopathology 86(11): S 50 (Abstract).

Dutkiewicz, J., Tucker, J., Burrell, R., Olenchock, S. A., Schwegler-Berry, D, Keller, G. E., Ochalska, B., Kaczmarski, F., and Skorska, C. 1992. Ultra structure of the endotoxin produced by gram-negative bacteria associated with organic dusts. Systematic and Applied Microbiology 15(3): 474-485.

Dye, D. W. *Erwinia*: The "Amylovora" and "Herbicola" Groups. In: Plant Bacterial Diseases - A Diagnostic Guide. P. C. Fahy and G. J. Persley (eds.), Academic Press Australia, pp. 67-86.

Dye, D. W., Bradbury, J. F., Goto, M., Hayward, A. C., Lelliott, R. A., and Schroth, M. N. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. Rev. Plant Path. 59: 153-168.

El-Banoby, F. E., and Rudolph, K. 1979. A polysaccharide from liquid cultures of *Pseudomonas phaseolicola* which specifically induces water-soaking in bean leaves (*Phaseolus vulgaris* L.). Phytopathol. Z. 95: 38-50.

Endert, E. and Ritchie, D. F. 1984. Detection of pathogenicity, measurement of virulence, and determination of strain variation in *Pseudomonas syringae* pv. *syringae*. Plant Dis. 68: 677-680.

Ewing, W. H. and Fife, M. A. 1972. *Enterobacter agglomerans* (Beijerinck) comb. nov. (the Herbicola-Lathyri Bacteria). Int. J. Syst. Bacteriol. 22(1): 4-11.

Expert, D. and Toussaint, A. 1985. Bacteriocin-resistant mutants of *Erwinia chrysanthemi*: Possible involvement of iron acquisition in phytopathogenicity. J. Bacteriol. 163(1): 221-227.

Farrand, S. K., Piper, K. R., Sackett, R., Ping, G., Shaw, P. D., and Kim, K. S. 1996. Homoserine lactone-mediated microbial signaling: A communication system common to plant-associated bacteria. In: Biology of Plant-Microbe Interactions. G. Stacey, B. Mullin, and P. M. Gresshoff (eds.). Proceedings of the 8th International Symposium on Molecular Plant-Microbe Interactions, Knoxville, Tennessee, July 14-19, 1996. International Society for Mol. Plant-Microbe Interactions, St. Paul, Minnesota, USA, pp. 173-179.

Feistner, G. J. and Ishimaru, C. 1996. Proferrioxamine profiles of *Erwinia herbicola* and related bacteria. BioMetals 9: 337-344.

Finlay, B. B. and Falkow, S. 1997. Common themes in microbial pathogenicity revisited. Microbiology and Molecular Biology Reviews 61(2): 136-169.

Forster, R. 1997. Bacterial Blight. In: Compendium of barley diseases. Second edition. D. E. Mathre (ed.). APS Press. St. Paul, Minnesota. pp. 7-9.

Fuqua, C., Winans, S. C., and Greenberg, E. P. 1996. Census and consensus in bacterial ecosystems: The LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. 50: 727-751.

Gavini, F., Mergaert, J., Beji, A., Mielcarek, C., Izard, D., Kersters, K., and de Ley, J. 1989. Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea* gen. nov. as *Pantoea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov. Int. J. Syst. Bacteriol. 39: 337-345.

Gebhardt, D. J., Rasmusson, D. C., and Wilcoxson, R. D. 1992. Cyclic breeding used to incorporate kernel discoloration resistance into malting barley. Crop Sci. 32:352-356.

Georgakopoulos, D. G. and Sands, D. C. 1992. Epiphytic populations of *Pseudomonas syringae* on barley. Can. J. Microbiol. 38: 111-114.

Gibbins, L. N. 1978. *Erwinia herbicola*: A review and perspective. In: Proceedings of the 4th International Conference on Plant Pathogenic Bacteria. S. d. p. v. e. phytobacteriologie (ed.). Angers, France. Vol.2, pp. 403-431.

Goto, M. 1992. Fundamentals of bacterial plant pathology. Academic Press, Inc., San Diego, CA.

Goto, S., Sakai, S., Kera, J., Suma, Y., Soma, G.I., and Takeuchi, S. 1996. Intradermal administration of lipopolysaccharide in treatment of human cancer. Cancer Immunol. Immunother. 42(4): 255-261.

Gross, D. C. 1985. Regulation of syringomycin synthesis in *Pseudomonas syringae* pv. *syringae* and defined conditions for its production. Journal of Applied Bacteriology 58: 167-174.

Gross, D. C. 1991. Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. Ann. Rev. Phytopathol. 29: 247-278.

Gross, D. C. and Cody, Y, S. 1984. The effects of iron acquisition and uptake on syringomycin production by *Pseudomonas syringae* pv. *syringae*. In: Proceedings of the 2nd Working Group on *Pseudomonas syringae* Pathovars. Hellenic Phytopathological Society, Athens, Greece. pp. 77-79.

Gross, D. C. and Cody, Y. S. 1985. Mechanisms of plant pathogenesis by *Pseudomonas species*. Can. J. Microbiol. 31: 403-410.

Grothues, D. and Tümmler, B. 1991. New approaches in genome analysis by pulsed-field gel electrophoresis: application to the analysis of *Pseudomonas* species. Molecular Microbiology 5 (11): 2763-2776.

Gvozdyak, R. I., and Pasichnik, L. A. 1991. The peculiarities of strain serogrouping of *Pseudomonas syringae* pv. *atrofaciens*. Proc. of the 4th International Working Group on *Pseudomonas syringae* Pathovars. Florence, Italy, June 10-13. pp. 117-123.

Hattingh, M. J., Beer, S. V., and Lawson, E. W. 1986. Scanning electron microscopy of apple blossoms colonized by *Erwinia amylovora* and *Erwinia herbicola*. Phytopathology. 76: 900-904.

Hendson, M., Hildebrand, D. C., and Schroth, M. N. 1992. Relatedness of *Pseudomonas syringae* pv. *tomato*, *Pseudomonas syringae* pv. *maculicola* and Pseudomonas syringae pv. *antirrhini*. Journal of Applied Bacteriology 73: 455-464.

Hildebrand, D. C., Schroth, M. N., and Sands, D. C. 1988. *Pseudomonas*. In: Laboratory Guide for Identification of Plant Pathogenic Bacteria, 2nd edition, ed. Schaad, N. W. APS Press, St. Paul, Minnesota, pp. 60-80.

Hirano, S. S. and Upper, C. D. 1983. Ecology and epidemiology of foliar bacterial plant pathogens. Annu. Rev. Phytopathol. 21:243-269.

Hirano, S. S. and Upper, C. D. 1985. Ecology and physiology of *Pseudomonas syringae*. Bio/Technology 3: 1073-1078.

Hirano, S. S. and Upper, C. D. 1990. Population biology and epidemiology of *Pseudomonas syringae*. Annu. Rev. Phytopathol. 28: 155-177.

Holt, J. G., Krieg, N. R., Sneath, P.H.A., Staley, J.T., and Williams, S.T. (eds.) 1994. Bergey's manual of determinative bacteriology. Ninth edition. Williams & Wilkins, Baltimore, MD.

Horino, O. 1976. Induction of bacterial leaf blight resistance by incompatible strains of *Xanthomonas oryzae* in rice. In: Biochemistry and Cytology of Plant Parasite Interactions. K. Tomiyama, J. Daly, I. Uritani, H. Oku, and S. Ouchi (eds.). Kodanska, Tokyo. pp. 43-55.

Hsieh, S. P. Y. and Buddenhagen, I. W. 1974. Suppressing effects of *Erwinia herbicola* on infection by *Xanthomonas oryzae* and on symptom development in rice. Phytopathology. 64: 1182-1185.

Hutchison, M, Tester, M. A., and Gross, D. C. 1995. Role of biosurfactant and ion channel-forming activities of syringomycin in transmembrane ion flux: a model for the mechanism of action in the plant-pathogen interaction. Molecular Plant-Microbe Interactions 8(4): 610-620.

Hwang, B. K. and Heitefuss, R. 1982. Induced resistance in spring barley to *Erysiphe graminis* f. sp. *hordei*. Phytopath. Z. 103: 41-47.

Iacobellis, N. S., Lavermicocca, P., Grgurina, I., Simmaco, M., and Ballio, A. 1992. Phytotoxic properties of *Pseudomonas syringae* pv. *syringae* toxins. Physiological and Molecular Plant Pathology 40: 107-116.

Inagawa, H., Nishizawa, T., Noguchi, K., Minamimura, M., Takagi, K., Goto, S., Soma, G., and Mizuno, D. 1997. Anti-tumor effect of lipopolysaccharide by intradermal administration as a novel drug delivery system. Anticancer Res. 17(3C): 2153-2158.

Isenbeck, M. and Schulz, F. A. 1985. Biological control of fire blight (*Erwinia amylovora* [Burr.] Winslow et al.) on ornamentals. I. Control of the pathogen by antagonistic bacteria. Phytopath. Z. 113:324-333.

Ishimaru, C. A., Klos, E. J., and Brubaker, R. R. 1988. Multiple antibiotic production by *Erwinia herbicola*. Phytopathology. 78: 746-750.

Iwamoto, I., Goto, S., Kera, J., Soma, G., Takeuchi, S., Nagata, Y. 1996. Mechanistic analysis of high antitumor effect of intradermal administration of lipopolysaccharide from *Pantoea agglomerans*. Med. Oncol. 13(2): 103-109.

Jacobs, C. 1997. Life in the balance: Cell walls and antibiotic resistance. Science 278: 1731-1732.

Jacobsen, B. J. 1997. Role of plant pathology in integrated pest management. Annu. Rev. Phytopathol. 35: 373-391.

Ji, P., Campbell, H. L., Lindow, S. E., and Wilson, M. 1996. Determination of the importance of nutritional similarity between biocontrol agents and the target pathogen in biocontrol of *Pseudomonas syringae* pv. *tomato* on tomato. Phytopathology. 86(11): S50 (Abstract).

Johnson, K. B., Stockwell, V. O., McLaughlin, R. J., Sugar, D., Loper, J. E., and Roberts, R. G. 1993. Effects of antagonistic bacteria on establishment of honey bee-dispersed *Erwinia amylovora* in pear blossoms and on fire blight control. Phytopathology. 83: 995-1002.

Johnston, R. 1997. Black Point (Kernel Blight). In: Compendium of barley diseases. Second edition. D. E. Mathre (ed.). APS Press. St. Paul. Minnesota. pp. 54-55.

Kamei, J., Iwamoto, Y., Suzuki, T., Misawa, M., Kasuya, Y., Okutomi, T., Soma, G.I., and Mizuno, D. 1994. Suppression of hyperalgesia in streptozotocin-induced diabetic mice by a lipopolysaccharide from *Pantoea agglomerans*. Biol. Pharm. Bull. 17(5): 632-634.

Kearns, L. P. and Hale, C. N. 1993. Biological control of fire blight by *Erwinia herbicola*: Survival of applied bacteria in orchard and glasshouse trials. Acta Horticulturae 338: 333-339.

Kearns, L. P. and Hale, C. N. 1996. Partial characterization of an inhibitory strain of *Erwinia herbicola* with potential as a biocontrol agent for *Erwinia amylovora*, the fire blight pathogen. J. of Applied Bacteriology 81: 369-374.

Kempf, H. J. 1988. Biologische Bekämpfung von pflanzenpathogenen Pilzen, insbesondere *Fusarium culmorum*, durch *Erwinia herbicola* und andere mikrobielle Antagonisten. Dissertation, Universität Göttingen, Germany.

Kessmann, H., Staub, T., Hofmann, C., Maetzke, T., Herzog, J., Ward, E., Uknes, S., and Ryals, J. 1994. Induction of systemic acquired diseases resistance in plants by chemicals. Annu. Rev. Phytopathol. 32: 439-459.

Khetmalas, M. B., Bal, A. K., Noble, L. D., and Gow, J. A. 1996. *Pantoea agglomerans* is the etiological agent for black spot necrosis on beach peas. Can. J. Microbiol. 42: 1252-1257.

Kietzell von, J. M. 1995. Untersuchungen zur Epidemiologie und Spezifität von *Pseudomonas syringae* pv. *atrofaciens* ((McCulloch), Dye, Young, Wilkie), dem Erreger der basalen Spelzenfäule des Getreides. Dissertation, Universität Göttingen, Germany.

Kietzell von, J. M. 1991. Variation in virulence of strains of *Pseudomonas syringae* pv. *atrofaciens* causing basal glume rot of cereals. Proc. of the 4th International Working Group on *Pseudomonas syringae* Pathovars. Florence, Italy, June 10-13. pp. 117-123.

Kinkel, L. L. 1997. Microbial population dynamics on leaves. Annu. Rev. Phytopathol. 35: 327-347.

Klement, Z., Rudolph, K., and Sands, D. C (eds.). 1990. Methods in phytobacteriology. Akademia Kiado, Budapest.

Kloepper, J. W., Leong, J., Teintze, M. and Schroth, M. N. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286: 885-886.

Koch, M. F., Taanami, Z. and Levy. E. 1996. Damage to garlic crops caused by *Erwinia herbicola*. Phytoparasitica 24: 125-126.

Kogel, K. H., Beckhove, U., Dreschers, J., Münch, S. and Romme, Y. 1994. Acquired resistance in barley. Plant Physiol. 106: 1269-1277.

Kuc, J. and Richmond, S. 1977. Aspects of the protection of cucumber against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. Phytopathology 67: 533-536.

Legard, D. E., Aquadro, C. F. and Hunter, J. E. 1993. DNA sequence variation and phylogenetic relationships among strains of *Pseudomonas syringae* pv. *syringae* inferred from restriction site maps and restriction fragment length polymorphism. Applied and Environmental Microbiology 59 (12): 4180-4188.

Lelliott, R. A. and Dickey, R. S. 1984. Genus VII. Erwinia Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith 1920, 209^{AL}. In: Bergey's Manual of Systematic Bacteriology. Vol. 1. N. R. Holt, J. G. and Krieg, N. R. (eds.), Williams & Wilkins, Baltimore, MD, pp. 469-476.

Leong, J. 1986. Siderophores: Their biochemistry and possible role in the biocontrol of plant pathogens. Annu. Rev. Phytopathol. 24: 187-209.

Liang, L. Z., Sobiczewski, P., Paterson, J. M., and Jones, A. L. 1994. Variation in virulence, plasmid content, and genes for coronatine synthesis between *Pseudomonas syringae* pv. *morsprunorum* and *P. s. syringae* from *Prunus*. Plant Dis. 78: 389-392.

Lindemann, J., Arny, D. C., Upper, C. D. 1984. Use of an apparent infection threshold population of *Pseudomonas syringae* to predict incidence and severity of brown spot of bean. Phytopathology 74: 1334-1339.

Lindgren, P. B. 1997. The role of *hrp* genes during plant-bacterial interactions. Annu. Rev. Phytopathol. 35: 129-152.

Lindow, S. E. 1985. Integrated control and role of antibiosis in biological control of firelight and frost injury. in: Biological control on the phylloplane. C. E. Windels and S. E. Lindow (eds.). p. 83-115.

Lindow, S. E. 1987. Competitive exclusion of epiphytic bacteria by Ice$^-$ *Pseudomonas syringae* mutants. Appl. Environ. Microbiol. 53(10): 2520-2527.

Lindow, S. E. 1988. Lack of correlation of in vitro antibiosis with antagonism of ice nucleation active bacteria on leaf surfaces by non-ice nucleation active bacteria. Phytopathology: 78: 444-450.

Lindow, S. E., Arny, D. C., and Upper, C. D. 1983. Biological control of frost injury: Establishment and effects of an isolate of *Erwinia herbicola* antagonistic to ice nucleation active bacteria on corn in the field. Phytopathology: 73: 1102-1106.

Liu, S., Lee, L., Tai, C., Hung, C, Chang, Y., Wolfram, J.H., Rogers, R, and Goldstein, A.H. 1992. Cloning of an *Erwinia herbicola* gene necessary for gluconic acid production and enhanced mineral phosphate solubilization in *Escherichia coli* HB101: Nucleotide sequence and probable involvement in biosynthesis of the coenzyme pyrroloquinoline quinone. J. Bacteriol. 174(18): 5814-5819.

Lloyd-George, I. and Chang, T.M.S. 1995. Characterization of free and alginate-polylysine-alginate microencapsulated *Erwinia herbicola* for the conversion of ammonia, pyruvate, and phenol L-tyrosine. Biotechnology and Bioengineering 48(6): 706-714.

Loper, J. E. and Buyer, J. S. 1991. Siderophores in microbial interactions on plant surfaces. Molecular Plant-Microbe Interactions 4(1): 5-13.

Loper, J. E. and Lindow, S. E. 1987. Lack of evidence for in situ fluorescent pigment production by *Pseudomonas syringae* pv. *syringae* on bean leaf surfaces. Phytopathology 77: 1449-1454.

Loper, J. E. and Lindow, S. E. 1994. A biological sensor for iron available to bacteria in their habitats on plant surfaces. Appl. Environ. Microbiol. 60(6): 1934-1941.

Loper, J. E., Orser, C. S., Panopoulos, N. J., and Schroth, M. N. 1984. Genetic analysis of fluorescent pigment production in *Pseudomonas syringae* pv. *syringae*. Journal of General Microbiology 130: 1507-1515.

Louws, F. J., Fulbright, D. W., Stephens, C. T., and de Bruijn, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Applied and Environmental Microbiology 60 (7): 2286-2295.

Manceau, C. and Horvais, A. 1997. Assessment of genetic diversity among strains of *Pseudomonas syringae* by PCR-restriction fragment polymorphism analysis of rRNA operons with special emphasis on *P. syringae* pv. *tomato*. Appl. Environ. Microbiol. 63(2): 498-505.

Martinez-Miller, C. 1994. Etiology and characterization of two *Pseudomonas syringae* pathovars causing two bacterial kernel blights of barley. PhD Thesis. Montana State University, Bozeman, MT.

Martinez-Miller, C. and Braun, A. 1997. Bacterial kernel blights. In: Compendium of barley diseases. Second edition. D. E. Mathre (ed.). APS Press. St. Paul, Minnesota.

Martinez-Miller, C., Braun, A., Siemsen, S., and Sands, D. C. 1998. Etiology of basal kernel blight of barley caused by *Pseudomonas syringae* pv. *syringae*. Can. J. of Plant Pathology, in press.

Mathre, D. E. 1997. Bacterial and phytoplasma diseases. In: Compendium of barley diseases. Second edition. D. E. Mathre (ed.). APS Press. St. Paul, Minnesota.

Menn, J. J., Lumsden, R. D., and Vaughn, J. L. 1993. Preface. In: Proceedings of Beltsville Symposium XVIII, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland, May 2-6, 1993. R. D. Lumsden and J. L. Vaughn (eds.). American Chemical Society, Washington, D.C., pp. xi-xii.

Milanowski, J., Sorenson, W. G., Lewis, D. M., and Dutkiewicz, J. 1995. Chemotaxis of alveolar macrophages and neutrophils in response to microbial products derived from organic dusts. Journal of Investigational Allergology and Clinical Immunology 5(4): 221-227.

Miles, M. R., Wilcoxson, R. D., Rasmusson, D. C., Wiersma, J., and Warnes, D. 1987. Influence of genotype and environment on kernel discoloration of Midwestern malting barley. Plant Dis. 71: 500-504.

Mo, Y. Y. and Gross, D. C. 1991a. Expression in vitro and during plant pathogenesis of the *syrB* gene required for syringomycin production by *Pseudomonas syringae* pv. *syringae*. Molecular Plant-Microbe Interactions. 4(1): 28-36.

Mo, Y.Y. and Gross, D. C. 1991b. Plant signal molecules activate the *syrB* gene, which is required for syringomycin production by *Pseudomonas syringae* pv. *syringae*. J. Bacteriol. 173(18): 5784-5792.

Moores, J. C., Magazin, M., Ditta, G. S., and Leong, J. 1984. Cloning of genes involved in the biosynthesis of pseudobactin, a high-affinity iron transport agent of a plant growth-promoting *Pseudomonas* strain. J. Bacteriol. 157(1): 53-58.

Okutomi, T., Nishizawa, T., Inagawa, H., Soma, G., Minami, M., Satoh, M., and Mizuno, D. 1992. Inhibition of morphine dependence by a lipopolysaccharide from *Pantoea agglomerans*. Eur. Cytokine. Netw. 3(4): 417-420.

Oostendorp, M., Kessmann, H., Friedrich, L., Geissmann, A., Görlach, J., Hengy, G., Nordmeyer, D., Reist, R., Ruess, W., and Staub, T. 1996. Einfluß des Pflanzenaktivators Bion^{R} und von Triazol-Fungiziden auf pflanzliche Abwehrmechanismen. Gesunde Pflanzen, 48. Jahrg., Heft 7, pp. 260-264.

Ouchi, S., Oku, H., Hibino, C., and Akiyama, I. 1974. Induction of accessibility and resistance in leaves of barley by some races of *Erysiphe graminis*. Phytopath. Z. 79: 24-34.

Palleroni, N. J. 1984. Genus I. *Pseudomonas* Migula 1894. In: Bergey's Manual of Systematic Bacteriology Vol. 1. N. R. Krieg (ed.), Williams & Wilkins, Baltimore, p. 141

Panopoulos, N. J. and Schroth, M. N. 1974. Role of flagellar motility in the invasion of bean leaves by *Pseudomonas phaseolicola*. Phytopathology 64: 1389-1397.

Parker, W. L., Rathnum, M. L., Wells, Jr., J. S., Trejo, W. H., Principe, P. A., and Sykes, R. B. 1982. SQ 27,860, a simple carbapenem produced by species of *Serratia* and *Erwinia*. The Journal of Antibiotics 35(6): 653-660.

Paul, V. H., and Smith, I. M. 1989. Bacterial pathogens of Gramineae: Systematic review and assessment of quarantine status for the EPPO region. Bulletin OEPP/EPPO Bulletin 19: 33-42.

Paulraj, L. and O'Garro, L. W. 1993. Leaf blight of onions in Barbados caused by *Xanthomonas campestris*. Plant Dis. 77: 198-201.

Peters, R. A., Timian, R.G., and Wesenberg, P. 1983. A bacterial kernel spot of barley caused by *Pseudomonas syringae* pv. *syringae*. Plant Dis. 67: 435-438.

Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E. T. 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. The EMBO Journal 12(6): 2467-2476.

Poschenrieder, G. 1986. Eine wenig bekannte Getreidebakteriose. Pflanzenschutz-Praxis. 1: 18.

Press, C. M., Wilson, M., Tuzun, S. and Kloepper, J. W. 1997. Salicylic acid production by *Serratia marcescens* 90-166 is not the primary determinant of induced systemic resistance in cucumber or tobacco. Molecular Plant-Microbe Interactions 10(6): 761-768.

Quigley, N. B. and Gross, D. C. 1994. Syringomycin production among strains of *Pseudomonas syringae* pv. *syringae*: Conservation of the *syrB* and *syrD* genes and activation of phytotoxin production by plant signal molecules. Molecular Plant-Microbe Interactions 7(1): 78-90.

Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G., and Ausubel, F. M. 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science 268: 1899-1902.

Rich, J. J., Hirano, S. S., and Willis, D. K. 1992. Pathovar-specific requirement for the *Pseudomonas syringae* lemA gene in disease lesion formation. Appl. Environ. Microbiol. 58(5): 1440-1446.

Rich, J. J., Kinscherf, T. G., Kitten, T., and Willis, D. K. 1994. Genetic evidence that the gacA gene encodes the cognate response regulator for the lemA sensor in *Pseudomonas syringae*. J. Bacteriol. 176(24): 7468-7475.

Riggle, J. H. and Klos, E. J. 1972. Relationship of *Erwinia herbicola* to *Erwinia amylovora*. Can. J. Bot. 50: 1077-1083.

Romantschuk, M., Nurmiaho-Lassila, E. L., Roine, E., and Suoniemi, A. 1993. Pilus-mediated adsorption of *Pseudomonas syringae* to the surface of host and non-host plant leaves. J. Gen. Microbiol. 139: 2251-2260.

Romantschuk, M. & Bamford, D. H. 1986. The causal agent of halo blight of bean *Pseudomonas syringae* pv. *phaseolicola*, attaches to stomata via its pili. Microb. Pathog. 1: 139-148.

Roos, I. M. M., and Hattingh, M. J. 1987. Pathogenicity and numerical analysis of phenotypic features of *Pseudomonas syringae* strains isolated from deciduous fruit trees. Phytopathology 77:900-908.

Rosales, A. M., Vantomme, R., Swings, J., de Ley, J, and Mew, T. W. 1993. Indentification of some bacteria from paddy antagonistic to several rice fungal pathogens. J. Phytopathol. (Berl.). 138(3): 189-208.

Ruppel, S., Wache, H., and Höflich, G. 1989. Wirkprinzipien zur Wachstumsstimulierung von Winterweizen durch N_2-fixierende assoziative Rhizosphärenmikroorganismen. Forschungsbericht des FZB Müncheberg. FO 999.

Sands, D. C., Schroth, M. N., and Hildebrand, D. C. 1970. Taxonomy of phytopathogenic pseudomonads. J. Bacteriol. 101: 9-23.

Schaffrath, U., Freydl, E. and Dudler, R. 1997. Evidence for different signaling pathways activated by inducers of acquired resistance in wheat. Molecular Plant-Microbe Interactions 10(6): 779-783.

Scholz-Seidel, C. and Ruppel, S. 1992. Nitrogenase- and phytohormone activities of *Pantoea agglomerans* in culture and their reflection in combination with wheat plants. Zentralbl. Microbiol. 147: 319-328.

Schweizer, P., Hunziker, W., and Mösinger, E. 1989. cDNA cloning, in vitro transcription and partial sequence analysis of mRNAs from winter wheat (*Triticum aestivum* L.) with induced resistance to *Erysiphe graminis* f. sp. *tritici*. Plant Mol. Biol. 12: 643-654.

Sequeira, L. 1978. Lectins and their role in host-pathogen specificity. Annu. Rev. Phytopathol. 16: 453-481.

Sigee, D. C. 1993. Bacterial plant pathology: cell and molecular aspects. Cambridge University Press, Cambridge, U.K.

Smedegaard-Petersen, V., Brandt, J., Ho Cho, B., Collinge, D. B., Gregersen, P. L., and Thordal-Christensen, H. 1991. Molecular and energetic aspects of induced resistance in barley. In: J. H. Riesselman (ed.). Proc. of the international symposium on biotic stress of barley in arid and semiarid environments. Montana State University, Bozeman, MT, pp. 123-131.

Smith, J. J. and Mansfield, J. W. 1982. Ultrastructure of interactions between pseudomonads and oat leaves. Physiological Plant Pathology. 21: 259-266.

Smith, J. A. and Metraux, J. P. 1991. *Pseudomonas syringae* pv. *syringae* induces systemic resistance to *Pyricularia oryzae* in rice. Physiological and Molecular Plant Pathology 39: 451-461.

Stein, J. I. and Beer, S. V. 1980. Partial purification of a bacteriocin from *Erwinia herbicola*. Phytopathology 70(5): 469 (Abstract).

Steiner, U., Oerke, E. C. und Schönbeck, F. 1988. Zur Wirksamkeit der induzierten Resistenz unter praktischen Anbaubedingungen. Z. Pflanzenkr. Pflanzenschutz 95: 506-517.

Sticher, L., Mauch-Mani, B., and Metraux, J. P. 1997. Systemic acquired resistance. Annu. Rev. Phytopathol. 35: 235-270.

Sujkowski, L. S., Ophir, Y., Fry, W. E., and Beer, S. V. 1994. Antibiotic-producing strain of *Erwinia herbicola* as a potential control agent for *Alternaria solani*. Phytopathology. 84: 1082 (Abstract).

Suoniemi, A., Björklöf, K., Haahtela, K., and Romantschuk, M. 1995. Pili of *Pseudomonas syringae* pathovar *syringae* enhance initiation of bacterial epiphytic colonization of bean. Microbiology 141: 497-503.

Suzuki, T., Funada, M., Sugano, Y., Misawa, M., Okutomi, T, Soma, G.I., and Mizuno, D. 1994. Effects of a lipopolysaccharide from *Pantoea agglomerans* on the cocaine-induced place preference. Life Sciences 54(6): 75-80.

Taylor, C. B. 1996. Inside avirulence. Plant Cell 8: 1091-1093.

Teintze, M., Hossain, M. B., Barnes, C. L., Leong, J., and van der Helm, D. 1981. Structure of ferric pseudobactin, a siderophore from plant growth-promoting *Pseudomonas*. Biochemistry 20: 6446-6457.

Tenning, P., Van Rijsbergen, R., Zhao, Y., and Joos, H. 1993. Cloning and transfer of genes for antifungal compounds from *Erwinia herbicola* to *Escherichia coli*. Mol. Plant-Microbe Interact. 6: 474-480.

Toben, H., Mavridis, A., and Rudolph, K. 1989. Basal glume rot (*Pseudomonas syringae* pv. *atrofaciens*) on wheat and barley in FRG and resistance screening of wheat. Bulletin OEPP/EPPO Bulletin 19:119-125.

Toben, H., Mavridis, A. and Rudolph, K. 1991. On the occurrence of basal glume rot of wheat and barley caused by *Pseudomonas syringae* pv. *atrofaciens* in West Germany. Journal of Plant Diseases and Protection. 98: 225-235.

Vanneste, J. L. and Yu, J. 1990. Control of fire blight by *Erwinia herbicola* Eh 252 in an experimental orchard in Dax (Southwest of France). Acta Hort. 273: 409-410.

Vanneste, J. L. and Yu, J. 1996. Cloning of the genes from *Erwinia herbicola* Eh 252 necessary for production of an antibacterial compound which inhibits *Erwinia amylovora*. Acta Horticulturae 411: 347-349.

Vanneste, J. L., Yu, J., and Beer, S. V. 1992. Role of antibiotic production by *Erwinia herbicola* Eh 252 in biological control of *Erwinia amylovora*. J. Bacteriol. 174: 2785-2796.

Vassilev, V., Lavermicocca, P., Giorgio Di, D., and Iacobellis, N. S. 1996. Production of syringomycins and syringopeptins by *Pseudomonas syringae* pv. *atrofaciens*. Plant Pathology 45: 316-322.

Vera Cruz, C. M., Ardales, E. Y., Skinner, D. Z., Talag, J., Nelson, R. J., Louws, F. J., Leung, H., Mew, T. W., and Leach, J. E. 1996. Measurement of haplotypic variation in *Xanthomonas oryzae* pv. *oryzae* within a single field by rep-PCR and RFLP Analyses. Phytopathology 86: 1352-1359.

Vidaver, A. K.1983. Bacteriocins: The lure and the reality. Plant Dis. 67: 471-475.

Vivian, A. and Gibbon, M. J. 1997. Avirulence genes in plant-pathogenic bacteria: signals or weapons? Microbiology 143: 693-704.

Wei, G., Kloepper, J. W., and Tuzun, S. 1991. Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. Phytopathology 81: 1508-1512.

Wei, G., Kloepper, J. W., and Tuzun, S. 1996. Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. Phytopathology 86: 221-224.

Weller, D. M. and Cook, R. J. 1986. Suppression of root diseases of wheat by fluorescent pseudomonads and mechanisms of action. In: Iron, Siderophores, and Plant Diseases. T. R. Swinburne (ed.). NATO ASI Series, Series A: Life Sciences Vol. 117, pp. 99-107.

Wilson, M., Epton, H. A. S., and Sigee, D. C. 1990. Biological control of fire blight of hawthorn (*Crataegus monogyna*) with *Erwinia herbicola* under protected conditions. Plant Pathol. (Oxf.). 39: 301-308.

Wilson, M., Epton, H. A. S., Sigee, D. C. 1992. Interactions between *Erwinia herbicola* and *E. amylovora* on the stigma of hawthorn blossoms. Phytopathology. 82: 914-918.

Wilson, M. and Lindow, S. E. 1994. Ecological similarity and coexistence of epiphytic ice-nucleating (ice+) *Pseudomonas syringae* strains and a non-ice-nucleating (ice-) biological control agent. Appl. Environ. Microbiol. 60(9): 3128-3137.

Winkelmann, G. und Adam, W. 1980. Herbicoline - neue acylpeptidantibiotika mit antimyzetischer Aktivität gegen Dermatophyten. Mykosen 23(6): 290-294.

Winkelmann, G., Lupp, R. and Jung, G. 1980. Herbicolins-new peptide antibiotics from *Erwinia herbicola*. The Journal of Antibiotics 33(4): 353-358.

Wodzinski, R. S., and Paulin, J. P. 1994. Frequency and diversity of antibiotic production by putative *Erwinia herbicola* strains. J. Appl. Bact. 76: 603-607.

Wodzinski, R. S., Umholtz, T. E., Garrett, K., and Beer, S. V. 1987. Attempts to find the mechanism by which *Erwinia herbicola* inhibits *Erwinia amylovora*. Acta Hortic. 217: 223-227.

Wodzinski, R. S., Umholtz, T.E., Rundle, J. R., and Beer, S. V. 1994. Mechanisms of inhibition of *Erwinia amylovora* by *Erwinia herbicola* in vitro and in vivo. J. Appl. Bact. 76: 22-29.

Wright, S. A. I. 1997. The genetics of antibiotic production and the role of antibiotics in biological control of *Erwinia amylovora* by *Erwinia herbicola*. PhD thesis. Cornell University, Ithaca, N.Y.

Wright, S. A. I., and Beer, S. V. 1997. Genetics of Pantocin A and Pantocin B production and their role in biocontrol of the fireblight pathogen, *Erwinia amylovora*. Proc. of Molecular Approaches in Biological Control. Delemont, Switzerland 15-18 September 1997. IOBC/OILB workshop. in press.

Xu, G. W. and Gross, D. C. 1988. Evaluation of the role of syringomycin in plant pathogenesis by using Tn5 mutants of *Pseudomonas syringae* pv. *syringae* defective in syringomycin production. Appl. Environ. Microbiol. 54(6): 1345-1353.

Young, J. M., Bradbury, J. F., Davis, R. E., Dickey, R. S., Ercolani, G. L., Hayward, A. C., Vidaver, A. K. 1991. Nomenclatural revision of plant pathogenic bacteria and list of names 1980-1988. Rev. Plant Path. 70(4): 211-221.

Young, J. M., Saddler, G. S., Takikawa, Y., De Boer, S. H., Vauterin, L., Gardan, L., Gvozdyak, R. I., and Stead, D. E. 1996. Names of plant pathogenic bacteria 1864-1995 - The ISPP accepted list of bacterial names. Review of Plant Pathology 75(9): 721-763.

Young, J. M., Takikawa, Y., Gardan, L., and Stead, D. E. 1992. Changing concepts in the taxonomy of plant pathogenic bacteria. Ann. Rev. Phytopathol. 30: 67-105.

Yuen, G. Y., Craig, M. L., Kerr, E. D., and Steadman, J. R. 1994. Influences of antagonist population levels, blossom development stage, and canopy temperature on the inhibition of *Sclerotinia sclerotiorum* on dry edible bean by *Erwinia herbicola*. Phytopathology 84: 495-501.

Yuen, G. Y. and Schroth, M. N. 1986. Inhibition of *Fusarium oxysporum* f. sp. *dianthi* by iron competition with an *Alcaligenes* sp. Phytopathology 75: 171-176.

Zhang, J. H., Quigley, N. B., and Gross, D. C. 1995. Analysis of the *syrB* and *syrC* genes of *Pseudomonas syringae* pv. *syringae* indicates that syringomycin is synthesized by a thiotemplate mechanism. J. Bacteriol. 177(14): 4009-4020.

CHAPTER 2

LIGHT- AND ELECTRON MICROSCOPIC LOCALIZATION OF *PSEUDOMONAS SYRINGAE* IN BARLEY KERNEL TISSUE

Introduction

Pseudomonas syringae is an ubiquitous epiphytic plant pathogen found on a multiplicity of hosts, including many dicots, as well as most small grains (Bradbury, 1986; Young, 1992; Georgakopoulos & Sands, 1992; Martinez-Miller & Braun, 1997). *P. s.* pv. *syringae* is, with *Xanthomonas campestris* pv. *translucens* and *P. s.* pv. *atrofaciens*, the most important bacterial plant pathogen on cereals worldwide (Paul & Smith, 1989; Von Kietzell, 1995; Mathre, 1997). *P. s.* pv. *syringae* can cause severe damage to barley kernels, resulting in black discoloration on the kernel base, reduced germination and embryo damage (Martinez-Miller, 1994; Martinez-Miller & Braun, 1997). We have previously reported the etiology and significance of basal kernel blight of barley caused by *P. s.* pv. *syringae* (Martinez-Miller et al., 1998). The disease varies in severity with cultivars, environmental conditions, and virulence of the pathogen. The critical period of infection escalated from early milk to soft dough stages of kernel development, with free moisture being necessary for infection and disease development (Martinez-Miller et al., 1998).

Despite numerous studies devoted to specific host-parasite interactions of pathogenic or saprophytic pseudomonads with host or non-host plants, information concerning the localization and pathogenesis of *Pseudomonas syringae* in barley kernels is missing.

However, the interactions between oat (*Avena sativa* L.) and the foliar pathogen *Pseudomonas coronafaciens* and the non-pathogens *P. coronafaciens* var. *atropurpurea*, *P. fluorescens* and *P. tabaci* were examined by Smith & Mansfield (1982) using ultrastructural analyses. Regarding the hypersensitivity reaction (HR) on non-host plants and pathogenicity on host plants, the authors described several types of associations between bacteria and plant cells: (1) incompatible bacteria were encapsulated by fibrillar materials on plant cell walls (physical entrapment), (2) incompatible bacteria were attached to modified, often degraded plant cell walls, and (3) compatible but not incompatible bacteria were surrounded by extracellular polysaccharides (EPS), which prevented the attachment to oat cell walls. Recently, cytological studies of plant-microbe interactions have been facilitated by the use of genetically modified microorganisms harboring gene fusions with the promoter of a constitutively expressed gene. Vasse et al. (1995) compared the temporal and spatial patterns of infection and colonization of tomato roots by a pathogenic strain of *Pseudomonas solanacearum* with those of a spontaneous nonpathogenic strain. The use of an *E. coli lacZ* reporter gene fusion and histochemical blue staining of the bacterial β-galactosidase activity revealed that pathogenic bacteria intercellularly infect the inner cortex and invade proto xylem vessels degrading cell walls, while nonpathogenic strains were able to infect intercellular spaces of the inner cortex, but were never observed in the vascular cylinder. Furthermore, Boher et al. (1995) performed cytochemical investigations on the interactions between cassava and *Xanthomonas campestris* pv. *manihotis*, the cassava bacterial blight pathogen, to gain insight into molecular and cellular mechanisms involved in leaf cell wall degradation by this pathogen. The use of anti-pectin monoclonal antibodies demonstrated

that the plant middle lamellae were damaged during the infection process, in addition to alterations of primary and secondary cell walls as indicated by the application of β-1,4-exoglucanase-gold probes.

Furthermore, Luebeck et al. (1998) used fluorescently labeled rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy for the spatio-temporal localization of a *Pseudomonas fluorescens* biological control strain. Their research focused on the distribution and activity of colonizing bacteria over time, from one day old germinating barley roots to one week old roots. Alternatively, Chin-A-Woeng et al. (1997) described the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* emphasizing spatio-temporal distribution patterns along the root system based on scanning electron microscopy. The authors proposed a model for root colonization after seed inoculation by several biocontrol strains, in which the colonization of more distant root zones progresses downward to the root tip, initially as single cells, and results in the establishment of micro-colonies. They further hypothesized that micro-colonies are the sites where the bacterial intracellular N-acyl-L-homoserine lactone concentration becomes sufficiently high as a consensus signal to influence regulatory proteins, which in turn transcriptionally regulate the expression of target genes, such as the phenazine genes for antibiotic synthesis. Following inoculation with the endophytic bacterium *Bacillus pumilus* prior to infection with the root-infecting fungus *Fusarium oxysporum* f. sp. *pisi*, Benhamou et al. (1996 a) found wall appositions containing large amounts of callose and phenolic compounds, suggesting the induction of defense-related ultrastructural modifications in the prebacterized pea root tissues. Furthermore, host reactions included a strengthening of

epidermal and cortical cell walls and a deposition of newly formed barriers beyond the infection sites. Similar modifications in pea root tissues were observed when *Pseudomonas fluorescens* was used to induce host responses prior to challenge with *Fusarium oxysporum* f. sp. *pisi* and *Pythium ultimum* (Benhamou et al., 1996 b).

However, bereft of information regarding the infection process of barley kernels by *P. syringae*, the objective of this study was the detection and localization of pathogenic bacteria in and on barley kernels following inoculation using light-, scanning and transmission electron microscopy. In addition, I examined the cellular interactions between barley kernels and the pathogen following inoculation when the first water-soaked symptoms became visible at the kernel base. The localization of the pathogen and investigations with respect to disease development may provide a better understanding of barley - *P. syringae* interactions. The knowledge may also help derive control management strategies, including the use of biological control agents, such as *Pantoea agglomerans*, which has been used successfully to control basal kernel blight (Braun et al., 1996, 1997 a,b).

Materials and Methods

Bacterial cultures and inoculum preparation.

Two *Pseudomonas syringae* isolates, one from barley kernels showing symptoms of basal blight (strain Pss 552) and one from kernels with spot blight symptoms (Ps 418) were stored in nutrient broth and 15 % glycerol at -70°C. Strain Pss 552 was identified as belonging to the pathovar *syringae* based on the utilization of quinate, trigonelline and L-

lactate (Hildebrand et al., 1988), whereas strain Ps 418 could not be clearly identified on the pathovar level, since it utilized quinate, but not trigonelline or L-lactate and was negative in tabtoxin production, traits indicative of pathovar *coronafaciens* (Martinez-Miller, 1994). Furthermore, Pss 552 was positive in syringomycin production, while Ps 418 was negative (Martinez-Miller, 1994).To obtain cells for barley head inoculation, bacteria were retrieved from storage, streaked on King's B medium (King et al., 1954), and incubated at 28°C. Two days later, bacterial cells were suspended in 10 ml PBS buffer (0.85% NaCl, 0.03 M NaOH, 0.05 M KH_2PO_4; pH 7.0) and the density of the suspension adjusted to 1 x 10^8 cfu/ml by measuring the absorbance at 580 nm on a Spectronic 20 (Bausch & Lomb, Rochester, NY) and comparing it with a standardized growth curve developed previously. The suspension was then diluted 1:10 in 90 ml sterile tapwater containing 0.025% Tween 20 (polyoxyethylenesorbitan monolaurate) as a surfactant to give a final concentration of 1 x 10^7 cfu/ml.

Plant inoculation.

Three barley plants (cv. B 2601) each having one spike were grown per pot in the Montana State University Plant Growth Center (PGC) in Aqua Gro soil (PGC mix). Each *P. syringae* strain was inoculated individually at soft dough stage of barley development (EC 85; Zadoks et al., 1974), the stage conducive to basal blight development (Martinez-Miller et al., 1998). One pot (19 cm diameter) with three spikes at soft dough stage represented one replication, and treatments were replicated five times (n=5). Inoculum was applied to spikes with a hand air brush sprayer (Model Paaschi D500 1/10 H.P.) until runoff (approx. 5 ml per

spike). Control plants were sprayed with an aqueous solution of 0.025% Tween 20 alone. After inoculation, plants were left in a mist chamber (80-100 % r.h.) providing continuous plant wetness at 22/18 ± 1 °C (day/night) and a 12 h photoperiod, for 72 h before being placed on the greenhouse bench. Kernels were sampled for microscopy seven days after inoculation when water-soaked symptoms and kernel necrosis at the base of the kernels in a spike became visible.

Tissue processing for scanning electron microscopy (SEM).

Scanning electron micrographs of the barley kernel surface were analyzed 1 and 7 days following inoculation with the pathogen Pss 552. Five kernels per head were randomly removed from the barley spikes with forceps, air-dried, and cut longitudinally. Palea and lemma sides were mounted on specimen holders. Particular care was taken while handling the kernels to avoid excess touching and possible damage to the kernel surface. Samples were directly sputter-coated with 25 nm Au/Pd and examined with a JEOL 6100 scanning electron microscope operating at 8-10 kV.

Tissue processing for light- and transmission electron microscopy (LM and TEM).

Samples (3 mm^3) were carefully excised from control and pathogen-inoculated barley kernels at sites of occurrence of water-soaking and beginning basal necrosis. Tissues were fixed in a solution of 2% glutaraldehyde and 2% formaldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) containing 1 mg/ml ruthenium red and 3 mM $CaCl_2$. Penetration of fixative solution into intercellular spaces was promoted by air evacuation under vacuum for 4 h at

20 C, after which the samples were left in fresh fixative overnight at 4 C. Cacodylate buffer amended with $CaCl_2$ was used for membrane preservation and ruthenium red, a hexavalent cation that precipitates a large variety of polyanions by ionic interaction, was used according to Smith & Mansfield (1982) to demonstrate a possible presence of acidic polysaccharides, such as alginate, around bacteria. Furthermore, ruthenium red reacts with osmium tetroxide (OsO_4) and amplifies the electron density resulting in stronger contrast for electron microscopy. After fixation, tissue samples were washed three times for 15 min in 0.2 M sodium cacodylate buffer (pH 7.4). Samples were postfixed in 2% OsO_4 at 4 C for 2 h. In specimen preparations for immunogold labeling procedures OsO_4 was not used because it reacts aggressively with proteins (Hamacher, 1992). Samples were subsequently dehydrated in a graded ethanol series (15%, 30%, 50%, 70%, 90% [2x], 100% [2x]; 30 min each stage) prior to embedding in either Spurr's or LR-White (for immunogold labeling) resin, according to the method described by Hamacher (1992).

For infiltration in Spurr's low-viscosity epoxy resin, samples were left in Spurr's:propyleneoxide (1:3 v/v) for 16 h, in 1:1 for 4 h, in 3:1 for 4 h and in pure Spurr's for 18 h. The solution was then changed to fresh Spurr's and specimens embedded in latex flat bottom boats (agar aids) or plastic capsules. Polymerization of the resin was done at 70°C overnight. For infiltration in LR-White resin, samples were left in LR-White:ethanol (1:3 v/v) for 16 h, in 1:1 for 4 h, in 3:1 for 4 h, and in pure LR-White for 18 h. The solution was changed subsequently to fresh LR-White and cast in gelatin capsules, in which specimens were embedded and polymerized at 50°C for 72 h. Thick sections (1.0 -2.0 μm) were cut from trimmed tissue blocks with glass knives attached to an Ultracut E

ultramicrotome (Reichert-Jung, Vienna, Austria) and mounted on a microscope slide. Sections were stained with 1% toluidine blue and observed under the light microscope to select appropriate blocks for electron microscopy. Thin sections (about 500 nm) were cut with a diamond knife, collected on Butvar-coated nickel grids and were either contrasted with 2% uranyl acetate (8 min) for direct examination with a Zeiss EM 109 transmission electron microscope operating at 60 KV or processed for immunogold labeling. For each treatment (inoculated and non-inoculated kernels), ten kernels from six heads were examined, using five sections per kernel.

In addition to cytochemical investigations, ten *P. syringae* strains including strains Ps 418 and Pss 552 were tested biochemically for enzyme activity in plate bioassays. Protease activity was determined by gelatin liquefaction according to Hankin & Anagnostakis (1975), cellulase activity on CMC medium described by Andro et al. (1984), and starch hydrolysis on starch agar plates flooded with Lugol's iodine solution (Leary & Chun, 1988). β-Glucanase activity was determined by formation of clear zones around bacterial colonies grown on minimal medium containing 1% β-D-glucan from barley trim (Nurture Inc., Missoula, MT) as sole carbon source.

Preparation of primary antibodies (Ig) from antiserum.

Polyclonal antibodies (primary) were developed in rabbits injected with heat-treated whole cells of *P. syringae* strain Ps 554, a strain similar in characteristics to strain Ps 418, according to the method described by Schaad et al. (1990). The antisera solutions were maintained at - 20°C. The γ-globulins were partially purified by precipitation with sodium

sulfate (Clark & Adams, 1977; Lister, 1978). Eight ml of sterile distilled water were added to 2.0 ml of antiserum in a sterile tube. Ten milliliter of a saturated sodium sulfate solution (36 % Na_2SO_4) was added dropwise with stirring at room temperature, left for 1.5 h for precipitation, then stirred gently, and centrifuged for ten minutes at 8000 x g to collect the precipitate. The pellet was washed once in 18% sodium sulfate solution, centrifuged again, the supernatant discarded, and the pellet resuspended in 2 ml half-strength PBS buffer (8 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4 anhydrous, 0.2 g KCl, 0.2 g NaN_3; pH 7.4). This solution was dialysed (Spectra/Por dialysis membrane MWCO: 6-8.000, Spectrum, Houston, Tx) three times against 1 liter half-strength PBS, first for 1 h at 20°C, then for 1 h at 4°C, and finally for 12 h at 4°C. After dialysis, the optical density was measured at A_{280} (DU-50 Spectrophotometer, Beckmann Instruments, Inc., Fullerton, CA) and the immunoglobulin (Ig) protein concentration adjusted to approximately 1.4 OD (about 1 mg protein/ml). Igs were stored at -20°C for several months.

Immunogold labeling of antibodies raised against *P. syringae*.

Nickel grids containing thin sections were laid specimen side down onto 20 μl droplets of 0.05 M Glycine-IGL-buffer (0.5 M glycine, 8 g NaCl, 1.44 g Na_2HPO_4, 0.2 g KCl, 0.15 g K_2HPO_4 per liter distilled water, pH 7.25) for 10 min. Grids were drained on filter paper, then incubated on IGL-T-BSA blocking buffer (2 x IGL buffer, 0.5% w/v bovine serum albumin, 0.025% v/v Tween 20, 0.02% sodium azide, pH 7.25) for 3 h. Subsequently, grids were washed with 5 drops of washing buffer (10 x IGL-buffer, pH 7.25) and drained on filter paper prior to primary antibody incubation for 3 h. Antibodies were diluted 1:250

in IGL-T-BSA buffer. Grids were washed again with 50 drops of washing buffer and drained on filter paper, then incubated on droplets of IGL-T-BSA buffer diluted secondary antibody (immunogold conjugate EM GAR 20, EM goat anti-rabbit IgG complexed to colloidal gold: 20 nm, Goldmark Biologicals, Phillipsburg, NJ; dilution 1:20) for 3 h. Finally, grids were washed with 25 drops of washing buffer and 50 drops of double distilled water, drained, contrasted with 2% uranyl acetate for 8 minutes and specimens analyzed in the transmission electron microscope either directly or after contrasting.

Results

Light micrographs.

While no bacteria were visible in uninoculated kernels (Fig. 2.1 A & B), *P. syringae* Ps 418 were located in vicinity of vascular bundles present in the husk (palea) and in the pericarp at the ventral furrow of the kernel base seven days after inoculation (data not shown). Bacteria could be visualized by ruthenium red staining and were present also in intercellular spaces of the aleurone (Fig. 2.1 C) and amyloplast cells (Fig. 2.1 C & D).

Scanning electron microscopy.

Pseudomonas syringae pv. *syringae* Pss 552 cells were located at bases of trichomes (Fig. 2.2 A & B) on the barley kernel surface seven days after inoculation or close to stomata (Fig. 2.2 D), which extend down each side of the ventral furrow on the palea (Miskin & Rasmusson, 1970). In addition, bacteria were found close to stomata-like cells on the

external epidermis of the palea (Fig. 2.2 E & F). No bacteria were observed at these sites on uninoculated kernels or 24 h after inoculation (data not shown). However, a few randomly distributed cells were located at kernel surface ridges on the lemma 24 h after inoculation (Fig. 2.2 C), suggesting initial loss of the inoculated pathogen after introduction, but multiplication and establishment after seven days of plant incubation.

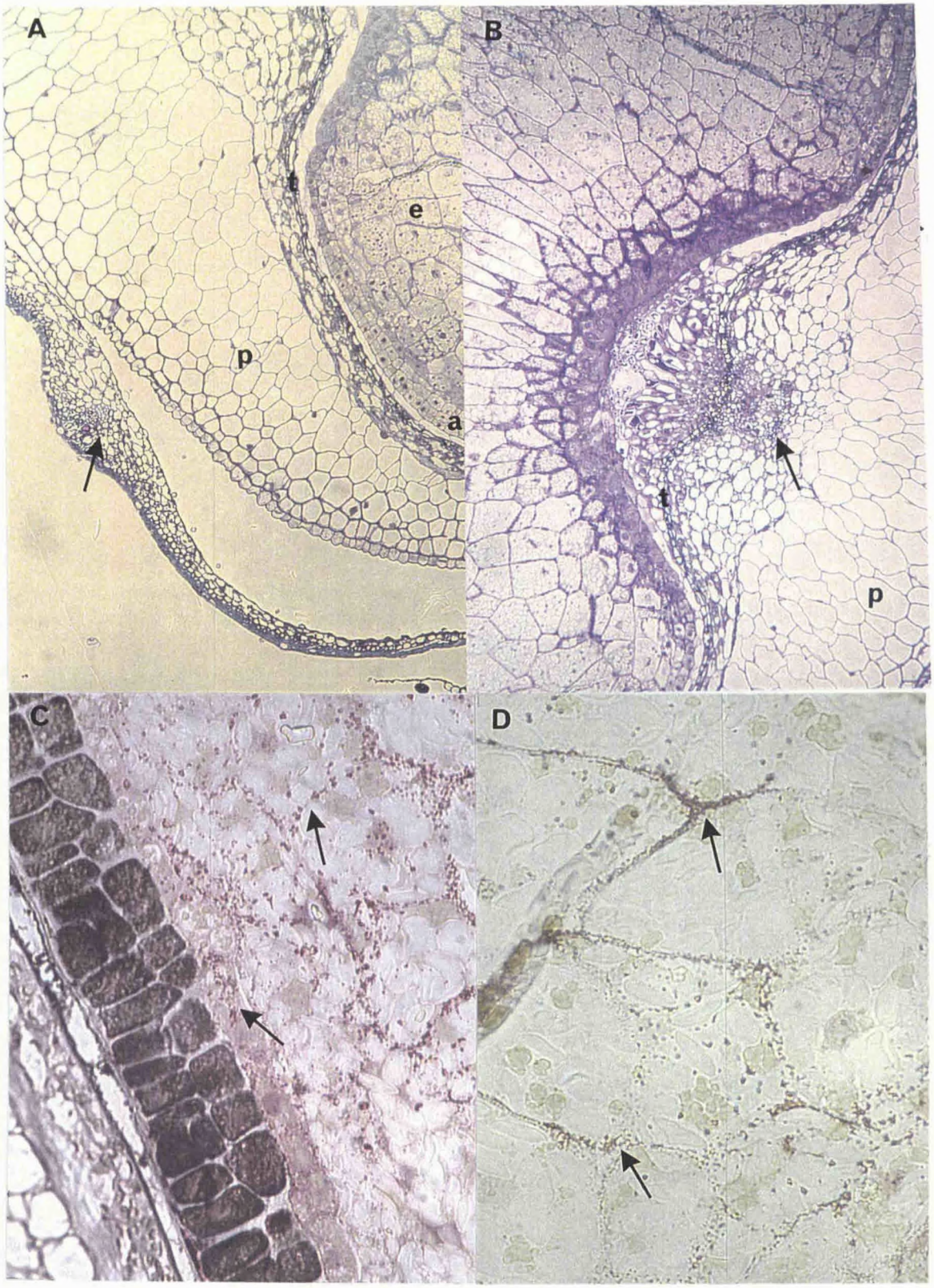
Electron micrographs

confirmed the presence of Ps 418 in pericarp cells (Fig. 2.3 A & B) and in vascular tissue cells (Fig. 2.3 B) in vicinity of the pigmented strand at the ventral furrow, suggesting vertical spread of the pathogen with the transpiration stream in mature xylem vessels. Furthermore, bacteria were consistently surrounded by electron translucent halos within a fibrillar matrix of extracellular polysaccharides (Fig. 2.3 B, arrows). Figure 2.3 C revealed the disruption of primary cell walls of a xylem element and horizontal spread of *P. syringae* Ps 418 to nearby unlignified pericarp cells. Disruption occurred in the vicinity of the pathogen and cellulosic portions of the degraded primary wall were detached from the main cell frame. The host plant's cell wall appeared to inlay a single bacterial cell (Fig. 2.3 D, arrow) in association with a pronounced wall modification, such as an erosion of the cell wall beneath the embedded bacterium. The mass of bacteria apparently induced cell wall degradations (Fig. 2.3 E & F), most likely due to toxin and/or extracellular enzyme action. Colonies of *P. syringae* Ps 418 in pericarp cells at the ventral furrow demonstrated pronounced cell wall disruptions and induced the formation of dark staining appositions at the sites of wall degradation in the collapsed plant cells (Fig. 2.3 E & F).

Immunogold labeling. The use of polyclonal antibodies in combination with goat anti-rabbit gold labels confirmed the presence of the inoculated Ps 418 pathogen in barley kernel cells (Fig. 2.4). Gold particles were found surrounding bacterial cell walls (Fig. 2.4, arrows) and were seen over the fibrillar matrix of extracellular polysaccharides that adhere to electron translucent halos, indicating specificity of primary antibodies to polysaccharide capsules of the bacteria.

Enzyme activity tests performed on ten *P. syringae* strains including strains Ps 418 and Pss 552 (data not shown) revealed that all strains tested positive for cellulase, β-glucanase and protease activity, as well as for starch hydrolysis.

Fig. 2.1. A & B: Light micrographs showing transsection of an uninoculated barley kernel 7 days after inoculation (dpi). C & D: Light micrographs showing transsection of a *P. syringae* Ps 418 inoculated barley kernel 7 dpi. A: Structures revealed a vascular bundle (arrow) in the spongy parenchyma of the palea, the pericarp (p) at the ventral furrow, the testa (t), the aleurone (a) and starchy endosperm (e) in the center of the kernel (x 100). B: Details of structures found at the ventral furrow, particularly the unlignified pericarp (p), comprising a vascular bundle (arrow), and the testa (t), which merges with the pigment strand (x 100). C: Ruthenium red enhanced resolution of Ps 418 cells (arrows) located in intercellular spaces of the aleurone layer and endosperm (x 400). D: Ps 418 (arrows) located in intercellular spaces of amyloplast cells in the starchy endosperm (x 400).


A
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D

Fig. 2.2. Scanning electron micrographs of surface structures found on a barley kernel and localization of *P. s.* pv. *syringae* Pss 552 seven days after head inoculation (7 dpi). A: A colony of Pss 552 (arrow) close to a trichome base on the lemma of the barley kernel (7dpi). B: Close up of the Pss 552 colony at the trichome base (7 dpi). C: Single cells (arrows) of randomly distributed Pss 552 on surface ridges on the lemma of a barley kernel one day after inoculation. D: Cells of Pss 552 (arrows) close to and inside a stoma on the palea of a barley kernel (7dpi). E: Stomata-like cells (arrows) on the external epidermis of the palea (7dpi). F: Pss 552 (arrows) found close to a stoma-like cell on the external epidermis of the palea (7dpi).

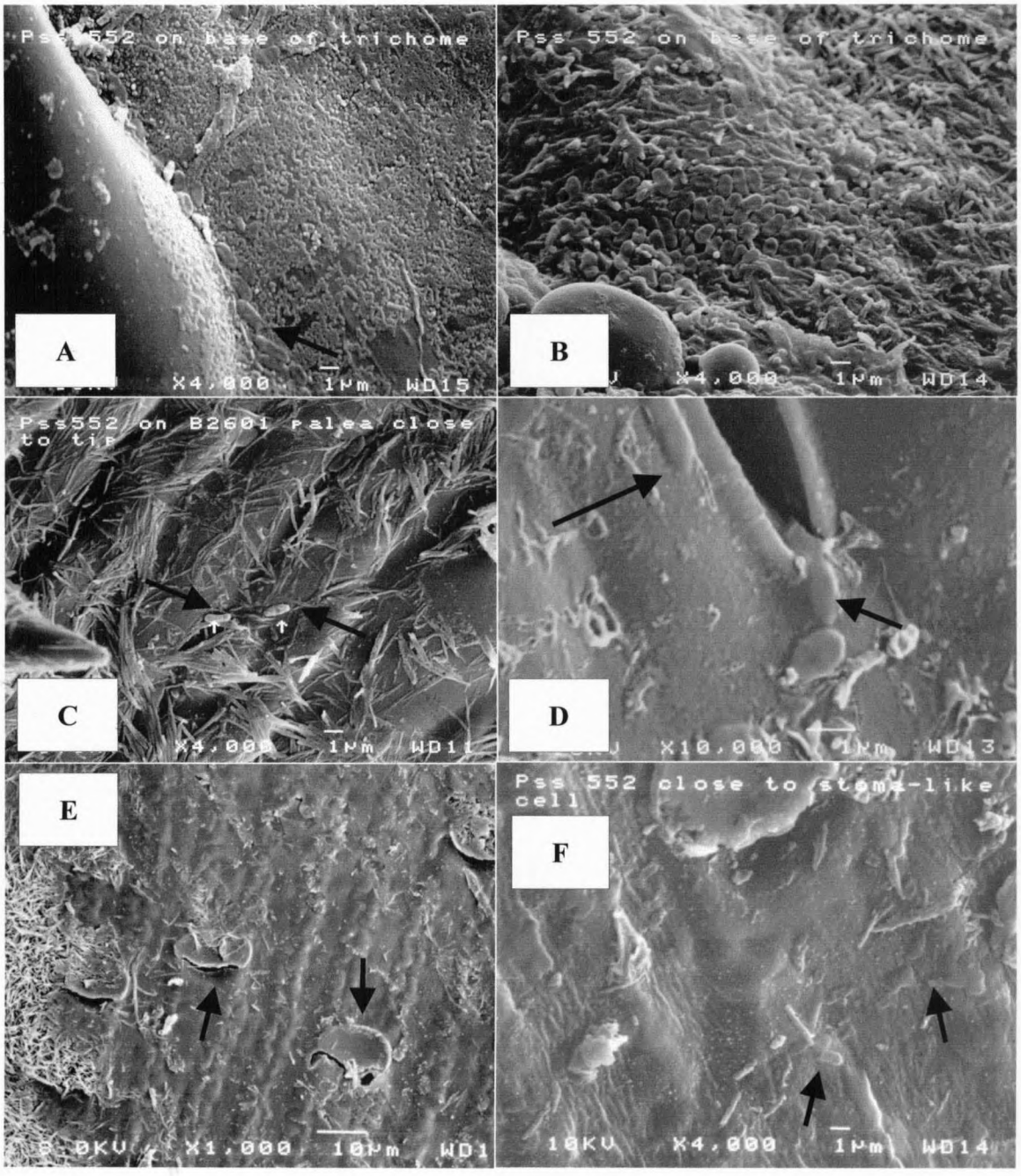
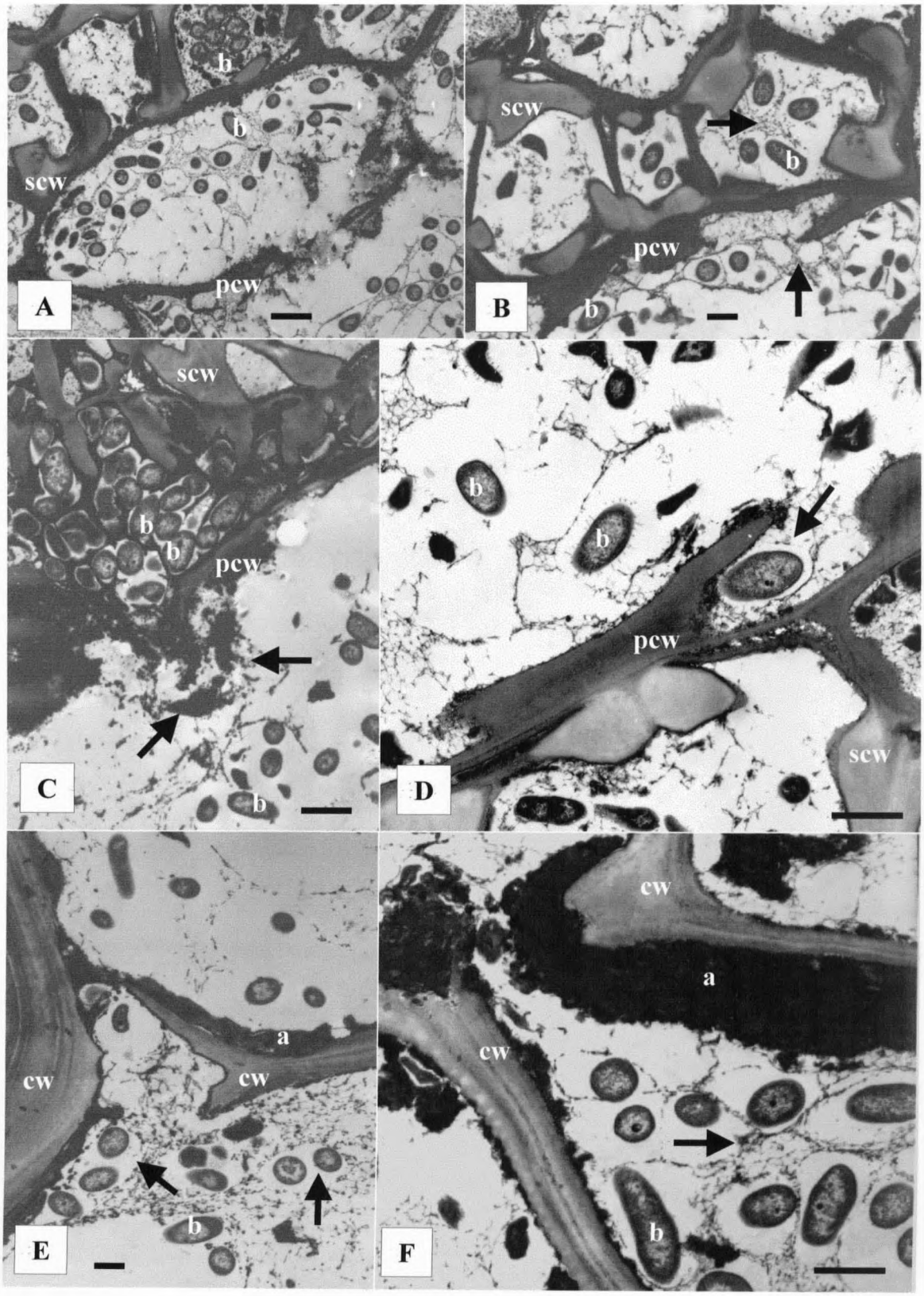

Pss 552 on base of trichome
A
X4,000 1µm WD15
Pss 552 on base of trichome
B
X4,000 1µm WD14
Pss552 on B2601 palea close to tip
C
X4,000 1µm WD11
D
X10,000 1µm WD13
E
X1,000 10µm WD1
Pss 552 close to stoma-like cell
F
10KV X4,000 1µm WD14

Fig. 2.3. Transmission electron micrographs of *P. syringae* Ps 418 infected barley kernels seven days after inoculation. A: Ps 418 in parenchyma and vascular tissue cells of the pericarp near the pigmented strand at the ventral furrow. Scale bar = 2 μm. B: Three mature xylem cells viewed in transverse section showing electron-dense lignified wall thickenings: the cytoplasm has been digested and the primary walls are almost broken down. Note: Bacteria are surrounded by electron-translucent halos within a fibrillar matrix of extracellular polysaccharides (arrows). Scale bar = 1 μm. C: Bacteria located in a xylem vessel. The primary cell wall appears disrupted in the vicinity of the pathogen, while the secondary walls seem thicker and intact. Note: Portions of the primary cell wall are detached from the main cell frame (arrows). Scale bar = 1 μm. D: Bacterial cells located in xylem parenchyma. Note: Presence of a single bacterial cell (arrow) accompanied by localized cell wall modification. Scale bar = 1 μm. E & F: Disruption of pericarp cell walls in the vicinity of the pathogen, leading to horizontal spread of bacteria to neighboring cells. Note the formation of dark staining wall appositions at sites of cell wall disruptions. Bacteria were surrounded by electron-translucent halos within a fibrillar matrix (arrows). Scale bar = 1μm. cw = cell wall, b = bacterium, a = wall apposition, pcw = primary cell wall, scw = secondary cell wall.


A
b
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b
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a
cw
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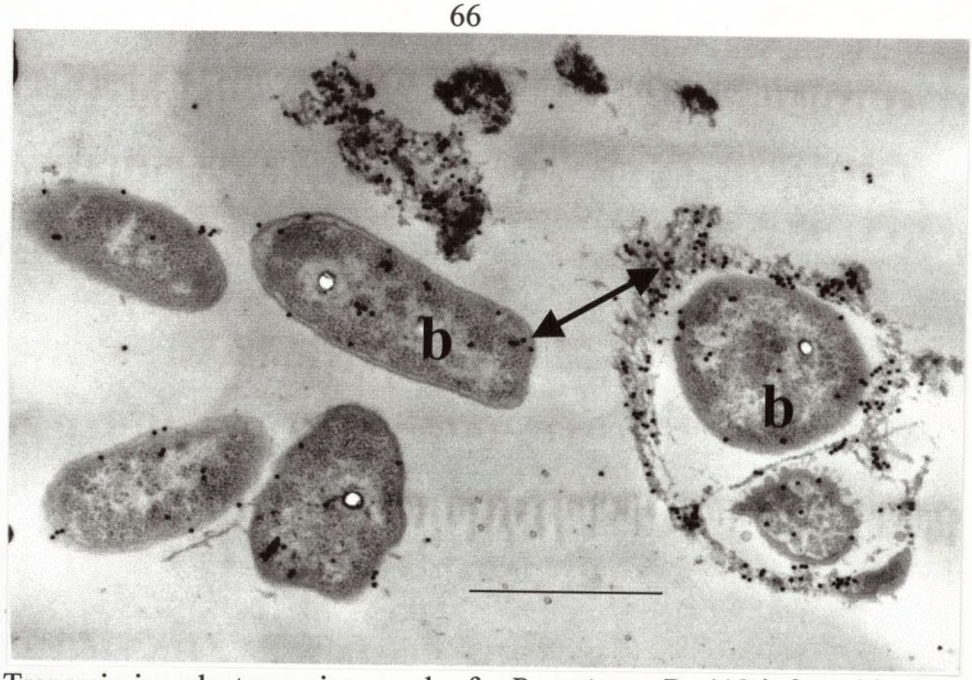

Fig. 2.4. Transmission electron micrograph of a *P. syringae* Ps 418 infected barley kernel seven days after inoculation. The infected kernel section was incubated with polyclonal antibodies and labeled with anti-rabbit immunogold-IgG conjugate. Note: The primary antibody reacted with peripheral cell surface epitopes as well as with a fibrillar matrix, (arrows), most likely extracellular polysaccharides from bacterial cell capsules, as shown by the localized distribution of gold particles; b = bacterium. Scale bar = 1 μm.

Fig. 2.5. Pss 552 inoculated barley kernels showing symptoms of basal kernel blight after harvest. Pss 552 was inoculated at soft dough stage of kernel development. Non-inoculated kernels are shown as controls (arrows). Note: Symptoms start at the kernel base and extend upward along the central vascular bundle located on the kernel lemma.

Discussion

Seven days after inoculation of *P. s.* pv. *syringae* Pss 552, bacterial cells were located near or in stomata or stomata-like cells on barley kernels, as well as at bases of trichomes suggesting site-specific colonization of barley kernels and invasion through natural openings. The presence of stomata-like cells on the surface of the palea in mature and immature barley were not surprising to Pomeranz & Sachs (1972), since husks are remnants of the protective envelope of the flower and are ontologically leaf tissue. The diameter of about 12 μm of stomata-like cells was also within the range of stomatal cells of cereal leaves (Pomeranz & Sachs, 1972), which could be confirmed in my studies (Fig. 2.2 E & F). Unlike many fungal pathogens, most plant pathogenic bacteria enter plants only via wounds and natural openings, since they are not able to pass directly through the epidermis due to a lack of esterases or peroxidases that could degrade the outer layer of the cuticle or surface waxes (Huang, 1986). Stomata and hydathodes are the most important portals of entry for bacterial pathogens, such as *P. syringae* and *Xanthomonas campestris* pathovars, that are epiphytic residents of the leaf surface and cause foliar diseases (Huang, 1986; Hirano & Upper, 1990; Goto, 1992; Sigee, 1993). Bacteria frequently multiply in substomatal cavities and subsequently migrate into surrounding intercellular spaces (Huang, 1986; Goto, 1992). Although stomata are not found on the lemma of the kernel (Miskin & Rasmusson, 1970), they were located on either side of the midvein on the outer face of the awn and as single rows down each side of the crease on the palea. Considering the importance of stomata for *P. syringae* to penetrate and infect the plant, in addition to the

large size of a stoma compared to the small size of the pathogen, the significance of stomatal number and size in resistance to the bacteria becomes apparent and may require further examination relative to basal kernel blight disease development.

Furthermore, many foliar bacterial plant pathogens, including *P. cichori*, *P. s.* pv. *tomato*, *Clavibacter michiganensis* subsp. *michiganensis* and *Erwinia amylovora* invade their hosts through trichomes (Huang, 1986; Goto, 1992). Studying the epiphytic survival of *P. s.* pv. *syringae* on tomato and seeds Mariano & McCarter (1991) found that *P. s.* pv. *syringae* existed primarily around trichomes on foliage of tomato, spurred anoda, and smooth vetch, in depressions between epidermal cells and along the veins. Goto (1992) claimed that susceptibility of leaves to bacterial infection was generally correlated with the density of trichomes; thus young leaves with the greatest density of hairs were most susceptible to infection. The decreasing number of trichomes on older leaves/kernels and the fact that stomata closure is more evident in mature kernels might explain why I found less basal kernel blight disease on plants that were inoculated at hard dough stage of kernel development while early milk and soft dough stage inoculation resulted in highest susceptibility to the bacterial pathogen (Martinez-Miller, 1994; Martinez-Miller et al., 1998). Furthermore, ectodesmata extending from the cuticle through the wall to the lumen of epidermal cells are confined to special sites such as trichomes (Goto, 1992). Since they function as foliar absorption and excretion sites of aqueous organic and inorganic substances, phylloplane residents preferentially colonized the leaf surface where trichomes and ectodesmata were concentrated (Goto, 1992). Another evidence for site-specific colonization of foliar pathogens was presented by Mew & Vera Cruz (1986), who studied

the epiphytic behavior of *X. campestris* pv. *oryzicola*. These bacteria invaded the parenchymatous tissues and were found in the vicinity of stomata, trichome bases, and other parts of the leaf surface covered with wax particles. They did not, however, colonize hydathodes, which are preferentially colonized by *X. campestris* pv. *oryzae*. Therefore, site-specificity appeared related to the nature of pathogenesis and the natural openings that bacteria can enter. The fact that only single cells of Pss 552 bacteria were found randomly distributed over the kernel surface one day after inoculation whereas colonies of Pss 552 were visible close to natural portals of entry seven days after inoculation might be explained by spatial-temporal colonization patterns not examined in my studies. Chin-A-Wong et al. (1997), who studied the daily colonization pattern of *P. fluorescens* on tomato roots, pointed out that colonization progressed down the root, initially as single bacterial cells. After 7 days, micro-colonies had developed at positions where only single cells were observed 1-3 days after inoculation. Alternatively *P. s.* pv. *syringae* Pss 552 could have demonstrated an extended lag phase prior to exponential growth on barley kernels. This hypothesis was confirmed by greenhouse reisolation studies (chapter 5; Fig. 5.4), in which the Pss 552 population declined 31-fold to 3.2×10^5 cfu/kernel one day after inoculation of 1×10^7 cfu/ml but recovered thereafter (10-fold increase in population size) and remained consistent at 3.2×10^6 cfu/kernel over a period of 11 days (chapter 5; Fig. 5.4).

My observations after spray inoculation of unwounded barley kernels, suggested two possible invasion strategies : (1) The pathogen may have entered through stomata on the palea and subsequently colonized intercellular spaces in the bast layer and thin-walled spongy parenchyma below the bast. Bacteria could have further migrated intercellularly to

the vascular elements located in the husk or left the husk through stomata located on the inner epidermis of the husk and entered the outer thin-walled epidermal cell layer of the pericarp (Briggs, 1978; Peters et al., 1983). Pericarp cells are compressed during development and not lignified, since lignin is confined to the husk in completely mature grain (Briggs, 1978). (2) Bacteria might have penetrated the kernel through the kernel base, which is attached to the nodes of the rachis. Water drops containing cells of the pathogen usually collect at these sites, particularly in 6-row barley at soft dough stage. Bacteria could have invaded the kernels through natural openings on the palea, spread intercellularly through the lignified husk tissue until they encountered vascular bundles. Vertical spread inside the kernel possibly occurred once the bacteria entered xylem vessels. Moreover, vascular bundles run inside the rachis and interconnect the barley stem with the spike (Briggs, 1978), indicating a potential for systemic spread of the pathogen through stem and kernel tissues with the transpiration stream. Horizontal cell-to-cell spread inside the pericarp tissues was probably accomplished by enzyme activities.

A careful observation of the disease symptomatology supported the second scenario since basal blight symptoms spread upward on the kernel, starting out as black discoloration (necrosis) on the vascular bundle located in the middle of the lemma and then spreading over the entire kernel base (Fig. 2.5).

Plant cell walls represent physical barriers to pathogen invasion, but previous work on *Pseudomonas* and *Xanthomonas*-infected plants mentioned that host cell walls were altered during the infection process (Sequeira et al., 1977; Smith & Mansfield, 1982; Boher et al., 1995). The present ultrastructural investigation clearly demonstrated that cell walls

were highly degraded in barley cultivar B 2601 infected with *P. syringae* Ps 418. Host cell walls displayed extensively disrupted areas seven days after inoculation regardless of the tissue colonized [xylem vessels (Fig. 2.3 C), xylem parenchyma (Fig. 2.3 A) or other pericarp cells (Fig. 2.3 E & F)]. Degradation affected mainly the primary cell wall allowing some bacteria to penetrate into uninvaded cells (Fig. 2.3 A,B,C&D). Subsequently, the lumen of the cells were colonized by actively multiplying bacteria. The colonized cells were filled with an extracellular fibrillar material except for an electron-transparent halo surrounding each bacterium (Fig. 2.3 A ,B, E & F). Consequently, the bacterial progression in host tissue resulted in kernel necrosis and embryo damage probably due to the loss of tissue cohesion.

Boher et al. (1995) also reported cell wall damage caused by *X. c.* pv. *manihotis* in cassava leaves. Their studies, using anti-pectin monoclonal antibodies and β-1,4-exoglucanase-gold probes, demonstrated that middle lamellae and primary and secondary cell walls were degraded during the infection process. This indicated that enzymes such as pectinases and cellulases may be required for host colonization, although their role in pathogenicity remained unclear (Boher et al., 1995). The production of glycoside hydrolases, such as cellulases and hemicellulases in addition to xylosidase and pectinase activity was found to be a common phenomenon among soft rot bacteria as well as among bacteria that cause tissue necrosis and vascular wilt diseases (Sigee, 1993). The role of cellulases in pathogenesis has been studied in *X. campestris*, *E. chrysanthemi*, and *P. solanacearum* by cloning their genes. Mutational analyses demonstrated that cellulases were not essential for disease development, i.e. they were thought to be more advantageous to saprophytic life and

degradation of dead plant material than to parasitic life (Goto, 1992). Ultrastructural evidence of primary cell wall degradation by *P. solanacearum* in tobacco mesophyll cells was reported by Sequeira et al. (1977). Four hours after inoculation, compatible bacteria were actively dividing (exponential growth phase) in intercellular spaces and host cell walls appeared eroded and disrupted in close proximity to the bacterial cells. Loose fibrillar material was also visible in areas where the cell wall had been weakened. Disruption of the wall was accompanied by a slight separation of the plasmalemma from the cell wall and granular material was seen between the plasmalemma and the cell wall. Furthermore, *P. solanacearum* produced pectic and cellulytic enzymes in diseased tissues enabling the bacterium to invade tissues and obtain nutrients (Buddenhagen & Kelman, 1964). Thus, cell-wall degrading enzymes of *P. syringae* may serve a nutritional purpose to provide nutrients that can be used as energy source or an invasive role for enhanced penetration (Gross & Cody, 1985).

Relative to enzyme activity I found that all *P. syringae* strains were positive for cellulase, β-glucanase and protease activity as well as for starch hydrolysis. Although I do not know the relevance of these enzymes in barley pathogenesis at this point, the enzymes may be important for growth adaptation of *P. syringae* to barley kernels. Gross & Cody (1985) suggested that because most strains of *P. syringae* pv. *syringae* cannot utilize cellobiose as an energy source, cellulase activity may function only in enhancing penetration of host tissue. Since all *P. syringae* strains displayed β-glucanase activity, I hypothesize that cellulose present in primary plant cell walls might be degraded stepwise, first by bacterial endocellulase which cleaves β-glucosidic bonds at random in the middle of cellulose

molecules, releasing cellobiose and cellodextrins, with the latter being cleaved by the bacterial exo-β-glucosidase activity into glucose (Goto, 1992). These results are consistent with those of Hayward (1977), who observed differences in the reactions of fluorescent pseudomonads in tests for α-glucosidase, β-glucosidase and β-xylosidase. Whereas weak and delayed reactions for α-glucosidase were found among both plant pathogenic and saprophytic fluorescent pseudomonads, none of the saprophytes gave positive reactions in either the β-glucosidase or β-xylosidase tests. None of the 18 *P. phaseolicola* isolates examined gave a positive reaction in the test for β-xylosidase activity whereas six of the seven *P. syringae* isolates did. Many of the fluorescent plant pathogens gave positive reactions in the β-glucosidase test, except for *P. fluorescens*. The hydrolyzation of plant β-glucosides may result in tissue disorganization and intercellular spread of the pathogen (Gross & Cody, 1985).

The accumulation of fibrillar material surrounding *P. syringae* Ps 418 was observed in almost all tissues (Fig. 2.3 A, B, E& F). This extracellular material is most likely of pathogen origin since a positive labeling was obtained with antibodies made to *P. syringae* whole cell preparations (Fig. 2.4), which consisted mainly of extracellular polysaccharides (EPS). The many functions of bacterial EPS in plant pathogenesis have been reviewed (Sigee, 1993; Roberts, 1996). Hydrophilic EPS have been implicated in induction and maintenance of water-soaking, which is an important step in the development of the susceptible reaction for many leaf spot diseases caused by pseudomonads and xanthomonads. With plants kept under high humidity, El-Banoby and Rudolph (1979) were able to directly induce water-soaking in leaf tissue by infiltration of EPS preparations from

P. s. pv. *phaseolicola.* These EPS contained four macromolecular fractions: levan, alginate, lipopolysaccharide and protein. EPS therefore may provide some bacterial species, such as *E. coli, Erwinia stewartii, P. aeruginosa* and others with advantages for survival since the formation of a hydrated gel around the cell surface may protect them from desiccation (Roberts, 1996). Furthermore, EPS may promote the adherence of bacteria to surfaces, thereby facilitating the formation of a biofilm and the colonization of various ecological niches (Costerton et al., 1987). EPS have also been shown to be important in promoting the ability of virulent pathogenic bacteria to avoid plant recognition and thus triggering a hypersensitive reaction (HR) by masking receptor sites on the bacterial surface (Sigee, 1993). In animal systems capsular polysaccharides were found to confer resistance to phagocytosis by masking the underlying C3b recognition sites form C3b receptors on the phagocyte cell surface (Roberts, 1996). Studying the interaction between *Pseudomonas* pathogens and oat leaves, Smith & Mansfield (1982) discovered that in incompatible interactions pathogens became attached to non-host plant cell walls, whereas in compatible interactions bacteria were always surrounded by EPS. My observations with *P. syringae* and barley kernel cells were similar to this compatible interaction (Fig. 2.3 A, B, E & F). In addition, dark staining appositions were found at sites where bacteria were close to the cell wall. This response, although more frequently observed in incompatible interactions in combination with the HR (Smith & Mansfield, 1982; Politis & Goodman, 1978), was thought to represent part of the process of host resistance, but it was not clear whether it might influence bacterial growth. I also found dark staining appositions accumulating at inner plant cell wall layers, predominantly in cells that demonstrated cell wall disruptions

(Fig. 2.3 E & F). The observation of two layers surrounding bacterial cells, one electron-translucent halo and one electron-dense fibrillar matrix was investigated by Fletcher & Floodgate (1973), who studied the adherence of marine pseudomonads to solid surfaces. They proposed that EPS comprised 2 layers, an electron translucent inner layer of primary polysaccharide and an outer layer of fibrillar polysaccharides. Smith & Mansfield (1982) hypothesized it may be comprised of only a single type of polysaccharide that shrinks away from the bacterial wall during preparation for microscopy. Recently, it was discussed that it may result from synergistic interactions between bacterial EPS and plant cell wall polysaccharides (Boher et al., 1995). Finally, I observed the embedding of a single bacterial cell by the host cell wall (Fig. 2.3 D), which most likely was an attempt of the plant to locally immobilize an individual bacterium and avoid its multiplication, but this was in general the exception rather than the rule. Since this reaction was accompanied by host cell wall modifications resulting in the embedding of the bacterium, it appeared similar to one of the two incompatible interactions described by Smith & Mansfield (1982), in which the attachment of bacteria to plant cell walls involved a localized modification of the underlying wall. In addition, Kunoh (1996) reported that defense-related reactions can occur only in the cells that are attacked by individual microbes but not in all cells within the same tissue. Alternatively, the picture (Fig. 2.3 D) might show the process of cell wall degradation by the individual bacterium.

In conclusion, the present study provides insights into invasion strategies of barley kernels and cell wall degradation by *Pseudomonas syringae*. My results favor the hypothesis that lytic enzymes, presumably of bacterial origin, weaken and loosen the cell walls of barley

during host colonization by *P. syringae*. In this compatible interaction, bacteria were seldom seen in close contact with the plant cell wall, which appeared to be prevented by the presence of EPS around the pathogen. Also, EPS fibrils were closely associated with detached portions of host cell walls, likely providing a more accessible nutrient supply for the pathogen (Fig. 2.2. C, E, F). Moreover, EPS could be responsible for the appearance of water-soaked symptoms on kernel bases seven days after inoculation and in combination with lytic enzymes account for kernel necrosis and basal blight symptoms. However, whether EPS and such enzymes are required for pathogenicity remains unknown and could be studied in detail using EPS-minus mutants, enzyme-truncated or deficient mutants, and a resistant barley cultivar to investigate and compare cell wall degradation.

Literature Cited

Andro, T., Chambost, J. P., Kotonjansky, A., Cattano, J., Bertheau, Y., Barras, F., van Gijsegem, F., and Coleno, A. 1984. Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and cellulase. J. Bacteriol. 160: 1199-1203.

Benhamou, N., Kloepper, J. W., Quadt-Hallmann, A., and Tuzun, S. 1996 a. Induction of defense-related ultrastructural modifications in pea root tissues inoculated with endophytic bacteria. Plant Physiol. 112: 919-929.

Benhamou, N., Belanger, R. R., and Paulitz, T. C. 1996 b. Induction of differential host responses by *Pseudomonas fluorescens* in Ri T-DNA-transformed pea roots after challenge with *Fusarium oxysporum* f. sp. *pisi* and *Pythium ultimum*. Phytopathology. 86(11): 1174-1185.

Boher, B., Kpemona, K., Nicole, M., Luisetti, J. and Geiger, J. P. 1995. Ultrastructure of interactions between cassava and *Xanthomonas campestris* pv. *manihotis*: cytochemistry of cellulose and pectin degradation in a susceptible cultivar. Phytopathology. 85(7): 777-788.

Bradbury, J. F. 1986. *Pseudomonas*. In: Guide to plant pathogenic bacteria. CAB International Mycological Institute, Kew, United Kingdom. pp. 154-187.

Braun, A., Rose, A., Kaliyeva, G.T., Martinez-Miller, C., and Sands, D.C. 1996. Biocontrol of bacterial kernel blight of barley by beneficial *P. agglomerans*. Phytopathology. 86(11): S79 (Abstract).

Braun, A., Jacobsen, B. J., and Sands, D. C. 1997 a. Possible biocontrol mechanisms in the *P. syringae-Pantoea agglomerans* interaction. Phytopathology. 87(6): S11. (Abstract)

Braun, A., Jacobsen, B. J., and Sands, D. C. 1997 b. Suppression of bacterial kernel blight of barley by antagonistic *Pantoea agglomerans*, 1995. Biological and Cultural Tests for Control of Plant Diseases. 12: 105.

Briggs, D. E. 1978. The morphology of barley; the vegetative phase. In: Barley. D. E. Briggs (ed.), Wiley and Sons, Inc., NY, p. 1-14.

Buddenhagen, I. and Kelman, A. 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. Ann. Rev. Phytopathol. 2: 203-230.

Chin-A-Wong, T. F. C., de Priester, W., van der Bij, A. J., and Lugtenberg, B. J. J. 1997. Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365, using scanning electron microscopy. Mol. Plant Microbe Interactions. 10(1): 79-86.

Clark, M. F. and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. gen. Virol. 34: 475-483.

Costerton, J. W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C. 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. 41: 435-464.

El-Banoby, F. E., and Rudolph, K. 1979. A polysaccharide from liquid cultures of *Pseudomonas phaseolicola* which specifically induces water-soaking in bean leaves (*Phaseolus vulgaris* L.). Phytopathol. Z. 95: 38-50.

Fletcher, M., and Floodgate, G. D. 1973. An electron microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. Journal of General Microbiology. 74: 325-334.

Georgakopoulos, D. G. and Sands, D. C. 1992. Epiphytic populations of *Pseudomonas syringae* on barley. Can. J. Microbiol. 38: 111-114.

Goto, M. 1992. Fundamentals of bacterial plant pathology. Academic Press, Inc., San Diego, CA.

Gross, D. C. and Cody, Y. S. 1985. Mechanisms of plant pathogenesis by *Pseudomonas* species. Can. J. Microbiol. 31: 403-410.

Hamacher, J. 1992. Licht und electronenmikroskopische Untersuchungen an Laubbäumen unter verschiedenen Streßeinwirkungen. Habilitationsschrift Landwirtschaftliche Fakultät der Rheinischen Friedrich Wilhelms Universität zu Bonn.

Hankin, L. and Anagnostakis, S. L. 1975. The use of solid media for isolation of enzyme production by fungi. Mycologia. 67: 597-607.

Hayward, A. C. 1977. Occurrence of glycoside hydrolases in plant pathogenic and related bacteria. J. Appl. Bacteriology. 43: 407-411.

Hildebrand, D. C., Schroth, M. N., and Sands, D. C. 1988. *Pseudomonas*. In: Laboratory Guide for Identification of Plant Pathogenic Bacteria, 2nd edition, ed. Schaad, N. W. APS Press, St. Paul, Minnesota, pp. 60-80.

Hirano, S. S. and Upper, C. D. 1990. Population biology and epidemiology of *Pseudomonas syringae*. Annu. Rev. Phytopathol. 28: 155-177.

Huang, J. S. 1986. Ultrastructure of bacterial penetration in plants. Annu. Rev. Phytopathol. 24: 141-157.

Kietzell von, J. M. 1995. Untersuchungen zur Epidemiologie und Spezifität von *Pseudomonas syringae* pv. *atrofaciens* ((McCulloch), Dye, Young, Wilkie), dem Erreger der basalen Spelzenfäule des Getreides. Dissertation, Universität Göttingen, Germany.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 434: 301-307.

Kunoh, H. 1996. Cytological approaches in understanding host-parasite interactions. In: Molecular Aspects of Pathogenicity and Resistance: Requirements for Signal Transduction D. Mills, H. Kunoh, N. T. Keen, and S. Mayama (eds.). The American Phytopathological Society, St. Paul, Minnesota, pp. 15-29.

Leary, J. V. and Chun, W. W. C. 1988. *Bacillus*. Diagnostic media and tests. Starch hydrolysis. In: Plant Pathogenic Bacteria. N. W. Schaad (ed.). APS Press, St. Paul, MN. p. 125.

Lister, R. M. 1978. Application of the enzyme-linked immunosorbent assay for detecting viruses in soybean seed and plants. Phytopathology. 68: 1393-1400.

Luebeck, P. S., Hansen, M., and Sorensen, J. 1998. In situ localization of *Pseudomonas* sp.on barley roots with fluorescently labelled, rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy. In: Proc. of molecular approaches in biological control. Delemont, Switzerland, 15-18. Sept. 1997. IOBC/OILB, European Foundation for Plant Pathology, Biological Control Working Group. 31:58. in press.

Mariano, R. D. L. R. and McCarter, S. M. 1991. Epiphytic survival of *Pseudomonas syringae* pv. *syringae* on tomato and seeds. Fitopatologia Brasileira 16 (1): 92-97.

Martinez-Miller, C. 1994. Etiology and characterization of two *Pseudomonas syringae* pathovars causing two bacterial kernel blights of barley. PhD Thesis. Montana State University, Bozeman, MT.

Martinez-Miller, C. and Braun, A. 1997. Bacterial Kernel Blights. In: Compendium of barley diseases. Second edition. D. E. Mathre (ed.). APS Press. St. Paul, Minnesota.

Martinez-Miller, C., Braun, A., Siemsen, S., and Sands, D. C. 1998. Etiology of basal kernel blight of barley caused by *Pseudomonas syringae* pv. *syringae*. Can. J. of Plant Pathology. In press.

Mathre, D. E. 1997. Bacterial and phytoplasma diseases. In: Compendium of barley diseases. Second edition. D. E. Mathre (ed.). APS Press. St. Paul, Minnesota.

Mew, T. W. and Vera Cruz, C. M. 1986. Epiphytic colonization of host and non-host plants by phytopathogenic bacteria. In: Microbiology of the phyllosphere. N. J. Fokkema and J. Van den Heuvel (eds.). Cambridge University Press, NY. p. 269-282.

Miskin, K. E., and Rasmusson, D. C. 1970. Frequency and distribution of stomata in barley. Crop Science. 10: 575-578.

Paul, V. H., and Smith, I. M. 1989. Bacterial pathogens of graminae: Systematic review and assessment of quarantine status for the EPPO region. Bulletin OEPP/EPPO Bulletin 19: 33-42.

Peters, R. A., Timian, R.G., and Wesenberg, P. 1983. A bacterial kernel spot of barley caused by *Pseudomonas syringae* pv. *syringae*. Plant Dis. 67: 435-438.

Politis, D.J., and Goodman, R.N. 1978. Localized cell wall appositions: Incompatibility response of tobacco leaf cells to *Pseudomonas pisi*. Phytopathology. 68: 309-316.

Pomeranz, Y. and Sachs, I. B. 1972. Determining the structure of the barley kernel by scanning electron microscopy. Cereal Chemistry. 49(1): 1-19.

Roberts, I. S. 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. Annu. Rev. Microbiol. 50: 285-315.

Schaad, N. W., Suele, S., van Vuurde, J. W. L., Vruggink, H., Alvarez, A. M., Benedict, A. A., De Wael, L., and van Laere, O. 1990. Serology. In: Methods in Phytobacteriology. Z. Klement, K. Rudolph, and D. C. Sands (eds.). Akademiai Kiado, Budapest. p. 153-190.

Sequeira, L., Gaard, G., and De Zoeten, G. A. 1977. Interaction of bacteria and host cell walls: its relation to mechanisms of induced resistance. Physiological Plant Pathology. 10: 43-50.

Sigee, D. C. 1993. Bacterial plant pathology: cell and molecular aspects. Cambridge University Press, Cambridge, U.K.

Smith, J. J. and Mansfield, J. W. 1982. Ultrastructure of interactions between pseudomonads and oat leaves. Physiological Plant Pathology. 21: 259-266.

Vasse, J., Frey, P., and Trigalet, A. 1995. Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by *Pseudomonas solanacearum*. Mol. Plant Microbe Interactions. 8(2): 241-251.

Young, J. M., Takikawa, Y., Gardan, L., and Stead, D. E. 1992. Changing concepts in the taxonomy of plant pathogenic bacteria. Annu. Rev. Phytopathol. 30: 67-105.

Zadoks, J. C., Chang, T. T., and Konzak, C. F. 1974. A decimal code for the growth stages of cereals. Weed Res. 14: 415-421.

CHAPTER 3

DIVERSITY OF *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* STRAINS ASSOCIATED WITH BARLEY KERNEL BLIGHT: CORRELATION OF PATHOGENICITY, TOXIN PRODUCTION, NUTRITIONAL PROFILE AND RESTRICTION-FRAGMENT LENGTH POLYMORPHISM

Introduction

Pseudomonas syringae pv. *syringae* (Pss) van Hall 1902 is an important bacterial plant pathogen found throughout the world on a multiplicity of hosts (Bradbury, 1986; Young, 1992; Georgakopoulos & Sands, 1992; Martinez-Miller & Braun, 1997). *P. s.* pv. *syringae* damage to barley kernels, results in black discoloration on the kernel lemma (spot blight) or base (basal blight) including the embryo (Martinez-Miller, 1994; Martinez-Miller & Braun, 1997). We have reported upon the etiology and significance of basal kernel blight of barley caused by *P. s.* pv. *syringae* (Martinez-Miller et al., 1998). Furthermore, electron microscopy revealed data on kernel invasion, pathogenesis and compatibility in this host-parasite interaction (Braun et al., 1997 a; chapter 2). The only control measure to date is the avoidance of irrigation during the most susceptible kernel developmental stage at soft dough and the use of resistant cultivars (Martinez-Miller et al., 1998), since no bactericides are available on small grains that might provide control of the pathogen.

The heterogeneity of strains within the pathovar *syringae* has been described (Roos & Hattingh, 1987; Denny et al., 1988; Cooksey & Graham, 1989; Louws et al., 1994) using strains isolated from a limited number of hosts and/or from diverse geographical areas. RFLP analyses and DNA-hybridization successfully differentiated strains of *Pseudomonas syringae* between and within pathovars (Denny et al., 1988; Legard et al., 1993; Quigley & Gross, 1994; Martinez-Miller, 1994). In addition, rare-cutting restriction endonucleases have been used to generate distinctive patterns of large DNA fragments which could be separated by field inversion (Cooksey & Graham, 1989) or pulsed-field gel electrophoresis (Grothues & Tümmler, 1991). Such macrorestriction fingerprints of the entire bacterial genome were shown to be very sensitive to mutations at a specific restriction site, thus exposing polymorphism between and within genetically distinct bacterial species and pathovars. Furthermore, PCR-RFLP analysis of rRNA operons and rep-PCR-based amplification of variable DNA regions between conserved bacterial repetitive elements have been useful techniques for the rapid and reproducible identification of plant pathogenic bacteria at the species, pathovar and even strain level (Jensen et al., 1993; Louws et al., 1994; Manceau & Horvais, 1997). Amplified Fragment Length Polymorphism (AFLP) was employed by Bragard et al. (1997) to characterize 68 xanthomonad strains from small grains and found the results to be consistent with pathogenicity tests and RFLP analysis. Thus AFLP, has become a method of choice not only for taxonomy, but also for epidemiological studies. Each of the afore mentioned analytical techniques based on DNA characterization alone may indicate whether different field isolates represent a single evolutionary line (common ancestor) or are composed of several genetically distinct lines that might have converged to

a similar pathogenic phenotype. However, the combination of biochemical, pathological and genetic analyses has been proposed to assess the phylogenetic relatedness more appropriately in relation to the host plant (Denny, 1988; Hendson et al., 1992).

Earlier attempts to determine relationships between the host plant barley and two *P. syringae* groups, which appeared to cause distinct symptoms (spot and basal kernel blight), using RFLPs with the *syrB* gene as DNA probe, revealed the distribution of Pss strains isolated from basal blight in closely related clusters, while a few *P. syringae* strains isolated from spot blight symptoms were in a divergent cluster (Martinez-Miller, 1994). However, a large variability among strains was recorded when RFLPs also included the gene probes *hrp10* and *hrp14*, two individual fragments of a 30 kb insert containing the *hrp* region from *P. s.* pv. *syringae* 61 (Martinez-Miller, 1994).

This study was done to describe the phylogenetic relatedness of *P. s.* pv. *syringae* strains based on more phenotypic and genotypic parameters, including cultivar-strain interactions, since the genome characteristics for pathogenicity and virulence occurring at the strain level are uncertain. Isolates of Pss were selected from barley kernels obtained from resistance screening experiments in Fairfield, MT, consisting of twelve barley cultivars. Several *P. s.* pv. *syringae* strains from hosts other than barley and five other cereal pathovars of *P. syringae* were included to detect possible differences between host species and pathovars. Traits examined consisted of pathogenicity on barley cultivar B 2601, hypersensitivity on tobacco, syringomycin toxin production, nutritional profiles and genome organization, which is thought to be shaped by selection and evolution (Louws et al., 1994). The knowledge may be valuable since preliminary studies indicated that cultivar preferences

may have developed between Pss and barley (Braun & Sands, 1995), in a similar way as has been reported for Pss and *P. s.* pv. *phaseolicola* and snap bean or Pss and cherry trees (Hirano & Upper, 1990). Additionally, data on pathogen diversity may provide important information for the development of an effective biological control system based on the application of antagonistic *Pantoea agglomerans* prior to the infection window (Braun et al., 1996; Braun et al., 1997 b; chapter 4).

Materials and Methods

Bacterial isolation and identification.

Twenty-two Pss isolates were obtained from individual barley kernels after harvest in 1994 and 33 in 1995. Kernels used for pathogen isolation either exhibited basal or spot blight disease symptoms, or no symptoms at all. In addition, two strains were isolated from healthy Canada thistles (*Cirsium arvense*) surrounding the barley field in 1994 (Tables 3.1; 3.2). Isolates were collected from resistance screening experiments in Fairfield, Montana, in which 12 barley cultivars were tested for susceptibility to basal and/or spot kernel blight, respectively (Martinez-Miller et al., 1998). The semi-selective KBC medium was used for isolation of *P. s.* pv. *syringae* (Mohan & Schaad, 1987). Kernels were individually soaked in 1 ml sterile PBS buffer (0.85 % NaCl, 0.03 M NaOH, 0.05 M KH_2PO_4; pH 7.0) for 2 h at 4 C, then transferred to a rotary shaker at 150 rpm at 22±2 C for an additional 30 min. The suspension was diluted ten-fold in PBS and 0.1 ml portions were spread on KBC plates amended with 75 μg/ml cycloheximide and 80 μg/ml cephalexin. Plates were incubated in

the dark at 28 C for 48 h. Fluorescent pseudomonad colonies were selected visually for further analysis based on their fluorescence under 366 nm UV light (Model UVL-21, BLAK-RAY, Ultra-Violet Products, Inc., San Gabriel, California). Putative *P. syringae* isolates were identified according to standard tests, including oxidase reaction (Kovacs, 1956), hypersensitivity (HR) on tobacco leaves (*Nicotiana tabacum* cv. *Xanthi*) (Klement, 1963), and arginine dihydrolase activity (Hildebrand et al., 1988). The *syringae* pathovar identification was based on trigonelline, L-lactate and quinate utilization as sole carbon source (Hildebrand et al., 1988). Additionally, 1994 isolates were identified using the Biolog system (BIOLOG, Inc. Hayware, CA) and 1995 isolates were identified by the Microbial Identification System (MIDI, Microbial ID Inc. Newark, Delaware). Strains were also tested for syringomycin-like toxin production by inhibition of the toxin-sensitive fungus *Geotrichum candidum* in a SRM-plate bioassay described by Gross et al. (1985). This test detects the production of syringomycin or its amino acid derivatives, syringostatin, syringotoxin (Gross, 1991) and pseudomycin (Harrison et al., 1991), but not syringopeptin (Iacobellis et al., 1992; Vassilev et al., 1996).

Pathogenicity tests on barley.

Pathogenicity of isolates was examined by inoculating a bacterial suspension (1 x 10^7 cfu/ml sterile tapwater) containing 0.025% of Tween 20 as surfactant on spikes of the susceptible barley cultivar B 2601 at soft dough stage. Inoculum was applied until runoff (approx. 5 ml per spike) by using a hand air brush sprayer (Model Paaschi D500 1/10 H.P.). A pot (19 cm diameter) with three 3 barley heads (one spike per plant) at the appropriate

stage was inoculated per strain and pots were replicated. Pots were incubated in a mist chamber (approx. 95% r.h.) for 3 days following inoculation before being transferred to the greenhouse bench. The greenhouse was maintained at a temperature of 22/18 ± 2 C (day/night) and a 12 h photoperiod. After harvest, all kernels of one replicate (pot) were combined, and the percentage of kernels with basal blight infection was calculated by the following formula (Martinez-Miller et al., 1998):

$$\% \text{ kernel blight} = (a/b) \times 100,$$

where a = number of blighted kernels per sample; b = total number of kernels per sample.

Eleven *P. s.* pv. *syringae* reference strains and five other cereal pathovars, including *P. s.* pv. *coronafaciens*, *P. s.* pv. *atrofaciens*, and *P. s.* pv. *striafaciens* from several bacterial collections were included in the study for comparison. Their sources and test reactions are listed in Table 3.3. All bacteria were stored at -70 C in nutrient broth with 15 % glycerol and recovered on King's B medium (King et al., 1954) prior to analysis.

Nutritional profiles.

P. s. pv. *syringae* isolates obtained in 1994 and reference strains Pss 552, Ps 418, Pss 529, Psc 564, Psa 570, 579, and Psst 565 (Table 3.3) were subjected to metabolic fingerprinting on GN MicroPlates (Biolog, Inc., Hayward, CA). Briefly, bacteria were grown on Tryptic Soy Agar (TSA) for 24 hours and a bacterial suspension (OD_{A580} = 0.35, Spectronic 20, Milton Roy Company) prepared in sterile saline solution (0.85% NaCl). All

wells of a GN MicroPlate were filled with 150 μl of this solution, plates covered and incubated at 28 C. Since all strains required overnight incubation to give an adequate nutritional profile, the overnight reading was recorded, and the MicroLog 1 computer software program (Biolog's MicroLog™ QC Software, Release 3.5) was used to evaluate visual readings. Nutritional diversity of strains was determined by cluster analysis from a 0/1 data matrix, based on the utilization or non-utilization of distinctive carbon and nitrogen sources.

Genetic profiles.

The intrapathovar variation of *P. s.* pv. *syringae* field isolates was determined by comparing genomic DNA-profiles of strains isolated from kernels of different cultivars randomly distributed in the field. Twenty-four strains from 8 cultivars were investigated in 1994 and 33 from 6 cultivars in 1995. Cultivar source and relevant information on bacterial isolates are listed in Tables 3.1 and 3.2, respectively. DNA preparation of bacterial cells for pulsed-field gel electrophoresis (PFGE) was done according to Megeed & Sherwood (1995). Agar plugs containing lysed bacterial cells were digested with 40 units of the restriction endonuclease *XbaI* [TCTAGA] or 10 units of *SpeI* [ACTAGT], respectively and stored in 0.5 M Na-EDTA (pH 8.0) at 4 C. PFGE was carried out in a 1% agarose gel using the BIO-RAD CHEF DR-II system. Lambda DNA concatomers (BIO-RAD Laboratories) were used as molecular size markers. A voltage of 200 V was applied for a running time of 22 h for XbaI and 23 h for SpeI in 0.5x TBE buffer at 18 C. Optimal separation of DNA fragments between 100-300 kb was achieved by ramping the switch time from $\tau = 8$ to $\tau = 20$ s during

the running time. DNA was visualized by staining the gel with ethidium bromide and subsequent UV transillumination.

Analysis of DNA fingerprints and estimation of phylogeny.

The size of restriction fragments was determined by comparison with lambda DNA size standards. For each strain binary data, coding 1 for the presence or 0 for the absence of a band, were scored at each position along a lane in the DNA-fragment size range of 100-300 kb. A total of maximal 17 band positions were obtained for *XbaI* and 18 band positions for *SpeI* haplotypes. The combined restriction enzyme digest data for the field isolates represented a total of 35 band positions. Dice similarity coefficients were calculated for each strain pair by the following equation:

$$D = 2n_{xy}/(n_x + n_y),$$

where n_{xy} is the number of fragments shared by the two strains and n_x and n_y are the numbers of fragments in strain x and y, respectively. Cluster analysis was conducted and dendrograms constructed by the unweighted pair group method with averages (UPGMA) using the NTSYSpc-program (version 2.01c; Applied Biostatistics Inc.). For the computation of dependencies between two parameters, simple correlations/regressions analyses were performed using the MSTATc program (Michigan State University), and the degree of the relationship was described as correlation/regression coefficient (r) at the probability (p) (Köhler et al., 1992).

Results

No spot blight symptoms were detected on kernels in 1994 field experiments, possibly due to hot and dry weather conditions during the infection window (Martinez-Miller et al., 1998). Therefore, all 1994 isolates came from kernels exhibiting basal blight symptoms (Table 3.1). Three isolates were obtained from the susceptible to moderately susceptible cultivars B 2601, Morex, Excel, and Robust and two isolates from the moderately resistant cultivars B 2912, B 1202, and Mn 567. Four isolates from the resistant cultivar Chevron and two isolates from Canada thistle (*Cirsium arvense*) leaves were included for comparative studies on the presence of potential pathogenic Pss strains on healthy plants and non-host plants (Table 3.1). In 1995, a year with average rainfall and extended overhead irrigation (Martinez-Miller et al., 1998), basal as well as spot blight symptoms occurred on barley kernels. Four isolates from kernels exhibiting basal symptoms were collected from cultivar B 2601, B 5133, Karl, and three from Steptoe (Table 3.2). Five isolates from spot blight symptoms were isolated from cultivar Steptoe, four from B 5133, three from Karl and two from B 1202. Four isolates from symptomless kernels of the resistant cultivar Harrington were included for comparative purposes.

Pathovar identification.

All isolates tested, except for reference strain *P. s.* pv. *striafaciens* 565, grew on quinate as the sole carbon source (Table 3.3). Isolates from kernels exhibiting basal blight symptoms (Table 3.1, 3.2) grew on trigonelline and L-lactate as sole carbon sources and

were consequently identified as pathovar *syringae* (Hildebrand et al., 1988). Isolate 779 from leaf blight symptoms and all isolates from healthy barley kernels also tested positive on these two carbon sources and were identified as *P. s.* pv. *syringae*. The isolates from spot blight symptoms were more variable in their growth on these minimal media. Fifty percent of isolates grew on trigonelline and L-lactate and were subsequently identified as pathovar *syringae*. Thirty-six percent did not utilize these two carbon sources as sole carbon source and 14 % grew on L-lactate but not trigonelline (Table 3.2) and could therefore not clearly be identified on the pathovar level by this method. Commercially available identification systems, such as Biolog and MIDI identified all isolates as *syringae* on the species level, but were inconsistent on the pathovar level. Forty-six percent of 1994 isolates (Table 3.1) were identified by the Biolog system as pathovar *syringae* ($p = 0.54$-0.87), 17% as pathovar *aptata* ($p = 0.71$-0.85), 13% as pathovar *pisi* ($p = 0.68$-0.72) and pathovar *hibisci* ($p = 0.62$-0.78), respectively, 8% as pathovar *morsprunorum* ($p = 0.60$-0.79), and 4% as pathovar *atrofaciens* ($p = 0.91$). Fifty-six percent of isolates tested in 1995 were identified as pathovar *pisi* ($p =$ 0.5-0.69) by cellular fatty acid analysis (MIDI), including all isolates from basal blight symptoms. Nineteen percent of isolates were identified as pathovar *delafieldii* ($p = 0.67$-0.73), 13% as pathovar *lachrymans* (0.59-0.68), and 6% as pathovar *maculicola* ($p = 0.57$) and species *pseudoflava* ($p = 0.35$), respectively (Table 3.2).

Table 3.1. Name and source of 1994 *Pseudomonas syringae* pv. *syringae* isolates, symptom type at isolation, commercial identification based on Biolog, trigonelline (TRI) and L-lactate (LA) utilization, hypersensitivity reaction (HR) on tobacco, syringomycin-like toxin production measured in a *Geotrichum candidum* inhibition bioassay, and pathogenicity on barley cv. B 2601 expressed as percentage of basal blight infected kernels, when inoculated at soft dough stage.

Name	Source/ cultivar	Symptom	Biolog	TRI[1)]	LA[1)]	HR[1)]	Zone size [2)] (mm)	% Basal blight [3)]
789	2601	basal	*syringae*	+	+	+	2	3.8
793	2601	basal	*pisi*	+	+	+	8	44.6
791	2601	basal	*pisi*	+	+	+	10	63.7
1254	2912	basal	*morsprun.*	+	+	+	2	--
1213	2912	basal	*syringae*	+	+	+	10	68.4
1214	1202	basal	*syringae*	+	+	+	8	30.4
1224	1202	basal	*hibisci*	+	+	+	8	--
1259	Morex	basal	*syringae*	+	+	+	2	--
1221	Morex	basal	*aptata*	+	+	+	6	--
1252	Morex	basal	*syringae*	+	+	+	4	--
1249	Excel	basal	*syringae*	+	+	+	2	--
1251	Excel	basal	*morsprun.*	+	+	+	8	--
1262	Excel	basal	*syringae*	+	+	+	2	--
1217	Mn567	basal	*syringae*	+	+	+	0	31.2
1261	Mn567	basal	*hibisci*	+	+	+	8	--
1218	Robust	basal	*syringae*	+	+	+	10	61.0
1219	Robust	basal	*hibisci*	+	+	+	2	--
1222	Robust	basal	*aptata*	+	+	+	0	--
779	Chevron	leaf blight	*atrofaciens*	+	+	-	0	12.9
1212	Chevron	no sympt.	*aptata*	+	+	+	4	33.9
1247	Chevron	no sympt.	*syringae*	+	+	+	8	--
1250	Chevron	no sympt.	*syringae*	+	+	+	4	--
780	Thistle	no sympt.	*pisi*	+	+	+	0	18.4
781	Thistle	no sympt.	*aptata*	+	+	+	0	13.5

[1)] + indicates growth on trigonelline (TRI) or L-lactate (LA) as sole carbon source and a positive hypersensitivity reaction (HR) on tobacco. - indicates a negative HR reaction.
[2)] Values are means of 4 replications per Pss strain, and represent millimeters of *Geotrichum candidum* inhibition around 5 day old colonies on SRM medium (Gross, 1985).
[3)] Values represent mean percentages of basal blight infected kernels obtained from 6 replicate barley spikes inoculated at soft dough stage; -- indicates not determined.

Table 3.2. Name and source of 1995 *Pseudomonas syringae* pv. *syringae* isolates, symptom type at isolation, commercial identification based on MIDI, trigonelline (TRI) and L-lactate (LA) utilization, hypersensitivity reaction (HR) on tobacco, syringomycin-like toxin production measured in a *Geotrichum candidum* inhibition bioassay, and pathogenicity on barley cv. B 2601 expressed as percentage of basal blight infected kernels, when inoculated at soft dough stage.

Name	Source/ cultivar	Symptom	MIDI	TRI [1)]	LA [1)]	HR [1)]	Zone size [2)] (mm)	% Basal blight [3)]
852	2601	basal	*pisi*	+	+	+	10	18.9
853	2601	basal	--	+	+	+	10	14.8
854	2601	basal	--	+	+	+	10	7.6
849	2601	basal	*pisi*	+	+	+	10	32.8
860	5133	basal	*pisi*	+	+	+	10	11.9
861	5133	basal	--	+	+	+	10	9.9
862	5133	basal	--	+	+	+	10	25.4
864	5133	basal	--	+	+	+	4	42.0
874	5133	spot	--	-	+	+	0	2.7
875	5133	spot	--	+	+	+	0	10.8
876	5133	spot	*lachrymans*	+	+	-	0	0.0
877	5133	spot	--	+	+	-	0	0.0
829	Steptoe	basal	*pisi*	+	+	+	4	21.0
831	Steptoe	basal	--	+	+	+	6	13.4
832	Steptoe	basal	--	+	+	+	6	19.5
822	Steptoe	spot	*delafieldii*	+	+	-	0	0.0
823	Steptoe	spot	*maculicola*	+	+	-	0	0.0
825	Steptoe	spot	*pisi*	+	+	-	0	0.0
826	Steptoe	spot	--	-	-	+	14	13.7
827	Steptoe	spot	*lachrymans*	-	-	+	14	32.4
836	Karl	basal	--	+	+	+	8	4.4
837	Karl	basal	--	+	+	+	8	4.2
839	Karl	basal	--	+	+	+	8	3.9
843	Karl	basal	--	+	+	+	6	11.9
833	Karl	spot	*pisi*	-	-	+	10	22.0
834	Karl	spot	*delafieldii*	+	+	+	6	0.0
835	Karl	spot	*delafieldii*	-	-	+	0	0.0
881	1202	spot	--	-	+	-	0	40.7
882	1202	spot	--	-	-	-	0	14.1
884	Harrington	no sympt.	*pisi*	+	+	-	0	33.1
885	Harrington	no sympt.	*P.pseudoflava*	+	+	-	0	6.4
886	Harrington	no sympt.	*pisi*	+	+	+	0	54.1
887	Harrington	no sympt.	*pisi*	+	+	+	0	17.6

[1)] + indicates growth on trigonelline (TRI) or L-lactate (LA) as sole carbon source and a positive hypersensitivity reaction (HR) on tobacco. - indicates a negative HR reaction.

[2)] Values are means of 4 replications per Pss strain, and represent millimeters of *Geotrichum candidum* inhibition around 5 day old colonies on SRM medium (Gross, 1985).

[3)] Values represent mean percentages of basal blight infected kernels obtained from 6 replicate barley spikes inoculated at soft dough stage; -- indicates not determined.

Table 3.3. Name and source of *Pseudomonas syringae* reference strains, symptom type at isolation, commercial identification based on Biolog and/or MIDI, trigonelline (TRI) and L-lactate (LA) utilization, hypersensitivity reaction (HR) on tobacco, syringomycin-like toxin production measured in a *Geotrichum candidum* inhibition bioassay, and pathogenicity on barley cv. B 2601 expressed as percentage of basal blight infected kernels, when inoculated at soft dough stage.

Name	Cultivar/source	Symptom	Biolog	MIDI	TRI [1)]	LA [1)]	HR [1)]	Zone size [2)] (mm)	% Basal blight [3)]
Pss 552	B 2601, MT, D.C. Sands	basal	*syringae*	*pisi*	+	+	+	10	62.2
Pss 537	B 2601, MT, D.C. Sands	basal	--	--	+	+	+	10	--
Pss 541	B 5133, MT, D.C. Sands	basal	--	*glycinea*	+	+	+	10	--
Pss 542	B 5133, MT, D.C. Sands	basal	--	*pisi*	+	+	+	10	--
Ps 418	B 1202, MT, D.C. Sands	spot	*pisi*	*coronafaciens*	-	+	+	0	15.4
Ps 421	B 1202, MT, D.C. Sands	spot	--	*coronafaciens*	-	+	+	0	--
Ps 428	B 1202, MT, D.C. Sands	spot	--	--	-	+	+	0	--
Ps 492	B 1202, MT, D.C. Sands	spot	--	--	-	+	+	0	--
Pss 529	SD202, wheat, SD, D.C. Gross	leaf blight	*aptata*	--	+	+	+	10	12.9
Pss 530	W4N9, bingcherry, OR, D.C. Gross	canker	--	--	+	+	+	10	15.4
Pss 531	W4N53, d'Anjou pear, OR, D.C. Gross	canker	—	--	+	+	+	0	0.0
Psa 570	pv. *atrofaciens*, barley, 30/1742, K. Rudolph, F.R.G.	glume rot	*syringae*	--	+	+	+	6	20.2
Psa 579	pv. *atrofaciens*, wheat, CB168, E. Duveiller,MX	glume rot	*tabaci*	--	+	+	+	4	8.8
Psc 564	pv. *coronafaciens*, oats, Pc27, K. Willis, WI	halo blight	*pisi*	--	-	+	+	4	0.0
Psc 566	pv. *coronafaciens*, oats, K. Willis, WI	halo blight	--	--	-	+	+	--	--
Psst 565	pv. *striafaciens*, barley, 1515, K. Willis, WI	stripe blight	*tabaci*		-	+	+	4	1.8

[1)]+ indicates growth on trigonelline (TRI) or L-lactate (LA) as sole carbon source and a positive hypersensitivity reaction (HR) on tobacco. - indicates a negative HR reaction. [2)]Values are means of 4 replications per Pss strain, and represent millimeters of *Geotrichum candidum* inhibition around 5 day old colonies on SRM medium (Gross, 1985). [3)] Values represent mean percentages of basal blight infected kernels obtained from 6 replicate barley spikes inoculated at soft dough stage; -- indicates not determined.

In addition to symptom type at isolation, *P. s.* pv. *syringae* strains were examined for syringomycin-like toxin production and pathogenicity based on the hypersensitivity reaction (HR) on tobacco and percentage of kernel infection on the susceptible cultivar B 2601, inoculated at soft dough stage of kernel development. Ten of 57 strains (=18%) were HR-negative and 82% HR- positive. Of the HR-negative strains none produced syringomycin or its amino acid derivatives *in vitro* (Table 3.1, 3.2), indicating a positive correlation between toxin production and pathogenicity on tobacco. However, five of these HR-negative, toxin-negative strains caused between 6 to 41% basal blight infection on cv. B 2601, while the other five were non-pathogenic to this cultivar, suggesting factors other than toxin production and pathogenicity on tobacco are involved in pathogenicity on barley cultivar B 2601. Seven out of 10 (70%) HR-negative strains were isolated from spot blight symptoms, one from leaf blight symptoms and two from symptomless kernels, while the other seven isolates from spot blight symptoms were HR-positive. Out of the HR-positive strains, 81% (9/47) demonstrated varying degrees of toxin production (2-14 mm) and were pathogenic to B 2601 with varying infection levels. Nevertheless, 19% of HR-positive strains did not produce any visible amounts of syringomycin-like toxins *in vitro*, but were still pathogenic to B 2601 (Tables 3.1, 3.2). It was apparent, however, that all isolates from basal blight symptoms (32) were HR-positive, of which only 2 (6.3%) were toxin-negative and all of the tested basal isolates (22) were pathogenic to B 2601, indicating a positive correlation of HR-reaction, toxin production and pathogenicity to B 2601 with isolates of basal type symptoms. While there was an obvious overall correlation between toxin production *in vitro* and pathogenicity on tobacco, I detected only a weak correlation between toxin production and

pathogenicity on B 2601, since 67% of the toxin-negative strains were pathogenic on this cultivar and only 33% were non pathogenic, indicating again that factors other than toxin production must be involved in symptom appearance. In contrast, 96% (25/26) of the tested toxin producers were pathogenic to B 2601 with varying degrees of infection, whereas only one toxin-positive (HR-positive) strain was non-pathogenic to this cultivar. Over all levels of toxin production, I could demonstrate a weak, significant positive relationship ® = 0.31, p = 0.046) between syringomycin-like toxin production and infection level on cultivar B 2601 (Fig. 3.1).

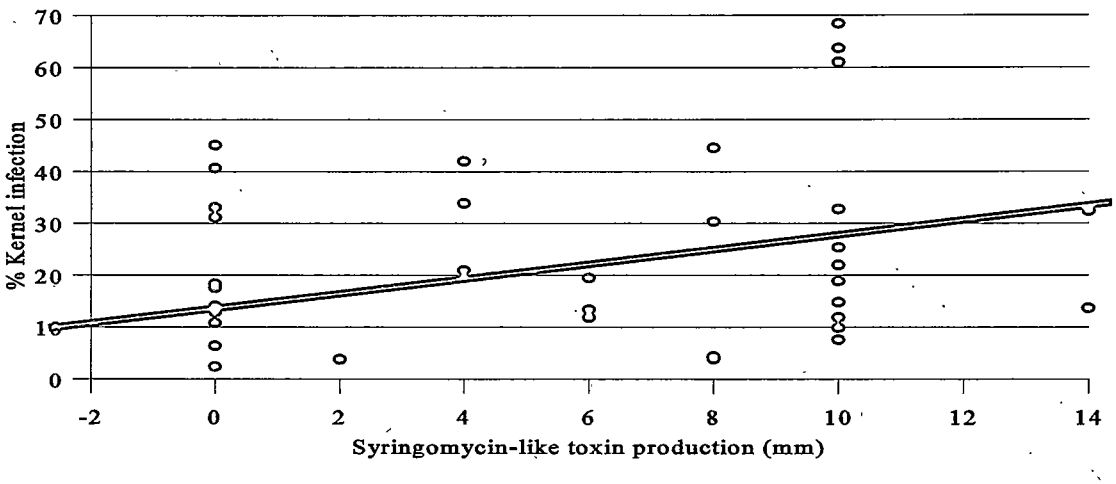

Fig.3.1. Linear regression of amount of syringomycin-like toxin production (X) versus percentage of basal kernel blight infection (Y) obtained on barley cv. B 2601, when heads were inoculated at soft dough stage

Nutritional diversity.

The nutritional diversity of 23 Pss strains isolated from barley kernels, in addition to reference strains Pss 552, Pss 529, Ps 418, Psa 570, Psa 579, Psstria 565, and Psc 564 was determined by metabolic fingerprinting after oxidation of 95 different carbon and nitrogen sources using Biolog GN plates. The phenotypic dendrogram (Fig. 3.2) created after cluster analysis demonstrated a 76-100% overall nutritional similarity among strains based on the *in vitro* nutritional overlap. However, reference strains Pss 552 and Psa 570 from barley of different geographical origins were nutritionally 100% identical. Strains 1249 and 1262, both from cultivar Excel, were also identical, and another identical pair was 1247 and 1217, one from Chevron, the other from Mn567. I detected a grouping of strains into 3 major clusters. Strains within group I revealed 92% similarity and strains within groups II and III 90% nutritional similarity. The similarity between groups was more diverse (approx. 87%). Group I included mainly isolates of cultivars B 2601, Morex, and B 2912, group II isolates of Chevron, Excel, Robust, Mn567, B 1202, and group III the strains Ps 418 from spot type symptoms, Psa 579 from wheat and Psstria 565 from Wisconsin barley. Strains 1251 from Excel, Psc 564 from oats, and strain 780 from thistle were outliers and presented only 76-86% similarity to the other groups of strains. Isolates from more susceptible cultivars in group I demonstrated an overall higher pathogenicity on B 2601 (52.9%) than isolates from moderate to resistant cultivars in group II (36.5%) or isolates from spot blight symptoms or pvs. *atrofaciens* and *striafaciens* in group III (8.6%), based on averages of B 2601 infection from the cultivars tested.

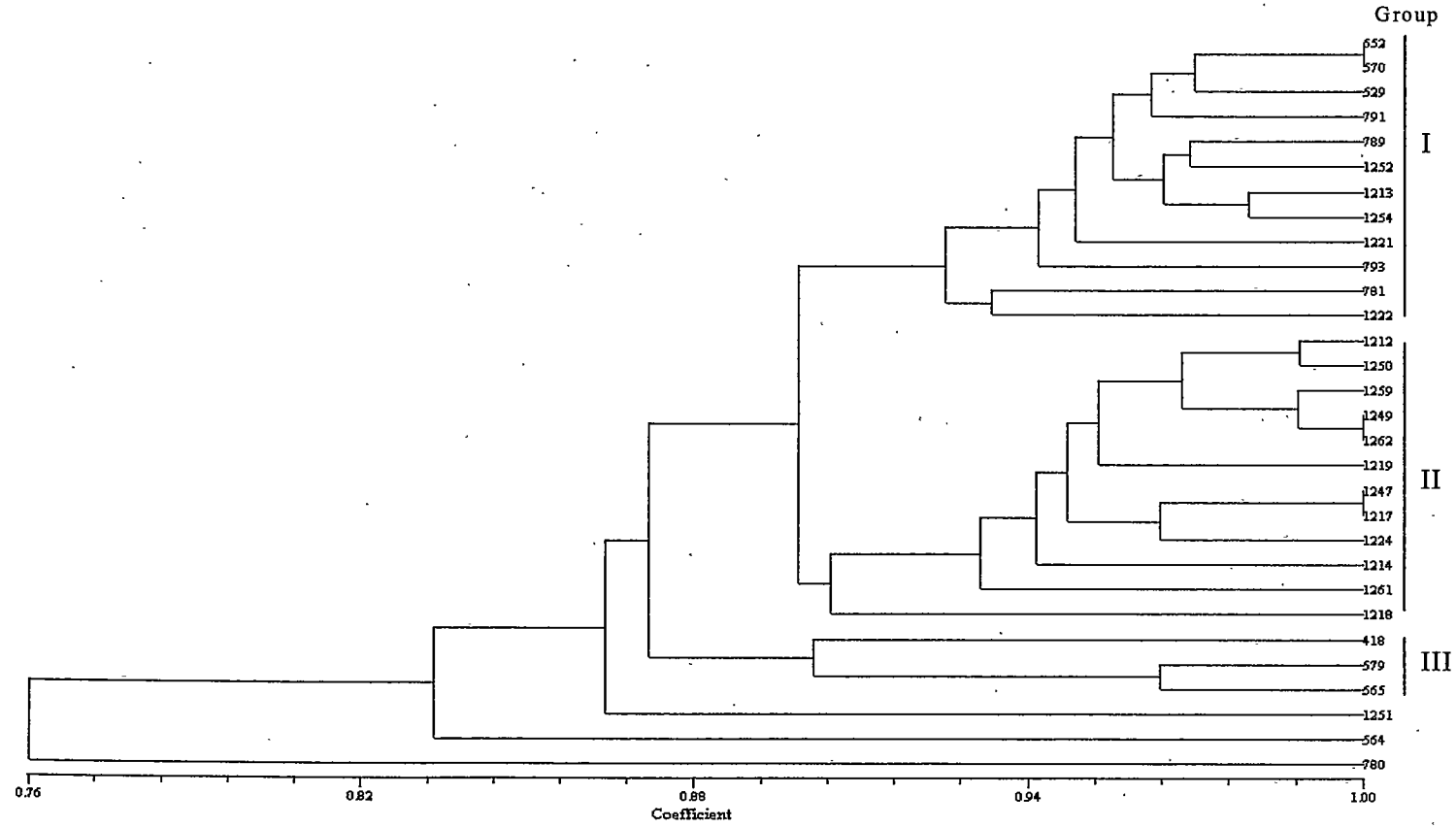

Fig.3.2. Phenotypic dendrogram based on 95 nutritional properties of 23 Pss strains isolated from kernels of different barley cultivars in 1994. Two strains (780, 781) isolated from Canada thistles (*Cirsium arvense*), one isolate from wheat (529), and 4 isolates from pathovars *atrofaciens* (570, 579), *striafaciens* (565), and *coronafaciens* (564) were included for comparison. See Table 3.1 for strain source and characteristics. The clusterlevel is based on Dice similarity coefficients and three major clusters (I - III) of strains were obtained, which did not correlate with either cultivar of isolation, symptom type at isolation, amount of toxin production, or HR reaction on tobacco.

Genetic analysis.

To determine the intrapathovar diversity of DNA fingerprints, I examined 24 *P. s.* pv. *syringae* isolates from 8 cultivars of the same geographic area in 1994 and 33 isolates from 6 cultivars in the same area in 1995 (Table 3.1, 3.2). Genomic fingerprinting resulted in, at most, 17 DNA fragments in the range of 100-300 kb after digestion with *XbaI* and 18 DNA fragments after digestion with *SpeI*. *P. syringae* pathovars isolated from different hosts and geographical origin revealed differences in their banding pattern (Fig. 3.3). Strains within the pv. *syringae* were still diverse, with a 40-80% similarity between most strains or group of strains (Fig. 3.3, 3.4). However, a higher similarity (86-100%) was apparent in 48% of the strains isolated from spatially separated kernels of the same cultivar (Fig. 3.4). The dendrogram (Fig. 3.4) demonstrated 100% similarity between strains 881 and 882 from spot blighted kernels of cultivar B 1202, between strains 874 and 875 from spot blighted kernels of cultivar B 5133, and between strains 823 and 825 from spot blighted kernels of cultivar Steptoe. Furthermore, strains 884 and 885 from symptomless kernels of cultivar Harrington, strains 836 and 837 from basal blighted kernels of cultivar Karl, strains 860,861, and 862 from basal blighted kernels of cultivar B 5133 and strains 849,852, and 853 from basal blight symptoms of cultivar B 2601 demonstrated 100% similarity in their genomic profile. Moreover, strains 886 and 887 from symptomless kernels of cultivar Harrington revealed 96% similarity, strains 831 and 832 from basal blighted kernels of cultivar Steptoe 91% and strains 822, 823, and 825 from spot blighted kernels of cultivar Steptoe 86% similarity. While these clusters of similarity were striking, no obvious grouping of the other strains in relation to their cultivar of origin, symptom type at isolation or pathogenicity on cultivar B 2601 was observed. The

dendrogram demonstrated an overall heterogeneous genomic population of *P. s.* pv. *syringae* strains isolated from barley kernels of different cultivars within the same geographical area.

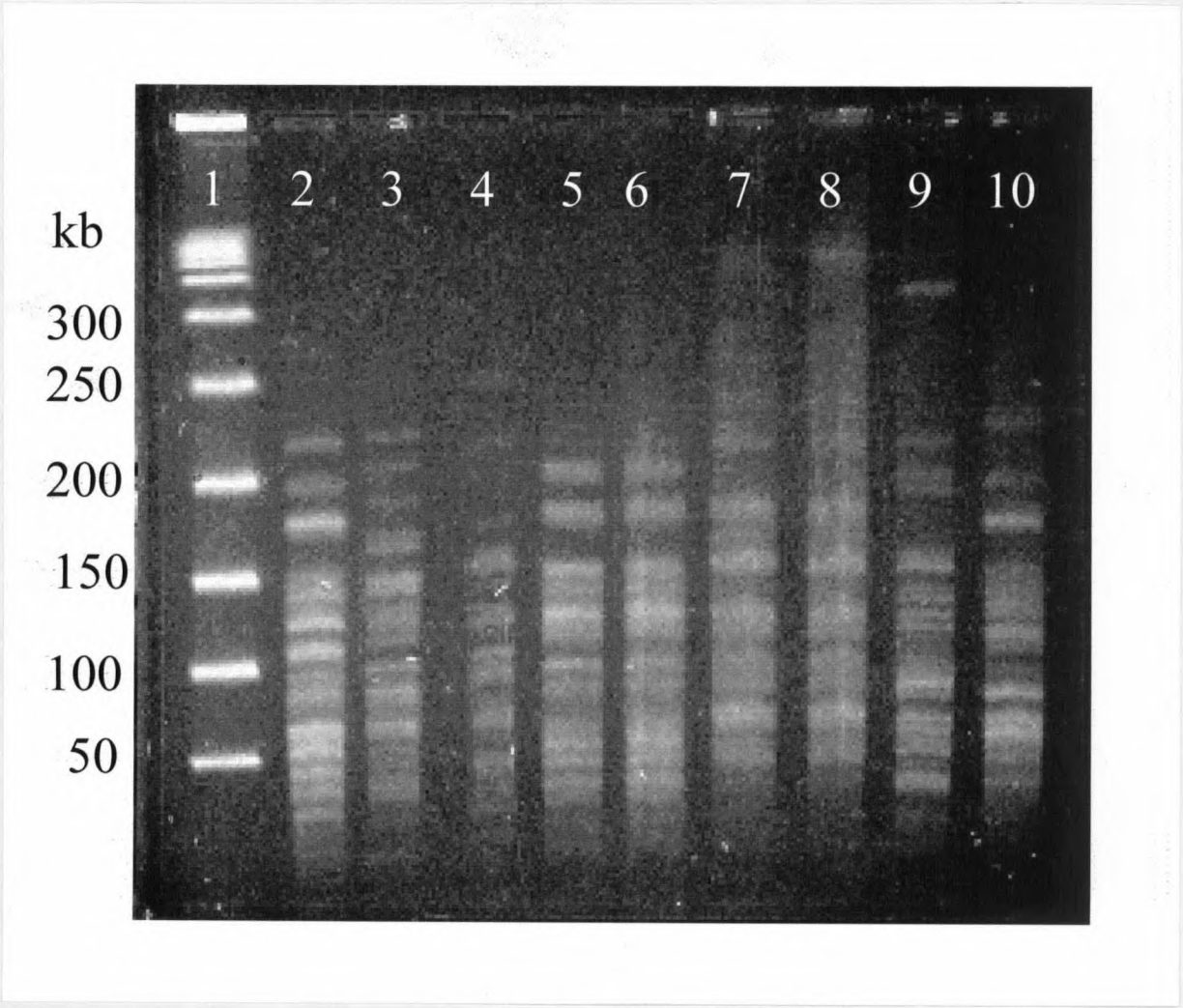

Fig.3.3. Genomic DNA-fingerprints of *P. syringae* isolates. DNA embedded in agarose was digested with *XbaI* and fragments separated by pulsed field gel electrophoresis for 22 h with ramping from 8-20 s at 200 V. Lane 1= λ DNA size markers, lane 2 = 529 (Pss from wheat, SD), lane 3 = 530 (Pss from cherry, OR), lane 4 = 531 (Pss from pear, OR), lane 5 = 552 (Pss from B 2601, MT), lane 6 = 537 (Pss from B 2601, MT), lane 7 = 541 (Pss from B 5133, MT), lane 8 = 542 (Pss 542 from B 5133, MT), lane 9 =579 (*P.s. atrofaciens* from wheat, MX), lane 10 = 570 (*P.s. atrofaciens* from barley, F.R.G.).

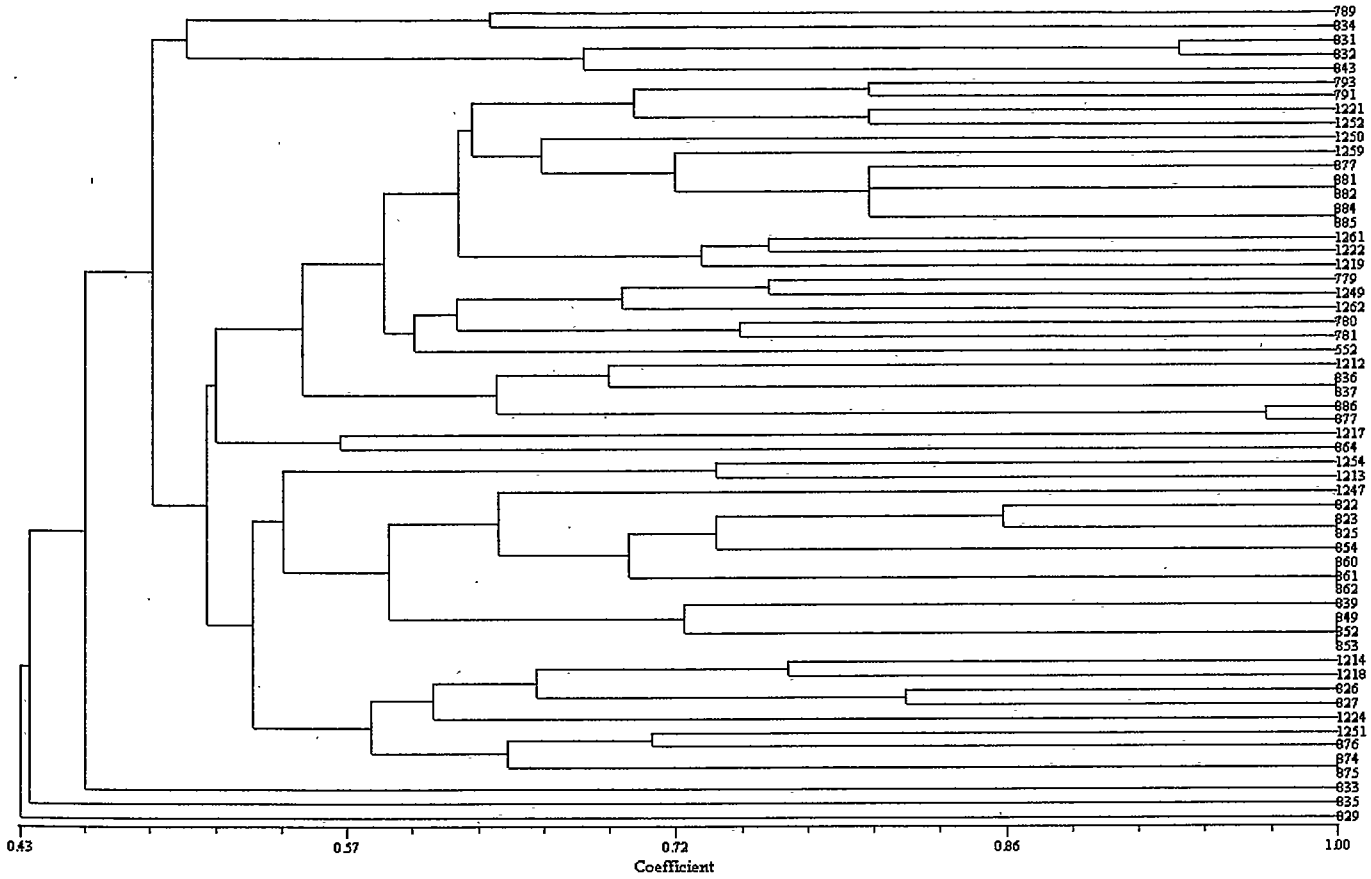

Fig. 3.4. Dendrogram of genetic relationships between 56 *P. s.* pv. *syringae* strains isolated from kernels of twelve barley cultivars in 1994 and 1995, and 2 strains from Canada thistle (*Cirsium arvense*). See Tables 3.1 & 3.2 for source and characteristics of strains. The dendrogram was obtained from cluster analysis of macrorestriction DNA-fingerprints by using UPGMA and Dice similarity coefficients. Each strain of Pss was digested with the restriction enzymes *XbaI* and *SpeI* and run on a pulsed-field gel at 200 V for 24 h and 22 h, respectively.

Correlation analyses.

The relationship between nutritional similarity and genetic similarity based on the Dice coefficients was performed on the data obtained for 1994 isolates. Despite the overall heterogeneity of Pss populations obtained from both analyses, there was only a low but significant correlation between nutritional and genetic data ($r = -0.19$; $p = 0.039$), possibly because strains responded differently to both analyses (Fig. 3.2; 3.4). However, the overall similarity of Pss strains obtained from the nutritional profiles (average similarity = 0.89) was greater than the similarity of Pss strains derived from the genetic analysis (average = 0.56).

Discussion

The Biolog system proved useful to identify bacterial isolates on the genus and species level but was inconsistent on the subspecies or pathovar level. Despite this disadvantage it provided valuable information on the nutritional characteristics of a particular strain, which might separate it from others. The MIDI system also was advantageous on the genus and species level, but had demonstrated inconsistencies on the pathovar level. Similar insensitivities using only biochemical and pathogenicity tests have been reported by others, who therefore recommended the use of more rapid and less expensive techniques to identify and classify closely related bacteria on the pathovar and strain level (Denny, 1988; Legard et al., 1993; Louws et al., 1994).

The nutritional diversity of 1994 strains (76-100% similarity) based on *in vitro* nutritional profiles (Fig. 3.2) confirmed the overall genetic heterogeneity (43-100% similarity) among Pss

populations within this pathovar (Fig. 3.4). However, there was no strong correlation between nutritional and genetic similarity (r = -0.19; p = 0.039), since strains, which were nutritionally similar (>90%) were only 69-77% similar based on their DNA fragment patterns. The discrepancy between diversity detected by carbohydrate utilization tests and genome analyses was also reported by Denny (1988) and Denny et al. (1988). For instance, despite their genetic diversity (Fig. 3.3; 3.4), no obvious phenotypic differences were detected between Pss 552 isolated from kernels of cv. B 2601 in MT and Psa 570 isolated from barley kernels in Germany. Both strains were pathogenic to cv. B 2601 and both demonstrated *in vitro* toxin production (10 mm versus 6 mm, respectively). Syringomycin toxin production by *P. s.* pv. *atrofaciens* strains 570 and 579 was supported by Southern analysis of *PstI*-cut genomic DNA and hybridization to the 1.1 kb *syrB* gene fragment (Braun & Sands, 1995). Interestingly, strains 564, 566, and 565 from pvs. *coronafaciens* and *striafaciens*, which also demonstrated syringomycin-like toxin production were non-pathogenic to B 2601 and did not hybridize to the *syrB* fragment (Braun & Sands, 1995). The production of syringomycins and syringopeptins by *P. s.* pv. *atrofaciens* was demonstrated recently by Vassilev et al. (1996). Moreover, the fungus *Geotrichum candidum* used in my bioassay, although not sensitive to syringopeptins (Iacobellis et al., 1992; Vassilev et al., 1996), might still be sensitive to toxins such as tabtoxin (= monocyclic β-lactam), reported to be produced by several *P. syringae* pathovars, including *P. s.* pv. *coronafaciens* (Gross, 1991), which could explain the zones of inhibition, at least in pv. *coronafaciens*.

Furthermore, isolates from thistle which were phenotypically and genotypically distinct and did not produce visible syringomycin levels *in vitro* were pathogenic to cv. B 2601, indicating that

nutritional versatility probably enabled Pss populations to multiply on hosts other than barley until favorable conditions induced the barley infection. Similar results were obtained by Roos & Hattingh (1987), who investigated the pathogenicity and phenotypic features of *P. syringae* strains isolated from fruit trees in South Africa. Most strains within the pv. *syringae* were heterogeneous due to nutritional versatility, origin of isolation, and pathogenicity to several pome and stone fruit hosts. The authors found that survival of Pss on weeds during the winter and its nutritional diversity favored its multiplication on many hosts, complicating epidemiological studies and the development of disease control strategies. According to the authors, each host fruit variety supported a heterogeneous population of Pss and some of these strains were more virulent on other host trees. Symptoms were expressed only if conditions promoted the particular strain, since strains colonized plum tissue without causing visible symptoms, whereas on apple, pear, and cherry cankers were developed. Similar interactions between the Pss pathogen and host plants seem to be present in my barley system, since most isolates caused typical disease symptoms on B 2601 with differences in severity, probably reflecting the virulence of individual strains. Strains pathogenic to B 2601 were also isolated from symptomless kernels of cultivars Chevron and Harrington, indicating again the ubiquitous existence of the pathogen, even on more resistant cultivars.

Three groups of clusters were found among 1994 Pss strains based on nutritional profiles but this clustering did not correspond to the grouping based on toxin production, confirming earlier RFLP studies, which revealed no apparent clustering of strains in relation to syringomycin production when *syrB*, *hrp10kb*, *hrp14kb* were used as DNA probes (Martinez-Miller, 1994). However, I could demonstrate a significant but weak correlation ($r = 0.31$, $p = 0.046$) between

amount of syringomycin production and the percentage of kernel infection on cv. B 2601, probably reflecting the importance of syringomycin as virulence factor in Pss populations, hither to mentioned by Xu and Gross (1988) and Gross (1991).

Macrorestriction analysis estimated the genetic relatedness of *P. syringae* isolates within the pv. *syringae* from the distribution of infrequent oligonucleotide sequences in the bacterial chromosome (Fig. 3.4), which should be similar in closely related strains (Corich et al., 1991). The intrapathovar variation of 57 isolates used in my study could be roughly divided into two groups, isolates that had identical genomic DNA fingerprints and shared multiple bands of equal mobility (48% of 1995 strains), and isolates that had unique profiles (72% of all strains) and thus could be divided into separate lines that did not share common banding patterns. A high similarity of fragment profiles (100%), which may define a lineage of evolutionary related strains (Louws, 1994; Grotheus & Tümmler, 1991), was observed for strains isolated from spatially separated kernels of the same barley cultivar distributed randomly in the field screening experiment in 1995. Grotheus & Tümmler (1991) observed a high relatedness of DNA fragment patterns for *P. aeruginosa* isolates from individual cystic fibrosis patients and *P. syringae* strains from the pvs. *glycinea* and *phaseolicola*, confirming the definition of a pathovar as an infrasubspecific rank. Furthermore, some isolates were demonstrated to have originated from the same source, because 6 identical strains of *P. s.* pv. *phaseolicola* were isolated from the same host plant.

In my studies, Pss strains with identical DNA fingerprints (Fig. 3.4) could have originated from the same source (single evolutionary line, common ancestry), i. e. barley seeds, which emphasizes the assumption of seed transmittance of the pathogen. In addition, the strains displayed

similar virulence patterns, since also toxin production and the HR reaction were identical, possibly reflecting their phenotypic adaptation to certain host cultivars. On the other hand, cultivar-specificity in Pss appeared unlikely, because most strains demonstrated pathogenicity on the susceptible cultivar B 2601, even when they were isolated from cultivars other than B 2601 or from thistles surrounding the barley field (Tab. 3.1;3.2). Most variability among barley-infecting strains of Pss was due to differences in levels of virulence. In fact, barley seeds for 1995 field experiments only came from two nurseries, one in Fort Collins, CO and one in Bozeman, MT, while seeds for 1994 field experiments came from various nurseries within the US, also supporting seed transmission. Similar results have been found by Denny et al. (1988), who speculated that, although Pss strains occupy a variety of habitats in the biosphere throughout the world with many lineages and a wide genetic diversity is expected to exist within this pathovar, genetic divergence is low in a population within a pathovar which is highly adapted to a specialized environment. Legard et al. (1993) obtained genetic data on the phylogeny and heterogeneity of lima bean *P. s.* pv. *syringae* strains which appeared very scattered, unlike bean strains, which were more similar, possibly due to seed transmission. The authors speculated that heterogeneous populations may have moved to or from other hosts multiple times and a single origin may not have occurred.

Alternatively, the overall existence of many heterogeneous genomic populations of pv. *syringae* strains isolated from barley kernels of different cultivars within the same geographical area, might indicate a high rate of mutational and/or recombinational events between bacterial cells in the same environment to occur in otherwise clonal populations. Horizontal gene transfer of genes involved in pathogenicity could have taken place among strains or pathovars, resulting in strains

with wide host ranges (Hendson et al., 1992). The horizontal exchange of *hrp*-gene sequences between pathovars of *P. syringae* has been demonstrated in laboratory experiments and seems likely considering its sequence conservation, structural homology, and functional complementation (Lindgren et al., 1986, 1997; Scholz et al., 1992; Alfano & Collmer, 1996).

However, according to Cohan (1996) bacterial evolution and diversity between populations seem to be determined by adaptive mutations and periodic (natural) selection rather than by genetic exchange, since recombination is rare (10^{-6} to 10^{-7} per gene segment per genome per generation) in nature for most groups of bacteria, and recombinants are usually less well adapted to either population's ecological niche, unless recombination occurs in functionally interchangeable alleles.

In my system, the heterogeneity of nutritional profiles and DNA-fingerprints might reflect the frequent divergence of Pss populations into different niches, representing many distinct ecological populations in a barley field composed of several cultivars. Furthermore, a stable coexistence of diverse populations may represent the three-dimensional nature of an ecological niche (resource, habitat, time) and its differential exploitation (Bianchi & Bianchi, 1995). Diverse populations might depend on each other for degradation of complex materials, hence physical or chemical stresses, such as starvation conditions, can increase bacterial diversity. Accordingly, any organism dying out can thus be replaced by a "hidden" organism functionally able to fill in for the lost organism. Although these biodiversity mechanisms seem to be important in ecosystem maintenance and adaptation and survival of microorganisms in continuously changing environments (Bianchi & Bianchi, 1995), plant pathologists need to be aware of these stability processes when thinking about effective and long-lasting disease management practices.

Finally, it should be noted that the comparison of macrorestriction fingerprints is confined to fragment size as its only quantifiable parameter. Fragment patterns per se do not provide conclusive information as to whether mutations in the restriction enzyme recognition sites or genomic insertions, deletions or rearrangements account for differences in restriction fragment size (Grotheus & Tümmler, 1991). The order of homology of DNA fragments has to be elucidated by supplementary experiments, such as cloning and sequencing or hybridization, since transposable elements can cause the above mentioned mutations in bacterial genomes with otherwise identical restriction site maps, leading to changes in fragment sizes and thus an overestimation of divergence (Denny et al., 1988; Legard et al., 1993).

It appears that the differentiation and relatedness among strains of a single heterogeneous pathovar such as *P. s.* pv. *syringae* requires more representative strains from a single cultivar and host range studies in addition to genomic profiles using macrorestriction fingerprints, RFLP analyses with selected DNA hybridization probes, repPCR or AFLP studies. The results of additional diversity studies, including mutational and recombinational events within and between marked populations, could finally lead to a better understanding of bacterial evolution, changes in virulence patterns of pathogen and improved disease control strategies for phytopathogenic bacteria.

Literature Cited

Alfano, J. R. and Collmer, A. 1996. Bacterial Pathogens in Plants: Life up against the wall. The Plant Cell 8 (10): 1683-1698.

Bianchi, A. and Bianchi, M. 1995. Bacterial diversity and ecosystem maintenance: an overview. In: Microbial Diversity and Ecosystem Function. (eds D. Allsopp, R. R. Colwell, and D. L. Hawksworth) CAB International, pp. 185-198.

Bradbury, J. F. 1986. Pseudomonas. In: Guide to plant pathogenic bacteria. CAB International Mycological Institute, Kew, United Kingdom. pp. 154-187.

Bragard, C., Singer, E., Alizadeh, A., Vauterin, L., Maraite, H., and Swings, J. 1997. *Xanthomonas translucens* from small grains: diversity and phytopathological relevance. Phytopathology 87: 1111-1117.

Braun, A. and Sands, D. C. 1995. Identification of two bacterial blights and their suppression by *Pantoea agglomerans*. Phytopathology 85 (10): 1163 (Abstract).

Braun, A., Rose, A., Kaliyeva, G.T., Martinez-Miller, C., and Sands, D. C. 1996. Biocontrol of bacterial kernel blight of barley by beneficial *Pantoea agglomerans*. Phytopathology 86 (11): S79 (Abstract).

Braun, A., Brumfield, S., Hamacher, J., Sands, D. C. 1997 a. Ultrastructural localization of *Pseudomonas syringae* in barley kernel tissue. Phytopathology. 87(6): S11 (Abstract).

Braun, A., Sands, D. C., and Jacobsen, B. J. 1997 b. Suppression of bacterial kernel blight of barley by antagonistic *Pantoea agglomerans*. Biol. Cult. Tests Control Plant Dis. 12: 105.

Cohan, F. M. 1996. The role of genetic exchange in bacterial evolution. ASM News. 62 (12): 631-636.

Cooksey, D. A. and Graham, J. H. 1989. Genomic fingerprinting of two pathovars of phytopathogenic bacteria by rare-cutting restriction enzymes and field inversion gel electrophoresis. Phytopathology 79: 745-750.

Corich, V., Giacomini, A., Ollero, F. J., Squartini, A., and Nuti, M. F. 1991. Pulsed-field electrophoresis in contour-clamped homogeneous electric fields (CHEF) for the fingerprinting of *Rhizobium* spp. FEMS Microbiology Letters 83: 193-198.

Denny, T. P. 1988. Phenotypic Diversity in *Pseudomonas syringae* pv. *tomato*. Journal of General Microbiology. 134: 1939-1948.

Denny, T. P., Gilmour, M. N. and Selander, R. K. 1988. Genetic diversity and relationships of two pathovars of *Pseudomonas syringae*. Journal of General Microbiology. 134: 1949-1960.

Georgakopoulos, D. G. and Sands, D. C. 1992. Epiphytic populations of *Pseudomonas syringae* on barley. Can. J. Microbiol. 38: 111-114.

Gross, D. C. 1985. Regulation of syringomycin synthesis in *Pseudomonas syringae* pv. *syringae* and defined conditions for its production. Journal of Applied Bacteriology 58: 167-174.

Gross, D. C. 1991. Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. Ann. Rev. Phytopathol. 29: 247-278.

Grothues, D. and Tümmler, B. 1991. New approaches in genome analysis by pulsed-field gel electrophoresis: application to the analysis of *Pseudomonas* species. Molecular Microbiology 5 (11): 2763-2776.

Hendson, M., Hildebrand, D. C., and Schroth, M. N. 1992. Relatedness of *Pseudomonas syringae* pv. *tomato*, *Pseudomonas syringae* pv. *maculicola* and Pseudomonas syringae pv. *antirrhini*. Journal of Applied Bacteriology 73: 455-464.

Hildebrand, D. C., Schroth, M. N., and Sands, D. C. 1988. *Pseudomonas*. In: Laboratory Guide for Identification of Plant Pathogenic Bacteria, 2nd edition, ed. Schaad, N. W. APS Press, St. Paul, Minnesota, pp. 60-80.

Iacobellis, N. S., Lavermicocca, P., Grgurina, I., Simmaco, M., and Ballio, A. 1992. Phytotoxic properties of *Pseudomonas syringae* pv. *syringae* toxins. Physiological and Molecular Plant Pathology 40: 107-116.

Jensen, M. A., Webster, J. A. and Straus, N. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl. and Environ. Microbiol. 59(4): 945-952.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 434: 301-307.

Klement, Z. 1963. Rapid detection of the pathogenicity of phytopathogenic pseudomonads. Nature 199: 299.

Köhler, W., Schachtel, G., und Voleske, P. 1992. Biostatistik-Einführung in die Biometrie für Biologen und Agrarwissenschaftler. Springer-Verlag, New York. pp. 47-71.

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

Legard, D. E., Aquadro, C. F. and Hunter, J. E. 1993. DNA sequence variation and phylogenetic relationships among strains of *Pseudomonas syringae* pv. *syringae* inferred from restriction site maps and restriction fragment length polymorphism. Applied and Environmental Microbiology 59 (12): 4180-4188.

Lindgren, P. B., Peet, R. C. and Panopoulos, N. J. 1986. Gene cluster of *P. s.* pv. "*phaseolicola*" controls pathogenicity on bean plants and hypersensitivity on nonhost plants. Journal of Bacteriology 168: 512-522.

Lindgren, P. B. 1997. The role of *hrp* genes during plant-bacterial interactions. Annu. Rev. Phytopathology. 35: 129-152.

Louws, F. J., Fulbright, D. W., Stephens, C. T., and de Bruijn, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Applied and Environmental Microbiology 60 (7): 2286-2295.

Manceau, C. and Horvais, A. 1997. Assessment of genetic diversity among strains of *Pseudomonas syringae* by PCR-restriction fragment polymorphism analysis of rRNA operons with special emphasis on *P. syringae* pv. *tomato*. Appl. Environ. Microbiol. 63(2): 498-505.

Martinez-Miller, C. 1994. Etiology and characterization of two *Pseudomonas syringae* pathovars causing two bacterial kernel blights of barley. PhD Thesis. Montana State University, Bozeman, MT.

Martinez-Miller, C. and Braun, A. 1997. Bacterial kernel blights. in: Compendium of barley diseases. Second edition. APS Press. St. Paul, Minnesota.

Martinez-Miller, C., Braun, A., Siemsen, S., and Sands, D. C. 1998. Etiology of basal kernel blight of barley caused by *Pseudomonas syringae* pv. *syringae*. Can. J. of Plant Pathology, in press.

Megeed, E. and Sherwood, J. E. 1995. Pulsed-field gel electrophoresis fingerprinting for identification of *Azospirillum* species. Current Microbiology 30: 127-131.

Mohan, S. K. and Schaad, N. W. 1987. An improved agar plating assay for detecting *Pseudomonas syringae* pv. *syringae* and pv. *phaseolicola* in contaminated bean seed. Phytopathology 77: 1390-1395.

Quigley, N. B. and Gross, D. C. 1994. Syringomycin production among strains of *Pseudomonas syringae* pv. *syringae*: Conservation of the *syrB* and *syrD* genes and activation of phytotoxin production by plant signal molecules. Molecular Plant-Microbe Interactions 7(1): 78-90.

Roos, I. M. M., and Hattingh, M. J. 1987. Pathogenicity and numerical analysis of phenotypic features of *Pseudomonas syringae* strains isolated from deciduous fruit trees. Phytopathology 77:900-908.

Scholz, B. K., Jakobek, J. L., and Lindgren, P. B. 1992. RFLP analysis of the hrp region of *Pseudomonas syringae* pv. *tabaci* using hrp sequences of *Pseudomonas syringae* pv. *phaseolicola*. Phytopathology 82: 1100 (Abstract).

Vassilev, V., Lavermicocca, P., Giorgio di, D., and Iacobellis, N. S. 1996. Production of syringomycins and syringopeptins by *Pseudomonas syringae* pv. *atrofaciens*. Plant Pathology 45: 316-322.

Vera Cruz, C. M., Ardales, E. Y., Skinner, D. Z., Talag, J., Nelson, R. J., Louws, F. J., Leung, H., Mew, T. W., and Leach, J. E. 1996. Measurement of haplotypic variation in *Xanthomonas oryzae* pv. *oryzae* within a single field by rep-PCR and RFLP Analyses. Phytopathology 86: 1352-1359.

Xu, G. W. and Gross, D. C. 1988. Evaluation of the role of syringomycin in plant pathogenesis by using Tn5 mutants of *Pseudomonas syringae* pv. *syringae* defective in syringomycin production. Appl. Environ. Microbiol. 54(6): 1345-1353.

Young, J. M., Takikawa, Y., Gardan, L., and Stead, D. E. 1992. Changing concepts in the taxonomy of plant pathogenic bacteria. Ann. Rev. Phytopathol. 30: 67-105.

CHAPTER 4

BIOLOGICAL CONTROL OF *PSEUDOMONAS SYRINGAE PV. SYRINGAE* THE CAUSAL AGENT OF BASAL KERNEL BLIGHT OF BARLEY BY *PANTOEA AGGLOMERANS*

Introduction

Barley kernel blight has been reported to be associated with *Bipolaris sorokiniana*, *Fusarium graminearum*, and *Alternaria spp.* predominantly in the Upper Midwest (Banttari et al., 1975; Johnston, 1997) and with *Pseudomonas syringae* pathovars in the Northern Great Plains, such as Montana and Idaho (Peters et al., 1983; Martinez-Miller, 1994). The disease varies in severity with cultivars and environmental conditions regardless of the pathogen involved. Kernel blight is most severe during years of higher than average rainfall or barley production under sprinkler (Johnston, 1997; Martinez-Miller & Braun, 1997). We have reported previously about the etiology and significance of basal kernel blight of barley caused by *Pseudomonas syringae* pv. *syringae* (Martinez-Miller et al., 1998). In that study, we determined that the critical period of infection is from late milk to soft dough stages of kernel development and that high moisture is necessary during this period of infection and disease development. The disease is important because malting and brewing industries prefer barley free of discoloration. Discolored barley can be discounted or rejected. Kernel discoloration involves weight, yield and quality loss no matter which pathogen is involved

(Anderson & Banttari, 1976; Basson et al., 1990), however, weight reduction is less severe when bacteria are associated with the disease. Breeding programs in the Midwest have lead to agronomically desirable barley lines with resistance to *B. sorokiniana* (Banttari et al., 1975; Gebhardt et al., 1992). Since no bactericides are available on small grains that might provide control of bacterial pathogens, the only mitigating measure to date is the avoidance of irrigation during the most susceptible kernel developmental stage at soft dough and the use of less susceptible cultivars (Martinez-Miller et al., 1998). Over the last three years I have developed an effective biological control system based on the application of the common phyllosphere colonist *Pantoea agglomerans* prior to the *P. s.* pv. *syringae* infection window (Braun et al., 1996; Braun et al., 1997).

Pantoea agglomerans, syn. *Erwinia herbicola* (Gavini et al., 1989; Holt et al., 1994) has been used as biological control agent (BCA) against several fungal and bacterial diseases. Seed treatment with *E. herbicola* protected wheat from infection by *F. culmorum* (Kempf & Wolf, 1989), *F. nivale* and *Pythium ultimum* (Adetuyi, 1992; Nelson, 1988). Seed bacterization with an *E. herbicola* isolate also inhibited *Rhizoctonia solani* thus providing sheath blight protection (Rosales et al., 1993). Furthermore, a strain of *E. herbicola* isolated from sclerotia of *Verticillium dahliae* was found to inhibit this pathogen *in vitro* (Berg & Ballin, 1994). In the phyllosphere, *E. herbicola* was reported to control *Puccinia recondita* f. sp. *tritici* on wheat (Kempf & Wolf, 1989), *Alternaria solani* on tomato (Sujkowski et al., 1994), and *Sclerotinia sclerotiorum* on dry edible beans (Yuen et al., 1994). Indirect plant growth promoting effects of *P. agglomerans* strains from the winter wheat phyllosphere were noted by Ruppel et al.(1989) and Scholz-Seidel & Ruppel (1992). In addition to

nitrogenase- and phytohormone activity in some strains, the authors detected wheat and barley yield increases of up to 500 kg/ha in field experiments with prebacterized seeds.

Control of bacterial diseases by *E. herbicola* has involved antagonism against ice nucleation active (ice^+) bacteria (Lindow et al., 1983 a,b) and several other bacterial pathogens. These include protection of cotton seeds from *Xanthomonas campestris* pv. *malvacearum* (Randhawa et al., 1987), rice seedlings from attack by *X. oryzae* (Hsieh & Buddenhagen, 1974), mungbeans from *X. c.* pv. *vingnaeradiatae* (Bora et al., 1993) and control of the fire blight pathogen *E. amylovora* on apples (Beer et al., 1984; Vanneste, 1990; Kearns & Hale, 1993; Wright, 1997) pear (Johnson et al., 1993; Stockwell et al., 1994), and hawthorn (Wilson et al., 1990).

The objectives of the present study were to develop an effective and practical biological control system using *Pantoea agglomerans* to suppress *P. s.* pv. *syringae* causing basal kernel blight of barley. In addition to field and glasshouse experimental results with several *Pantoea agglomerans* strains, I present data on time of application and concentration needed for successful disease prevention. Furthermore, I investigated the establishment of antibiotic resistant biocontrol bacteria in the greenhouse and field and evaluated the effectiveness of several formulations for long-term preservation of these gram-negative biocontrol agents (BCA). Data on possible mechanisms involved in the biocontrol performance have been investigated but will be discussed in more detail in chapter 5.

Materials and Methods

Bacterial cultures and inoculum preparation.

P. agglomerans were isolated from non-symptomatic barley kernels in a field that had endemic basal kernel blight in Fairfield, Montana in 1990, 1993 and 1994. *P. s.* pv. *syringae* (Pss) were isolated from barley kernels showing symptoms of basal blight in 1993-1995. Isolates were identified according to standard tests (Hildebrand et al., 1988; Klement et al., 1990) and based on the Biolog (Biolog, Inc., Hayward, CA) and MIDI (Microbial ID Inc., Newark, Delaware) identification systems. They were stored at -70°C in nutrient broth containing 15 % glycerol. To produce bacterial cells for barley inoculation, bacteria were retrieved from storage, streaked onto King's B medium (*P. s.* pv. *syringae*) or Tryptic Soy Agar (TSA, *P. agglomerans*), and incubated at 28 C for 48 or 24 hours, respectively. Cells were suspended in 10 ml PBS buffer (0.85 % NaCl, 0.03 M NaOH, 0.05 M KH_2PO_4; pH 7.0) and the density of the suspension adjusted to 10^8 cfu/ml by measuring the absorbance at 580 nm on a Spectronic 20 (Bausch & Lomb, Rochester, NY) and comparing it with a standardized growth curve developed previously. The suspension was diluted 1:10 in 90 ml sterile tapwater containing 0.025% Tween 20 (polyoxyethylenesorbitan monolaurate) as surfactant to a final concentration of 10^7 cfu/ml. In the greenhouse *P. s.* pv. *syringae* was inoculated on barley heads at soft dough stage (EC85), the most critical period of infection (Martinez-Miller et al., 1998). In field experiments natural infection by *P. s.* pv. *syringae* was enhanced by brief periods of daily overhead sprinkler irrigation for 5 to 10 days during the window of infection (Martinez-Miller et al., 1998).

Application of *P. agglomerans* in field experiments.

In 1994, four *P. agglomerans* strains (Eh 236, 239, 454, and 460) were sprayed at a concentration of 1 x 10^9 cfu/ml on barley cultivars B 2601 and B 1202 prior to the expected infection window for *P. s.* pv. *syringae* attack at late milk stage (EC 77) in Fairfield, MT. Natural infection was favored by providing overhead sprinkler irrigation for approximately 4 h in the morning and 4 h in the evening for 5 days during soft dough stage (EC 83) from July 14th to 18th delivering an average of 15 mm of water per 4 h of irrigation (Martinez-Miller et al., 1998). Traces of precipitation (0.6 mm) fell from July 14th to 23rd in addition to the 150 mm provided by irrigation. Fifty milliliter of biocontrol bacterial suspensions were sprayed per 1.5 m row (0.4 liter/3.5 m^2) on barley heads on July 10th until run-off using a compressed air sprayer (KGRO all purpose plant sprayer, K-mart Corp., Troy, MI). Assuming an average of 100 plants with 153 spikes per row and about 30 kernels per spike, the application rate was 1.1 x 10^7 cfu/kernel. A water/Tween 20 (formulation) check, heat-killed Eh 236, 239, 454, 460 and Tilt® (propiconazole; Novartis Crop Protection, Greensboro, NC) applied at 120 ml/A on June 17th were used as controls. At harvest fifteen randomly marked barley heads representing one sample per replication were picked and evaluated for basal kernel blight presence. The experiment was conducted as a two-factorial split plot design with cultivars as main plots (2 levels) and treatments as subplots (10 levels) with 4 blocks. Each treatment was applied to a 1.5 m long row within a plot consisting of 6 rows, 30 cm apart. The two border rows remained uninoculated and a 60 cm alley separated each plot from the adjacent ones. Percentage of basal kernel blight infection was calculated after harvest (August 2nd) according to the following formula:

$$\% \text{ kernel blight} = (a/b) \times 100,$$

where a = number of blighted kernels per sample; b = total number of kernels per sample. All treatments were compared to untreated water controls of each cultivar to derive blight reduction data.

In 1995, the same four *P. agglomerans* strains used in 1994 were sprayed at a concentration of 4.0×10^8 cfu/ml on the four barley cultivars, B 2601, B 2912, B 5133, and B 1202 prior to the infection window at early milk stage (EC 73). Two-hundred milliliter of biocontrol bacterial suspensions were sprayed per 1.5 m row (3.0 liter/5 m^2) on barley heads on July 13th until run-off. Assuming an average of 100 plants with 153 spikes per row and about 30 kernels per spike, the application rate was 1.7×10^7 cfu/kernel. Control treatments included the water/Tween 20 check, the autoclaved biological control agents (BCA), a 50 mM L-tartaric acid (pH 3.5) treatment, different *Pantoea* strains in combination and the Tilt® application on June 20th. Sprinkler irrigation provided 30 mm of precipitation from early milk to hard dough stage (July 15th-28th). Additionally, 16 mm rain fell during the irrigation period. The evaluation of the disease was completed after harvest (August 22nd) in the same way as in 1994. The experiment was conducted as a two-factorial split plot design with cultivars as main plots (4 levels) and treatments as subplots (16 levels) with 4 blocks.

Performance of *P. agglomerans* in glasshouse tests.

Two *P. agglomerans* strains (Eh 454, Eh 239) were sprayed on cvs. B 1202 and B 2601, respectively, at soft dough stage 3 days prior to inoculation with *P. s.* pv. *syringae*

strain 552. A water/Tween 20 application, the positive pathogen control (Pss 552), and the heat-killed strain Eh 239 were included as controls in each experiment. One pot (19 cm diameter) with 3 barley spikes per replication (n=5) was inoculated, and both biocontrol bacteria and the pathogen Pss 552 were sprayed on heads until run-off (approx. 5 ml per spike) using a hand air brush sprayer (Model Paaschi D500 1/10 H.P.). Plants were incubated in a mist chamber (95% r. h.) for 3 days after BCA application and again for 72 h after pathogen inoculation before being transferred to the greenhouse bench. The greenhouse was maintained at 22/18 ± 2 C (day/night) with a 12 h photoperiod. Basal kernel blight percentages were calculated after harvest by the formula described previously and blight reduction data obtained by comparing treatments with the positive control. Each experiment (cultivar) was conducted as a one-factorial completely randomized design. The same experiment was performed with strains Eh 236 and Eh 460 instead of strains Eh 454 and Eh 239 to see differences between different biocontrol strains applied.

A possible correlation between *in vitro* antibiosis and *in vivo* suppression of basal kernel blight was examined in glasshouse assays using *P. agglomerans* strains tested previously in plate bioassays against *P. s.* pv. *syringae* Pss 552 (Table 4.3). Strains Eh 239, Eh 460, Eh 926, Eh 981, Eh 906, and Eh 907 were sprayed on B 2601 heads 3 days prior to Pss 552 at EC 85. BCA and pathogen inoculum preparation, inoculation, incubation and disease evaluation were done as previously described.

To investigate the performance of biocontrol strains against several *P. s.* pv. *syringae* strains, another glasshouse experiment was conducted as a 2 x 4 factorial randomized complete block design with 4 replications. The biocontrol strains Eh 460, Eh 237, and Eh

234 were sprayed on B 2601 heads 3 days prior to pathogen Pss 552 or Pss 793, respectively, at soft dough stage. Controls included a water treatment and the Pss 552 and Pss 793 positive checks. Plant incubation and disease evaluation were carried out as described previously.

In vitro biotests.

P. agglomerans strains were tested for antibiotic activity against *P.s.* pv. *syringae* and other indicator bacteria in plate bioassays. Biocontrol isolates were spotted on Potato dextrose agar plates (PDA, 20 ml per 9 cm plate) with sterile toothpicks, 6 cm apart from each other and 3 cm from the center of the plate. Two spots were made per isolate per plate and plates were duplicated (= 4 replications per strain). Plates were incubated in the dark at 28 C for 5 days and colonies subsequently scraped off the plate with sterile cotton swabs after zones of colony growth had been marked. Plates were than exposed to $CHCl_3$ vapor for 20 minutes, kept open under the laminar flow hood for 20 min. to remove $CHCl_3$ vapors and sprayed with a suspension (10^7 cfu/ml) of each indicator strain using an aerosol sprayer (Sigma Spray Aerosol Propellant, Sigma, St. Louis, MO). Zones of inhibition of indicator strains around marked areas where biocontrol strains had been grown previously were recorded after 24 h incubation at 28 C. For testing antifungal activity, a centered line of biocontrol bacteria was streaked onto a PDA plate. On each side of this streak (3 cm apart), an agar plug (5 mm) containing mycelium of a test fungus (2 plugs/plate) was transferred and plates were incubated for 3 days at 28 C, then at 22 C. Plates containing only the test fungi served as controls and plates were replicated 4 times per biocontrol strain and fungus tested. Fungal growth on plates was compared in the presence and absence of biocontrol

bacteria, and zones of inhibition of test fungi recorded as 0 = no inhibition, + = slight zones of inhibition (weak antifungal activity), and ++ = clear zones of inhibition (strong antifungal activity). Bioassay data were pooled over two experiments.

Time and rate of application of *P. agglomerans*.

In most glasshouse and field experiments the biocontrol agents (BCAs) were applied three days prior to the pathogen or enhanced natural infection, respectively. To determine whether time of BCA application is critical, two glasshouse tests were conducted. In one experiment the chloramphenicol resistant (chl^R) *P. agglomerans* strain Eh 460 chl^R was applied to barley heads (cv. B 2601) at soft dough stage 3 days prior to the rifampicin resistant (rif^R) *P. s.* pv. *syringae* strain Pss 552 rif^R (= 3d, conventional application). A second treatment consisted of the application of strain Eh 460 chl^R immediately before inoculation of Pss 552 rif^R (= 0d). A third treatment included the control applications of Eh 460 chl^R or Pss 552 rif^R, respectively, or the water check. Strain Pss 552 rif^R was still pathogenic and strain Eh 460 chl^R demonstrated biocontrol activity after mutagenesis as determined by plate bioassays (data not shown). Plant inoculation, incubation and disease evaluation after harvest were done as described previously. Reisolations of the pathogen and the biocontrol agent were done in this experiment to evaluate the influence of the introduction of the biocontrol agent on the pathogen population. The results of this experiment are mentioned but discussed in more detail elsewhere (chapter 5; Fig. 5.4).

The question of whether the biocontrol agent could be introduced earlier than soft dough stage was examined in a separate glasshouse experiment by applying strain Eh 239

at EC 49, the last stage of booting before seedhead emergence. The pathogen Pss 552 was inoculated as usual at soft dough stage (EC 85). Other treatments included the conventional Eh 239 application at EC 85 three days prior to the pathogen, the water control, and the Pss 552 positive control. The experiment was conducted as a completely randomized design with 4 treatments and 5 replications. Plant inoculation, incubation in the mist chamber, and disease evaluation was performed in the same way as described above.

To estimate the dose (cfu/ml) of biocontrol agents needed for basal kernel blight disease control, *P. agglomerans* strain Eh 460 was sprayed on cv. B 2601 barley heads at EC 85 at concentrations of 10^3, 10^5, and 10^7 cfu/ml, respectively, three days prior to Pss 552. The water check and the Pss 552 treatment without BCAs served as controls. The experiment was performed as a completely randomized design, with 5 treatments and 6 replications. Plant inoculation, incubation and disease evaluation was done as previously described.

Establishment of the biocontrol agents in the barley phyllosphere.

In a greenhouse experiment, Eh 239 rif^R was sprayed at a concentration of 6.3×10^6 cfu/ml on barley heads (cv. Karl) at watery ripe stage (EC 71). Samples were taken for reisolation 1, 5, 10, 15, 21, and 35 days after application. Three heads per pot were taken as individual samples per isolation date and samples replicated 4 times (n = 12). The heads were placed in tubes containing 20 ml sterile PBS buffer and vortexed. After 30 minutes with periodical vortexing, appropriate dilutions were spiral plated (Model C spiral plater, Spiral Biotech, Bethesda, MD) on TSA plates containing 100 μg/ml rifampicin. Plates were

incubated at 28 C overnight and colonies counted and expressed as log cfu/head.

In the field, rif^R-marked *P. agglomerans* strains Eh 236, 239, 454, and 460 were sprayed at a concentration of 4.0×10^8 cfu/ml on cv. B 2601 and cv. B 5133 on July 13th 1995, when plants were at early milk stage (EC 73) as described previously. Three barley heads were sampled at random from each replicate row, placed in a new Ziploc plastic bag, kept on ice, transported to the laboratory, and stored at 4 C until processing the following morning. Reisolations of the rif^R strains were done 1, 7, and 18 days after foliar application. Samples for reisolation were taken at 11:00 a.m. to minimize population size changes. A final isolation was done from kernels 2 months after harvest using 50 randomly picked kernels per replication (n = 4). Kernels were shaken in 100 ml PBS buffer at 150 rpm for 2 hours at 4 C and appropriate dilutions (to 10^{-7}) spiral plated on TSA containing 100 μg/ml rifampicin. Presence of rif^R-marked biocontrol strains was calculated as log cfu/kernel.

Formulation of *P. agglomerans*.

Formulations (Fig. 4.1) of biocontrol agents were prepared using the oil/starch/sugar encapsulation method developed by Zidack et al. (1995) and Quimby et al. (1996). Briefly, this involved growing the BCA on TSA plates overnight at 28 C. Plates were overlaid with 10 ml 1 M sucrose solution for membrane stabilization and bacteria scraped off the plates into 100 ml sterile beakers using a sterile cotton swab. Five g of the water-absorbent starch Water-lock® (Grain Processing Corporation, Muscatine, IA) and 5 ml unrefined corn oil (Spectrum Naturals Inc., Petaluma, CA) were added to each beaker. These primary components were blended into a dough-like mass in a Sigma mixer. 17.5 g of the silica-

based granulating agent Hi-Sil® (Cabot Corp., Tuscola, IL) was added and blending continued until the dough crumbed, thereby producing a granular product. The wettable powder granules were spread in foil pans and air dried in a laminar flow cabinet for 48 h at room temperature. The dried formulation was then sieved in the size range of 250-710 μm and viable bacterial colony counts recorded over a period of 1.5 years for *P. agglomerans* strains Eh 239 and EhL13 stored at room temperature (22 C) and 4 C, respectively. For lyophilization, strains Eh 239 and Eh 460 were grown in 200 ml 2 % Potato dextrose broth (PDB) for 20 h, centrifuged at 6000 x g for 15 min. and the cell pellet resuspended in 10 ml PBS buffer. 10 % dry milk powder (Carnation pasteurized non-fat dry milk, fortified with Vitamins A&D, but no preservatives, Los Angeles, CA) was added as a food base and the suspension vortexed. Samples were freeze-dried (Labconco Freeze dry system/Lyphlock 4.5, Kansas City, Missouri) for 24-48 h following freezing in liquid nitrogen for 10 min. Strains were kept at 4 C and viable colony counts recorded over time. For dry milk formulation of strain EhL13, the oil/starch/sugar encapsulation method (Zidack et al., 1995) was slightly modified by replacing starch with 10 g dry milk powder. The powder was stored at room temperature over a period of 1.5 years. Viable colony counts were performed by adding 0.1 g of powdered bacteria/formulation to 9.9 ml of PBS buffer. Serial dilutions were prepared from this stock solution to 10^{-7} and spiral plated on TSA plates. Bacterial numbers were calculated as log cfu/g powdered formulation and isolations were done 1 day, 2 weeks, 4 weeks, 3 months, 6 months, 1 year, and 1.5 years after formulation.

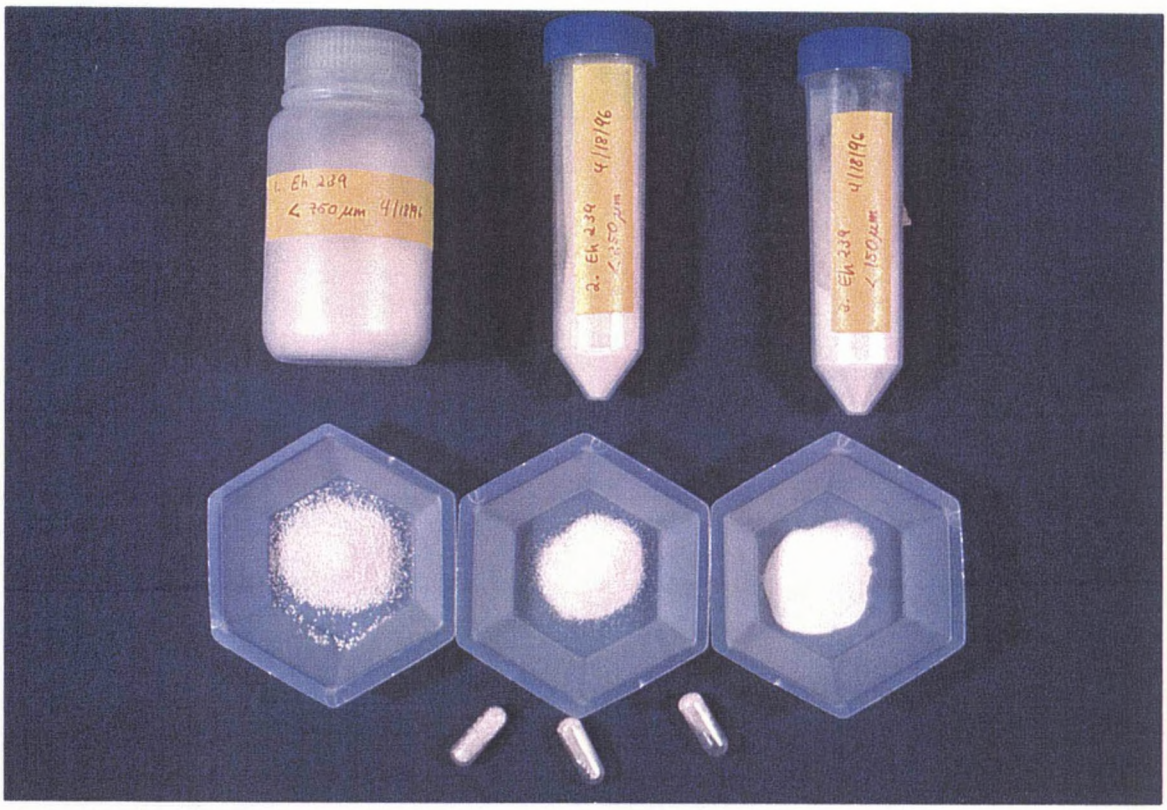

Fig. 4.1. Photo of *Pantoea agglomerans* strain Eh 239 formulated in the oil/starch/sugar encapsulation method (USDA) developed by Quimby et al., 1996. Bacterial formulations were sieved after air drying in granule sizes of 250-750 μm, 150-249 μm, and <150 μm and stored as wettable powders in plastic containers or gelatin capsules.

The biocontrol activity of formulated *P. agglomerans* strain Eh 239 after long-term storage against Pss 552 was tested in a glasshouse trial, which included the treatments Eh 239 non-formulated (NF), Eh 239 freeze-dried (FD) and stored at 4 C, Eh 239 (USDA) formulated and stored at 4 C or 22 C, respectively. Bacteria were recovered from formulations by diluting 0.1 g in 9.9 ml PBS buffer and streaking suspensions on TSA plates. After overnight incubation at 28 C, bacterial suspensions were prepared as previously described and all treatments sprayed on barley cv. B 2601 three days prior to Pss 552 inoculation at soft dough stage. The experiment was conducted as completely randomized

design with 4 replications per treatment. Plants were incubated and percentage of basal kernel blight assessed after harvest as described above.

Statistical analyses.

All statistical analyses were performed as appropriate to the experimental design using the MSUSTAT program (Lund, 1993). Data were analyzed by analysis of variance (ANOVA), and the sources and amount of variation were compared using an F test. To compare differences between more than two treatment means, least significant differences were calculated at $\alpha = 5\%$ unless stated otherwise. Correlation/regression analyses between two variables were performed and the degree of the relationship determined as coefficient (r) using the statistical analysis package MSTATC (Michigan State University). All calculations involving bacterial population densities were log-transformed prior to analysis and means were presented as log cfu/unit.

Results

Suppression of basal kernel blight by *Pantoea agglomerans* in field experiments.

In 1994, the *P. s.* pv. *syringae* induced disease levels in Fairfield, MT were below 4% for most cultivars tested due to hot and dry weather conditions (0.6 mm rainfall) and limited levels of overhead sprinkler irrigation (5 days = 150 mm precipitation) during the window of infection from heading to soft dough (Martinez-Miller et al., 1998). Nonetheless, all 4 biocontrol *Pantoea agglomerans* strains significantly reduced the basal kernel blight

percentage by 45-68% on cv. B 2601 when compared to non-treated controls (Table 4.1). On B 1202 the blight reduction ranged between 30-58% depending on the bacterial strain used. The Tilt® treatment and heat-killed biocontrol strains were not significantly different from the non-treated water control. In 1995, a year with average rainfall (16 mm) and extended overhead irrigation (10 days = 300 mm precipitation) during the infection window, significant disease developed with infection percentages of 9.5 % and 8.9% for the six-row cultivars B 2601 and B 2912, respectively, and 7.1% and 5.5% for the two-row cultivars, B 1202 and B 5133, respectively. Disease reduction by the biocontrol bacteria was 36-74% over all cultivars when compared to untreated controls (Table 4.2). On B 2601 the reduction was 49-68%, on B 2912 60-74%, on B 1202 53-68%, and on B 5133 36-61%, depending on the biocontrol strain used. Disease reductions by strain combinations were also significant in most cases (42-64%), but inferior compared to the application of individual strains. Reductions due to Tilt® application ranged between 0-43%, which were only significant on B 2601 (Table 4.2). Tartaric acid treatments ranged between 20-35% disease reduction, but were never significantly different compared to the H_2O controls.

Table 4.1. Percentage of the basal blight disease incidence over all cultivars and percentage of kernel blight reduction on barley cultivars (cv.) B 2601 and B 1202 at Fairfield, MT in 1994. The water/Tween 20 check, biocontrol *P. agglomerans* strains Eh 236, 239, 454, and 460, heat-killed bacteria (D), or Tilt®, respectively, were applied to plant heads at late milk stage prior to the *P. s.* pv. *syringae* infection window.

Treatment	Disease incidence all cultivars (%)	% Basal kernel blight reduction	
		cv. B 1202	cv. B 2601
H_2O	2.3 a [2)]		
Eh 236	1.1 c	33	59 *[3)]
Eh 239	0.9 c	50	68 *
Eh 454	1.4 bc	30	45 *
Eh 460	0.9 c	58	65 *
Eh 236 D [1)]	2.2 a	0	26
Eh 239 D	2.5 a	0	17
Eh 454 D	2.7 a	0	27
Eh 460 D	2.0 ab	0	27
Tilt®	2.3 a	0	25
LSD (0.05)	0.82		

[1)] Heat-killed bacteria; [2)] Values are means of 4 replicates and 2 cultivars; and values followed by the same letter are not significantly different at $p \leq 0.05$; [3)] * represents significant reduction values of treatments compared with untreated controls within a cultivar at $p \leq 0.05$, using single-degree-of-freedom contrasts.

Table 4.2. Percentage of the basal kernel blight disease incidence over all cultivars and percentage blight reduction on barley cultivars B 2601, B 2912, B 1202, and B 5133 at Fairfield, MT in 1995. The water check, *P. agglomerans* strains Eh 236, 239, 454, and 460, heat-killed bacteria (D), biocontrol strain combinations, a 50 mM L-tartaric acid treatment and Tilt® were applied to barley heads at early milk stage prior to the *P. s.* pv. *syringae* infection window.

Treatment	Disease incidence all cultivars (%)	% Basal kernel blight reduction			
		B 2601	B 2912	B 1202	B 5133
H_2O	7.7 a [2)]				
Eh 236	3.4 ghi	48.9 *[3)]	66.5 *	52.86 *	51.16
Eh 239	2.7 hi	60.3 *	73.8 *	67.14 *	48.83
Eh 454	2.6 i	68.9 *	64.2 *	68.57 *	61.39 *
Eh 460	3.5 ghi	60.3 *	60.0 *	64.3 *	36.7
Eh 236 D [1)]	6.5 abcd	23.9	0.0	28.6	12.1
Eh 239 D	7.2 ab	0.0	0.0	11.4	20.5
Eh 454 D	5.9 bcd	0.0	45.6	52.9 *	18.1
Eh 460 D	6.6 abc	0.0	13.8	38.6 *	26.5
Eh 239+454	5.2 cdef	48.4 *	40.7	64.3 *	33.5
Eh 460+236	5.1 def	44.2 *	46.8	34.3 *	58.1 *
Eh 239+460	4.1 fgh	42.1 *	26.9	42.9 *	10.7
Eh 236+239	4.2 fg	42.1 *	25.9	62.9 *	20.0
all Eh	4.4 efg	45.5 *	52.3 *	45.7 *	16.3
Tartaric Acid	6.5 abcd	26.0	20.3	35.7 *	20.5
Tilt®	5.7 cde	40.3 *	0.0	25.7	43.3
LSD (0.05)	1.4				

[1)] Heat-killed bacteria; [2)] Values are means of 4 replicates and 4 cultivars; data were log transformed prior to analysis and values followed by the same letter are not significantly different at $p \leq 0.05$; [3)] * represents significant reduction values of treatments compared with untreated controls within a cultivar at $p \leq 0.05$, using single-degree-of-freedom contrasts.