



Etiology and characterization of two *Pseudomonas syringae* pathovars causing two bacterial kernel blights of barley
by Concepcion Martinez-Miller

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

Kernel blight of barley, an important disease for malting and brewing industry, exhibits two types of symptoms in Montana. The most common is basal kernel blight consisting of dark brown discoloration on the embryo end of the kernel. Spot kernel blight, on the other hand, has a well defined spot on the lemma of the kernel. Two different pathovars of *Pseudomonas syringae* were associated with the different Symptom types. The first group, *P. s. pv. syringae*, produced syringomycin and utilized L-lactate and trigonelline. The second group, an undefined pathovar designated as *P. syringae* 554, did not produce syringomycin nor utilize L-lactate or trigonelline. *P. s. , pv. syringae* strains were strongly associated with basal kernel blight while *P. syringae* 554 was associated with spot kernel blight. Strains were characterized by RFLP analysis using three different probes. A dendrogram showed that most of the *P. s. pv. syringae* strains were distributed in closely related clusters while *P. syringae* 554 strains were in a completely divergent cluster. Along with the genetic analysis of these two pathovars, aspects of disease development were also studied. A different window of infection was observed for the two types of blight. The window of infection for basal kernel blight was during late milk and dough stages while that of spot kernel blight was at early milk. High moisture was determined to be necessary during the window of infection for successful development of the disease. Screening methods revealed that screening for resistance to kernel blight must be performed separately for each type of blight.

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by

Concepcion Martinez-Miller

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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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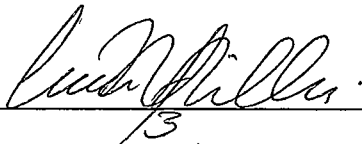
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TABLE OF CONTENTS

	Page
LIST OF TABLES	vii
LIST OF FIGURES	ix
ABSTRACT	xii
1. LITERATURE REVIEW	1
Kernel Blight of Barley	1
Importance of the Disease	1
Disease Description and Causal Agents	2
The Bacterium <i>Pseudomonas syringae</i> van hall 1902.	3
Toxin Production	4
Syringomycin Production by <i>P.s.</i> pv. <i>syringae</i>	5
Characterization of Phytopathogenic Bacteria	7
Biochemical and Physiological Tests	7
RFLP Analysis	7
Factors Involved in Development of the Disease	12
Kernel Developmental Stage	13
Environmental Conditions	14
Genetic Resistance	15
2. SYMPTOMATOLOGY AND STUDY OF THE CAUSAL AGENTS OF KERNEL BLIGHT OF BARLEY IN MONTANA	17
Introduction	17
Materials and Methods	20
Samples.	20
Fungal Isolation	21
Bacterial Isolation	21
Bacterial Inoculation in the Greenhouse	23
Syringomycin Assay	25
Biochemical Tests on Bacterial Strains	25
Reaction to a Polyclonal Antiserum	26
RFLP Analysis	26
Strains	26
Preparation of Total Genomic and Plasmid DNA	27
Digestion of DNA, Separation of Restriction Fragments, and Alkaline Transfer	27
DNA Probes	28
DNA Hybridization	29
Genetic Relationships of <i>P. syringae</i> Strains Based on RFLP Profiles	29

TABLE OF CONTENTS (CONTINUED)

Results	30
Symptoms	30
Fungal Isolation	31
Bacterial Isolation	32
Bacterial Inoculation in the Greenhouse	34
Syringomycin Production	35
Pathovar Identification	38
Reaction to a Polyclonal Antiserum	42
RFLP Analysis	43
Genetic Relationships of <i>P. syringae</i>	
Strains Based on RFLP Profiles	43
Discussion	49
3. HOST-RELATED AND ENVIRONMENTAL FACTORS	
INVOLVED IN THE DEVELOPMENT OF KERNEL BLIGHT	59
Introduction	59
Materials and Methods	62
Greenhouse Experiments	62
Effect of Kernel Developmental Stage	62
Effect of Duration of Free Moisture	
and Temperature	64
Screening Method	65
Field Experiments	66
Effect of Kernel Developmental Stage	
and Moisture	66
Screening Method	67
Results	68
Effect of Kernel Developmental Stage	68
Effect of Moisture and Temperature	73
Screening Method	77
Discussion	81
SUMMARY	95
LITERATURE CITED	98
APPENDICES	107

LIST OF TABLES

Table	Page
1. Malting barley samples obtained from Northcentral Montana and North Dakota, South Dakota, and Minnesota during the growing seasons 1991, 1992, and 1993	22
2. Relative frequency with which <i>Pseudomonas syringae</i> (P.s), <i>Cochliobolus sativus</i> (C.s), and <i>Alternaria</i> sp. (A.sp) were isolated from blighted kernels of malting barley produced in Montana, North Dakota, South Dakota, and Minnesota in 1991, 1992 and 1993	33
3. Characterization of <i>Pseudomonas syringae</i> utilized in this study	40
4. Analysis of Variance for effect of kernel developmental stage at the time that additional sprinkler irrigation was provided to two barley cultivars on the incidence of kernel blight under field conditions in 1992 and 1993	72
5. Effect of kernel developmental stage, moisture and temperature on the development of kernel blight under greenhouse conditions. Percentages of kernel blight obtained on barley plants inoculated with a combination of <i>P. syringae</i> pv. <i>syringae</i> and <i>P. syringae</i> 554	76
6. Effect of moisture on the development of kernel blight under field conditions for barley grown under different irrigation conditions at Fairfield MT during 1992 and 1993	77
7. Effect of barley cultivars on the percentage of the different types of kernel blight obtained in the greenhouse when inoculated with <i>P. s.</i> pv. <i>syringae</i> strain 552 and <i>P. syringae</i> 554 strain 554	79

LIST OF TABLES (CONTINUED)

Table	Page
8. Interaction of cultivar and <i>P. syringae</i> strains and cultivar and stage on development of the different kernel blight types obtained in the greenhouse	80
9. Percentage of basal kernel blight obtained on 6 barley cultivars supplied with additional sprinkler irrigation during different kernel developmental stages at Fairfield MT in 1993	81
10. <i>Pst</i> I restriction fragment patterns that resulted by hybridization to the <i>syrB</i> probe. . .	109
11. <i>Pst</i> I restriction fragment patterns that resulted by hybridization to the 10 Kbp <i>hrp</i> probe	110
12. <i>Pst</i> I restriction fragment patterns that resulted by hybridization to the 14 Kbp <i>hrp</i> probe	111

LIST OF FIGURES

Figure		Page
1.	Symptom types of kernel blight. (a) Basal kernel blight characterized by dark brown discoloration on the embryo end of the kernel. (b) Spot kernel blight characterized by tan to dark brown necrotic spots with distinct margins on the lemma of the kernel	31
2.	Syringomycin assay. Syringomycin production by <i>P. syringae</i> strains was tested for antifungal activity against <i>Geotrichum candidum</i> . Zone of inhibition around a bacterial colony was considered indicative of syringomycin production. The 5 colonies in the top of the plate were positive for syringomycin production, while the 5 colonies in the bottom of the plate were clearly negative. Arrows indicate the position of the non-syringomycin producing strains	36
3.	Percentage of <i>P. syringae</i> pv. <i>syringae</i> (<i>Pss</i>) and <i>P. syringae</i> 554 (<i>Ps</i>) strains isolated from samples of malting barley showing basal kernel blight. Samples were obtained from northern Montana, North Dakota, South Dakota and Minnesota in the growing seasons of 1991, 1992 and 1993. . .	37
4.	Percentage of <i>P. syringae</i> pv. <i>syringae</i> (<i>Pss</i>) and <i>P. syringae</i> 554 (<i>Ps</i>) strains isolated from samples of malting barley showing spot kernel blight. Samples were obtained from Northern Montana during 1991, 1992 and 1993	39
5.	Restriction fragment length polymorphism patterns of ten <i>P. syringae</i> strains, lanes A to H are toxigenic and I and J non-toxigenic. Total DNAs were digested with <i>Pst</i> I and probed with (a) <i>SyrB</i> , (b) 10-Kbp <i>hrp</i> , and (c) 14-Kbp <i>hrp</i> . A=552, B=592, C=541, D=542, E=643 , F=645, G=647, H=644, I=554, J=418	45

LIST OF FIGURES (CONTINUED)

Figure	Page
6. Dendrogram obtained from cluster analysis of restriction fragments by using UPGMA. Each <i>P. syringae</i> DNA was digested with <i>Pst</i> I and hybridized to the <i>SyrB</i> probe and the 10-Kbp and 14-Kbp <i>hrp</i> fragments	47
7. Percentage of basal kernel blight obtained in the greenhouse by inoculation of barley heads at early milk (EM) late milk (LM) soft dough (SD) and hard dough (HD). Inoculation was done with a combination of bacterial suspensions of <i>P. s. pv. syringae</i> strain 552 and <i>P. syringae</i> 554 strain 554. Bars with different letters are significantly different at $p = 0.05$	69
8. Percentage of basal kernel blight obtained under field conditions when additional overhead irrigation was supplied to barley heads at early milk (EM), late milk (LM), soft dough (SD), and hard dough (HD). Experiment was performed in Fairfield MT 1992. Bars with different letters are significantly different at $p = 0.05$	70
9. Percentage of basal kernel blight obtained under field conditions when additional overhead irrigation was supplied to barley heads at early milk (EM), late milk (LM), and soft dough (SD). Experiment was performed in Fairfield MT 1993. Bars with different letters are significantly different at $p = 0.05$	71
10. Percentage and type of kernel blight obtained in the greenhouse when <i>P. s. pv. syringae</i> (Pss) and <i>P. syringae</i> 554 (Ps) were inoculated individually on barley heads at two different kernel developmental stages. Values are averages of two experiments. ** = statistically significant at $p = 0.05$; n.s. = non significant at $p = 0.05$	74

LIST OF FIGURES (CONTINUED)

Figure		Page
11.	Effect of the kernel developmental stage on kernel blight percentage of six barley cultivars. Disease was enhanced by overhead sprinkler irrigation at early milk (EM) or soft dough stages (SD). Fairfield MT, 1993. Cultivars with different letter were statistically different at $P = 0.05$	82
12.	Rainfall obtained during the month of July 1989, Fairfield MT.	113
13.	Rainfall obtained during the month of July 1990, Fairfield MT.	113
14.	Rainfall obtained during the month of July 1991, Fairfield MT.	114
15.	Rainfall obtained during the month of July 1992, Fairfield MT.	114
16.	Rainfall obtained during the month of July 1993, Fairfield MT.	115
17.	Rainfall obtained during the month of August 1993, Fairfield MT.	115
18.	Temperature data collected during the month of July in Fairfield MT., in 1989 and 1993	116

ABSTRACT

Kernel blight of barley, an important disease for malting and brewing industry, exhibits two types of symptoms in Montana. The most common is basal kernel blight consisting of dark brown discoloration on the embryo end of the kernel. Spot kernel blight, on the other hand, has a well defined spot on the lemma of the kernel. Two different pathovars of *Pseudomonas syringae* were associated with the different symptom types. The first group, *P. s. pv. syringae*, produced syringomycin and utilized L-lactate and trigonelline. The second group, an undefined pathovar designated as *P. syringae* 554, did not produce syringomycin nor utilize L-lactate or trigonelline. *P. s. pv. syringae* strains were strongly associated with basal kernel blight while *P. syringae* 554 was associated with spot kernel blight. Strains were characterized by RFLP analysis using three different probes. A dendrogram showed that most of the *P. s. pv. syringae* strains were distributed in closely related clusters while *P. syringae* 554 strains were in a completely divergent cluster. Along with the genetic analysis of these two pathovars, aspects of disease development were also studied. A different window of infection was observed for the two types of blight. The window of infection for basal kernel blight was during late milk and dough stages while that of spot kernel blight was at early milk. High moisture was determined to be necessary during the window of infection for successful development of the disease. Screening methods revealed that screening for resistance to kernel blight must be performed separately for each type of blight.

CHAPTER 1

LITERATURE REVIEW

Kernel Blight of Barley.

Importance of the Disease. 'Kernel blight' and 'kernel discoloration' have been used to describe a barley disease of importance to the malting and brewing industries. Both will be referred to as kernel blight in this study. Since malting is a germination and growth process, and good malt can only be obtained by rapid and uniform germinating barley, any reduction in germination produced by diseased kernels will ultimately result in lower brew-house extract and may adversely affect quality of the final product (Burger and LaBerge, 1985). Microbial infection of kernels can also affect the brewing process by excretion of substances such as germination inhibitors which, besides affecting the malting process, also produce off-flavor metabolites that carry over to the beer (Burger and LaBergue, 1985). Another brewing problem known as gushing involves the overfoaming of beer caused by a sudden release of CO₂ when packed beer is opened which seems to be directly attributable to microbial infection of barley seed (Burger and LaBergue, 1985). Severely affected malting barley can be downgraded or rejected by the industry. It can then only

be sold for animal feed resulting in large economic losses for growers. Kernel blight is commonly present in the upper Midwestern region of the United States and has recently become a problem in the state of Idaho and in the North central part of Montana, varying in frequency and severity from year to year. The Northcentral part of Montana includes a large region where malting barley is one of the primary crops and in some years such as 1989, the disease caused large economic losses.

Disease Description and Causal Agents. Kernel blight in Montana has been characterized by the appearance of two symptom types. The most common type consists of a dark brown discoloration of the embryo end of the kernel (hereafter referred to as **basal kernel blight**) (Fig. 1a). This type of symptom has been termed 'black point' in wheat, barley and rye (Machacek and Greaney, 1938) and is also known as 'kernel discoloration' for barley (Anderson and Banttari, 1976). *Alternaria* sp. and *Cochliobolus sativus* (Ito and Korib) have been reported as the most common causal agents of black point (Machacek and Greaney, 1938) with *C. sativus* as the primary causal agent of kernel discoloration in barley in the midwest (Anderson and Banttari, 1976). Up to now no one has reported bacteria as one of the causal agents of this type of symptom.

The second type of blight, reportedly caused by the bacterium *Pseudomonas syringae* pv. *syringae* (Peters et al.,

1983), occurs more sporadically and is characterized by tan to dark brown necrotic spots with distinct margins on the lemma of the kernel (hereafter referred to as **spot kernel blight**) (Fig. 1b). This symptom type is called 'bacterial kernel spot of barley' and was first observed in large quantities on sprinkler irrigated "Klages" barley in southern Idaho in 1977 (Peters et al., 1983). Spot kernel blight has been observed to develop mainly in two-row barley cultivars rather than in six-row cultivars (personal communication Bob Miller, 1993 and Darryl Wesenberg, 1994) but the reason for this phenomenon is unknown. A very similar symptom to spot kernel blight has been reported to occur in wheat, barley, rye and grasses and is called "basal glume rot" reportedly caused by *P. syringae* pv. *atrofaciens* McCulloch 1920 (Toben et al., 1989).

The Bacterium Pseudomonas syringae van Hall 1902.

One of the main groups of phytopathogenic pseudomonads are those that produce diffusible fluorescent pigments, particularly in iron-deficient media such as King's B (King et al., 1954). Strains in this group usually do not accumulate poly- β -hydroxybutyrate (PHB) and do not utilize D-arabinose (Hildebrand et al., 1988). *Pseudomonas syringae* belongs to this group, and it is characterized as a gram-negative rod of ca. 0.7 to 1.2 by 1.5 to 3 μ m, occurring singly or in long chains or filaments. It is an obligate aerobe and is motile with polar multitrichous flagella.

Cultures of most strains produce slime in media containing two to four percent sucrose as a result of levan formation. Cytochrome *c* is not detectable and it is arginine dehydrolase negative. Optimal temperature for growth is ca. 25 to 30 C with no growth at 41 C. *P. syringae* is found on various plants and almost all strains produce a hypersensitive reaction in tobacco. The G + C content of the DNA is ca. 59-61 mole percent (Buchanan and Gibbons, 1974; Hildebrand et al., 1988).

Toxin Production. Plant pathogenic bacteria are equipped with an arsenal of virulence factors that condition the host for colonization as well as being involved in symptom expression. Nonhost-specific phytotoxins are generally acknowledged to be an element of virulence for many bacteria and this is especially evident among the pathovars of *P. syringae* (Gross, 1991). *P. syringae* represents a wide range of plant associated bacteria, including pathogens, weak pathogens or saprophytes. The nonhost-specific phytotoxins produced by pathovars of this bacterium are a family of structurally diverse compounds, usually peptide in nature, that in some cases display a wide-spectrum of antibiotic activity (Mitchell, 1981). Several structurally distinct classes of toxins that cause either chlorotic or necrotic symptoms in infected plant tissue are known to be produced by pathovars of *P. syringae* (Gross 1991). The chlorosis-inducing toxins are the most

common and are classified broadly as either tabtoxin (Barta *et al.*, 1992), phaseolotoxin (Staskawicz and Panopoulos, 1979), coronatine (Palmer and Bender, 1993; Young *et al.*, 1992) or tagetitoxin (Mitchell *et al.*, 1989). The necrosis-inducing toxins seem to be restricted to the pathovar *syringae* and encompass a family of structurally related lipopeptides including, syringomycin (Bidwai and Takemoto, 1987; Fukuchi *et al.*, 1990; Gross, 1991; Iacobellis *et al.*, 1992;), syringotoxin (Ballio *et al.*, 1990), syringopeptin (Ballio *et al.*, 1991; Iacobellis *et al.*, 1992), and pseudomycins (Harrison *et al.*, 1991). This discussion will be limited to syringomycin due to the apparent association of syringomycin producing strains with kernel blight.

Syringomycin Production by *P. s. pv. syringae*. Toxin synthesis and secretion are prevalent among the pathovars of *P. s. pv. syringae*, suggesting that strong selective pressure for toxigenic strains exists in the plant environment (Gross, 1991). One of these toxins, syringomycin, is the most common and most studied. Syringomycin is a lipodepsinopeptide consisting of a macrocyclic ring of nine amino acids with a fatty acid side chain. Three of the amino acids are uncommon: 4-chlorothreonine, 3-hydroxyaspartic acid, and 2,3-dehydrothreonine (Serge *et al.*, 1989; Fukuchi *et al.*, 1990). Although the biosynthetic pathway for syringomycin production has not been elucidated, it is expected to be

complex due to the unusual amino acids and the macrocyclic ring structure (Xu and Gross, 1988a; Hrabak and Willis, 1993). The mode of action of syringomycin is thought to involve increased plant cell uptake of calcium, resulting in disruption of ion transport across the plasmalemma and activation of a cascade of physiological events leading to cell death (Takemoto, 1992). Studies to determine syringomycin involvement in virulence or pathogenicity were performed by isolation of non-toxigenic mutants generated with the transposon Tn5. Mutants were quantitatively evaluated for their ability to multiply and cause disease in immature sweet-cherry fruits (Xu and Gross, 1988a). Results of these studies indicate that syringomycin is not essential for pathogenicity, but significantly contributes to virulence (Xu and Gross, 1988a). Following the genetic characterization of syringomycin production by *P. s. pv. syringae*, Xu and Gross (1988b) reported the existence of two genes, *syrA* and *syrB*, that are involved in syringomycin production and are required for the formation of proteins SR4 and SR5. These proteins are believed to be components of the syringomycin synthase complex. Another gene, *syrD* is thought to encode a protein that belongs to the ATP-binding cassette (ABC) superfamily of transporter proteins (Quigley et al., 1993). It is proposed that the *syrD* gene product is embedded in the bacterial cytoplasmic membrane and functions as an ATP-driven efflux pump for the secretion of

syringomycin (Quigley and Gross, 1994). The *lemA* gene has also been reported to be involved in production of syringomycin (Hrabak and Willis, 1993).

Characterization of Phytopathogenic Bacteria.

Biochemical and physiological tests. More than one criterium is necessary for the genetic characterization of phytopathogenic bacteria such as *P. syringae*. The use of pathogenicity and biochemical tests are important in the systematics of phytopathogenic bacteria (Hildebrand et al., 1988). Systems such as Biolog MicroStation™ (Biolog, Inc. Hayward, CA, 94545), based on the utilization of 95 carbon sources and fatty acid profiles, that combine cellular fatty acid analysis with computerized high resolution gas chromatography such as that performed by Microbial Identification Inc. (MIDI, Newark, DE) have been useful tools in the characterization of some pathogenic bacteria. Although these tests are useful in distinguishing most pathovars (Hildebrand et al., 1988), they cannot distinguish strain differences within a given pathovar (Legard et al., 1993).

RFLP Analysis. Combining biochemical and pathogenicity tests with analysis of restriction fragment length polymorphism (RFLP) of chromosomal DNA has become more and more useful in determining phylogenetic relationships among phytopathogenic bacteria. Hartung and Civerolo (1991)

utilized *in vitro* aggressiveness, RFLP analysis, and carbon source utilization profiles to study variation among strains of *Xanthomonas campestris* causing citrus bacterial spot. These studies showed that most aggressive strains of *X. campestris* belonged to a single RFLP group although members of this RFLP group varied in carbon source utilization (Hartung and Civerolo, 1991). The less aggressive strains comprised a continuum of RFLP types and usually could be separated from the more aggressive strains by carbon source utilization profiles. Similar results were obtained with *Xanthomonas campestris* pv. *citrumelo* Gabriel pv. Nov. using virulence tests, reaction to a panel of monoclonal antibodies and RFLP analysis. The highly aggressive strains of *Xanthomonas campestris* pv. *citrumelo* were correlated with RFLP and serological reaction patterns (Gottwald et al., 1991). Similarly, races and biovars among *Pseudomonas solanacearum* strains could be distinguished by RFLP analysis (Cook et al., 1989).

In the case of *P. syringae*, several studies have utilized RFLP analysis to differentiate strains both within and between pathovars, (Hendson et al. 1992; Legard et al., 1993, Denny et al., 1988; Quigley and Gross, 1994). Hendson et al., (1992) compared strains of *P. syringae* pathovars *tomato*, *maculicola*, *antirrhini*, and *apii* by RFLP patterns, nutritional characteristics, host origin and host ranges. Results of this study indicated that phylogenetic

relationships cannot be assessed solely on the basis of pathogenicity, nutritional characteristics alone, or RFLP analysis alone. The combination of multiple criteria would provide better characterization of bacterial strains (Hendson et al., 1992).

Genes involved in toxin production may be useful as probes for characterizing and identifying strains of *P. syringae* by RFLP analysis. Toxin synthesis and secretion are prevalent among the pathovars of *P. syringae*, and it appears that toxigenesis may reflect overall genetic differences (Hildebrand et al., 1982; Gross, 1991). Pathovars of *P. syringae* that produce a particular toxin appear to constitute distinct taxonomic clusters that share a high degree of genomic DNA relatedness (Hildebrand, 1982; Denny et al., 1988). For instance, genes required for phaseolotoxin production by *P. s. pv. phaseolicola* and coronatine production by *P. s. pv. tomato* and related pathovars have been used as specific probes for disease diagnosis (Cuppels et al., 1990; Shaad et al., 1989; Prosen et al., 1993). Sections of the tabtoxin biosynthetic region have been used in studies on the mechanisms of toxin regulation, and as specific probes for tabtoxin-producing strains of *P. syringae* including pathovars *tabaci*, *coronafaciens*, and *syringae* (Kinscherf et al., 1991). Quigley and Gross (1994) postulated that *syrB* and *syrD* genes encode proteins that function in the synthesis and export of

syringomycin, respectively, and are conserved among a broad spectrum of *P. s. pv. syringae* strains that produce syringomycin or one of its analogs, syringotoxin and/or syringostatin. These genes were absent in all *P. syringae* strains tested that did not produce syringomycin or syringomycin analogs (Quigley and Gross 1994). RFLP analyses were used to construct a dendrogram that revealed subclusters of strains that appear to share specific qualities relevant to plant-pathogen interactions (Quigley and Gross, 1994). A large proportion of the strains originally obtained from dicots exhibited the same RFLP profile while the few strains tested from monocots fell into two genetically distinct clusters that were quite divergent from the dicot strains (Quigley and Gross, 1994). Similarly, Denny et al. (1988) assessed the genetic diversity among a sampling of monocot and dicot strains of *P. s. pv. tomato* by RFLP analysis and found two clusters that were restricted to either monocot or dicot strains.

Another class of genes that appear useful in RFLP analysis to study genetic relationships among *P. syringae* strains are the *hrp* (hypersensitive reaction and pathogenicity) genes. The hypersensitive response (HR) is a rapid, localized necrosis of plant tissue that is observed when many phytopathogenic bacteria are inoculated into nonhost plant species or resistant varieties of susceptible plant species (Klement et al. 1990). Very little is known

about the biological mechanisms involved in this plant-microbe interaction, stimulating several studies on the genes, which have been designed *hrp*, that are involved in these mechanisms (Willis et al., 1991). The *hrp* genes have been found in many phytopathogenic bacteria including *Pseudomonas solanacearum* (Boucher et al., 1987), *Xanthomonas campestris* pathovars (Bonas et al., 1991; Daniels et al., 1988), *Erwinia amylovora* (Bauer and Beer, 1987) and several pathovars of *P. syringae* (Willis et al., 1991). Additional studies have shown that the *hrp* genes are conserved with respect to homology and function within *P. syringae* pathovars. Liang et al. (1993) compared *hrp* gene restriction maps of *P. s. pv. morsprunorum*, *P. s. pv. phaseolicola* and *P. s. pv. syringae*. This comparison revealed that there are greater similarities in restriction sites between DNA from pathovars *phaseolicola* and *morsprunorum* than between DNA from pathovars *syringae* and *morsprunorum*. Relatedness of *hrp* genes has also been reported among other pathogenic bacteria (Arlat et al., 1991). The use of low-stringency hybridization conditions revealed hybridization between the chromosomal inserts within pCPP430 and pHIR11, which contain the *hrp* clusters from *Erwinia amylovora* and *P. s. pv. syringae* (Laby and Beer, 1992). It has also been reported that predicted protein sequences of three *hrp* genes from *P. solanacearum* show remarkable sequence similarity to key virulence

determinants of animal pathogenic bacteria of the genus *Yersenia* (Cough et al., 1992). Similarly, Huang et al. (1993) characterized the *Pseudomonas syringae* pv. *syringae* 61 *hrpJ* and *hrpI* genes and the predicted encoded proteins and found that HrpI belongs to a superfamily of proteins that are found in *Yersenia petis* Lcr D.

In *P. s.* pv. *syringae* the *hrp* genes were identified by obtaining mutants of *P. s.* pv. *syringae* strain 61 that failed to induce HR in tobacco. A cosmid clone pHIR11 which had a 31-Kb chromosomal insert was reported to contain all genes necessary to restore HR not only in the mutants but also in non pathogenic bacteria such as *P. fluorescens* and *Escherichia coli* (Huang et al., 1988). This cosmid was used along with other random genomic fragments as probes in RFLP analysis for the construction of a dendrogram to study genetic relationships among *P. s.* pv. *syringae* strains (Legard et al. 1993). By these means, Legard et al. (1993) were able to show that strains of *P. s.* pv. *syringae* pathogenic to beans belonged to a taxonomic group that was distinct from strains of *P. syringae* pv. *syringae* pathogenic to other hosts.

Factors Involved in Development of Kernel Blight.

The uncertainty of the presence or absence of kernel blight and its variation in severity from year to year

suggest that environmental conditions and the physiological stage of the host may have an effect on this disease.

Kernel Developmental Stage. Basal kernel blight is normally difficult to observe in barley heads in the field and symptoms can usually only be observed after harvest. In contrast, spot kernel blight symptoms can be observed much easier and earlier during kernel development in the field. This may be due to the morphology of the barley head or to the time of onset of infection. Studies on wheat seed infection by *Pyrenophora tritici-repentis* (Died) indicated that the kernel developmental stage of the plant affected the incidence of infection (Schilder and Bergstrom, 1994). Wheat seeds were susceptible to infection throughout most of their development, from the end of anthesis through the soft dough stage. However, an increase in the incidence of *P. tritici-repentis*-infected seeds occurred when inoculation took place at milk stage (Schilder and Bergstrom, 1994). Teviotale and Hall (1976) reported that barley seeds were susceptible to *Pyrenophora graminea* (Ito and Kurib) from head emergence through the soft dough stage, but there was less transmission of fungus through infected seed the later the infection took place. In the case of kernel discoloration of barley, caused by *C. sativus*, the stage of kernel development when infection occurred did not appear to be an important factor (Luthey, 1962) and infection was observed to take place from the end of flowering through

maturity (Stevenson, 1981). However, some studies on the microbial population in barley heads have shown that the number of bacteria and filamentous fungi increase greatly from milk to late dough stage and that the greatest increase of infection by bacteria and yeast comes just before maturity (Follstad 1961). Peters et al. (1983) working with bacterial kernel spot (spot type of blight) caused by *P. syringae*, found that infection occurred at the beginning of kernel development, just before the lemma was attached to the seed. The possibility that the presence of a different window of infection for the different symptoms of kernel blight observed in Montana seemed likely and research in that area was included in this study.

Environmental Conditions. The amount of available moisture after heading has often been associated with kernel blight. Kernel discoloration and bacterial kernel spot have always been reported to be most severe after abundant rain or in fields with overhead irrigation (Follstad 1961, Lutey 1962, Stevenson 1981, Wilcoxson, et al., 1980; Miles et al., 1987; Wesenberg, 1994, personal communication; Peters et al., 1983). It has also been reported that the frequency with which fungi were isolated from barley kernels was increased during years with above normal rainfall (Follstad 1961, Lutey 1962). In Montana, the presence of both symptom types of kernel blight has also been associated with wet years but specific experiments have not been done in this

area. While the effect of moisture on the development of kernel blight has been studied extensively in other states, no information is available about the effect of temperature. Since *P. syringae* is known to have an optimum growth temperature around 28 C, temperature may also have an effect on the development of this disease.

Genetic Resistance. The degree of infection and discoloration in barley kernels seems to be influenced by different factors including the pathogen, plant genotype and environment (Miles et al., 1987). Genetic resistance is considered the most practical tool for control of kernel blight and breeding studies for kernel discoloration caused by *C. sativus* have been in progress for some time. (Bantary et al., 1975; Wilcoxson et al., 1980; Miles et al., 1987; Miles et al., 1989; Gebhardt et al., 1992). The standard screening process involves enhancing development of the disease by overhead irrigation with concomitant inoculation with *C. sativus* (Miles et al., 1987). In one of these studies it was found that irrigation significantly increased black stain severity. On the other hand, even though inoculation may have increased black stain severity, the effect was not statistically significant (Miles et al., 1987). Draper (1985), looking for genetic resistance to bacterial kernel spot, reported differences among ten barley cultivars suggesting the existence of genes for resistance. Differences were reported between two cultivars ("Liberty"

and "Moore") in the number of isolatable bacteria per gram of grain (Follstad, 1961). These differences seemed consistent and large enough to lend experimental evidence that there actually are differences among cultivars in susceptibility to invasion by bacteria (Follstad, 1961). A common limitation in some screening programs is the lack of reliability of the screening method used, a factor that has been one of the goals of this project. Symptommatological and etiological studies of kernel blight allow a better understanding of this disease and the results can be utilized in the development of a reliable method of screening for genetic resistance.

CHAPTER 2

SYMPTOMATOLOGY AND STUDY OF THE CAUSAL AGENTS OF
KERNEL BLIGHT OF BARLEY IN MONTANAIntroduction

'Kernel blight' and 'kernel discoloration' have been used to describe a barley disease of importance to the malting and brewing industries, referred to as kernel blight in this study. Microorganisms present in infected kernels can detrimentally affect different steps of the malting and brewing processes and thereby the quality of the final product. This disease is commonly present in the upper Midwestern region of the United States (Miles *et al.*, 1987, Gebhardt *et al.*, 1992). In the past several years it has occurred with some severity in Northcentral Montana, where it has caused concern to local growers and users of malting barley.

Two symptom types of kernel blight have been observed in Montana. The most common type consists of dark brown discoloration of the embryo end of the kernel, hereafter referred to as basal kernel blight (Fig. 1a), and generally attributed to fungal infection. This symptom has previously been called 'black point' or 'kernel discoloration' and has been observed on wheat, barley, and rye (Machacek and Greaney, 1938; Anderson and Bantary, 1976, Mathre, 1982).

Several fungi have been reported to cause this symptom including *Alternaria* spp., *Cochliobolus sativus* (Ito and Korib), and *Fusarium* spp. (Machacek and Greaney, 1938; Anderson and Banttari, 1976; Banttari et al., 1975; Mathre, 1982). Until now, no one has proposed bacteria as the causal agent of this symptom. The second type of blight, reportedly caused by the bacterium *Pseudomonas syringae* pv. *syringae* (Peters et al., 1983), occurs more sporadically and is characterized by tan to dark brown necrotic spots with distinct margins on the lemma of the kernel, hereafter referred to as spot kernel blight (Fig. 1b). Results of this research indicated that species of *P. syringae* were associated with both types of blight. Preliminary evidence suggested that these strains were phylogenetically different.

For assessment of possible phylogenetic relationships of phytopathogenic bacteria such as *P. syringae*, more than one criterium is necessary. The use of pathogenicity and biochemical tests have traditionally been important in the systematics of phytopathogenic bacteria (Hildebrand et al., 1988). These tests are not always adequate to define pathovars, nor do they appear useful in distinguishing strain differences within a pathovar (Legard et al., 1993). However by combining biochemical and pathogenicity tests with other tools, such as analysis of restriction fragment length polymorphism (RFLP) and serological techniques, it

should be possible to ascertain and assess phylogenetic relationships among phytopathogenic bacteria (Hartung and Civerolo, 1991; Gottwald et al., 1991).

In the case of *P. syringae*, several studies have utilized RFLP analysis in the characterization of strains between and within pathovars, (Hendson et al., 1992; Legard et al., 1993; Denny et al., 1988; Quigley and Gross, 1994). Toxin synthesis and toxin secretion are common characteristics among pathovars of *Pseudomonas syringae*, and it appears that peculiarities of toxigenesis may reflect overall genetic differences (Gross, 1991; Hildebrand et al., 1982). Thus, whole genes or fragments of genes involved in toxin production have proven useful as probes for characterizing toxigenic strains of *P. syringae* (Cuppels et al., 1990; Shaad et al., 1989). Sections of DNA of the tabtoxin biosynthetic region have been used as specific probes for tabtoxin-producing strains of different pathovars of *P. syringae* including *tabaci*, *coronafaciens*, and *syringae* (Kinscherf et al., 1991). Quigley and Gross (1994) demonstrated that *syrB* and *syrD* genes are conserved among a broad spectrum of *P. s. pv. syringae* strains that produce syringomycin or one of its analogs, syringotoxin or syringostatin. Thus, these probes proved to be very useful in studies of genetic relationships among syringomycin-producing strains (Quigley and Gross, 1994). Other genes that also appear useful in RFLP analysis are those in the

hrp (hypersensitive reaction and pathogenicity) region. By using these genes as probes, Legard et al. (1993) constructed a dendrogram showing that *P. s. pv. syringae* strains that were pathogenic to beans belonged to a taxonomic group distinct from the strains of *P. syringae* pv. *syringae* pathogenic in other hosts.

The present study was initiated to determine the causal agent of kernel blight of barley in Montana. When *P. syringae* was found to be the primary causal agent, two types of this bacterium were identified, each appearing to cause a distinct symptom and the molecular methodologies described above were applied to these bacteria to determine their relationships to each other and to other phytopathogens.

Materials and Methods

Samples.

Field samples showing kernel blight symptoms were collected from different barley cultivars grown in Northcentral Montana and from the Midwestern states of North Dakota, Minnesota, and South Dakota. A total of 91 samples were obtained from the 1991, 1992, and 1993 growing seasons. A description of these samples is presented in Table 1. Each sample consisted of approximately 500 g of kernels and was obtained from a single field. Symptomatic kernels showing basal or spot kernel blight were analyzed for the presence of bacteria and fungi.

Fungal Isolation.

Fifty symptomatic kernels were chosen from each sample in 1991 and 10 each from the 1992 and 1993 samples. Each kernel was considered a subsample and the type of symptom was recorded. Symptomatic kernels were surface sterilized in a solution of 0.5% (v/v) sodium hypochlorite (household bleach) and 10% (v/v) ethyl alcohol for 5 minutes followed by three rinses in sterile distilled water. Kernels were placed on potato dextrose agar (PDA) containing 10 ug/ml of streptomycin dissolved in 70 percent ethanol and added after autoclaving. All laboratory chemicals were obtained from Sigma Chemical Company, St. Louis MO., unless otherwise noted. After 4 to 6 days, fungal colonies were purified by the obtention of monosporic cultures and when possible identified under the microscope. Frequency of fungi present in symptomatic kernels was determined.

Bacterial Isolation.

Subsamples were obtained as described for fungal isolation. Symptomatic kernels were soaked individually in sterile phosphate buffer solution (PBS) (0.5M potassium phosphate, 0.75% sodium chloride) for two hours at 4 C, and then transferred to a rotary shaker at 250 rpm at room temperature for an additional 30 minutes. A ten-fold dilution series was prepared in PBS and spread onto the semi-selective medium KBBC, consisting of King's B medium

Table 1. Malting barley samples obtained from Northcentral Montana and North Dakota, South Dakota, and Minnesota during the growing seasons 1991, 1992, and 1993.

Locality	Year	Cultivar	Blight type	# samples
Montana				
	1991	B1202	Spot	9
		B1202	Basal	2
		B2601	Basal	11
		Klages	Spot	2
	1992	B5133	Spot	1
		B1202	Spot	2
		B1202	Basal	4
		B2601	Basal	6
	1993	B1202	Mixture ^a	10
		Harrington	Mixture	2
		Harrington	Mixture	2
		Morex	Mixture	4
		B2601	Mixture	3
North Dakota				
	1991	Robust	Basal	2
	1992	Robust	Basal	2
		Morex	Basal	3
	1993	Robust	Basal	7
		B2912	Basal	1
		Morex	Basal	2
South Dakota				
	1991	Morex	Basal	1
	1992	Morex	Basal	1
Minnesota				
	1991	Campbell	Basal	1
		Crookstan	Basal	1
	1992	Robust	Basal	2
	1993	Robust	Basal	7
		Excel	Basal	3

^a Mixture = Samples that had kernels with spot and kernels with basal kernel blight.

(KB) (King et al., 1954) amended with 1.5 ug/ml boric acid (autoclaved separately), and 80 ug/ml of cephalexin (Mohan and Shaad, 1987) and 20 ug/ml of cycloheximide. Antibiotics were dissolved in 70 percent ethanol and added after autoclaving. Plates were incubated in the dark at 28 C for 48 hours and *Pseudomonas*-like colonies were selected visually by pigment production and/or fluorescence under UV (Model UVL-21, BLAK-RAY, Ultra-Violet Products, Inc., San Gabriel, California) for further analysis. Isolates were tested for oxidase activity (Kovacs, 1965); hypersensitivity (HR) in tobacco leaves (*Nicotiana tabacum* cv. *Xanthi*) by infiltration of ca. 10^9 cfu/ml bacterial suspensions in sterile distilled water (Huang et al., 1988); and for arginine dihydrolase activity (Hildebrand et al., 1988). Other non-*Pseudomonas*-like bacteria were also purified and tested for their hypersensitive reaction in tobacco plants to detect potential pathogens.

Bacterial Inoculation in the Greenhouse.

Pathogenicity tests were performed under greenhouse conditions at temperatures of 22 to 24 C during the day and 12 C during the night with 14 hr light periods. In all greenhouse experiments, each replication consisted of three plants in a 19 cm pot. Each experiment was conducted in a completely randomized design with three replications per treatment. Data were analyzed by Analysis of Variance using

the MSU statistical program (Montana State University, Bozeman MT.). Each experiment was repeated twice.

Two barley cultivars, B2601 (six-row) and B1202 (two-row) from Bush Ag Resources were planted in the greenhouse. The heads of two sets of plants were inoculated during kernel development. One set was inoculated with *P. s. pv. syringae*, strain 552 isolated from kernels with basal kernel blight and the other set was inoculated with *P. syringae* strain 554 obtained from kernels with spot kernel blight. For inoculum, the bacteria were grown for 24 hrs on KB and a 1:10 dilution was made of a suspension yielding a turbidity of 80 Klett units on a Klett-Summerson spectrophotometer with a green filter (ca. 10^7 cfu/ml) was prepared in sterile distilled water with 0.025% Tween 20 (polyoxyethylenesorbitan monolaurate). Plants were inoculated by spraying barley heads at the milk stage with the bacterial suspension using a hand air brush (Model Paaschi D500 1/10 H.P.) until runoff. Control plants were sprayed with a water solution of 0.025% Tween 20. After inoculation, plants were incubated for 48 hrs in a mist chamber in the greenhouse providing continuous wetness and then transferred to the greenhouse bench. At maturity, inoculated heads were harvested and kernel blight was visually evaluated and percentage of blighted kernels by weight was obtained by comparison to the weight of the total amount of kernels.

Syringomycin Assay.

The putative *Pseudomonas syringae* isolates were tested for production of syringomycin *in vitro* by measuring antifungal activity against *Geotrichum candidum* Link following the technique of Gross (1985). Bacteria were transferred with a sterile toothpick onto plates of either potato dextrose agar (PDA) supplemented with 1% glucose or the defined toxin production medium SRM and incubated at 25 C for 5 days (Gross, 1985). Toxin production was determined by spraying the plates with a suspension of the toxin-sensitive fungus *G. candidum* strain F-260 (provided by D.C. Gross, Washington State University). After 24 to 48 hours of incubation, a zone of inhibition of *G. candidum* around a bacterial colony was considered indicative of syringomycin production. The zone of inhibition was measured from edge of colony to edge of zone to give a semi-quantitative evaluation of the amount of toxin produced.

Biochemical Tests on Bacterial Strains.

A sample of 60 oxidase negative, HR (+) strains isolated from kernels showing the two different types of blight symptoms were selected from the large collection of bacterial strains obtained during initial isolations. Two known *P. s. pv. syringae*, strains B301D (pear pathogen) and SD202 (wheat pathogen), supplied by D.C. Gross, were included for comparison. The strains were tested for

utilization of carbon sources including glucose, lactic acid, trigonelline and quinate (Hildebrand et al., 1988). A subsample of these strains was also tested by using the Biolog MicroStation™ (Biolog, Inc. Hayward, CA, 94545), which is based on the utilization of 95 carbon sources, and by fatty acid profiles which combines cellular fatty acid analysis with computerized high resolution gas chromatography performed by the Microbial Identification System (MIDI, Newark, DE).

Reaction to a Polyclonal Antiserum.

The same 60 strains described above were also tested for the reaction to a polyclonal antiserum. The antiserum was produced against strain 384 which belongs to the *P. syringae* 554 group. Reaction to the antiserum was performed by indirect ELISA test employing goat anti-rabbit antibodies conjugated to horseradish peroxidase and detected with azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and hydrogen peroxide (Harlow and Lane, 1988).

RFLP Analysis.

Strains. The same 60 strains were also utilized for genetic characterization studies. *P. syringae* and *E. coli* strains were grown in Luria-Bertani (LB) broth (Sambrook et al. 1989) and stored in 15% glycerol at -80 C for long term storage.

Preparation of Total Genomic and Plasmid DNA. *P.*

syringae strains were grown overnight in LB broth on a rotary shaker at 28 C. Three ml of bacterial suspension were used for preparation of total DNA by obtaining a pellet by centrifugation. Pelleted cells were washed twice in sterile deionized water and genomic DNA was prepared using the S&S Elu-Quik DNA purification Kit (Schleicher & Schuell, Kleene, NH). The protocol followed was as suggested by the manufacturer with the only modification being that lysed cells were treated with 2 ng of RNase at 37 C for 15 min. All the plasmids used were maintained in *E. coli* strain DH5 and multiplied by growing the bacteria overnight at 37 C in LB broth with the appropriate antibiotics. Plasmid DNA was prepared by the standard boiling method (Sambrook et al. 1989).

Digestion of DNA, Separation of Restriction Fragments, and Alkaline DNA Transfer.

Total genomic DNA was digested to completion with the restriction enzyme *Pst*I as recommended by the supplier (New England BioLabs). Approximately 0.5 to 1.0 ug of digested DNA was separated by gel electrophoresis on 0.8 % agarose gel in Tris-acetate buffer at 50 to 60 volts for 3-4 hours. Molecular size standards of lambda DNA digested with *Hind*III were run concurrently on each gel to allow size comparisons. DNA fragments were transferred to Hybond-N+ nylon membranes (Amersham International plc, Amersham UK) under alkaline

conditions (0.4 M KOH) overnight and baked at 80 C for 30 minutes, following the manufacturer's instructions.

DNA Probes. Four DNA probes were utilized in this study. Probes consisted of excision fragments obtained from four different plasmids. One probe was excised from p91 and consisted of the 1.1-Kbp *SalI* internal fragment of the *SyrB* gene, reported to be present in all *P. syringae* pv. *syringae* strains that produce syringomycin or its amino acid analogs. This plasmid was provided by D. C. Gross (Quigley and Gross, 1994). Two probes were individual fragments from the 30 Kbp insert containing the *hrp* region from *P. s.* pv. *syringae* (Huang et al., 1988). These fragments were 10-Kbp and 14-Kbp *EcoRI* inserts excised from pLY9 and pLY14, respectively, provided by S. W. Hutcheson of the University of Maryland (Heu and Hutcheson, 1993). DNA regions included in these fragments are known to be essential for the production of HR activity in *P. s.* pv. *syringae* (Huang et al 1991). The fourth probe was a 5.3 Kbp *PvuII* fragment excised from pQZ4100, a plasmid provided by D. K. Willis, University of Wisconsin (Kinscherf et al., 1991). After plasmid extraction, each vector was digested with the appropriate enzyme and separated by electrophoresis in low melting temperature agarose gels containing ethidium bromide using standard procedures (Sambrook et al., 1989). Inserts were excised from the gel with a razor blade and the DNA was eluted by using the MagicTM PCR Preps DNA purification

system for rapid purification of DNA fragments (Promega, Madison, WI).

DNA Hybridization. DNA probes were labelled with the non-radioactive ECL (enhance chemiluminescence) direct nucleic acid labelling and detection system of Amersham International plc (Amersham, UK). The hybridization buffer was adjusted to 0.25 M NaCl and after one hour pre-hybridization, the labelled probe was added to the solution and hybridization was carried overnight at 42 C. Hybridized membranes were washed in two changes of primary wash buffer (6M urea, 0.4% SDS and 0.1x SSC) at 42 C for 20 min each followed by two five minute washes in 2x SSC at room temperature. Procedures for signal generation and detection were done as described by the manufacturer. Membranes were then exposed to autoradiography film (Hyperfilm-ECL, Amersham) for 1 to 20 min at room temperature. Each probe was individually hybridized to the membranes, scored, and the membrane stripped as recommended by the membrane manufacturer before reprobing with the next probe.

Genetic Relationships of *P. syringae* Strains Based on RFLP Profiles. The size of restriction fragments hybridizing with each probe was determined by comparison with the lambda DNA size standards. Strains were evaluated for the presence or absence of fragments of specific sizes. Data from separate hybridizations of *Pst*I digests with the

three of the DNA probes were combined for analysis as reported by Legard *et al.* (1993). Pairwise comparisons were made by the NTSYS-pc program version 1.8 (Exeter Publishing Ltd., Setauket, N.Y.) using the following equation: $F = 2n_{xy} / (n_x + n_y)$, where n_{xy} is the number of hybridizing fragments shared by the two strains and n_x and n_y are the total number of fragments in strain x and y, respectively (Legard *et al.*, 1993). A similarity matrix of F values was constructed using the NTSYS program and cluster analysis of distances resulted in a dendrogram using the unweighted pair group method with averages, UPGMA.

Results

Symptoms.

Both basal and spot kernel blight symptoms (Fig. 1a and 1b) occurred in Montana samples in 1991 and 1992, yet all symptomatic kernels from a single sample or field had only one type of blight. In 1993, a year with more rainfall, every Montana sample had kernels with basal and kernels with spot kernel blight. Only basal kernel blight was observed in all symptomatic kernels from the upper midwest samples for the same three years.

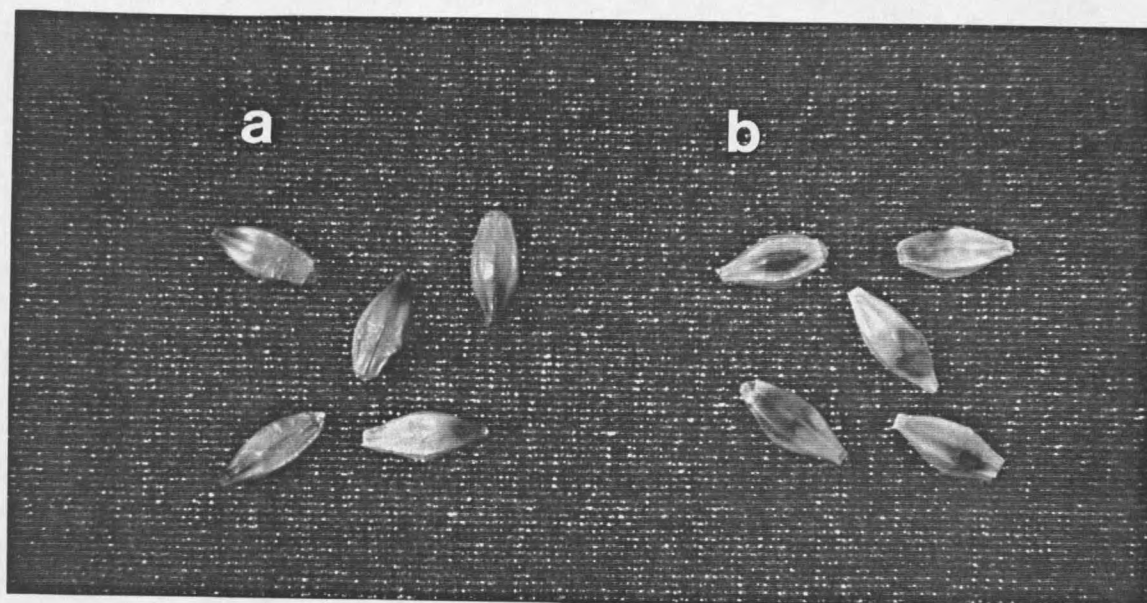


Figure 1. Symptom types of kernel blight. (a) Basal kernel blight characterized by dark brown discoloration on the embryo end of the kernel. (b) Spot kernel blight characterized by tan to dark brown necrotic spots with distinct margins on the lemma of the kernel.

Fungal Isolation.

Several fungi were obtained from symptomatic kernels but no single fungus was isolated consistently. When possible, fungi were identified microscopically by morphological characteristics. The most common fungi isolated were different types of *Alternaria* but no single type was consistently found in diseased kernels. Other fungi sporadically found in the Montana samples were *Pyrenophora teres*, *C. sativus*, *Fusarium* spp. and *Arthrinium*

arundinis. Since *C. sativus* and *Alternaria* sp. are the most common pathogenic fungi reported to cause this disease, the percent of these fungi present in analyzed samples was determined (Table 2). In all Montana samples examined *C. sativus* was found to occur in low frequency (2 to 12 percent) or not at all as in 1993. In the case of *Alternaria* spp., due to its variability and large number of saprophytic species, no definitive conclusions were made. In the samples from the upper midwest, *C. sativus* was found in the 1991 samples at significant percent but not at all in the 1992 samples (Table 2). In 1993, *C. sativus* was isolated from only four percent of the blighted kernels obtained from North Dakota and Minnesota (Table 2), while *Fusarium* spp. occurred in almost 100 percent of the tested kernels. It should be noted that 1993 was an unusually severe year for scab caused by *Fusarium* spp. in the midwest but not in Montana.

Bacterial Isolation.

Bacterial isolates obtained from blighted kernels were tested for oxidase, arginine dihydrolase activity and for hypersensitivity reaction (HR) in tobacco leaves. Only fluorescent *Pseudomonas*-like isolates that were HR (+), oxidase (-), and arginine dehydrolase (-) (traits indicative of *P. syringae*), were used to determine percentage occurrence and to suggest further characterization. All

Table 2. Relative frequency with which *Pseudomonas syringae* (P.s), *Cochliobolus sativus* (C.s), and *Alternaria* sp. (A.sp) were isolated from blighted kernels of malting barley produced in Montana, North Dakota, South Dakota, and Minnesota in 1991, 1992 and 1993.

State	1991			1992			1993		
	P.s	C.s	A.sp	P.s	C.s	A.sp	P.s	C.s	A.sp
Montana ^a	90 ^e	2	24	89	12	32	100	0	47
N. Dakota ^b	5	16	71	43	0	90	20	4	34
S. Dakota ^c	10	38	28	20	0	90	ND ^f	ND	ND
Minnesota ^d	30	22	20	75	0	89	15	4	16

^a 24 samples in 1991, 13 in 1992, and 21 in 1993.

^b 2 samples in 1991, 5 in 1992, and 10 in 1993.

^c 1 sample in 1991 and 1 in 1992.

^d 2 samples in 1991, 2 in 1992, and 10 in 1993.

^e Percentage of subsamples from which each organism was isolated. Fifty subsamples per sample in 1991 and 10 subsamples from 1992 and 1993 samples were analyzed.

^f ND = Non determined.

non-*P. syringae* isolates found in symptomatic kernels were HR(-) and were identified as saprophytes including *Erwinia herbicola*, *Erwinia* spp., and some coryneform types (Hildebrand et al., 1988). *P. syringae* was present in approximately 90 percent of the subsamples analyzed from Montana in 1991 and 1992 and in 100 percent of the subsamples in 1993 (Table 2). In the samples obtained from the upper midwest, *P. syringae* strains were present to a lesser extent than in Montana samples. A higher percentage of symptomatic kernels from these states had *P. syringae* in 1992 than in 1991 or 1993 (Table 2).

Bacterial Inoculation in the Greenhouse.

Koch's Postulates were fulfilled in greenhouse studies with each of the two types of *P. syringae*, *P. s.* pv. *syringae* and *P. syringae* strain 554. Either type of kernel blight occurred in inoculated plants depending mainly on the kernel developmental stage at the time of inoculation (see chapter 3). Plants inoculated with either type of bacterium at early milk stage primarily developed spot kernel blight and plants inoculated after late milk stage developed basal kernel blight. Blighted kernels obtained from these plants were assessed *in vitro* for the presence of the inoculated organism. The inoculated bacterium was isolated from 100% of the blighted kernels and from less than 1% of the

asymptomatic kernels. Symptoms occurred occasionally on control plants sprayed with sterile distilled water. Nonetheless, inoculated *P. syringae* strains were always found on these kernels and it was assumed that such infection resulted from air-borne contamination from adjacent inoculated plants in the mist chamber.

Syringomycin Production.

A total of 1621 *P. syringae* isolates obtained from kernels with either basal or spot kernel blight from the three years were characterized based on the production or lack of production of syringomycin (Figure 2). The number of isolates analyzed from each year and type of symptom were as follows: From 1991, 279 isolates from basal kernel blight and 432 from spot type; from 1992, 572 from basal and 192 from spot; and from 1993, 67 from basal and 79 from spot kernel blight. Two major groups or pathovars of *P. syringae* were established based on syringomycin production. Members of one group produced the toxin, syringomycin, a trait that indicates that this group of strains is *P. s. pv. syringae* (*Pss*) (Quigley and Gross 1994). A rough measurement of syringomycin production was estimated by the size of the zone of inhibition to *G. candidum* which resulted in two subgroups of toxigenic strains. Strains of one subgroup produced large zones of inhibition (8 to 10 mm), while

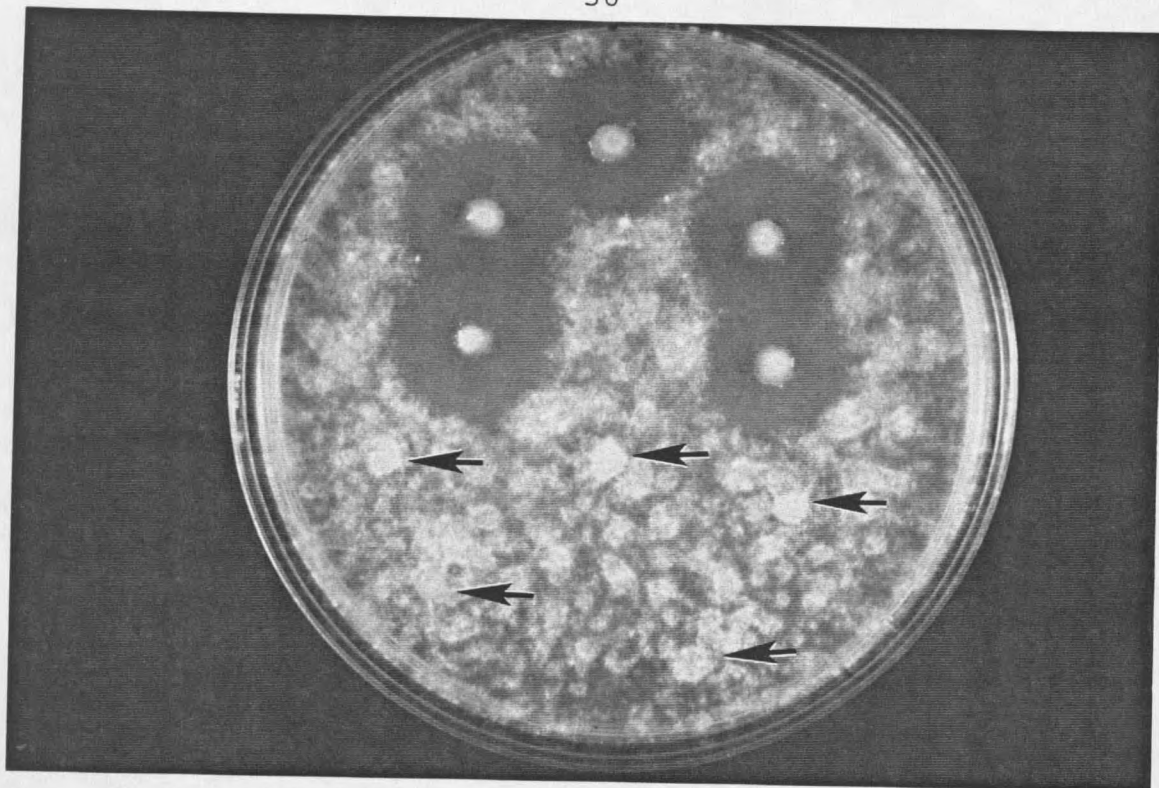


Figure 2. Syringomycin assay. Syringomycin production by *P. syringae* strains was tested for antifungal activity against *Geotrichum candidum*. Zone of inhibition around a bacterial colony was considered indicative of syringomycin production. The 5 colonies in the top of the plate were positive for syringomycin production, while the 5 colonies in the bottom of the plate were clearly negative. Arrows indicate the position of the non-syringomycin producing strains.

strains of the other subgroup produced small zones of inhibition (2-4 mm). All syringomycin-producing strains were found to occur primarily in those kernels with basal kernel blight (Fig. 3). In the 1991 and 1992 samples, more

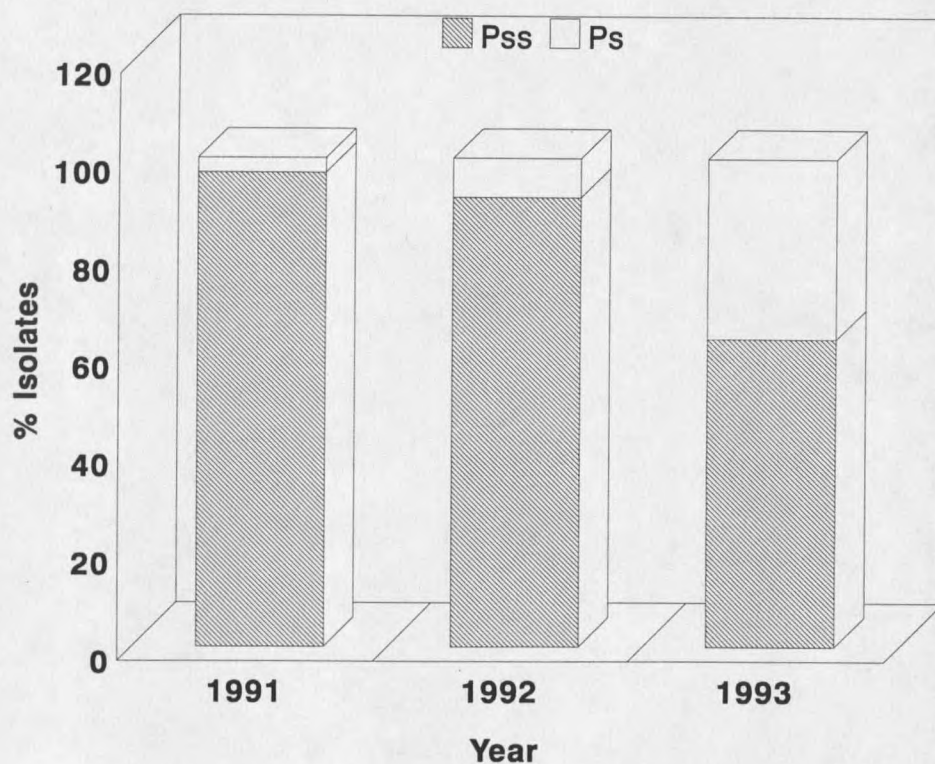


Figure 3. Percentage of *P. syringae* pv. *syringae* (Pss) and *P. syringae* 554 (Ps) strains isolated from samples of malting barley showing basal kernel blight. Samples were obtained from northern Montana, North Dakota, South Dakota and Minnesota in the growing seasons of 1991, 1992 and 1993.

than 90 percent of the *P. syringae* strains isolated from basal kernel blight were *P. s. pv. syringae* (Fig. 3), while in 1993 this percentage dropped to 63. All samples from the upper midwest were basal kernel blight and 100 percent of the *P. syringae* strains isolated from these samples produced syringomycin thus placing them in the *P. s. pv. syringae* group.

The second group of *P. syringae* (*Ps*) strains did not produce syringomycin. This group of strains was associated with spot kernel blight (Fig. 4). One hundred percent of the isolates from 1991 and 1992 samples with spot kernel blight were identified as *P. syringae* belonging to this second group, while in 1993 the percentage was 74 percent (Fig. 4).

Pathovar Identification.

Carbohydrate utilization comparisons using the Biolog system and fatty acid profiles using MIDI were conducted for a subsample of strains from both groups of bacteria and the results are presented in Table 3. In addition to the above mentioned systems, individual tests were also performed for utilization of glucose, L-lactate, trigonelline and quinate as carbon sources. These tests were done on a total of 60 strains, 33 that produced syringomycin (group 1) and 27 that did not produce syringomycin (group 2). The presence of two

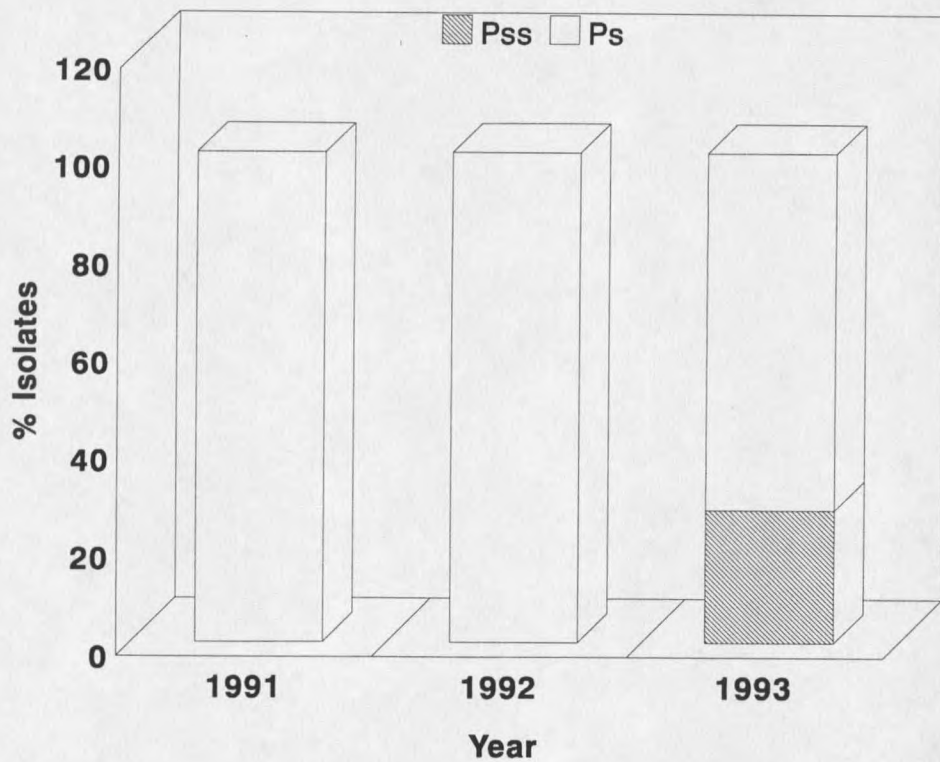


Figure 4. Percentage of *P. syringae* pv. *syringae* (Pss) and *P. syringae* 554 (Ps) strains isolated from samples of malting barley showing spot kernel blight. Samples were obtained from Northern Montana during 1991, 1992 and 1993.

Table 3. Characterization of *Pseudomonas syringae* strains utilized in this study.

Strain Number	Toxin (mm) ^a	Anti-serum	Taxonomic Identification ^b	
			Biolog	MIDI

<i>P. syringae</i> pv. <i>syringae</i> strains from different hosts ^c				
B301D	10	-	ND ^d	ND
SD202	10	-	ND	ND

Strains isolated from basal kernel blight ^e				
552	10	-	<i>P.s. syringae</i> B	<i>P. syringae</i>
537	10	-	<i>P.s. hibisci</i>	ND
592	10	-	<i>P.s. morsprunorum</i>	ND
594	10	-	<i>P.s. morsprunorum</i>	ND
605	10	-	ND	ND
330	10	-	ND	ND
334	10	-	ND	ND
393	10	-	ND	ND
351	10	-	ND	ND
360	10	-	ND	ND
489	10	-	ND	ND
593	10	-	<i>P.s. morsprunorum</i>	ND
610	10	-	ND	ND
607	10	-	<i>P.s. syringae</i> A	ND
346	10	-	ND	ND
392	10	-	<i>P.s. morsprunorum</i>	ND
595	10	-	<i>P.s. syringae</i> A	ND
348	10	-	ND	ND
490	10	+	<i>P.s. apata</i>	<i>P.s. atrofaciens</i>
491	10	+	ND	ND
541	10	+	<i>P.s. syringae</i> B	ND
542	10	+	ND	ND
648	2	+	ND	ND
652	2	+	ND	ND
651	2	+	ND	ND
600	2	-	ND	ND
645	2	+	ND	ND
646	2	+	ND	ND
649	2	+	ND	ND
643	2	+	ND	ND
650	2	+	ND	ND
647	2	+	ND	ND
644	2	+	ND	ND

Table 3. Continuation.

Strain Number	Toxin (mm)	Anti-serum	Taxonomic Identification	
			Biolog	MIDI
Strains isolated from spot kernel blight ^f				
418	0	+	ND	ND
536	0	+	<i>P.s. apata</i>	<i>P. syringae</i>
384	0	+	<i>P.s. tabaci</i>	<i>P.s. tabaci</i>
419	0	+	ND	ND
421	0	+	ND	ND
423	0	+	<i>P.s. tabaci</i>	<i>P.s. coronafaciens</i>
427	0	+	ND	ND
428	0	+	ND	ND
429	0	+	ND	ND
492	0	+	<i>P.s. hibisci</i>	ND
494	0	+	ND	ND
498	0	+	ND	ND
500	0	+	<i>P.s. tabaci</i>	<i>P.s. coronafaciens</i>
504	0	+	ND	ND
510	0	+	ND	ND
448	0	+	ND	ND
517	0	+	<i>P.s. porri</i>	ND
520	0	+	ND	ND
588	0	+	<i>P.s. hibisci</i>	ND
590	0	+	<i>P.s. helianthi</i>	ND
597	0	+	<i>P. syringae</i>	ND
598	0	+	ND	ND
599	0	+	ND	ND
601	0	+	ND	ND
602	0	+	<i>P.s. hibisci</i>	ND
603	0	+	ND	ND
604	0	+	ND	ND

^a Zones of inhibition of *G. candidum* as measured from the margin of the bacterial colony to the point of fungal growth on PDA and on SRM. A value of 10 for zone between 8-10 mm and 2 for zones between 2-4 mm. Each value is the average of two trials conducted on separate occasions.

^b Some strains were analyzed for identification by the Biolog system and some were sent to MIDI (Microbial Identification System Inc) to be identified.

^c Strain B301D originated from cherry tree (*Prunus avium* L) and SD202 from wheat (*Triticum aestivum*); both of them identified as *P.s. pv. syringae*. These strains were kindly provided by D. C. Gross.

^d ND = Not determined.

^e All syringomycin-producers were characterized by utilization of L-lactate and trigonelline.

^f All non-syringomycin producers were characterized for non utilization of L-lactate or trigonelline.

groups coinciding with the grouping based on syringomycin production was observed. All 60 strains utilized both glucose and quinate, however, only the syringomycin-producing strains also utilized L-lactate and trigonelline. The production of syringomycin and carbon source utilization confirmed the classification of group one as strains of *P. s. pv. syringae* (Quigley and Gross, 1994; Hildebrand et al., 1988). All of the strains from group 2 (non-syringomycin-producing strains), which did not utilize L-lactate or trigonelline, apparently would be closely related to *P. s. pv. coronafaciens* (Hildebrand et al., 1988). But no pathovar was defined for these strains. Therefore, this group is being called *P. syringae* 554.

Reaction to a Polyclonal Antiserum.

Results of reactions to a polyclonal antiserum are presented in table 3. All strains belonging to the *P. syringae* 554 group reacted positively to the antiserum. Positive reactions were also observed among all the *P. s. pv. syringae* strains, except strain 600, that produced small zones of syringomycin production. Only four toxigenic strains producing large zones of inhibition, strains 490, 491, 541 and 542, reacted positively to the antiserum. A more purified antiserum, obtained by subtraction absorption to an acetone powder (Harlow and Lane, 1988) of strain 541,

resulted in negative reactions to strains 541 and 542, but retained positive reactions to other syr (+) strains that had reacted to the unpurified antiserum. Efforts to absorb the antiserum to additional reacting strains of *P. s. pv. syringae* resulted in almost complete loss of the antiserum reaction for all strains including the *P. syringae* 554 group.

RFLP Analysis.

RFLP analysis was performed with the thirty-three *P. s. pv. syringae* strains isolated from basal kernel blight which produced syringomycin and utilized glucose, L-lactate, trigonelline and quinate. Also analyzed were the twenty-seven *P. syringae* 554 strains isolated from spot kernel blight that did not produce syringomycin, utilized glucose and quinate but not L-lactate or trigonelline (Table 3).

Genetic Relationships of *P. syringae* Strains Based on

RFLP Profiles. None of the 60 strains hybridized to the 5.3 Kbp probe, reportedly specific to tabtoxin-producing strains, a toxin that is known to be normally produced by *P. s. pv. coronafaciens* (Barta et al., 1992). Thus, the cluster analysis and resulting dendrogram were performed only with data obtained from the other three probes. The

fragment from *syrB*, a 1.107 Kbp probe, readily hybridized to all the *P. s. pv. syringae* strains (lanes A through H, Fig. 5a) but to none of the non-syringomycin producing strains (lanes I and J, Fig. 5a). Nine groups of RFLP profiles (1 to 9) were found distributed among all hybridizing strains (Appendix A, Table 10), examples of four of these patterns are shown in lanes A through H, (Fig. 5a). RFLP groups were compared to the zone of inhibition produced by each strain in the syringomycin test. The largest RFLP group designated number 1 included 54% of all the strains that produced large zones of inhibition (10mm) and strain SD202 also belonged to this group (Examples in lanes A and B Fig. 5a) (Appendix A, Table 10). The other 45% of the strains that produced large zones were distributed in 4 groups (groups 2 to 5, Appendix A, Table 10). Group 4 enclosed most of these strains and examples of this group are shown in lanes C and D (Fig. 5a). Eight out of the eleven strains that produced small zones of inhibition (2mm) presented a single RFLP group (group 6, Appendix A, Table 10) and examples of this group are shown in lanes E and F (Fig. 5a). Two of the other three strains resulted in a single group (group 8) shown in lanes G and H (Fig. 5a). Strain B301D, originally from a cherry tree, was found to belong to an individual RFLP group designed 9. With exception of the last three strains that produced small zones of inhibition (644, 647, and 650) and strain B301D,

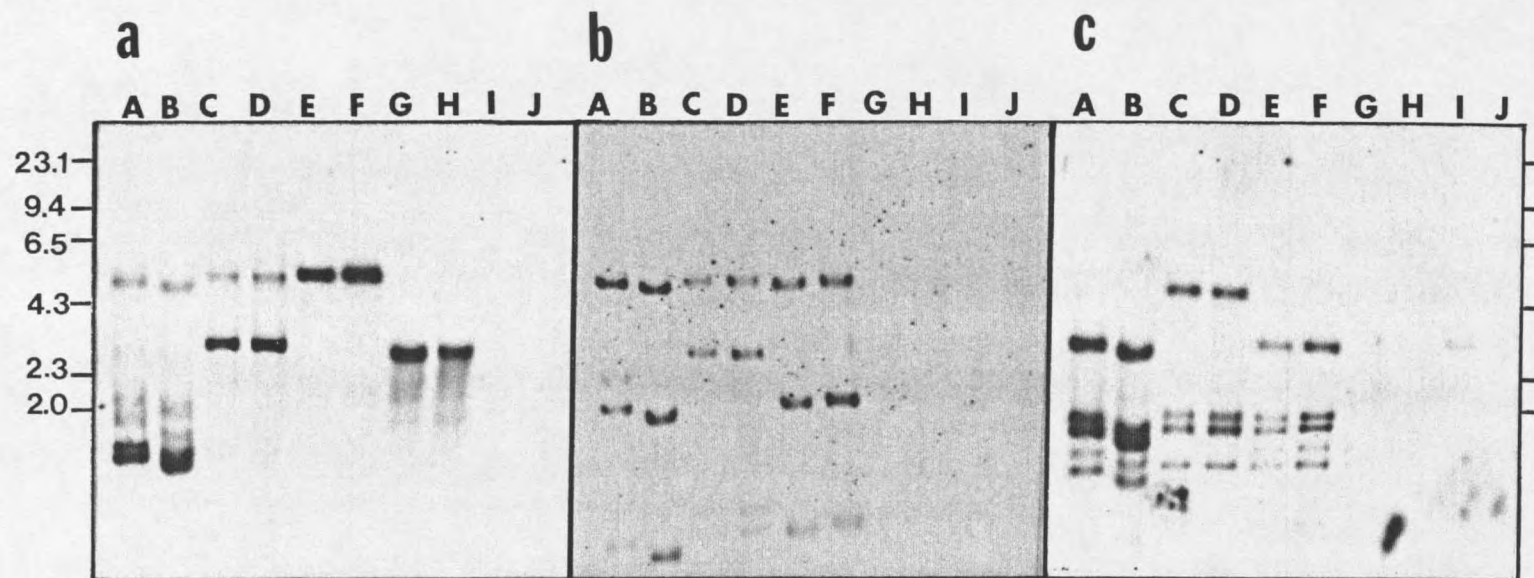
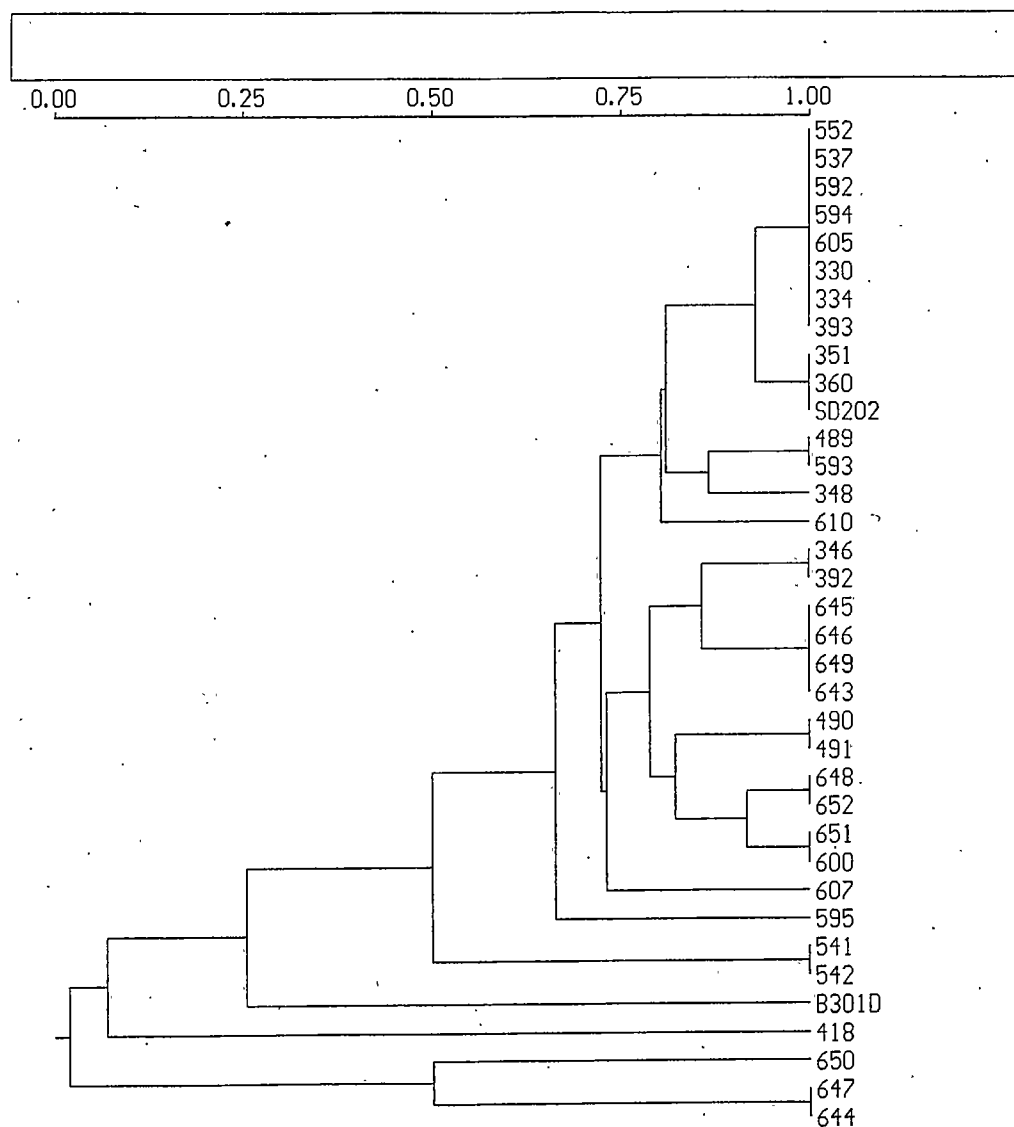


Figure 5. Restriction fragment length polymorphism patterns of ten *P. syringae* strains, lanes A to H are toxigenic and lanes I and J are non-toxigenic. Total DNAs were digested with *Pst*I and probed with (a) *SyrB*, (b) 10-Kbp *hrp*, and (c) 14-Kbp *hrp*. A=strain 552, B=592, C=541, D=542, E=643, F=645, G=647, H=644, I=554, and J=418.

all the other strains contained a 5.6-Kbp *Pst*I fragment that hybridized to the *syxB* probe.

The two probes obtained from the *hrp* region, a 10-Kbp and a 14-Kbp, hybridized to all of the *P. s. pv. syringae* with the exception of the same last three strains 644, 647, (lanes G and H, Fig. 5b and 5c) and strain 650 (Appendix A, Tables 11 and 12). As a result, in the dendrogram these three strains formed a cluster divergent from the rest of the *P. s. pv. syringae* strains (Fig. 6). With the *hrp* 10-Kbp probe, 9 different RFLP groups (10 to 18) were obtained (Appendix A, Table 11). Examples of group 10 can be observed in lanes A and B. Group 15 includes only strains 541 and 542 whose pattern can be observed in lanes C and D. Examples of group 17 are in lanes E and F (Fig. 5b). Lanes G and H show strains 644 and 647 which did not hybridize to this probe (Fig. 5b). With this probe, no apparent groupings were observed that correlated with the zone of inhibition, since all strains were distributed among different groups regardless of the zone of inhibition. The same 5.6-Kbp *Pst*I fragment was shared in all hybridizing strains, which in this case included strain B301D (Appendix A, Table 11). When hybridizing with the *hrp* 14-Kbp probe, only 4 RFLP groups were obtained among all hybridizing strains plus the group of the strains that did not hybridize (644, 647, 650 and B301D). This was the only probe that

Figure 6. Dendrogram obtained from cluster analysis of restriction fragments by UPGMA. Each *P. syringae* DNA was digested with *Pst*I and hybridized to the *SyrB* probe and the 10-Kbp and 14-Kbp *hrp* fragments.



hybridized to strains of the *P. syringae* 554 producing a single 3.6 Kbp band for all of them (lane I and lane J, Fig. 5c, Appendix A, Table 12). The band in lane J is not clearly visible due to difference in DNA concentration and time of exposure. Twenty-seven out of the 32 *P. s. pv. syringae* hybridizing strains belonged to a single RFLP group (Group 19, lanes A, B, E, and F, Fig. 5c). Strains 541 and 542 were again grouped (group 20) by themselves as they were with the *hrp* 10-Kbp probe (lanes C and D, Fig. 5c). *P. s. pv. syringae* strains 610 and 607 formed another separate cluster, group 21 (Appendix A, Table 12). All *P. syringae* 554 strains are represented by strain 418 and all of them contained a single faint 3.6-Kbp *Pst*I fragment that can be observed in lane I (Fig. 5c). This fragment was also shared by all hybridizing *P. s. pv. syringae* strains with the exception of strains 541 and 542. A set of four *Pst*I fragments 1.7, 1.4, 1.2, and 0.6-Kbp were present in all hybridizing *P. s. pv. syringae* strains with exception of strains 541 and 542.

The data obtained by hybridization with all three probes were combined and analyzed to obtain a dendrogram. The three probes produced a total of 36 bands from all strains which were used in the production of the cluster analysis and the dendrogram (Fig. 6). When data from the three probes was combined, many clusters were obtained. *P.*

s. pv. syringae strains were distributed among several clusters and yet only strains 650, 647 and 644 were placed in a very distant group from the rest. Strain SD202 originally obtained from wheat by D. C. Gross was determined to be very closely related to the majority of strains. Strain B301D originally isolated from cherry, also supplied by D. C. Gross, was placed in an isolated cluster very divergent from most of the rest of the *P. s. pv. syringae* strains. Strain 418, a type culture from *P. syringae* 554 was found to be completely different from *P. syringae* *pv. syringae* as were all the *P. syringae* 554 strains since they all displayed the same RFLP pattern.

Discussion

Although fungi previously reported to cause barley kernel blight including *C. sativus*, *Alternaria* spp. and *Fusarium* spp. (Machacek and Greaney, 1938; Anderson and Banttari, 1967; Mathre, 1982) were found in the Montana samples, they were in such a low frequency that these fungi appear unimportant in the development of this disease in Montana. This may be due to the normally drier and cooler environmental conditions prevalent in the state of Montana compared to the upper midwest states, limiting the establishment and build-up of fungal inoculum. Another

reason may be the possible antagonism of *P. s. pv. syringae* against these fungi since it produces syringomycin which is known to be an antifungal toxin (Iacobellis et al., 1992). This antifungal activity may be maintaining low levels of fungal organisms on the heads. In the upper midwest samples, *C. sativus* was isolated in 16 to 38% of the samples from 1991 but not at all from 1992 samples, and only in very low frequency (4%) from 1993 samples (Table 2). However, the limited number of samples analyzed from these states, and the high occurrence of *Fusarium spp.* in 1993, may lead to an underestimation of *C. sativus* occurrence in the upper midwest states. The absence of these fungi in samples of kernel blight from Montana led to the focus of subsequent research on the role and characterization of the bacteria involved in the disease.

In these studies it was determined that two types of kernel blight symptoms occurred in Montana corresponding to kernel discoloration and to bacterial kernel spot (Machacek and Greaney, 1938; Anderson and Banttari, 1967; Mathre, 1982; Peters et al., 1983). In this work, bacteria were found to cause both symptom types. Therefore, a different terminology is proposed based on the types of symptoms observed on the kernel. These types are referred to as basal and spot kernel blight (Fig. 1). Isolation of bacteria from symptomatic kernels indicated that *P. syringae*

strains were in approximately 90 percent of all subsamples from Montana showing basal or spot kernel blight. This suggested a high association of *P. syringae* strains with both types of kernel blight in this state. *P. syringae* has never been considered a problem in the upper midwest. However, these results showed that in certain wet years such as 1992, *P. syringae* may occur with some severity and should be considered in future studies on basal kernel blight or kernel discoloration in these states. Inoculation in the greenhouse confirmed the hypothesis that both types of kernel blight in Montana could be caused by strains of *P. syringae*. This is important especially in the case of the basal kernel blight, as this is the most common type of kernel blight in most areas of North America and it has always been attributed to fungal pathogens rather than bacteria (Machacek and Greaney, 1938, Anderson and Banttari, 1976; Mathre, 1982). This study constitutes the first report of *P. syringae* as a causal agent of basal kernel blight also referred to as kernel discoloration.

Two major groups or pathovars of *P. syringae* isolated from infected kernels were established based on their ability or inability to produce a compound that inhibited *G. candidum* *in vitro*. Based on this inhibition and on the hybridization with the *syrB* probe, it was assumed that this toxin was syringomycin (Gross, 1985; Quigley and Gross,

1994). However, the toxin was not chemically characterized, therefore the possibility that this toxin may be one of the syringomycin analogs cannot be precluded. Members of one group were identified as *P. s. pv. syringae* based on the production of syringomycin (Quigley and Gross, 1994) and isolates of this group were obtained primarily from kernels with basal kernel blight. This association was observed especially in 1991 and 1992 samples and to a lesser extent in 1993 samples (Fig. 3). This may have been due to the fact that 1991 and 1992 samples had only one type of blight while all samples from 1993 had kernels showing basal kernel blight and kernels showing spot kernel blight. This could have resulted in co-infection and/or cross contamination with both types of bacteria from kernels expressing only one type of blight (Fig. 3). The fact that all samples from the upper midwest had only basal kernel blight and that 100% of the isolated strains belonged to the *P. s. pv. syringae* group, supports the association of this bacterium with the basal kernel blight. The effect of syringomycin production on virulence and/or on the symptom type development is not known and should be studied to gain a better understanding of this disease. It would also be interesting to determine if the difference in amount of syringomycin production is in any way correlated to the type or severity of the symptom.

The second group, *P. syringae* 554, did not produce syringomycin and appeared to be associated with spot kernel blight (Fig. 4): Similar to that described above, *P. syringae* 554 strains were clearly associated with spot kernel blight in 1991 and 1992 but not as clear in the 1993. In 1993, 77% of the strains isolated from spot kernel blight belonged to *P. syringae* 554, but 23% were *P. s. pv. syringae*, which again may be due to the presence of both types of blight in the samples from this year. The association of *P. syringae* 554 strains with spot kernel blight in Montana appears to differ from the report of Peters et al., (1983) who noted that *P. s. pv. syringae* was the causal agent of 'bacterial kernel spot' whose symptomatology corresponds to the spot kernel blight. Unfortunately, the strains that were originally described for 'bacterial kernel spot' by Peters et al., (1983) were not available and no comparison could be made with them.

Efforts to determine the pathovar designation of both groups of *P. syringae* strains were not very successful when Biolog and MIDI systems were utilized (Table 3). Although *P. syringae* was consistently identified for all of these strains, pathovar determinations were inconsistent. The variability in the pathovar identification between the two systems for the same or different strains demonstrated the difficulty of designating a pathovar based on either of

these identification systems. Other traditional individual tests such as use of L-lactate, trigonelline and quinate and the production of syringomycin appeared to be more helpful in their identification at least of the first group of strains as *P. s. pv. syringae* (Quigley and Gross, 1994; Hildebrand et al., 1988). Characteristics of the second group of strains placed them as being related to *P. s. pv. coronafaciens* (Hildebrand et al., 1988). This relatedness was also reported by Peters et al. (1983) who used biochemical tests and found that the causal agent of 'bacterial kernel spot' was closely related to *P. s. pv. coronafaciens*. Thus, it appears likely that we are dealing with the same organism when describing 'spot kernel blight' and 'bacterial kernel spot' even if the taxonomic identification of this bacterium by Peters et al. (1983) differs from the one described in this study.

The taxonomic classification of *P. syringae* pathovars has always been difficult due to the large variability of this species and to the fact that some strains do not fit in existent pathovars but rather appear as points in a continuum. Thus, extensive characterization of such organisms is necessary and useful. The use of biochemical and pathogenicity tests in combination with other tools, such as RFLP analysis and serological techniques, has resulted in a clearer characterization and determination of

genetic relationships among phytopathogenic bacteria (Hartung and Civerolo, 1991; Gottwald et al., 1991).

In the present study, RFLP analysis of the *P. s. pv. syringae* strains with the *syrB* probe revealed the presence of a possible correlation between zone of inhibition and RFLP profiles. Most *P. s. pv. syringae* strains were fairly closely related to each other with the exception of 3 strains that inexplicably were completely different from the rest. These analyses also revealed that, although most strains were closely related, there was a large variability among *P. s. pv. syringae* strains involved in the same disease. Hybridizations with the *syrB* probe showed that the majority of the strains that produced large zones of inhibition, presumably a result of a large amount of syringomycin, were clustered in the largest group (RFLP 1, Appendix A, Table 10). It was also observed that all the strains that produced a small amount of syringomycin (with the exception of strains 647, 644, and 650) were placed in a single RFLP group (RFLP 6, Appendix A, Table 10). Analysis of a larger number of strains would probably give more information to confirm or deny the presence of a relationship between amount of syringomycin produced and RFLP pattern. Since the *syrD* gene is reported to be involved in syringomycin excretion (Quigley and Gross, 1994), it would be interesting to test these strains with

this probe. This may reveal the possible changes of the *syrD* gene in strains that excrete large amounts of syringomycin in comparison to those that excrete small amounts. However, another aspect to consider is that the zone of inhibition observed on the PDA and SRM media can be considerably altered by adding specific metabolites to the media (Quigley and Gross, 1994). Thus, studies to determine the possible genetic relationships among syringomycin producers would have to take in consideration other physiological aspects of the bacteria such as precursors, regulation factors, degradation, plasmids, etc.

When the results of all three probes were used to construct the dendrogram, there was no apparent clustering of strains in relation to syringomycin production. The presence of a 5.6 Kbp *Pst*I band that hybridized to the *syrB* probe and to the 10Kbp *hrp* probe was highly conserved among most of the syringomycin-producing strains (Appendix A, Tables 10 and 11) and may indicate a relationship between the *syrB* gene and the *hrp* region. Mapping studies utilizing these results would give information on whether or not these genes are close to each other in the genome. This would be valuable to know since regulation and functional properties of these genomic regions are still in process (Heu and Hutcheson, 1993; Quigley and Gross, 1994). The fact that strain SD202 (wheat) was closely related to most of the

barley strains and the individuality of strain B301D in the dendrogram appears to agree with the results of Quigley and Gross (1994). They found that a large proportion of the *P. s. pv. syringae* strains originally obtained from dicots exhibit the same RFLP profile as strain B310D (cherry tree) while strains tested from monocots appeared to form two genetically distinct clusters very divergent from the dicot strains (Quigley and Gross, 1994). Similarly, Denny et al. (1988) assessed the genetic diversity among a sampling of monocot and dicot strains of *P. s. pv. tomato* by RFLP analysis. Two distinct clusters were observed that were restricted to either monocot or dicot strains (Denny et al., 1988).

All *P. syringae* 554 strains formed a cluster very divergent from all *P. s. pv. syringae* strains (represented by strain 418 in the dendrogram, Fig. 6). These results were most unexpected since strains from the two pathovars were found in the same crop causing similar or even identical symptoms. However, much less genetic information was obtained from strains of *P. syringae* 554 since they hybridized only to the 14 Kbp *hrp* probe. Although this hybridization was to a single 3.6-Kbp band, it suggested that all the strains from this pathovar are very closely related. This band was shared with 83 percent of the *P. s. pv. syringae* strains (Appendix A. Fig. 12) which can be

interpreted as a type of relatedness between strains of the two pathovars. More biochemical studies, host range tests and probably a larger number of probes for RFLP analysis are necessary for a more complete characterization or pathovar identification for the group of strains belonging to the *P. syringae* 554 group.

These studies revealed, as have some other studies (Hartung and Civerolo, 1991; Gottwald et al., 1991) how relatedness among pathogenic bacteria requires more than just RFLP patterns for accurate analysis. Several clusters were found among *P. S. pv. syringae* by RFLP analysis but it was also clear that this clustering did not completely correspond to the grouping by size of inhibition zone of *G. candidum*. Thus, differentiation and relatedness among strains would be incomplete if only RFLP results are used. Other characteristics intrinsic to the bacteria such as the amount of syringomycin produced, virulence, and host range would give a more accurate analysis of genetic.

CHAPTER 3

HOST-RELATED AND ENVIRONMENTAL FACTORS INVOLVED
IN THE DEVELOPMENT OF KERNEL BLIGHTIntroduction

'Kernel blight' is a barley disease of importance to the malting and brewing industries since microorganisms present in infected kernels can detrimentally affect the quality of the final product. This disease is commonly present in the upper midwestern region of the United States (Miles *et al.*, 1987; Gebhardt *et al.*, 1992) and in the past several years has occurred in northcentral Montana.

Two different symptom types of kernel blight have been observed to occur in Montana and will be referred to as basal and spot kernel blight. Basal kernel blight and spot kernel blight correspond to kernel blight symptoms previously described to be caused by fungi and bacteria, respectively (Machacek and Greaney, 1938; Anderson *et al.*, 1976; Banttari *et al.*, 1975; Peters *et al.*, 1983).

In Montana it was found that both types of kernel blight can be caused by *Pseudomonas syringae* strains and characterization of these strains revealed the existence of two separate pathovars, or groups of bacteria, that appeared

to be correlated with each type of symptom. Results of this characterization are reported in chapter 2.

The variation in the presence and severity of kernel blight from year to year, and place to place, suggests that environmental factors, such as moisture and temperature and host-related characters might influence the development of this disease. Kernel developmental stage has been found to directly affect the incidence of infection of some fungi, such as *Pyrenophora graminea* in barley seeds and *P. tritici-repentis* in wheat seeds (Teviotale and Hall, 1976; Schilder and Bergstrom, 1994). Comparable studies of microbial populations in barley heads have shown that the number of bacteria and filamentous fungi increase greatly from the milk to the late dough stage and that an increase in infection by bacteria comes just prior to maturity (Follstad 1961). The severity of kernel discoloration caused by *Cochliobolus sativus*, which causes basal kernel blight, appeared not to be affected by the stage of kernel development (Lutey, 1962; Stevenson, 1981). In contrast, Peters et al. (1983) working with bacterial kernel spot, whose symptomatology is equivalent to spot kernel blight, found that infection occurred at the beginning of kernel development prior to attachment of the lemma to the seed. Results of these studies suggested the need for research on the effect of kernel developmental stage for both basal and

spot kernel blight caused by the two pathovars of *P. syringae* in Montana.

The amount of moisture available after heading has often been associated with these symptoms. Kernel discoloration and bacterial kernel spot have always been found to be most severe after abundant rain or in fields with overhead irrigation (Follstad, 1961; Lutey, 1962; Stevenson, 1981; Wilcoxson et al., 1980; Miles et al., 1987; Miles et al., 1989; Wesenberg, 1994 personal communication; Peters et al., 1983). In Montana the appearance of both basal and spot kernel blight have also been associated with wet years (Miller, 1990 personal communication).

Genetic resistance is considered the most practical means of control of kernel blight and breeding studies for kernel discoloration caused by *C. sativus* have been in process for several years (Banttary et al., 1975; Wilcoxson et al., 1980; Miles et al., 1987; Miles et al., 1989; Gebhardt et al., 1992). Draper (1985) looked for genetic resistance to the 'bacterial kernel spot' (spot kernel blight) and reported differences among ten barley cultivars. Studies on microbial populations of different cultivars suggested the existence of differences among varieties in susceptibility to invasion by bacteria (Follstad, 1961).

The purpose of the present study was to determine how environmental conditions and host-related factors affect the

development of kernel blight of malting barley in Montana. The effects of kernel developmental stage, humidity and temperature were studied under greenhouse and field conditions. Results of this study were essential in the development of a reliable method of screening for resistance.

Materials and Methods

Greenhouse Experiments.

Effect of Kernel Developmental Stage. The effect of kernel developmental stage on kernel blight was determined using B2601 (six-row) and B1202 (two-row) Bush Ag Resources cultivars. Seeds were planted at weekly intervals in the greenhouse to synchronize different kernel developmental stages. Barley heads were marked and inoculated at different kernel developmental stages (Zadoks et al., 1974): early milk (Zadoks Growth Stage 73), late milk (ZGS 77), soft dough (ZGS 85) and hard dough (ZGS 87). A combination of two *P. syringae* types, strain 552 isolated from basal kernel blight and identified as *P. s. pv. syringae* and *P. syringae* 554 strain 554, isolated from spot kernel blight (chapter 2), were used for inoculation. These bacteria were grown for 24 hrs on King's B media (King et al., 1954) and a bacterial suspension of ca. 10^7 cfu/ml of each strain, based

on a 1:10 dilution of suspensions with turbidity of 80 Klett units on a Klett-Summerson spectrophotometer with a green filter, was prepared in sterile distilled water plus 0.025% Tween 20 (polyoxyethylenesorbitan monolaurate). Inoculum was prepared by mixing equal volumes of each bacterial suspension and marked heads were inoculated and incubated in a mist chamber as previously described (chapter 2).

A similar experiment examined the effect of inoculation with each strain separately. Cultivars B2601 and B1202 were planted at two week intervals and barley heads were marked and inoculated at the early milk (Zadoks Growth Stage 73), and at the soft dough stages (ZGS 85). One set of plants was inoculated with *P. s. syringae*, strain 552. The other set of plants was inoculated with *P. syringae* 554, strain 554. Bacteria were grown as previously described and a 10^7 cfu/ml bacterial suspension was prepared from each strain and barley heads were inoculated and incubated in the mist chamber as described.

In both experiments control plants for each stage were sprayed with a water solution plus 0.025% Tween 20. After incubation for 48 hrs in the mist chamber, plants were transferred to the greenhouse bench. At maturity, inoculated heads were harvested individually and kernel blight was evaluated by visualization obtaining a percentage of blighted kernels by weight. The greenhouse was

maintained at 22 to 24 C during the day and 12 C during the night with 14 hr light per day. The 14 hr light per day were maintained by artificial light for 4 hr in the morning and 4 hr in the evening plus day light. In all greenhouse experiments an experimental unit consisted of three plants in a 19 cm pot. Each experiment was analyzed as a factorial with inoculum and stage as the two factors in a completely randomized design with three replications per treatment. Experiments were repeated twice and data were studied by analysis of variance in the MSU STAT program..

Effect of Duration of Free Moisture and Temperature.

Barley heads of cultivar B2601 were inoculated at early milk and soft dough stage with a combination of the two types of bacteria. Inoculum preparation and inoculation were performed as described in the first experiment. Three sets of plants were inoculated and immediately after inoculation one set was placed on the greenhouse bench; a second set was incubated in the mist chamber for 24 hrs and then transferred to the bench; a third set was incubated for 48 hours in the mist chamber and then transferred to the bench.

To test the effect of temperature, a similar design was followed and three sets of plants from each moisture level were transferred to three different temperatures selected based on averages of climatological data from Fairfield MT.

One set was at 18 C day 14 C night, another set at 28-30 C day and 22 C night, and a third set at 22-24 C day 12 C night, all of them with 14 hours light. The percentage of kernel blight was obtained and data were analyzed as above as a factorial with humidity, temperature, and stage as the three factors in a completely randomized design with three replications per treatment.

Screening Method. To develop a reliable method for screening, the effect of kernel developmental stage on the presence of the disease was studied on seven different barley genotypes in the greenhouse. The planted cultivars were: Bush Ag Resources genotypes B2601, B2912, B3380, B1202, and B5133, the commercial cultivar Klages, and the genotype M567 from Minnesota that has been shown to be resistant to kernel discoloration caused by *C. sativus* (this genotype was obtained from R. D. Wilcoxson at the University of Minnesota). Two sets of cultivars were planted in two lots 10 days apart and during kernel development barley heads for each cultivar were marked at early milk and at soft dough. One set of plants was inoculated with strain 552 and another set with strain 554 as previously described. After 48 hours of incubation in the mist chamber, plants were transferred to the greenhouse bench until maturity. Individual heads were harvested and kernel blight was

evaluated. The effect of stage and cultivar were analyzed as the two factors in a completely randomized design with three replications per treatment.

Field Experiments.

Effect of Kernel Developmental Stage and Moisture.

Field experiments were performed at Fairfield, MT, which is located in the northcentral part of the state, during the growing seasons of 1992 and 1993 under flood type irrigation. The effect of kernel developmental stage and moisture on the presence of kernel blight were studied. Natural infection was enhanced by supplying additional overhead sprinkler irrigation at the different kernel developmental stages (ZGS), previously described EM (ZGS 73), LM (ZGS 77), SD (ZGS 85), and HD (ZGS 87). Cultivars B2601 and B1202 were planted at four weekly intervals (only three intervals in 1993). On July 27th, 1992 and August 6th 1993, fifteen individual heads per experimental unit were marked for each of the kernel developmental stages. A fine mist generated by using rainbird impact head sprinklers (Rainbird Sprinklers) was applied to the plots for approximately 4 hours in the morning and 4 hours in the evening for five consecutive days. During the irrigation periods, sprinklers were on for 5 minutes and off for 30

minutes to maintain free moisture on the barley heads. Water measuring cups were placed in each experimental unit to confirm uniformity of the irrigation system. The same experiment was also performed without additional overhead sprinkler irrigation in an adjacent plot. At maturity marked heads were harvested and kernel blight percentage was obtained by weight. The experiment was planted in a randomized split-plot design with six replicates per treatment. Each replicate (experimental unit) consisted of 6 rows 1.5 m long. Data were analyzed as above in a factorial with stage and humidity as the two factors.

Screening Method. A reliable method for screening barley genotypes was developed by studying the effect of kernel developmental stage on the presence of the disease in different genotypes. This experiment was done in the 1993 growing season. The same barley cultivars used in the greenhouse experiment (with exception of B2601) were planted at two different times 10 days apart to synchronize the early milk and the soft dough kernel developmental stages. On August 6th, fifteen heads per experimental unit were marked at either early milk or soft dough stages and overhead sprinkler irrigation was supplied for 5 days in the same way as previously described. Marked heads were harvested at maturity and the percentage of kernel blight by

weight was determined. This experiment was planted in a randomized split-plot design with three replicates and experimental units consisted of 3 rows each 1.5 m in length. Data were statistically analyzed as a factorial with stage and cultivar as the two factors.

Results

Effect of Kernel Developmental Stage. When a mixture of the bacterial strains (552 and 554) was inoculated in the greenhouse only the basal kernel blight symptom occurred. Disease levels were compared in plants inoculated at the different kernel developmental stages. A window of infection was found to occur in greenhouse experiments when plants inoculated at late milk stage, soft dough or hard dough stage developed statistically higher levels of basal kernel blight than those inoculated at early milk (Fig. 7). Similarly in field experiments only basal kernel blight was observed in 1992 and 1993 growing seasons. The same window of infection was observed in 1992 when statistically higher levels of basal kernel blight were found in those heads which were supplied with additional overhead irrigation at late milk, soft dough and hard dough stages as compared to those at early milk (Fig. 8). In 1993 the same trend was observed where plants submitted to high moisture late in the kernel development had more basal kernel blight (fig. 9) but

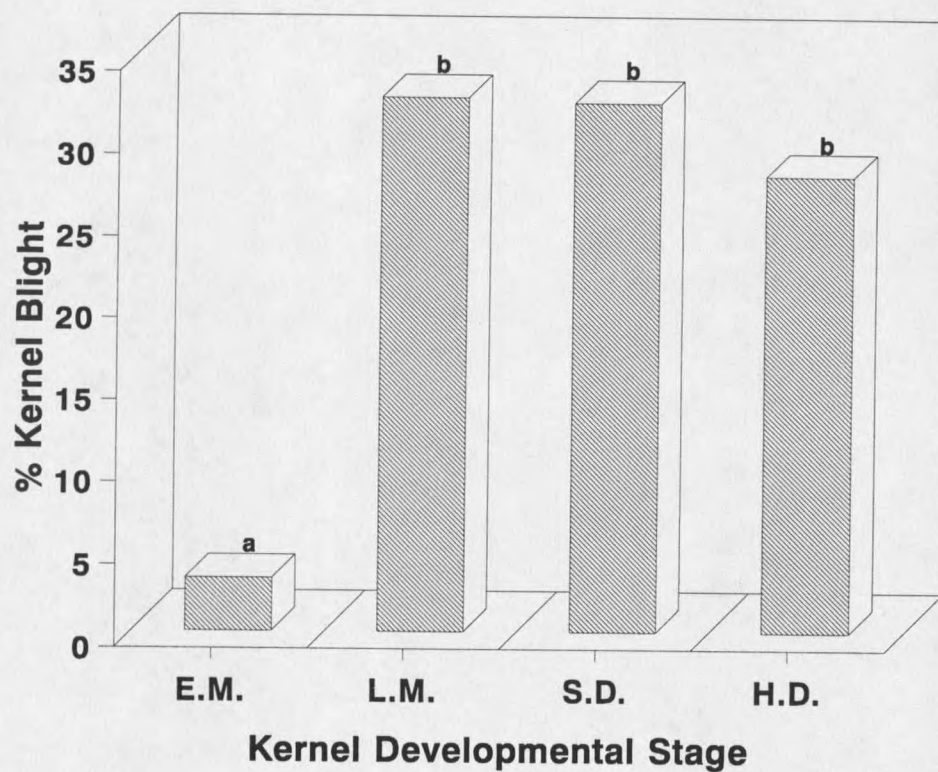


Figure 7. Percentage of basal kernel blight obtained in the greenhouse by inoculation of barley heads at early milk (EM) late milk (LM) soft dough (SD) and hard dough (HD). Inoculation was done with a combination of bacterial suspensions of *P. s. pv. syringae* strain 552 and *P. syringae* 554 strain 554. Bars with different letters are significantly different at $p = 0.05$.

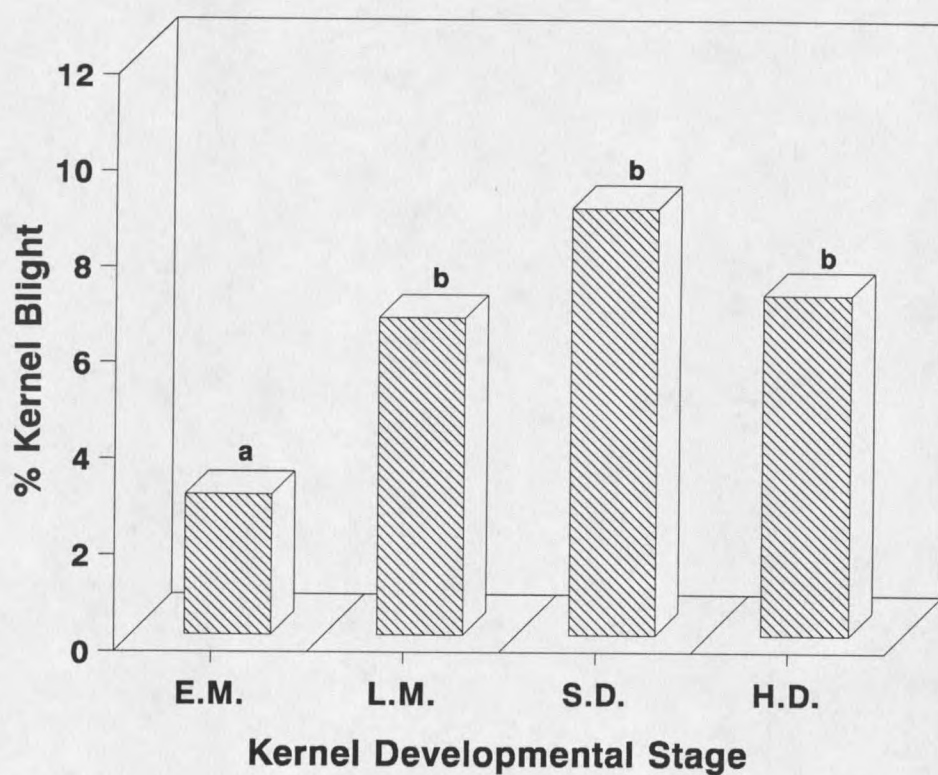


Figure 8. Percentage of basal kernel blight obtained under field conditions when additional overhead irrigation was supplied to barley heads at early milk (EM), late milk (LM), soft dough (SD), and hard dough (HD). Experiment was performed in Fairfield MT 1992. Bars with different letters are significantly different at $p = 0.05$.

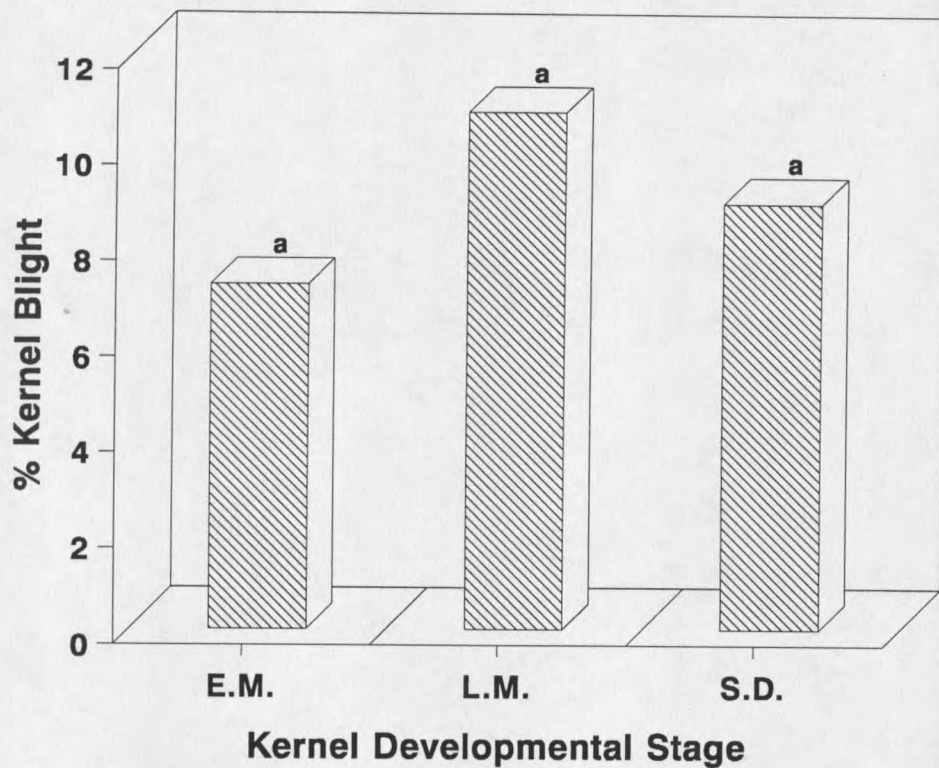


Figure 9. Percentage of basal kernel blight obtained under field conditions when additional overhead irrigation was supplied to barley heads at early milk (EM), late milk (LM), and soft dough (SD). Experiment was performed in Fairfield MT 1993. Bars with different letters are significantly different at $p = 0.05$.

there were no statistical differences between treatments. When data from the two years were combined and analyzed as a factorial with year, stage, and cultivar as the 3 factors (Table 4), the differences attributed to year were not significant ($p = 0.05$), while differences due to growth stage were significant ($p = 0.05$). Plants submitted to high moisture at and after late milk stage showed statistically higher basal kernel blight levels than those supplied with moisture at the early milk stage. The two cultivars proved to be different at $p = 0.05$, and cultivar B2601 had statistically higher levels of disease than cultivar B1202.

Table 4. Analysis of Variance for effect of kernel developmental stage at the time that additional sprinkler irrigation was provided to two barley cultivars on the incidence of kernel blight under field conditions in 1992 and 1993^a

Source	DF	S.S.	M.S.	F-Value	P-Value
Block	2	14.76	7.38		
UNITS					
Year ^b	1	55.25	55.25	4.09	0.053
Stage	2	115.30	57.65	4.27	0.024*
Year*Stage	2	33.68	16.84	1.25	0.303
Cultivar	1	213.16	213.16	15.80	0.000*
Residual	27	364.38	13.49		
Total	35	796.53			

^a Analysis performed on data of percentage of kernel blight.

^b Data from 1992 and 1993 were combined.

* Significant at $p=0.05$

When *P. s. pv. syringae* and *P. syringae* 554 were inoculated separately on barley heads at two different kernel developmental stages in the greenhouse, both types of kernel blight developed depending on the time of inoculation. A different window of infection was observed for the development of each type of blight. Spot kernel blight was obtained on both cultivars when either bacterium was inoculated at early milk but not in those plants inoculated at soft dough (Fig. 10). Basal kernel blight symptoms were also obtained when either strain was inoculated but the window of infection for basal kernel blight was observed to be at soft dough stage of the kernel development (Fig. 10). However, it was also observed that the different levels of basal kernel blight obtained by inoculation of *P. syringae* 554 were not statistically significant at $p = 0.05$.

Effect of Free Moisture and Temperature. Barley plants inoculated in the greenhouse at early milk and soft dough were submitted to free moisture for 0, 24 and 48 hrs of incubation in a mist chamber after inoculation. The experiment was repeated twice and data were analyzed separately and in combination. Only basal kernel blight was obtained and the same window of infection was observed at all moisture and temperature levels. Plants inoculated at

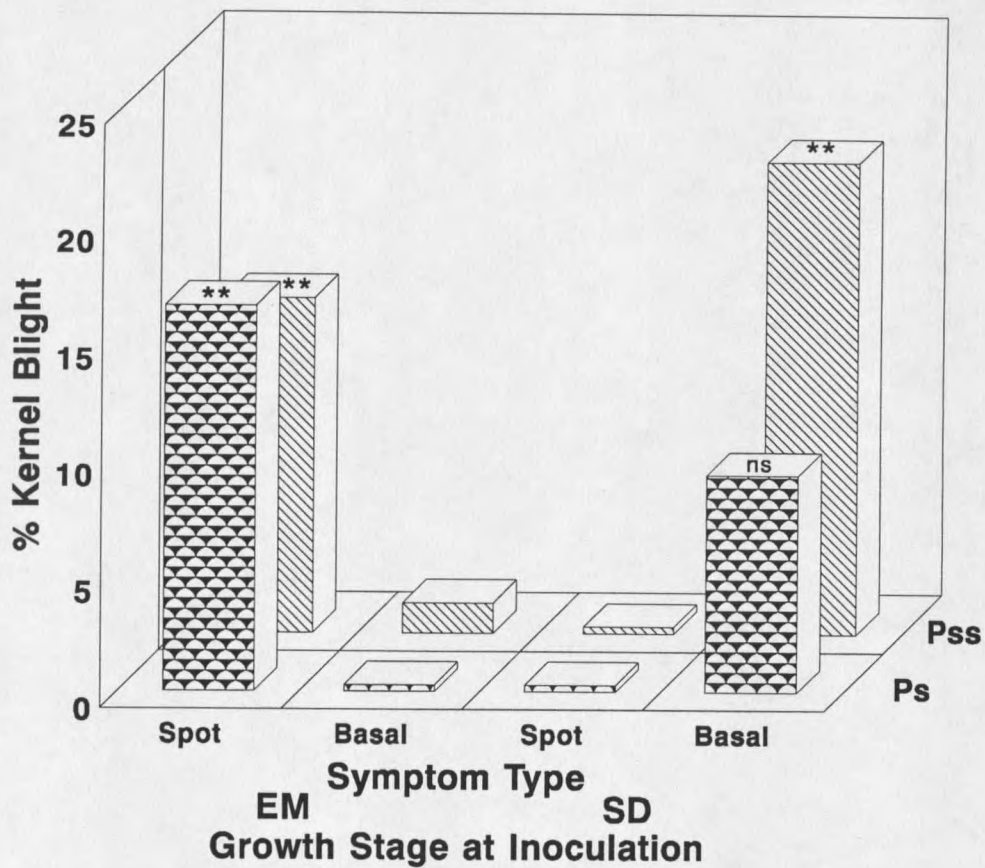


Figure 10. Percentage and type of kernel blight obtained in the greenhouse when *P. s. pv. syringae* (Pss) and *P. syringae* pv. 554 (Ps) were inoculated individually on barley heads at two different kernel developmental stages. Values are averages of two experiments. ** = statistically significant at $p = 0.05$; n.s. = not significant at $p = 0.05$

soft dough stage developed statistically more basal kernel blight than those inoculated at early milk stage (Table 5). Different periods of exposure to high moisture were statistically different both times that the experiment was performed and similar results were obtained. The longer the humidity period, the greater the basal kernel blight percentage (Table 5). After incubation in the humidity chamber, three sets of plants were transferred to three different temperatures, 18, 22-24 and 28 C. The effect of different temperatures on the development of the disease varied from one experiment to the other. No effect on the development of disease symptoms was found when data from the first experiment was analyzed (Table 5). Results from the second experiment revealed that different temperatures affected the levels of kernel blight. Plants submitted to 28 C showed statistically higher levels of kernel blight than those exposed to 18 or 22-24 C (Table 5). When data from the two experiments were analyzed together, temperature levels were significant but at a lower level of significance ($p = 0.06$).

To study the effect of moisture in the field, the percent of kernels showing basal blight was determined on plants supplemented with additional overhead sprinkler irrigation and from plants without additional irrigation. As in the results obtained in the greenhouse experiments,

Table 5. Effect of kernel developmental stage, moisture and temperature on the development of kernel blight under greenhouse conditions. Percentages of kernel blight obtained on barley plants inoculated with a combination of *P. syringae* pv. *syringae* and *P. syringae* 554.

Variable	Treatment	Exp. I ^d	Exp. II	Combined
<u>Kernel stage^a</u>				
	Early Milk	1.9 a ^e	3.7 a	2.8 a
	Soft Dough	19.2 b	21.0 b	20.7 b
<u>Moisture^b</u>				
	0 Hrs	0.7 a	4.5 a	2.6 a
	24 Hrs	13.3 b	13.4 b	13.3 b
	48 Hrs	17.7 c	19.1 c	18.4 c
<u>Temperature^c</u>				
	18 C	10.8 a	11.5 a	11.3 ab
	22-24 C	10.2 a	7.5 a	8.8 a
	28-30 C	10.6 a	18.1 b	14.4 b

^a Differences attributed to stage of kernel development were significant ($p=0.05$) in both experiments.

^b Differences attributed to moisture were significant in both experiments ($p=0.05$).

^c Differences due to temperature were significant only in experiment II. When data from both experiments were combined, effect of temperature was not significant at $p=0.05$ but it was significant at $p = 0.1$

^d Same experiment was performed twice (Exp. I and Exp. II) and data were analyzed individually and in combination.

^e Values in a variable and in a column with different letters are significantly different at $p=0.05$ according to LSD test.

field results revealed that levels of basal kernel blight obtained in the plots under additional sprinkler irrigation during the window of infection were statistically higher than those under normal irrigation (Table 6).

Table 6. Effect of moisture on the development of kernel blight under field conditions for barley grown under different irrigation conditions at Fairfield MT during 1992 and 1993.

Treatment ^{a,b}	1992	1993	Mean
No Sprinkler	1.3 a ^d	2.2 a	1.8 a
Sprinkler	6.4 b	8.8 b	7.6 b
Year mean ^c	3.9	5.8	

^a Differences attributed to irrigation treatment were significant ($p=0.05$).

^b Additional overhead sprinkler irrigation was supplied during five days to provide mist on the barley heads during different kernel developmental stages. Normal irrigation was performed by flooding according to farmer's schedule.

^c Difference attributed for year were not significant ($p=0.05$).

^d Values in a column with different letters are significantly different at $p=0.05$ according to LSD test.

Screening Method. Several features of symptom specificity were revealed when seven barley cultivars were screened in the greenhouse by inoculation at early milk or at soft dough with *P. s. pv. syringae* strain 552 and *P. syringae* 554 strain 554. First, strain 552 (*P. s. pv.*

syringae) caused significantly higher levels of basal blight than those caused by *P. syringae* 554 in all cultivars (Table 7). Similarly strain 554 caused statistically higher levels of spot kernel blight than the levels caused by strain 552 of *P. s. pv. syringae* (Table 7). Second, the optimum developmental stage for infection was statistically different ($p = 0.05$) for spot and basal kernel blight. Early milk stage was the window of infection for spot type while soft dough is optimal for basal type (Table 7). Third, there were statistical differences among cultivars for both basal and spot kernel blight and their ranking is shown in Table 7. Another observation was that in the case of all 6-row cultivars that developed spot kernel blight, the symptom was on the opposite side of the kernel than where the spot type is usually observed. The spot blight observed in 2-row cultivars was on the lemma, exactly as was observed in field samples under natural infection. Fourth, the interactions between cultivar and growth stage and cultivar and strain were statistically significant ($p = 0.05$) (Table 8).

When the six cultivars were evaluated for the presence of kernel blight under field conditions, only basal kernel blight was observed. Statistical differences in severity were found for cultivars and for kernel developmental stage. Plants supplied with overhead irrigation at soft dough

Table 7. Effect of barley cultivars on the percentage of the different types of kernel blight obtained in the greenhouse when inoculated with *P. s. pv. syringae* strain 552 and *P. syringae* 554 strain 554.

Source	Treatment	Means %	
<u>Basal Kernel Blight</u>			
Strain*	554 ^a	1.0	a ^c
	552 ^b	17.5	b
Stage*	EM	2.6	a
	SD	15.9	b
Cultivar*	Klages (2-row)	2.4	a
	B5133 (2-row)	2.9	a
	B2912 (6-row)	6.1	ab
	MN567 (6-row)	6.6	ab
	B1202 (2-row)	7.6	ab
	B3380 (6-row)	13.8	b
	B2601 (6-row)	25.2	c
<u>Spot Kernel Blight</u>			
Strain*	552	3.5	a
	554	14.5	b
Stage*	SD	4.3	a
	EM	13.7	b
Cultivar*	Klages (2-row)	2.5	a
	B2912 (6-row)	2.7	a
	B2601 (6-row)	6.9	ab
	B1202 (2-row)	7.0	ab
	MN567 (6-row)	7.7	ab
	B5133 (2-row)	14.4	bc
	B3380 (6-row)	21.9	c

* Statistically significant at $p = 0.05$

^a Strain 554: type isolate obtained from spot blight.

^b Strain 552: type isolate obtained from basal blight.

^c Values in a single source with different letter are significant different at $p = 0.05$

Table 8. Interaction of cultivar and *P. syringae* strains and cultivar and stage on development of the different kernel blight types obtained in the greenhouse.

Cultivar	Stage		Strain	
	Early Milk	Soft Dough	552	554
<u>Basal Kernel Blight^a</u>				
B2601	18.5 ^c	31.8	48.1	2.1
MN567	0.0	13.3	13.0	0.3
B2912	0.0	12.7	12.1	0.0
B3380	7.2	20.5	27.7	0.0
Klages	0.0	9.1	4.9	0.0
B1202	1.0	14.3	15.3	0.0
B5133	0.0	9.5	1.6	4.2
<u>Spot Kernel Blight^b</u>				
B2601	11.4	2.4	1.7	12.1
MN567	13.0	2.4	0.0	15.4
B2912	3.9	1.5	1.1	4.4
B3380	22.8	21.0	10.1	33.7
Klages	4.3	0.6	0.6	4.3
B1202	13.9	0.0	5.3	8.7
B5133	26.6	2.2	5.6	23.2

^a Interactions Cultivar/stage and Cultivar/strain are statistically significant at $p = 0.05$

^b Interactions Cultivar/stage and Cultivar/strain are statistically significant at $p = 0.05$

^c Levels of disease expressed in percentage of kernel blight; average of three replicates.

showed a statistically higher level of disease than those that were irrigated at early milk (Table 9). The interaction of cultivar and stage was not statistically significant ($p = 0.05$), thus all cultivars showed the same response to kernel blight regardless the kernel developmental stages at the time of irrigation (Fig. 11).

Table 9. Percentage of basal kernel blight obtained on 6 barley cultivars supplied with additional sprinkler irrigation during different kernel developmental stages at Fairfield MT in 1993.

Source	Treatment	Means %	
Stage*	EM	6.6	a ^b
	SD	13.6	b
Cultivar*	MN567 (6-row)	2.8	a
	Klages (2-row)	3.4	a
	B1202 (2-row)	4.9	ab
	B2912 (6-row)	8.9	b
	B5133 (2-row)	9.9	b
	B3380 (6-row)	27.5	c

* Statistically significant at $p = 0.05$

^b Values in a single source with different letter are significant different at $p = 0.05$

Discussion

Greenhouse experiments showed that only basal kernel blight symptoms and not spot type symptoms were obtained when the combination of *P. s. pv. syringae* strain 552 and *P.*

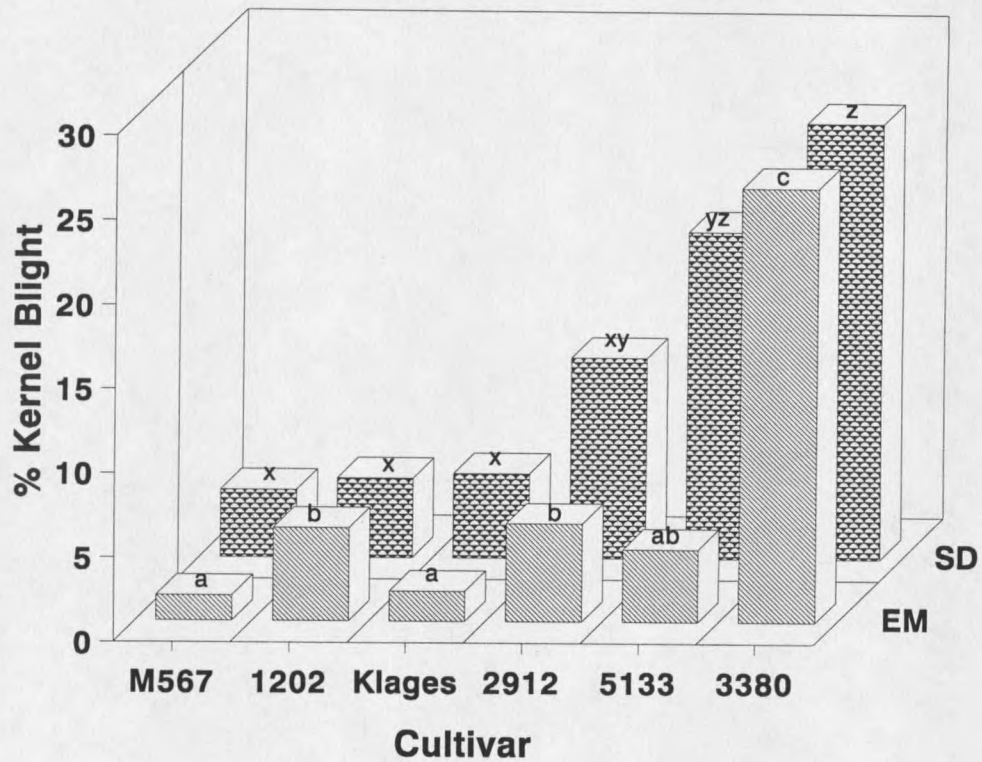


Figure 11. Effect of the kernel developmental stage on kernel blight percentage of six barley cultivars. Disease was enhanced by overhead sprinkler irrigation at early milk (EM) or soft dough stages (SD). Fairfield MT., 1993. Cultivars with different letter were statistically different at $P = 0.05$

syringae 554 strain 554 were inoculated together. Only when these two strains were inoculated individually did both symptom types develop. The reasons for this are unknown but one explanation may be the possible antagonism of one bacterium to the other. Since strain 552 has been shown to cause basal kernel blight and strain 554 spot kernel blight, the presence of strain antagonism or competition might be considered. This may be due to the production of toxic substances but also might be a result of nutritional competition. Observations in the laboratory have shown that strains from the *P. s. pv. syringae* group such as strain 552, have a faster growth rate than strains from the *P. syringae* 554 group such as strain 554 (personal observation). Thus it might be that once both strains are on the plant, strain 552 may multiply faster depleting nutrients resulting in the death or reduced growth of strain 554. Likewise, there may be virulence genes that are involved in the production of one symptom or the other. Another aspect to consider is that when both bacteria were inoculated together the actual inoculum concentration of each strain was decreased in half (approx 5×10^6 cfu/ml) compared to the inoculum used when inoculations were done separately (1×10^7 cfu/ml). This fact may suggest that development of spot kernel blight has a higher inoculum threshold than that required for the development of basal

type. All these are different possibilities that need to be explored before there is a complete understanding of these two symptom types.

Studies on the effect of the kernel developmental stage on the development of kernel blight revealed the existence of a different window of infection for each type of kernel blight. Both greenhouse and field results showed that even though infection for basal kernel blight can occur early during the kernel development, the level of the disease is clearly higher if plants are submitted to high disease pressure at or after late milk stage (Figures 7, 8, 9, and 10). Likewise, Schilder and Bergstrom (1994) reported that the incidence of *P. tritici-repentis*-infected seed increased when inoculation took place at the milk stage. Similarly, microbial ecology studies in barley plants have shown that the population of bacteria present in/on kernels increases from the milk to the late dough stage and that the greatest infestation by bacteria comes just before maturity (Follstad, 1961). The presence of this window of infection may be explained by the large seed size and high moisture content favoring access to and infection of the seeds at this stage (Schilder and Bergstrom, 1994). Biochemical composition changes that occur in the barley head during kernel development may also influence the optimal time of infection. It has been found that over 90% of toxigenic *P.*

S. pv. syringae strains produced larger quantities of toxin when the plant signal molecules, arbutin and D-fructose, were added to syringomycin minimal medium (Quigley and Gross, 1994). Thus, Quigley and Gross (1994) reported that almost all *P. syringae* *pv. syringae* strains have a regulatory mechanism whereby specific plant metabolites modulate the production of syringomycin. This may also imply that there might be other plant metabolites that differ, depending on the host. Therefore, it would be necessary to study which metabolites are present in barley and if their composition varies during the kernel development that could consequently influence the window of infection. However, in the case of kernel discoloration caused by *C. sativus* where the symptomatology visually corresponds to basal kernel blight, it has been reported that the stage of kernel development did not have an important effect on the disease. Infection was observed to take place any time from flowering through maturity (Lutey, 1962; Stevenson, 1981). Thus, the effect of the kernel developmental stage is much more relevant to the development of basal kernel blight when caused by bacteria than when caused by fungi.

In comparing the basal kernel blight window of infection with that of spot kernel blight, the latter was observed to be at early milk since this symptom developed

only in plants that were inoculated at this stage (Fig. 10). These results agree with the reports of Peters et al. (1983) who found that barley plants were more susceptible to "bacterial kernel spot" when they were inoculated at early stages during kernel development just before the lemma attached to the caryopsis. Lemma attachment occurs shortly after the caryopsis has reached its maximum length (water ripe stage), corresponding to the early milk stage. Peters et al., (1983) suggests that since only the inner epidermis of the barley lemma contains stomata, the bacteria enter the lemma primarily through the stomata before lemma-caryopsis attachment. Another factor to consider is that in Montana, as well as in Idaho, this type of symptom develops mainly in two-row cultivars rather than in six-row cultivars (personnel communications Miller, 1993 and Wesenberg, 1994). Thus differences in the head morphology of two-row versus six-row barley cultivars may help in elucidating the possible bacterial mode of action.

There is no clear explanation for why the two types of blight have differences in their windows of infection and only further studies will answer these questions. Studies should involve use of the scanning electron microscope to determine the bacterial mode of action. Since spot kernel blight is observed on the central part of the lemma of the kernel, it could be that the bacterium needs an entrance and

that at early milk stage the lemma is very tender. Since this tissue is highly exposed to environmental factors, the tenderness may enable wounds to occur in the central part of the lemma. These wounds may be used by the bacteria as a mode of entrance and infection resulting in the spot type of blight. In Fairfield, for instance, spot kernel blight has been observed to develop in high levels in those fields that had been hit by a hail storm (personal communication Miller 1993). This suggests that hail may be causing the necessary wounds on the lemma which may be used by the bacteria for infection. Conversely, in the case of basal kernel blight it might be that after the milk stage the tissue is hardened and natural entries and wounds are less abundant. Therefore only those bacteria that might migrate to the base of the kernel where there is accumulation of moisture would multiply. Hence infection would develop primarily at the basal end of the kernel. These mechanisms could be tested by following the infection process during the development of each type of blight. Again, one of the most efficient methods to do this may be with the scanning electron microscope.

The strong association of strains of *P. syringae* 554 group with the spot kernel blight obtained in field isolation experiments (chapter 2), was also observed in greenhouse experiments. *P. syringae* 554 caused

statistically significant levels of spot kernel blight in plants inoculated at early milk stage but did not cause statistically significant levels of basal kernel blight at any stage (Fig. 10). However, low levels of basal kernel blight were observed in plants inoculated at soft dough stage and even though these levels were not statistically significant, it does appear that this bacterium is capable of causing both types of blight. The association of *P. s.* pv. *syringae* strains with basal kernel blight observed in the field isolations (chapter 2) was not observed in the greenhouse experiments. In this case, *P. s.* pv. *syringae* strain 552 caused statistically significant levels of both basal and spot kernel blight depending on the kernel developmental stage of the plants at the time of inoculation. Barley plants inoculated at soft dough developed basal kernel blight while plants inoculated at early milk resulted in spot kernel blight (Fig. 10). One of the reasons for this situation may be that in the greenhouse both pathovars are capable of causing both symptom types when environmental conditions are optimal. In comparison, in the field there might be some bacterial-related characteristics, such as pathogen virulence and/or mode of action and biotic and abiotic environmental factors that influence each pathovar to cause one type of blight rather than the other. There are still numerous unknown factors

when referring to the specificity of the two pathovars of *P. syringae* and the type of blight, and more research into the peculiarities of each pathovar and the host-related factors influencing symptomatology is necessary.

It was clear that humidity after inoculation in the greenhouse played a major role in the severity of the disease (Table 5). Likewise in the field, levels of kernel blight were clearly higher in plants supplied with additional overhead irrigation at the window of infection compared to the levels obtained in those plants that were not so irrigated (Table 6). Similarly, Miles *et al.*, (1987), reported that irrigation significantly increased kernel discoloration severity. In addition, climatological data for the Fairfield area for the last five years have shown a strong association of rainfall and kernel blight levels in commercial fields. These observations were based on the rainfall data from the month of July since it is during this month that most of the fields in the area exhibit their window of infection. In 1989 kernel blight levels were extremely high (above 6-8%) in a large number of the fields. This was associated with high moisture during the whole month of July. Rainfall was present during a continuous period of 5-6 days (July 9th-15th) (Appendix B Figure 12). In 1990, 1991, and 1992 the levels of kernel blight in commercial fields were low. In 1990 and 1991

these low levels may be explained by dry weather conditions during July (Appendix B Figures 13 and 14). For 1992 (Appendix B, Fig. 15) there was rainfall during the month of July which might normally be expected to influence high levels of kernel blight. However, in that year, due to drought at seeding time and early aphid epidemics, most of the fields were not very uniform in their kernel developmental stage. This non-uniformity in kernel development may have decreased the number of kernels susceptible to disease during infection cycles resulting in a lower percentage of kernels with blight. In 1993, early seeded fields were at the window of infection during the month of July and showed high levels of kernel blight. This was probably due again to continuous rainfall for 5-6 days during July (11th-17th) (Appendix B, Fig. 16). In the same year, fields that were seeded later in the season were at their window of infection in August, resulting in very low levels of kernel blight. In August 1993 there was considerable rainfall (Appendix B, Fig. 17), but the duration of continuous rainfall was shorter than in July and rain was scattered throughout the month. This association of rainfall with kernel blight is not surprising since high available moisture after heading has often been associated with the disease (Follstad, 1961; Lutey, 1962; Stevenson, 1981; Wilcoxson et al., 1980; Peters et al., 1983).

Temperatures above 26 C were observed during the month of July 1989, a year with high levels of kernel blight (Appendix B, Fig. 18). July 1993 had very low temperatures (15-18 C), but fields that were at the window of infection during that month developed higher levels of disease (Appendix B, Fig. 18). This may suggest that temperature is not an important factor in the development of kernel blight. However the effect of temperature on the presence of the disease is not clear since different results were obtained when different temperature levels were assessed in the greenhouse in two separate experiments. No effect was found in the first experiment, but a direct effect was found in the second where high temperature after incubation increased the levels of basal kernel blight ($p = 0.05$). When data from both experiments was combined in an analysis of variance, the temperature levels gave significantly different effects but at a slightly lower level of significance ($p = 0.06$). One of the possible reasons for such differences found in results in one experiment versus the other is that these experiments were done at different times of the year. This is important because these studies were performed on greenhouse benches where the temperature and humidity are often affected by outside environmental conditions. Since *P. syringae* strains have an optimum growth at 28 C and a maximum of only 30 C and at lower

temperatures the growth rate of this bacterium decreases, the effect of temperature on kernel blight should be reconsidered. This could be done by repeating these experiments under growth chamber conditions to assure the desired temperature levels. However, the problems of fluctuations in the greenhouse did not affect the results of the effect of free moisture periods since same results were obtained both times that the experiment was performed. This may be due to the fact that the effect of free moisture for long periods is so significant that changes in the greenhouse did not affect the results, whereas the effect of temperature is less obvious and hence more easily masked by the greenhouse fluctuations. Besides, humidity levels are a factor for only one to two days during the incubation periods, while temperature levels can be irregular for any time during a three to four week period.

The results of these studies were found to be highly important in the development of a reliable screening method. By the inoculation of the two types of bacteria separately it was found that the two symptoms should probably be treated as two different diseases. These results corroborated the different windows of infection for the two types of blight and the differences in each strain's ability to cause one symptom rather than the other. It was also revealed that cultivars behave differently according to the

type of inoculated strain (Table 8). For instance, cultivar B5133 had very low levels of basal blight caused by strain 552 while it showed the highest levels of basal blight when inoculated with strain 554. This large difference, for example, was much greater than the one observed in cultivar B1202 (5.3% versus 8.7%). A similar situation is observed with the different kernel developmental stages which interact with the different cultivars (Table 8). This means that the resulting ranking among cultivars will be influenced by the time at which inoculation is performed and this would depend on the type of symptom that is being evaluated. Thus, greenhouse results clearly pointed out the importance of performing cultivar screening taking in consideration the type of symptom, the type of bacteria inoculated and the time of inoculation. These statistically significant results involving these interactions were not observed in the field. However, in the field it was also observed that at least for basal kernel blight, enhancement of disease pressure by overhead irrigation at soft dough stage resulted in higher levels of disease increasing the differences between cultivars (Fig. 9). Greenhouse and field results showed the existence of genetic resistance among cultivars and also the importance of the different factors that have to be considered when screening programs are in progress.

Greenhouse and field experiment observations have shown that there are three major factors to consider in the etiology of kernel blight in Montana: The bacterium *P. syringae* as primary causal agent, the kernel developmental stage of the plant at time of infection, and the duration of moisture during this time. The type of bacteria seems highly correlated to the type of symptom and the type of symptom appears to be strongly influenced by the morphology of the barley head at different kernel developmental stages. This seems to be also associated with genetic characteristics, such as 2-row versus 6-row cultivars. These results are of direct importance to the farmer because with this information, sprinkler irrigation can be avoided during the window of infection and the appropriate cultivar can be selected. Also, although of a lesser value since weather conditions are highly variable, planting dates could also be chosen based on averages of climatological data from several years in hope of escaping the most likely rainfall season during the window of infection.

SUMMARY

Kernel blight of barley exhibits two types of symptoms in Montana. These symptoms are referred to as basal kernel blight consisting of dark brown discoloration on the embryo end of the kernel, and spot kernel blight with symptomatology of a well defined spot on the lemma of the kernel. Strains of *Pseudomonas syringae* were found to cause both types of blight. Two different pathovars were associated with the differing symptoms types. One group corresponded to *P. s. pv. syringae*, characterized by the production of syringomycin and utilization of L-lactate and trigonelline. The second group, an undefined pathovar designated as *P. syringae* 554, was differentiated from the previous group as not producing syringomycin or utilizing L-lactate or trigonelline. Strains from *P. s. pv. syringae* were strongly associated with basal kernel blight while *P. syringae* 554 strains were associated primarily with spot kernel blight. This constitutes the first report of *P. syringae* as a causal agent of basal kernel blight.

Using RFLP analysis, a dendrogram was constructed to ascertain possible genetic relatedness among the *P. s. pv. syringae* strains and *P. syringae* 554. Three probes were utilized in these studies including a 1.07-Kbp internal

fragment of the *syrB* gene (involved in syringomycin production) and two internal fragments, one 10-Kbp and one 14-Kbp, from the *hrp* region of *P. s. pv. syringae* 61. Most of the *P. s. pv. syringae* strains tested hybridized to all three probes while strains of *P. syringae* 554 only hybridized to the 14-Kbp *hrp* probe. RFLPs obtained with the *syrB* probe suggested a possible relationship between amount of syringomycin produced by a strain and its RFLP pattern. When RFLP profiles from the three probes were combined in the dendrogram, most of the *P. s. pv. syringae* strains were distributed in closely related clusters while *P. syringae* 554 strains were in a very divergent cluster.

Host-related and environmental conditions involved in development of the different types of kernel blight were studied under greenhouse and field conditions. These experiments indicated that the kernel developmental stage was an important factor in the disease. A different window of infection was observed for the two types of blight. Basal kernel blight occurred when infection onset was during late milk and dough stages while early milk was the optimum stage for infection for spot kernel blight. High moisture was determined to be necessary during the window of infection for successful development of the disease. Screening methods revealed that resistance of cultivars differed for the two types of blight. Therefore, screening

for resistance to kernel blight must be performed separately for each type of blight, taking into consideration the differences in their window of infection. This may serve as the basis for developing resistant cultivars of malting barley to both types of blight.

LITERATURE CITED

LITERATURE CITED

- Anderson, W. H., and Banttary, 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.
- Arlat, M., Gough, C. L., Barber, C. E., Boucher, C., and Daniels M. J. 1991. *Xanthomonas campestris* contains a cluster of *hrp* genes related to the larger *hrp* cluster of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 6:593-601.
- Banttari, E. E., Anderson, W. H., and Rasmusson, D.C. 1975. Helminthosporium head blight resistance in six-row spring barleys. Plant Dis. Rep. 59:274-277.
- Ballio, A., Bossa, F., Collina, A., Gallo, M., Iacobellis, N. S., Paci, M., Pucci, P., Scaloni, A., Segre, A., and Simmaco, M. 1990. Structure of syringotoxin, a bioactive metabolite of *Pseudomonas syringae* pv. *syringae*. FEBS Letters 269:377-380.
- Ballio, A., Barra, D., Bossa, F., Collina, 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 291:109-112.
- Barta, J. M., Kinscherf, T. G., and Willis, D. K. 1992. Regulation of tabtoxin production by the *lemA* gene in *Pseudomonas syringae*. J. Bacteriol. 174:3021-3029.
- Bauer, D. W., and Beer, S. V. 1987. Cloning of gene a gene from *Erwinia amylovora* involved in induction of hypersensitivity and pathogenicity. Pages 425-429 in: Plant Pathogenic Bacteria. E. L. Civerolo, A. Collmer, R. E. Davis, and A. G. Gillaspie, eds. Martinus Nijhoff, Boston.
- Buchanan, R. E. and N. E. Gibbons (ed.). 1974. Bergey's manual of determinative bacteriology, eighth edition, pp. 217-253. Waverly Press, Inc., Baltimore, Maryland.

- Bidwai, A. P., and Takemoto, J. Y. 1987. Bacterial phytotoxin, syringomycin, induces a protein kinase-mediated phosphorylation of red beet plasma membrane polypeptides. *Proc. Nat. Acad. Sci.* 84:6755-6759.
- Bonas, V., Schulte, R., Fencelau, S., Minsavage, G. V., Staskawicz, B. J., and Stall, R. E. 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. *Mol. Plant-Microbe Interact.* 4:81-88.
- Boucher, C. A., van Gijsegem, F., Barberis, P. A., Arlat, M., and Zischek, C. 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. *J. Bacteriol.* 169:5626-5632.
- Burger, W. C. and LaBerge, D. E. 1985. Malting and brewing quality. *Agron-A-Ser-Monogr-Am-Soc-Agron. Madison Wis., Soil Science Society of America* (26) p. 367-401.
- Cook, D., Barlow E., and Sequeira L. 1989. Genetic diversity of *Pseudomonas solanacearum*: Detection of restriction fragment length polymorphism with DNA probes that specify virulence and the hypersensitive response. *Mol. Plant-Microbe Interact.* 3:113-121.
- Cuppels, D. A., Moore, R. A., and Morris, V. L. 1990. Construction and use of a nonradioactive DNA hybridization probe for detection of *Pseudomonas syringae* pv. *tomato* on tomato plants. *Appl. Environ. Microbiol.* 56:1743-1749.
- Cough, C. L., Genin, S., Zischek, C., and Boucher, C. A. 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. *Mol. Plant-Microbe interact.* 5:384-389.
- Daniels, M. J., Dow, J. M., and Osborn, A. E. 1988. Molecular genetics of pathogenicity in phytopathogenic bacteria. *Annu. Rev. Microbiol.* 26:285-312.

- Denny, T. P., Gilmour, M. N., and Selander, R. K. 1988. Genetic diversity and relationships of two pathovars of *Pseudomonas syringae*. J. Gen. Microbiol. 134, 1949-1960.
- Drapper, M. A. 1985. The interaction of *Pseudomonas syringae* pv. *syringae* and barley cultivars in inciting leaf blight and kernel spot. M.S. thesis. North Dakota State University, Fargo, N.D.
- Follstad M. N. 1961. The microflora of barley in the field, during malting, and in storage. M.S. thesis. University of Minnesota, St. Paul. 82 pp.
- Fukuchi, N., Isogai, A., Yamashita, S., Suyama, K., Takemoto, J. K., and Suzuki, A. 1990. Structure of phytotoxin syringomycin produced by a sugar cane isolate of *Pseudomonas syringae* pv. *syringae*. Tetrahedron Lett. 31:1589-1592.
- Gebhardt, D. J., Rasmusson, D. C., and Wilcoxon, R. D. 1992. Cyclic breeding used to incorporate kernel discoloration resistance into malting barley. Crop Sci. 32:352-356.
- Gottwald, T. R., Alvarez, A. M., Hartung, and Benedict, A. A. 1991. Diversity of *Xanthomonas campestris* pv. *citrumelo* strains associated with epidemics of citrus bacterial spot in Florida citrus nurseries: Correlation of detached leaf, monoclonal antibody, and restriction fragment length polymorphism assays. Phytopathology 81:749-753.
- Gross, D. C. 1985. Regulation of syringomycin synthesis in *Pseudomonas syringae* pv. *syringae* and defined conditions for its production. J. Appl. Bacteriol. 58:167-174.
- Gross, D. C. 1991. Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. Annu. Rev. Phytopathol. 29:247-278.
- Harlow E. and D. Lane. 1988. Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, 726pp.
- Harrison, L., Teplow, D. B., Rinaldy, M., and Strobel, G. 1991. Pseudomycins, a family of novel peptides from *Pseudomonas syringae* possessing broad-spectrum antifungal activity. J. Gen. Microbiol. 137:2857-2865.

- Hartung, J. S., and Civerolo, E. L. 1991. Variation among strains of *Xanthomonas campestris* causing citrus bacterial spot. *Plant Dis.* 75:622-626.
- 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*. *J. Appl. Bacteriol.* 73, 455-464.
- Heu S., and Hutcheson, S. W. 1993. Nucleotide sequence and properties of the *hrmA* locus associated with the *Pseudomonas syringae* pv. *syringae* 61 *hrp* gene cluster. *Mol. Plant-Microbe Interact.* 6:553-564.
- Hildebrand, D. C., Schroth, M. N., and Huisman, O. C. 1982. The DNA homology matrix and non-random variation concepts as the basis for the taxonomic treatment of plant pathogenic and other bacteria. *Annu. Rev. Phytopathol.* 20:235-256.
- Hildebrand, D. C., Schroth, M. N., and Sands, D. C. 1988. *Pseudomonas*. In: Laboratory guide for identification of plant pathogenic bacteria. Edited by Schaad, N. W. 2nd Edition. The American Phytopathological Society. St. Paul, Minnesota.
- Hrabak, E. M., and Willis, D. K. 1993. Involvement of the *lemA* gene in production of syringomycin and protease by *Pseudomonas syringae* pv. *syringae*. *Mol. Plant-Microbe Interact.* 6:368-375.
- Huang, H. C., Schuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutcheson, S. W., and Collmer, A. 1988. Molecular cloning of *Pseudomonas syringae* pv. *syringae* gene that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants. *J. Bacteriol.* 170:4748-4756.
- Huang, H. C., Hutcheson, S. W., and Collmer, A. 1991. Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and *TnphoA* tagging of genes encoding exported or membrane-spanning Hrp proteins. *Mol. Plant-Microbe Interact.* 4:469-476.
- Iacobellis, N. S., Lavermicocca, P., Grgurina, I., Simmaco, M., and Ballio, A. 1992. Phytotoxin properties of *Pseudomonas syringae* pv. *syringae* toxins. *Physiol. Molec. Plant Pathol.* 40:107-116.

- King, E. O. M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301-307.
- Kinscherf, T. G., Coleman, R. H., Barta, T. M., and Willis, D.K. 1991. Cloning and expression of the tabtoxin biosynthetic region from *Pseudomonas syringae*. *J. Bacteriol.* 13:4124-4132.
- Klement, Z., Stall, R. E., Novacky, A. J., Ersek, T., Fett, W. F., Huang, J. S., and Beckman, C. H. 1990. Mechanisms of resistance. In: *Methods in Phytopathology*, ed by Klement Z., Rudolph, K., and Sands, D. C. Academiai Kiado Budapest.
- Kovacs, N. 1956. Identification of *Pseudomonas pyocyan*a by the oxidase reaction. *Nature* 178:703.
- Laby, R. J., and Beer, S. V. 1992. Hybridization and functional complementation of the *hrp* gene cluster from *Erwinia amylovora* strain Ea 321 with DNA of other bacteria. *Mol. Plant-Microbe Interact.* 5:412-419.
- 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. *Appl. Environ. Microbiol.* 59:4180-4188.
- Liang, L. Z., Chiou, C.-S., and Jones, A. L. 1993. Organization of a gene cluster from *Pseudomonas syringae* pv. *morsprunorum* responsible for necrosis in cherry and hypersensitivity in tobacco. *Abstr. Phytopathology* 83:1416.
- Lutey, R. W. 1962. Studies on the microflora of barley kernels. Ph.D. thesis. University of Minnesota, St. Paul. 111 pp.
- Machacek, J. E., and Greaney, F. J. 1938. The 'black point' or 'kernel smudge' disease of cereals. *Can. j. Res., Sect. C.*, 16:84-113. Botanical Sciences.
- Mathre, D. E. 1982. *Compendium of barley diseases*. APS Press. St. Paul, Minnesota

- 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.
- Miles, M. R., Wilcoxson, R. D., and Rasmusson, D. C. 1989. Inheritance of resistance to kernel discoloration of barley. *Plant Dis.* 73:711-715.
- Miller, B. 1993. Bush Agricultural Resources. Fairfield, MT. Personal communication.
- Mitchell, R. E. 1981. Structure: Bacterial. In *Toxins in plant diseases*, ed. R. D. Durbin, pp. 259-293. New York Academic. 515 pp.
- Mitchell, R. E., Coddington, J. M., and Young, H. 1989. A revised structure for tagetitoxin. *Tetrahedron Lett.* 30:501-504.
- Mohan, S. K., and N. W. Shaad. 1987. Semiselective agar medium for isolating *Pseudomonas syringae* pv. *syringae* and pv. *phaseolicola* from bean seed. *Phytopathology* 77:1390-1395.
- Palmer, D. A., and Bender, C. L. 1993. Effects of environmental and nutritional factors on production of the polyketide phytotoxin coronatine by *Pseudomonas syringae* pv. *glycinea*. *Appl. Environ. Microbiol.* 59:1619-1626.
- 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.
- Prosen, D., Hatziloukas, E., Schaad, N. W., and Panopoulos, N. J. 1993. Specific detection of *Pseudomonas syringae* pv. *phaseolicola* DNA in bean seed by polymerase chain reaction-based amplification of a phaseolotoxin gene region. *Phytopathology* 83:965-970.
- 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. *Mol. Plant-Microbe Interact.* 1:78-90.

- Quigley, N. B., Mo, Y. Y., and Gross, D. C. 1993. SyrD is required for syringomycin production by *Pseudomonas syringae* pv *syringae* and is related to a family of ATP-binding secretion proteins. *Mol. Microbiol.* 9:787-801.
- Sambrook, J., Fritsch, E. T., and Maniatis, T. 1989. Molecular cloning, a laboratory manual, second edition. Cold spring Harbor Laboratory Press.
- Schilder A. M. C., and Bergstrom, G. C. 1994. Infection of wheat seed by *Pyrenophora tritici-repentis*. *Can J. of Botany*. In Press.
- Segre, A., Bachmann, R. C., Ballio, A., Bossa, F., Grgurina, I., Iacobellis, N. S., Marino, G., Pucci, P., Simmaco, M., and Takemoto, J. Y. 1989. The structure of syringomycins A₁, E and G. *FEBS Lett.* 255:27-31.
- Shaad, N. W., Azad, H., Peet, R. C., and Panopoulos, N. J. 1989. Identification of *Pseudomonas syringae* pv. *phaseolicola* by a DNA hybridization probe. *Phytopathology* 79:903-907.
- Staskawicz, B. J., and Panopoulos, N. J. 1979. A rapid and sensitive microbial assay for phaseolotoxin. *Phytopathology* 69:663-666.
- Stevenson, I. L. 1981. Timing and nature of seed infection of barley by *Cochliobolus sativus*. *Can. J. Plant Pathol.* 3:76-85.
- Takemoto, J. Y. 1992. Bacterial phytotoxin syringomycin and its interactions with host membranes. Pages 247-260 in: *Molecular Signals in Plant-Microbe Communications*. D. P. S. Verma, ed. CRC Press, Boca Raton, FL.
- Teviotdale, B. L., and Hall D. L. 1976. Factors affecting inoculum development and seed transmission of *Helminthosporium gramineum*. *Phytopathology* 66:295-301.
- Toben, H., Mavridis, A., and Rudolph, K. W. E. 1989. Basal glume rot (*Pseudomonas syringae* pv. *atrofaciens*) on wheat and barley in FRG and resistance screening on wheat. *EPPO Bulletin* 19:119-125.
- Wesenberg, D. M. 1994. Personal communication.

- Wilcoxson, R. D., Rasmusson, D. C., Banttari, E. E., and Johnson, D. A. 1980. Feasibility of selecting for resistance to kernel discoloration in barley. *Plant Dis.* 64:928-930.
- Willis, D. K., Rich, J. J., and Hrabak, E. M. 1991. hrp genes of phytopathogenic bacteria. *Mol. Plant-Microbe Interact.* 4:132-138.
- Xu, G.-W., and Gross, D. C. 1988a. 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.* 54:1345-1353.
- Xu, G.-W., and Gross, D. C. 1988b. Physical and functional analysis of the *syrA* and *syrB* genes involved in syringomycin production by *Pseudomonas syringae* pv. *syringae*. *J. Bacteriol.* 170:5680-5688.
- Young, S. A., Park, S. K., Rodgers, C, Mitchell, R. E., and Bender, C. L. 1992. Physical and functional characterization of the gene cluster encoding the polyketide phytotoxin coronatine in *Pseudomonas syringae* pv. *glycinea*. *J. Bacteriol.* 174:1837-1843.
- Zadoks, J. C., Chang, T. T., and Konzak, C. F. 1974. A decimal code for the growth stages of cereals. *Weed Research*, 14:415-421.

APPENDICES

APPENDIX A

RESTRICTION FRAGMENT PATTERNS

Table 10. *Pst*I restriction fragment patterns that resulted by hybridization to the *syrB* probe.

Strain	Toxin Zone ^a	RFLP ^b	<i>Pst</i> I Fragment (Kbp)					
			7.4	5.9	5.6	3.2	1.4	1.2
B301D	10	9	-	-	-	+	+	+
SD202	10	1	-	-	+	-	+	+
552	10	1	-	-	+	-	+	+
537	10	1	-	-	+	-	+	+
592	10	1	-	-	+	-	+	+
594	10	1	-	-	+	-	+	+
605	10	1	-	-	+	-	+	+
330	10	1	-	-	+	-	+	+
334	10	1	-	-	+	-	+	+
393	10	1	-	-	+	-	+	+
351	10	1	-	-	+	-	+	+
360	10	1	-	-	+	-	+	+
489	10	1	-	-	+	-	+	+
593	10	1	-	-	+	-	+	+
610	10	2	-	-	+	+	+	+
607	10	3	+	-	+	-	-	-
346	10	4	-	-	+	+	-	-
392	10	4	-	-	+	+	-	-
595	10	4	-	-	+	+	-	-
541	10	4	-	-	+	+	-	-
542	10	4	-	-	+	+	-	-
348	10	4	-	-	+	+	-	-
490	10	5	-	+	+	-	-	-
491	10	5	-	+	+	-	-	-
648	2	6	-	-	+	-	-	-
652	2	6	-	-	+	-	-	-
651	2	6	-	-	+	-	-	-
600	2	6	-	-	+	-	-	-
645	2	6	-	-	+	-	-	-
646	2	6	-	-	+	-	-	-
649	2	6	-	-	+	-	-	-
643	2	6	-	-	+	-	-	-
650	2	7	+	-	-	+	-	-
647	2	8	-	-	-	+	-	-
644	2	8	-	-	-	+	-	-
418	0	-	-	-	-	-	-	-

Table 11. *Pst*I restriction fragment patterns that resulted by hybridization to the 10 Kbp *hrp* probe.

[illegible]

Table 12. *Pst*I restriction fragment patterns that resulted by hybridization to the 14 Kbp *hrp* probe.

Strain	Toxin Zone ^a	RFLP ^b	<i>Pst</i> I Fragment (Kbp)						
			5.6	3.6	1.9	1.7	1.4	1.2	0.6
B301D	10	-	-	-	-	-	-	-	-
SD202	10	19	-	+	+	+	+	+	+
552	10	19	-	+	+	+	+	+	+
537	10	19	-	+	+	+	+	+	+
592	10	19	-	+	+	+	+	+	+
594	10	19	-	+	+	+	+	+	+
605	10	19	-	+	+	+	+	+	+
330	10	19	-	+	+	+	+	+	+
334	10	19	-	+	+	+	+	+	+
393	10	19	-	+	+	+	+	+	+
351	10	19	-	+	+	+	+	+	+
360	10	19	-	+	+	+	+	+	+
489	10	19	-	+	+	+	+	+	+
593	10	19	-	+	+	+	+	+	+
346	10	19	-	+	+	+	+	+	+
392	10	19	-	+	+	+	+	+	+
595	10	19	-	+	+	+	+	+	+
348	10	19	-	+	+	+	+	+	+
490	10	19	-	+	+	+	+	+	+
491	10	19	-	+	+	+	+	+	+
648	2	19	-	+	+	+	+	+	+
652	2	19	-	+	+	+	+	+	+
651	2	19	-	+	+	+	+	+	+
600	2	19	-	+	+	+	+	+	+
645	2	19	-	+	+	+	+	+	+
646	2	19	-	+	+	+	+	+	+
649	2	19	-	+	+	+	+	+	+
643	2	19	-	+	+	+	+	+	+
541	10	20	+	-	+	+	-	+	+
542	10	20	+	-	+	+	-	+	+
610	10	21	+	+	+	+	+	+	+
607	10	21	+	+	+	+	+	+	+
418	0	22	-	+	-	-	-	-	-
650	2	-	-	-	-	-	-	-	-
647	2	-	-	-	-	-	-	-	-
644	2	-	-	-	-	-	-	-	-

^a Zones of inhibition of *G. candidum* as measured from the margin of the bacterial colony to the point of fungal growth on PDA and on SRM. A value of 10 when zone was between 8-10 mm and of 2 for zones between 2-4 mm. Each value represents the average of two trials conducted on separate occasions.

^b Strains within a RFLP group had the same pattern with a specific probe. The order was assigned arbitrary.

APPENDIX B

CLIMATOLOGICAL DATA, FAIRFIELD MT.

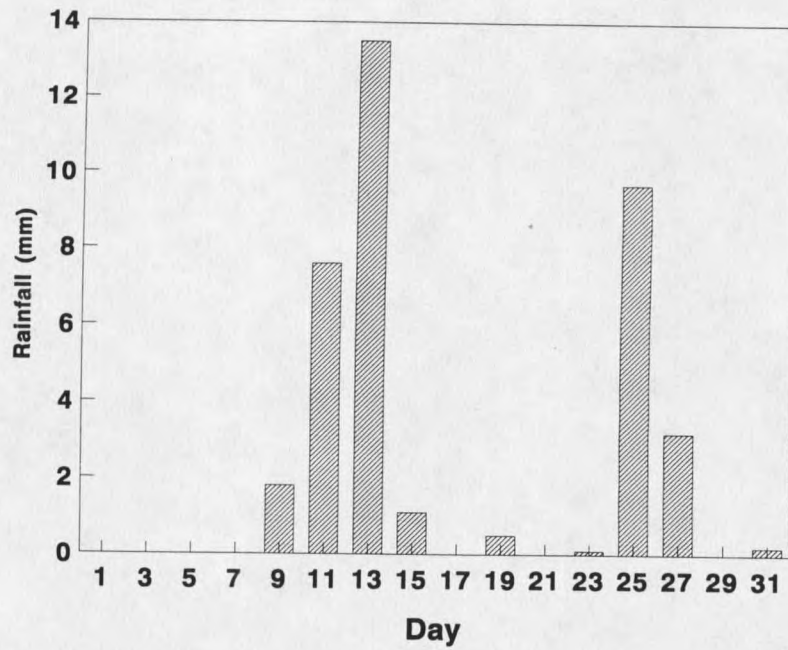


Figure 12. Rainfall obtained during the month of July 1989, Fairfield MT.

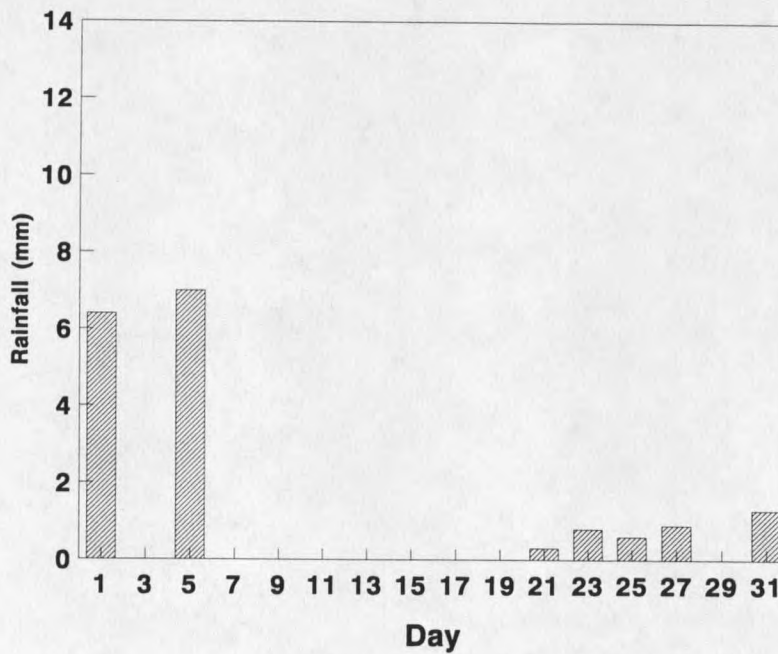


Figure 13. Rainfall obtained during the month of July 1990, Fairfield MT.

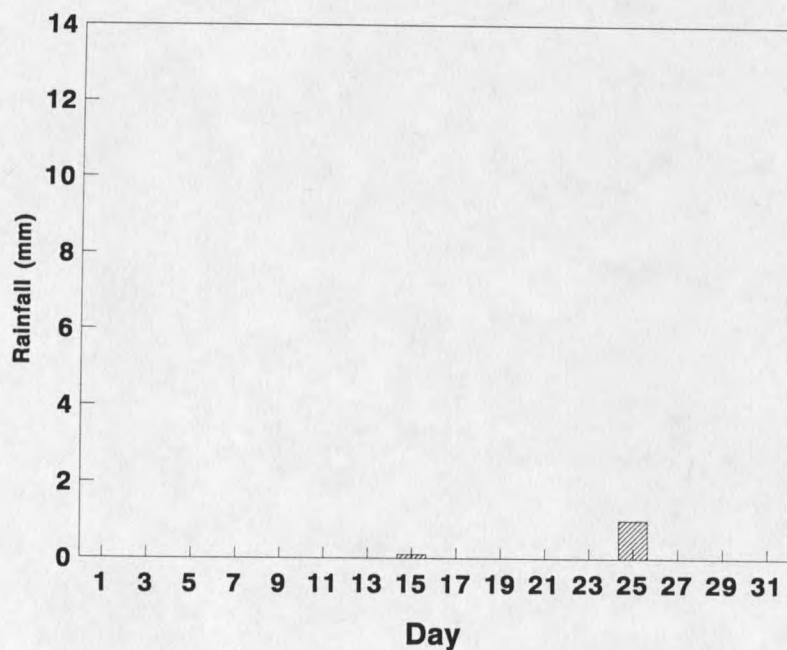


Figure 14. Rainfall obtained during the month of July 1991, Fairfield MT.

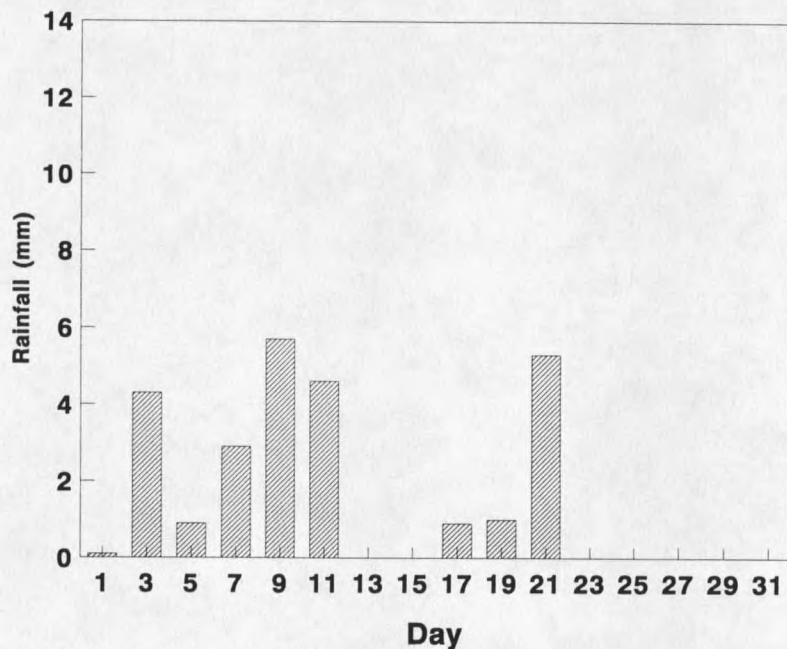


Figure 15. Rainfall obtained during the month of July 1992, Fairfield MT.

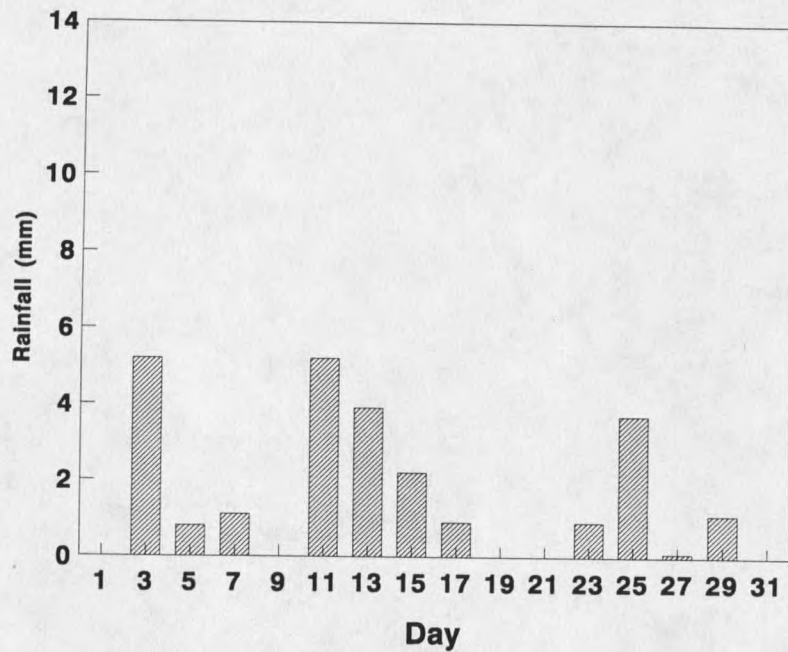


Figure 16. Rainfall obtained during the month of July 1993, Fairfield MT.

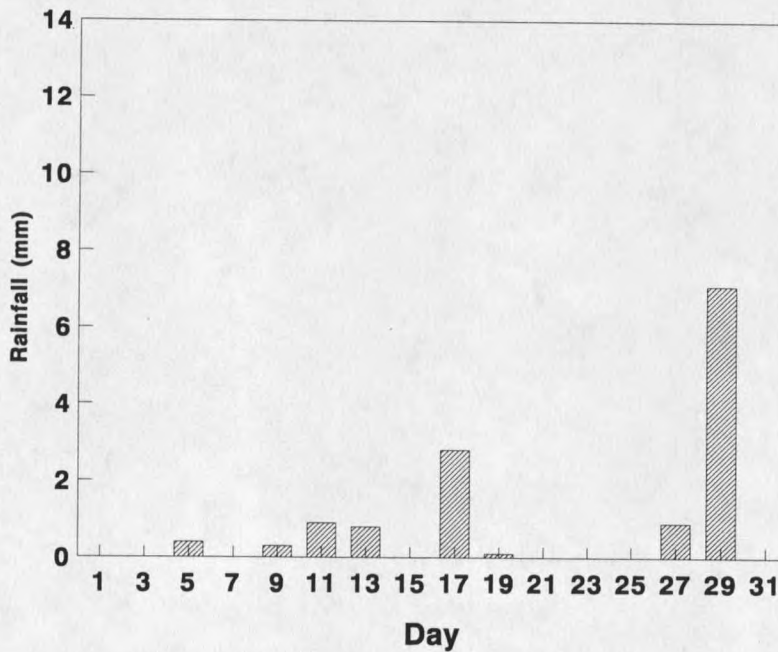


Figure 17. Rainfall obtained during the month of August 1993, Fairfield MT.

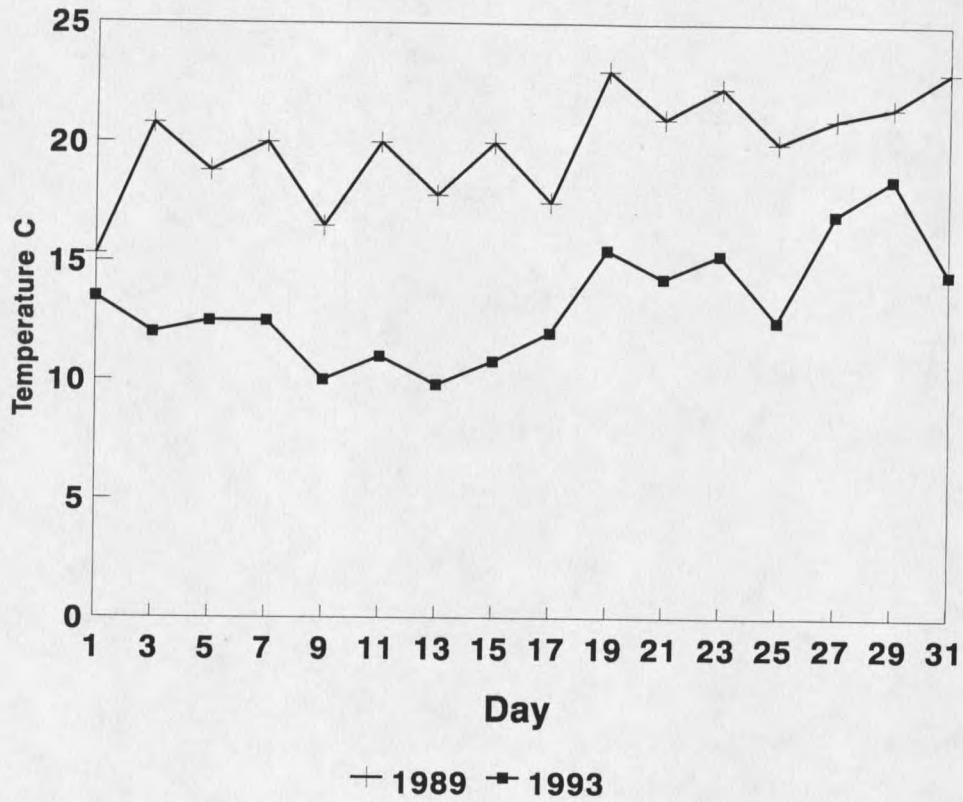


Figure 18. Temperature data collected during the month of July in Fairfield MT., in 1989 and 1993.

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