An Abstract of the Thesis of

M. Margaret Roche for the degree of Master of Science in Horticulture presented on July 19, 2001. Title: Development of an *In Vitro* and Modification of an *In Vivo* Bioassay to Screen Cherry Genotypes for Response to Inoculation with *Pseudomonas syringae* pv. *syringae*

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Anita N. Azarenko

The bacterium *Pseudomonas syringae* affects different crops worldwide. In the Willamette Valley of Oregon, *P. syringae* causes bacterial canker in sweet cherry, severely limiting its production. High grafting of susceptible sweet cherry cultivars to resistant rootstocks is practiced in the Willamette Valley to reduce incidence of this disease. The research objective was to screen for potential resistance to bacterial canker using a modified *in vivo* and an *in vitro* bioassay.

The severity of infection of sweet cherry genotypes inoculated with *Pseudomonas syringae* pv. *syringae* was rated, using both an *in vitro* excised leaf bioassay and an *in vivo* twig bioassay. The response of *in vitro* excised leaves after inoculation with a mixture of four highly virulent *P. syringae* pv. *syringae* strains (SD443, SD447, W4N54, and W4N108), at two concentrations (10^6 and 10^8 cfu/ml) was evaluated. The response to inoculation with water and a *P. syringae* pv. *syringae* strain (JL2000) with low virulence was also rated using the *in vitro* bioassay. Necrosis of leaves was recorded on a scale from 0 to 4 with a score of 4 indicating complete leaf necrosis.

The *in vivo* bioassay used twigs as the plant material. A browning response to inoculation with water and a mixture of four pathogenic strains (SD443, SD447, W4N54, and W4N108) at 10^8 cfu/ml was rated on a scale from 1 to 4. A score of 1 indicated

yellow pith while a 4 indicated completely brown pith. Gummosis and callus were also noted in the *in vivo* bioassay.

Results from the *in vivo* and *in vitro* bioassays showed Mazzard x Mahaleb (MxM) genotypes had the smallest response to inoculation with the mixed pathogenic treatments. The *in vitro* bioassay further indicated that Pi-Ku genotypes 4-11, 4-22, 4-20 and 4-17; and Giessen clones 148-1 (GiSelA 6), 192-2 (GiSelA 12), 148-9 (GiSelA 8) 148-8 (GiSelA 7), 196-4, 318-17,195-20, 497-8 and 473-10 (GiSelA 4) might be regarded as potential resistant rootstocks. Weiroot genotypes, tested only in the *in vitro* bioassay, showed a large necrotic response. In the *in vivo* bioassay Giessen clone 169-15 showed a low browning response and a high gummosis response.

This investigation identified genotypes possessing potential resistance to bacterial canker. These genotypes should be evaluated under field conditions to determine disease resistance in the orchard environment. Results from field trials will help growers determine the optimal rootstock for their production system. Ultimately, these assays may be useful in screening sweet cherry scion genotypes for resistance to bacterial canker.

Development of an *In Vitro* and Modification of an *In Vivo* Bioassay to Screen Cherry Genotypes for Response to Inoculation with *Pseudomonas syringae* pv. *syringae*

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APPROVED:

Redacted for privacy

Major Professor, representing Horticulture

Redacted for privacy

Chair of Department of Horticulture

Redacted for privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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M. Margaret Roche, Author

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Dedication

This work is dedicated to the fond memory of my devoted grandmother Evelyn Roche -- a true and unwavering inspiration.

Development of an *In Vitro* and Modification of an *In Vivo* Bioassay to Screen Cherry Genotypes for Response to Inoculation with *Pseudomonas syringae* pv. *syringae*

Chapter 1

Introduction

Throughout the world, the bacteria *Pseudomonas syringae* is a serious threat to fruit growing regions. Economic losses due to diseases caused by the pathogen span wide geographical areas and affect many different crops. In Oregon, bacterial canker, caused by *P. syringae* pv. *syringae*, is a major limiting factor in sweet cherry production, especially in the Willamette Valley region where economic losses include reduced yield and damaged trees (Cameron, 1962). Further, losses in the form of frost injury are also attributed to the bacteria, resulting from its ice nucleation capabilities (Baca and Moore, 1987; Lindow, 1983).

Many symptoms are associated with the disease, the most destructive being cankers on the trunk and scaffold limbs. These cankers, sometimes accompanied by gumming, often lead to girdling and hence the death of the area above the canker. Death of dormant buds, which can be severe in Oregon, is also destructive. Of somewhat lesser significance are symptoms on the leaves, fruit, and blossoms (Cameron, 1962).

The bacterial canker disease cycle includes prominent winter and summer phases (Cameron, 1962). The winter phase resides in the bark of stems and branches while the summer phase occupies the green tissues, including the leaves, blossoms, and shoots (Crosse, 1966). Cankers develop from fall to spring, with reduced expansion during the

cold season and more accelerated growth following the cold season. Growth and development of the tree characterize the summer phase. At this time, the tree is capable of surrounding the bacteria with callus tissue effectively halting their growth and further canker development (Cameron, 1962). It is during the winter phase that infection poses the highest risk.

Overwintering of the bacteria takes place in active cankers, infected tissues, xylem vessels and, epiphytically, in buds and on weeds (Agrios, 199; Malvick, 1987; Sundin *et al.,* 1988). Dissemination of the bacteria is by wind and rain (Agrios, 1997). The several infection routes include flowers, limbs, buds, and leaves (Crosse, 1954; Cameron, 1962; Agrios, 1997). Often symptoms of these sites include necrosis that is induced by the bacterial phytotoxin syringomycin (Sinden *et al.,* 1971; Bender *et al.,* 1999; Quigley and Gross, 1994a).

In addition to causing plant necrosis, syringomycin is toxic to microorganisms (Sinden *et al.,* 1971; Bender *et al.,* 1999). In plants, the toxin primarily acts at the plasma membrane where inserts and forms pores. The pores act to disrupt the ionic balance of the membrane, via fluxes of K^+, H^+ and Ca^{2+}, creating a nutrient rich environment for the bacteria, in intercellular spaces, and initiating cell death (Quigley and Gross, 1994a; Bender *et al.,* 1999).

Chemical control of bacterial canker in sweet cherries has been inconsistent in Oregon. Ineffective control is attributed to both the development of copper and streptomycin resistant strains and to the systemic properties of the bacteria (Cameron, 1970; Cameron 1962; Scheck *et al.,* 1996; De Boer, 1980; Young, 1977). Cultural control practices that reduce winter injury, create optimal growing environments, reduce

wounding, and utilize healthy plant material are recommended to reduce the incidence and severity of bacterial canker (Hawkins, 1976; Spotts *et al.,* 1990; Agrios, 1997; Crosse, 1954). The most touted cultural control is the use of resistant plant material as a rootstock to which a scion is high grafted onto scaffold limbs (Grubb, 1944; Cameron, 1962; Shanmuganathan and Crosse, 1963; Cameron 1971).

The demand for resistant plant material has resulted in the introduction of many rootstocks. Some of the rootstocks being considered for the Willamette Valley include the Weiroot series, *Prunus cerasus* clonal selections from Germany; interspecific hybrids, the Giessen and Pi-KU series; and genotypes belonging to the Mazzard x Mahaleb (MxM) series (Wertheim, 1998). *Prunus cerasus* genotypes in the past have been described as having "high field resistance" to bacterial canker and MxM genotypes have showed no to low browning in a previous bacterial canker bioassay (De Vries,1965, Krzesinska and Azarenko, 1992). Therefore, in this study Weiroot and MxM genotypes were expected to have small necrotic responses. In contrast, the Giessen and Pi-KU genotypes were expected to show a range of responses due to their interspecific hybrid status.

Field observation has been used to determine field resistance to bacterial canker but has often yielded inconsistent results. Recently, more reliable assessments identifying resistance to bacterial canker in cherry have been achieved using an *in vivo* twig bioassay (Krzesinska and Azarenko, 1992). Refinement of *in vivo* bioassays have resulted in the development of *in vitro* bioassays which, when *in vitro* plant material is available, can be advantageous due to quicker symptom development, season independence, and faster procurement of tissue.

In this study an *in vitro* excised leaf bioassay was developed to screen cherry plantlets for a response to inoculation with *P. syringae* pv. *syringae*. Additionally, an *in vivo* twig bioassay, determined to accurately reflect genotypic field resistance, was modified to better resemble the protocol used in the excised leaf bioassay (Krzesinska and Azarenko, 1992). The modified twig bioassay was used to evaluate cherry genotypes both already and not already screened using the excised leaf method. Some genotypes that were evaluated in a twig bioassay, prior to modification, were screened again using the modified twig bioassay. The results from the excised leaf and the modified twig bioassay were compared and agreement was found. It was concluded that both the excised leaf bioassay and the modified twig assay have useful and valuable applications.

Chapter 2

Literature Review

Bacterial canker of stone fruit, caused by the bacterium *Pseudomonas syringae*, has been detected in fruit growing regions throughout the world. In addition to its wide geographical range, pathovars of this plant pathogen are known to incite disease on several different crops including pome fruits, citrus, ornamentals, vegetables and small grains (Agrios, 1997). Resulting from its ubiquity, many common names have been assigned to this disease. These names include bacterial soursap, bacteriosis, blast of stone fruit, blossom blast, bud blast, cherry gumnosis, die-back, gummosis, shoot blight, soursap, spur blight, twig blight, and wither tip (Cameron, 1962; Agrios, 1997). Despite these descriptors, the common name bacterial canker is preferred since it denotes the presence of necrotic lesions or cankers (Wilson, 1953).

The first recorded description of the organism causing bacterial canker appeared in 1902 when C. J. J. van Hall noted that it caused bacterial blight on lilac. Van Hall has been recognized as being the first researcher to demonstrate the pathogenicity of *P. syringae* (Wilson, 1953). The first account of the organism in the United States did not appear, however, until 1911 when F. L. Griffith, studying bacterial gummosis of cherry, conducted several inoculations with the pathogen and, after describing the organism, named it *Pseudomonas cerasus*. Additional United States' pioneers include Barss, who offered control suggestions and demonstrated that, in Western Oregon, peaches, apricots, and Italian prunes are also affected by the pathogen, and H. R. Cameron, who in 1962

wrote a comprehensive technical bulletin reviewing the diseases incited by *P. syringae* (Barss, 1918; English and Hansen, 1955).

Because the pathogen causing bacterial canker has been identified worldwide, has several hosts, and has been studied by several researchers, it has been known by no less than twelve species names originating with van Hall's designation *Pseudomonas syringae* van Hall (Wilson, 1953). Presently, the recognized identity of the organism is *Pseudomonas syringae*. Two pathovars of *P. syringae*, causing disease on sweet cherry have been observed: pv. *syringae* and pv. *morsprunorum*. Incidence of disease caused by *P. syringae* pv. *morsprunorum* has occurred in England (Crosse, 1963), Poland (Lyskanowska, 1976; Melakeberhan *et al.,* 1993), South Africa (Roos and Hattingh, 1986), Eastern United States and Michigan (Cameron, 1962; Jones 1971). Despite the widespread presence of *P. syringae* pv. *morsprunorum*, *P. syringae* pv. *syringae* is thought to be the organism responsible for inciting disease on sweet cherry trees in Oregon (Cameron, 1962).

In Oregon, bacterial canker has been recognized as a major limiting factor in sweet cherry production. Economic losses attributed to bacterial canker include damage to trees and reduced yields. Although economic losses vary in Oregon orchards, they have been up to 80% in devastating years where dead-bud frequency was significantly high (Cameron, 1962).

The most deleterious symptoms of bacterial canker in sweet cherry are cankers on the trunk and scaffold limbs. Cankers, moderate to severe in Oregon, usually appear long and narrow as they tend to favor upward spread over downward or lateral spread. Cankers are first observed in late winter and early spring, although they begin developing

in fall and winter. Often in spring, as the trees break dormancy, gum that has built up in tissues, surrounding the canker, may break through the bark and exude from the canker. However, cankers are not always accompanied by gumming, which is a common wound response in cherry trees. If the canker girdles the tree then death of the limb occurs. Once girdled, curling or drooping leaves may be noted by spring or midsummer. Eventually the leaves will turn yellow and appear strongly rolled. At this time, death of the affected area approaches. In extreme cases, where the canker is located beneath the scaffold limbs, the entire top of the tree may die. Cankers have been observed to be a greater threat in trees one to eight years old (Cameron, 1962).

Death of spurs and dormant buds can be as injurious as cankers. Death of dormant buds can be severe in Oregon, especially on mature trees. In the Willamette Valley, 70-80% of buds may be killed, resulting in economic loss and, in some cases, the removal of entire orchards. Profound demonstration of losses can be observed during full bloom when scantly blooming diseased trees are overshadowed by abundant blooms on healthy trees. Sectioned buds in late February to early March, that reveal brown tissue at the base of the bud scales, can provide the first indication of bud infections. Bud infections occur indiscriminately in both flower and leaf buds (Cameron, 1962).

Other less severe symptoms of bacterial canker on sweet cherry include symptoms on the leaves, fruit, and blossoms. Symptoms on leaves usually originate as angular water soaked spots, dark green in color. Leaf spots, mild in Oregon, are typically 1-2 mm or larger in diameter. As the disease progresses, the spots turn necrotic and eventually become dry and brittle. At this time the spots may fall out of the leaf leaving a shot-hole appearance to the leaf. Diseased fruit, occurring occasionally in Oregon, are

associated with irregular brown to black infections that appear flat. Lesions on fruit are typically 2-3 mm in diameter. Fruit lesions can also appear depressed and may harbor gum pockets (Cameron, 1962).

Infected blossoms are not of large consequence on sweet cherry and occur only occasionally in Oregon. However, under favorable environmental conditions, severe blossom infection can occur. Hanging blossoms, with brown water soaked regions, can indicate blossom infection. Infection initiating in the blossom is capable of spreading into the spur possibly giving rise to canker formation, or into the twig leading to twig wilt (Cameron, 1962).

The virulence of *P. syringae* pv. *syringae* is attributed, in part, to the necrosis inducing phytotoxin, known as syringomycin. Syringomycin belongs to a small class of antibiotics termed polypeptins (Quigley and Gross, 1994a). Inoculations with the toxin have yielded similar symptoms as inoculations with the bacterium. Peach shoots inoculated with various concentrations of syringomycin have produced symptoms similar to those observed in shoots inoculated with *P. syringae* (Sinden *et al.,* 1971). Likewise, peach trees injected with preparations of syringomycin express symptoms resembling those caused by *P. syringae* pv. *syringae* inoculations (DeVay *et al.,* 1968). Stone fruit ecotypes of *P. syringae* pv. *syringae* exhibit reduced virulence in the absence of syringomycin production (Gross and DeVay, 1977). In addition to plant tissue, syringomycin has demonstrated toxicity to a wide spectrum of bacterial and fungal microorganisms, and for this reason its use as a fungicide has been contemplated (Sinden *et al.,* 1971; Bender *et al.,* 1999).

Syringomycin primarily acts at the plasma membrane where, because of its lipophilic property, it can insert within the lipid layers of the membrane and form pores. The most accepted model asserts that the toxin disrupts the ionic balance of membranes, thus initiating a cascade of events that eventually manifests in rapid cell death (Quigley and Gross, 1994a). This disruption results from a deadly fluxes of K^+, H^+, and Ca^{2+} ions across the membrane, the end result of which is a nutrient rich environment for the bacterium in the intercellular spaces of the host tissue (Bender *et al.,* 1999).

The K^+/H^+ exchange results in a collapse of the plasma membrane pH gradient, leading to cytoplasmic acidification. Further chaos ensues from an unchecked influx of Ca^{2+}, that stimulates events connected to plant cellular signaling. The discovery of these ion channels, formed in the plasma membrane by syringomycin, is the first of its kind to be associated with a plant pathogenic bacterium. Also considered a powerful surfactant, syringomycin facilitates the dissemination of bacteria over plant surfaces by lowering the surface tension of water (Bender *et al.,* 1999).

Three significant factors involved in the synthesis of syringomycin are the recognition of plant signals by the bacteria, the induction of genes involved in syringomycin production, and the synthesis and secretion of the phytotoxin (Quigley and Gross, 1994a). Six genes, closely linked in a cluster, are involved in the synthesis (*syrB1*, *syrB2*, *syrC*, and *syrE*), secretion (*syrD*), and regulation (*syrP*) of syringomycin. The *syrB* and *syrD* genes can be used as pathovar-specific gene probes, as evidenced by the presence of DNA homologs to these genes in strains of *P. syringae* pv. *syringae* but which are lacking in other pathovars (Scheck *et al.,* 1997; Quigley and Gross, 1994b).

Nutritional factors and plant signal molecules are involved in regulating and activating the production of syringomycin. Iron is known to positively regulate the production of the plant toxin while inorganic phosphate concentrations have been shown to negatively regulate syringomycin production. Activation of syringomycin production occurs in the company of plant signal molecules, such as phenolic glycosides, which are present in the leaves, bark, and flowers of plants susceptible to *P. syringae* pv. *syringae* infection. Furthermore, activation of syringomycin production is improved in the presence of sugars, especially sucrose and fructose. In the case of cherry, the leaves possess two flavonol glycosides, and one flavanone glycoside, whereas large amounts of sucrose and fructose are also present. The high availability of these plant signal molecules in cherry, and their involvement in syringomycin production, may explain the susceptibility of cherry to *P. syringae* pv. *syringae* (Bender *et al.,* 1999).

In addition to its toxin producing capabilities, *P. syringae* also initiates ice formation in plants, contributing to frost injury. *Pseudomonas syringae* has been identified as one of two pathogens considered to be the most efficient naturally occurring ice nuclei. Specifically, *P. syringae* is capable of initiating ice formation at temperatures as high as -1C (Lindow, 1983). Frost injury is recognized as favoring the infection of buds, blossoms and young leaves (Agrios, 1997). In the Pacific Northwest, nursery workers have observed that *P. syringae* infections in woody plants often occur after exposure to low temperatures of 0 to -5C (Baca and Moore, 1987).

The most active seasons for bacterial canker are fall, winter, and spring when the cankers are known to develop. Infected areas enlarge rapidly in the fall and then experience reduced expansion during the cold season, after which more significant

enlargement is noted just before the tree begins accelerated growth (Wilson, 1953). Following the transition to spring, cankers begin to appear in the infected regions (Cameron, 1962).

Generally, *P. syringae* pv. *syringae* is most insidious during the dormant period. The infection period spans from late autumn to early spring. Following this time, the branches and stems enter an interval, corresponding with the growing season, where the tree is actively resisting the infection. Trees typically resist infection from the summer to early autumn (Cameron, 1962). Inoculations during the growing season have shown that the growth of bacteria is checked by a plant response to surround the bacteria with callus tissue, hence restricting its growth (Crosse, 1966). This ability to arrest the pathogen's growth may correspond to varietal differences in resistance. While most cankers are considered to be annual, some cankers may persist and resume activity for subsequent years (Cameron, 1962).

Overwintering of the bacteria occurs in active cankers, infected tissues, such as buds, in the xylem of hosts, and epiphytically (Agrios, 1997). In an Oregon pear orchard and maple nursery, epiphytic populations of *P. syringae* pv. *syringae* were isolated from perennial ryegrass, orchard grass, red fescue grass, annual ryegrass, brome grass, pear trees and maple trees (Malvick, 1987). Additionally, *P. syringae* pv. *syringae* has been recovered from apparently healthy dormant buds on sweet cherry trees (Sundin *et al.*, 1988).

Dissemination of bacteria to new plant growth, from winter cankers, is via wind and rain. Although insects have been implicated as being involved in dissemination, their role is considered inconsequential (Cameron, 1962). There are many possible infection

routes associated with bacterial canker. These routes include floral, limb, bud, and leaf infections. Generally, floral infection routes are considered rare, but under humid conditions they may cause canker formation where bacteria may progress, from natural openings and wounds, into the spur or twig (Agrios, 1997).

Limbs offer several entry points for bacteria including the bases of infected spurs, the bases of infected buds, pruning cuts, and leaf scars. After infection, the bacteria invade the bark, intercellularly, and proceed to attack the ray parenchyma of the phloem and xylem. However, bacteria in the xylem seldom travel far in the vessels. If infection continues unchecked, the bacteria may break down the parenchyma cells creating voids which in turn become occupied by the pathogen (Agrios, 1997).

Bud infections initiate from the outside scales. Timing of bud infection in Oregon is between November and March and is speculated to be on a warm day, after the chilling requirement has been met. Under these conditions, it is probable that buds have begun to swell, enabling bacteria to be washed in between the bud scales, and hence enter the bud (Cameron, 1962). From the base of the scales the bacteria proceed to the base of the bud, causing bud death. In some instances, the bacteria may invade the stem tissues, surrounding the base of the bud, often causing additional tissue death. The infection of buds is compounded by frost injury which is facilitated by *P. syringae* pv. *syringae*'s ice nucleation characteristic (Agrios, 1997).

Leaves are infected through the stomata or areas of the cuticle that have been subjected to insect damage (Crosse, 1954). Evidence suggests that leaf scars are not involved in *P. syringae* pv. *syringae* infections, although this avenue is important in *P. syringae* pv. *mors-prunorum* infections (Cameron, 1962). Young leaves are particularity

susceptible to infection, especially if the spring is cool and wet. Like the limb infections, the bacteria invade intercellularly where they break down and kill cells. These dead regions on the leaves are noted as small angular necrotic lesions. Timing of leaf infection is early in the season when the leaves are young (Agrios, 1997).

Control of bacterial canker is at best inconsistent. Chemical control has been attempted through the use of spray programs that include streptomycin and/or copper (Hawkins, 1976). However, historically in Oregon, control of the phases of bacterial canker with chemicals has yielded mixed results. In 1962 it was reported that bactericides provided successful control for leaf-spot and dead bud but only erratic control for the canker phase (Cameron, 1962). The lack of effective control has been attributed, in part, to the systemic properties of *P. syringae* pv. *syringae*, as illustrated by the recovery of bacteria from within both diseased and apparently healthy sweet cherry trees (Cameron, 1970). The recovery of bacteria from apparently healthy buds indicates that even in cases where chemical efficacy and coverage is optimal the bacterial population can not be eradicated. In more recent years, chemical options have further lost effectiveness due to the selection of copper resistant and streptomycin resistant bacteria (Scheck *et al.,* 1996; De Boer, 1980; Young, 1977).

Cultural control practices have been purported to reduce the severity of bacterial canker in sweet cherry. Early cultural control of bacterial canker, that included cutting out diseased tissue, was often ineffective due to incomplete removal of infection. In New Zealand, cauterization with a hand held propane burner has halted the canker infections after one treatment (Hawkins, 1976).

Cultural practices that reduce winter or frost injury of sweet cherry trees, such as painting trunks white, and employing heating devices or sprinklers, have been implicated in reducing the incidence of trunk cankers (Hawkins, 1976; Spotts *et al.,* 1990). In addition to reducing winter injury, suboptimal environments such as waterlogged soils and drought conditions should be avoided (Agrios, 1997). Further, avoidance of wounding during wet weather, such as with post-harvest pruning, has been shown to contribute to effective disease control (Crosse, 1954). When establishing new plantings, only healthy nursery material and budwood should be used. Also care should be taken to plant new orchards away from older orchards to lower disease incidence (Spotts *et al.,* 1990).

Perhaps the most promising cultural control is the use of resistant plant material. Barss encouraged the use of resistant rootstocks, in Oregon, as early as the 1910s (Cameron, 1962; Barss, 1918). It has been noted that scion wood appears less susceptible when grafted to resistant material, as demonstrated in plum scion cultivars and also in cherry trees (Schofield and Clift, 1959; Grub, 1944). Further, in Oregon it has been shown that when the scion is grafted on scaffold limbs, as opposed to low budded on rootstock, a 40% tree survival rate and 30% reduction in severely infected trees was achieved (Cameron, 1971).

When considering the use of resistant or tolerant rootstocks for control of bacterial canker, it is essential to identify genotypes that exhibit resistance to the disease in the field. Field resistant sweet cherry, *Prunus avium*, plant material is very infrequent whereas sour cherry, *Prunus cerasus,* often appears to have "high field resistance" (De Vries, 1965). Interspecific crosses between *P. avium* (Mazzard) and *P. cerasus* yield trees

possessing "very high field resistance" (De Vries, 1965). Rootstocks used for cherry belong to the formerly mentioned species, interspecific hybrids of these and other species, and also to *Prunus mahaleb*.

Rootstocks were originally collected to promote rootstock uniformity, because variability can be eliminated via vegetative propagation. Presently, rootstocks are used to reduce tree vigor, which enables trees to be planted in higher densities. Reduced tree vigor can facilitate both earlier and higher cropping and can also make possible the establishment of bird netting and rain covers. High budding or grafting onto rootstocks has the distinct advantage of eliminating stem cankers if done on resistant rootstock material (Wertheim, 1998).

Mazzard seedling and clonal Mazzard rootstocks, the first of which was F 12/1, are commonly found in the Willamette Valley. Mazzard seedlings give rise to large trees that are late bearing. F 12/1, selected for at the East Malling research station in England, is as vigorous and non-precocious as the Mazzard seedlings; however, it is acknowledged to possess bacterial canker "field resistance" (Wertheim, 1998).

In contrast to Mazzard, *P. cerasus* is not as significant a rootstock in the Willamette Valley. *Prunus cerasus* is propagated from summer cuttings with ease but has limited use as a sweet cherry rootstock due to potential graft incompatibility and to virus sensitivity. *Prunus cerasus* clonal selections of interest include the Weiroot series from Germany. Weiroot 10, 13, 53, 72, 154, and 158 have been recommended as rootstocks for sweet cherry but little is recorded about their performances outside of Germany (Wertheim, 1998). Of these selections, 53, 72, and 158, have shown to be more

graft compatible with cultivars, although 53 and 72 may require support due to weak growth (Webster and Schmidt, 1996).

Prunus mahaleb, including the vegetatively propagated selection Saint Lucie, is better adapted to soils that are light, gravelly, or calcareous and also to arid or continental climates than *P. avium* roostocks (Wertheim, 1998). Additionally, *P. mahaleb* has demonstrated "tolerance" to bacterial canker (Webster and Schmidt, 1996; Webster 1984). Despite these advantages, *P. mahaleb,* would be an inferior choice for the Willamette Valley due to its poor performance in wet and heavy soils and its susceptibility to *Phytopthora* root rot (Wertheim, 1998).

Interspecific hybrids, the best known being Colt, the Giessen clones, and genotypes belonging to the Mazzard x Mahaleb (MxM) series, attempt to combine desirable traits from within *Prunus*. The most sought after traits have been reduced vigor and increased precocity. Vigor in Colt (*P. avium* x *P. pseudocerasus*) released from the East Malling research station, is sometimes rated 30% less than Mazzard clonal and seedling rootstocks. However, Colt's vigor reduction is greatly dependent on the growing conditions and in some cases growth can exceed that of Mazzard rootstocks. Further benefits of Colt are that it is easily propagated from hardwood and softwood cuttings, laboratory and greenhouse studies have shown it to be "tolerant" to bacterial canker, and trees on Colt are more precocious than trees on F 12/1 (Wertheim, 1998; Webster, 1980; Garrett, 1986). However, a disadvantage of Colt is that it develops crown gall (Pscheidt, personal communication).

The German Giessen clones (interspecific crosses among *P. fructicosa*, *P. cerasus*, *P. canescens*, and *P. avium*) promise more consistent vigor reduction than Colt.

The most encouraging selections of these clones are GiSelA 5, 6, and 12 (Azarenko, personal communication). Vigor of each of these selections has been rated less than F 12/1. GiSelA 5 (148-2), 45% the size of Mazzard, and GiSelA 6 (148-1), 50% the size of Mazzard, are precocious, provide dwarfing, are tolerant to Prune Dwarf Virus and Prunus Necrotic Ringspot Virus, and develop wide branch angles (Wertheim, 1998). Additionally, GiSelA 6 (148-1) and 7 (148-8) received low browning scores when twigs were inoculated with *P. syringae* pv. *syringae* suggesting that they may have bacterial canker resistance (Krzesinska and Azarenko, 1992).

In addition to the Giessen clones, the MxM series, from Oregon, has also been recently reintroduced. The MxM series includes vigorous, semi-vigorous, and dwarfing rootstocks. They have been shown to be productive, sturdy, well-anchored, free from suckering, and are "more resistant" to bacterial canker than Mazzard or Mahaleb (Cummins, 1984). MxM 14 has been touted as the most valuable rootstock of the series, due to its semi-dwarfing capabilities and MxM 60 is popular in Oregon due to its resistance to Phytophtora root rot and improved productivity over Mazzard seedling.

A lesser known series resulting from interspecific hybrids is the Pi-KU series, from Eastern Germany. The Pi-KU selections that have been presented are easily propagated and take well to bud grafting. However, they do not all appear to be consistently dwarfing and therefore their recommendation is contingent on the release of further information (Wertheim, 1998).

Faced with many novel rootstocks, researchers and breeders employ methods of determining which genotypes can offer disease resistance. Often the most common way of determining whether a genotype has resistant tendencies is by simple field observation.

Unfortunately field observation, due in part to environmental variability and pathogen variation, can occasionally provide conflicting evidence of bacterial canker resistance.

In addition to field observations, methods exist to evaluate a material's response to a pathogen. These inoculations and bioassays can be helpful in evaluating the response of cherry genotypes to inoculation with *P. syringae* pv. *syringae*. Evaluating genotypes is valuable since it facilitates the selection of potentially resistant genotypes that may be suitable for cherry production in Oregon. *In vivo* methods of screening plant material have been used for many pathogens and species (see Table 2.1).

In vitro methods of evaluating resistant genotypes improve on the *in vivo* bioassays because plant tissue culture bioassays typically have required less time for symptoms to develop, are season independent, and can reduce the time that it takes to procure new plant material for subsequent inoculations (see Table 2.1). It should be cautioned, however, that not all cherry genotypes have been successfully initiated into plant tissue culture. Even in cases where initiation has been successful current tissue culture media are not always optimized for specific genotypes. Until both protocol ensuring successful initiation into plant tissue culture and versatile media are developed *in vivo* methods of determining resistance will be relied upon.

In addition to plant tissue culture assays that can be used to evaluate resistance, plant tissue culture methods also enable a researcher to select for plant material that is resistant to disease. Typically, the researcher will use the pathogen or the pathogen metabolites as the selecting agent (Daub, 1986). Researchers adopting this approach have shown peach regenerants to possess increased levels of resistance to bacterial canker (Hammerschlag, 1988a).

Table 2.1 Comparison of *in vitro* and *in vivo* assays.

Method	Host(s)	Pathogen(s)	Tissue(s) Assayed	Reference
In vivo	Cherry	*Pseudomonas syringae* pv. *syringae*	Twig	Krzesinska and Azarenko, 1992
	Cucumber, Pear, Potato	*Pseudomonas lachrymasn*	Leaves	Hass and Rotem, 1976
	Cherry, Pear, Apple, Peach	*Pseudomonas syringae* pv. *syringae*	Leaves and twigs of trees, immature fruit, seedlings	Endert and Ritchie, 1984
	Peach	*Xanthomonas campestris* pv. *pruni*	Young detached leaves of seedlings	Randhawa and Civerolo, 1985
	Apple	*Erwinia amylovora*	Excised leaves	Donovon, 1991
	Apple	*Erwinia amylovora*	Greenhouse plants	Donovon *et al.*, 1994
	Pear	*Pseudomonas syringae* pv. *syringae*	Detached leaves of seedlings, seedlings	Yessad *et al.*, 1992
	Plum, Cherry	*Pseudomona syringae* pv. *morsprunorum*, *Pseudomonas syringae* pv. *syringae*	Stems, branches	Cross and Garrett, 1966
In vitro	Peach	*Xanthomonas campestris* pv. *pruni*	Callus	Hammerschlag, 1990
	Apple, Pear	*Erwinia amylovora*	Tissue culture plantlets	Duron, 1987
	Apple	*Erwinia amylovora*	Tissue culture plantlets	Norelli *et al.*, 1988
	Apple	*Erwinia amylovora*	Excised tissue culture leaves	Donovon, 1991
	Apple	*Erwinia amylovora*	Tissue culture plantlets	Donovon *et al.*, 1994
	Peach	*Xanthomonas campestris* pv. *pruni*	Callus	Hammerschlag, 1994
	Peach	*Xanthomonas campestris* pv. *pruni*	Tissue culture plantlets	Hammerschlag, 1988b
	Poplar	*Septoria musiva*	Leaf discs of tissue culture plantlets	Ostry *et al.*, 1988
	Lilac	*Pseudomonas syringae* pv. *syringae*	Tissue culture plantlets	Scheck *et al.*, 1997
	Pear	*Pseudomonas syringae* pv. *syringae*	Detached leaves of tissue culture plantlets, tissue culture plantlets	Yessad *et al.*, 1992

Chapter 3

In Vitro Excised Leaf Assay to Screen Cherry Genotypes for a Necrotic Response to Inoculation with *Pseudomonas syringae* pv. *syringae*

Abstract

An excised leaf bioassay was developed to test for a necrotic response, in sweet cherry tissue culture plantlets, to inoculation with *Pseudomonas syringae* pv. *syringae*. The four treatments included in this bioassay were a water control, a low virulence treatment, a 10^6 cfu/ml mixed pathogenic treatment, and a 10^8 cfu/ml mixed pathogenic treatment. Evaluation of the necrotic response used a rating scale of 0-4 with a 0 rating denoting necrosis surrounding the wound site only and a 4 rating denoting necrosis of the entire leaf. None of the evaluated genotypes tested exhibited any necrosis when inoculated with the water control yet a few genotypes exhibited negligible necrosis when inoculated with a low virulence strain, JL2000. Several of the genotypes tested had necrotic responses statistically the same as the susceptible check 'Corum' when inoculated with either mixed pathogenic treatment. In general, Weiroot genotypes showed a high necrotic response, Pi-KU and Giessen genotypes showed a range of responses, and Mazzard x Mahaleb (MxM) genotypes showed low necrotic responses. A dose response was observed in many genotypes.

Introduction

Sweet cherry production in the Willamette Valley of Oregon is limited by the disease bacterial canker, caused by the bacterium *Pseudomonas syringae* pv. *syringae*.

Economic losses, however, are not limited to Oregon as the disease is a problem in fruit growing regions worldwide. Losses are especially severe when the disease manifests as cankers on the trunk and scaffold limbs or dead dormant buds (Cameron, 1962).

The disease remains unchecked in Oregon due to ineffective chemical control, attributed to the evolution of copper and streptomycin resistant strains, and to the systemic properties of the bacteria (Cameron, 1970; Cameron 1962; Scheck *et al.,* 1996; De Boer, 1980; Young, 1977). Despite the lack of chemical efficacy, some control has been obtained through the application of cultural control practices, most notably the use of resistant rootstocks, shown to diminish the incidence of bacterial canker (Schofield and Clift, 1959; Grubb, 1944).

Introduction of new sweet cherry genotypes for size control may also facilitate control of bacterial canker by providing resistant rootstock material. Novel material considered for the Willamette Valley includes the Giessen clones, genotypes belonging to the Mazzard x Mahaleb (MxM) series, Weiroot series, and Pi-KU series. This access to new rootstocks has stimulated investigations aimed at elucidating which rootstocks may confer resistance to bacterial canker.

In vitro excised leaf assays have been used to rate susceptibility of tissue culture plantlets to various diseases (Hammerschlag, 1990; Yessad *et al.,* 1992; Donovan, 1991). *In vitro* assays are preferred over *in vivo* assays because they are season independent and because the disease response can be rated in a shorter period of time. The objective of this study was to utilize a modified version of a previous assay used to determine the virulence of bacterial strains on *in vitro* pear excised leaves, to screen *in vitro* sweet

cherry excised leaves for a necrotic response caused by inoculation with *P. syringae* pv. *syringae* (Yessad *et al.*, 1992).

Materials and Methods

The low virulence (JL2000) *Pseudomonas syringae* pv. *syringae* strain and four highly virulent *P. syringae* pv. *syringae* strains included in this assay (W4N54, W4N108, 5D443, and 5D447) were provided by Ms. Marilyn Miller of Oregon State University and Dr. Dennis Gross of Washington State University, respectively. Virulence is defined as the range of damage producing capabilities of a pathogen (Zadoks and Schein, 1979). The pathogenic strain W4N54 was used in a previous cherry twig assay (Krzesinska and Azarenko, 1992). The low virulence strain was included to test if there was a difference in necrotic response between leaves inoculated with a mixture of strains known to cause high browning in twigs and leaves inoculated with a strain known to cause little or no browning. A difference would suggest that the *in vitro* bioassay differentiates between high and low virulent bacteria. The original sources of the highly virulent strains are provided in Table 3.1.

Table 3.1. Source of *Pseudomonas syringae* pv. *syringae* strains pathogenic on cherry.

Strain	Original Source
W4N54	Anjou Pear
W4N108	*P. avium*
5D443	*P. avium*
5D447	*P. avium*

Prior to the inoculation date, both the high virulence and the low virulence cultures, stored at -80C in Luria-Bertani medium (LB) supplemented with 50% sterile glycerol, were cultured on King's B medium agar and incubated for 36h at 26C (King *et al.*, 1954). After the incubation period, the strains were suspended in sterile double deionized water and the inocula concentration, 10^8 colony forming units (cfu)/ml, was determined spectrophotometrically (Klement *et al.*, 1990). Confirmation of the inocula concentration was attained using a standard dilution plate assay following each inoculation (Klement *et al.*, 1990). The excised leaf assay included four treatments, a 10^8 cfu/ml mixed pathogenic treatment, a 10^6 cfu/ml mixed pathogenic treatment, a 10^8 cfu/ml low virulence (JL2000) treatment, and water. The 10^8 cfu/ml mixed pathogenic treatment was prepared by combining equal volumes (250µl) of high virulence suspensions of W4N54, W4N108, 5D443, and 5D447 while the 10^6 cfu/ml mixed pathogenic treatment was prepared by dilution of the 10^8 cfu/ml inoculum.

The excised leaves were obtained from tissue culture plantlets, maintained on DKW media, in the multiplication stage (Bonga and Durzan, 1987). A total of 60 fully expanded leaves per genotype were excised from the plantlets with a scalpel using sterile technique. The leaves were assumed to be sterile and hence were not disinfected. After wounding the midrib with a sterile scalpel, the excised leaves were inoculated by depositing a 2µl drop of the bacterial suspension on the wound. A total of 15 leaves were inoculated per genotype per treatment. After inoculation, leaves were placed in a growth chamber (16h of light at 25C; 8h of darkness at 20C), in sterile parafilm-wrapped petri dishes containing water agar, 7g/l, and a water saturated sterile filter paper disc (Yessad *et al.*, 1992). Each petri dish contained 5 leaves per genotype per treatment. Following

a seven day incubation, the excised leaves were evaluated for necrosis on a scale of 0-4 (0 = no necrosis, 1 = necrosis restricted to midrib wound, 2 = necrosis < 50% leaf area and not restricted to midrib wound, 3 = necrosis ≥ 50% leaf area and not restricted to midrib wound, and 4 = total necrosis of leaf).

The genotypes assayed were inoculated on several dates between August 2000 and May 2001. Up to six genotypes were assayed on a single day, including the susceptible check ('Corum'), and a total of 25 genotypes were assayed in all. The evaluated sweet cherry genotypes included representatives of the Giessen clones, MxM series, Weiroot series, Pi-KU series, 'Corum', Mazzard seedling, and 'Rainier' (Table 3.2).

Table 3.2. Parentage of cherry genotypes used in the *in vitro* excised leaf assay.

Genotype	Species or Hybrid
Scion (ASHS, 1997)	
Corum	*P. avium* (Chance seedling)
Rainier	*P. avium*
Rootstock (Wertheim, 1998)	
Mazzard seedling	*P. avium*
Giessen Series	
148-1/GiSelA 6	*P. cerasus x P. canescens*
148-2/GiSelA 5	*P. cerasus x P. canescens*
148-8/GiSelA 7	*P. cerasus x P. canescens*
148-9/GiSelA 8	*P. cerasus x P. canescens*
195-2/GiSelA 12	*P. canescens x P. cerasus*
195-20	*P. canescens x P. cerasus*
196-4	*P. canescens x P. avium*
209-1	*P. cerasus x P. canescens*
318-17	*P. canescens x P. avium*
473-10/GiSelA 4	*P. avium x P. fruticosa*
M x M series	
M x M 14	*P.avium x P. mahaleb*
M x M 39	*P.avium x P. mahaleb*
Weiroot Series	
10	*P.cerasus*
53	*P.cerasus*
154	*P.cerasus*
158	*P.cerasus*
Pi-KU	
4-20	*P. avium* x (*P. canescens x P. tomentosa*)
4-22	(*P. canescens x P. tomentosa*) x *P. avium*

Due to the restriction of the number of genotypes assayed in a single day, by both insufficient time and plant tissue culture material, statistical analysis was performed using an augmented design. In this design, blocks represented the dates and the susceptible check genotype, present in each date, was 'Corum'. There were three replicates of 'Corum' per treatment per date enabling three checks per treatment per block. An ANOVA was run on the responses generated from 'Corum' and used to calculate the least significant increase (LSI) and standard errors for genotypes within the same block and between different blocks. Further, the responses generated from 'Corum' were used to calculate mean necrotic response adjustments, distinct for each date, so that the genotypes in different blocks could be pooled together and ranked according to their adjusted mean necrotic response. To simplify the statistical analysis, the 'Corum' mean responses across dates, for both high virulence treatments, were tested using an F-test to discern any differences between 'Corum' replications across blocks. When the statistical analysis revealed the replications to be statistically the same the necrotic responses were averaged to represent one mean response for each high virulence treatment. In addition to blocking, randomization of the excised leaves was used to reduce experimental error. The leaves were randomized by pooling them together in a sterile petri dish containing sterile water and withdrawing them for inoculation by chance.

Results

Excised leaves inoculated with water did not produce a necrotic response (rating=0) while varieties 'Corum' and 'Rainier'; Giessen clones 148-8 (GiSelA 7) and 318-17; Pi-KU genotypes 4-20, 4-17, and 4-22; and Weiroot genotypes 10, 154, and 53

all had at least one leaf that showed a necrotic response (rating≤1) when inoculated with JL2000 at 10^8 cfu/ml. Leaves inoculated with both mixed pathogenic treatments showed a range of responses between no necrosis and total leaf necrosis.

After adjusting necrotic response means for differences between inoculation dates the necrotic response means were ranked separately for the 10^6 cfu/ml and the 10^8 cfu/ml mixed pathogen treatments. Results from the 10^6 cfu/ml treatment indicated that 'Rainier' had the greatest mean adjusted necrotic response, 2.93, while Pi-KU 4-22 had the smallest mean adjusted necrotic response, -0.13 (see Table 3.3 and Figure 3.1).

Table 3.3. Comparison of adjusted necrotic responses in leaves inoculated with the 10^6 and 10^8 cfu/ml of mixed pathogenic treatment.

Genotype	Block	Adjusted Response 10^6cfu/ml	Adjusted Response 10^8cfu/ml
'Rainier'	I	2.93	2.93
Weiroot 53	V	2.34	2.47
'Corum'	I, II, III, IV, V, VI	2.20	2.67
Mazzard seedling	VI	2.13	2.00
Weiroot 158	V	1.80	2.33
Weiroot 154	V	1.80	2.27
Weiroot 10	V	1.74	2.00
Pi-KU 1-10	III	1.60	1.80
Gi 195-20	III	1.53	1.00
Pi-KU 4-11	VI	1.40	1.40
Gi 497-8	VI	1.33	1.33
Gi 473-10/GiSelA 4	III	1.13	1.20
MxM 39	III	0.93	0.40
Gi 318-17	IV	0.80	1.53
Gi 148-8/GiSelA 7	II	0.60	1.20
Gi 196-4	III	0.60	0.47
Gi 148-9/GiSelA 8	II	0.53	1.13
Gi 195-2/GiSelA 12	II	0.53	1.20
Gi 148-2/GiSelA 5	I	0.40	2.26
Gi 209-1	II	0.40	2.07
Pi-KU 4-20	IV	0.27	0.66
Pi-KU 4-17	IV	0.20	1.19
MxM 14	IV	0.13	0.13
Gi 148-1/GiSelA 6	I	-0.07	1.00
Pi-KU 4-22	III	-0.13	0.33

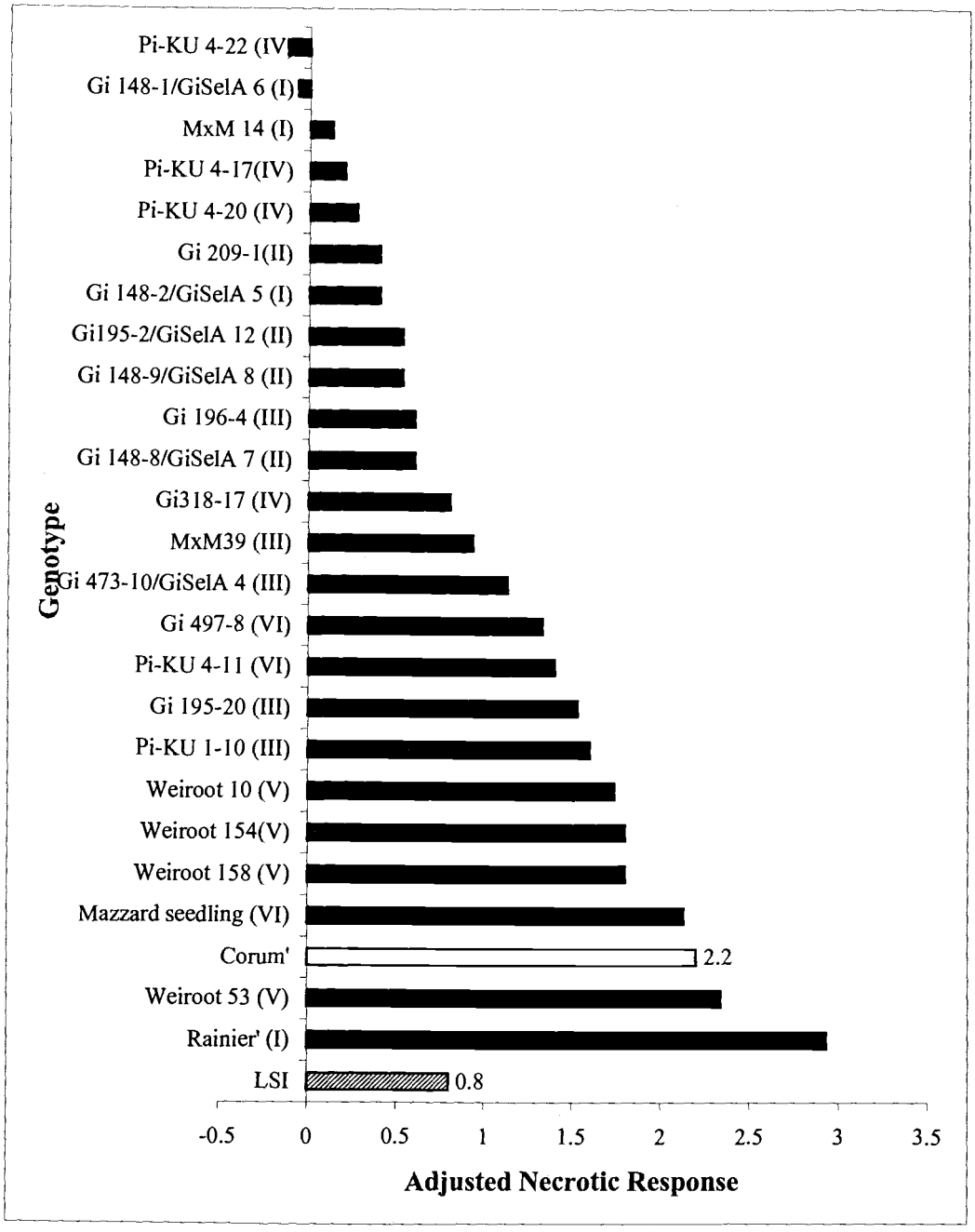

Figure 3.1 Comparison of necrotic response in cherry excised tissue culture leaves inoculated with 10^6 cfu/ml of a mixture of four pathogenic *Pseudomonas syringae* pv. *syringae* strains, one week after inoculation. (Note, the Roman numerals indicate blocks). The white bar indicates the check adjusted mean response. The striped bar shows the least significant increase (LSI) determined using an augmented design. The standard error between blocks is 1.02 and within blocks is 0.88.

Genotypes that responded statistically the same as 'Corum' (2.20), when inoculated with 10^6 cfu/ml of mixed pathogenic treatment, included 'Rainier' (2.93), Weiroot 53 (2.34), Mazzard seedling (2.13), Weiroot 158 (1.80), Weiroot 154 (1.80), Weiroot 10 (1.74), Pi-KU 1-10 (1.60) and Giessen clone 195-20 (1.53). In addition Pi-KU 4-11 (1.40), and Giessen clones 497-8 (1.33) and 473-10 (1.13) responded statistically the same as Mazzard seedling (2.13) when inoculated with 10^6 cfu/ml of mixed pathogenic treatment. Genotypes that responded the same as the Pi-KU 4-22 (-0.13), which scored the lowest in the 10^6 cfu/ml of mixed pathogenic treatment, include Giessen clones 318-17, 148-8 (GiSelA 7), 196-4, 148-9 (GiSelA 8), 195-2 (GiSelA 12), 148-2 (GiSelA 5), 209-1, and 148-1 (GiSelA 6); Pi-KU clones 4-20, 4-17, and 4-22; and MxM 14. The Least Significant Increase (LSI) in the 10^6 cfu/ml treatment was 0.80. The standard error between blocks was 1.02 and within blocks was 0.88 (see Table 3.3).

Consistent with the 10^6 cfu/ml treatment, 'Rainier' was also the genotype with the greatest adjusted mean necrotic response, (2.93), after inoculation with the 10^8 cfu/ml treatment (see Table 3.3 and Figure 3.2). The genotype with the smallest mean adjusted necrosis, when inoculated with the 10^8 cfu/ml treatment, was MxM 14 (0.13). Five genotypes, 'Rainier' (2.93), Weiroot 53 (2.47), 158 (2.33), and 154 (2.27), and Giessen clone 148-2, also GiSelA 5, (2.26), inoculated with 10^8 cfu/ml mixed pathogenic treatment responded statistically the same as 'Corum' (2.67). Genotypes that responded statistically the same as Mazzard seedling (2.00), after inoculation with the 10^8 cfu/ml treatment, included 'Corum' (2.67), Weiroot genotypes 53 (2.47), 158 (2.33), 154 (2.27) and 10 (2.00); Giessen clones, 148-2, also GiSelA 5, (2.26), 209-1 (2.07), 318-17 (1.53); and Pi-KU 1-10 (1.80). Genotypes that responded the same as MxM 14 (0.13), which

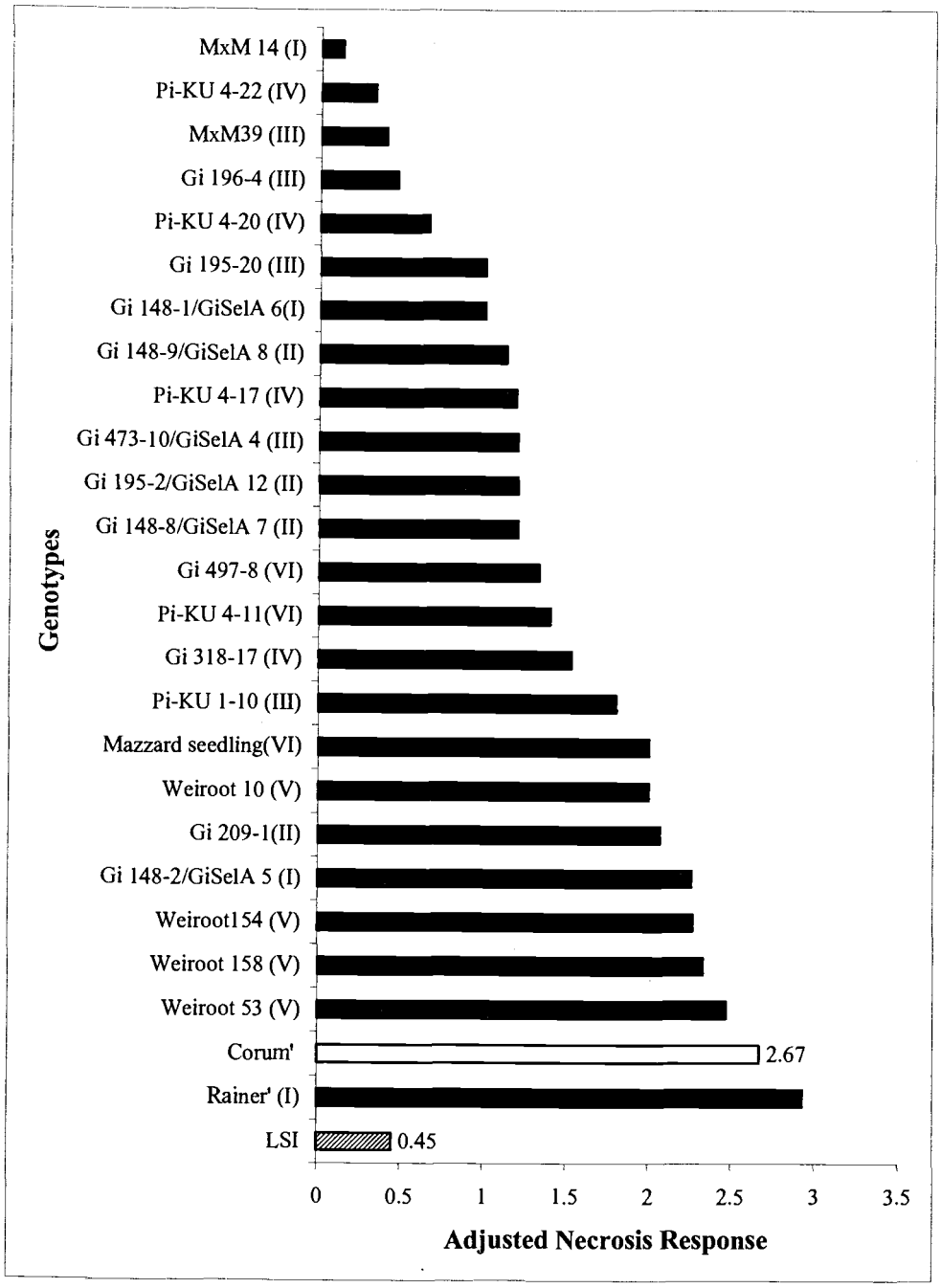

Figure 3.2 Comparison of necrotic response in cherry excised tissue culture leaves inoculated with 10^8 cfu/ml of a mixture of four pathogenic *Pseudomonas syringae* pv. *syringae* strains, one week after inoculation. (Note, the Roman numerals indicate blocks). The white bar indicates the check adjusted mean response. The striped bar shows the least significant increase (LSI) determined using an augmented design. The standard error between blocks is 0.58 and within blocks is 0.50.

scored the lowest in the 10^8 cfu/ml of mixed pathogenic treatment, include Giessen clone 196-4, Pi-KU genotypes 4-20 and 4-22, and MxM 39. The Least Significant Increase (LSI) in the 10^8 cfu/ml treatment was 0.45. The standard error between blocks was 0.58 and within blocks was 0.50 (see Table 3.3).

Results of the inoculation with the 10^6 cfu/ml mixed pathogenic treatment indicated Weiroot genotypes to have the greatest necrotic response; Pi-KU genotypes and Giessen clones to show a broad range of responses; and the MxM genotypes to be best categorized as having lower necrotic responses, especially MxM 14. Results of inoculation with the 10^8 cfu/ml mixed pathogenic treatment suggested further that the Weiroot genotypes typically showed a greater necrotic response while Giessen clones and Pi-KU genotypes showed widespread necrotic responses, and MxM genotypes displayed the smallest necrotic responses. All but four of the genotypes (Mazzard seedling, Giessen clone 195-20, MxM 39, and Giessen clone 196-4) had an equal or greater necrotic response when inoculated with the 10^8 cfu/ml mixed pathogenic treatment as when inoculated with the 10^6 cfu/ml mixed pathogenic treatment.

Discussion

The lack of necrosis when inoculated with the water control and the range in necrotic responses when inoculated with the mixed pathogenic treatments was predicted. However, based on preliminary work that suggested JL2000 was non-pathogenic on cherry, the largely negligible necrotic responses in select genotypes resulting when excised leaves were inoculated with JL2000, were unexpected and demonstrates that this strain, at a concentration of 10^6 cfu/ml, is capable of eliciting a necrotic response in specific genotypes (Bassil, personal communication).

The validity of the assay is confirmed by the agreement between the two mixed pathogenic treatments. Results from both mixed pathogenic treatments showed that the Weiroot genotypes had a greater necrotic response than the Pi-KU genotypes and many of the Giessen clones. Likewise, the MxM genotypes had the smallest necrotic response to inoculation with the mixed pathogenic treatments. The Weiroot ratings were somewhat surprising since the Weiroot genotypes are derived from *Prunus cerasus*, which has been shown to possess "high resistance" to bacterial canker (De Vries, 1965). Although this result may be accurate, one factor that may have influenced a high rating was the tendency of the Weiroot genotypes to be chlorotic in tissue culture.

While only healthy green leaves were selected for this assay, the majority of Weiroot leaves on the DKW media were chlorotic and did not green up until a liquid layer of DKW media was added on top of the semi-solid DKW media. Despite the exclusive use of green leaves in this assay, the chlorosis of the rejected leaves may have indicated that the selected green leaves might have also experienced stress thus making them susceptible to the bacteria. It would be worthwhile to optimize a tissue culture medium to better meet the nutritional requirements of the Weiroot genotypes. Once the Weiroot genotypes have been established on a new medium and appear healthy they should be assayed again to confirm the results of this bioassay.

The high necrotic rating given to Mazzard seedling, which having descended from *P. avium* is know to be susceptible to bacterial canker, unlike F12/1 which was selected for resistance to bacterial canker, was expected (Wertheim, 1998). Likewise, the MxM genotypes were expected to show low or no necrosis since the MxM genotypes have received low ratings to inoculation with *P. syringae* pv. *syringae* in a previous

biossay (Krzesinska and Azarenko, 1992). The range in Giessen and Pi-KU responses was also predicted since they include a wide array of interspecific hybrids. Additionally, it was anticipated that the necrotic responses associated with the 10^8 cfu/ml treatment would meet or exceed the rating of the necrotic responses associated with the 10^6 cfu/ml treatment. This concentration effect is consistent with a dose response.

Genotypes, such as Giessen clone 209-1, that had greater necrosis as the concentration of the mixed pathogenic treatments increased had not reached their highest necrotic potential, given the conditions of this assay, when inoculated with the 10^6 cfu/ml treatment. In contrast, genotypes that showed little or no change in necrosis as the concentration of the pathogenic mixture increased, such as 'Rainier', had already reached their highest necrotic potential when inoculated with the 10^6 cfu/ml mixed pathogenic treatment. The necrotic responses exhibited by genotypes in this bioassay are specific to the parameters of this experiment and to inoculation with *P. syringae* pv. *syringae* strains W4N54, W4N108, 5D447, and 5D443 and might change as conditions change.

Of the four genotypes that showed a decrease in the necrotic rating as the concentration was increased from 10^6 cfu/ml to 10^8 cfu/ml mixed pathogenic treatment only two, MxM 39 and Giessen clone 195-20, showed a moderate decrease (0.53). These decrease in adjusted ratings all fell within the standard error range and therfore are of little concern.

The 10^6 cfu/ml mixed pathogenic treatment did not discriminate as well as the 10^8 cfu/ml mixed pathogenic treatment. This is demonstrated by the much higher MSE for the 10^6 cfu/ml mixed pathogenic treatment (0.393) which exceeded the MSE (0.124) for the10^8 cfu/ml mixed pathogenic treatment by over 2.5 times (see Appendix). The larger

MSE, an indicator of experimental error, for the 10^6 cfu/ml mixed pathogenic treatment resulted in a large standard error for mean necrotic responses across inoculation dates and hence the standard error was not sensitive to separating small differences between genotypes. However, for the purpose of selecting material from this assay for inclusion in a field study separating small differences is not essential.

When conducting a more focused investigation, it would be useful to employ a completely randomized design, which is possible, if the inoculation technique can be standardized so that there are no differences between the procedures of distinct dates. Differences between dates can be measured by including internal checks in the study, as 'Corum' was included in this study, and randomization can be achieved by randomly assigning dates to different replications prior to experimentation. Disadvantages of a completely randomized design are that growth media, growth chambers, and bacterial strains have to be meticulously monitored to reduce the chance for error and the genotypes must be at the proper growth stage when the day for inoculation arrives. The major advantage of this approach is that statistical analysis of this kind of assay would better differentiate between small differences in mean necrotic response which although not essential for the purpose of this study might be advantageous for other investigations using this protocol.

The appearance of necrosis in genotypes that were consistently rated 1 or less, Pi-KU 4-22 and 4-20, MxM 14 and 39, and Giessen 196-4 and 148-1 (GiSelA 6) might be explained by the possible occurrence of the hypersensitive reaction (HR), which is not an indication of a susceptible response. The HR often becomes visible in tissues inoculated with high concentrations of bacteria, 10^8 cfu/ml or greater (Klement *et al.*, 1990). In

inoculations with concentrations of bacteria not exceeding 10^7 cfu/ml the HR does not usually occur and hence necrosis can be regarded as a susceptible response (Klement *et al.*, 1990). Since the HR occurs within the first 24h following inoculation, determining whether genotypes were actually exhibiting the HR in this assay can be easily done (Klement *et al.*, 1990).

The purpose of this experiment was to assay available plant tissue culture material for a necrotic response to inoculation with *P. syringae* pv. *syringae*. The next logical step in this process is to run a more detailed analysis in the field environment with fewer genotypes, more replications, and a more detailed rating system. Genotypes that showed necrosis that was less significant than 'Corum' should be included in future screening. Further, of the genotypes that scored less than 'Corum' the ones that had consistently low necrotic responses (response$\leq$1.5) in both mixed pathogenic treatments should be given first consideration in a field planting. These candidates include MxM 14 and 39; Pi-KU 4-22, 4-20, 4-17, and 4-11; and Giessen clones 148-1 (GiSelA 6), 192-2 (Gisela 12), 148-9 (GiSelA 8) 148-8 (GiSelA 7), 196-4, 318-17,195-20, 497-8 and 473-10 (GiSelA 4). Until more is known regarding the Weiroot genotypes they should only be included in field studies if space permits.

This study provided new information regarding the necrotic responses of MxM, Pi-KU, Weiroot, and Giessen clones. At this time it would be premature to make recommendations to growers based on *in vitro* research. Although the assay is limited to finding rather large differences in mean necrotic response, especially when inoculated with the 10^6 cfu/ml mixed pathogenic treatment, it can be successfully employed to select genotypes for further, more in-depth, resistance screening. Field-testing, although

variable, would be the ultimate next step before making sweeping generalizations regarding the responses of excised plant tissue culture leaves, since there is little that authentically mimics the field environment in the laboratory.

Chapter 4

In Vivo Excised Twig Assay to Screen Cherry Genotypes for a Browning Response to Inoculation with *Pseudomonas syringae* pv. *syringae*

Abstract

An excised twig assay was modified to include a mixed pathogenic treatment of *Pseudomonas syringae* pv. *syringae* strains, at a concentration of 10^8 cfu/ml. Cherry twigs were inoculated with a water control and *Pseudomonas syringae*. A browning response was evaluated following 3 weeks incubation, at 15-20C in darkness with high relative humidity. A scale of 1-4 was used to rate browning. A rating of 1 indicated normal yellow pith and a rating of 4 indicated dark brown pith (Krzesinska and Azarenko, 1992). Callusing and gummosis was also observed. None of the 13 genotypes tested exhibited any browning when inoculated with water alone, a rating of 1.0. In contrast, 9 of the genotypes exhibited a mean browning response greater than 1.0 when inoculated with the pathogenic mixture. Giessen clone 209-1, 'Royal Ann', Mazzard seedling, and 'Sweetheart' showed the greatest mean browning responses while MxM 14, MxM 60, MxM 2 and Giessen clone 169-15 showed no browning when inoculated with *Pseudomonas syringae*. A high gummosis response was observed in Giessen clone 169-15. All the genotypes exhibited a callus response when inoculated with either treatment.

Introduction

The bacterium *Pseudomonas syringae* is a problem in fruit growing regions worldwide causing economic loss for growers in numerous geographical areas. In the

Willamette Valley of Oregon, sweet cherry production is severely limited by the disease bacterial canker, caused by *P. syringae* pv. *syringae*. The most destructive symptoms associated with the disease are cankers on the trunk and scaffold limbs and the death of dormant buds (Cameron, 1962).

Chemical control in Oregon has not been consistently effective due to both the development of copper and streptomycin resistant strains and to the systemic properties of the bacteria (Cameron, 1970; Cameron 1962; Scheck *et al.,* 1996; De Boer, 1980; Young, 1977). The industry has used cultural control practices, especially the use of resistant rootstocks, that have reduced the incidence of this disease (Schofield and Clift, 1959).

In an effort to meet the demand for resistant plant material many novel rootstocks have been developed. Rootstocks being considered for the Willamette Valley include the Giessen clones, and genotypes belonging to the MxM series. This availability of new rootstocks has spurred an interest to determine the susceptibility of plant material to bacterial canker. Resistance to bacterial canker in cherry has been documented using an *in vivo* twig bioassay (Krzesinska and Azarenko, 1992).

The objective of this study was to repeat a previous twig assay that had been validated by field observation for the purpose of relating necrotic responses measured in an *in vitro* excised leaf assay to browning responses measured in an *in vivo* twig assay (Krzesinska and Azarenko, 1992). Achieving consistent results between the former twig assay, this twig assay, and the excised leaf assay might help us predict how genotypes included in the excised leaf assay will respond to bacterial canker in a field environment. Consistent findings would also suggest that the excised leaf assay is an accurate tool.

The previous twig assay was modified in this study to more closely resemble the excised leaf assay by including a mixture of pathogenic strains (W4N54, W4N108, 5D443, 5D447), as opposed to only one strain (W4N54).

Materials and Methods

The pathogenic *Pseudomonas syringae* pv. *syringae* strains in this assay, W4N54, W4N108, 5D443, and 5D447, were provided by Dr. Dennis Gross of Washington State University. Of special note is the strain W4N54 which in a previous cherry twig assay demonstrated high virulence (Krzesinska and Azarenko, 1992).

Each of the strains, stored at -80C in Luria-Bertani medium (LB) supplemented with 50% sterile glycerol, were cultured on King's B medium agar for 36h at 26C and subsequently suspended in sterile double deionized water at a concentration of 10^8 cfu/ml (King *et al.,* 1954). The concentration of the inoculum was determined spectrophotometrically and validated after the inoculation using a standard dilution plate assay. Equal volumes of each of the individual bacterial suspensions were combined to form a mixed pathogenic treatment, which was used to inoculate cherry twigs.

On 18 December 2000, cherry twigs were obtained from the Lewis-Brown Horticulture Research Farm, Corvallis, Oregon. Twigs were collected in December so that results could be compared to an earlier twig assay that used December twigs (Krzesinska and Azarenko, 1992). Twigs evaluated at another time would be expected to respond differently to inoculation since they would represent different stages from within the dormancy period. Twig samples taken at the end of the dormancy period are less

susceptible to disease and would have a lower browning response (Krzesinska and Azarenko, 1992).

The assay included 10 twigs 20 cm long per genotype per treatment, representing ten replications. A total of 260 one-year old twigs, representing 13 genotypes, were surfaced sterilized with ethanol (75%, 30 sec) followed by sodium hypochlorite (0.05%, 10 min) (Krzesinska and Azarenko, 1992). The two treatments consisted of a double deionized sterile water control and a 10^8 cfu/ml mixture of four pathogenic bacteria strains.

After sterilization, and prior to inoculation, the basal 2cm were removed from each twig with a sterile razor blade and discarded. Along each twig a longitudinal incision, revealing cambium tissue, was made using a sterile razor blade. Each twig was then inoculated with either a 20µl drop of inocula, containing all four pathogenic strains, or sterile water, which was deposited in the seam of the longitudinal incision, and its resultant 0.5x2cm bark appendage using a sterile pipette. The inoculated twigs were kept in sterile glass culture tubes (2.5x25cm), covered with plastic caps, that contained sterile water saturated cotton and incubated for 3 weeks at 15-20C in the dark. Following incubation, the twigs were evaluated for browning on a scale of 1-4 (1= normal yellow pith, 2 < than 50% browning, 3 $\geq$ 50% browning, 4 = total browning) and observations regarding callus and gummosis were recorded (Krzesinska and Azarenko, 1992). The evaluated sweet cherry genotypes included 'Royal Ann', 'Corum' and cherry rootstocks from the Giessen clones and MxM series. Twigs from one Mazzard seedling tree were used as an industry standard due to the unavailability of F12/1.

Results

Twigs inoculated with water did not produce a browning response while 9 out of the 13 genotypes did produce a browning response when inoculated with 10^8 cfu/ml of the pathogenic mixture (Table 4.1). Genotypes MxM 14, MxM 60, MxM 2, and Giessen clone 169-15, exhibited no browning and therefore received a mean browning rating of 1.0. The genotypes displaying the greatest browning response, Giessen clone 209-1,

Table 4.1. Mean separation of genotype browning response.

Genotype	Browning Response[z]
Gi 209-1	2.8a
'Royal Ann'	2.8a
Mazzard seedling	2.7a
'Sweetheart'	2.6a
'Corum'	2.0b
Gi 195-20	1.7bc
Gi 195-2 (GiSelA12)	1.7bc
MxM 46	1.7bc
MxM 39	1.2dc
MxM 14	1.0d
MxM 60	1.0d
MxM 2	1.0d
Gi 169-15	1.0d

[z] Each value represents a mean of 10 replicate twigs. Mean separation by genotype was by the Waller-Duncan k-ration t-test, k=100. (0.53 = Minimum Significant difference).

'Royal Ann', Mazzard seedling, and 'Sweetheart', had mean responses ranging from 2.8-2.6 and all showed a significantly greater browning response than 'Corum' which had a mean response of 2.0. Genotypes that had the same browning response as 'Corum', Giessen clone 195-20, 195-2 (GiSelA 12) and MxM 46, all had a mean browning response of 1.7. Whereas, genotypes with a lesser browning response than 'Corum', MxM 39, MxM 14, MxM 60, MxM 2, and Giessen clone 169-15, had mean responses ranging from 1.0 to 1.2.

Genotypes belonging to the MxM series typically had a lesser browning response than the Giessen clones. Within the MxM series, MxM 46 had more browning (1.7) than MxM 2, MxM 14, and MxM 60, which exhibited no browning, but did not show a significantly different browning response from MxM 39 (1.2). In contrast, the Giessen clones, 195-20, and 195-2 (GiSelA 12) had a higher browning response, each earning a 1.7 rating, than 169-15, which had no browning. (See Appendix for ANOVA results.)

The sole genotype that produced a gummosis response when inoculated with water was Giessen clone 169-15, which also demonstrated a gummosis response to inoculation with 10^8 cfu/ml of the pathogenic mixture. Other genotypes, including 'Corum', MxM 39, Mazzard seedling, 'Sweetheart', Giessen clone 209-1, and 'Royal Ann' also exhibited a gummosis response when inoculated with 10^8 cfu/ml of pathogenic mixture. The genotypes having the least gummosis included 'Corum' and MxM 39 while Giessen clone 169-15 displayed the greatest gummosis response. Additionally, all 15 of the genotypes assayed were observed to callus in response to inoculation by both water and 10^8 cfu/ml of bacteria mixture inocula.

Discussion

The highest browning group did not include 'Corum' which was selected for this assay as a susceptible control and which was found to have browning ratings ranging from 1.2 to 3.2 using the same scale when inoculated with W4N54 in a previous assay (Krzesinska and Azarenko, 1992). However, 'Corum' did receive a higher browning response than 8 of the assayed genotypes and so it may, with caution, be included in future assays as a susceptible response, although Giessen clone 209-1, 'Royal Ann', Mazzard seedling, or 'Sweetheart' might better serve this purpose. Consistent with this study, Mazzard seedling and Giessen clone 209-1 were shown to have a high response to inoculation with the same mixture of pathogenic *P. syringae* pv *syringae* when using the *in vitro* excised leaf assay. However, in the excised leaf assay the response of Giessen clone 209-1 dramatically increased when inoculated with the 10^8 cfu/ml mixed pathogenic treatment and hence if Giessen clone 209-1 is selected as a susceptible control then concentrations lower than 10^8 cfu/ml might not be appropriate.

Consistent with former twig data (Krzesinska and Azarenko, 1992), this study finds MxM 2, MxM 39 and MxM 60 to exhibit low browning responses, less than 2.0. Additionally, this study reports that MxM 14 and MxM 46 show no to low browning tendency and should be considered as potential resistant rootstock material. Further, MxM clones 14 and 39 were shown to have a low necrotic response when *in vitro* excised leaves were inoculated with a the same mixture of pathogenic *P. syringae* pv. *syringae*. In this assay, two of the Giessen clones, 195-2 (GiSelA 12), and 195-20 had the same browning response as 'Corum' (2.0). The browning response of Giessen clone 195-20 is consistent with results from the *in vitro* excised leaf assay where 195-20 received a rating

statistically the same as 'Corum' after inoculation with the 10^6 cfu/ml mixed pathogenic treatment. Assays that include the widely planted Giessen clones 148-1 (GiSelA 6) and 148-2 (GiSelA 5) should be done to determine if one or both of these rootstocks might show low or no browning, resembling the MxM responses, when inoculated with *P. syringae* pv. *syringae.*

The only inconsistent result between the two twig assays was the response of Giessen clone 169-15. In this assay Giessen clone 169-15 showed no browning and a high degree of gummosis but in the previous twig assay exhibited browning rates of 1.8 and 2.0 (29 October 1989 and 20 December 1989, respectively). Further, Giessen clone 169-15 showed very little to no gummosis when inoculated with bacteria strain W4N54 in the previous assay (Krzesinska and Azarenko, 1992). Perhaps the high incidence of gumming in this study affected the browning response of Giessen clone 169-15. However, authors Krzesinska and Azarenko have concluded that the incidence of gumming is a poor predictor of cherry resistance to bacterial canker (1992). Differences between the two assays might better be explained by environmental variation between the two years when the twigs were collected, despite being collected in December both years. However it is also possible that inoculation with a mixture of bacteria instead of with one strain was responsible for this difference. This possibility could be tested by inoculating Giessen clone 169-15 with both the mixed treatment and with a W4N54 bacterial suspension at 10^8 cfu/ml and comparing the results.

This assay is recommended when season or time is not limiting and when plant material has not been initiated into tissue culture, eliminating the possibility of an *in vitro* assay. New insights regarding the browning responses of MxM and Giessen genotypes to

inoculation with *P. syringae* pv. *syringae* have been provided by this sudy. However, before drawing firm conclusions regarding resistance and susceptibility, field studies should be considered to determine the authenticity of information resulting from this assay since orchard environments differ greatly from the laboratory setting and offer unique challenges for trees.

Chapter 5

Conclusion

'Corum' and 'Rainier', observed to have susceptible reactions to *Pseudomonas syringae* pv. *syringae* in the field (Azarenko, Personal Communication), had the highest necrotic rating in the *in vitro* excised leaf assay. The reliability of this assay was validated by the absence of necrosis when inoculated with water and the negligible necrosis when inoculated with JL2000. The results from the excised leaf assay suggest that the Mazzard x Mahaleb (MxM) genotypes; Pi-KU genotypes 4-22, 4-20, 4-17 and 4-11; and Giessen clones 148-1 (GiSelA 6), 192-2 (GiSelA 12), 148-9 (GiSelA 8), 148-7, 196-4, 318-17, and 473-10 (GiSelA 4) might offer the best resistance as rootstocks. As expected, results from the *in vivo* twig assay further indicate that the MxM genotypes potentially might be regarded as resistant rootstock material. The broad range in response ratings given to Giessen and Pi-KU excised leaves was also expected since they represent many different interspecific hybrid combinations. In contrast, the Weiroot genotypes did not respond to inoculation of excised leaves as expected. The Weiroot genotypes were expected to rate low due to their *Prunus cerasus* parentage but instead they exhibited high necrotic ratings in the excised leaf assay. While this may be an accurate assessment of the Weiroot genotypes, there is some concern that nutritional deficiencies in the tissue culture media may have stressed the leaves possibly predisposing them to a high necrotic response.

There was good agreement between the results of the modified twig and the previous twig bioassays. Both twig bioassays rated 'Corum' as having higher browning

than MxM 2, 39 and 60. The excised leaf assay also agreed with the modified twig assay as demonstrated by the low ratings (less than 'Corum') for MxM 14 and 36 in both bioassays. Additionally, Mazzard seedling was rated high (the same or greater as 'Corum') in both the modified twig and excised leaf bioassay as was Giessen clone 209-1. The browning response of Giessen clone 169-15 was the sole inconsistency between the two twig bioassays and can probably be explained by environmental differences between the two years twigs were assayed.

The statistical design of the excised leaf assay limits the power to differentiate between small rating differences due to reduced degrees of freedom associated with error variation. The ability to distinguish among small genotypic differences being contingent on degrees of freedom suggests that a statistical design such as a completely randomized design might be more appropriate. However for the purposes of selecting potentially resistant material for field studies small differences need not be detected and so the augmented design is acceptable.

The modification of the twig assay and the development of the excised leaf assay allow for more accessible bacterial canker resistance screening. The excised leaf assay is especially advantageous because it can be performed independent of season, unlike the twig assay. However, because all cherry genotypes are not available in tissue culture the twig assay is still a valuable tool. The purpose of both assays was to identify possible resistant material using reproducible screening protocols. Following the screening procedure it is envisioned that genotypes showing low responses (1 for the excised leaf assay and less than 2 for the twig assay) will be included in a more thorough field investigation involving several years of data. The evaluation of scion genotypes,

contingent on their initiation into tissue culture, via the excised leaf assay would be a logical evolution.

Results from these assays should be considered when planning a field experiment evaluating bacterial canker incidence. Based on this research it is recommended that MxM 14 and 39; Pi-KU genotypes 4-11, 4-20, 4-22, and 4-17; and Giessen clones 196-4, 192-2 (GiSelA 12), 148-1 (GiSelA 6), 148-9 (GiSelA 8), 148-8 (GiSelA 7), 318-17, 195-20, 497-8 and 473-10 (GiSelA 4) be included in a Willamette Valley field study. Space permitting, the Weiroot genotypes might also be considered for further field-testing. Although, field studies are challenging and tend to vary across years they are worthy explorations as they attempt to elucidate genotype resistance in an authentic and dynamic environment.

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Appendix

Table A1. ANOVA table for the *in vitro* excised leaf assay, showing results from the 10^6 cfu/ml pathogenic treatment, analyzed using an augmented design with blocking by inoculation dates. Note the check genotype is 'Corum', which was replicated three times on each inoculation date.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
'Corum' Replications	1.56	2	0.78	1.98 NS	0.19
Blocks	7.15	5	1.43	3.63 *	0.04
Error	3.93	10	0.39		
Total	12.64	17			

Table A2. ANOVA table for the *in vitro* excised leaf assay, showing results from the 10^8 cfu/ml pathogenic treatment, analyzed using an augmented design with blocking by inoculation dates. Note the check genotype is 'Corum', which was replicated three times on each inoculation date.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
'Corum' Replications	0.76	2	0.38	3.07 NS	0.09
Blocks	3.2	5	0.64	5.16 **	0.01
Error	1.24	10	0.124		
Total	5.2	17			

Table A3. ANOVA table for the *in vivo* twig assay analyzed using a completely randomized design.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Genotype	64.37	12	5.36	12.65 **	<0.0001
Error	49.60	117	0.42		
Total	113.97	129			