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John Bryan Webber III for the degree of Master of Science in Horticulture presented on August 13, 2019.

Title: Characterization and Cultivar Susceptibility Assessment of Bacterial Blight in Hazelnut Caused by *Xanthomonas arboricola* pv. *corylina*

Abstract approved:

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Nik G. Wiman

Bacterial blight (*Xanthomonas arboricola* pv. *corylina*) (*Xac*) of hazelnut (*Corylus avellana* L.) was described first in Oregon in 1915 and is now recognized as a damaging disease of young hazelnut trees worldwide. Stressed hazelnut trees in conditions such as planting on marginal sites, and trees between 1 and 4-years-old are most susceptible to bacterial blight. The Willamette Valley of Oregon is where 99% of the U.S. hazelnut crop is grown. Bacterial blight was the most impactful disease in the Pacific Northwest (PNW) until the devastating fungal canker disease eastern filbert blight (EFB) made its way to the hazelnut growing region in the mid-1970s. However, since the release of a series of cultivars with genetic EFB resistance starting in 2005, there has been a surge in new hazelnut plantings. Widespread planting of new hazelnut cultivars has created a resurgence in interest in bacterial blight throughout the PNW growing region. No research has been conducted on the disease in Oregon since the 1970s. In this thesis, a comprehensive review of the literature was conducted on bacterial blight research over the history of the Oregon hazelnut industry, and the more recent history of the disease in Europe. Experimental research began with a characterization study on *Xac* utilizing morphological, biochemical, and molecular techniques on field-collected bacterial populations. In other experiments, hazelnut cultivars were assessed for their response to

inoculation of *Xac in vitro* with hazelnut explants cultured in micropropagation media, as well as in young potted hazelnut trees grown in a greenhouse and inoculated under orchard conditions. The bacteria recovered from symptomatic hazelnut tissue in commercial orchards were positively identified as *Xac*, and the phylogenetic diversity of the pathogen present in European countries was confirmed to also be present in Oregon. The disease progression *in vitro* on explants and on potted tree inoculations was documented and found consistent with symptomology and disease incidence reports from the field. Tissue culture was demonstrated to be an effective research environment to evaluate the infection efficiency of *Xac* on hazelnut cultivars. It was found from inoculations of both explants and potted trees that each hazelnut cultivar was susceptible to bacterial blight infection from artificial inoculations, though some cultivars were more severely affected. The bacterial blight susceptibility of each of the EFB resistant hazelnut cultivars evaluated indicates that management of the disease must rely on maintaining healthy cultural practices, sanitation of pruning tools, and timely copper sprays to minimize the spread, until bacterial blight resistance can be discovered and bred into hazelnut cultivars. This research on characterization of *Xac* and its application to assess hazelnut cultivars to bacterial blight has laid a foundation for further applied research on the disease. Genomic characterization of *Xac* will be the next step to increase the understanding of the molecular interactions of *Xac* with the host plant. Populations of *Xac* isolates are available for use in further applied research to investigate cultural bacterial bight management strategies.

Characterization and Cultivar Susceptibility Assessment of Bacterial Blight in Hazelnut Caused by *Xanthomonas arboricola* pv. *corylina*

by
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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.

John Bryan Webber III, Author

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CONTRIBUTION OF AUTHORS

Chapter 1: John Bryan Webber conducted the literature search to compile this review. The Oregon Hazelnut Commission provided access to document files on bacterial blight. Jay Pscheidt and Melodie Putnam allowed access to the OSU Plant Clinic records. Nik Wiman copy-edited the chapter and provided direction on making the figures.

Chapter 2: The diseased plant material was submitted to the OSU Plant Clinic by Nik Wiman. Melodie Putnam, Maryna Serdani, and the Clinic provided isolates that were recovered when bacterial blight was suspected. Virginia Stockwell was involved in planning, designing, interpreting the characterization analysis, and edited the chapter. John Bryan Webber conducted the lab work, carried out the analysis, was involved in planning and designing the characterization, and authored the chapter. Nik Wiman edited the chapter and assisted in the analysis.

Chapter 3: Sugae Wada co-conceived the idea and provided tissue culture mentorship and plant and lab materials. Virginia Stockwell aided in developing the procedure. John Bryan Webber co-conceived the idea, carried out the experiment, evaluation, analysis, and authored the chapter. Nik Wiman co-conceived the idea, aided in the analysis, and edited the chapter.

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Chapter 5: John Bryan Webber co-conceived the idea and authored the chapter. Nik Wiman co-conceived the idea and edited the chapter

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Chapter 1.
Bacterial Blight Literature Review

Introduction

Production of European hazelnut (*Corylus avellana* L.) is an important industry worth up to $3.3 billion on the world market. The United States is responsible for about 5% of the world's hazelnut production (National Agricultural Statistics Service, 2019). The Willamette Valley in western Oregon has an ideal growing climate for hazelnut production with its mild winters and cool, long growing season. The many family-owned farms in this region are responsible for producing 99% of the U.S. hazelnut crop. Recently, an unprecedented number of farming operations in the Willamette Valley have been transitioning to hazelnut production as hazelnuts are recognized as a low input, high return crop. The total acreage devoted to hazelnut production in Oregon has reached 78,603, with nearly 40,000 acres of immature plantings from one to five years old (Pacific Agricultural Survey LLC, 2019).

The surge in acreage planted throughout the Willamette Valley has been due to the popularity of hazelnut cultivars resistant to eastern filbert blight (EFB) from the Oregon State University Hazelnut Breeding program. While these EFB resistant hazelnut cultivars have given the U.S. hazelnut industry new life, hazelnut trees are increasingly being planted in sites not inherently suited for optimal hazelnut growth. Hazelnut trees become larger and more productive when planted on deep soils of medium-textured bottomland than when planted on soils that are shallow, sandy, or high in clay content (Olsen, 2013a). Abiotic and biotic stress factors such as drought stress, standing water in high clay content soil, nutrition imbalance, sunscald, herbicide damage, insects and diseases on top of the increased scale of production of the new hazelnut cultivars

have exposed production knowledge gaps. Some of these fundamental issues have been a part of the industry since it has existed.

One biotic factor impacting the industry is bacterial blight, which has always been an issue in hazelnut production in the Pacific Northwest (PNW). The causal pathogen of bacterial blight was not given a name until 1940 when it was recognized as a unique bacterium and was called *Phytomonas corylina* (Miller *et al.*, 1940). The name was later changed to *Xanthomonas corylina* (Starr and Burkholder, 1942), then to *Xanthomonas campestris* pv. *corylina* (Dye, 1962; Dye *et al.*, 1980). Today, the bacterium is called *Xanthomonas arboricola* pv. *corylina* (*Xac*) (Vauterin *et al.*, 1995).

Bacterial blight had been the most economically important disease of hazelnuts in the PNW but was eclipsed after introduction and spread of EFB, which is a more devastating disease. No new research has been conducted in Oregon on bacterial blight since the 1970s, and many of the current recommendations for bacterial blight control originated before this time period. In many cases, the origin of that knowledge has been forgotten in time. The OSU Plant Clinic diagnosis records from 1955 to 2017 (Figure 1.1) were searched for the number of submissions of hazelnut tissue and the number of bacterial blight diagnosis. Bacterial blight diagnosis was given to 100 out of the 365 symptomatic hazelnut submissions received by the OSU Plant Clinic between 1955 and 2017. The bulk of those submissions were in the 1960s, 1970s and 1980s, after which bacterial blight diagnosis tapered off through the 1990s and early 2000s as EFB became the predominant disease reported in the Oregon hazelnut industry. The EFB diagnoses in the records tapered off in the late 1990s and 2000s as growers became more familiar with the signs and symptoms and EFB resistance cultivars became available. Bacterial blight remains a major factor today and is of greatest concern in the young hazelnut plantings. There is renewed interest in learning how to manage the disease more effectively given the current planting trends.

The aim of this review is to uncover, condense, and summarize information that was conducted during the peak of bacterial blight research, but

has been lost or scattered since it was the only major disease in the Oregon hazelnut industry. Modern bacterial blight research that has been conducted worldwide is also summarized to illuminate where future research can be directed most effectively.

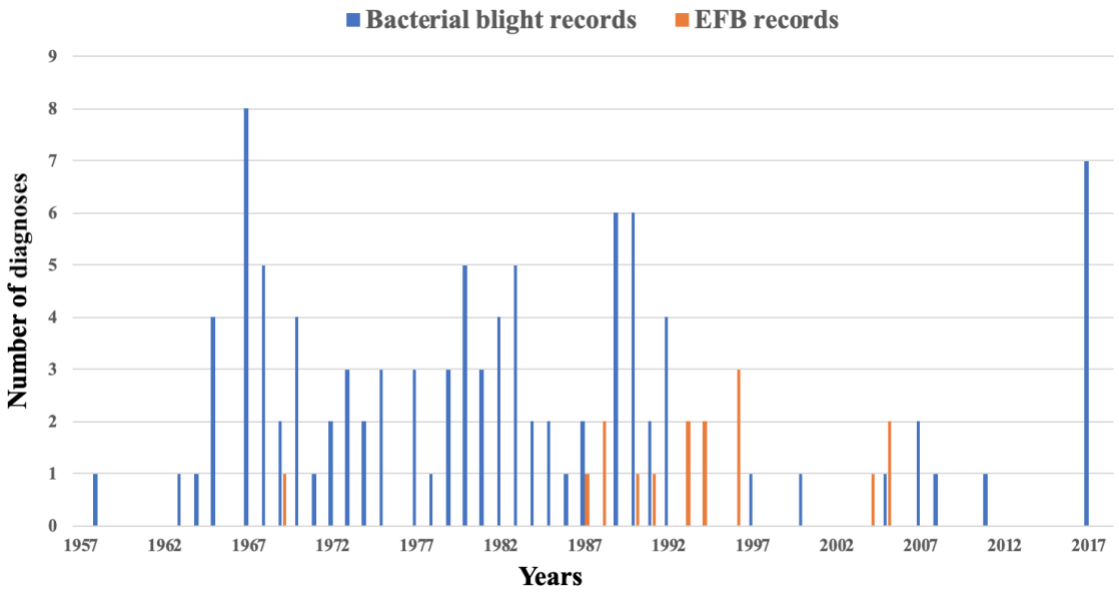

Figure 1.1. Bacterial blight of hazelnuts incidence records (1955 to 2017)

Incidences of bacterial blight of hazelnuts in the Willamette Valley diagnosed by the OSU Plant Clinic. Incidences recorded from the archives of results from samples submitted for diagnosis.

Bacterial Blight in the Early Years of the Oregon Hazelnut Industry (1913-1949)

European hazelnut trees (*Corylus avellana* L.) were first planted in Oregon at the end of the 19th century after being introduced to the Pacific Northwest (PNW) by Felix Gillet (Mehlenbacher and Miller, 1989). Hazelnuts, originally known as filberts in the PNW, were planted by pioneering growers in small orchards on a “gamble” as no one knew whether the European hazelnut would grow in the region (Dorris, 1927). George Dorris planted the first commercial hazelnut orchard in 1903 comprised of 200 acres of ‘Barcelona’ near Springfield, Oregon. Over the next 20 years, more growers throughout the valley were

planting hazelnuts as it became apparent that the tree was well-suited for the climate. There was great certainty by this time that hazelnuts would be an important crop in the Oregon horticultural market. Hazelnuts were recognized to hold a major advantage compared to nearly all other orchard crops being grown at the time, requiring relatively few inputs and having few pests and diseases (Barss, 1927; Dorris, 1927). Only one disease was reported on hazelnuts at the time and its cause, biology, and control were unknown.

In 1915, H. P. Barss, a researcher at Oregon State College in the Botany and Plant Pathology department published the first report that described the new disease in Oregon later referred to as bacterial blight of hazelnut. Bacterial blight in hazelnuts was the only infectious disease present in the industry and many resources were invested into learning more about the disease over the next 60 years until the introduction of EFB. The early reports published on bacterial blight of hazelnuts primarily focused on observations of the disease. Many of these observations are still relevant today to help identify the pathogen. The insights from the early reports provided the basis for research that would be conducted on the disease in the following years, and that research is the root source of current management recommendations. Research was focused on spread of the disease, timing of infection, control measures, and the specific characteristics of the bacterium responsible for infection.

In the first published report, Barss (1915) recorded the disease was first noticed as dead areas of bark girdling twigs that caused dieback in commercial orchards in Woodburn, Oregon in 1913. Within a year of these first reports, the same symptoms were reported in multiple locations throughout the growing region (Barss, 1915). Dilution plating of the inner tissue of these symptoms yielded pure cultures of distinctive bacteria (Barss, 1915). Repeated inoculation experiments were conducted that verified that the bacteria recovered from these symptoms were in fact the agents causing the blight (Barss, 1927). Barss (1927) documented all the visible aspects of the disease throughout hazelnut orchards with careful observations. No common factor of transmission was apparent

among the reports of these infected orchards, such as source of nursery stock. This led investigators to believe that bacterial blight was present throughout the growing region in the Willamette Valley, and it likely had been there for a long time.

Symptoms of Bacterial Blight in Hazelnuts

Early research showed that the first detectable symptom of the disease occurred in early spring with blighted buds that fail to expand or break. Later in the season, the proximal ends of new shoots would become shrunken and necrotic. Necrotic, dark brown cankers in the bark would start to appear and begin to girdle the rest of the tree first with a wilted appearance in the leaves and branches, then a yellow chlorotic appearance. The leaves clinging on to the branches would then turn from dried green to a reddish brown as the tree fully died. The growing points of new shoots and suckers could also become water-soaked or darkened when infected. Green leaves could become speckled with small necrotic spots that could become more numerous and closer together making the leaves dry up and die. The greatest damage of bacterial blight resulted from girdling cankers on the trunk, main branches, and young shoots. This mainly occurred when shoots were less than an inch in diameter or the trunk of trees from 1 to 4 years old. The wood of branches or the trunks would be weakened so much in the area of the bacterial cankers that breakage would occur in strong winds. Branches that were larger and trees that were more developed often were not detrimentally affected by infection (Barss, 1927).

It was determined that the bacteria were most likely carried over from one season to the next on branch and stem cankers or in infected buds (Barss, 1915). Bacterial ooze could be found exuding from cankers and blighted shoots in the early part of the season. This ooze was suspected to drip and splash during damp, rainy weather to spread and collect in branch angles producing new infections on young succulent growth and that could then be carried over to the next season. Barss (1915) also suspected that insects contributed to the

spread of infection by feeding on the bacterial ooze and moving to other trees in the field. This hypothesis for insect involvement in the spread was discarded in a later report published by P.W. Miller (1931) as there was no reported insect attraction to the exudate.

There was uncertainty as to whether bacterial blight directly affected the nuts. After repeated attempts to artificially inoculate nuts, Miller (1936) reported the first successful inoculation of nuts, and the same symptoms were reported in the field that year. Infection of the nut was characterized by the presence of dark brown to black spots in the outer portion of the shell and sometimes into the tissue of the kernel. Lesions were irregular shaped and ranged from 0.5 mm to a quarter or half the area of the nut with a water-soaked zone surrounding them. Bacterial ooze was found on some of them during high humidity. High levels of the bacterial blight pathogen was recovered from a large percentage of sample collections, though in most cases the disease was confined to the shell of the nut making the effects of bacterial blight on the nuts of little consequence (Miller, 1937; Miller *et al.*, 1940). Nuts with water-soaked spots on the surface with drops of brownish exudate were commonly observed, but researchers did not suspect bacterial blight to be the cause of these nut symptoms because no organism was ever recovered from symptomatic nuts (Barss, 1915). This condition was later described as brown stain (Miller, 1945; Miller and Thompson, 1935).

The impact of bacterial blight mainly depended on the part of the hazelnut that was infected, the time of infection, and even the cultivar of hazelnut. Young orchards in their first 3 to 4 years were most susceptible to infection, but once an orchard was past this point bacterial blight infection was of little consequence and growers were encouraged to divert all resources into maintenance of soil fertility, moisture supply, and other cultural management to keep the production of nuts as high as possible. Disease symptoms in young orchards could kill trees in a single season or cripple developing trees in the first few years before they would succumb to infection in their third or fourth year.

The Host Range of the Pathogen

The seemingly ubiquitous nature of bacterial blight in Oregon made many believe that the bacteria were being transferred to orchards from the native hazel vegetation. The California hazel (*Corylus cornuta* Marshall var. *californica* (A. DC.) Sharp) is native throughout the PNW and is a common understory brush in Oregon's Willamette Valley (Miller, 1934). Barss (1927) repeatedly experimented with artificial inoculation of the California hazel with bacteria from commercial orchards, and found that it is a susceptible host. The California hazel brush throughout the area was scoured and searched for signs and symptoms of naturally occurring bacterial blight disease, but at this time bacterial blight was not recovered (Barss, 1927). In 1934, symptoms of bacterial blight were found on California hazel near Oregon City, Oregon. Plant pathologist P.W. Miller recovered bacteria from the tissue and found it was indistinguishable in culture from the pathogen causing the disease in cultivated hazelnuts. Pathogenicity of the newly recovered bacteria was demonstrated on the leaves of *C. avellana*, making this the first definitive report of *C. cornuta* var. *californica* as a host for bacterial blight (Miller, 1934).

The host range of the pathogen causing bacterial blight in hazelnut (*Xanthomonas arboricola* pv. *corylina,* or *Xac)* is highly host-specific and limited to the genus *Corylus*. Members of the species that have been determined to be viable hosts of this pathogen include *C. cornuta* var. *californica*, *C. pontica* Koch, *C. maxima* Mill., and *C. colurna* Spach, but the disease is most significant on *C. avellana* which is the primary commercially grown species for nut crop production (EPPO, 2004; Caballero *et al.*, 2013; Miller, 1934; Miller *et al.*, 1949). The narrow host range of this plant pathogen has been shown to be one of its most distinctive phenotypic characteristics.

P.W. Miller (1940) compared the phenotypic characteristics of the causal pathogen of walnut blight (*Xanthomonas arboricola* pv*. juglandis*) and the causal pathogen of bacterial blight in hazelnut (*Xanthomonas arboricola* pv. *corylina*). At the time, the pathogen causing walnut blight was named *Phytomonas juglandis*

and the pathogen causing bacterial blight had yet to be named or recognized as a unique pathogen. The two bacterial pathogens were found to be nearly identical in the morphological, cultural, and biochemical characteristics evaluated at the time until the pathogenicity testing (Miller *et al.*, 1940).

Numerous studies of cross-infection pathogenicity were conducted using hazelnuts and walnuts with the respective pathogens under field conditions and under greenhouse conditions. Studies were done comparing infection on leaves, on the fruits, on the stems of young shoots of current growth, and on older branches of both hazelnuts and walnuts with the other pathogens. Slight symptoms of infection were reported in each of the cross-infection combinations, however the symptoms of infection from each bacterium were consistently more aggressive on its specific host with the greatest difference seen during the twig and branch inoculation tests of each host (Miller *et al.*, 1940). This difference in pathogenicity was the first characterization on record that suggested the causal pathogen of bacterial blight should be named as its own species. The suggested name was *Phytomonas corylina* (Miller *et al.*, 1940). This high host specificity was again seen later in a species susceptibility inoculation experiment using *X. arboricola* pv. *juglandis* on 6 *Juglans* species when the *Xac* pathotype strain was included as a non-host control. No disease was detected from *Xac* when inoculated on any of the walnut species (Belisario *et al.*, 1999).

Early Observations on Susceptibility of Hazelnut Cultivars

It was noticed early on that some hazelnut cultivars would succumb to infection much more easily than others, although no cultivars were seen to be immune to infection (Miller *et al.*, 1949). Cultivar differences were most apparent in how much trunk cankers would stunt the growth of the trees. ‘Barcelona’ was the most commonly planted hazelnut cultivar at the time, and the Oregon hazelnut industry favorite (Mehlenbacher and Miller, 1989). ‘Barcelona’ was considered to be moderately susceptible to bacterial blight compared to more susceptible cultivars such as the ‘Aveline’ types, ‘DuChilly’ and ‘Brixnut’. Infection

was most problematic in susceptible orchards between the first and fourth year they had been planted and poor site conditions seemed to predispose young trees to bacterial blight infection and other problems such as shot-hole borer attack (Miller *et al.*, 1949; Miller and Thompson, 1935). Seemingly more resistant cultivars such as pollinizers 'Daviana' and 'Hall's Giant', as well as *Corylus colurna* (Turkish tree hazel), were not as widely planted as main nut producing cultivars due to their inferior and inconsistent nut quality (Miller, 1936). Most of the early growers thought it was worth the risk of bacterial blight infection to plant the more susceptible cultivars that yielded better quality nuts than to plant resistant trees with poor nut-set and quality (Dorris, 1927).

Early Management and Control (1915 to 1935)

The top suggestions in the early literature for managing bacterial blight were planting hazelnut cultivars that showed signs of resistance, pruning out infected branches and disinfecting pruning tools, growing the hazelnuts in tree form by clearing away suckers and spraying copper solutions as a bactericide in conjunction with the other recommended management methods (Barss, 1915; Miller, 1936; Miller and Thompson, 1935). It was also added that managing cultural conditions of the orchards represented another important consideration in bacterial blight management. Paying attention to aspects such as sunscald, winter injury, drought, proper site selection, nutrition, and the use of quality nursery stock could reduce plant stress and maintain a strong orchard (Dorris, 1927; Miller and Schuster, 1938). All these management suggestions laid the groundwork for the research that would be conducted. The control measures would then be improved and solidified over the years.

One of the best ways to minimize the spread of infection was to prune out all signs of infected twigs and canker tissue in order to reduce the source of inoculum for future spread, ideally in midsummer after the rainy season ends (Miller and Thompson, 1935). Sterilizing pruning tools between trees, especially when pruning out infected material, was recommended the best way of

controlling the human-assisted spread of bacterial blight within an orchard. Until the mid-1940s the disinfectant suggested consisted of cyanide of mercury, bichloride of mercury and water. Today, a 70% alcohol or dilute bleach solution is recommended as a disinfectant. This alcohol treatment of tools works best with prolonged contact time (Pscheidt and Ocamb, 2019).

Early Research Conducted on Bacterial Blight (1930 to 1949)

Throughout the 1930s, bacterial blight in hazelnuts was studied and observed intensively to increase knowledge of the disease life cycle and improve management strategies. Barss (1927) had suggested sanitation, which involved removal of infected plant material through pruning it to reduce primary inoculum. In 1932, an experiment was carried out to verify if pruning out infected plant material could be the sole management task to control bacterial infection from spreading through an orchard. All detectable sources of infection were removed during the summer from an infected block of hazelnuts. The disease the following season was greatly reduced, but new symptoms were still present indicating sanitation should be supplemented with other management strategies (Miller and Thompson, 1935). In conjunction with this experiment, the pruning wounds of a sample of these cuts were treated with a painted-on disinfectant solution over the wounds to see if the spread of disease would be halted. The disinfectant proved ineffective as infections in the treated wounds were still present the following season (Miller *et al.*, 1949). These results showed that more information was needed on the mode of entry by which the bacteria penetrate the hazelnut tissue.

In response to these questions, researchers experimented with artificially inoculating trees through cutting wound and leaf spraying under various conditions. These inoculations were observed to establish symptomology and learn key life cycle components including how the bacteria enters the tissue, the general timing and conditions of infection, and locations where the bacteria can overwinter and be re-isolated. Inoculations were made at weekly intervals

throughout the year from August 1935 through September 1936 during periods of high humidity. A sterile needle was used to make wounds in the tissue and a pure water suspension of the causal bacteria was sprayed onto the fresh wounds (Miller, 1937). The first symptoms to appear from these inoculations were apparent in mid-April as the buds began to open. From then on, the disease became increasingly conspicuous and reached a peak in the latter part of May after which new infections were few and disease development slowed.

The bacteria were found to readily enter the hazelnut trees through wounded tissue in fall and early spring, but inoculations made in late spring through the summer were mainly negative. Microscopic examination of inoculation points revealed aggregation of bacteria in tissue cavities in and among the stomata of the leaf surfaces after being sprayed on the plants and emerging inoculated buds. This revelation of the mode of entry into healthy plant tissue and the timing of bacterial blight infection confirmed the idea that the bacteria could likely be controlled using foliar sprays. The timing and frequency needed for these sprays was still unknown (Miller, 1936).

The knowledge of where the bacteria would aggregate on the leaf surfaces led to the first experiments to determine the timing and frequency for Bordeaux mixture spray applications. Bordeaux consists of a mixture of copper sulphate, hydrated lime, and water in varying quantities that could have a spreader-sticker added. A Bordeaux mixture concentration of 6-4-100 (6 lbs of copper sulphate, 4 lbs of hydrated lime, and 100 gal of water) was often recommended. Experiments were conducted in multiple plots within an 8 year old ‘Barcelona’ orchard located near Chehalis, Washington, and was repeated that same year in a 6 year old ‘Barcelona’ orchard near Woodburn, Oregon, achieving similar results. In this field study, plot 1 received two treatments of Bordeaux with one treatment application in the early fall before the first fall rains occurred (Sept. 5, 1935) and a second treatment application in the spring when buds were opening with from one to two leaves showing (Apr. 4, 1936). A second plot adjoining the first received only one treatment application in the early fall, and a

third adjoining plot received only one treatment application in the spring when buds were opening. A fourth large plot was left non-sprayed as a control. Final data were collected and shown as the percentage of the total trees per plot that showed a small amount of bud and twig blight, moderate amount of bud and twig blight, or a great amount of bud and twig blight. The results seen from this study showed a reduction in the incidence and severity of the disease in all plots that received an early fall copper application compared to the non-treated plot. The single spring application reduced disease incidence and severity somewhat but not as much as the fall application.

These results suggested that a second application of copper in the late fall after the early fall application would extend the protection that the bactericide would provide. A variety of copper treatments were also included in the evaluation for the control of bacterial blight. It was found that a mixture of Bordeaux 6-4-100 worked just as well as stronger concentrations, or that a mixture of yellow cuprous oxide dust could be used with the same results in their ability to reduce infection.

Over the next 12 years these experiments were repeated under a wide range of local and seasonal conditions through the involvement and cooperation with growers throughout the Willamette Valley. The goals of these experiments over the long term were to determine the ideal timing of spray applications and the number of spray applications that would have the greatest impact on minimizing bacterial blight incidence and severity. Each year between the fall of 1935 and the spring of 1947, plots in hazelnut orchards throughout the Oregon growing region were treated with 0, 1, 2, 3, or 4 applications of Bordeaux copper mixture. These applications were applied in all combinations of timing and frequency in the early fall, late fall, early spring, late spring and mid-summer (Miller *et al.*, 1949). The results from the seasons of 1939 to 1947 are shown in Figure 1.2.

Based on this data, researchers confirmed that control of bacterial blight in hazelnut orchards could be aided with spray applications of a copper treatment.

This data suggests that nearly any application timing of copper treatments would provide greater control of infection than no treatment. The application timing that shows the greatest bacterial blight control based on the knowledge of the disease cycle and the results of these trials was one application in the early fall before the rains began. If the early fall was particularly wet and there was greater risk of bacterial blight, then this data shows that a second application in the late fall once 75% of the leaves have fallen, and a third application in the early spring as the buds were just beginning to open would provide sufficient control.

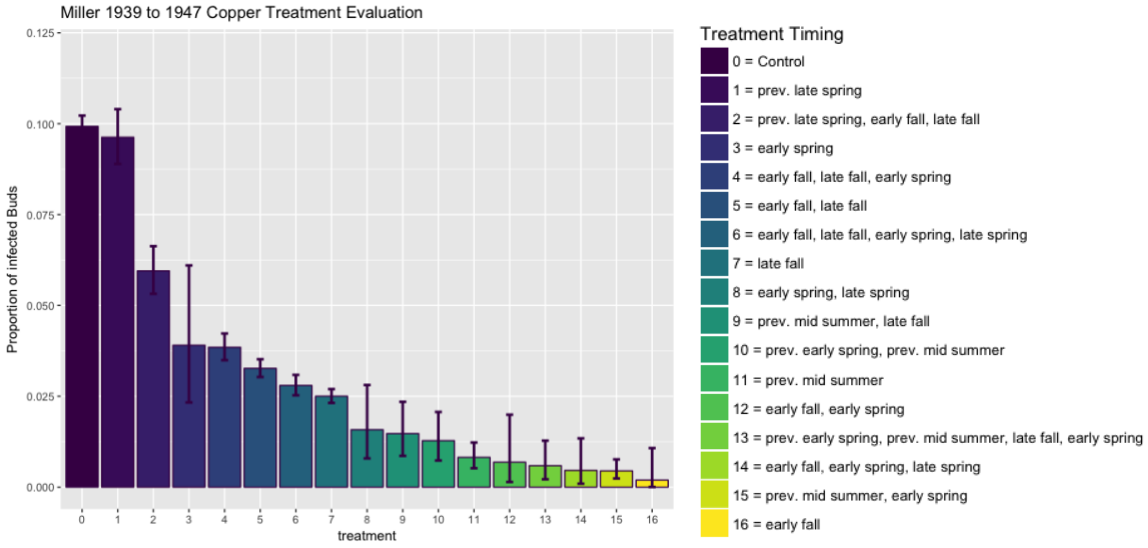

Figure 1.2. Miller (1949) Copper Treatment Application Timing Combinations

The proportion of infected buds compared to the total number of buds examined for each of the copper treatment timing combinations evaluated the summer after the treatment timings were applied. Sample sizes varied for each treatment combination.

Oregon State University Research in the 1970s

By the late 1960s, interest increased in re-examining bacterial blight and answering new questions that had arisen since Miller *et al.* (1949) had released their comprehensive report on the disease. In 1969, Oregon State University researcher Larry Moore reported that a substantial number of hazelnut growers in Oregon had suffered from severe losses in young hazelnut orchards attributed

to bacterial blight. It was conservatively assumed that bacterial blight was responsible for 7% of the hazelnut tree losses each year in orchards from two to five years old. An association of increased disease severity was observed under harsh environmental conditions coupled with marginal cultural practices. Events such as frost injury, improper pruning, sunscald, and drought appeared to affect the trees' ability to withstand bacterial infection (Moore, 1969). Oregon growers were faced with the loss of trees, planting and management costs, replanting costs, and the intangible costs of delayed production and uneven tree height and growth. The estimated cost of bacterial blight to the hazelnut industry in losses over a three-year period was calculated based on crop planting values from 1970 to 1972 at a time when the cumulative value of the hazelnut industry was $10.5 million. During this three-year period, $600,000 was invested toward planting new orchards ($200,000 per year). The estimated loss from bacterial blight in first year of new orchard plantings across the whole industry was $19,600 and that value would rise to $58,800 by the third year (Moore, 1972).

A cooperative research endeavor was initiated at the nursery level with the aim of answering questions that could improve management strategies and reduce the cost of the disease to growers. Research questions involved investigating how proper management of nursery trees could reduce the incidence of bacterial blight in young orchards, the association of pruning wounds and stem canker production, the effects of pruning timing to reduce bacterial infection, and how sealing pruning wounds immediately after cutting might prohibit later ingress of the pathogen. The hypothesis that young trees can "outgrow" bacterial blight infection given good nursery management and good orchard cultural practices was put to the test.

In the spring of 1969, isolates of *Xanthomonas arboricola* pv. *corylina* were easily cultured from numerous orchards around the growing region. It was also evident that nursery trees pruned with shears that were contaminated by the pathogen did not callus well and caused a dead and discolored wound. It was

determined that cutting with contaminated pruning shears was an effective tool to administer an inoculation with the bacterial blight pathogen (Moore, 1974, 1969).

The first study, conducted in June 1969, investigated the effects of sealed pruning wounds on inhibiting bacterial blight infection. A population of 'Barcelona' trees were used under nursery conditions. A portion of trees were pruned with shears that had been dipped in an *Xanthomonas arboricola* pv. *corylina* suspension, with half receiving paint to seal the pruning wounds and half left unsealed. Another portion of trees were pruned with clean shears with half of the wounds sealed and the other half not sealed. The trees were evaluated in September 1969. It was determined that sealing the pruning wounds was not an effective strategy to inhibit growth of the bacteria. The bacteria treated pruning wounds did not callus over and bacteria were readily isolated from those areas whether they were sealed or not. All the non-inoculated pruning wounds callused over and healed, and no bacteria was recovered. This experiment showed that sealing pruning wounds is not necessary when the natural populations of *X. arboricola* pv. *corylina* are low and the trees are pruned in the winter using disinfected tools (Moore, 1969).

Dr. Larry Moore and cooperators took this research a step further to investigate areas of cultural practices including the effects of trunk sunscald protection and irrigation on bacterial blight during the first three years of an orchard (Moore, 1974). The trunk guards used during this time were a newsprint mat guard that wrapped around the trunk. These trunk guards were compared to latex paint. Both trunk protection treatments were applied to trees that had received the bacterial inoculation or had received a sterile water control. One portion of the trial saw mortality in both inoculated and non-inoculated trunk guard treatment due to the trees overheating inside the guards. In the other trials, the effects of the bacterial treatment showed no difference between protective guards, painted trunks, or the bare trunk controls.

The effects of irrigation on bacterial blight infection were the most notable results from this study. Irrigated trees received water three times during the

summer of each growing season for the course of three years and were compared to non-irrigated trees after each were inoculated with *Xac* during the first season. There was the most infection on the non-irrigated trees and the effects were most apparent the second season after inoculation. These experiments showed that non-irrigated trees were more likely to be killed by bacterial blight infection. The irrigation increased the ability of young trees to withstand infection even though irrigation also allowed for the growth of asymptomatic bacteria on those trees. *Xac* can reside in apparently healthy tissue for 48 months or longer, but young hazelnuts will not suffer too greatly from bacterial blight if necessary precautions are taken to maintain good cultural practices (Moore, 1974).

These results supported claims made by Miller, Barss, and experienced growers in the early years of the industry that said stressed trees are more likely to be impacted by bacterial blight than trees grown under good cultural conditions (Barss, 1927; Dorris, 1927; Miller *et al.*, 1949). Thus, the risk of bacterial blight infection could be reduced by planting new orchards in the fall or winter in sites with deep well-drained soil, and using trunk protection, disinfected tools, and copper treatment sprays in the early fall, along with minimizing moisture stress during the growing season (Barss, 1927; Miller *et al.*, 1949; Moore, 1974).

Bacterial Blight Inoculation Experiments and Applied Research (1915 to 1974)

Significant improvements were made in the ability to artificially inoculate hazelnut trees as these early researchers learned more about *Xac* and its disease cycle. Artificial inoculations to verify the causal pathogen of bacterial blight done by Barss led to inoculations to learn more about the life cycle and timing of the disease conducted by Miller, which all paved the way for applied research to test how the pathogen interacted with the production system in experiments conducted by Moore (Barss, 1927; Miller *et al.*, 1949; Moore, 1974). Many experiments were carried out to optimize the inoculation process and

determine conditions in a controlled environment that would most resemble and replicate the natural systems where infections occur.

The first inoculation experiments carried out by Barss to establish pathogenicity had a very low success rate. Barss first started his inoculations by introducing pure cultures of the pathogen to hazelnut tissue using needle punctures. These experiments were conducted in late December and early January. After failed attempts during the winter, inoculations were attempted again on fresh succulent tissue twice during the summer, at the end of June and at the end of July. It was hot and dry during these inoculations, the results were meager, and further testing was required (Barss, 1915). Infection was finally successfully induced in the fall during conditions of greater moisture. It was determined that this bacterial blight requires very particular timing and conditions to generate disease, but there was no doubt that the bacteria recovered from the infection tissue in the field was the causal agent of the disease (Barss, 1927).

Miller (1937) then conducted inoculation testing to determine the timing of infection in the life cycle of the bacteria. In learning more about the life cycle of the bacterium this experiment also illuminated the optimum timing of inoculation that would best resemble natural infection conditions. Hazelnut buds were mist sprayed with a bacterial suspension every 1 to 2 weeks from August to June in 1935 and 1936. Symptoms first became apparent in mid-April of 1936 and no new symptoms developed later than mid-May. These experiments showed that infection typically occurs between the late fall and early spring, making these the best times of the year to artificially inoculate hazelnut trees for applied research (Miller, 1937). This information was also invaluable for developing the timing to control the disease with bactericidal and preventative sprays.

As the knowledge of bacterial blight increased over the years, Miller *et al.* (1940) began to compare *Xac* with its closely resembled walnut blight counterpart, *Xanthomonas arboricola* pv. *juglandis* (Xaj). In a publication comparing the attributes of the two pathogens, a cross-infection study was conducted to determine the pathological relationship on the two pathogens under

controlled artificial inoculations of leaf tissue, fruit tissue, young shoots growth, and on older branches (Miller *et al.*, 1940).

Inoculations on the leaf tissue were administered to trees held in a greenhouse by a spray inoculation with suspensions of each isolate being tested. The trees were given a 24 hour pre-inoculation moist exposure time in a saturated atmosphere and were kept under high-humidity conditions for 48 hours post-inoculation. Results from these cross-infection leaf tissue tests with Xaj and *Xac* suggest that leaf tissue is not as ideal to test pathogenicity as other portions of the hosts as there were few significant differences between the lesions caused by the two pathogens on each host. The role of moisture in this inoculation experiment may have made each host more susceptible to infection that made all the symptoms too similar to distinguish differences in pathogenicity (Miller *et al.*, 1940).

Inoculations of half-grown fruits from the walnut host were carried out under field conditions and inoculations on young fruits in the early post-bloom stage on potted hazelnuts were carried out in greenhouse conditions. The bacterial suspensions for each isolate was sprayed onto the fruits of each host. The potted trees under greenhouse conditions were exposed to high humidity conditions for 28 hours post-inoculation. The *Xac* caused only small lesions to form on the walnut fruits while Xaj generated much larger lesions on the walnut fruits. Xaj produced only small and superficial lesions on the developing hazelnut fruits. *Xac* did not produce lesions on hazelnut any more substantial than those caused by Xaj. Bacterial blight is rarely an issue on the nuts of its natural host. Walnut blight was consistently more virulent on its natural host than on hazelnut.

Puncture inoculation wounds were made on the young shoots of current growth to administer the treatment of the different isolates on walnut shoots growing in the field. No lesions developed when isolates from hazelnut were applied to walnut. Xaj generated substantial infection when lesions over 50 mm long were formed. Inoculation of Xaj on potted hazelnuts under greenhouse conditions were negative while *Xac* generated well-defined lesions up to 12 mm

long. Similar results were found on the older branches when puncture wounds were made on one year old hazelnut twigs with Xaj and *Xac*. Xaj did not produce infection on hazelnuts, while *Xac* created stem-girdling cankers that were well-defined.

Xaj and *Xac* were each found to be much more pathogenic on their natural host than in cross-infection. It was also determined that the stage of growth and the type of plant tissue inoculated affects clarity of the pathogenicity testing. Young shoots and older stems showed the clearest results from the pathogenicity testing. Moisture levels and temperature also seemed to make plant tissue much more susceptible to infection, which led to further investigation into inoculation conditions. The results from this experiment allowed *Xac* to be recognized as a unique pathogen of hazelnuts (Miller *et al.*, 1940).

The role of temperature during the infection period was evaluated on one year old 'Barcelona' whips planted in pots under controlled greenhouse conditions. The trees were inoculated by spraying a suspension of *Xac* over the whole tree. The trees were then placed in a humidity chamber where the temperature could be controlled. The temperatures evaluated ranged from 7.5 ^{o}C to 25 ^{o}C. Infection symptoms appeared with great severity during experiments at temperature of 22 ^{o}C and higher. Experiments done at temperatures that were below 20 ^{o}C showed less disease symptoms and took a longer time to develop than at warmer temperatures. It was determined that warmer temperatures are more favorable for infection than lower temperatures, and inoculation temperature plays a role in the infection of the plant tissue, but not as great a role as moisture (Miller *et al.*, 1949).

A prominent role of moisture in the incidence of bacterial blight infections had long been assumed as the disease had been noticed as being more severe when the infection period had a seasonably high amount of rainfall (Miller, 1939, 1937). The role of moisture in these observations were supported in tests where no infection occurred from smearing bacterial cultures directly to the trees with no exposure moisture. Incubation in a moisture chamber following inoculation

generated abundant infection with a noticeable increase in the number and size of lesions accompanying prolonged moist periods. Exposure to moist conditions before inoculation also expedited the infection process. Longer periods of moisture allow for bacterial penetration, and water-soaked tissue aids in the growth and migration of the bacteria through the tissues. The minimal period of continuous wetting needed for infection varied greatly with the age of the host tissue, the extent of opening of the stomata, and the degree of water-soaking of the tissues (Miller *et al.*, 1949). Young and stressed plants were generally most susceptible.

The development of this work to replicate natural environmental conditions to induce infection in young hazelnut trees helped researchers learn a great deal about the bacterial blight infection. Orchards surveyed during the season of 1969 showed a considerable number of bacterial blight infections that indicated the role that injuries in the hazelnut orchards (mechanical, sunburn, frost, chemical) play in predisposing trees to infection. In many cases it was reported that the worst impacts of infection occurred two years after planting (Moore, 1974, 1970). In 1970 a study was done to measure the effects of wounding during bacterial blight inoculations on bud tissue of older trees. It was found that wounding was required for the bacteria to penetrate the inoculated tissue and the result was a clear expression of disease. Moore (1974) also attempted to inoculate 'Barcelona' trees in the field by dipping pruning shears in inoculum prior to making pruning wound. This was used as a reliable method for inoculation during the multiyear field studies carried out on moisture stress, pruning timing and wound sealing, and the effects of tree guards on disease development.

Inoculation methods had been developed to learn about the disease cycle of *Xanthomonas arboricola* pv. *corylina*, for general pathogenicity tests, and for multiyear applied research on the effects of the disease. However, there was still a gap in the knowledge of how to incorporate bacterial blight disease resistance screening into hazelnut breeding as well as how to determine the effects that bacterial blight has on the crop yields of full-grown orchards. Regrettably, all the

lessons that had been learned through the many years of bacterial blight research applying to these two topics of research were never formally published. The Oregon Hazelnut Commission funded the project for two years and the proposal plus two progress reports were recently discovered in a tucked-away file.

It was noticed that while trees between 1 and 4 years old were most readily killed by bacterial blight infection, symptoms that would result in the dieback of nut bearing twigs were frequently seen in orchards of older trees. A project was developed to verify whether nut yields were affected by bacterial blight infection by inoculating a mature orchard with *Xac* and measuring the effect on yields. The treatments were based on inoculation timing made each month during September, November, January, and March on untreated control tree, and a tree protected with bactericide, for a total of seven treatments applied to each of eight trees in three different plots. One of the plots was given irrigation and the other two were not irrigated. *Xac* was found to be present in the orchard prior to inoculation along with the commonly found saprophytic bacteria. An antibiotic-resistant mutant strain of *Xac* was developed and used in the inoculation to make re-isolation more reliable and discern the inoculated bacteria from the natural population of bacteria.

The applications of the inoculum at the different timing points in each treatment were successful, however no infection or signs of disease developed in the treatment blocks as the seasons progressed. Re-isolation from the plant tissue recovered very few pathogenic bacteria and those that were recovered were not mutants, indicating that the mutants survived poorly in nature. The results of this experiment showed a more significant impact on yield from the effects of irrigation vs. non-irrigation than from a treatment effect for the inoculated *Xac* bacteria in the orchard. This field study was ended and never published likely due to these negative results.

Contained in the same proposal and progress reports was an investigation by Larry Moore with collaborator Maxine Thompson, the lead of the OSU

Hazelnut breeding program. Some hazelnut cultivars were observed to be considerably more susceptible to bacterial blight infection than other cultivars, and some showed a considerable amount of resistance to the disease. It was suggested to devise a bacterial blight resistance screening method to include in the progeny evaluation from hazelnut breeding crosses. The resistance screening method evaluation examined the different moisture and temperature regimes to induce the most rapid symptom expression. Different inoculation methods were tested and evaluated, and the difference between greenhouse-grown plants and field-grown plants was examined. The results from all the previous work on setting up inoculation systems provided a great jumping off point for the role that moisture, temperature, and wounding would play in the inoculation system (Miller *et al.*, 1949).

Several hundred whips of ‘Barcelona’ and open-pollinated seedlings of ‘Tonda Romona’ × ‘Barcelona’ were used in testing the various methods for disease screening. Several of the most successful methods were presented in the final report on the subject. A post-inoculation incubation period in a high relative humidity environment was best for the largest number of water-soaked lesions per leaf. Some trees were kept in the dark during this incubation period and some were kept in the light. The trees in the dark required 72 hours of incubation to achieve the same amount of infection as trees kept in the light produced under just 48 hours of incubation. A pre-inoculation incubation at high humidity in addition to the post inoculation incubation was found to maximize the number of plants infected. Some inoculations were done using a Tween 80 surfactant mixed with the inoculum, and were only found to be effective when the plants were incubated in the dark at a high relative humidity. Trees that were inoculated with the Tween 80 inoculum and put directly into the greenhouse showed greater infection than tree inoculated without the Tween 80. However, even with the use of the surfactant the total number of plants infected was lower than when post-incubated after inoculations. These resistance screening methods were never fully integrated into the OSU Hazelnut Breeding program

progeny evaluation because, just after this period, all research efforts were diverted to learning how to cope with the new problem of eastern filbert blight (EFB), which had recently been discovered in Lewis County, Washington and was spreading (Johnson *et al.*, 1996).

European Research on Bacterial Blight of Hazelnuts

The first observed incidence of bacterial blight-like symptoms on hazelnuts in Europe occurred in 1931 and 1932 in Italy, near Rome, where a bacterial infection that resembled walnut blight was observed on hazelnuts (Noviello, 1968). Similar symptoms were observed somewhat sporadically again in Italy in 1958, but it was not until a severe infection outbreak in 1965 that a characterization study was conducted to verify the presence of the causal pathogen *Xanthomonas arboricola* pv. *corylina* (Noviello, 1968). Although the European hazelnut industry had disease pressures that were not present in Oregon, bacterial blight was still considered one of the most insidious and difficult to manage. The disease continued to be an issue and was appearing in more locations by the 1970s (Luisetti *et al.*, 1976; Noviello, 1968; Prunier *et al.*, 1976).

The first reported incidence of bacterial blight in France came in 1974, as trees in production orchards and nurseries in the southwest were showing symptoms. The cause of these symptoms was verified in 1975 to be due to *Xac* (Prunier *et al.*, 1976). The origin of the disease was unknown, but the nursery where the disease was first found was growing some propagation beds with trees that had been transported directly from Oregon (Luisetti *et al.*, 1976). It is still uncertain if the disease had already existed in France, but many believed the pathogen to have been disseminated on infected propagation material of U.S. origin that was not properly quarantined prior to being introduced into nurseries (Prunier *et al.*, 1976). Over the next 10 years the economic impact of bacterial blight was being recognized throughout the world and the European Plant Protection Organization (EPPO) classified *Xanthomonas arboricola* pv. *corylina*

as an EPPO phytosanitary A2 List no. 134 quarantine pathogen (Gardan and Devaux, 1987). This designation recognized the pathogen as present in the EPPO regions and recommended that *Xac* be regulated as a quarantine pest within the EPPO countries (EPPO, 2004, 1986). By this time the pathogen had also been reported in Australia, Algeria, Turkey and the United Kingdom, and what at the time was Yugoslavia and the USSR (EPPO, 1986; Gardan and Devaux, 1987; Locke and Barnes, 1979; Wimalajeewa and Washington, 1980).

The only research being conducted on bacterial blight up until the 1970s was in Oregon (Luisetti *et al.*, 1976; Noviello, 1968). The methods for disease characterization and disease management that were utilized at this point in Europe were based on observations seen in nurseries and orchards as well as the research synopsis from Miller (1949) (Gardan and Devaux, 1987; Luisetti *et al.*, 1976; Noviello, 1968; Prunier *et al.*, 1976). Gaps in the knowledge still existed and European researchers proposed a program to further the research on bacteriological studies, differences in susceptibility of tissues and their age of development in the host, detection of pathways of the bacterium, sources of inoculum, cultivar susceptibility, and control treatments (Prunier *et al.*, 1976).

Outcomes of the research being conducted on bacterial blight in Europe included the EPPO phytosanitary risk designation, the archiving of strains collected throughout the European growing region, the creation of a proposed diagram for the disease life cycle of *Xanthomonas arboricola* pv. *corylina*, and further description of the signs and symptoms of the disease (EPPO, 1986; Fischer-Le Saux *et al.*, 2015; Gardan and Devaux, 1987). The life cycle and bacterial blight descriptions proposed by Gardan and Devaux (1987) were developed based on previously recorded observations as well as their current production techniques. The disease life cycle was segmented into how the bacteria caused infection in the leaves, buds and trunk of the hazelnut throughout the four seasons.

Briefly, in the winter, layer propagated trees were suspected to have been contaminated from their mother tree. These young trees could have discrete

existing trunk cankers or could carry epiphytic bacteria asymptomatically that could spread during the early spring. The bacteria from these young trees could contaminate emerging buds and leaves to cause bacterial infection with necrotic lesions on the leaves or cause dieback of new shoots in the spring and summer. Epiphytic bacteria on the leaves could spread to the buds during the fall to overwinter. Already necrotic buds could develop into cankers on the trunk throughout the spring and summer that would then provide a place to overwinter where the cycle could repeat the following year (Gardan and Devaux, 1987).

Modern-day Bacterial Blight and *Xanthomonas* Research

From the late 1980s through the early 2000s, very little applied research was conducted on bacterial blight of hazelnut anywhere in the world. Eastern filbert blight was spreading through the heart of the Oregon hazelnut industry, and all research efforts were being devoted to the biology of EFB, potential cultural and chemical control measures, and development of resistant hazelnut cultivars (Johnson *et al.*, 1996). This intensive research in Oregon left no room for research on the less-aggressive bacterial blight pathogen (Thompson *et al.*, 1996).

While less research was being conducted on bacterial blight worldwide, more research was being conducted on *Xanthomonas* spp. *Xac* was increasingly being included in studies designed to identify characteristics in the *Xanthomonas* genus because of its similarities to other economically impactful pathogens like *X. arboricola* pv. *pruni* and *X. arboricola* pv. *juglandis* (Fischer-Le Saux *et al.*, 2015; Lee *et al.*, 1992; Vauterin *et al.*, 1995). Characteristics of these *Xanthomonads* were investigated based on morphological, biochemical and molecular indicators (Fischer-Le Saux *et al.*, 2015; Gardan and Devaux, 1987a; Lamichhane and Varvaro, 2014; Luisetti *et al.*, 1976; Miller *et al.*, 1940; Prokić *et al.*, 2012; Puławska *et al.*, 2010; Scortichini *et al.*, 2002; Vauterin *et al.*, 1995; Young *et al.*, 2008).

Xanthomonas arboricola pv. *corylina* is a gram-negative, rod shaped bacterium with a single polar flagellum. The bacterium grows brilliantly yellow on GYCA (glucose, yeast extract, calcium carbonate agar) media, which can help to distinguish it from other yellow saprophytic bacteria collected during field isolations (Lamichhane and Varvaro, 2014; Prokić *et al.*, 2012). The growth and growing conditions of *Xac* is nearly indistinguishable from the pathovars pv. *pruni* and pv. *juglandis*, and the phenotypic characteristic that sets these pathovars apart the most are their host specificity (Fischer-Le Saux *et al.*, 2015; Miller *et al.*, 1940).

A comprehensive DNA-DNA hybridization study was conducted using 183 strains from the genus *Xanthomonas* which included the pathovar *corylina* (Vauterin *et al.*, 1995). The genus was found to comprise 20 DNA homology groups which were considered different species (Lee *et al.*, 1992). *Xac* was found to be in *Xanthomonas* DNA homology group 6, which contained seven pathovars *X. arboricola* pv. *celebenis*, *X. arboricola* pv. *corylina*, *X. arboricola* pv. *fragariae, X. arboricola* pv. *juglandis*, *X. arboricola* pv. *poinsettiicola*, *X. arboricola* pv. *populi,* and *X. arboricola* pv. *pruni* (Fischer-Le Saux *et al.*, 2015; Lee *et al.*, 1999, 1992; Vauterin *et al.*, 1995). Species identification was based on differentiating biochemical characteristics for each of the individual homology groups. A database of these characteristics was compiled and systems such as the Biolog GN microplate system could utilize the differing metabolic activity on a range of carbon sources that could link back to the characterizing database as a rapid method for *Xanthomonas* species identification (Vauterin *et al.*, 1995).

The use of single metabolic biochemical assays could also be used to identify suspected individual pathovars for routine diagnostic purposes. The use of quinate metabolism was determined to be an indicator of *Xanthomonas* DNA homology group 6. When a bacterial isolate was plated on the succinate-quinate semi-selective media, members of *Xanthomonas* DNA homology group 6 produce a diffusion of dark green pigmentation that is clearly distinguishable from other pathovars (Lee *et al.*, 1992). It was later determined that the ability for

quinate metabolism was linked to a specific gene, *qumA*, that could also be used to identify pathovars of *X. arboricola* DNA homology group 6 when present in polymerase chain reaction (PCR) assays (Lee *et al.*, 1999).

This gene that is involved in quinate metabolism was later utilized in a duplex PCR assay developed in a study by Pothier *et al.* (2011). This duplex PCR assay was first developed to generate an *Xanthomonas arboricola* pv. *pruni* (Xap) specific PCR identification assay (Pothier *et al.*, 2011). Pothier *et al.* (2011) used the primer *Xarb*Q to amplify the quinate metabolism gene, *qumA*, to produce a band at 402 bp as an indicator of *Xanthomonas arboricola* species level identification (Lee *et al.*, 1999, 1992; Pothier *et al.*, 2011). Primer *Xap*Y17 was used to generate an Xap specific band at 943 bp amplifying the gene *ftsX*. Pothier *et al.* (2011) found there was consistently a cross-reaction with each of the 10 *Xanthomonas arboricola* pv*. corylina* strains tested along with the Xap strains tested. This duplex PCR method has since been used as a tool for rapid identification to narrow down the pathovar level to pv*. pruni* or pv. *corylina* when there are positive bands at 402 bp and 943 bp (Lamichhane and Varvaro, 2014; Prokić *et al.*, 2012; Puławska *et al.*, 2010).

Assessment of *Xac* strain collections also have been conducted through means of Repetitive PCR DNA fingerprinting with ERIC-, BOX-, and REP- primer sets (Puławska *et al.*, 2010; Scortichini *et al.*, 2002). These Rep-PCR DNA fingerprints have been used to identify pathovars and strains of *Xanthomonas* isolates through a reflection of their genomic structures as a repeatable and reliable method (Louws *et al.*, 1994; Versalovic *et al.*, 1991). Scortichini *et al.* (2002) found that the *Xac* clustered into five groups using DNA fingerprinting with UPGMA cluster analysis. There was a very high level of congruence and little diversity between the groups. The most distinctive group was the pathotype strain NCPPB 935 (isolated from Oregon), but there was no relationship based on geographic origin (Scortichini *et al.*, 2002). Puławska (2010) found a similar result with *Xac* isolates obtained from Polish hazelnut orchards collected in two different years. Rep-PCR revealed two distinct clusters of *Xac* strains collected in

the different years with the pathotype strain creating the most distinct DNA fingerprint (Puławska *et al.*, 2010). The pathotype strain was also found to be consistently weakly pathogenic during pathogenicity testing in each of these studies, causing the representative nature of the pathotype strain to be put into question (Puławska *et al.*, 2010; Scortichini *et al.*, 2002).

The standard in bacterial phylogenetic analysis has shifted towards multi-locus sequence analysis (MLSA) as a means of characterizing the identity of specific bacterial pathogens and to establish the phylogenetic relationships between species and pathovars as the technology for genetic sequencing has become more cost effective (Almeida *et al.*, 2010; Fargier *et al.*, 2011; Maiden *et al.*, 1998). MLSA is done by sequencing sets of genes known as housekeeping genes. Mutations and polymorphisms within the housekeeping genes are assumed to be selectively neutral and may reflect the phylogeny of the strains when analyzed against one another and outgroups (Fargier *et al.*, 2011). MLSA allows for an easy, robust, and reproducible approach to the identification of pathogens that have diverging traits and species boundaries that are not well known (Almeida *et al.*, 2010; Fischer-Le Saux *et al.*, 2015; Maiden *et al.*, 1998).

Up to seven concatenated DNA house-keeping genes have been used to perform MLSA on pathovars of *Xanthomonas arboricola* (Fargier *et al.*, 2011; Fischer-Le Saux *et al.*, 2015; Young *et al.*, 2008). The claims of the nonrepresentative nature of the *Xac* pathotype strain in previous work were challenged in a study that utilized MLSA to characterize and compare *Xac* isolates along with isolates of other *Xanthomonas* pathovars from around the world (Fischer-Le Saux *et al.*, 2015). This study included eight isolates of *Xac* from four different global regions. The analysis showed that the isolates separated into two distinct phylogenetic clades with one of the clades including the pathotype strain (Fischer-Le Saux *et al.*, 2015). A reduced model using only two concatenated housekeeping genes proved to be enough to distinguish pv. *corylina* from the closely resembling pathovars *juglandis* and *pruni*. It was recommended that at least two reference isolates, one from each clade, be

included in any identification studies. Thus, the pathotype strain is representative of the pathovar *corylina* on a molecular level as a reference tool for identification and comparison among a multiple pathovar of *Xanthomonas* sp. (Fischer-Le Saux *et al.*, 2015). The concatenated sequences of *gyrB* and *rpoD* are recommended by the International Center for Microbial Resources-French Collection for Plant-associated Bacteria (CIRM-CFBP) for pathovar identification for *Xac*.

It has been suggested that the use of a single housekeeping gene, such as *gyrB*, could work for pathovar identification (Parkinson, 2007; Parkinson *et al.*, 2009; Puławska *et al.*, 2010). Others have recommended that reliance on a single housekeeping gene could be problematic as there are reports of inter- and intraspecies recombination that has played a role in generating similar polymorphisms between different pathovars (Fischer-Le Saux *et al.*, 2015). Some of the *gyrB* sequences of pathovars *juglandis* and *pruni* were identical to the isolates that fell into one of the clades of *Xac*, but a reliable discrimination was determined between pathovars when the *rpoD* sequence was included (Fischer-Le Saux *et al.*, 2015). Another molecular technique that has been used to identify and characterize *Xac* is genomic sequencing. There is currently only one annotated genomic sequence that has been published for *Xac* (Jorge Ibarra Caballero *et al.*, 2013). The sequence was done using an isolate of *Xac* that was collected from *Corylus colurna* L. (Turkish tree hazel). Further utilization of genomic sequencing in strains of *Xac* in conjunction with *Corylus* sp. genome sequencing could greatly improve our understanding of the molecular basis of pathogenesis and potential plant resistance mechanisms (Lamichhane, 2014).

Modern Applied Research on Bacterial Blight (2012 to present)

The impact of bacterial blight on hazelnut production continues to be felt in hazelnut producing areas throughout the world. A severe threat of bacterial blight was reported in Chile as the cultivation of hazelnuts became more prevalent

there, and incidence of the disease ranged from 60 to 90% in both nurseries and orchards, with cultivars ‘Barcelona’ and ‘Tonda di Giffoni’ being most affected by the disease (Lamichhane *et al.*, 2012). The cultivar ‘Tonda di Giffoni’ had also been recently reported to have been severely affected by an outbreak of *Xac* in a major hazelnut growing region in Italy. A survey of 3 orchards (5 hectares total) of 4 year old hazelnut trees showed an 80 to 100% bacterial blight incidence of the trees with symptoms that led to trunk girdling cankers (Lamichhane *et al.*, 2012). Additionally, an investigation of bacterial blight-like symptoms reported from a 1-hectare hazelnut planting in central Poland showed nearly 100% of the trees showing leaf lesions (Puławska *et al.*, 2010). These incidences of bacterial blight throughout the world have initiated a renewed effort in updating the knowledge of the disease and performing applied research in hazelnut orchards.

In central Italy, a study was conducted to survey orchards around the province of Viterbo (Lamichhane *et al.*, 2013). Factors such as amount of rainfall, total nitrogen in the soil, the soil Mg/K ratio, levels of aluminum in the soil, thermal shock, and soil pH were evaluated. These pedoclimatic variables were analyzed using geostatistical analysis to gain an understanding of the correlation between these factors and the occurrence and spread of bacterial blight. The analysis showed the strongest correlation of bacterial blight incidence with high rainfall, a higher content of nitrogen in the soil, higher thermal shock values and a lower Mg/K ratio in the soil. Levels of aluminum and the pH in the soil showed less impact on the disease occurrence compared to the other factors (Lamichhane *et al.*, 2013). These findings support previous predisposing factor claims that contribute to disease incidence such as rainfall and stress conditions, and highlights the importance of proper site selection when beginning new orchards and monitoring orchard nutrition (Miller, 1937; Moore, 1974; Olsen, 2013b, 2013a).

Knowledge of the symptomologies and disease cycle, and the use of a timely and reliable detection procedure once the disease is confirmed are crucial

factors in maintaining disease-free plant material and understanding the hazelnut-bacterial blight pathosystem.

Lamichhane and Varvaro (2014) compiled the most recent and up-to-date symptomology report including photos, a simplified representation of the disease cycle, and current management recommendations. The symptoms of bacterial blight seen in nurseries and the field often differ as a result of the different growing systems and the age of the trees. Densely crowded, moist environments of nurseries are ideal for the proliferation and spread of the pathogen, with necrotic lesions on the leaves and trunks often most noticeable under these conditions. Under field conditions symptoms can range from lesions on the leaves, necrotic buds, canker formation, dieback in the canopy and tree death (Puławska *et al.*, 2010). During the spring, cankers may ooze bacterial exudate that serves as a secondary inoculum, and cankers may weaken the tree trunk to be blown over in a strong wind. The beginning stages of cankers cause a slight, water-soaked discoloration that can easily go unnoticed. These cankers appear sunken as they develop and the surrounding tissue continues to grow. These cankers will turn a reddish brown over time with the infection moving into the xylem until the trunk or branch is girdled (Lamichhane and Varvaro, 2014). This study also provides the only other disease cycle diagram besides the one developed by Gardan and Devaux (1987). The updated disease life cycle connects the mechanisms of bacterial blight spread with the development of the disease (Lamichhane and Varvaro, 2014).

The chemical treatment to manage bacterial blight is still limited to copper-based compounds applied in the fall or early spring. These applications do not penetrate the tissue, so are aimed at targeting the epiphytic populations at a time when the possibility of infection is high (Lamichhane and Varvaro, 2014). The use of a nonpathogenic bacterial antagonist to out-compete the pathogenic *Xac* has been suggested and used to control bacterial spot and canker of stone fruits (Biondi *et al.*, 2008), but no similar work has been conducted with *Xac*.

Prevention and sanitation remained the best form of management for bacterial blight in hazelnuts.

A recent study investigated the use of hot water treatment of hazelnut nursery stock to prevent the spread of *Xac* on latently infected propagation material (Pisetta *et al.*, 2016). This study determined the ideal temperature and exposure time to minimize the presence of bacterial blight without causing damage to the trees. It was found that 45 minutes in a water bath set to 42 ^{o}C was the optimal exposure time to ensure the proper development of trees. The populations of *Xac* were significantly reduced as a result of this treatment and the application of this treatment would minimize the risk of spreading *Xac* to countries where it is of greater concern (Pisetta *et al.*, 2016).

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Chapter 2.
Characterization of *Xanthomonas arboricola* pv. *corylina* causing Bacterial Blight of Hazelnut

Abstract

Bacterial blight (*Xanthomonas arboricola* pv*. corylina*) (*Xac*) of hazelnut (*Corylus avellana* L.) was described first in Oregon in 1915 and is recognized as a damaging disease of young hazelnut trees worldwide. Thousands of acres of hazelnut cultivars resistant to the canker disease eastern filbert blight (*Anisogramma anomala*) were planted in the Willamette Valley of Oregon, where 99% of the US hazelnut crop is grown. Stressed trees are more susceptible to bacterial blight in conditions such as planting on marginal sites. There has been an increased incidence of bacterial blight in young hazelnut plantings, but no research on the disease has been done in Oregon since the 1970's. We characterized bacterial isolates from symptomatic tissues for growth on semi-selective media, nutrient utilization profiles with Biolog Gen II, quinate metabolism, copper resistance, hypersensitive response (HR) on tobacco, and pathogenicity on hazelnut. Additionally, isolates were identified with a duplex PCR assay (*ftsX* and *qumA*), 16S rRNA sequence, and multilocus sequence analysis (MLSA) using *rpoD* and *gyrB*. Pathogenic isolates were identified as *Xac* using morphological, biochemical, and molecular assays. With MLSA, *Xac* isolates separated into two clades, one clade with the type strain and a second clade previously described using isolates from France and Poland. Thus, the phylogenetic diversity of *Xac* observed in other countries also is present in Oregon. Future studies will evaluate sensitivity of the newly released OSU hazelnut cultivars to bacterial blight with *Xac* isolates from the two clades.

Introduction

Production of European hazelnut (*Corylus avellana* L.) is an important industry worth up to $3.3 billion on the world market. The United States is responsible for about 5% of the world's hazelnut production (National Agricultural Statistics Service, 2019). The Willamette Valley in western Oregon provides an ideal growing climate in the US for hazelnut production with its mild winters and long growing season. The many family-owned farms in this region are responsible for producing 99% of the United States hazelnut crop. An unprecedented number of farming operations in the Willamette Valley have transitioned to hazelnut production as hazelnuts are recognized as a low input, high return crop. The total acreage of hazelnuts in Oregon has reached 78,603 with nearly 40,000 acres of that total being 1-5 years old plantings (Pacific Agricultural Survey LLC, 2019). The surge in acreage planted throughout the Willamette Valley has been made possible by the release of eastern filbert blight (EFB) resistant hazelnut cultivars from the Oregon State University Hazelnut Breeding program. While these new hazelnut cultivars have given new life to the Oregon hazelnut industry, hazelnuts are increasingly being planted in field sites not inherently suited for optimal hazelnut tree growth (Olsen, 2013a). Stress factors such as drought stress, standing water in high clay content soil, nutrition imbalance, sunscald and herbicide damage on top of the increased genetic diversity among the new hazelnut cultivars has left a gap in the knowledge on how to make the most informed management decisions to protect young orchards and set them on a trajectory for success. This lack of knowledge and the contributing stress factors has increased the incidence of susceptible young hazelnut trees to a whole host of damaging insects and other disease pressures. One of the biggest problems for orchard establishment is bacterial blight of hazelnuts.

Bacterial blight of hazelnuts has been an issue since hazelnuts were first planted in Oregon at the beginning of the twentieth century. The disease was first reported in 1913 as a new filbert (hazelnut) disease in Oregon in a preliminary report published by H. P. Barss (1915) of Oregon State College Botany and Plant

Pathology Department. Bacterial blight has since been described as present in nearly every hazelnut producing country in the world including: Turkey, Italy, Spain, France, Iran, Portugal, Netherlands, Poland, southern Russia, Serbia, Montenegro, Switzerland, United Kingdom, Algeria, Chile, and Australia (Victoria, Western Australia) (EPPO, 2004; Kazempour *et al.*, 2006; Locke and Barnes, 1979; Puławska *et al.*, 2010; Wimalajeewa and Washington, 1980).

Bacterial blight was the most important economic disease in the Oregon hazelnut industry until the accidental introduction of eastern filbert blight (EFB) caused by *Anisogramma anomala* (Peck.) to the Pacific Northwest in the early 1970s (Johnson *et al.*, 1996). No research has been conducted on bacterial blight in Oregon since that time and there is a renewed need for information on this disease. With the current surge of hazelnut plantings, orchards are more frequently planted on marginal sites where trees may experience more stress. It has been shown that stress conditions, particularly water stress, may predispose young hazelnut plantings to bacterial blight infection (Moore, 1974).

Bacterial blight is caused by the bacterium *Xanthomonas arboricola* pv. *corylina*. This pathogen is highly host specific and only affects *Corylus* spp. Bacterial blight infection can affect the leaves, twigs, sometimes the nuts and has the greatest impact when infections occur on the trunks of hazelnut trees. Disease symptoms can range from oily lesions that may go unnoticed to stem girdling cankers that may kill the trees outright or stunt tree growth and delay production (Lamichhane and Varvaro, 2014).

The first thorough characterization of the causal pathogen of bacterial blight, *Xanthomonas arboricola* pv. *corylina*, was conducted in 1940 when the bacterium was first proposed to be unique enough to be given a pathovar name (Miller *et al.*, 1940). Miller *et al.* (1940) would then go on to characterize the pathovar type-strain (pathotype) from a bacterial isolated from *Corylus maxima* in Oregon as the first representative strain for this pathovar. Since then, *Xanthomonas arboricola* pv. *corylina* has been characterized in multiple studies and included in the characterization of closely related *Xanthomonads* conducted

mainly in European countries (Karahan *et al.*, 2013; Belisario *et al.*, 1999; EPPO, 2004; Fargier *et al.*, 2011; Frutos, 2010; Gardan and Devaux, 1987b; Lamichhane and Varvaro, 2014; Lee *et al.*, 1992; Parkinson, 2007; Pisetta *et al.*, 2016; Puławska *et al.*, 2010; Scortichini *et al.*, 2002; Young *et al.*, 2008).

Many methods have been developed for characterizing *Xanthomonad* species utilizing phenotypic variations, biochemical attributes, and host pathogenicity evaluation (Dye *et al.*, 1980). The pathovar type-strain isolated from Oregon in the 1940s has routinely been included in these studies. However, it has been argued that this type strain is a poor representation of the pathovar due to its consistently weak pathogenicity (Puławska *et al.*, 2010; Scortichini *et al.*, 2002). Results at the molecular level revealed that the pathotype strain is representative of the pathovar *Xanthomonas arboricola* pv. *corylina* when molecular characterization technology such as polymerase chain reaction (PCR) and genetic sequencing analysis are used (Fischer-Le Saux *et al.*, 2015).

This incongruity among research results regarding the pathovar level representation of the Oregon isolated *Xac* type strain and the isolates originating from Europe call to question how other bacterial strains isolated from symptomatic tissue in Oregon hazelnuts compare to isolates previously described in these European studies. In this research, the characterization of these bacterial isolates was performed using phenotypic, biochemical and molecular characteristics. These attributes were used to definitively identify the Oregon bacterial strains and to compare the isolates found in the Oregon hazelnut growing region with those previously described in European growing regions.

Materials and Methods

Isolation of Strains and Initial Characterization

Symptomatic hazelnut tissue samples were submitted to the Oregon State University Plant Clinic (Corvallis, OR) from mid spring through the fall of 2017 by hazelnut growers, farm managers, and extension agents. Bacteria were recovered from symptomatic hazelnut tissues that included water-soaked angular spots and lesions on leaves, necrotic buds, necrotic woody tissue, stem cankers, or from bacterial exudate produced from stem lesions. Symptomatic tissue was surface-sterilized and blotted dry. Small pieces of tissue were excised with a sterile razor blade from the margin of healthy tissue and diseased tissue. These excised pieces were homogenized in 2 mL of phosphate buffer solution with a glass rod and vortexed for 10 seconds. The resulting homogenate was plated out in a dilution series on semi-selective GYCA media (yeast extract 5.0 g, D-glucose 10 g, $CaCO_3$ 30 g, agar 20 g, distilled water to 1 L with 50 M/mL cycloheximide) (EPPO, 2004; Gardan and Devaux, 1987; Prokić *et al.*, 2012). Colonies of *Xanthomonas arboricola* pv. *corylina* were differentiated from commonly occurring saprophytic bacteria with brilliant yellow glossy colony formation on this media. Colonies produce large amounts of mucoid polysaccharides (Xanthan gum) and become visible after approximately 72 hours of incubation at 27 ^{o}C. Selected colonies of isolated strains from each source were subjected to further identity screening (Prokić *et al.*, 2012; Puławska *et al.*, 2010; Vauterin *et al.*, 1995). Isolates were stored, backed up in a -80 ^{o}C freezer and cataloged in the lab naming system before undergoing further screening. Two known reference strains of *Xanthomonas arboricola* pv*. corylina*, isolates RIPEX09 and RIPEX23, were provided from the Research Institute of Pomology and Floriculture, Department of Plant Pathology, ul. Pomologiczna 18, Skierniewice, Poland.

Carbon Source Utilization

A Biolog Gn II microplate system (Biolog, Inc. Hayward, California) was used by the OSU Plant Clinic to the manufacturer's instructions in determining the carbon source utilization profiles of each bacterial isolate (Vauterin *et al.*,

1995). The output provided the measured carbon source utilization as a positive, negative, or intermediate reaction for the isolate's response to each compound. This information was compared to the Biolog Gn II database to obtain a genus level identification for each of the bacterial isolates. The output was also compared to previous carbon utilization results from studies that has previously characterized this pathogen.

Quinate Metabolism

A colony suspension was made for each isolate by transferring an aliquot of bacteria into 2 mL of phosphate buffer and vortexed for 15 seconds until homogeneous. The suspension was adjusted to an optical density ($O.D._{600}$) of 0.2. Succinate-quinate (SQ) medium was used for the biochemical reaction (10 g of succinic acid (disodium salt, hexahydrate), 5 g of quinic acid, 1.5 g of K_2HPO_4, 1.0 g of $(NH_4)_2SO_4$, 0.5 g of yeast extract, and 15 g of agar). The pH of the medium was adjusted to 7.2- 7.5 with 10 M NaOH and autoclaved for 20 minutes. After autoclaving, 7.5 mL of 0.2-micron filter sterilized 20% $MgSO_4$ was added (Lee *et al.*, 1992). PCR tubes were used to hold 100 μl of the media and the final reaction was contained within these tubes. Ten microliters of bacterial suspension from each isolate were added to the SQ medium filled tubes. The tubes were left ajar and placed at 27 ^oC. Isolate JL2602 had been tested in preliminary experiments so it was used as a positive control and *Pseudomonas syringae* isolate JL2455 was used as a negative control. Each isolate was evaluated with three replications. Diffusion of greenish-brown through the media was considered a positive reaction and was apparent after three days.

Copper Resistance Assay

In vitro copper resistance assays were performed on the *Xanthomonas* isolates to determine the efficiency of cupric ions at preventing the growth of the bacteria on culture media. A disease management program for one or two

applications of copper products in the late summer and mid fall is recommended for control of bacterial blight in hazelnut orchards (Miller *et al.*, 1949; Wiman *et al.*, 2019). The method for performing the copper resistance assay was adapted from Andersen *et al.* (1991) who developed the test for *Pseudomonas syringae* pv. *syringae* (Andersen *et al.*, 1991). Copper resistance assays included 13 isolates: JL2600, JL2602, JL2603, JL2604, JL2605, JL2606, JL2607, JL2609, JL2610, JL2611, JL2613, JL2614, JL2615. Solidified Casitone Yeast Extract medium (CYE) amended with copper was used in the resistance assay. Copper in the form of $CuSO_4$ was tested at four concentrations: 0, 0.16, 0.32, 0.8 mM. An aqueous bacterial suspension from each isolate was adjusted to 0.2 OD_{600} (10^8 cfu/ml). Each bacterial isolate was grown out on yeast nutrient agar media (YNA) and incubated overnight at 27 ^oC prior to adjusting the OD. Ten μl of each bacterial isolate suspension were pipetted on the copper incorporated plates in a grid pattern. *Pseudomonas syringae* pv. *syringae* strains JL2455 and JL2456 were used as control copper resistant and copper sensitive verification, respectively, at each concentration level. This copper resistance assay was repeated three times for each isolate. Each plate was examined daily for growth, which was assessed on a 0-5 scale based on the amount of colony development on the media.

Colony Cell Lysis Procedure

Colony cell lysis was done to prepare the bacterial isolates for DNA amplification during the polymerase chain reaction (PCR). A single aliquot of the bacteria was transferred into 2.5 mL of LB liquid broth culture medium. This liquid culture was grown overnight at 27 ^oC in a shaker-incubator. Members of the *Xanthomonas* spp. often produce large amounts of the mucoidal xanthan gum so this procedure was adjusted to a larger volume to clean the bacterial cells more efficiently. All centrifugation steps were done at 8000 rpm for 2 minutes. One mL of overnight broth cultures was placed in 1.5 mL tubes and centrifuged for 2 minutes at 8000 rpm. The supernatant was removed, and the pellet was re-

suspended in 1 mL of LB broth and vortexed. The suspension was spun down, and supernatant removed. The pellet was resuspended in 1 mL of LB broth and vortexed. Two hundred μl of the washed LB broth was transferred to a PCR tube. The PCR tube was centrifuged, and supernatant removed. Then 25 μl of lysis buffer solution was added to the bacterial pellet. Lysis buffer solution was made by combining 1 mL of sterile milli-Q water, 10 μl of 5M NaOH, and 25 μl of 10% SDS. Once the lysis buffer solution was added to the pellet, the suspension was boiled in a thermocycler at 100 ºC for 10 minutes. PCR tubes were removed from the thermocycler and diluted with 175 μl of sterile milli-Q water. This final product was used as a template during PCR reactions.

Genomic DNA Extraction

Genomic DNA extractions were performed using the DNeasy® Blood & Tissue Kit (Qiagen Sciences, Germantown, MD, USA) according to the manufacturer's instructions. Genomic DNA was used in every gene sequencing protocol. Briefly, a single bacterial colony from each isolate was suspended in 5 mL of LB broth. The liquid broth culture was incubated on a shaking table overnight at 27 ºC. One mL of the overnight broth solution was centrifuged and washed in LB broth. The washed pellet was then resuspended in 180 μl of ATL buffer, incubated for 30 minutes at 37 ºC and vortexed. Then 25 μl of proteinase K and 200 μl of AL buffer were added, vortexed and incubated for 30 minutes at 56 ºC. Four μl of RNase were added, vortexed and allowed to incubate for 2 minutes at room temperature. After 15 seconds of vortexing, 200 μl of ethanol was added and mixed thoroughly. The mixture was then pipetted into the DNeasy® columns and centrifuged at 8000 rpm for 1 minute, supernatant discarded. With new tubes, 500 μl of AW1 buffer was added to the columns and centrifuged, supernatant was discarded. With new tubes, 500 μl of AW2 buffer was added to the columns and centrifuged, supernatant discarded. 100 μl of AE

buffer was then added to the columns for elution, incubated for 5 minutes and centrifuged. This last step was repeated by replacing the flow through back on the column, incubating and centrifuging. The concentration and purity of the genomic DNA recovered was verified using a nanodrop and the resulting product was stored at -20 ^{o}C. Working stock tubes were calculated to contain 50 ng/μl of genomic DNA.

Duplex Polymerase Chain Reaction (PCR)

Duplex PCR was performed using the Qiagen Multiplex PCR kit (Qiagen Sciences, Germantown, MD, USA) according to manufacturer's instructions. Briefly, 3 μl of colony cell lysed bacterial isolate DNA templates was added to PCR tubes, each containing 22 μl of a PCR mixture composed of 12.5 μl of 2x Qiagen Multiplex PCR Master Mix, 2.5 μl of 10x primer mix, 2.5 μl of 5x Q-solution, and 4.5 μl of RNase-free sterile water. The primer mix was composed from prepared 10 μmol solutions of the primers *XapY17-F*, *XapY17-R*, *XarbQ-F*, and *XarbQ-R* (Table 2.1) (Pothier *et al.*, 2011). The primers *XarbQ-F*, and *XarbQ-R* serve as an indicator of *Xanthomonas arboricola* species level when the band has a specific length of 402 bp. The primers *XapY17-F* and *XapY17-R* serve as an indicator at the pathovar level of either pv. *pruni* or pv. *corylina* when a band has a length of 943 bp (Lamichhane and Varvaro, 2014; Pothier *et al.*, 2011; Puławska *et al.*, 2010). The 25 μl solution was placed in a thermocycler for the PCR. After an initial activation step for 15 minutes at 95 ^{o}C, the DNA was amplified for 35 cycles. Each cycle consisting of denaturation for 30 seconds at 94 ^{o}C, annealing for 90 seconds at 55 ^{o}C, and extension for 90 seconds at 72 ^{o}C. The final extension was for 10 minutes at 72 ^{o}C. A reference isolate was used as a positive control and *Pseudomonas syringae* pv. *syringae* isolate JL2455 was used as a negative. Three μl of PCR product, 2 μl of 6x dye, and 7 μl of sterile milli-Q water where mixed to produce 12 μl of PCR product that was

electrophoresed in a 1% agarose gel using 1x Tris-acetate-EDTA buffer stained with ethidium bromide.

16S rRNA PCR

All amplifications for 16S were carried out at the final concentration volume of 25 μl using ThermalAce DNA polymerase (Invitrogen Corporation, Carlsbad, CA) used according to manufacturer's instructions. Briefly, 1 μl of genomic DNA template at the concentration 50 ng/μl was added to PCR tubes, each containing 17.5 μl of sterile Milli-Q water, 2.5 μl of 10x ThermalAce Buffer, 2.5 μl of dNTP, 0.5 μl of forward primer *fD1*, 0.5 μl of reverse primer *rP2*, and 0.5 μl of ThermalAce DNA polymerase. The initial activation step in the thermocycler was set for 3 minutes at 98 ^{o}C, the reactants were amplified for 30 cycles. Each cycle consisting of denaturation for 30 seconds at 98 ^{o}C, annealing for 30 seconds at 54 ^{o}C, and extension for 90 seconds at 72 ^{o}C. The final extension step was for 10 minutes at 72 ^{o}C. The PCR product was then viewed using gel electrophoresis on a 1% agarose gel containing one drop of ethidium bromide. Three μl of PCR product were mixed with 2 μl of 6x dye and 7 μl of milli-Q water. The gel was run at 100V for 30 minutes. After 30 minutes the gel was viewed under UV light and the bands for 16S were expected in the 1500 bp range.

PCR and Sequencing of Housekeeping Genes

Amplifications of partial gene sequences of two protein-coding genes, *gyrB* (DNA gyrase subunit B) and *rpoD* (RNA polymerase sigma-70 factor), were sequenced to be concatenated and analyzed as previously described (Fargier *et al.*, 2011; Fischer-Le Saux *et al.*, 2015; Young *et al.*, 2008). The primer set used to amplify the *gyrB* housekeeping gene were *gyrB1F* and *gyrB1R*. The two primer sets used to sequence the *rpoD* housekeeping gene were *emirpo11F* and *emirpo13R* and *rpoDX-SoF4* and *rpoDX-SoR6* (Table 2.1). Primers were purchased through Sigma-Aldrich Co. LLC. All amplifications were carried out at

the final concentration volume 25 μl using ThermalAce DNA polymerase (Invitrogen Corporation, Carlsbad, CA) used according to manufacturer's instructions. Briefly, 1 μl of genomic DNA template was added to PCR tubes, each containing 17.5 μl of sterile Milli-Q water, 2.5 μl of 10x ThermalAce Buffer, 2.5 μl of dNTP, 0.5 μl of forward primer, 0.5 μl of reverse primer, and 0.5 μl of ThermalAce DNA polymerase. For the *gyrB* primers, after an initial activation step for 3 minutes at 98 ^{o}C, the DNA was amplified for 30 cycles. Each cycle consisting of denaturation for 30 seconds at 98 ^{o}C, annealing for 30 seconds at 54 ^{o}C, and extension for 60 seconds at 72 ^{o}C. The final extension step was for 10 minutes at 72 ^{o}C. For the *rpoD* primers, after an initial activation step for 3 minutes at 98 ^{o}C, the DNA was amplified for 30 cycles. Each cycle consisting of denaturation for 30 seconds at 98 ^{o}C, annealing for 90 seconds at 60 ^{o}C, and extension for 60 seconds at 72 ^{o}C. The final extension step was for 10 minutes at 72 ^{o}C.

Table 2.1. Nucleotide sequences of PCR primers and expected amplicon sizes used in the study.

Primer	Gene	Sequence (5'–3')	Amplicon (bp)	Consensus sequence used for trimming	Source
*Xap*Y17-F	*ftsX*	GACGTGGTGATCAGCGAGTCATTC	943		Pothier *et al.*, 2011
*Xap*Y17-R	*ftsX*	GACGTGGTGATGATGATCTGC			Pothier *et al.*, 2011
*Xarb*Q-F	*qumA*	GCGCGAGATCAATGCGACCTCGTC	402		Pothier *et al.*, 2011
*Xarb*Q-R	*qumA*	GGTGACCACATCGAACCGCGCA			Pothier *et al.*, 2011
*Xgyr*PCR2F	*gyrB*	AAGCAGGGCAAGAGCGAGCTGTA	700		Parkinson, N., 2007
*X.gyrrsp*1	*gyrB*	CAAGGTGCTGAAGATCTGGTC			Parkinson, N., 2007
X-gyrB1F	gyrB	ACGAGTACAACCCGGACAA	904	CACATCCGB	Young *et al.*, 2008
X-gyrB1R	gyrB	CCCATCARGGTGCTGAAGAT		GCCGARCAG	Young *et al.*, 2008
emirpo11F	*rpoD*	ATGGCCAACGAACGTCCTGC	1313	GAAATGGGY	Fargier *et al.*, 2011
emirpo13R	*rpoD*	AACTTGTAACCGCGACGGTATTCG		TTCATYCGY	Fargier *et al.*, 2011
rpoDX-SoF4	*rpoD*	GGAGCAGATCGAAGACATCATCAGC	951		Fischer-Le Saux *et al.*, 2015
rpoDX-SoR6	*rpoD*	CATCTCGATCGAGCCCTGC			Fischer-Le Saux *et al.*, 2015

Sequencing

The amplified PCR products from each of the positive PCR reactions were purified using ExoSAP-IT (Affymetrix Inc. Santa Clara, CA) according to the manufacturer's instructions. Briefly, a combined reaction volume of 23.5 μl was reached by adding 1.5 μl of ExoSAP-IT to each PCR product. The remaining primers and nucleotides were degraded from the PCR products during an incubation period at 37 ^{o}C for 2.5 hours. A further incubation step at 80 ^{o}C for 15 minutes was done to inactivate the ExoSAP-IT. After the completion of these incubation steps, the purified PCR products adjusted to the correct concentrations for DNA sequencing with forward and reverse primers added, respectively. Both strands of each PCR product for genes 16S rRNA, *rpoD* and *gyrB* were sequenced using Sanger Sequencing through the Oregon State University Center for Genome Research and Biocomputing Core facilities (Corvallis, OR).

Sequence Alignment and Phylogenetic Analysis

The forward and reverse nucleotide sequences were assembled, edited, aligned and trimmed using Geneious bioinformatics software 11.0.3 (Biomattters, Auckland, New Zealand). Each finished sequence was compared to the NCBI Genebank using nBlast for initial identity comparisons.

Further phylogenetic analysis was computed for individual gene sequences and for the concatenated data sets. The *gyrB* and *rpoD* sequences were concatenated in alphabetical order of the two genes making a sequence of 1,344 bp (bp 1 to 735 for *gyrB* and 736 to 1,344 for *rpoD*). Allele sequences for reference isolates of *Xanthomonas arboricola* were included in the analysis, in addition to 10 strains of *Xanthomonas arboricola* pv. *corylina* reference isolates, which were obtained from sequences deposited in the Plant Associated Microbes Database (PAMDB) (Fargier *et al.*, 2011; Fischer-Le Saux *et al.*, 2015; Young *et al.*, 2008).

The single gene sequences and the concatenated sequences were each aligned using MAFFT multiple sequence alignment software version 7 (Katoh *et al.*, 2002; Katoh and Standley, 2013) as a plug-in of Geneious 11.0.3. These aligned sequences were made into maximum-likelihood analysis trees which were obtained using PhyML 3.0 Phylogeny software (Guindon *et al.*, 2010) as a plug-in of Geneious 11.0.3. The trees were generated using the Kimura substitution model and 1,000 bootstrap replicates. These trees were rooted using single and concatenated sequences, respectively, of the *Xanthomonas campestris* pv. *campestris* pathotype strain ICMP 13. Further analysis was as previously described (Fischer-Le Saux *et al.*, 2015).

Inoculum Preparation

Inoculum suspensions were prepared from freeze-dried bacteria for 11 isolates: JL2600, JL2602, JL2603, JL2604, JL2605, JL2606, JL2607, JL2609, JL2613, JL2614, JL2615. The bacterial isolates were cultured for 5 days at 27 ^{o}C on GYCA. The bacteria were then scraped from the media surface with a spatula and mixed with powdered skim milk [38% (w/v)]. The bacterial suspension underwent lyophilization using FreeZone^{6} (Labconco Co. Kansas City, MO), was then ground to a fine powder, and stored at -80 ^{o}C (Rothleutner *et al.* 2014; Johnson *et al.*, 1993). The titer was calculated through dilution plating. The stock freeze-dried bacteria were calculated to be at a titer of 10^{12} colony forming units (CFU)/g. Ten milligrams of stock freeze-dried bacteria were re-suspended into 100 mL of sterile deionized water then diluted one more tenfold series to reach a concentration of 10^{8} colony forming units (CFU)/mL in a sterile phosphate buffer for inoculation. The titer of the freeze-dried inoculum was routinely verified and was consistent in all tests.

Hypersensitive response

The ability of the bacterial isolates to produce a hypersensitive response (HR) in the non-host plant *Nicotiana tabacum* L. was evaluated. In this assay, approximately 0.5 mL of a 10^8 CFU/mL bacterial suspension of each treatment was infiltrated into mesophyll through the lower epidermis of the leaves using a syringe without a needle. An adjacent leaf on the same plant was also selected to administer a negative water control. *Pseudomonas fluorescens* isolate A506 was included as a negative control. Replications on three different plants per isolate were peformed.

Inoculation of host plant

Pathogenicity of the *Xanthomonas* isolates was evaluated on *Corylus avellana* over two seasons following the procedure of Scortichini *et al.* (2002). The first eight bacterial strains to be collected in the 2017 field season (JL2600, JL2602, JL2603, JL2604, JL2605, JL2606, JL2607, JL2609) were evaluated for their ability to cause infection. This test was conducted under field conditions in a commercial orchard using an OSU breeding program numbered cultivar 'OSU 688.010', a sister of the cultivar 'Jefferson', which is a standard cultivar in the industry (Mehlenbacher *et al.*, 2011). At the time of inoculation in mid-October of 2017, the population of trees were in their sixth leaf year and had been harvested and were going into dormancy. One tree was designated for each of the isolates plus a tree for a negative sterile phosphate buffer control for a total of 9 trees for the 9 treatments. Two branch tips of one year old growth containing 10 buds on new growth were selected and labeled on each tree. A total of twenty buds for each treatment were then inoculated using a hypodermic syringe to inject 10 μl of a 10^8 CFU/mL bacterial suspension inoculum under the bud scales until runoff.

Additionally, during the 2017 fall inoculation, 20 separate one year old twigs were also selected and marked on each tree. An incision was made using a scalpel on each of the twigs and 10 μl of a 10^8 CFU/mL bacterial suspension of

each treatment was pipetted into the wounds. The incisions were monitored from mid-October throughout the dormant season.

Pathogenicity testing on *Corylus avellana* was repeated for a second year using isolates JL2600, JL2602, JL2603, JL2605, JL2606, JL2607 with the addition of isolates JL2613, JL2614, JL2615 which had been collected later in the 2017 season. These bacterial treatments plus a sterile phosphate buffer control were applied on a population of two year old potted 'Jefferson' trees in the first week of November in 2018. Each treatment was randomly assigned 4 trees from the population and 20 buds on each tree were injected with 10 μl of 10^8 CFU/mL for a total of 80 buds inoculated of each assigned treatment. The trees were maintained and monitored in a shade house at the North Willamette Research and Extension Center (Aurora, OR).

The inoculated buds from each replication were monitored throughout the dormant season. Symptoms first became apparent in mid-April as the buds began to burst and leaves expand. Both populations were evaluated in the first week of May in their respective years. Inoculated buds were rated as either affected or unaffected based on the presence of necrotic tissue and lesions on buds, petioles and emerging leaves. Five randomly selected buds from each treatment were collected at the time of evaluation to attempt re-isolation of bacteria. Results from these experiments were statistically analyzed using logistic regression models with the response variable representing the number of buds infected out of total number of buds inoculated for each treatment. The data were presented in bar graphs with error bars based on the binomial confidence intervals for the pathogenicity of each treatment. All statistical analyses and associated figures for the inoculation experiments were conducted in the open-source statistical environment R with R packages: multcomp, binom, ggplot2, dplyr (R Core Team, 2019). A two-way binomial generalized linear model (glm) was followed by Chi-squared test. When the Chi-squared test was significant a Dunnetts Contrast was used to analyze the 2017 inoculations compared to the

water control and Tukey's Contrasts were used to further analyze the 2018 inoculations.

Results

Isolation of Strains and Initial Characterization

Thick yellow colonies were easily distinguishable from other bacteria and yellow saprophytic bacteria isolated from symptomatic hazelnut tissue when plated on the GYCA media. There were 15 isolates: JL2600, JL2602, JL2603, JL2605, JL2606, JL2607, JL2609, JL2610, JL2611, JL2613, JL2614, JL2615, JL2616, JL2617 and JL2618 that produced this characteristic pigmentation and growth when plated on GYCA media. One isolate, JL2604, did not show the characteristic growth on the GYCA media. (Table 2.2).

Carbon Source Utilization

Biolog Gen II microplate system analysis showed what compounds each isolate could utilize as a potential carbon source. Results showed that 14 isolates: JL2600, Jl2602, JL2603, JL2605, JL2606, JL2607, JL2609, JL2610, JL2611, JL2613, JL2614, JL2615, JL2616, and JL2617 were able to utilize: tween40, N-acetyl-D-glucosamine, cellobiose, D-fructose, D-galactose, a-D-glucose, D-mannose, sucrose, trehalose, methyl pyruvate, mono-methyl succinate, cis-aconitic acid, a-keto glutaric acid, succinic acid, bromo succinic acid, and L- alanyl-glycine. The MicroLog database showed that each of these isolates belonged to the genus *Xanthomonas* based on these carbon source reactions*. Xanthomonas arboricola* pv. *corylina* is always able to utilize cellobiose, D-galactose a-D-glucose, D-mannose, sucrose and trehalose. *Xac* are not able to utilize the sources: adonitol, D-mannitol, L-rhamnose, D-sorbitol, l-

xylose, inulin, dulcitol, and erythritol. Further analysis of each isolate was needed for species and pathovar level identification.

Quinate Metabolism

Bacterial isolates that showed the diffusion of deep green as a positive reaction for quinate metabolism (Figure 2.1) included: positive control JL2602, JL2600, JL2603, JL2605, JL2606, JL2607, JL2610, JL2611, JL2613, JL2614, JL2615, JL2616, JL2617, and JL2618. Negative reaction isolates included*:* *Pseudomonas syringae* negative control, JL2455, JL2604, and JL2609 (Table 2.2).

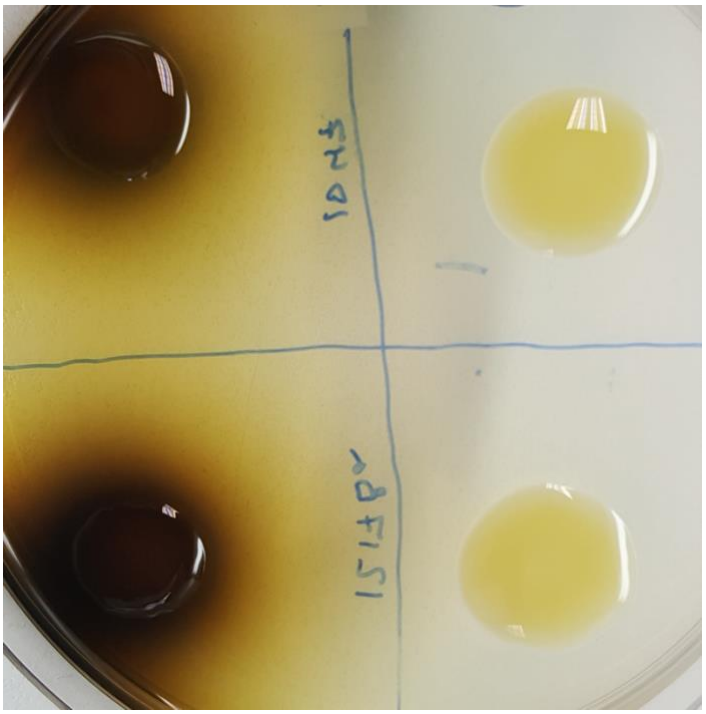

Figure 2.1. Quinate metabolism reaction.

A positive reaction for quinate metabolism shown by diffusion of a deep green pigment surrounding the growing bacterial mass.

Copper Resistance Assay

The control copper resistant isolate JL2456 showed a growth rating of 5 at all $CuSO_4$ concentration levels with concentration level 0.32 mM $CuSO_4$ or greater considered copper resistance. The control copper sensitive isolate JL2455 showed a growth scale rating of 5 on concentration 0 mM $CuSO_4$ and a rating of 2 at the 0.16 mM $CuSO_4$ but no growth on the 0.32 mM or 0.8 mM $CuSO_4$ plates (Table 2.2). Isolates JL2602, JL2603 JL2605, JL2607, JL2610, JL2611, and JL2613 showed a growth scale rating of 5 on concentration 0 mM $CuSO_4$ and a rating of 3 at the 0.16 mM $CuSO_4$ but had no growth at the resistance threshold. Isolates JL2600, JL2606, JL2614, and JL2615 showed a growth scale rating of 5 on concentration 0 mM $CuSO_4$, but no growth on any copper amended plates. Isolates JL2604 and JL2609 showed a growth rating of 5 at concentration levels of 0mM, 0.16 mM and resistance threshold 0.32 mM but showed no growth at the higher 0.8 mM level.

Duplex PCR

Out of the 16 isolates collected, 14 displayed strong bands in this duplex PCR reaction at both 402 bp and at 943 bp (JL2600, JL2602, JL2603, JL2605, JL2606, JL2607, JL2610, JL2611, JL2613, JL2614, JL2615, JL2616, JL2617, and JL2618) and two isolates were negative for both bands (JL2604 and JL2609) (Table 2.2). *Xac* reference isolates RIPEX23 and RIPEX09 included as positive controls both exhibited strong bands at 402 bp and 943 bp. *Pseudomonas syringae* isolate JL2455 was used as the negative control which showed no amplification at either band. The presence of the 402 bp band indicates the presence of the *QumA* gene and the ability to metabolized quinate media while also placing those isolates at the *Xanthomonas arboricola* species level. The presence of the additional band at 943 bp with the band at 402 bp suggests that the identity of those 14 isolates are either *X. a*. pv. *corylina or pruni*.

16S rRNA Sequencing

The 16S rRNA gene sequences provided very little discrimination for verifying the identity of the *Xanthomonas* isolates suspected to be in the pathovar *corylina*. The comparison of all the sequences available on GenBank showed that isolate JL2604 belonged to the genus *Sphingamonas* sp., a common non-pathogenic yellow saprophytic bacterium, while JL2609 was *Xanthomonas campestris.*

Multi-locus Sequence Analysis

When the single *rpoD* sequences were aligned and assembled into a phylogenetic tree with *Xac* reference isolates, there was very little separation due to a general homogeneity among the sequences. There were only a few single nucleotide polymorphisms (SNPs) within the sequence which caused some weak separation in the phylogenetic tree (Figure 2.2). With a bootstrap of 61.8 isolate JL2606 and reference isolate 1484 were separated from the main group and with a bootstrap of 64.7 isolates JL2616 and JL2617 were also separated from the main group. However, when these single *rpoD* sequences were aligned and assembled into a tree with single *rpoD* sequences from outgroups of pathovars *pruni* and *juglandis* there was clear separation of the three pathovars but no separation within the pathovars themselves (Figure 2.3).

When the single *gyrB* sequences were aligned and assembled into a tree with *Xac* reference isolates there was much clearer segregation of the phylogenetic clades than seen using the single *rpoD* (Figure 2.4). SNPs in the alignment revealed two distinct phylogenetic clades. One clade with a bootstrap of 99.7 included the pathotype strain along with isolates from France and Poland and isolate JL2607. This clade had a subgroup with a bootstrap of 62.3 that consisted of isolates only found in Oregon (isolates JL2602, JL2603, JL2605, JL2610, JL2611, JL2613, JL2616, JL2617, and JL2618) showing that there is slight variation in these housekeeping DNA sequences between the Oregon and European isolates. The other distinct clade had previously been described in

France (Fischer-Le Saux *et al.*, 2015) and had a bootstrap value of 99.8. This group contained 4 isolates from Europe and 4 isolates from Oregon (JL2600, JL2606, JL2614, JL2615).

When the single *gyrB* sequences were aligned and assembled into a tree with single *gyrB* sequences of outgroups pv. *pruni* and pv. *juglandis*, the separation of the pathovars was not as evident (Figure 2.5). The *Xac* clade with the pathotype strain was distinct from the other pathovars, but the second clade was mixed with strains of pv. *juglandis* and pv. *pruni*. Thus, the *gyrB* sequence alone will not discriminate the pv. *corylina* isolates of each clade from the other pathovars.

When the *gyrB* and *rpoD* sequences were concatenated and the sequences were aligned and assembled into a phylogenetic tree there was the clearest separation of the two phylogenetic clades within the *corylina* pathovar (Figure 2.6). When these concatenated sequences were aligned and assembled with outgroups of pv. *juglandis* and pv. *pruni* there was a clear distinction between groupings of all three pathovars (Figure 2.7). The *Xac* clade with the pathotype strain was separated with a bootstrap value of 99.6. The second phylogenetic clade was separated with a bootstrap of 67.4.

Hypersensitive Response

The hypersensitive response of the *Xanthomonas* isolates was tested on *Nicotiana tabacum.* A positive response began when the area which was infiltrated turned from chlorotic after 3 days to necrotic after 7 days. The control and the negative control *Pseudomonas fluorescens* A506 infiltrations did not induce symptoms on any replicate. Isolates JL2600, JL2602, JL2603, JL2605, JL2606, JL2607, JL2613, JL2614, and JL2615 showed positive reactions indicating bacterial plant pathogenesis. Isolates JL2604 and JL2609 did not induce any pathogenic response.

Field Bud Inoculations

Symptoms from the fall 2017 field bud inoculations became apparent by mid-April 2018 as the buds began to swell and break. By late April and early May, infected buds were showing necrotic symptoms and the buds that were not affected continued to develop (Figure 2.8). There were no symptoms noted among the buds subjected to the control treatment, and the buds continued to develop normally into shoots throughout the growing season. There was a significant difference between the isolates and the water control ($\chi^2_{8,0}$ =0, p < 0.001). Isolates JL2604 and JL2609 were later identified to be *Sphingomonas sp*. and *Xanthomonas campestris*, respectively, and were not pathogenic on hazelnut. Isolates JL2602 and JL2603 had the lowest percentage of infected buds with 13 infected out of the buds 20 inoculated (65%). Isolate JL2605 had a very similar percentage with 12 infected out of the 18 buds inoculated (66%). Isolates JL2606 and JL2607 each had 15 infected out of the 20 buds inoculated (75%). Isolate JL2600 had the highest proportion of infected buds with 19 out of the 20 buds inoculated (95%). Using Dunnetts contrast there was found to be a significant difference between the controls compared to isolate JL2600.

The symptoms from the fall 2017 twig incision inoculation wounds healed over similarly to the control incisions leaving no lesions that could be measured. Under the conditions tested the incision inoculations showed no discrimination among the treatments. *Xac* was recovered from the inoculation points at a lower concentration (10^4 CFU/ incision) than was initially applied.

Symptoms from the fall 2018 potted tree bud inoculation became apparent in mid-April of 2019 as the buds were breaking and the leaves were beginning to expand. Symptom development occurred rapidly, over the course of about 3 weeks, from the time buds first began to show signs of infection to when there were no more new infections among the inoculated buds. The final bud evaluation was completed on May 6, 2019. There were no symptoms seen in the water control buds and the shoots developed normally with only a few tattered holes in the leaves from where the syringe pierced the developing leaves inside

the dormant buds during inoculation (Figure 2.9). Each of the *Xac* isolates induced significantly more infection than the water control ($\chi^2_{9,30}$ =43.7, p < 0.001). Isolate JL2613 induced the greatest percentage of symptomatic buds with 67 out of the 78 buds inoculated (85.8%) but was hardly significantly different than the percentage of infection caused by the isolates that caused the lowest amount of infection, isolates JL2607 and JL2605 (53.7%; p = 0.05 and 56.7%; p < 0.09, respectively) The other 6 isolates all produced similar proportions of infection with the median infection inducing isolate being JL2600 with 54 out of the 80 buds inoculated showing infection (67.5%).

The symptoms observed in these pathogenicity tests were consistent with bacterial blight disease symptoms observed in the field during early spring. These trials over the course of the two seasons were both in agreement with each other on the pathogenicity of the isolates tested based on the proportion of infected buds. Bud samples were collected from each treatment in both years with consistent results. No *Xanthomonas arboricola* pv. *corylina* was recovered from the asymptomatic controls, while *Xanthomonas arboricola* pv. *corylina* were recovered from the infected buds at a rate of 10^9 CFU/bud. The isolates collected were verified to be the originally inoculated isolates by repeating the methods used to initially characterize the isolates, thus fulfilling Koch's postulate.

Table 2.2. Characteristics of the isolates studied.

Isolate	Host	ID	Growth on GYCA	Quinate metabolism	Copper resistance	HR on tobacco	Duplex PCR	Hazelnut pathogenicity	MLSA *Xac*. I.D.
JL2600	*Corylus avellana* 'Dorris'	*Xac*	+	+	-	+	+	+	+
JL2602	*Corylus avellana* 'McDonald'	*Xac*	+	+	-	+	+	+	+
JL2603	*Corylus avellana* 'Wepster'	*Xac*	+	+	-	+	+	+	+
JL2604	*Corylus avellana* 'Wepster'	*Sphingomonas* spp.	-	-	+	-	-	-	-
JL2605	*Corylus avellana* 'Wepster'	*Xac*	+	+	-	+	+	+	+
JL2606	*Corylus avellana* 'McDonald'	*Xac*	+	+	-	+	+	+	+
JL2607	*Corylus avellana* 'Ennis'	*Xac*	+	+	-	+	+	+	+
JL2609	*Corylus avellana* 'Jefferson'	*X. campestris*	+	-	+	-	-	-	-
JL2610	*Corylus avellana* 'Wepster'	*Xac*	+	+	-	nt	+	nt	+
JL2611	*Corylus avellana* 'Wepster'	*Xac*	+	+	-	nt	+	nt	+
JL2613	*Corylus avellana* 'Jefferson'	*Xac*	+	+	-	+	+	+	+
JL2614	*Corylus avellana* 'Jefferson'	*Xac*	+	+	-	+	+	+	+
JL2615	*Corylus avellana* 'Jefferson'	*Xac*	+	+	-	+	+	+	+
JL2616	*Corylus avellana* 'Jefferson'	*Xac*	+	+	nt	nt	+	nt	+
JL2617	*Corylus avellana* 'Jefferson'	*Xac*	+	+	nt	nt	+	nt	+
JL2618	*Corylus avellana* 'Jefferson'	*Xac*	+	+	nt	nt	+	nt	+
A506	Negative control	*P. fluorescens*	nt	-	nt	-	nt	-	nt
JL2455	Copper negative control	*P. syringae* pv. *syringae*	nt	-	-	nt	-	nt	nt
JL2456	Copper positive control	*P. syringae* pv. *syringae*	nt	-	+	nt	nt	nt	nt

Table presenting the results of the characteristic evaluated for each of the bacterial strains used in the study, nt= not tested.

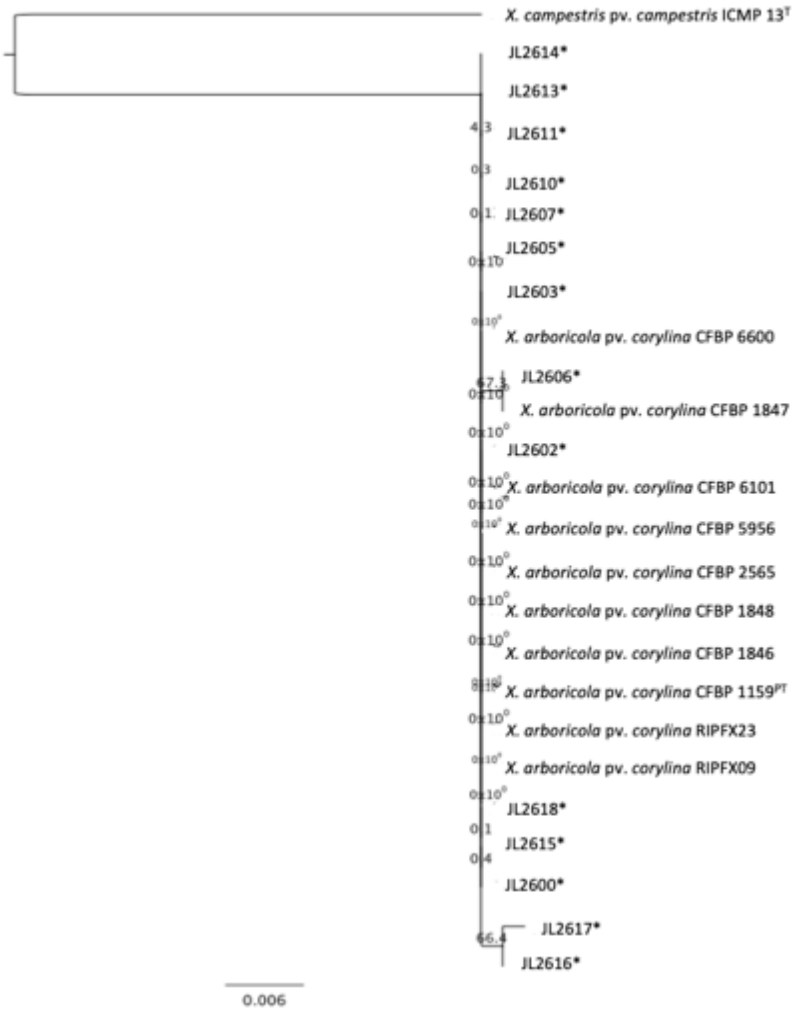

Figure 2.2. *rpoD* single sequence *Xanthomonas arboricola* pv. *corylina* with reference isolates.

*Sequences were aligned using MAFFT and the tree assembled using PhyML. Tree rooted with Xanthomonas campestris pv. campestris (Xcc) type strain ICMP13. Isolates identified in this study denoted with *, pathotype strain denoted with ^{PT}, type strain denoted with ^{T}.*

Figure 2.3. *rpoD* single sequence phylogenetic tree comparing pv. *juglandis* (Xaj), pv. *pruni* (Xap), and pv. *corylina* (*Xac*) isolates.

*Shows a distinct clade of pv. corylina from the other two pathovars but no resolution within itself. Sequences were aligned using MAFFT and tree assembled using PhyML. Tree rooted with Xanthomonas campestris pv. campestris (Xcc) type strain ICMP13. Isolates identified in this study denoted with *, pathotype strain denoted with ^{PT}, type strain denoted with ^{T}.*

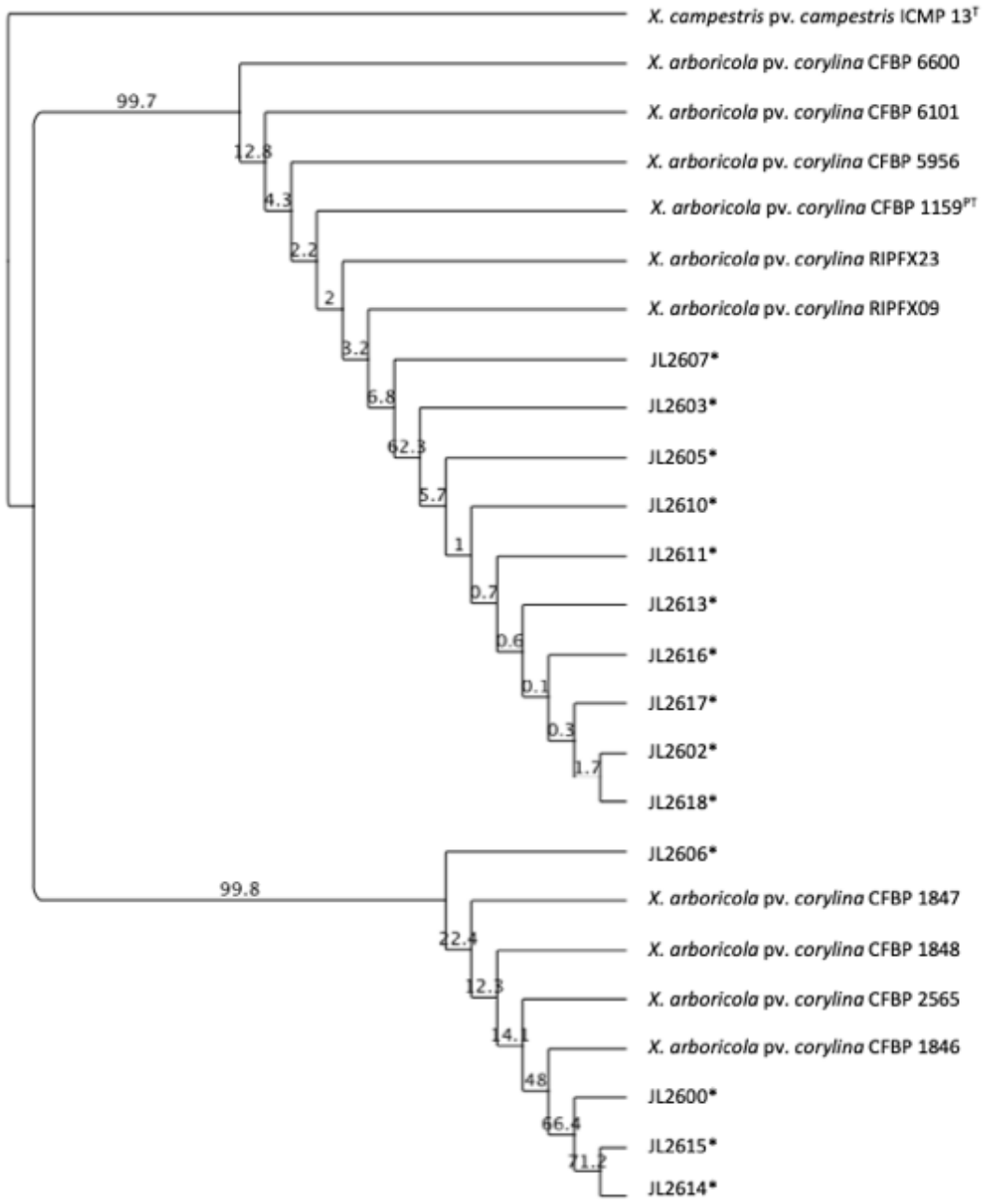

Figure 2.4. Single *gyrB* sequence *Xanthomonas arboricola* pv. *corylina* with reference isolates.

*Sequences were aligned using MAFFT and the tree assembled using PhyML. Tree rooted with Xanthomonas campestris pv. campestris (Xcc) type strain ICMP13. Isolates identified in this study denoted with *, pathotype strain denoted with ^{PT}, type strain denoted with ^{T}.*

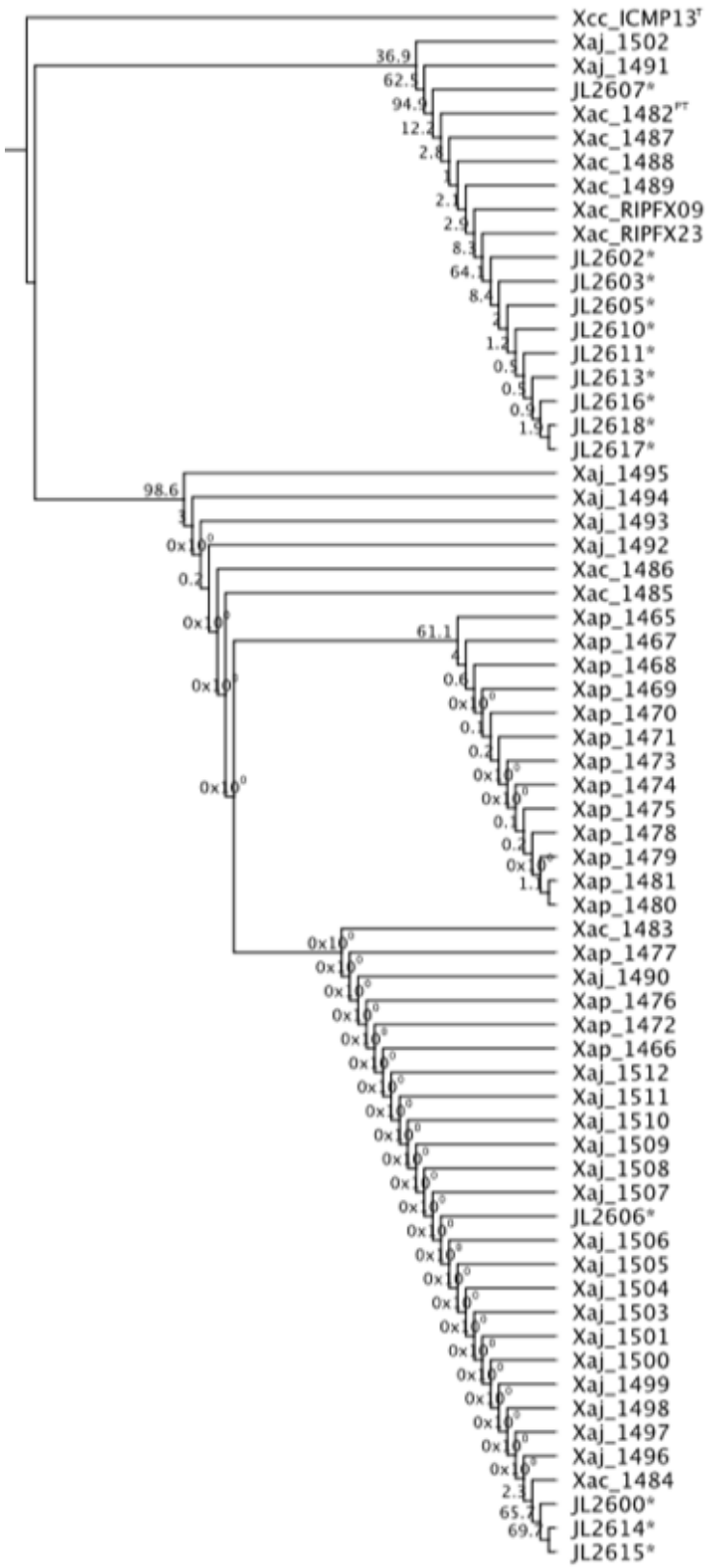
Xcc_ICMP13T
Xaj_1502
Xaj_1491
JL2607*
Xac_1482PT
Xac_1487
Xac_1488
Xac_1489
Xac_RIPFX09
Xac_RIPFX23
JL2602*
JL2603*
JL2605*
JL2610*
JL2611*
JL2613*
JL2616*
JL2618*
JL2617*
Xaj_1495
Xaj_1494
Xaj_1493
Xaj_1492
Xac_1486
Xac_1485
Xap_1465
Xap_1467
Xap_1468
Xap_1469
Xap_1470
Xap_1471
Xap_1473
Xap_1474
Xap_1475
Xap_1478
Xap_1479
Xap_1481
Xap_1480
Xac_1483
Xap_1477
Xaj_1490
Xap_1476
Xap_1472
Xap_1466
Xaj_1512
Xaj_1511
Xaj_1510
Xaj_1509
Xaj_1508
Xaj_1507
JL2606*
Xaj_1506
Xaj_1505
Xaj_1504
Xaj_1503
Xaj_1501
Xaj_1500
Xaj_1499
Xaj_1498
Xaj_1497
Xaj_1496
Xac_1484
JL2600*
JL2614*
JL2615*

Figure 2.5. Single gene *gyrB* with pv. *juglandis* (Xaj), pv. *pruni* (Xap), and pv. *corylina* (*Xac*).

*Concatenated sequences aligned using MAFFT and tree constructed using PhyML. Tree rooted with Xanthomonas campestris pv. campestris (Xcc) type strain ICMP13. Isolates identified in this study denoted with *, pathotype strain denoted with ^{PT}, type strain denoted with ^{T}.*

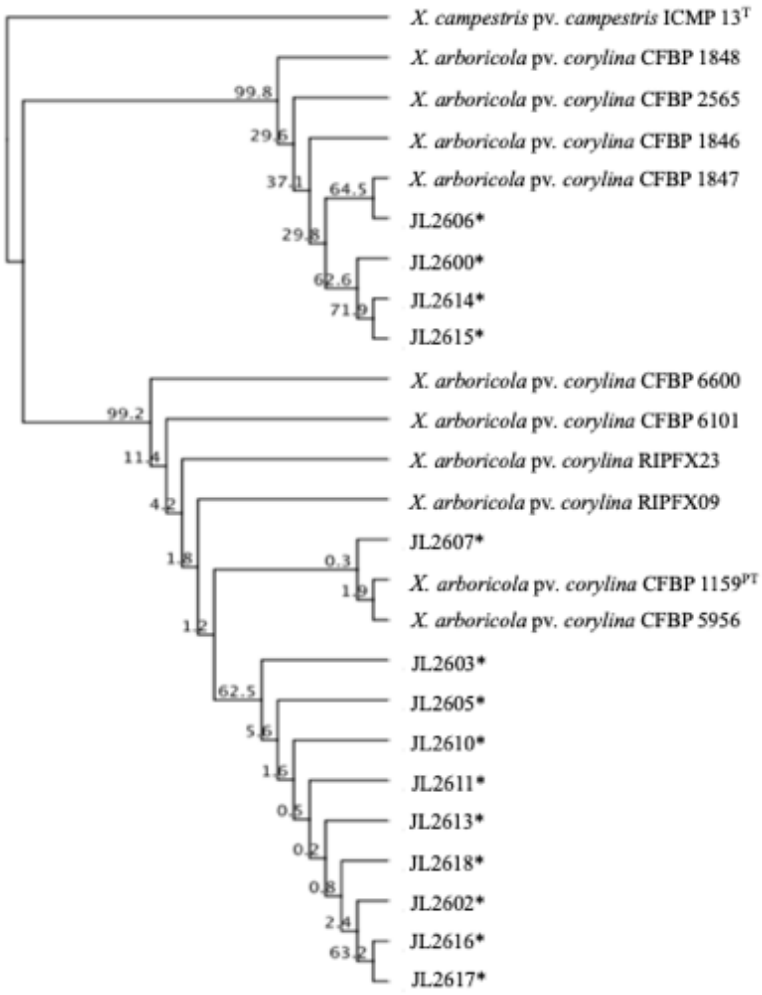

Figure 2.6. *Xanthomonas arboricola* pv. *corylina rpoD* and *gyrB* concatenated sequences.

*Sequences aligned using MAFFT and tree constructed using PhyML. Tree rooted with Xanthomonas campestris pv. campestris pathotype strain ICMP13. Isolates identified in this study denoted with *, pathotype strain denoted with ^{PT}, type strain denoted with ^{T}.*

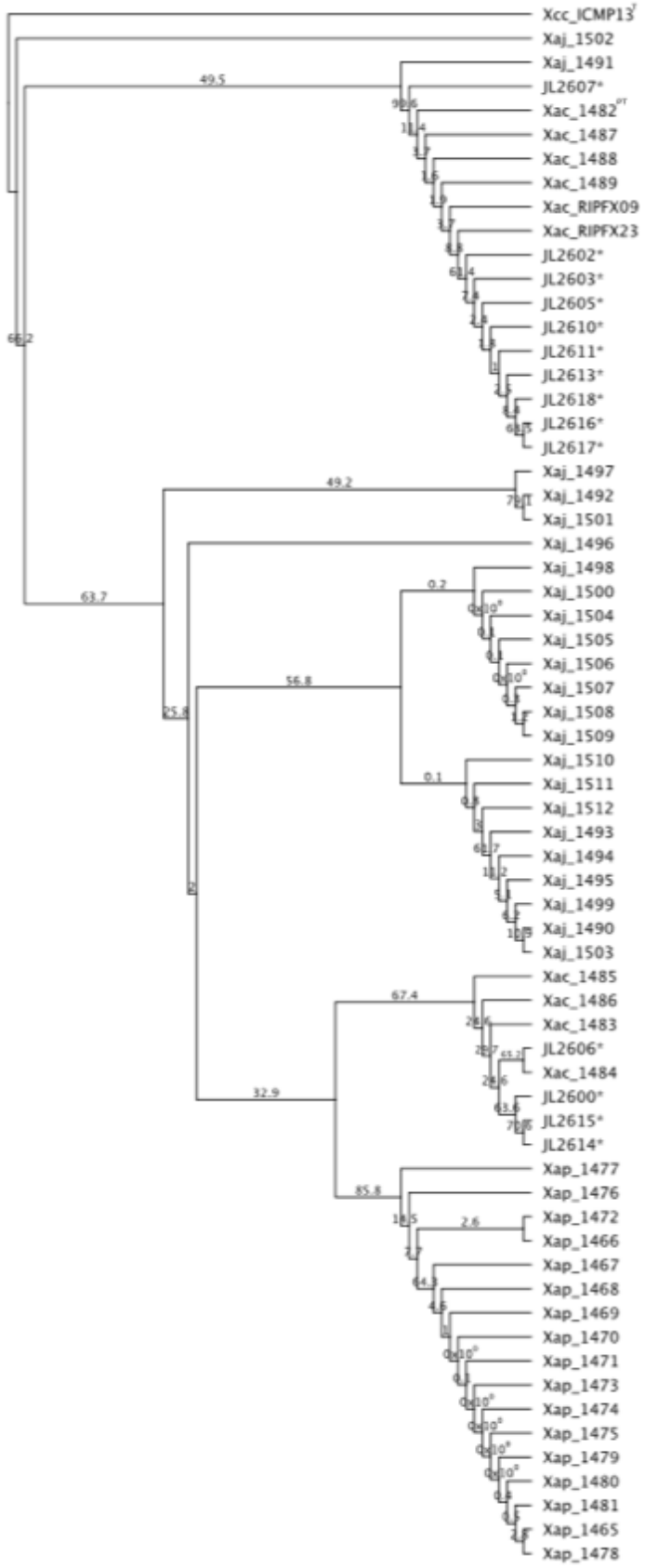
Xcc_ICMP13^T
Xaj_1502
Xaj_1491
JL2607*
Xac_1482^PT
Xac_1487
Xac_1488
Xac_1489
Xac_RIPFX09
Xac_RIPFX23
JL2602*
JL2603*
JL2605*
JL2610*
JL2611*
JL2613*
JL2618*
JL2616*
JL2617*
Xaj_1497
Xaj_1492
Xaj_1501
Xaj_1496
Xaj_1498
Xaj_1500
Xaj_1504
Xaj_1505
Xaj_1506
Xaj_1507
Xaj_1508
Xaj_1509
Xaj_1510
Xaj_1511
Xaj_1512
Xaj_1493
Xaj_1494
Xaj_1495
Xaj_1499
Xaj_1490
Xaj_1503
Xac_1485
Xac_1486
Xac_1483
JL2606*
Xac_1484
JL2600*
JL2615*
JL2614*
Xap_1477
Xap_1476
Xap_1472
Xap_1466
Xap_1467
Xap_1468
Xap_1469
Xap_1470
Xap_1471
Xap_1473
Xap_1474
Xap_1475
Xap_1479
Xap_1480
Xap_1481
Xap_1465
Xap_1478
49.5
49.2
63.7
56.8
25.8
0.2
0.1
67.4
32.9
85.8
2.6

Figure 2.7. *Xanthomonas arboricola* pathovars *corylina* (*Xac*), *juglandis* (Xaj), and *pruni* (Xap) *gyrB* and *rpoD* concatenated sequences.

*Sequences aligned using MAFFT and phylogenetic tree made using PhyML rooted with Xanthomonas campestris pv. campestris pathotype strain ICMP13. Isolates identified in this study denoted with *, pathotype strain denoted with ^{PT}, type strain denoted with ^{T}.*

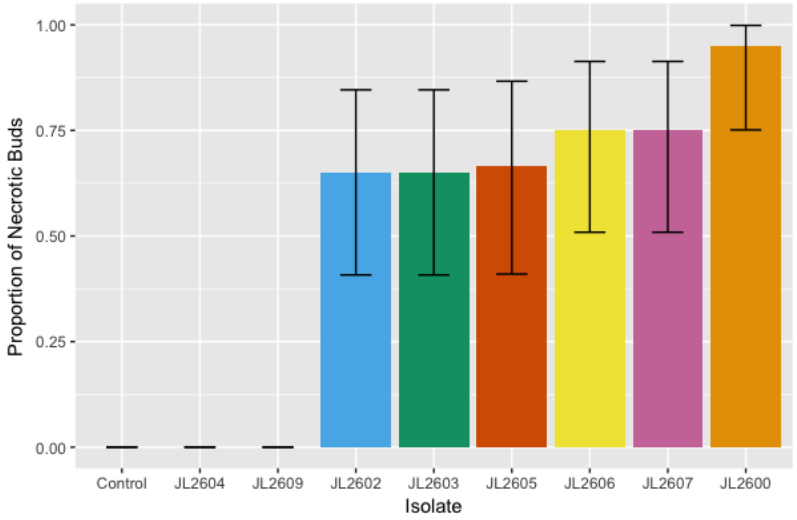

Figure 2.8. Evaluation of isolate pathogenicity on field hazelnut trees (2018)

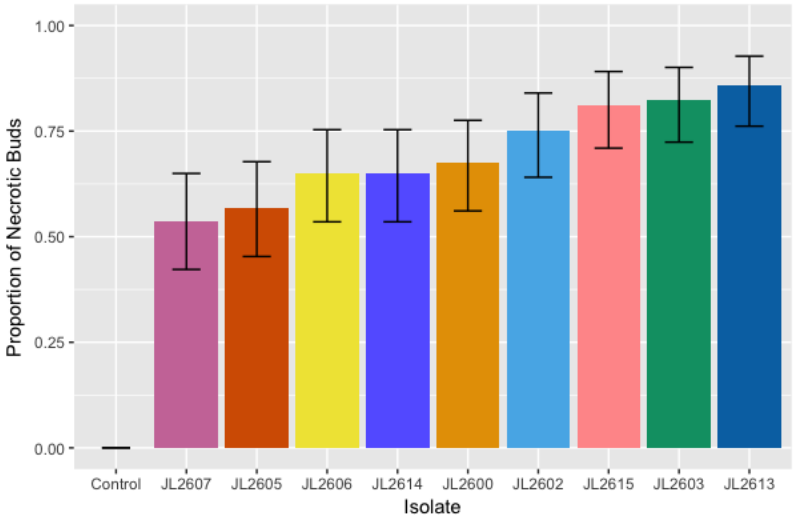

Figure 2.9. Evaluation of isolate pathogenicity on potted hazelnut trees (2019)

Discussion

Bacterial blight of hazelnut is a re-emerging disease in young orchards in Oregon. The current recommendation for management of bacterial blight of hazelnuts in Oregon is one or two applications of copper products in the late fall and early spring (Miller *et al.*, 1949; Wiman *et al.*, 2019). We isolated and characterized the causal agent *Xanthomonas arboricola* pv. *corylina* from hazelnut orchards in Oregon to examine its phylogenetic, pathogenicity, and sensitivity to copper. We found that the isolates from Oregon hazelnuts represent the known phylogenetic diversity of the pathovar and are sensitive to copper.

For many years, taxonomy of species within the genus *Xanthomonas* was determined based solely on phenetic approaches with the most important quality being host specificity (Dye *et al.*, 1980; Miller *et al.*, 1940; Vauterin *et al.*, 1995). This resulted in hundreds of species that encompassed the *Xanthomonas* genus, but these have since been grouped into different pathovars (Fischer-Le Saux *et al.*, 2015; Kałużna *et al.*, 2014; Vauterin *et al.*, 1995). The term *pathovar* is used to refer to a strain or sets of strains with the same or similar characteristics that are differentiated on the basis of pathogenicity to specific plant hosts (Dye, 1962; Dye *et al.*, 1980). Proper classification for strain designation is vital to avoid strains being given separate pathovar designations when they are actually phylogenetically identical, or given the same name when they belong to phylogenetically distinct groups (Almeida *et al.*, 2010). Ideally, the classification of pathovars within a given bacterial species should utilize phenotypic, chemotaxonomic, and genotypic approaches with the classification system being stable and converging to a reasonable degree (Vauterin *et al.*, 1995).

The causal agent behind bacterial blight of hazelnuts, *Xanthomonas arboricola* pv*. corylina*, is one of three pathovars within the *X. arboricola* species that frequently cause severe economic impacts in fruit and nut industries all over the world (Fischer-Le Saux *et al.*, 2015). The other significant disease inducing pathovars include *Xanthomonas arboricola pv. pruni* and *Xanthomonas arboricola pv. juglandis*, casual pathogens of bacterial spot in stone fruits and

walnut blight respectively (Boudon *et al.*, 2005; Fischer-Le Saux *et al.*, 2015; Kałużna *et al.*, 2014). There are striking similarities phenotypically as well as phylogenetically within these three pathovars that require a high level of resolution to accurately characterize and identify (Fischer-Le Saux *et al.*, 2015; Kałużna *et al.*, 2014). In the late 1930s, Miller *et al.* did a full investigation on the morphological and cultural characteristics of the pathogen *Xanthomonas arboricola* pv. *corylina* compared with pv. *juglandis* (Miller *et al.*, 1940, 1949). It was found that each of these pathovars would not infect the opposite host to any major effect, thus the ability to cause disease on a host was the main criteria used to recognize these two distinct pathogens.

Recent studies have demonstrated that the first isolate to be characterized, the pathotype strain, for the pathovar *corylina* is today weakly pathogenic on hazelnut. This consistent avirulence observation has led some to suggest that it is no longer representative of the pathovar (Belisario *et al.*, 1999; Lamichhane and Varvaro, 2014; Puławska *et al.*, 2010; Scortichini *et al.*, 2002). Other researchers have shown that the pathovar strain consistently displays characteristic phenotypic, biochemical and molecular characteristics that are representative of the strain and even with inconsistent pathogenicity that the presence of these other qualities warrants that it remains the pathotype strain (Fischer-Le Saux *et al.*, 2015).

Measuring the metabolic activity on a range of carbon sources and compounds as genus and species differentiating biochemical identification assays through tools such as the Biolog GnII system began as one of the more accurate biochemical assessments for pathogen characterization (Vauterin *et al.*, 1995). The Biolog GnII analysis showed that the hazelnut isolates were in the genus *Xanthomonas*, however further characterization was needed to narrow down the species and pathovar of the bacteria being examined.

To supplement this carbon source utilization profile, an assay specifically for Xanthomonads in the species *arboricola* that accesses quinate metabolism in each isolate was available. This served as a detection method of *Xanthomonas*

DNA homology group 6 which includes *Xanthomonas arboricola* pv*. celebensis*, *Xanthomonas arboricola* pv*. corylina*, *Xanthomonas arboricola* pv*. juglandis,* and *Xanthomonas arboricola* pv*. pruni* (Lee *et al.*, 1992). The quinate metabolism assay was done in conjunction with a multi-plex PCR assay that was developed to detect the genes *qumA* and *ftsX*. The presence of the gene *qumA* with a band at 402 bp is associated with quinate metabolism and is an indicator of the bacterial identity being *X. arboricola*. The additional presence of the gene *ftsX* with a band at 943 bp has been found to suggest the identity being either *X. arboricola* pv. *pruni* or pv. *corylina* (Lamichhane and Varvaro, 2014; Pothier *et al.*, 2011; Prokić *et al.*, 2012; Puławska *et al.*, 2010).

The standard in molecular methods for bacterial phylogenetic analysis has shifted towards multi-locus sequence analysis (MLSA) as the technology for genetic sequencing has become more cost effective (Almeida *et al.*, 2010). MLSA is done by sequencing sets of genes known as housekeeping genes. Housekeeping genes encode essential metabolic cellular functions and they are shared by all members of a species (Maiden *et al.*, 1998). Mutations and polymorphisms within the housekeeping genes are assumed to be selectively neutral and may reflect the phylogeny of the strains when analyzed against one another and outgroups (Fargier *et al.*, 2011). The concatenated sequences of the protein-coding genes can be compared for their relatedness through phylogenetic trees and analysis (Pérez-Losada *et al.*, 2013). MLSA allows for an easy, robust, and reproducible approach to the identification of pathogens that have diverging traits and species boundaries that are not well known as well as improving the descriptions their genetic population insights (Almeida *et al.*, 2010; Fischer-Le Saux *et al.*, 2015; Garita-Cambronero *et al.*, 2016; Maiden *et al.*, 1998).

The use of single locus housekeeping genes, such as *gyrB* or *rpoD,* as a cost effective scheme have been used to identify pathovars of *Xanthomonas arboricola* (Parkinson, 2007; Parkinson and Elphinstone, 2010; Prokić *et al.*, 2012; Puławska *et al.*, 2010). The housekeeping partial gene sequence of *gyrB*

encodes the subunit B protein of DNA gyrase. The single *gyrB* gene sequence reveals diverging alleles that form the two phylogenetic clades when isolates of pv. *corylina* are compared with other members of the pathovar. The second phylogenetic clade found in the pathovar *corylina gyrB* sequence is identical to several of the isolates of pathovars *juglandis* and *pruni*. Divergent alleles and inter- and intra- species recombination have been reported among these three pathovars making the use single gene sequences of *gyrB*, *atpD* or *rpoD* not as accurate for pathovar discrimination (Cesbron *et al.*, 2015; Fischer-Le Saux *et al.*, 2015; Kałużna *et al.*, 2014). The analysis of multiple concatenated genes provide a buffer against the distorting effects of recombination at a single locus resulting in the necessary resolution needed (Gevers *et al.*, 2005).

The housekeeping partial gene sequence of *rpoD* encodes for the RNA polymerase sigma 70 factor. Sigma factors are initiation factors that promote the attachment of RNA polymerase to specific initiation sites and are then released (Maciąg *et al.*, 2011). The *rpoD* sequence has been shown to produce a high haplotype diversity measure that can increase the discrimination between isolates (Fischer-Le Saux *et al.*, 2015). The use of *gyrB* and *rpoD* concatenated housekeeping gene sequences as a MLSA scheme provides enough resolution to determine the phylogenetic relationship within the *Xanthomonas arboricola* pv. *corylina*. The International Center for Microbial Resources-French Collection for Plant-associated Bacteria (CIRM-CFBP) recommends both *gyrB* and *rpoD* as authentication resources to identify the genus *Xanthomonas* at the species and pathovar level (Fischer-Le Saux *et al.*, 2015). Both were used in this study.

MSLA information databases, such as the Plant Associated Microbe Database (PAMDB), have been used as a platform where concatenated sequences can be uploaded, peer reviewed, and cited. Resources like PAMDB allow for collaboration and comparison of genetic material from all over the world. Most comparative phylogenetic studies to date investigating *Xanthomonas arboricola* pv. *corylina* have been in European countries using isolates obtained from those regions along with the pathovar type strain, NCPPB 935^{T}, which was

isolated in 1939 from *Corylus avellana* in Oregon (Miller *et al.*, 1949). Through PAMDB, 8 additional reference sequences of pv. *corylina* were included in this study. These reference sequences came from isolates originally collected by Gardan and Devaux (1987) with 5 isolates collected from France, 1 isolate collected from the United Kingdom and 1 isolate collected from Algeria. An additional 2 European reference isolates were included in the study that were collected and provided by Puławska *et al.* (2010) from Poland. The sequences of the pathotype strain were also obtained from this database from sequences uploaded by Fischer-Le Saux *et al.* (2015), Miller *et al.* (1940), and Young *et al.* (2008).

Puławska *et al.* (2010) first described *Xanthomonas arboricola* pv. *corylina* in Poland by defining the relationship between isolates collected in different years using the aligned sequence of *gyrB* following the work of Parkinson (2007) and the genetic fingerprints constructed on the basis of combined data of ERIC-, REP- and BOX-PCR used by Scortichini *et al.* (2002) (Parkinson, 2007; Puławska *et al.*, 2010; Scortichini *et al.*, 2002). The isolates formed two groups with the pathotype strain from Oregon producing the most distinctive fingerprint (Puławska *et al.*, 2010). Fischer-Le Saux (2015) similarly found that using a reduced MLSA scheme (two concatenated genes) that eight strains of pv. *corylina* were split into two distant groups (Fischer-Le Saux *et al.*, 2015). The groups were identified as two closely related subgroups forming a monophyletic clade in the complete MLSA scheme (seven concatenated genes). In each of these studies one group contained the pathotype strain and the other were all strains collected in Europe. It was recommended that in further studies at least two reference strains, one from each group, be included in the phylogenetic analysis. (Fischer-Le Saux *et al.*, 2015). The current study provides additional support for two distinct *corylina* clade subgroups using Oregon obtained isolates.

It has been suggested and hypothesized that bacterial blight first originated in Oregon and was transported throughout the world on infected propagation material (Fischer-Le Saux *et al.*, 2015; Lamichhane *et al.*, 2013;

Luisetti *et al.*, 1976; Scortichini *et al.*, 2002). In France it had been reported that hazelnut materials imported from Oregon were among the first trees in two nurseries to show bacterial blight symptoms (Luisetti *et al.*, 1976). These claims have further been supported by MLST analysis showing that French isolates were identical to the Oregon pathotype strain, or to single locus variants of the original strains (Fischer-Le Saux *et al.*, 2015). While this isolate similarity could be attributed to spread from Oregon to Europe, based on this evidence, bacterial blight also could have been transferred from Europe to Oregon when European hazelnuts were first introduced there at the end of the 19th century by Felix Gillet from France (Mehlenbacher and Miller, 1989).

The isolates used in these studies, however, came from a very narrow host range and geographic origins and there was a small sample size. While the transportation of infected propagation material is a very plausible means of dissemination of the disease from one country to another (Pisetta *et al.*, 2016), conclusions on the geographic evolutionary origins and subsequent spread of the disease would be more accurately supported through the genomic sequence analysis on isolates from a wider range of *Corylus* species and geographic locations. To date, the genomic sequence of *Xanthomonas arboricola* pv. *corylina* has only been reported once on a strain isolated from *Corylus colurna* (Turkish tree hazel) in Colorado, USA (Caballero *et al.*, 2013). Next steps for further characterization of *Xac* would be to sequence the genomes of isolates found from a wide geographic range.

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Chapter 3.
In Vitro Protocol for Evaluating Bacterial Blight Susceptibility of Hazelnut Cultivars

Abstract

Bacterial blight (*Xanthomonas arboricola* pv. *corylina*) (*Xac*) of hazelnut (*Corylus avellana* L.) was described first in Oregon in 1915 and is now recognized as a damaging disease of young hazelnut trees worldwide. Thousands of acres of new hazelnut cultivars are being planted in the Willamette Valley of Oregon where 99% of the U.S. hazelnut crop is grown. There has been an increased incidence of bacterial blight in young hazelnut plantings and no quantitative research on bacterial blight susceptibility of the different hazelnut cultivars. Increased hazelnut tree nursery production to meet the rising demand has been made possible through the development of the 2016 *Corylus* tissue culture medium optimized for hazelnut growth. In this study, tissue culture was used as a controlled environment to investigate the potential for developing a rapid screening technique to determine relative susceptibility of cultivars to bacterial blight infection. Culture medium, stress conditions, and disease symptom progression were evaluated to analyze the response of hazelnut explants in culture tubes to bacterial blight inoculation. The bacteria proliferated on the culture media, so explants were transferred to inert media for the duration of the evaluation. Symptoms consistent with those seen in the field such as leaf lesions, leaf chlorosis, leaf and shoot necrosis along with characteristic bacterial ooze were observed in inoculated hazelnut explants within two weeks of inoculation. No significant differences in relative susceptibility of cultivars was detected, as each of the five cultivars displayed symptoms similar in rate and severity over the course of evaluation. Each of the five cultivars had been reported to succumb to bacterial blight infection under field conditions. The *in*

vitro protocol reduced variability due to the environment, and saved considerable space and time compared to conventional pathogen screening using potted trees or field inoculations. Tissue culture as a technique for rapid screening and understanding bacterial blight disease progression and symptomology was demonstrated in susceptible hazelnut cultivars over time.

Introduction

Production of European hazelnut (*Corylus avellana* L.) is an important industry worth up to $3.3 billion on the world market. The United States is responsible for about 5% of the world's hazelnut production (National Agricultural Statistics Service, 2019). The Willamette Valley in western Oregon represents approximately 99% of the nation's supply. The two most notable diseases of hazelnuts grown in Oregon are eastern filbert blight (EFB) caused by the fungal pathogen *Anisogramma anomala* (Peck.) E. Müller, and bacterial blight caused by the bacterium *Xanthomonas arboricola* pv. *corylina* (Miller *et al.*, 1940; Vauterin *et al.*, 1995). Release of EFB-resistant hazelnut cultivars (Olsen *et al.*, 2013) has given the Oregon hazelnut industry an opportunity for growth. As the industry expands, there is increased interest in developing improved management strategies for bacterial blight and conducting research to learn more about this disease.

Xanthomonas arboricola pv. *corylina* is a highly host-specific pathogen that only causes bacterial blight in hazelnuts (*Corylus* spp.). Bacterial blight disease can affect the leaves, twigs, and trunks of hazelnut trees. Disease symptoms have also been reported on the nuts (Lamichhane and Varvaro, 2014; Miller *et al.*, 1949). Disease symptoms and the impact of infection can range from oily lesions that may go unnoticed to stem girdling cankers that may stunt tree growth or kill the trees outright (Lamichhane *et al.*, 2013; Puławska *et al.*, 2010; Scortichini *et al.*, 2002). Hazelnut orchards between 1 and 4 years old are most at risk of bacterial blight infection and stress conditions are known to

predispose young hazelnuts to infection (Moore, 1974). The disease can be difficult to detect in young hazelnut trees until the disease symptoms have had a detrimental effect in orchards making this a challenging disease to manage and study in the field.

Disease characterization studies have been carried out with this pathogen describing phenotypic, biochemical and molecular qualities of the bacterium as well as pathogenicity testing of the pathogen on hazelnuts (Lamichhane and Varvaro, 2014; Prokić *et al.*, 2012; Puławska *et al.*, 2010; Scortichini *et al.*, 2002; Chapter 2). Bacterial blight infection requires ideal environmental conditions and timing for specific growth stages of the hazelnut tissue to induce infection (Miller *et al.*, 1949; Moore, 1969). The primary infection period of bacterial blight is in the late fall and early winter during rainy and wet conditions (Miller *et al.*, 1949). During some growing seasons, the disease is highly problematic in young orchards. Symptoms of bacterial blight first appear from early to mid-April through early June in Oregon (Miller, 1937; Moore, 1974). Infections in spring may continue to develop and cause detrimental effects through the growing season, but there are no new infection points during the summer months (Lamichhane and Varvaro, 2014; Miller *et al.*, 1949; Prokić *et al.*, 2012; Scortichini *et al.*, 2002). Evaluating the effects of this pathogen is challenging with environmental variability and the long disease cycle. Greenhouse experiments with potted trees have been used to artificially produce the conditions needed for successful inoculation for small-scale experiments (Miller *et al.*, 1949; Prokić *et al.*, 2012), but the capacity for large scale experiments to achieve high replication of treatments is limited.

Micropropagation can been used to produce many plant replicates and a controlled environment for disease screening (Barlass *et al.*, 1986; Brisset *et al.*, 1988; Chandra, 2010; Duron, 1987; Scheck *et al.*, 1997; Tripathi *et al.*, 2008). Micropropagation is a technique for rapid plant propagation under sterile conditions. Tissue culture has had a great impact on the ability to produce large quantities of true-to-type, disease free plantlets in a relatively short period of time

with year-round application. Hazelnuts were first propagated in an *in vitro* system in 1975 (Radojevic *et al.*, 1975) and since then, many improvements have been made to optimize propagation. For years it was standard practice for researchers to use Murashige and Skoog (MS) media (Murashige and Skoog, 1962) with nutrient and plant growth regulator modifications for hazelnut micropropagation, but hazelnuts were difficult to produce on a large scale in tissue culture on MS media. Yu and Reed (1993) tested a variety of basal media and carbon sources and found that Driver-Kuniyuki Walnut (DKW) achieved optimal shoot multiplication for hazelnuts. The DKW micropropagation media was adjusted and improved over the years, and the most recent *Corylus* media was formulated in 2016 for optimal hazelnut growth with DKW as the basis (Akin et al, 2017; Bacchetta *et al.*, 2008; Nas and Reed, 2004).

The rate and demand for planting hazelnuts in the Willamette Valley in Oregon has surpassed the supply from the traditional means of propagating hazelnuts (Olsen and Smith, 2013). Many nurseries have relied heavily on micropropagation to rapidly produce true-to-type trees to keep up with the high demand for young trees (Thompson *et al.*, 1996). The 2016 *Corylus* media is used in most large-scale hazelnut tissue culture operations and makes research with tissue culture hazelnut explants possible. The *in vitro* hazelnut system has not been used for disease screening previously.

In this study, for the first time, tissue culture was used as a controlled environment to investigate the potential for developing a rapid screening technique to determine relative cultivar susceptibility of hazelnut cultivars to bacterial blight infection. Five hazelnut cultivars were propagated *in vitro* using the 2016 *Corylus* tissue medium and were used in inoculation experiments with *Xanthomonas arboricola* pv. *corylina*. ‘Barcelona’ is considered to be moderately to highly susceptible to bacterial blight infection and was included as an industry standard cultivar (Barss, 1927; Mehlenbacher and Miller, 1989; Miller *et al.*, 1949). Material for a known resistant cultivar, such as pollinizer ‘Hall’s Giant’, was unavailable for this experiment (Pscheidt and Ocamb, 2019). The other

evaluated hazelnut cultivars ‘Jefferson’, ‘McDonald’, ‘Wepster’, and ‘Dorris’ are among the new releases from the Oregon State University breeding program with single gene resistance to EFB (Mehlenbacher *et al.*, 2016, 2014, 2013, 2011; Olsen *et al.*, 2013). No information is available on their controlled response to bacterial blight infection. However, there have been field reports of bacterial blight symptoms on these cultivars from commercial orchards (Pscheidt and Ocamb, 2019). Preliminary experiments used ‘Barcelona’ to determine how the bacteria responded to the 2016 *Corylus* tissue culture media, and to different methods of inoculation. The effect of stress conditions on symptom development was examined by briefly exposing inoculated trees to damaging cold temperatures. Finally, we evaluated the relative susceptibility of hazelnut cultivars to bacterial blight with *in vitro* inoculations.

Materials and Methods

Plant Material

The hazelnut cultivars evaluated under tissue culture conditions included ‘Barcelona’, ‘Jefferson’, ‘McDonald’, ‘Wepster’, and ‘Dorris’. The plant materials were maintained in the Oregon State University Horticulture department tissue culture lab. The explants were propagated and prepared for the experiment using the 2016 *Corylus* micropropagation medium (NH_4No_3, $MgSO_4*7H_2O$,K_2SO_4,KH_2PO_4, DKW-MA micronutrients, DKW Vitamins, MS Thiamine, $CaCl_2*2H_2O$, $Ca(NO_3)_2*4H_2O$, Seq-Fe 138, BA-5 mg/L, adjusted to pH 5.2, agar 6 g/L) (PhytoTechnology Laboratories A1111) (Akin *et al.*, 2017).

Inoculations were performed on succulent apical shoots of hazelnut explants that had been grown on the *Corylus* medium for 6 weeks. The rootless explants were freshly cut and transferred to individual sterile culture tubes containing 10 mL of inert water-agar medium two days before inoculations (6 g of

agar and 1 L of sterile DI water) (PhytoTechnology Laboratories A1111). The explants remained on this inert medium for the duration of the evaluation.

Inoculum Preparation

Inoculations used virulent strains of *Xanthomonas arboricola* pv. *corylina*, JL2600 and JL2607, collected from commercial hazelnut orchards in the Willamette Valley, Oregon (Chapter 2). Inoculum suspensions were prepared from freeze-dried bacteria first cultured for 5 days on GYCA (glucose, yeast, calcium carbonate, agar) at 27 ^{o}C. The bacteria were then scraped from the media surface with a spatula and mixed with powdered skim milk [38% (w/v)] to make a suspension. The bacterial suspension underwent lyophilization using the FreeZone 6 system (Labconco Co. Kansas City, MO). The freeze-dried product was ground to a fine powder, and stored at -80 ^{o}C (Rothleutner *et al.* 2014; Johnson *et al.*, 1993). A concentration of 10^{7} colony forming units (CFU)/mL was prepared in sterile DI water from this stock as the inoculum for all experiments. The titer of the freeze-dried inoculum was routinely verified and was consistent in all tests.

Preliminary Test

Xac strains JL2607 and JL2600 were compared to sterile water controls in three treatment combinations. Inoculation methods included 1) dipping the explants in the bacterial suspension, and 2) wounding of leaf blades before dipping the explants. Explants of ‘Barcelona’ in culture tubes filled with 10 mL of 2016 *Corylus* media were used in this preliminary inoculation test. Additionally, 20 μl of inoculum from each treatment was pipetted directly onto the 2016 *Corylus* media.

For inoculation method 1 each explant was removed from the culture tube under sterile conditions in a laminar flow hood and was dipped and swirled for 10 seconds in the treatment solution with the apical meristem down. After the 10

seconds, any excess solution had dripped from the explants. Approximately 50 μl of inoculum was applied to each explant. Treated explants were then returned to culture tubes, with one inoculated explant per tube. For method 2 the procedure was the same, but prior to dipping a scalpel was used to remove one-third of the distal end of the top three leaves on each explant. Six explants were included for each method and treatment combination. The treated explants were maintained in a growth chamber at 25 ^{o}C with 14:10 L:D photoperiod for the duration of the experiment.

Inert Media Evaluation

Explants of 'Barcelona' were held in inert sterile water agar (500 mL DI H_2O + 3 g agar) and treated with *Xanthomonas arboricola* pv. *corylina* strain JL2600, water control or not treated. The explants were transferred to individual culture tubes containing 10 mL of inert sterile water agar media. The 20 replicates for each treatment were removed from their individual culture tubes using sterile forceps and were swirled in their respective solutions. After 10 seconds of swirling, the explants were gently shaken to remove excess solution and were replaced in their individual culture tubes. The treated explants were maintained in a growth chamber at 25 ^{o}C with a 14:10 L:D photoperiod for the duration of the experiment.

Temperature Stress Evaluation

Hazelnut explants were treated with either *Xanthomonas arboricola* pv. *corylina* strain JL2600 or a sterile water control. Hazelnut cultivars 'Barcelona', 'Dorris', 'McDonald', and 'Wepster' were maintained in tissue culture for 6 weeks on the 2016 *Corylus* tissue culture media prior to inoculation. Six replications of each cultivar were transferred to the culture tubes on inert sterile water agar medium. The explants were dipped and swirled for 10 seconds in their respective treatments before being placed back in their culture tubes. The treated explants

were then placed in a growth chamber at -5 ^{o}C for 10 minutes following the procedure outlined by Scheck *et al.* (1997). The exposure to negative temperatures was to simulate a frost event. After the cold exposure period was complete the explants were placed in a growth chamber at 25 ^{o}C with a 14:10 L:D photoperiod.

Cultivar Evaluation Test

Forty explants from 5 cultivars of hazelnut were transferred to culture tubes containing 10 mL of inert agar media. Hazelnut cultivars 'Jefferson', 'McDonald', 'Wepster', 'Dorris', and 'Barcelona' were used in this experiment. Two treatments were included in this experiment with 20 replications of each cultivar for each treatment. The explants were either dipped in a suspension of *Xanthomonas arboricola* pv. *corylina* strain JL2600 or dipped in a sterile water control. Each of the explants were removed from the culture tubes, the apical meristem the explants were removed with a scalpel to expose the vascular tissue and the explants were dipped in the respective treatment solutions. The explants were swirled for 10 seconds in solutions and the excess dripped off. The explants were then returned to the tubes they came from. The treated explants were maintained in a growth chamber at 25 ^{o}C with a 14:10 L:D photoperiod for the duration of the experiment.

Symptom Assessment

All changes in the appearance of the treated explants were observed and recorded to develop a screening method. Each explant was evaluated prior to inoculation for presence of lesions and blemishes naturally present in the tissue culture system. Symptoms were evaluated once a week for the duration of the experiment. The cultivar evaluation experiment was terminated at 8 weeks post inoculation (wpi). At this time, each of the explants was re-isolated using dilution series to verify the presence of the inoculated *Xac* and fulfill Koch's postulate.

Lesions were considered as any imperfections or blemishes present on the leaf surface (Figure 3.1). Leaves with lesions, chlorosis, or chlorotic patches were counted and recorded on each explant. Necrotic leaves or leaves with developing necrotic patches were also counted on each explant. The symptoms on each leaf were only classified in one category (lesions, chlorotic, or necrotic), making the sum of symptomatic leaves a proportion of the total number of leaves on each explant.

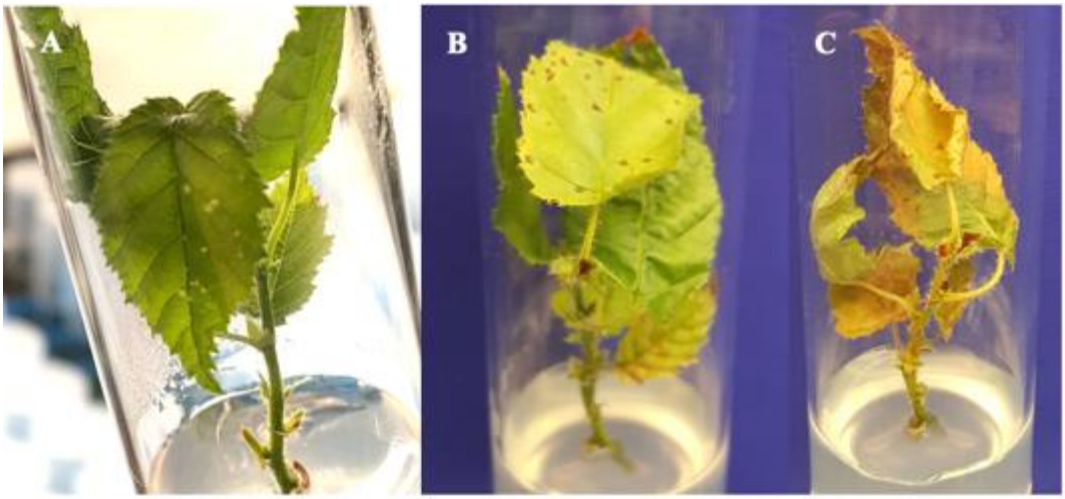

Figure 3.1. Symptoms of the *Xac* inoculation classified as present or absent in the leaves.

A: translucent water-soaked lesions. B: darkened lesions and leaf chlorosis. C: leaf chlorosis and lesions turned to leaf necrosis.

Statistical Analysis

Statistical analysis was performed, and figures were created using the open-source statistical environment R using R-packages: multcomp, binom, ggplot2, dplyr (R Core Team, 2019). A two-way binomial generalized linear model (glm) followed by chi-squared test. When the chi-squared test was significant, a Tukey's Contrast was used to further analyze treatment effects on bacterial blight symptoms in the cultivar evaluation test.

Results

Preliminary Test

When the *Xanthomonas arboricola* pv. *corylina* strains JL2600 and JL2607 were plated on the 2016 *Corylus* tissue culture medium they were observed growing on the media surface three days post inoculation. They began by forming many small single colonies, then grew to a mat that then morphed into one large mass. The bacteria proliferated on the tissue culture medium at the base of the inoculated explants and would engulf the base of the explants with a large mass of cells indicating that the tissue culture medium was not a suitable medium for cultivar evaluation.

Symptoms began appearing on the treated explants within 4 days post-inoculation (dpi). The initial observed symptoms were irregular speckled translucent lesions on the leaf surfaces. Water control explants showed no *Xac* symptom formation. At 9 dpi, the treated explants were showing marginal necrosis, slight chlorosis and speckled translucent lesion formation in patches on the leaf surfaces, whereas the water control explants showed no symptoms. New shoots appeared as branches growing from the base of both water control and inoculated explants, and bacteria proliferating on the media surface at the base of the explants were also noticed on all treated explants. At 25 dpi, there was a clear difference between the bacteria treated and water control explants. The main symptoms appearing were necrosis, chlorosis and lesion formation. Only one of these symptoms were affecting a given leaf at a time and some leaves were not affected. At 26 dpi, the number of leaves displaying one of the symptoms out of the total leaves on a single explant was calculated.

It was found that at 4 wpi and beyond there was a highly significant difference between the treated explants and the water control in the proportion of lesion development, necrosis, and chlorotic leaves to the total number of leaves on each explant ($p < 0.001$). No difference in symptoms was found between the

two bacterial strains, JL2600 and JL2607, in the treated ‘Barcelona’ explants (p = 0.9). No difference was found in the inoculation methods ($\chi^2_{1,32}$= 46.1, p >0.05).

Water Agar Media Evaluation

There was no sign of bacterial growth on the water agar media through the duration of the experiment. Signs and symptoms of the bacterial infection were confined to the tissue of the JL2600 treated explants and progressed in the explants over the course of evaluation (Figure 3.2). Symptoms on the treated explants began to appear at 1 wpi. Symptoms were consistent with the initial inoculation experiment and bacterial blight leaf symptoms observed under field conditions. Symptoms were initially characterized as translucent lesions that developed into chlorotic spots and over time became necrotic. By the end of evaluation, the tops of the treated explants were dark necrotic tissue that permeated down through the stem. The water controls showed slight chlorotic symptoms from growing in the inert media but showed none of the other symptoms present in the treated explants such as water-soaked lesions or necrosis (Figure 3.3).

Figure 3.2. The symptom progression in a ‘Barcelona’ explant treated with *Xac* isolate JL2600.

Week 0: freshly inoculated explant showing no symptoms. Week 2: Discrete water-soaked lesions forming on the upper leaf and on the newly emerging leaf.

Week 4: Water-soaked lesions merging to become more apparent and leaves showing signs of chlorosis. Week 6: Lesions and chlorosis beginning to give way to necrosis with severe water-soaked and chlorotic areas on the lower leaves. Week 8: Full necrosis in the leaves and infection spreading halfway down the stem of the explant.

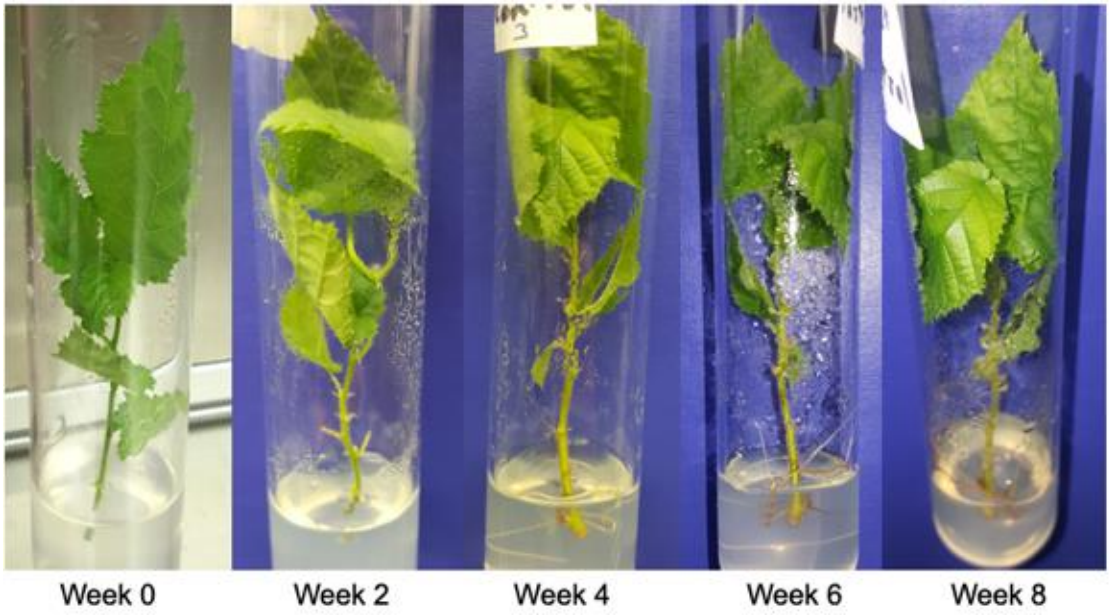

Figure 3.3. The progression of a water-control treatment 'Barcelona' explant from the week of inoculation to the final week of evaluation.

From week 0 to week 8, the water controls showed callus formation and root formation with slight chlorosis, but no visible infection.

After 1 wpi there was no difference in the symptoms on treated explants compared to the water control explants and no difference between the not treated and the water control ($\chi^2_{2,48}$= 65.1, $p > 0.05$). At 2 wpi, there was a significant difference between the total symptoms in the bacterial treated explants and the water control explants ($p < 0.01$) with still no difference between the water control and nontreated explants ($p = 0.9$). Between 2 and 4 wpi, there was a 0.4 proportion increase in the proportion of symptomatic leaves on the *Xac* treated explants. After 4 wpi, the *Xac* explant symptom development plateaued and the proportion of symptomatic leaves remained just under 0.8 for the remainder of the experiment with a highly significant difference between the *Xac* explants and the water controls ($p < 0.001$).

Temperature Stress Evaluation

Exposure to cold temperatures of -5 ^{o}C did not improve the uniformity of infection. There was no significant difference in the symptom development of the *Xac* inoculated hazelnut explants compared to the water controls at 4 wpi and for the remainder of the experiment ($\chi^2_{1,47}$= 64, p = 0.291). The exposure to the cold period after inoculations was not included in any further tests.

Cultivar Evaluation Test

The initial evaluation on the date of inoculation (0 wpi) showed blemishes on the leaves that were counted as lesions. The first symptoms from the treatment began to appear in each cultivar during the first wpi. Characteristic bacterial ooze was seen forming on the wound of the cut surfaces of each of the cultivars as early as 2 wpi (Lamichhane and Varvaro, 2014; Miller *et al.*, 1949).

All cultivars treated with *Xac* had an increasing proportion of leaves showing symptoms of bacterial blight over time (Figure 3.4). The proportion of leaves characterized by lesions peaked at 4 wpi. After 4 wpi, leaves with lesions began to turn chlorotic and then necrotic in the following weeks. There was a slight increase in symptoms observed for the water control treatments over time for each cultivar based on increasing nutrient deficiency. While these deficiency symptoms were also present in explants treated with *Xac*, blight infection symptoms occurred a greater rate than any symptoms in the water control explants. A significant difference in the proportion of disease symptoms in all the treated explants compared to their water control counterparts could be seen in each of the cultivars from 4 wpi and beyond (p <0.001). In trees treated with *Xac*, lesions steadily increased on leaf surfaces over the first 4 weeks. By 8 wpi, every leaf on the treated explants was showing symptoms of bacterial blight and there were no differences in the proportion of infection between any of the cultivars ($\chi^2_{4,295}$ = 336, p =0.997). In the water control treatment, ‘Dorris’ and ‘Jefferson’ showed just over half of the leaves with symptoms, while ‘Wepster’ and

‘Barcelona’ showed half of the leaves with symptoms, and ‘McDonald’ showed only a third of its leaves with symptoms. The bacterial re-isolations consistently showed *Xac* present on the treated explants from 1 $x10^4$ CFU/explant to $1x10^7$ CFU/explant. None of the water controls from the 3 trials had any outside contamination and no bacteria were isolated from the water control treated explants.

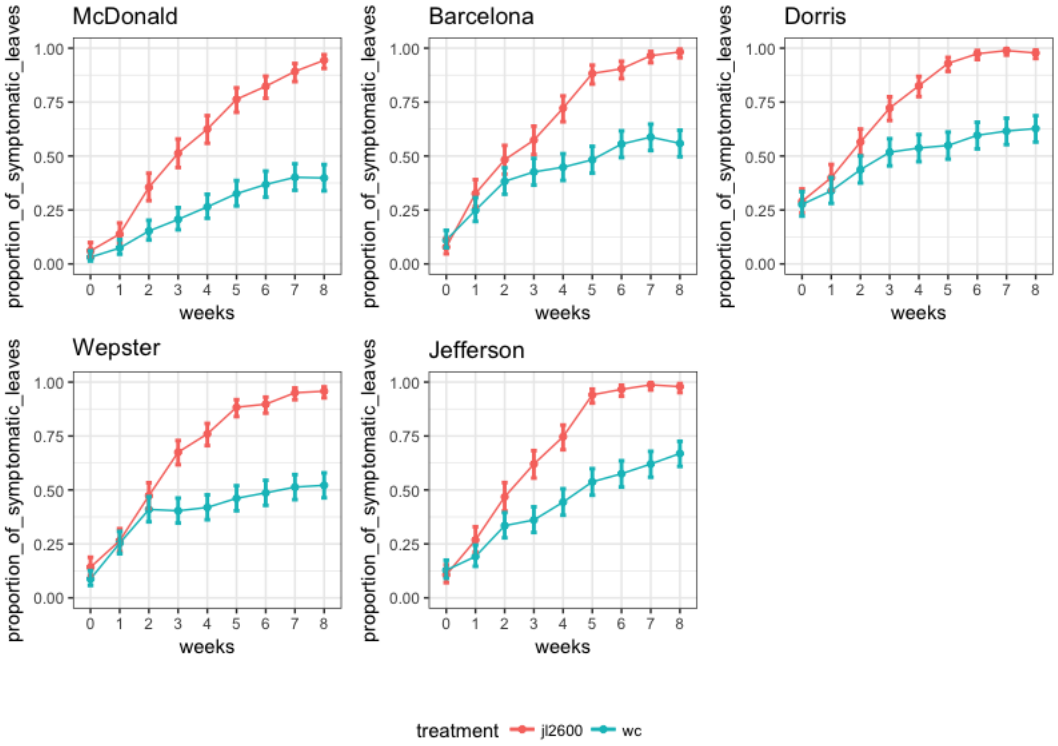

Figure 3.4. *In vitro* cultivar inoculation results.

Mean proportion of symptomatic leaves (± 95% binomial confidence interval) to total leaves, comparing the bacterial blight treated explants with the water controls for each hazelnut cultivar.

Root and Callus Formation

In each trial there was a consistent treatment effect on the explants' ability to induce rooting and callus formation. A larger proportion of water control explants formed roots than the *Xac* treated explants in the whole population ($p < 0.01$) (Figure 3.5). The water control explants formed callus at a significantly higher rate than the *Xac* treated explants from week 2 until the end of evaluation ($\chi^2_{1,198} = 231.8$, $p < 0.001$) (Figure 3.6). Callus formation was consistently less frequent when explants of any cultivar were treated with *Xac*.

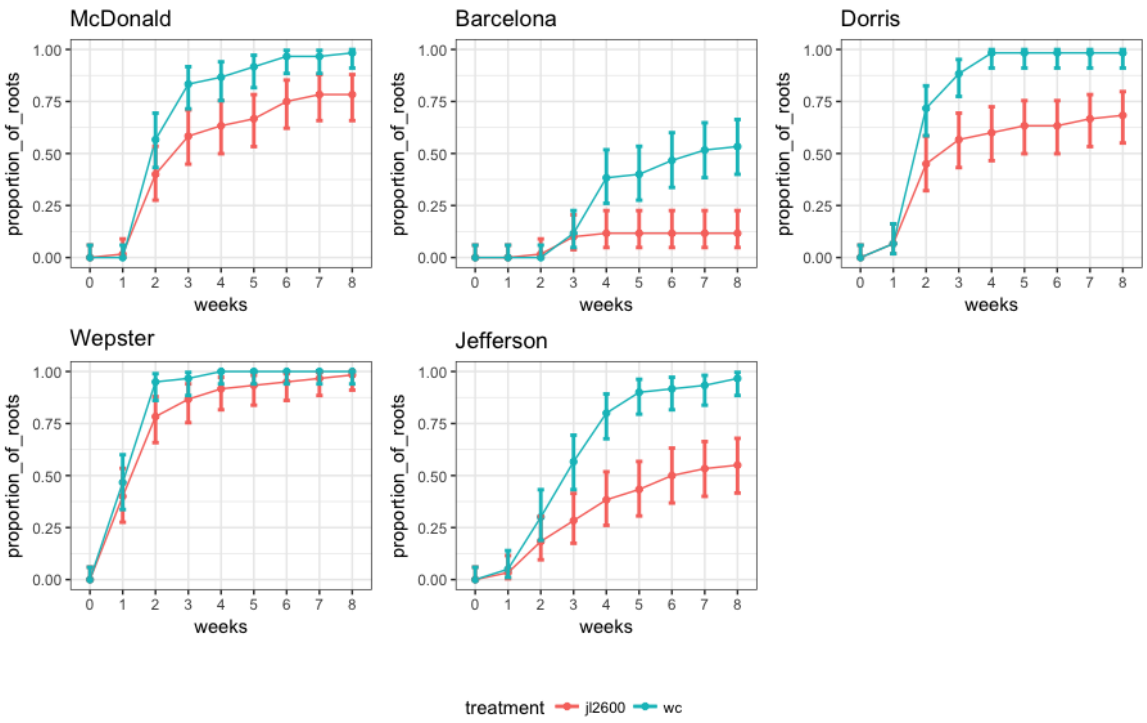

Figure 3.5. Root formation in the *in vitro* inoculation.

Mean proportion of explants showing root formation of the total number of explants, comparing the bacterial blight treated explants with the water controls for each hazelnut cultivar.

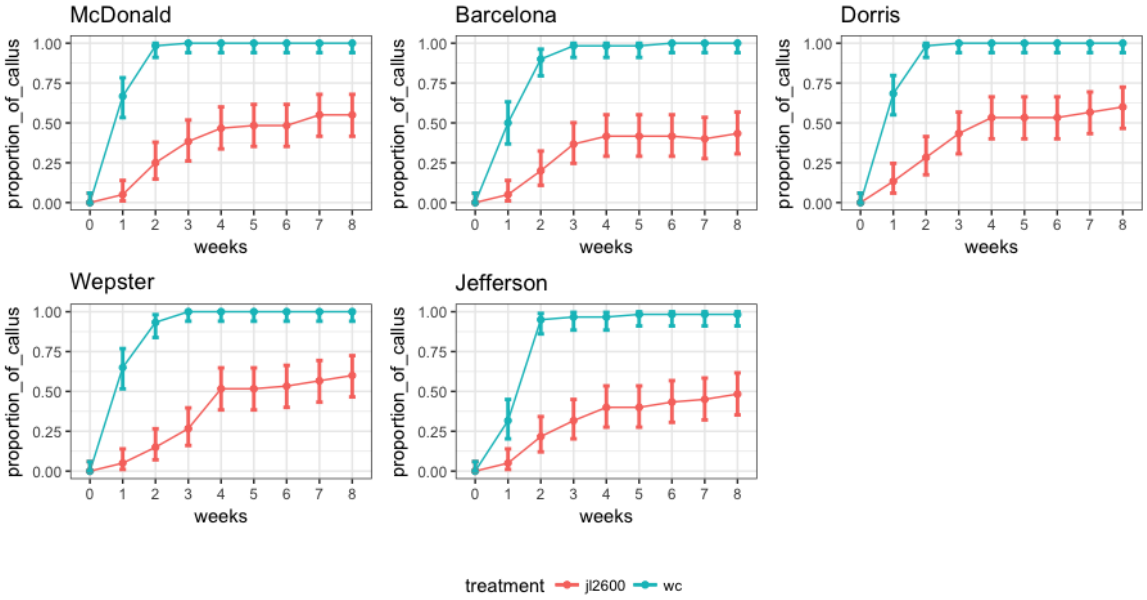

Figure 3.6. Callus formation in the *in vitro* inoculation.

Mean proportion of explants with callus formation of the total number of explants, comparing the bacterial blight treated explants with the water controls for each hazelnut cultivar.

Discussion

Hazelnut cultivars propagated and inoculated in tissue culture conditions were useful for examining pathogenicity of *Xanthomonas arboricola* pv. *corylina* and could form the basis for a protocol for rapid disease screening of susceptible hazelnut cultivars. The *in vitro* method is quick, low cost, easily replicated, and not dependent on seasonal conditions compared to field and potted tree inoculations. These are key requirements for developing a resistance screening method to bacterial blight, and perhaps other diseases in hazelnuts (Thompson *et al.*, 1996). All five of the hazelnut cultivars used in this trial showed uniform disease symptoms and were readily maintained in culture to produce a disease curve that was consistent in each replication. Symptoms began with lesion development on leaves, chlorosis of leaves and then necrosis of leaves. The disease then showed an ingress into the petioles and the stems of the explants as the disease progressed. Bacterial ooze formation was seen in wounded explants and on the undersides of leaves. Bacterial ooze is often noted as a sign of bacterial blight infection under field conditions during periods of high humidity (Lamichhane and Varvaro, 2014; Miller *et al.*, 1949).

Each of the five hazelnut cultivars demonstrated a consistent rate of symptom development that were each significantly different than their respective water controls but no differences in susceptibility were found among the cultivars. Bacterial blight has been seen under field conditions in each of the cultivars evaluated as reported by the OSU Plant Clinic, OSU Hazelnut extension, and in potted inoculation tests (Chapter 4). The results from this study under *in vitro* conditions supports those observations.

Use of tissue culture as a system for disease evaluation has been used for fungal infection evaluation, virulence detection and bacterial evaluation (Joint FAO/IAEA Programme, 2010). A few previous studies have evaluated bacterial pathogens in tissue culture. Plant resistance and pathogenicity screening have previously been tested using tissue culture in *Pseudomonas syringae* pv. *syringae* in lilacs, *Xanthomonas campestris* pv. *musacearum* causing

Xanthomonas wilt in bananas, and *Erwinia amylovora* causing fire blight on apples and pears (Brisset *et al.*, 1988; Duron, 1987; Scheck *et al.*, 1997; Tripathi *et al.*, 2008).

Scheck *et al.* (1997) evaluated *Pseudomonas syringae* pv. *syringae* strains for pathogenicity to determine if lilacs could be a universal host for pathogenicity testing with isolates of *P. syringae* pv. *syringae* from a wide range of host plants. In that assay, the *P. syringae* pv. *syringae* did not grow on the MS tissue culture media so no special adjustments were needed for the culture media. In this study, the *Xanthomonas arboricola* pv. *corylina* isolates grew on the 2016 *Corylus* media, requiring a change to water agar medium. The water control explants showed a degree of symptoms likely due to nutrient deficiency on the water agar. The symptom development did, however, level off due to the different cultivars' natural ability to tolerate nutrient deficiency in the *in vitro* system for the duration of the experiment.

Pathogenicity testing of *Pseudomonas syringae* pv. *syringae* produced uniform disease symptoms on the lilac explants that were readily maintained in culture. The disease symptoms on the lilac explants included water-soaked lesions on the leaves, vein and petiole necrosis and tip dieback. The symptoms appeared as little as 2 days post-inoculation with a complete disease response after 14 days (Scheck *et al.*, 1997). In the bacterial blight hazelnut system, symptoms began to appear as early as 5 days post-inoculation with a complete disease response greater than the water controls at 4 wpi. The bacterial blight assay in hazelnuts takes twice as long as the *Pseudomonas syringae* pv. *syringae* assay, but it is a major improvement on other previous methods of testing the disease response of *Xac* on hazelnut that can take up to 6 months, although there was no difference seen among cultivars (Prokić *et al.*, 2012; Scortichini *et al.*, 2002).

Some studies found tissue culture to be an effective method for screening plant resistance to bacterial pathogens and others found the results to be not comparable enough to field conditions to be an accurate screening tool (Brisset

et al., 1988; Tripathi *et al.*, 2008). A rapid technique for evaluating banana cultivars to *Xanthomonas* wilt was developed using tissue culture methods compared to potted plants. There was a significant cultivar effect in the disease symptoms in both systems. There were eight cultivars of bananas tested and a gradient of observed responses to infection in the field and polyploidy genetic variation (Tripathi *et al.*, 2008). In this hazelnut tissue culture system, there were five new cultivars with unknown susceptibility. The known moderately susceptible cultivar 'Barcelona' was included as a baseline for disease susceptibility. There were no clear differences in susceptibility between the different cultivars in our assay. The cultivar 'McDonald' showed the slowest progression of symptoms but there was still a significant treatment effect consistent with the other cultivars.

The tissue culture system is dependent on sensitive plantlets and there have been criticisms towards its validity as a resistance screening model. A study testing the feasibility of tissue culture as a resistance screening system for fire blight in *Pyrus* cultivars found that inoculations under *in vitro* conditions usually overestimated cultivar susceptibility. It was suggested that the gradient of susceptibility may not be accurately determined under these conditions (Brisset *et al.*, 1988). While the small differences in gradient between susceptible and highly susceptible could not be determined in the *Pyrus* explants, there was a clear difference in the highly resistant cultivar response compared to the susceptible cultivars that would be useful for obtaining a classification of susceptibility of cultivars for low cost in a short period of time (Duron, 1987; Visuer and Tapia y Figueroa, 1987). These studies suggest that *in vitro* inoculations may be useful in detecting individuals with high levels of resistance in *Pyrus* from a wider range of genetic variation or transgenic explants, or may be used to learn more about the virulence mechanisms of the pathogen.

The *in vitro* hazelnuts did not show differences in susceptibility of cultivars in tissue culture inoculations. In a potted tree inoculation experiment using seven different cultivars of hazelnut, the five that were evaluated under *in vitro* conditions plus two others ('Yamhill' and 'York'), it was found that all the cultivars

tested were susceptible to bacterial blight infection with each cultivar showing greater than or equal to the amount of infection as in the known susceptible cultivar (Chapter 4). The same susceptibility seen across cultivars in the *in vitro* study was also present in the difference between the *Xac* treated trees and the water control trees in the potted trees inoculation. However, two cultivars; Jefferson and Dorris, showed a greater infection efficiency to bacterial blight infection in one year old potted trees with just over 75% of all buds inoculated becoming symptomatic. This was 40% more infection than the other cultivars tested and greater than the known susceptible 'Barcelona'. 'Jefferson' and 'Dorris' showed clear infection symptoms under tissue culture conditions, but there was no sign in the symptom progress that suggested they were significantly more susceptible than the other three cultivars ('McDonald', 'Wepster' and 'Barcelona').

Environmental conditions for inoculated explants plays a critical role in the level and uniformity of symptom development (Brisset *et al.*, 1988; Duron, 1987; Scheck *et al.*, 1997; Tripathi *et al.*, 2008; Visuer and Tapia y Figueroa, 1987). In this study, preliminary experiments helped to optimize the conditions under which the hazelnut explants were exposed. The consistent results suggest that the recorded symptoms accurately represent the susceptibility found in the different cultivars. This protocol would be well-suited to evaluate a wider population of genetic variation in hazelnut cultivars to determine a resistant response. There may also be other symptoms, such as callus and roots formation, that would distinguish a highly resistant cultivar from a very susceptible cultivar and could help to understand the mechanism the bacteria used to cause infection.

Noticeable effects of the inoculation of *Xanthomonas arboricola* pv. *corylina* were apparent in the ability of explants to form callus and root tissue over the course of the evaluation. The formation of undifferentiated callus cells at the point of wounding is a natural defense response in most plants, and in tissue culture this callus formation is expected. The water control explants uniformly produced callus tissue by the second week post-inoculation with root formation to

follow. Callus formation on the *Xac* treated explants was slowed or prevented all together with only about half of the inoculated explants displaying any callus formation by the end of the evaluation. Prevented or delayed callus formation following *Xac* inoculations have been reported previously in a field trial to determine the effects of pruning timing on bacterial blight infection (Moore, 1974). Prevented and delayed callus formation on the pruning wounds in the presence of inoculated *Xac* was observed with the formation of bacterial blight infection. The lack of callus formation increased the exposure of these entry points making them more vulnerable to pathogen invasion (Moore, 1974).

Research in the suppression of callus formation caused by *Xanthomonas* spp. has been associated with the release of xanthan gum to induce plant susceptibility (Yun *et al.*, 2006). Xanthan gum is a commonly secreted exopolysaccharide in *Xanthomonas* spp. Cultured Xanthomonad isolates produce a thick mucoidal appearance because of the xanthan gum and is one of their most visible defining characteristics (Lamichhane *et al.*, 2013; Prokić *et al.*, 2012). The calcium in the GYCA culture media is incorporated to induce this characteristic feature as a means of identification. Xanthan gum is reported to be among the secreted exopolysaccharides associated with virulence (Ryan *et al.*, 2011). They play a role in masking the bacterium to prevent host recognition, contributing to epiphytic survival and enabling colonization of host tissue (Qian *et al.*, 2005). Research conducted with *Xanthomonas campestris* pv. *campestris* (Xcc) has suggested callus formation is necessary for resistance to Xcc and xanthan induces susceptibility in *Nicotiana benthamiana* and Arabidopsis by suppressing calcium deposition on a molecular level to inhibit the formation of callus tissue (Yun *et al.*, 2006). With the significant difference in the inhibition of callus formation in the bacteria treated explants in this study, there a possibility that this could be among the mechanisms by which *Xac* induces infection in hazelnuts. Further research is required to shed additional light on the mechanisms of *Xac* inducing pathogenicity in hazelnuts, on the effects of dose response in the symptoms observed on hazelnut explants, and if there would be

a difference in resistant and susceptible hazelnut cultivars given the different concentrations of inoculum.

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Chapter 4.
Evaluating Cultivars of Hazelnut for Resistance to Bacterial Blight of Hazelnut

Abstract

Bacterial blight (*Xanthomonas arboricola* pv. *corylina*) (*Xac*) of hazelnut (*Corylus avellana* L.) was first described in Oregon in 1915 and is now recognized as a damaging disease of young hazelnut trees worldwide. Thousands of acres of hazelnut cultivars that are resistant to eastern filbert blight (EFB) are being planted in the Willamette Valley of Oregon, where 99% of the U.S. hazelnut crop is grown. It has been reported that young hazelnuts under stress conditions, such as marginal planting sites or drought, are predisposed to bacterial blight infection. The bacterial blight resistance of the new hazelnut cultivars has never been quantified, but it has long been suggested that planting resistant cultivars is the most effective way to manage the disease. In the fall of 2018, a population representing 7 cultivars of hazelnut were inoculated by injecting individual buds on each tree with a suspension of *Xac* or a negative control. The cultivars included 6 EFB resistant cultivars that are currently being widely planted (‘Jefferson’, ‘Dorris’, ‘McDonald’, ‘Wepster’, ‘Yamhill’, and the pollinizer ‘York’), and a known susceptible cultivar ‘Barcelona’. The infection efficiency on each cultivar was measured as the number of infected buds compared as a proportion to the total buds inoculated. Results were recorded the following spring when the appearance of new symptoms reached its peak. Each cultivar was found to be susceptible, showing an infection efficiency equal to or greater than the known susceptible cultivar ‘Barcelona’. ‘Dorris’ and ‘Jefferson’ showed higher infection efficiency than the other cultivars in one year old potted trees. No bacterial blight resistance was found in the cultivars evaluated, but bud inoculations were successfully used to quantify the cultivar response to bacterial bight infection.

Introduction

Bacterial blight of hazelnut induced by *Xanthomonas arboricola* pv. *corylina* (*Xac*) (Miller *et al.*, 1940; Vauterin *et al.*, 1995) is one of the most economically impactful diseases in commercial European hazelnut (*Corylus avellana* L.) production worldwide (Fischer-Le Saux *et al.*, 2015; Lamichhane and Varvaro, 2014; Pisetta *et al.*, 2016). Bacterial blight is the second most important disease in the Oregon hazelnut industry behind the fungal disease eastern filbert blight (EFB), which was inadvertently introduced from its native range in the eastern U.S. The hazelnut production acreage in Oregon has more than doubled since the release of EFB resistant cultivars from 2007 to 2019 with more than 80,000 acres being cultivated (Pacific Agricultural Survey LLC, 2019). The rapid increase in planting made possible by the release of the EFB resistant cultivars has come with many biotic and abiotic challenges, including increased reports of bacterial blight in young orchards.

Bacterial blight symptoms are found on leaves, buds, twigs, trunks and occasionally nuts primarily on young hazelnut trees between 1 and 4 years old (Lamichhane and Varvaro, 2014; Miller *et al.*, 1949; Scortichini *et al.*, 2002). It has been shown that *Xac* may reside epiphytically on asymptomatic tissue and under bud scales for extended periods without inducing symptoms (Pisetta *et al.*, 2016). Suboptimal soil sites, dryland production, and excess nitrogen in the soil have been associated with bacterial blight infection on hazelnuts (Lamichhane *et al.*, 2013; Moore, 1974; Olsen, 2013; Pisetta *et al.*, 2016). Bacterial blight becomes less of an issue as orchards mature, so it is necessary to effectively manage the disease throughout the early orchard establishment period to set an orchard up for success (Moore, 1969; Wiman *et al.*, 2019).

Planting bacterial blight resistant cultivars has been suggested as the best control method for managing bacterial blight since the disease was first described, especially in conjunction with healthy cultural practices and timely copper sprays (Barss, 1915; Lamichhane and Varvaro, 2014; Miller *et al.*, 1949; Prunier *et al.*, 1976). The observed susceptibility reported in the older cultivars is

now of less applicable as they are no longer planted due to high EFB susceptibility (Olsen *et al.*, 2013). The genetic diversity of the recently released EFB resistant hazelnut cultivars has exposed knowledge gaps in how to best manage these trees. No data exists on the susceptibility of EFB resistant hazelnut cultivars to bacterial blight (Pscheidt and Ocamb, 2019).

Scortichini *et al.* (2002) included the evaluation of three Italian hazelnut cultivars for their response to bacterial blight with pathogenicity testing of 31 *Xac* isolates. They found no difference between the hazelnut cultivars. The inoculation methods done by Scortichini *et al.* (2002) were used in this study. This study represents the first potted tree inoculation and evaluation for the bacterial blight response in the new hazelnut cultivars that are being widely planted in the Pacific Northwest (PNW). The data were analyzed to quantify the relative bacterial blight susceptibility in the EFB resistant hazelnuts.

Materials and Methods

Plant Material

The trees used in this experiment represent 6 cultivars of hazelnut recently released from the hazelnut breeding program at Oregon State University that are widely planted in the Pacific Northwest including Oregon, Washington and British Columbia. Some of these cultivars are also being planted in Chile. These cultivars included 6 EFB-resistant cultivars: ‘Jefferson’, ‘McDonald’, ‘Wepster’, ‘Yamhill’, ‘Dorris’, and the pollinizer ‘York’. The industry standard ‘Barcelona’ was also included as a known bacterial blight susceptible cultivar. There were two groups of the 6 EFB resistant cultivars grown as micro-propagated trees purchased in plugs (North American Plants, LLC, McMinnville, OR) in 2017 and 2018 that were raised in pots under greenhouse conditions (16:8 L:D, 25 °C). Trees in the first group were two years old at the time of inoculation. They were potted under greenhouse conditions the spring of 2017

and were held outdoors the following winter and the summer before inoculation. These trees were regularly irrigated and were provided with 20 g slow-release fertilizer every 6 months (15-9-12, Osmocote®Plus, Maryville, OH). There were 40 trees of each cultivar from the 2017 potted trees. Trees in the second group were one year old at the time of inoculation. In the spring of 2018, one hundred trees of each cultivar were potted, given slow-release fertilizer and cared for under greenhouse conditions. In the fall of 2018, these trees were acclimated to the outdoors and went into dormancy. The cultivar 'Barcelona' was not available from micropropagation so 40 one year old layered trees were potted up and cared for in the same manner as the other trees. The total population consisted of 880 trees. There were 240 two year old trees, 600 one year old trees, and 40 layered 'Barcelona' one year old trees. The trees were held on an outdoor pad at the North Willamette Research and Extension Center in Aurora, Oregon.

Inoculum Preparation

Xanthomonas arboricola pv. *corylina* isolate JL2600 was used since characterization and pathogenicity of multiple isolates of *Xac* showed JL2600 to be consistently virulent on susceptible hazelnut cultivars (Chapter 2). Inoculum suspensions were prepared by using freeze-dried bacteria. The freeze-dried bacteria were prepared by first culturing bacterial isolates for 5 days on GYCA (glucose, yeast, calcium carbonate, agar) at 27 ^{o}C. The bacteria were then scraped from the media surface with a spatula and mixed with powdered skim milk [38% (w/v)] to make a suspension. The bacterial suspension underwent lyophilization using FreeZone^{6} (Labconco Co. Kansas City, MO). The freeze-dried product was then ground to a fine powder, and stored at -80 ^{o}C (Rothleutner *et al.* 2014; Johnson *et al.*, 1993). The titer was calculated through dilution plating. The stock freeze-dried bacteria were calculated to be at a titer of 10^{12} colony forming units (CFU)/g. A concentration of 10^{8} colony forming units (CFU)/mL was prepared as inoculum in a sterile phosphate buffer for all experiments. The titer of the freeze-dried inoculum was routinely verified and was

consistent in all tests. The control treatment contained only the sterile phosphate buffer.

Inoculation Procedure

Inoculations were carried out by administering the treatments with a needle syringe to individual buds on each tree using the method of Scortichini *et al.* (2002). Briefly, the cultivars and age groups were divided into *Xac* and control treatment groups with an equal number of trees in each. The trees were labeled with their respective treatments and the buds that would receive treatment were flagged with wire twist ties. Between 7 and 20 buds were marked on each tree depending on the number of buds available. A twist tie marker was placed at the bottom of the recorded number of buds that would receive treatment. Every bud above this marker was counted and inoculated. Once the hypodermic needle pierced the bud, the treatment solution was injected until right before runoff occurred. This method delivered approximately 10 μl of inoculum per bud (1×10^6 CFU/buds; Figure 4.1).

The inoculations were carried out during the first week of November 2018 over 4 consecutive days. Each day an equal number of *Xac* treatments and sterile phosphate buffer control treatments were administered for each cultivar and age. New inoculum and phosphate buffer controls were used each day and the concentration of bacteria was consistent. The treatments were kept separate during inoculation to avoid cross contamination while the inoculum was still wet. One week after inoculation, the *Xac* treated trees and the control trees of each cultivar and age were arranged into a completely randomized design. The population was monitored periodically throughout the dormant season and checked weekly as spring approached and the buds began to swell and break.

Figure 4.1. 2018 Fall bud inoculations.

A hypodermic needle was used to inject 10 µl of treatment solution into each individual bud selected for inoculation.

Symptom Analysis

Evaluation of symptoms was performed in the first week of May 2019. Inoculated buds were rated as infected by the presence of lesions and necrotic tissue on the buds, petioles and emerging leaves of each tree. A random sample of symptomatic and asymptomatic tissue from each cultivar and treatment was collected to re-isolate inoculated bacteria to fulfill Koch's postulate. The re-isolated bacteria were identified using dilution plating on the semi-selective growing medium GYCA (glucose, yeast extract, calcium carbonate, agar) and sequencing analysis of the DNA housekeeping gene *gyrB* for the detection of the unique polymorphisms in the *Xac* isolate JL2600 (Chapter 2).

Results were analyzed using generalized logistic regression models with the response variable representing the number of buds infected out of total number of buds inoculated for the explanatory variables: treatment, cultivar, and age of tree. An asymptotic chi-squared test based on the deviation was performed on the generalized linear model (glm) model. After a significant chi-

squared test, data was then analyzed with Tukey's multiple comparisons test. The data are presented in bar graphs with error bars based on the upper and lower limits of the binomial confidence intervals for the infection efficiency on each cultivar. All statistical analysis and associated figures for the inoculation experiments were conducted and produced in the open-source statistical environment R using packages: binom, ggplot, multcomp, dplyr (R Core team, 2019).

Figure 4.2. Bacterial blight infection symptoms.

Inoculated buds were rated as infected based on the presence of lesions and necrotic tissue on the buds (a), necrotic emerging buds (b) and dieback of emerging shoots (c-d).

Results

The first symptoms of bacterial blight were observed on April 16, 2019 when the trees were in the half-inch green leaf development stage, over 160

days after the inoculations were administered. Symptoms appeared as failure of buds to open, dieback of partially opening buds, and the beginning stages of shoot dieback. Symptoms progressed for another 3 weeks and the infection efficiency was evaluated May 6, 2019. In each cultivar, the proportion of symptomatic buds out of the total buds inoculated was significantly different than the respective water control treatment ($p < 0.001$) (Figures 4.3, 4.4). Only the cultivars 'Dorris' and 'Yamhill' showed a significant difference of infection between the ages of the trees where the 1 year old trees had a greater incidence than the 2 year old trees (($\chi^2_{1,68} = 88.2$, $p < 0.001$) , ($\chi^2_{1,67} = 87.1$, $p < 0.001$) , respectively).

The known susceptible cultivar 'Barcelona' had a total of 139 buds showing infection symptoms out of the 326 total buds (41%) inoculated with *Xac* strain JL2600 and only 9 buds were showing infection symptoms out of the 338 total buds on the controls. Both ages of the cultivars 'McDonald', 'Wepster', 'Yamhill' and 'York', and the two year old 'Dorris' and 'Jefferson' trees showed no difference in infection compared to 'Barcelona' ($p > 0.05$; Figure 4.5). The one year old 'Jefferson' and 'Dorris' showed significantly greater infection efficiency than 'Barcelona' ($p < 0.001$).

Bacteria were recovered from symptomatic buds and tissue at concentrations consistently 3-fold greater (1×10^9 CFU/bud) than the inoculum concentration applied in inoculations (1×10^6 CFU/buds). Asymptomatic phosphate buffer control tissue samples were also examined with re-isolation. No *Xac* was recovered from asymptomatic control tissue samples collected from two year old trees. Asymptomatic control tissue samples from one year old trees revealed low concentrations of bacteria detected (1×10^4 CFU/ sample). Genomic DNA sequencing of partial *gyrB* housekeeping genes from colonies each of the bacteria recovered the samples were consistent on a molecular level with the strain JL2600 of *Xac* originally applied. Phylogenetic analysis revealed the same unique polymorphisms in each of the samples analyzed that placed

strain JL2600 in the distinct phylogenetic clade compared to the pathotype strain (Chapter 2).

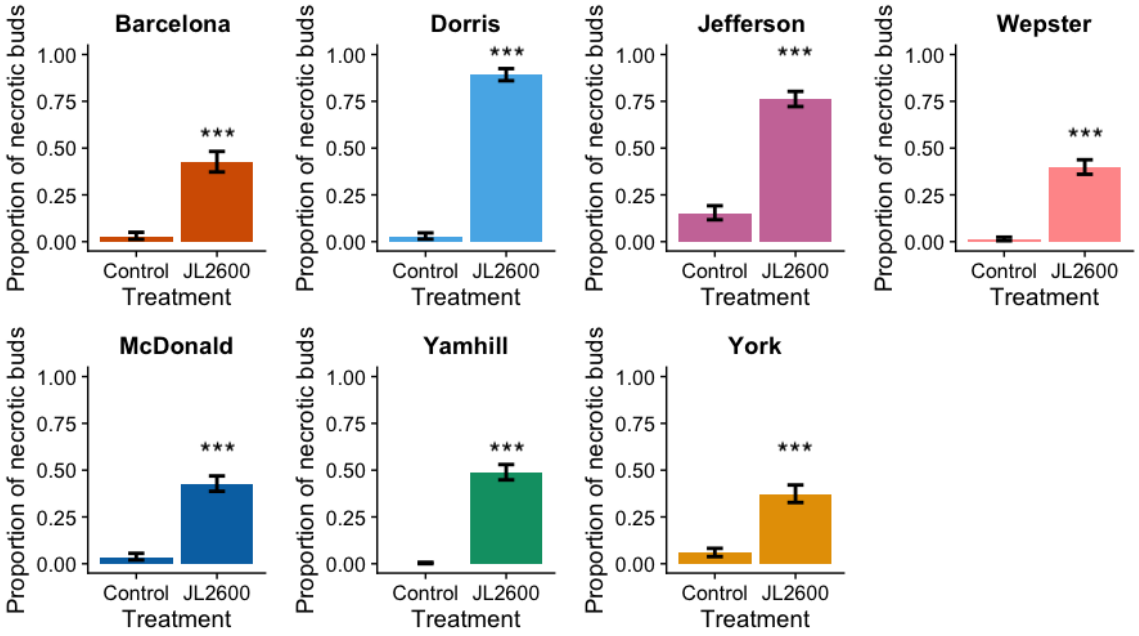

Figure 4.3. One year old trees *Xac* treatment compared to water controls.

The Xac treatments in each cultivar showed significant difference from their respective control ($p < 0.001$).

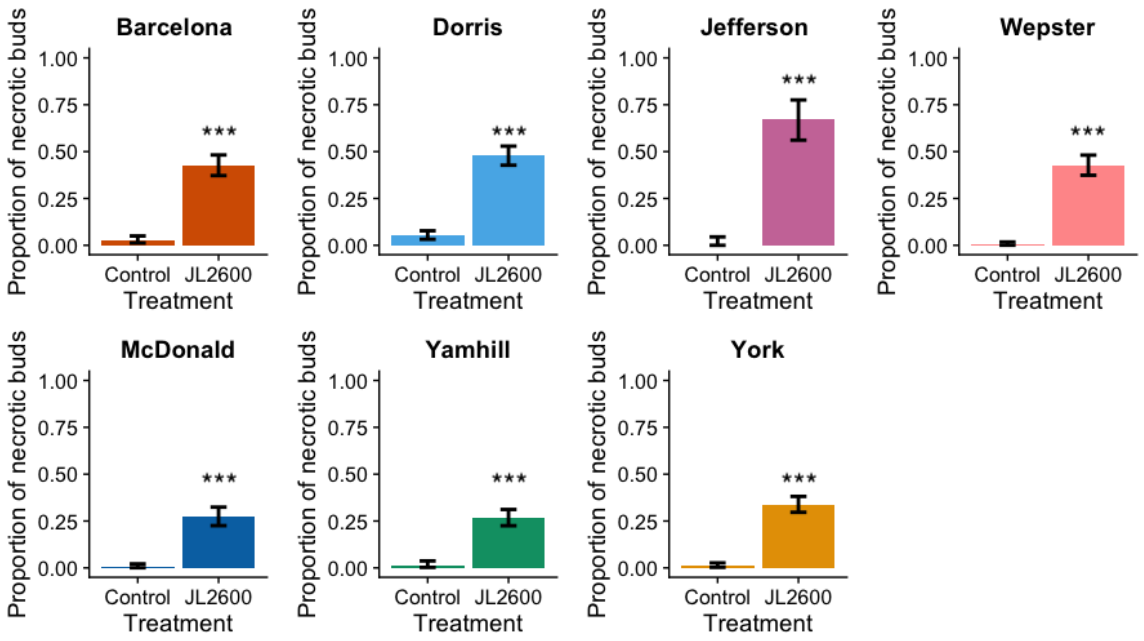

Figure 4.4. Two year old trees *Xac* treatment compared to water controls.

The Xac treatments in each cultivar showed significant difference from their respective control.

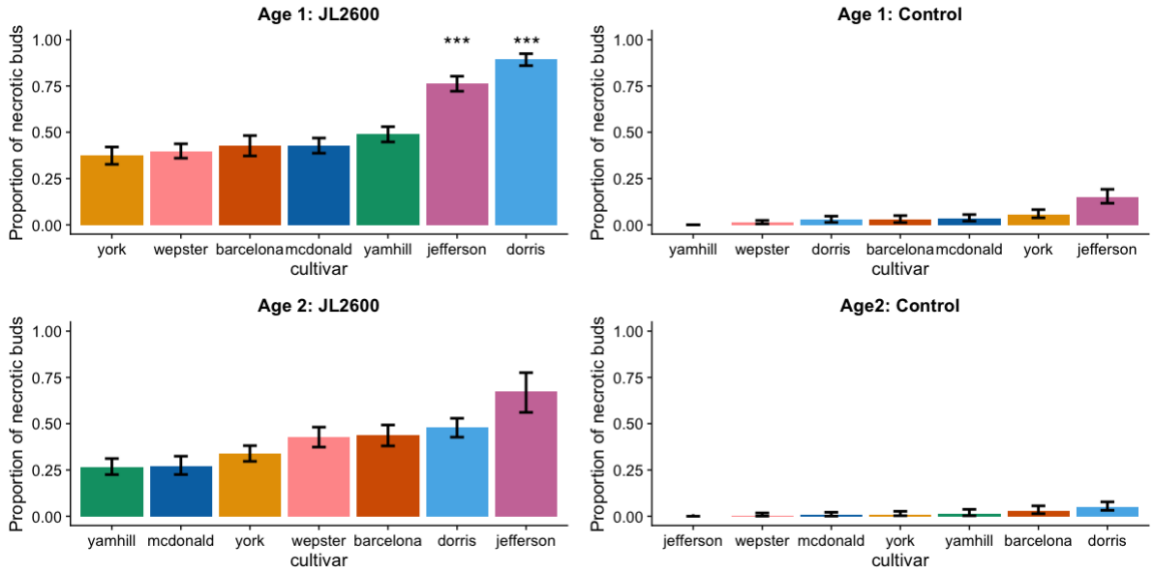

Figure 4.5. Cultivar comparisons for each age and treatment.

Each cultivar showed equal or greater infection efficiency compared to the known susceptible 'Barcelona'. This susceptibility was apparent in both age groups. Age 1: N = 100 trees per cultivar; Age 2: N = 40 trees per cultivar, on average.

Discussion

Each of the EFB resistant cultivars were found to be susceptible to bacterial blight infection with an infection efficiency equal to or greater than the known susceptible cultivar ‘Barcelona’. This study evaluated new hazelnut cultivars for resistance to bacterial blight. The assessment of the resistance was based on the infection efficiency from artificial inoculation on potted trees. Previous bacterial blight investigations reported the cultivar response to bacterial blight based on observations under natural conditions of infection, but with no formal quantification of disease. The inoculation used in this evaluation was meant to simulate bacteria overwintering in the bud scale with an added wound to localize the infection. The standard ‘Barcelona’ was classified under natural conditions as moderately to highly susceptible to bacterial blight and was included in this study as a known susceptible (Barss, 1927; Miller *et al.*, 1949; Pscheidt and Ocamb, 2019). The infection efficiency that ‘Barcelona’ showed from artificial inoculation supports these observations of susceptibility.

The bacteria recovered from tissue samples were verified to be JL2600 using morphological and molecular techniques. The low levels of JL2600 recovered from asymptomatic controls were only present on one year old trees but not the two year old trees. This is likely because *Xac* is spread through water splash and the smaller one year old trees were in the water splash zone below the older trees. The trees were open to the elements through the dormant season and rain water dripping through the canopy carried bacteria to the lower branches resulting in low levels of *Xac* present on some of the leaves. This is consistent with reported observations of the spread of *Xac* from infected branches higher in the canopy to lower branches causing the infection to move down (Miller *et al.*, 1949).

While each of the cultivars showed bacterial blight susceptibility, ‘Jefferson’ and ‘Dorris’ appeared to be more susceptible to infection than the others. Bacterial blight infection was reported at the initial release of the cultivar ‘Jefferson’ during a breeding trial in the OSU Hazelnut Breeding program

(Mehlenbacher *et al.*, 2011). The symptoms were described as a stressed appearance at the end of the growing season, liquid oozing from a crack where a large scaffold limb joined the main trunk, and some branch dieback in two of the four trees in the second breeding trial (Mehlenbacher *et al.*, 2011). Dorris had no reports of bacterial blight in the initial breeding trials (Mehlenbacher *et al.*, 2013). However, the *Xac* strain JL2600 used in this inoculation study was originally isolated from bud dieback found on a young 'Dorris' tree under nursery conditions (Chapter 2). Cultivars 'McDonald', 'Wepster', 'Yamhill', and 'York' had no signs of bacterial blight susceptibility during breeding trials, however, 'McDonald' and 'Wepster' have had numerous reports of bacterial blight infection throughout the Oregon growing region (McCluskey *et al.*, 2009; Mehlenbacher *et al.*, 2018, 2016, 2014). There have been no previous reports of bacterial blight infection in 'Yamhill' and 'York' making this the first reported incidence of bacterial blight in these two cultivars.

Several hazelnut cultivars have been reported under field conditions as having a degree of bacterial blight resistance such as pollinizers 'Daviana' and 'Hall's Giant' but even these are not believed to be immune to infection (Miller *et al.*, 1949; Prunier *et al.*, 1976; Thompson *et al.*, 1996). The suspected resistant cultivars are rarely planted today due to EFB susceptibility. They were also not widely planted as nut producing cultivars before the introduction of EFB due to less desirable nut qualities and low yields (Dorris, 1927; Lamichhane and Varvaro, 2014; Miller *et al.*, 1949). There were no known resistant cultivars included in this resistance trial due to a lack of available plant material.

The results obtained in this study were consistent with Scortichini (2002) who tested bacterial blight infection on three Italian hazelnut cultivars entering dormancy using the same bud injecting inoculation method. The cultivars included in that study were 'Tonda Gentile Romana', 'Tonda Gentile delle Langhe', and 'Nocchione'. Each of the cultivars were found to be susceptible to infection and there was no difference in infection using any of the 31 *Xac* isolates tested (Scortichini *et al.*, 2002).

The sample size for number of buds inoculated used by Scortichini (2002) on each cultivar was very low at 10 buds inoculated per isolate. During the characterization of the Oregon isolates (Chapter 2), the initial pathogenicity testing utilized 20 buds per isolate, and this level of replication was found to not have enough power to determine a clear difference between the bacterial isolates. For the current study, hundreds of buds were inoculated for each cultivar to increase the power of the experimental population.

As further research is conducted on the response of hazelnut cultivars to bacterial blight infection, it will become increasingly important to understand the molecular interactions between the bacterium and the host plant to determine what mechanisms confer resistance to infection (Lamichhane, 2014). The current understanding of pathogenicity initiation for most pathovars within the species *Xanthomonas arboricola* requires the use of the type III secretion system (Hajri *et al.*, 2012). The type III secretion system is a protein secretion system that is highly conserved among members of *X. arboricola* and contains structural components encoded by a cluster of hypersensitive response and pathogenicity (*hrp*) genes for cell entry (Alfano and Collmer, 1997). The effector proteins that are secreted into the host through the type III secretion system then interaction with receptors and defense compounds that the host. These compounds will induce a defense response such as a hypersensitive response or reactive oxygen species in a resistant host (Gürlebeck *et al.*, 2006). However, in a susceptible host the effectors from the bacteria will interrupt or bypass the host's defense activity to initiate infection (Alfano and Collmer, 2004). The *hrp* gene structure within the different pathovars of *X. arboricola* are what determines the high level of host specificity of the pathogens (Hajri *et al.*, 2012). More research is needed to understand the pathways involved in pathogenicity of *Xac* in hazelnut to inform breeding efforts to develop bacterial blight resistant hazelnut cultivars.

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Chapter 5.
Conclusions and Future Directions

This study was the first research conducted on bacterial blight of hazelnut in Oregon since the 1970s. The inception of this research was the re-ranking of bacterial blight as a high priority for research by the Oregon Hazelnut Commission in 2016 during the annual review of priorities based on industry needs. When the project began, the authors were not certain how big an issue bacterial blight was in the hazelnut industry. Growers would commonly discuss incidences of the disease, but further investigation showed that in many cases bacterial blight was not the culprit, but rather cultural issues or other disease pressures were to blame. At the same time, it was apparent that some growers were suffering from bacterial blight infection in their young orchards, and when the disease was confirmed, it was impacting 5 to 20% of an orchard, (Appendix 1). These incidences were verified with re-isolation and characterization of the bacteria.

This thesis set out to achieve four objectives: 1) collect, isolate, and genetically verify *Xanthomonas arboricola* pv. *corylina* cultures obtained throughout the Willamette Valley (the primary growing region for U.S. hazelnuts), 2) quantify the pathogenicity of the characterized *Xac* isolates, 3) assess and document of the rate and progression of disease symptoms on inoculated trees using tissue culture and potted trees, and 4) determine the response to bacterial blight in hazelnut cultivars by evaluating the progression of disease symptoms.

Chapter 1 takes a comprehensive look at the research and observations made on bacterial blight in hazelnuts from its first description in the early 1900's to present day. This work has shaped the current management practices for the disease, provided methods for characterization and inoculation, and provided a starting point for future research. A modern characterization of bacterial blight to reduce confusion on symptomology and an investigation into the response of

some of the cultivars currently in production was determined to be the logical starting point for the renewed research effort of bacterial blight of hazelnuts in Oregon.

Chapter 2 covers the characterization of the causal bacterium of bacterial blight, *Xanthomonas arboricola* pv*. corylina* (*Xac*), providing a foundation for further research to be conducted to develop management strategies for the disease and to learn new avenues of management by deepening the understanding of this pathogen. There were 14 isolates of *Xanthomonas arboricola* pv. *corylina* collected, isolated, and genetically verified. These isolates are securely stored in the USDA-ARS Plant Pathology Laboratory in Corvallis, Oregon. The isolates underwent characterization using morphological, biochemical, and molecular techniques. The molecular characterization completed during this study focused on using two housekeeping genes (*gyrB* and *rpoD*) for the identification of the bacteria. These were also used to establish a general understanding of the phylogenetic diversity present in Oregon compared to previously described isolates. The phylogenetic diversity in *Xac* found in Oregon has also been reported in Europe. It has been suggested that *Xac* may have been accidentally introduced to Europe from Oregon (Luisetti *et al.*, 1976), however it is just as likely that *Xac* was brought to Oregon from Europe when *Corylus avellana* (European hazelnut) was first planted (Mehlenbacher and Miller, 1989). A comparative analysis of genomic sequences from a wide geographic range of *Xac* isolates is the next step in a deeper understanding the genetic diversity and biogeography of bacterial blight through its evaluation and adaptation to the conditions where it is found.

There has only been one annotated genome sequence published for *Xac* to date (Caballero *et al.*, 2013). This genomic sequence in addition to others could be used to investigate the molecular basis of host pathogenesis for *Xac*. In a review, Lamichhane (2014) presents directions of further research in the management on diseases of fruit and nuts caused by *X. arboricola*. Lamichhane (2014) suggests that understanding the molecular infection mechanisms and

discovering genetic repertoires involved with inducing infection would be essential for breeding resistance to these pathogens (Lamichhane, 2014). Cultivar resistance is the most effective way to manage a disease, but very little was known about the response of the recently-released hazelnut cultivars to bacterial blight infection.

Nine of the 14 isolates characterized in Chapter 2 were evaluated for pathogenicity and were consistently virulent when inoculated on *Corylus avellana* L. There were no differences in pathogenicity among these isolates in field inoculations, potted tree inoculations, or, for two of the isolates, under tissue culture conditions. One of these isolates, JL2600, was selected for use in the inoculation experiments for its consistent virulence and unique polymorphisms in the housekeeping gene sequences. This virulent *Xac* isolate was utilized to evaluate the susceptibility of hazelnut cultivars in two different systems.

In Chapter 3, a protocol was developed for using tissue culture as a controlled *in vitro* environment to more efficiently analyze the response of hazelnut cultivars to *Xac* bacteria. In Chapter 4, a protocol for a large-scale potted tree bacterial blight inoculation was implemented to evaluate the susceptibility of 7 cultivars. The symptoms produced under tissue culture conditions and on potted trees were consistent with those seen in the field and the incidence previously reported on the cultivars evaluated.

In future studies, the effects of dose response should be tested on each of the cultivars to see if there are any resistance differences observed at various concentration levels of bacterial inoculum. Once the effects of dose response are determined, tissue culture and potted trees inoculations could be performed on a set of hazelnut cultivars that span a wider genetic diversity. The USDA-ARS National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon maintains a diverse world collection of the genus *Corylus*. Both the tissue culture and the potted tree evaluation methods could be applied to trees from this plant material to determine if there are any bacterial blight resistant cultivars to include in a breeding program.

There are many possibilities for applied horticultural research to be done examining the impact of cultural factors from the modern hazelnut production systems on bacterial blight infection in young orchards. Effects of drip irrigation on pathogen development, bacterial blight and trunk guard interactions, effects of herbicide on infection, the role of plant nutrition, and minimizing bacterial blight spread through pruning. There is also great opportunity to use mapping software to track the progression of bacterial blight through orchards over time and to survey the distribution of the disease on a regional scale.

Copper management has always been the main treatment protocol for bacterial blight, so future research should investigate the effectiveness of copper products and how the timing of copper treatments could be incorporated into EFB management protocols, such as using spring applications of copper during bud break. Research should also be conducted on the use of biological controls, antibiotics, and other active ingredients as potential control methods to reduce the risk of bacterial blight infection in young hazelnut plantings.

The insidious nature of bacterial blight is that it can become an economic problem rapidly and may have long term implications for orchard health. The anxiety, frustration, and loss that this disease has the potential to induce is a present threat to young trees in the Oregon hazelnut industry, which is currently comprised of mostly young trees planted within the last 10 years. Outreach to the growers communicating the signs and symptoms of the bacterial blight in a clear and concise format is vital for management of the disease. An emphasis on maintaining balanced cultural practices as a preventative measure against bacterial blight and helping growers to become more familiar with identifying the disease will help to demystify this long-standing disease in the Oregon hazelnut industry.

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APPENDICES

Appendix 1. Evaluating the Distribution of Bacterial Blight using Spatial Analysis

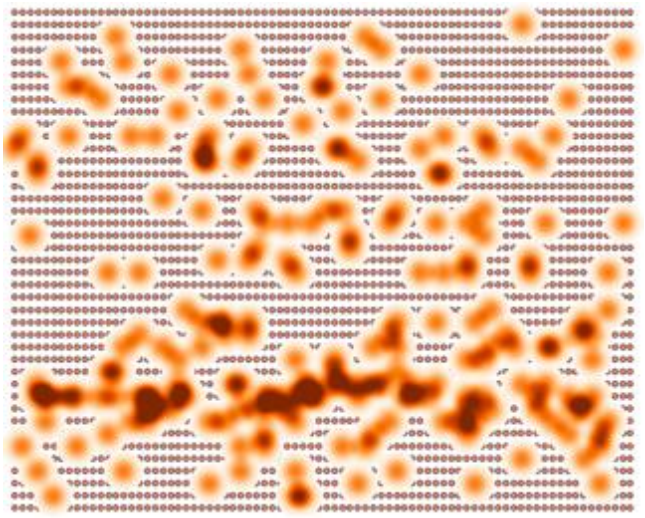
In the summer of 2017, the bacterial blight project was just beginning, and we were searching for bacterial isolates to use for characterization and further studies. Samples submitted to the OSU Plant Clinic showing symptoms from a newly planted 'Wepster' hazelnut orchard came back positive for *Xanthomonas* spp. Upon visiting the site on July 13, 2017, the hazelnuts throughout the field appeared either green and healthy, slightly yellow, desiccated but still green, desiccated and brown, or missing because the infected tree had been removed. The desiccated trees had visible trunk cankers that were girdling the trees. To gain an idea of the distribution of the infection across the field, each tree in the 48 x 80 tree plot (Large field) were recorded as healthy, yellow, crispy green, crispy brown, or missing by walking through the field and documenting the trees on a grid. A second 9 x 140 tree planting (skinny field) near the grower's house and shop was also showing symptoms and this field was evaluated with a walk through as well. Nearly a month later, August 10, 2017, the large field and the skinny field were evaluated for a second time. The incidence results are presented in Appendix Table 1.1. The software qGIS was used to generate a heatmap showing the spatial distribution of all the symptoms combined from the large field (Appendix Figure 1.1 and 1.2). A second orchard planted with cultivar 'Ennis' also had reports of significant bacterial blight so that orchard was evaluated on August 8, 2017 using the same ratings. Results are presented in Appendix Table 1.1. Heat map of symptoms from half of the field is presented in Appendix Figure 1.3.

In the 'Wepster' orchards, the initial amount of bacterial blight that effected the trees was 6% of the large field and 13% of the skinny field. These values increased to 7% and 16% over the next month during mid-summer. In the 'Ennis' orchard, 14% of the orchard was impacted by bacterial blight infection. These figures are consistent with reports from surveys conducted in the 1970s that

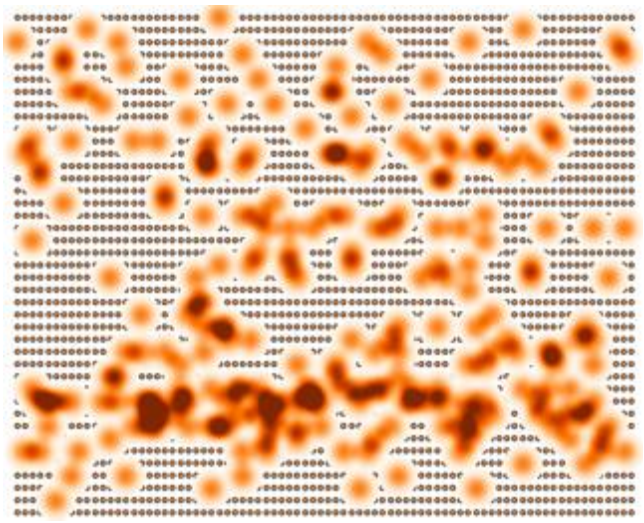
conservatively estimated that at least 7% of orchards with infection were impacted. Spatial imaging software on a larger scale could be used to determine the impact of bacterial blight infection in the modern production system by surveying orchards throughout the Valley. This type of analysis could be used to show trends in the distribution of the disease through an orchard during a season as well as across the valley over time.

Appendix Table 1.1. 'Wepster' and 'Ennis' Orchards symptom percentages.

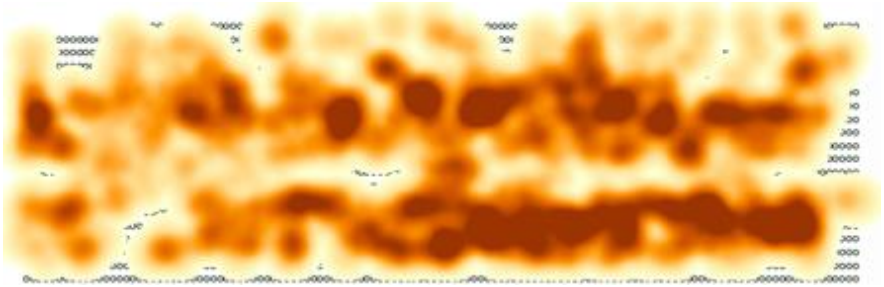
Field	Date	Total affected	Total Trees	Total percentage	Brown crispy/dead		Crispy green/ Recently dead		Yellow		Missing	
'Wepster' Large field	13-Jul-17	259	4231	6.10%	90	2.10%	50	1.20%	86	2.00%	33	0.80%
'Wepster' Large field	10-Aug-17	302	4231	7.10%	206	4.90%	18	0.40%	45	1.10%	33	0.80%
'Wepster' Skinny field	13-Jul-17	177	1340	13.20%	85	6.30%	26	1.90%	62	4.60%	4	0.30%
'Wepster' Skinny field	10-Aug-17	216	1340	16.10%	160	11.90%	17	1.30%	35	2.60%	4	0.30%
'Ennis' Orchard	8-Aug-17	629	4487	14%	305	6.70%	18	0.004%	293	6.50%	13	0.002%



Appendix Figure 1.1. 'Wepster' Orchard Large field symptom heat map distribution July 13, 2017.



Appendix Figure 1.2. ‘Wepster’ Orchard Large field symptom heat map distribution August 10, 2017.



Appendix Figure 1.3. Heat map distribution of bacterial blight symptoms in ‘Ennis’ orchard on August 8, 2017.

Appendix 2. Detached Shoot Pathogenicity Assay

Methods

Actively growing terminal shoots about 20 cm in length were collected from ‘Barcelona’ a known susceptible cultivar and ‘Hall’s Giant’ a known resistant cultivar. All leaves and petioles were removed, and the shoots were surface sterilized using a 10% bleach solution, 70% alcohol solution and a sterile DI water rinse. A 5 cm length was removed from the base of each shoot and 5 shoots were placed into each sterile 250 mL beaker containing 50 mL of sterile water. Inoculum from 9 strains of *Xac* was prepared to $1x10^8$ CFU/mL plus a non-pathogenic *Pseudomonas fluorescens* strain of A506 was used a negative control in addition to a sterile water control. A 3 cm length was removed from the shoot apex, and a 10 µl drop of each treatment was placed on the cut wound surface of each twig. The inoculum was absorbed in a few minutes and each beaker was covered with a polyethylene bag secured with a rubber band. Shoots in beakers were placed at room temperature in the lab at approximately 23 ^{o}C. The twigs were monitored daily. Lesion length was measured after 14 days. Measurements started at the cut surface going down.

Results and Discussion

After one week, a white mold began to grow on the twigs of each treatment and cultivar while they were under the polyethylene bags. The contamination affected the impact of the bacterial treatment on the twigs as well as the controls to a point where no clear symptoms could be attributed to bacterial infection. Some of the twigs had a bacterial film appearing at the cut surface for some of the isolates, including the negative control A506. After 14 days, the fungal contaminant was too great to glean any information from the

twigs. Some twigs were covered, and some were only sparsely contaminated, but no effect of the treatments was determined.

It was not clear from the methodology followed for this experiment whether the bags were supposed to be removed after an initial incubation period or left on for the duration of the observation period. However, should a detached stick assay be repeated using hazelnut twigs it is recommended that the sticks be given an incubation period with the bags for no longer than 72 hours and then the bags be removed for the remainder of the observations.

Appendix 3. Preliminary Leaf-Wounding and Cultivar Inoculation with Mist Chamber

Methods

On July 20, 2018, a population of fully leafed-out potted hazelnut trees maintained under greenhouse conditions were prepared for inoculation. The trees in the population were divided into three treatment groups. Groups received either sterile water control, strain A (JL2607) and strain B (JL2600). Three cultivars were included in the test. ‘Jefferson’ with a total of 12 trees (4 per treatment group), ‘Barcelona’ with a total of 9 trees (3 per treatment group), and ‘Hall’s Giant’ with a total of 15 trees (5 per treatment group). The leaves on each tree were subjected to one of three wounding treatments. The leaves were either: not wounded, had one-third cut with scissors at the distal end of the blade, or one-third of the distal end was roughed up using sand paper. The number of leaf replicates of each wounding treatment on the trees varied slightly on the individual but there were at least three replicates on each tree of each wounding treatment.

Once the wounding treatments were administered on a given tree, each leaf on the tree was sprayed with the assigned treatment solution making sure to achieve full coverage. Approximately 20 mL of inoculum was applied to each tree. The bacterial inoculum treatments were prepared using the freeze-dried inoculum preparation methods described in Chapter 2-4.The treated trees were left to air dry to let the inoculum create a film on the tree then they were placed in a mist chamber at about 25 ^{o}C for 72 hours with an intermittent 20 second mist every 16 minutes to maintain a high relative humidity environment for incubation. After the 72 hour incubation period, the trees were moved to a greenhouse maintained at 25 ^{o}C and monitored for bacterial symptoms on the leaves daily for 4 weeks.

Additionally, seven cultivars of hazelnut were selected for a preliminary inoculation. The population included ‘Barcelona’ (9), ‘McDonald’ (10), ‘Wepster’ (10), ‘Jefferson’ (10), ‘Dorris’ (9), ‘Yamhill (9), ‘York’ (3) for a total of 50 one year old potted trees. The trees from each cultivar were divided into three treatment groups: water control, JL2600, and JL2607. The 50 potted trees were put in the mist chamber with an intermittent spray of 20 seconds every 16 minutes to maintain a high relative humidity as a pre-incubation for 18 hours prior to inoculation. Trees were then removed from the chamber individually and sprayed using a squirt bottle with the respective treatments until runoff (between 10 and 50 mL per tree). Inoculum was prepared to $1x10^7$ CFU/mL as described in chapter 2. The trees were then put back in the mist chamber with a divider to separate the three treatments to avoid cross contamination. Post incubation lasted for 72 hours. Once post inoculation was complete, the trees from each treatment were arranged in a complete randomized design under a shade cloth outside. Trees were irrigated and cared for as needed. Trees were inspected for bacterial blight symptoms weekly.

Results and Discussion

In each of these preliminary inoculation experiment, no clear symptom formation could be distinguished between the treatment groups and the water controls. The wounding experiment did not improve the infection efficiency on any of the treatments or cultivars. In the multi-cultivar leaf spray experiment, there was very little symptom development that was clearly bacterial blight infection. On two of the ‘Barcelona’ trees (2/9), there were small leaf lesions forming on a couple of lower leaves 4 weeks post-inoculation. These leaf lesions were re-isolated, and it was determined a concentration of *Xac* was present in this tissue equal to what was originally applied ($1x\ 10^7$ cfu). Re-isolations of asymptomatic leaves from the other cultivars were also attempted, but no *Xac* bacteria was recovered. The low symptom formation see in these leaf

inoculations was consistent with reports of inoculation attempts using plant material of a similar growth stage. Miller (1949) found that as the leaves of young hazelnut trees mature into the mid to late summer, they are less susceptible to infection. Future experiments could utilize a mist chamber in the same way it was used in this experiment, but it is recommended that potted trees entering dormancy in the fall or succulent tissue in the early spring be evaluated with these methods when the tissue may be more susceptible.