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Bacterial Spot of Peach: Epidemiology and Chemical Sensitivity of Xanthomonas arboricola pv. pruni in South Carolina Orchards

Brodie Monroe Cox
Clemson University, brodiec@g.clemson.edu

BACTERIAL SPOT OF PEACH: EPIDEMIOLOGY AND CHEMICAL SENSITIVITY OF *XANTHOMONAS ARBORICOLA* PV. *PRUNI* IN SOUTH CAROLINA ORCHARDS

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Brodie Monroe Cox
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Accepted by:
Dr. Guido Schnabel, Committee Chair
Dr. Hehe Wang
Dr. Juan Carlos Melgar

ABSTRACT

Bacterial spot on peach, caused by *Xanthomonas arboricola* pv. *pruni* (Xap), is a major disease in the southeastern United States. The disease can cause substantial yield loss despite season long applications of copper. It is unknown whether selection of resistance over the course of the season contributes to disease development. Thus, we collect Xap from shoot cankers, leaves, and fruit over two years from cultivar O'Henry of three conventional and one organic farms in South Carolina and determined sensitivity to copper at the beginning (bud break), middle (pit hardening) and end (final swell) of production season. Four canker types were identified in both years, including bud cankers (infected flowering or leaf bud), tip cankers (necrotic tip of one year old shoot), concentric cankers (classic oval-shaped canker on one year shoot), and non-concentric cankers. Xap isolation rate was dependent on farm and canker type; more Xap were successfully isolated from the organic farm (24% of the canker) compared to two of the conventional farms and most (45%) came from bud cankers. Xap isolates were assessed for sensitivity to copper using two types of media, minimal glucose yeast agar and nutrient agar, amended with Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) at the discriminatory dose for tolerance at 150 µg/ml and for resistance at 200 µg/ml. In this study two phenotypes of copper tolerant Xap strains were discovered low copper tolerant (grew up to 150 µg/ml) and high copper tolerant (grew up to 200 µg/ml). Regardless of the farm and collection year, most Xap strains were sensitive to copper but tolerance was observed in 58 out of 298 strains. A total of 26 out of 139 and 32 out of 101 from the 2018 and 2019 collection, respectively, were tolerant to copper. The study illuminates shoot canker types and phenotypic diversity

among bacterial populations within and between farms and assesses the importance of copper tolerance phenotypes to the success of chemical management programs.

DEDICATION

First, I would like to dedicate all of this work to the Lord for he continuously loves and sustains me through life. I also would like to dedicate this work to my future wife, Kristi, who has stood beside me and loved me in the tough times and in the good times providing support mentally and spiritually. I also would like to dedicate this thesis to the many people who have pushed me to be a better man and person: my parents, Jeff and Angela Cox, who always pushed me to be the best I can be and to pursue my dreams, to my brother, Kyle, who showed determination and was an example of what it took to get to and graduate college with honors. To my grandparents, David and Juanita Cox; Billy and Doris Kirkendohl, who always supported me and gave me valuable wisdom in navigating school and life and to friends who provided a much-needed break and good laugh at times that were much needed.

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LITERATURE REVIEW

Section 1: The Peach Crop

The common peach (*Prunus persica*) originated from Southeast Asia in China (Cao et al. 2014). The climate is warm with mild winters and substantial rainfall (Janick 2010). Peaches are reported to be domesticated over 4,000 years ago and have made it from China, ancient Persia, Europe and finally to North America by the Spanish in the 17th century (South Carolina Encyclopedia 2016). By the 1700's peaches were being produced in the United States and by the mid 1800's peaches were being cultivated in South Carolina and being commercially grown to ship out of state (South Carolina Encyclopedia 2016). Today there are over 15,000 harvested acres of peaches in South Carolina alone (USDA/NASS 2019 State Agriculture Overview for South Carolina n.d.).

In 2018, 20 states produced peaches in the United States with the top two being California and South Carolina (USDA/NASS, 2019). There were over 690,100 tons of peaches produced in 2018 that were valued at 599 million United States dollars with the crop increasing in value over the past two decades (Noncitrus Fruits and Nuts 2018 Summary 06/18/2019 2019). More specifically, South Carolina produces 675,000 tons of peaches valued approximately at $93.8 million dollars (United States) making peaches a cash crop and a vital market in the state economy (USDA/NASS, 2019).

Section 2: Diseases of Peach

Considering the warm environment peaches are grown in and the perennial nature of the plant, there are a plethora of diseases that affect peaches. Accounting for the hot, humid South Carolina environment in the southeast United States the peach trees become even more susceptible to diseases. Peaches are infected by fungi, bacteria, viruses, viroids, and phytoplasmas. In the southeastern United States there is a group of major diseases that cause the most impact on production and tree health.

There are many fungal diseases that infect peaches ranging in severity from yield reductions to death of the entire tree. Brown rot is a common fungal disease, caused by *Monilinia fructicola*, on peaches that infect the fruit and is common in the later season when the fruit approaches ripening (Sinclair and Lyon 2005). *M. fructicola* also causes blossom blight in the early stages of the growing season. Leucostoma canker, also known as cytospora canker, is caused by a fungal complex of *Leucostoma cincta* and *Leucostoma personii* which enter through wounded or dead parts of the peach tree and cause cankers that lead to dieback of branches (Sholberg and Kappel 2008). Peach scab is another major fungal disease caused by *Cladosporium carpophilum* which causes lesions on the stem, leaf and fruit (Sholberg and Kappel 2008). Peach scab mainly causes economic loss on the fruit where it makes little black spots making the fruit unmarketable. Peach leaf curl on peach is caused by *Taphrina deformans* where it causes large, uneven galls or lesions on fruit and leaves (Sholberg and Kappel 2008). Peach leaf curl is not wide spread in the peach industry in the southeastern United States but does cause many issues for home growers (Peach Leaf Curl Management Guidelines--UC IPM n.d.).

Soil borne pathogens are also prominent to peaches around the world. Armillaria root rot, also known as oak root rot, is caused by *Desarmillaria tabescens* and causes many symptoms such as loss of limbs, weak tree, failure to produce leaves in the spring, and eventual premature death of the tree (Chandler, W. A. and Daniell, J. W. 1982). Armillaria root rot is a very important disease in the southeast of the United States and causes huge crop losses for the peach industry. Another soil borne pathogen that infects peach trees is Phytophthora root rot which is caused by *Phytophthora spp.* in the soil and is also known as "wet feet" (Sholberg and Kappel 2008). Phytophthora root rot attacks peach trees of all ages and causes decline of the canopy, dieback, chlorosis of foliage, overall withering, and death of tree (Sholberg and Kappel 2008).

Viruses and phytoplasmas are a unique set of diseases on peach but can have major impacts on tree health and production. Viral diseases in orchards can be very minor or so severe the entire block has to be pushed up. The most prominent peach viral diseases are peach mosaic virus, peach stunt disease, and plum pox disease. Peach mosaic is caused by peach mosaic virus (PMV) which is transmitted mainly by *Eriophyes insidiosus* or commonly known as peach bud mites (Gispert et al. 1998). Peach stunt disease (PSD) is actually caused by a virus complex of prune dwarf virus (PDV) and prunus necrotic ring spot virus (PNRSV) and is transmitted mainly by pollen transfer (Keitt, G. W. and Clayton, C. N. 1943). Plum pox virus (PPV) is caused by plum pox potyvirus and is mainly spread by aphid vectors or grafting of trees (Németh 1994).

Bacterial diseases on peach are of high concern in the industry and can cause major crop losses. *Pseudomonas syringae* causes bacterial canker and could lead to peach tree short life. *P. syringae* is prevalent in the fruit industry and causes high yield losses especially when conditions are right for the pathogen. The peach tree slowly declines as *P. syringae* makes its way, typically, from a pruning cut down the phloem which eventually takes out the entire scaffold branch and eventually the tree (Young 1988). Phony disease is caused by the bacterium *Xylella fastidiosa* (Wells et al. 1983). *X. fastidiosa* causes early bloom, delayed leaf senescence, and a reduction in fruit size (Jimenez, LG and M. J. Davis 1987). The economically most important, bacterial disease on peaches is bacterial spot which causes major yield losses and can lead to approximately $4,500 United States dollars per acre of damage (Stefani 2010). Bacterial spot is caused by *Xanthomonas arboricola* pv. *pruni* which is a gram-negative bacterial pathogen of peaches.

Section 3: *Xanthomonas arboricola* pv. *pruni*

Xanthomonas arboricola pv. *pruni* (Xap) is found in most major stone fruit producing areas such as the United States., Europe and Asia. Xap was first described in North America in 1903 and has since spread throughout the United States after (E.F. Smith 1903). Xap infects many hosts and is an economically important pathogen on peach, nectarine Japanese plum, apricot, and almond (Ritchie, D. F. 1995). Xap is most severe on peach and plum and causes major yield losses whereas it is not as aggressive on other stone fruits such as apricot (du Plessis 1988). The life cycle of Xap has evolved to match its host's life cycle.

The life cycle of Xap is interwoven with the peach tree's yearly life cycle (Appendix A). After the Xap has enters the peach tree it begins multiplying and syncing to the trees yearly cycle. In early spring the Xap begins to rapidly propagate in year old shoots of the tree. The cankers on the tree can harbor the bacteria and when bud break occurs Xap begins to follow suit by multiplying in the cankers (Appendix B) As the season progresses wind and rain events spread the Xap to the not fully expanded leaves and infects the foliage (Hugouvieux et al. 1998). The Xap also infects the small fruit around shuck split. Since Xap is a bacterium it does not have an active way to enter the tissue of the plant so it relies on injury or natural openings such as stomata or hydathodes (Hugouvieux et al. 1998). Wind-blown sand is key for the bacteria to enter the fruit and leaves causing micro abrasions. The fruit begin illustrating symptoms later in the spring as water soaked, small dark lesions. As the growing season continues Xap progresses through the field being spread by wind, rain and mechanical movement. Late infections occur on leaves and fruit causing smaller lesions on the leaves and freckles on the fruit. The infection on the leaves, if serious enough, leads to early senescence. The fruit that was infected earlier in the season with Xap have lesions that are sunken in and the fruit produces gummosis as a response. After final swell and harvest Xap persists in the field and continues to spread on foliage where it finds wounds and openings in the young shoots of the peach tree. After Xap enters the shoot the bacteria multiply causing cankers and overwinter as the tree enters dormancy (Battilani et al. 1999). Due to the life cycle of Xap, it is present in a field as long as the trees are present no matter the preventative spray or treatment that is applied.

More specifically, Xap has many methods and mechanisms that help it infect its host. When the bacteria are on the surface of the leaf they utilize the stomata and hydathodes to enter the leaf. After the bacteria enter the leaf they colonize mesophll with biofilm that is made up of hydrated polymeric matrix known as the extracellular polysaccharide (EPS) (Rickard et al. 2007). The biofilm that is produced by *Xanthomonas spp.* is unique to the bacteria and known as xanthan gum, and allows the bacteria to be protected from pH changes and plant defenses (Yun et al. 2006). The Xap had to attach to a portion of the leaf to ensure infection and *Xanthomonas spp.* have been shown to attach in the mesophyll tissue on the spongy parenchyma cells (Edward T. Cason Jr. et al. 1976). Control of Xap can be difficult but is possible with a combination of cultural practices and preventative sprays.

Section 4: Management of Bacterial Spot

Cultural practices are key to managing Xap. The first factor to consider is the cultivar that is planted in an orchard because some cultivars have shown tolerance to bacterial spot while others are highly susceptible. Site selection is very important when considering soil type, past orchards, diseases present, and amount of rain fall. Wind breaks are key to controlling bacterial spot because of how wind-blown sand is key in the spread of Xap (Goodman and Hattingh 1988). Controlling ground cover and weeds is not a necessity for the control of Xap because there is no evidence showing a link in ground cover/weeds leading to a higher infection rate of Xap (Lamichhane 2014). Plant spacing is

key to reduce canopy humidity which leads to environmental susceptibility to the disease (Zehr et al. 1996). Additionally, nutrient imbalance also leads to more susceptibility to bacterial spot so a fertilizer program that is thought out and applied correctly is crucial (Stefani 2010). Timing of pruning is a critical factor to the spread of many orchard diseases, and that includes Xap, so growers need to ensure pruning is practiced at a good time in relation to the stage of the tree and environmental factors (Goodman and Hattingh 1988).

Along with cultural practices, chemical products are available to help control bacterial spot. All chemical sprays for Xap are based off of preventative sprays and there is no curative spray that works for the bacterial infection. At the end of dormancy to around shuck split, copper-based compounds are used in cover sprays to help control the spread of Xap in the field (D Horton et al. 2020). Copper can cause phytotoxicity at recommended rates on the foliage of the peach trees so as the season progresses less copper is applied. Therefore, oxytetracycline-based sprays are used as a cover spray for bacterial spot since the chemical does not cause phytotoxicity to peach foliage at the recommended spray levels. There has been more interest and research in biocontrols for many pathogens including Xap. A study was conducted using *Pseudomonas aeruginosa* LV strain to control the spread of Xap on peach trees (Vasconcellos et al. 2014). The biocontrol study showed a significant difference in control when compared to a non-controlled plant (Vasconcellos et al. 2014). With the use of chemical sprays on bacteria there is always a concern of chemical resistance.

Section 5: Chemical Tolerance and Resistance of *Xanthomonas spp.*

Most bacteria have adapted or evolved ways to tolerate or mitigate active ingredients in common chemical products and antibiotics in the medical field and in agriculture. In *Xanthomonas spp.* chemical resistance is widespread, more specifically copper resistance found in *Xanthomonas campestris* pv. *viticola* on grapevine, *Xanthomonas campestris* pv. *Juglandis* on walnut trees, *Xanthomonas citri* on citrus trees, and *Xanthomonas* pathogens on tomato and pepper (Chand et al. 1994; Gardan et al. 1993; Behlau et al. 2011). Pepper and tomato *Xanthomonas spp.* have illustrated some of the earliest and most wide spread copper tolerance genotypes discovered (Marco and Stall 1983; Richard et al. 2017). In Italy, copper resistance has also been discovered in Xap recently in 2017 (Giovanardi et al. 2017). In addition to copper resistance, copper tolerance is reported in *Xanthomonas spp.* such as in Australia *Xanthomonas campestris* pv. *vesicatoria* on pepper plants and in Brazil *Xanthomonas citri* on citrus trees (Martin et al. 2004; Marin et al. 2019). In *Xanthomonas spp.* resistance and tolerance could be caused by different mechanisms and processes.

There are many ways bacteria sequester or overcome chemicals and induce resistance and *Xanthomonas spp.* are no exception. There are two known gene clusters in *Xanthomonas spp.* that induce resistance to copper which are the copLAB cluster and the copABCD cluster (Behlau et al. 2011; Pereira et al. 2015; Richard et al. 2017). The gene cluster copLAB was discovered in *Xanthomonas spp.* in 2011 and was determined that the gene cluster was necessary for copper resistance (Behlau et al. 2011, 2012). The copLAB

gene cluster controls how the bacterial cell sequesters the copper by conferring proteins to bind to the copper ions and accumulating the copper in the periplasm of the cell not allowing the ions to enter the cytoplasm (Cooksey 1990; Voloudakis et al. 2005). Each of the three genes in the copLAB cluster are vital and all have to be present for the sequestering process to work (Behlau et al. 2011). The copABCD cluster was discovered in *Xanthomonas spp.* in 2015 and identified again in 2017 (Pereira et al. 2015; Richard et al. 2017). The gene cluster copABCD encodes for copper-binding proteins that sequester the ions out of the cell, similar to the copLAB gene cluster (Adaikkalam and Swarup 2005). Both gene clusters illustrated that they are up regulated or activated in the presence of a high amount of copper ions suggesting the proteins are not produced in a large quantity until the bacterial cell needs to sequester the copper ions (Adaikkalam and Swarup 2005; Behlau et al. 2011). When growing the bacterial colonies out that have the cop resistant genes they turn a bluish color because of the accumulation of copper ions in the periplasm (Voloudakis et al. 2005).

In comparison to the resistant strains of *Xanthomonas spp.* some strains illustrate tolerance but not complete resistance. Tolerance is defined to be strains of bacteria that can tolerate a certain product at a lower level than the resistant strains but at a higher level than in sensitive strains. There is not a known specific genetic marker for tolerant strains but the leading theory is that there is a group of highly conserved genes that are responsible for tolerance (Fan et al. 2018). The suspected gene cluster are the cohLAB genes which have shown to increase in expression in the presence of copper (Marin et al. 2019). Also, the copper efflux regulator-like proteins are possible reasons for copper tolerant strains

because they have shown to cooperate with DNA and RNA polymerases which increase the transcription of the genes to transport copper ions out of the cell (Ma et al. 2009).

Oxytetracycline is an antibiotic that binds to the ribosome to inhibit the translation and binding of aminoacylated tRNA to the specific A site (Chopra and Roberts 2001). No published reports of any *Xanthomonas spp.* having tetracycline resistance has been reported yet (McManus et al. 2002). However, tetracycline resistance in other phytobacteria that infect the same host as Xap has been reported such as *Pseudomonas syringae* pv. *syringae* which causes bacterial canker of peach (RA Spotts and Cervantes 1995). The most common mechanism for tetracycline resistance are efflux pumps that sequester the antibiotic and is attributed to 28 known tet genes (Fan et al. 2007). Although none of these genes have been found in Xap they are still of a concern because of bacterial horizontal gene transfer.

Horizontal gene transfer is a form of genetic exchange of bacteria which is a key tactic to the bacteria adapting and surviving in the environment they are in. Horizontal gene transfer can occur between the same species of bacteria to completely different genera. Recent research has been done illustrating different xanthomonas transferring copper resistant genes to each other (Behlau et al. 2012). Additionally, the same study also showed that even different genera of bacteria such as *Stenotrophomonas maltophilia*, which is rarely a plant pathogen, could transfer the copper resistant genes to *Xanthomonas spp.* (Behlau et al. 2012). With chemical resistance on the rise, new and different ways to control the bacteria must be utilized and developed.

Section 6: Management for Chemical Resistance

Integrated pest management (IPM) is crucial in managing any disease in an orchard and in every other agricultural crop. IPM techniques allow for diverse control of many different pest and pathogens while using many diverse, unrelated regiments of control. For example, controlling different tree stresses, such as drought stress and nutrient deficiencies, allows the trees to be more resilient to different plant pathogens and pests. Many techniques from disease forecasting models to precision sprayers can be utilized in an IPM program. Since many bacterial diseases in orchards, including bacterial spot, persist and overwinter in the tree, many preventative measures must be taken (Xin and He 2013). The first approach to an IPM program for bacterial spot is cultivar choice and location of planting. Cultivar and location are extremely important due to the fact of how susceptible many peach cultivars are to Xap and how the disease thrives and spreads in certain environments with wind-blown sand (Goodman and Hattingh 1988). Preventative copper sprays are used to stop the spread of the bacteria when present in the field. Two main types of copper are sprayed for bacterial spot that show to work the best which are sulphate- and oxychloride-based formulations (Garcin et al. 2005). Oxytetracycline sprays are also used to prevent the spread of Xap in the field and can be sprayed in conjunction with copper to reduce phytotoxicity of the foliage. Supplemental control options have been used such as pruning out cankers in the field during the late winter and early spring (Stefani 2010) (Appendix B). New options for control are arising such as biological control with *Pseudomonas spp.*

outlined previously and forecast models used to predict when to spray or use preventative measures (Vasconcellos et al. 2014; Battilani et al. 1999). IPM for bacterial spot of peach is essential for controlling the disease because of its infectious potential and the little options available for control. With the advancement in technology better techniques will be developed to add to the arsenal to mitigate and control the spread of Xap throughout orchards.

Objectives of the Study

The objectives of this study are (1) determine correlation of number of spring cankers to bacterial spot disease incidence and severity, and (2) examine chemical sensitivity of the isolates and their progression through the growing season in relation to the spray programs from selected growers.

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SENSITIVITY OF *XANTHOMONAS ARBORICOLA* PV *PRUNI* FROM PEACH SPRING CANKERS, LEAVES, AND FRUIT TO COPPER

Introduction

Xanthomonas arboricola pv. pruni (Xap) is an economically important pathogen causing bacterial spot of peach and other stone fruits worldwide (E.F. Smith 1903), including the southeastern United States (EFSA Panel on Plant Health 2014). Bacterial spot causes increases cost in nursery production, and reduces orchard productivity and marketability of peach fruit (Stefani 2010). The disease can also lead to early defoliation, which can impact the tree resilience and lifespan. Xap favors climates that are temperate and humid with a higher amount of annual rainfall, making the southeastern US a prime climatic region for the bacteria to flourish (Garita□Cambronero et al. 2018; Lamichhane 2014).

In late winter/early spring, Xap begins to rapidly multiply in infected shoot cankers and buds (Appendix B). Wind and rain spread the disease to the leaves which are most susceptible when they are not fully expanded yet. Unlike fungi, bacteria do not have mechanism for forceful entry and have to enter passively (Hugouvieux et al. 1998). Xap enters the leaves through open stomates, hydathodes, and wounds caused by windblown sand and mechanical damage (Hugouvieux et al. 1998). Fruit are most susceptible to bacterial spot from ‘shuck split’ to ‘pit hardening’ and begin to show symptoms in the late spring and early summer. Xap spreads back and forth from leaves and fruit throughout the growing season by rain, windblown sand and mechanical spreading. Symptoms on ripened fruit range from deep crater lesions from early infections to small freckles from later infections. After harvest, Xap still persists on the leaves and finds wounds and openings from damage and leaf abscission zones on the young shoots where the bacteria enter and overwinter when the tree goes dormant in the early winter (Battilani et al. 1999) (Appendix A).

To be more specific, after the bacteria enter the leaf through natural openings or wounds, they congregate in the mesophyll (Kastelein et al. 2014). In the open-air spaces inside the mesophyll the bacteria multiply and start to produce biofilms in a matrix also known as the extracellular polysaccharide (EPS) (Allan□Wojtas et al. 2010). The EPS is comprised of polysaccharides, nucleic acids, and proteins and allows the bacteria cells to cling together and to produce the large structures inside the leaf (Allan□Wojtas et al. 2010). The production of EPS in a plant cell's mesophyll is what is thought to give the water-soaked look to foliage and tissue common for *Xanthomonas* spp. (Rudolph et al. 1994). *Xanthomonas* spp. also produce xanthan in the EPS giving it the yellow, puffy distinct look *in vitro* (Yun et al. 2006).

Management of bacterial spot in stone fruits is largely based on cultural practices, cultivar choice, and chemical control. Trees grown in sandy soils are more susceptible to bacterial spot (Lamichhane 2014). Orchards in locations with a warm and wet spring and little wind circulation are more at risk for infection. Trees that are stressed by other biotic factors such as nematodes or abiotic factors (e.g. heavy rainfall) are more susceptible to bacterial spot (Matthee and Daines 1968), but tree stress is not a prerequisite to infection or disease progression. Other cultural methods influencing tree health, which also plays a role to control bacterial spot, are fertilizer applications and the timing of pruning (Garita□Cambronero et al. 2018).Wind breaks and other methods to reduce the spread of inoculum are also helpful when controlling the disease (Garita□Cambronero et al. 2018; Ritchie 1995; 1999). Selection of disease resistant/tolerant cultivars is a key practice for bacterial spot management in stone fruits. However, in peach production, few cultivars are tolerant to bacterial spot, and every cultivar has shown bacterial spot symptoms when the environment is conducive for disease development (Yang et al. 2013).

In the southeastern United States, spray of copper-based compounds is designed for reduction of Xap inoculum and prevention of infection starting early in the season before bud break (Horton et al. 2020). As the season progresses, copper sprays are continued until three to four weeks prior to harvest. Although resistance to copper was reported in other

bacterial plant pathogens, no resistance has been reported in Xap in the United States. Copper resistance in bacteria was first reported in *Pseudomonas syringae* on tomato in 1986 and later in *Xanthomonas* strains (Trevors JT. 1986 ; Cooksey et al. 1990). There have been two gene clusters discovered in *Xanthomonas* spp. that confer copper tolerance, *copLAB* and copABCD with copLAB being the most prominent in *Xanthomonas* spp. (Behlau et al. 2011; Richard et al. 2017; Pereira et al. 2015). Each gene cluster must have every gene in order to express the proteins to sequester the copper ions (Behlau et al. 2011).

The objectives of this study were to isolate Xap from spring cankers, leaves, and fruit, and to examine their sensitivity to copper over the course of the season in a two-year period.

Materials and Methods

Experimental Layout

Four blocks of cultivar O'Henry were included in this study, each located at a different farm. The four farms were near McBee (one block), Ridge Spring (two blocks), and Monetta (one block) in South Carolina. The latter produced peaches organically (org.), while the other produced peaches conventionally (conv.). From each block, one-year old shoots were collected at phenological stage 'late dormancy' and leaf and fruit samples were collected at 'pit hardening' and 'final swell' (fruit were still hard but no longer green at the stem end). Sampling was conducted from three sets of 10 trees per block each set was separated by at least 10 trees. In total, 30 trees were used per block and the same 120 trees were sampled over a span of two years.

In-field Sampling

At 'bud break' 10 one-year-old shoots about 40 to 60 cm in length were collected arbitrarily from each experimental tree and brought back to the lab. The cut ends of the shoots were placed in 5 cm of water and incubated for two weeks with 12-hour photoperiod

at room temperature. Four canker categories were identified on the collected shoots. Bud canker, a necrotic bud surrounded by necrotic tissue; tip canker, the necrotic terminal end of a collected shoot; non-concentric cankers, irregularly shaped cankers with a water-soaked center; and concentric canker, a circular look with water-soaked center (Appendix B). The canker types were counted on the set number of shoots collected. At 'pit hardening' and 'commercial maturity', two symptomatic fruit and approximately five symptomatic leaves were harvested per tree. The same trees were used for every collection time including the canker collection. A two-way ANOVA analysis was used and a mixed model was run on the data using the JMP software at a 95% confidence level. The fixed effect was farm, and the random effect was sampling time.

Bacterial Extraction and Identification

The cankers taken from each shoot were surface sterilized for 3 minutes in a 10% bleach solution, rinsed with sterile water, and dried. Using forceps, the outer epidermal layer of the shoot was peeled back and two centimeters of tissue were removed from the discolored canker margin. The tissue was then plated on Pseudomonas agar F (Difco) (PA) plates. PA plates were used to easily identify contamination of Pseudomonas because the media enhances fluorescein production in many Pseudomonas, thus, making it easier to identify on the plate. The fruit and leaf samples were surface-sterilized with 10% bleach solution. A toothpick was used to puncture the bacterial lesion either on the fruit or leaf and placed into a 1.5 ml tube in sterile water. After five minutes the toothpick was removed from the 1.5 ml tube and a 25 µl loop was used to streak the suspension on PA plates. Suspected Xap colonies were streaked out on sucrose peptone agar (SPA) for single colony identification. Single Xap-like colonies were transferred by standard techniques outlined in Microbiological Applications (Benson 1967) and confirmed to the species level using qPCR as described by (Palacio-Bielsa et al. 2011). A two-way ANOVA analysis was used and a mixed model was run on the data collected above using the JMP software at a 95%

confidence level. The fixed effect was canker type and the random effect was sampling time.

Chemical Assay

To determine sensitivity to copper, bacterial strains were streaked on Mannitol-glutamate yeast extract (MGY) media in 90 mm petri dishes amended with 20 µg/ml $CuSO_4 \cdot 5H_2O$ (Copper sulfate pentahydrate, CSP, Chem-Impex Int'l Inc., Wood Dale, IL) at approximately 24 °C for one day to induce copper resistance genes that could be present (Marin et al. 2019). Then, a bacterial suspension of 10^8 CFU/ml (OD_{600} = 0.1) in sterilized water was transferred by a 5 µl drop in each well in a 24 well plate containing CSP at 0, 150, 200, or 500 µg/ml (Marin et al. 2019). The copper tolerant strains were then tested again on CSP amended MGY plates. The plates were incubated at 24 °C for four days before inspection. The bacteria plated on the CSP amended cells were rated as follows: copper sensitive (Cu^S) that did not grow at 150 µg/ml or higher, low copper tolerant (LCT) strains that grew up to 150 µg/ml, high copper tolerant (HCT) strains that grew up to 200 µg/ml, and copper resistant (Cu^R) strains that grew past 200 µg/ml (Marin et al. 2019).

Presence of known Copper Resistant Genes

PCR with previously-designed primers for copLAB (Behlau et al. 2011) and copABCD clusters (Richard et al. 2017) were used to detect known copper resistance genes in the Xap strains (Behlau et al. 2011; Richard et al. 2017). The primers used for copper resistant gene clusters are listed on table 2.2 below (Behlau et al. 2011; Richard et al. 2017). The samples were run with the following PCR protocol: 95°C for 5 minutes, then 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds, followed by 72°C for 10 minutes and 4°C for hold. A Xap resistant control was used (XAP-CU-R) that had the copLAB cluster and was collected in 2017 from an ornamental

plant. Also, a Xap sensitive strain was used as a negative control (XAP-1) collected in 2017 in a peach field.

Results

Four canker types, including bud cankers, tip cankers, non-concentric cankers, and concentric cankers, were found at all farms in each year (Appendix B). In both experimental years, bud cankers were the most prevalent ($P < 0.0001$; Fig. 2.1). No difference in prevalence was observed among the other, less prevalent canker types. Despite the higher prevalence, the recovery rate of Xap from bud cankers was not higher compared to the recovery rate from the three other cankers in conventional orchards (Fig. 2.2; $P = 0.1889$). In the organic orchard, however, more Xap was recovered from bud cankers than from any other canker (Fig. 2.2; $P = 0.0457$). Also, all cankers taken together from the organic farm yielded more Xap than cankers from two of the three conventional farms (Fig. 2.2). More cankers were present in 2018 than in 2019 (data not shown). In two of the three conventional farms tip cankers produced the most Xap numerically, but not statistically ($P = 0.4434$).

Three copper sensitivity phenotypes were discovered in this study, copper sensitive (sensitive), low copper tolerant (LCT), and high copper tolerant (HCT) (Table 2.1). Most strains (81%) from both years and all farms were sensitive to copper (Table 2.1). In 2019, fewer strains (101) were identified to be 'sensitive' compared to 2018 (139). A total of 43 strains (15%) were LCT with 23 and 20 strains isolated in 2018 and 2019, respectively. Only 13 strains were identified to be HCT with 3 and 10 strains collected in 2018 and 2019, respectively (Table 2.1). Remarkably, most HCT strains were recovered from the organic farm (Table 2.1). The presence or absence of copper resistance gene clusters copLAB and copABCD was evaluated in LCT and HCT isolates. Primers for the known copper resistant gene clusters copLAB and copABCD yielded the expected bands for the positive control strains but not for the strains collected in this study (Figure 2.3).

Discussion

Twig cankers can be formed by bacterial and fungal pathogens, including *Pseudomonas syringae*, *X. arboricola, Leucostoma personii*, and *Monilinia fructicola*. We focused on isolating Xap from cankers and did, therefore, not do a survey of pathogens potentially present in the cankers. The recovery rate of Xap in cankers was mostly in the range of 10 to 20%, indicating that most cankers were formed by pathogens other than Xap. But it is also possible that Xap initially caused some of the cankers but later died due to environmental conditions or during the isolation process when using sterilization techniques. Survival of the bacteria in cankers may also be affected by spray coverage and the copper dose applied. Although not the focus of this study, many of the cankers did reveal Pseudomonas species. *Pseudomonas syringae* pv *syringae* is a pathogen of peach and capable of producing cankers. The fact that we did isolate Xap from all four canker types indicates that the pathogen can cause tip, bud, and non-concentric cankers in addition to the 'text-book' concentric canker. Our study also indicates that the most common canker type caused by Xap was the bud canker. Xap forming primarily bud cankers is consistent with its disease cycle; bud development is initiated in the middle of summer when the bacteria are prevalent in orchards. The recovery rate of Xap from organic farm twig cankers was higher compared to two conventional farm twig cankers possibly due to the differences in management and chemical treatments the orchard used compared to the conventional orchards. Organic orchards are limited to the use of copper-based sprays and cannot supplement other sprays such as oxytetracycline based sprays. The latter may have had a detectable impact on survival of bacteria in those cankers. Also, in the organic block a bacterial canker disease epidemic in 2018 damaged many of the experimental trees and resulted the field not being managed as stringently as other blocks to reduce spray cost. This may have led to the greater number of cankers in the organic field, greater colonization of cankers, and greater survival of Xap in the cankers.

In this study no copper-resistant isolates were found, however, a significant number of LCF and HCF isolates was present in each of the four locations. The absence of copper-resistant Xap isolates contrasts with findings in other *Xanthomonas* pathogens in other crops such as walnut, citrus, and tomato (Gardan et al. 1993; RA Spotts and Cervantes 1995; Abbasi et al. 2015; Giovanardi et al. 2017). The copper resistance phenotype in other *Xanthomonas* pathogens has been characterized by the presence of the two plasmid-based resistance gene clusters copLAB and copABCD (Behlau et al. 2011; Richard et al. 2017) which were absent in the tolerant isolates in this study (table 2.2). Recent evaluation of copper sensitivity in *Xanthomonas spp.* reported a 200 µg/ml threshold for copper resistance on MGY (Basim, 2005). However, other studies using the similar dose but different media, such as nutrient agar (NA) or broth, referred to isolates growing on that dose as 'tolerant' to copper (Martin et al. 2004). Heavy metal resistance and tolerance is dependent on many variables and can change with media and ion exposure (Zevenhuizen et al. 1979). In this study two types of media were used, MGY and NA, in order to get a more precise number of the sensitivity of the Xap strains and to confirm if some strains were illustrating tolerance or resistance to copper. In a recent study analyzing copper resistance in Italy showed two isolates grow up on 200 µg/ml amended MGY agar plats and did not have the copLAB genes identified to be essential for copper resistance in Xap (Giovanardi et al. 2017; Behlau et al. 2011). This study illustrates that there is another possible mechanism of resistance that has not been discovered. Similarly, in this study there were 15 strains discovered that grew up to 200 µg/ml CSP-amended MGY agar plates but did not show copLAB genes using the primers designed by Behlau et al. (2011) There is a possibly that there is an unknown mechanism on a second plasmid, described in Giovanardi 2017, in the Xap strains (Giovanardi et al. 2017).

The copper tolerant phenotypes from our study were likely selected due to multiple years of frequent exposure to copper-based products. All growers reported copper applications starting at late dormancy all the way to about three weeks before harvest at 7 to 10-day intervals. Spray calendars were successfully obtained from two conventional farms (farms one and three) in this study and an average of 18.75 copper sprays for bacterial

spot were applied. In addition to frequency, the spray calendars illustrated that farms were using lower metallic copper equivalent (MCE) percentage sprays than recommended in the Southeastern Peach, Nectarine, and Plum Pest Management and Culture Guide, but within the label rate, thus possibly allowing the Xap strains to adjust to the copper applied. Tolerant Xap strains were not completely controlled at 150 µg/ml CSP *in vitro* but rather illustrated slower growth rates compared to known resistant strains of Xap. This suggests that copper tolerant isolates may cause disease in the field even though copper is applied preventatively and regularly.

Unlike other plant pathogens that develop resistance, bacteria do not alter the activation site but they can change their cellular membrane to enhance efflux of copper ions from the cells and prevent copper influx, or they can produce siderophores or copper-binding proteins to sequester copper in the extracellular or periplasmic space to prevent copper transport across the cell membrane (Braud et al. 2009, 2010; Cervantes and Gutierrez-Corona 1994; Cha and Cooksey 1991, 1993). The development of resistance in bacteria does not require evolving independently like many other organisms but is developed collectively by exchanging genetic material horizontally between pathovars, species, and even genera (Cooksey et al. 1990). Even though only tolerance was discovered studies show that bacteria can transfer genetic material, including resistant genes, to one another and even across different genera (Behlau et al. 2012). There have been recent studies speculating the conserved genes that help with homeostasis are attributed to copper tolerance known as the cohLAB genes (Marin et al. 2019). Also, Fan et al. (2018) found an unidentified gene in a *Xanthomonas spp.* that is induced by copper ions that could also lead to bacterial strains that are tolerant to copper and do not have the copLAB gene cluster (Fan et al. 2018). Copper efflux regulator-like proteins could be another possible reason for the copper tolerant strains found in this study because they have shown to cooperate with DNA and RNA polymerases which increase the transcription of the genes to transport copper ions out of the cell (Ma et al. 2009). Furthermore, *Xanthomonas spp.* have shown to obtain copper resistant genes from other epiphytic bacteria which could lead to future resistance in the field (Behlau et al. 2012).

Bacterial spot disease incidence in leaves and fruit from 2019 indicated significant differences in leaf to fruit disease incidence ratio between conventional farms 1 and 3 (data not shown). Specifically, farm one had significantly more bacterial spot on the fruit compared to farm 3 ($P = 0.0016$). The two farms and O'Henry blocks were within 10 miles from each other and thus environmental factors were similar. Spray records indicated differences in the two farms approaches for bacterial spot management, however. Farm one applied fewer copper sprays but used higher doses of MCE in each spray compared to farm 3. Whether there was a cause and effect between spray strategy and fruit disease incidence could not be confirmed beyond the one-year association, but this preliminary observation is worthwhile pursuing in future studies.

Conclusion

Xap can be found in many different types of cankers, including bud cankers, tip cankers, non-concentric cankers, and concentric cankers. This would imply that cutting out any canker in the orchard may reduce inoculum and be beneficial for bacterial spot control. Tolerance to copper was frequent in the populations sampled. More research is justified to determine whether the different copper-tolerant phenotypes can still be controlled with existing strategies.

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Table 2.1 Origin of copper and oxytetracycline phenotypes collected in two experimental years at three conventional and one organic South Carolina farms

		Tissue Type (#isolates)								
		2018				2019				
Phenotype[z]	Farm	Canker	Fruit	Leaf	Total	Canker	Fruit	Leaf	Total	Total
Sensitive	Conv. Farm 1	16	25	9		-	29	18		97
	Conv. Farm 2	3	38	25		-	8	7		81
	Conv. Farm 3	15	-	-		-	2	2		19
	Organic Farm	1	-	7		25	1	9		43
	Total				**139**				**101**	**240**
LCT	Conv. Farm 1	1	-	4		-	-	-		5
	Conv. Farm 2	1	2	3		-	3	3		12
	Conv. Farm 3	6	-	2		-	-	-		8
	Organic Farm	4	-	-		14	-	-		18
	Total				**23**				**20**	**43**
HCT	Conv. Farm 1	-	1	1		-	-	-		2
	Conv. Farm 2	-	-	-		-	-	-		
	Conv. Farm 3	1	-	-		-	-	2		3
	Organic Farm	-	-	-		3		5		8
	Total				**3**				**12**	**13**
Oxytet resistant + HCT	Conv. Farm 1	-	1	1		-	-	-		2
	Conv. Farm 2	-	-	-		-	-	1		1
	Conv. Farm 3	1	-	-		-	-	3		4
	Organic Farm	-	-	-		3	-	5		8
	Total				**3**				**12**	**15**
Overall Total										**298**

[z]LCT= Low Copper Tolerant; HCT= High Copper Tolerant; Oxytet= Oxytetracycline; - = Zero isolates from that origin

Table 2.2 List of primers used for the detection of known copper resistant gene clusters in Xap via conventional PCR

Gene Cluster	Prmer	Forward/Reverse	Primer Sequence
copLAB	copL	F	CCGTGTCAGCCTCCTCACTTCTAC
copLAB	copL	R	CAGCGGCATGACATCCAGGCC
copLAB	copA	F	CCTCCATGGCACGGACACTTCCATC
copLAB	copA	R	CCAGACATATCCATCGACCCATGATCCA
copLAB	copB	F	CTCAGGATCACTCTGCACATCAG
copLAB	copB	R	GCACGTAGCTCTTAATCGAGTTGTC
copABCD	copA_	F	GCCGTTCGCCATAGTTCAATC
copABCD	copA_	R	CGGTACTGACCTACGCAATGCTC
copABCD	copB_	F	TCAACACGCTCGGATTCGTCT
copABCD	copB_	R	ACTGCTGCTCACCAATCGTT
copABCD	copC_	F	TACTTCACACTAAACGAGATG
copABCD	copC_	R	ACTTGTGGTTTCCTCGCCTGT
copABCD	copD_	F	CGACACGGATCACCCACGTC
copABCD	copD_	R	TCTCCATCCGTCTCGCGCTCT

All primers above taken from following papers: Behlau et al. 2011 and Richard et al. 2017

Figure 2.1 Occurrence of four canker types collected from 300 shoots of 30 trees per farm and year at three conventional and one organic South Carolina farms in 2018 and 2019.

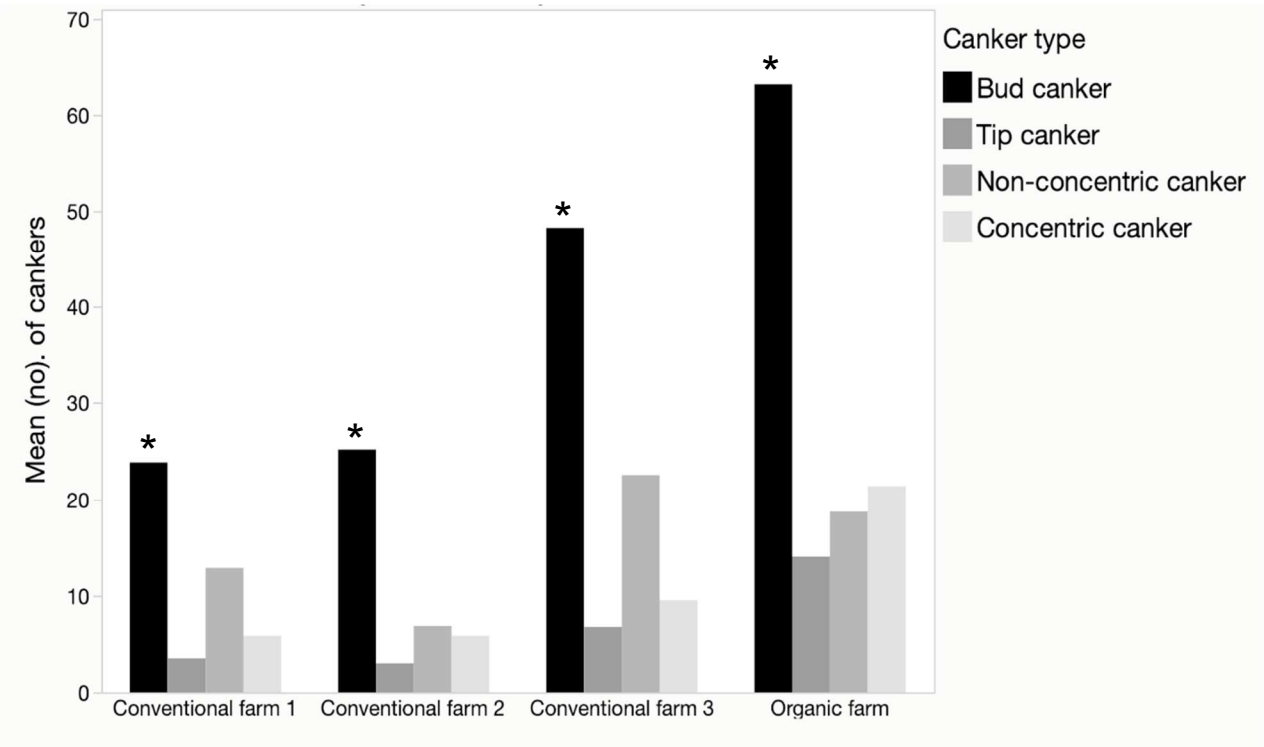
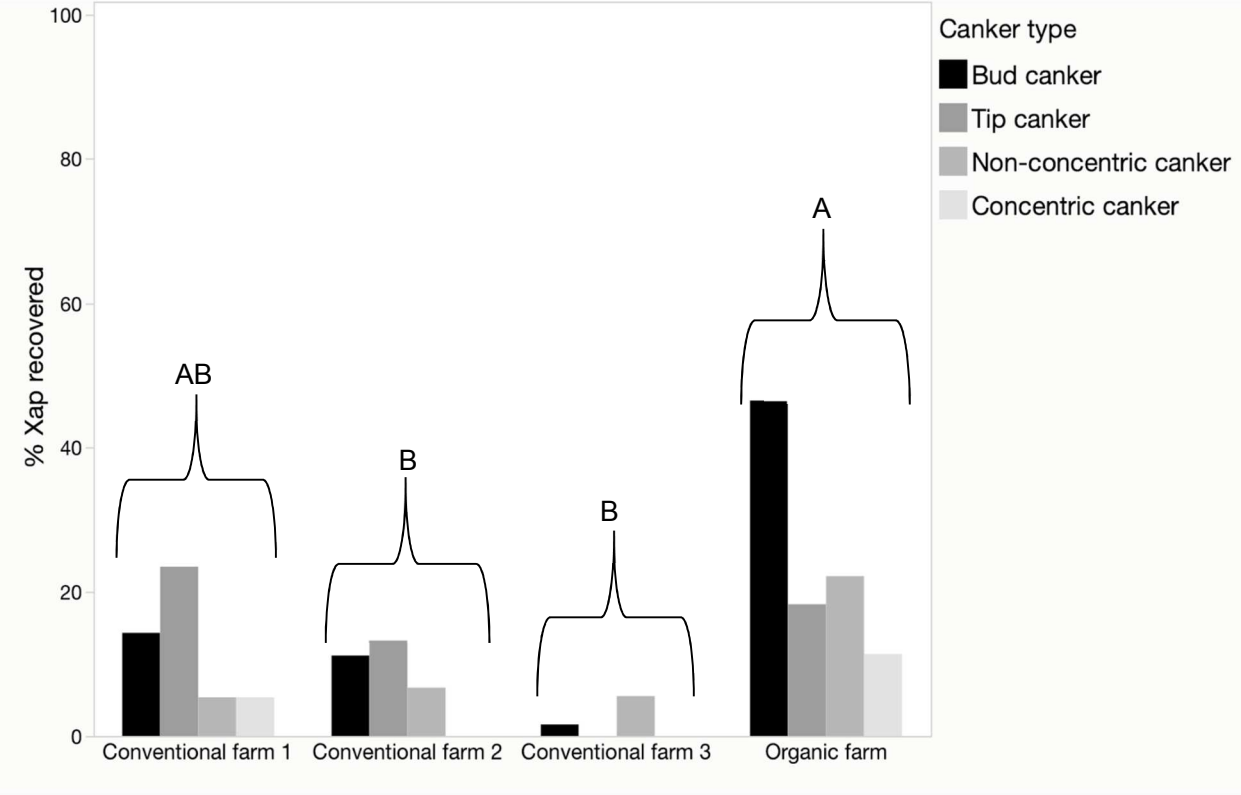


Figure 2.2 Percent of *Xanthomonas arboricola* pv. *pruni* (Xap) recovered from 300 shoots of 30 trees per farm in 2019 at three conventional and one organic farms in South Carolina



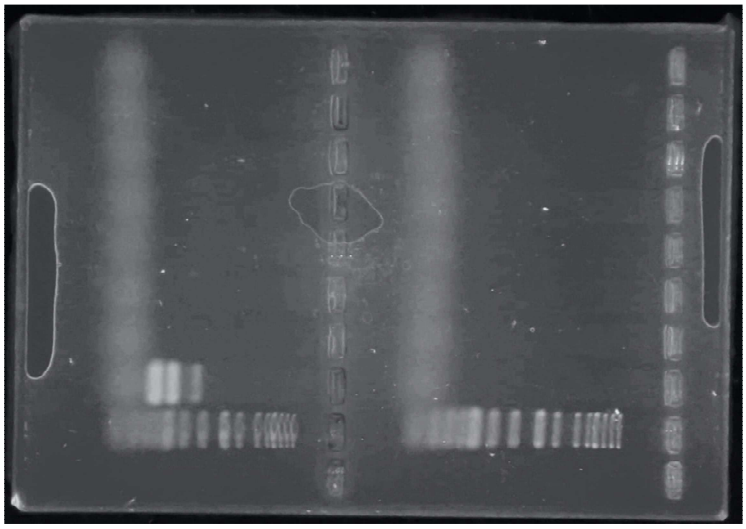
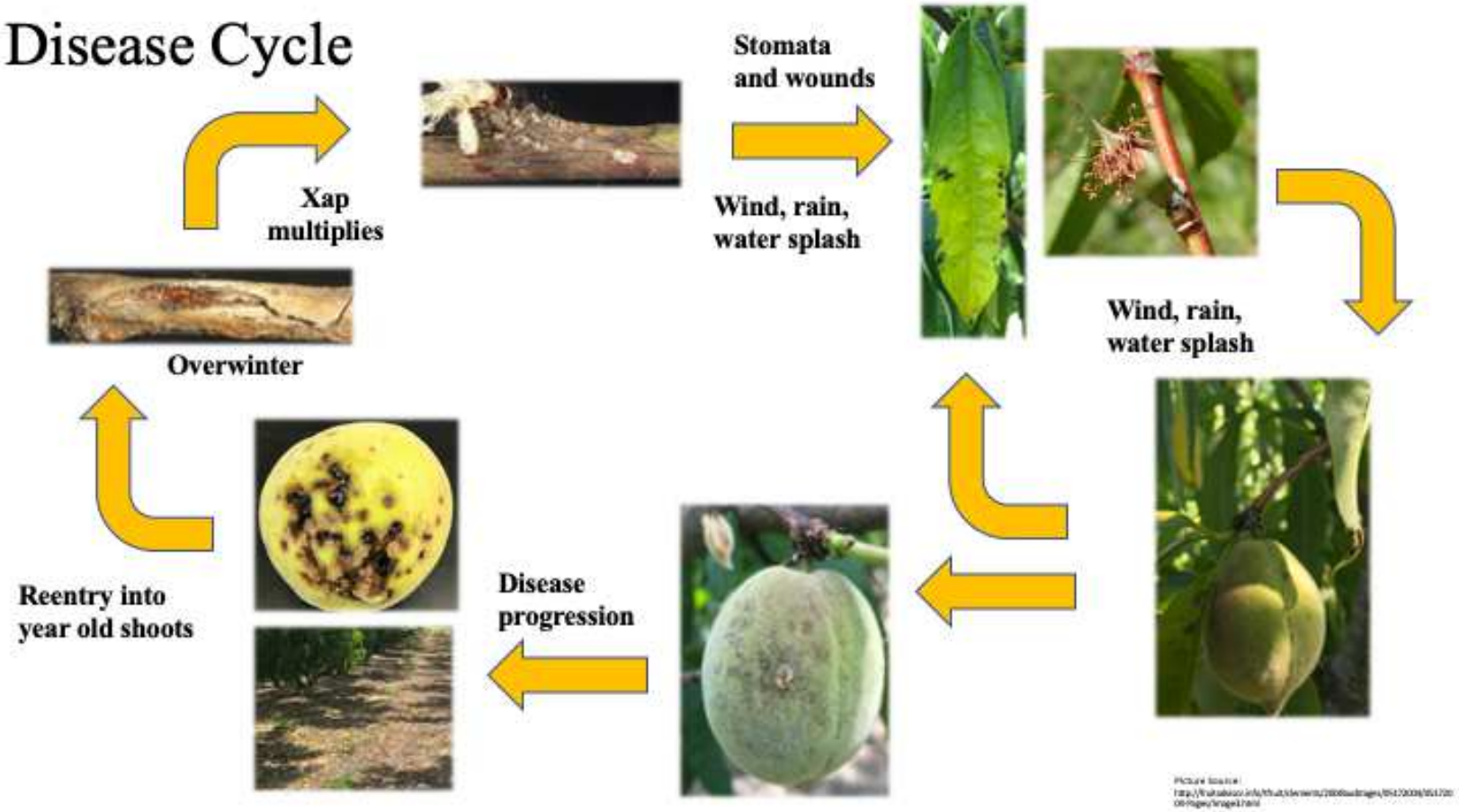


Figure 2.3 Gel electrophoresis on 1% agarose gel ran for 40 minutes and placed in gel red solution for 10 minutes. Lane 1 1 kb ladder, Lane 2 XAP-CU-R copper resistant reference strain, Lane 3 XAP-1 copper sensitive reference strain, lanes 4 to 17 strains HCT and LCT Xap strains collected from this study in 2018 and 2019 respectively.

APPENDICES

APPENDIX A

An illustration of the life cycle of *Xanthomonas arboricola* pv. *pruni*



APPENDIX B

Illustration of different canker types identified from one year old peach tree shoots in the study