A MICROBIOLOGICAL STUDY OF *ERWINIA AMYLOVORA* EXOPOLYSACCHARIDE OOZE

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ABSTRACT

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Fire blight, caused by the pathogen *Erwinia amylovora* (Burrill) Winslow et al, is the most devastating bacterial disease of pome fruits around the world. The primary dispersal of *E. amylovora* is through ooze, a mass of exopolysaccharides and bacterial cells that is exuded from infected host tissue. Over the 2013 and 2014 field seasons, 631 ooze droplets (201 in 2013 and 435 in 2014) were collected from field inoculated trees. Populations of *E. amylovora* in ooze droplets range from 10^8 to 10^{11} colony forming units per micro liter (cfu/µl). In the host tissue surrounding the droplets even larger populations of *E. amylovora* reside in the surrounding 1 cm of tissue. Three apple cultivars with varying levels of resistance were also infected with four Michigan *E. amylovora* strains. Using scanning electron microscopy, host tissue was examined for the origin of the ooze droplets and erumpent mounds and small (10 µm) tears were the only bacterial sources observed. Genetic expression analysis indicated that *E. amylovora* cells in stem sections located above ooze drops and in ooze drops were actively expressing virulence genes. If disseminated to susceptible host tissue, these cells would be primed for infection. The current study suggests the following: high populations of *E. amylovora* are present in ooze droplets which larger populations found in darker pigmented, smaller volume droplets. These droplets are rupturing out from the parenchyma and epidermis of the host, with evidence of immense pressure being involved from SEM observations. Ooze droplet volume and population can vary between host cultivar and the virulence of a specific *E. amylovora* strain. Genetic expression analysis of virulent factors in *E. amylovora* indicated that the bacteria in ooze were primed and ready to infect a new susceptible host.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW OF *ERWINIA AMYLOVORA* OOZE

1. Abstract

Thought to be the main dispersal method of *Erwinia amylovora*, the causal agent of fire blight, ooze has been neglected in the fire blight literature for many years, as the last fully dedicated paper to ooze was published in 1939 by E.M. Hildebrand (Hildebrand, 1939). Since then, most work directly involving ooze has been based more on the exopolysaccharide characterization (Bennett and Billings, 1979) and *E. amylovora* aerial strands (Eden-Green and Billing, 1972).Ooze droplets have been mentioned as a side note in disease dispersal (Eden-Green and Knee, 1974; Bennett and Billing, 1978; Vanneste, 2000) and in internal movement (Schouten, 1989; 1990; 1991).

This literature review seeks to address the available information on ooze droplets as well as other pertinent areas of *E. amylovora* biology that is related to exopolysaccharide ooze.

2. Introduction and Historical Importance of *Erwinia amylovora*

Erwinia amylovora (Burrill) Winslow et al., a rod-shaped, gram negative bacterium that is a member of the *Enterbacteriaceae* family, is the causal agent of fire blight, the most serious bacterial disease present in apples and pears. *E. amylovora* is thought to be native to North America, infecting hawthorns and other native Rosceae species. Commercial apples and pears are not native to North America and are typically susceptible to the disease, which can ultimately result in the death of the host (van der Zwet and Keil, 1979; Vanneste, 2000). Since the 1780s, fire blight has been reported along the east coast of the United States and the disease eventually spread West with the pioneers through the rest of the continent (van der Zwet and Keil, 1979). On the east coast of the United States fire blight still has a major presence; the disease is so severe in pear trees that the industry is non-existent (Vanneste, 2000). The apple industry in the East however, is successful, with Michigan, New York, Pennsylvania, and Virginia producing

large yields yearly. The main reason apple production is successful is that apples have higher rates of overall resistance to the disease than pears (Vanneste, 2000; Zamski et al., 2006). Even still, apple orchards can be hit with devastating fire blight epidemics as consumers favor varieties that tend to be more susceptible (van der Zewt and Beer, 1995; Vanneste, 2000). In 2000, Southwest Michigan experienced one of these epidemics causing around $42 Million in losses over five years (Longstorth, 2001). Unfortunately, fire blight epidemics are sporadic and mostly unpredictable (Schroth et al, 1974; Vanneste, 2000).

2.1 Introduction to ooze- The dispersion method

The main dispersal method of *E. amylovora* is via exopolysaccharide ooze. Ooze contains *E. amylovora* that forms external droplets on the host's tissues. This slimy secretion consists of bacterial cells and exopolysaccharides (Eden-Green and Knee, 1974; Bennett and Billing, 1979; Vanneste, 2000). However, others describe ooze as containing plant materials as well, thought the paper never defined what plant materials are present (Zamski et al, 2006). In addition to sticky droplets, the escaped cells may also form thin strings of bacteria called aerial strands (Keil and van der Zwet, 1972; Schouten, 1990). These external masses of tissue can be wind or insect dispersed throughout an orchard, possibly leading to epidemics. Aerial strands and ooze drops once they dry and the *E. amylovora* cells inside ooze drops can stay viable for at least 25 months (Hildebrand, 1939; Bauske, 1968; Keil and van der Zwet, 1972; Southey and Harper, 1972). In most weather conditions, the ooze dries and hardens in under 24 hours (Slack, observation). In the event of rain or hail storms, the ooze can be dissolved in the water and spread quickly. It is particularly dangerous to have ooze, fresh or dried, present during hail or wind storms as the damage to the tree allows for quick infection by the bacteria (Keil and van der Zwet, 1972).

2.2 *E. amylovora* creates External Ooze Drops

Ooze typically emerges from infected tissue showing symptoms of disease, but also can be observed occurring further from the infection area (Hildebrand, 1939). Aerial strands have also been observed originating from ooze droplets (Slack, personal observation). Though ooze drops offer protection from desiccation (Koczan et al., 2011) and provide bactericide resistance (Hildebrand, 1939; Sutherland, 1988), they do not protect from extreme heat (50^{o}C) (Hildebrand, 1939).

2.3 Importance of Ooze in the Disease Cycle

The primary inoculum for fire blight is thought to come from the overwintering cankers formed from infection during the previous season (Schroth et al., 1974; Vanneste, 2000). In the spring, these cankers begin the disease process with the release of ooze drops (Thomas and Ark, 1934). However, some cankers may not ooze until later in the season and some do not have any viable bacteria present (Schroth et al., 1974; Beer and Norelli, 1977; van der Zwet and Beer, 1995). Because cankers do not always produce ooze at bloom, it is thought that epiphytic *E. amylovora* could also act as primary inoculum (van der Zwet and Beer, 1995). The bacteria that do form ooze from viable cankers are thought to be dispersed via rain, wind, and insects until enough cells arrive at flowers to infect (Schroth et al., 1974). From the initially infected flower, the bacteria can be spread by pollinating insects to other flowers (Thomas and Ark, 1934; Schroth et al, 1974). Ooze droplets can have populations higher than $1x10^{10}$ cfu/µl, which is similar to other pathogenic bacteria (Vieira Lelis et al., 2013). Pollinating insects, in particular bees, are these secondary carriers of cells from infected flowers throughout an orchard (Schroth et al., 1974; Vanneste, 2000). The initial number of infected blossoms needed to create an

epidemic can be quite small (Billing, 2011). This is important because there only needs to be a minimum population of $1.04x10^2$ CFU/ml on the blossoms for disease to take hold (Schroth et al., 1974). However, once on the stigma of a flower, populations of *E. amylovora* can reach up to $1x10^6$cfu/µl (Kozan et al., 2009). From those numbers, if there is ample ooze available, the occurrence of an epidemic seems more likely. This stage is known as blossom blight which can seriously affect fruit yield and can establish *E. amylovora* in an orchard.

Secondary infections occur from the flowers or cankers and spreads to the shoots, causing shoot blight (Vanneste, 2000). During and following bloom, the ooze can appear all over infected blossom clusters and shoots and continue to spread blight through an orchard. Shoot Blight is known best by its symptom of a shepherd's crook, which is caused by the wiling of a shoot (van der Zwet and Beer, 1995; Vanneste, 2000). Bacteria can easily spread from shoot to shoot and quickly over take an orchard with blight, as only 38 cells are needed for injured shoots to become infected (Crosse et al., 1972; Schroth et al., 1974; Vanneste, 2000). This is partially due to fast growing, succulent shoots, large quantities of ooze, and early summer rains. Mass outbreaks of shoot blight are thought to occur from insects or weather events similar to blossom blight.

When *E. amylovora* becomes internal, entering either the infected flowers or shoots, the bacteria become systemic and eventually make their way down the scion and into the rootstock (Schroth et al., 1974; Norelli et al., 2000; Vanneste, 2000). This stage, called rootstock or collar blight, is the most devastating to an orchard , if the disease progresses to the collar blight stage it almost always causes tree death, especially in susceptible cultivars (van der Zwet and Beer, 1995). Rootstock blight is so devastating that an outbreak can cost up to $2,500 an acre with only a 10% infection rate (Norelli et al., 2000).

The last form of fire blight is called trauma blight (van der Zwet and Beer, 1995; Vanneste, 2000). Trama blight is associated with tree injury from external forces, such as storms or being near a dirt road, that cause damage to the tree (Schroth et al., 1974; Vanneste, 2000). Trauma blight occurring from a storm can be considered the most damaging, since the windblown rain disperses bacteria large distances and the trees can be heavily damaged, giving cells easy access to cause infection.

All four types of blight can lead to cankers (Vanneste, 2000). The tree can try to block off these cankers using natural defenses, however the cankers can stay active with viable bacteria for long periods of time (Schroth et al., 1974; Vanneste, 2000). When the cankers start to ooze in the spring, the cycle starts all over again.

3. *E. amylovora* Cell Movement in Internal Host Tissue

Understanding how *E. amylovora* cells moves through a shoot is vital for explaining how ooze is able to escape from inside the host. However, there is still a debate as to the spreading mechanism of cells (Zamski et al., 2006). In the symptomatic shoot tissue with necrosis present, bacteria can spread as fast as 2.5 cm a day (Momol et al., 1999; Blachinsky, 2003). It is also known that *E. amylovora* can be isolated internally some distance from symptomatic tissue (van der Zwet, 1969; Vanneste, 2000), which has led to the recommendation of removing infected tissue at least 30 cm beneath the symptoms of fire blight (Vanneste, 2000). This seems like a good recommendation, as ooze can be found as far as 11 cm beneath shoot symptoms (Vanneste, 2000).

3.1 *E. amylovora* Internal Movement Hypotheses

There are many hypotheses of how *E. amylovora* cells move through a host tree; over the last century many theories have been cast, and many have been discarded. However papers still debate on exactly how *E. amylovora* moves systemically though the plant. Literature suggests bacteria could move through either the vascular system via the xylem and/or phloem and/or the intracellular spaces (Crosse et al. 1972; Seemuller and Beer, 1977; Huang and Goodman, 1977; Ayers et al, 1979; Schouten, 1990; Boges et al., 1998; Koczan, 2011). The xylem hypothesis is further supported by a scanning electron microscopy study showing *E amylovora* blocking the xylem (Kozan et al., 2009). This seems to be most accepted theory on how the bacteria cells move though the host (Vanneste, 2000).

There is another theory, however, that cells only move through the intracellular space of the cortical parenchyma cells and do not travel through the vascular system at all (Bachmann, 1913; Tullis, 1929; Rosenburg, 1936; Huang and Goodman, 1976; Zamski et al., 2006). Zamiski et al. suggests that the reason why cells can be found in the xylem are because of embolisms allowing cells to enter the spaces. However, other sources suggest that the embolisms are a result of the *E. amylovora* population or exopolysaccharide build up and eventual cell wall destruction (Schouten, 1989). However, when these embolisms occur, they render the xylem useless as they no longer have turgor pressure and could not move bacteria (Zamski et al., 2006).

4. *E. amylovora* Escapes from the Host Tissue to Form Ooze Droplets

4.1 The Multiplication Hypothesis

There are also conflicting theories on how the ooze escapes from the host tissue. Some of the "pressure hypothesis" backers say that this pressure leads to the exudation of ooze droplets through natural openings such as lenticels or stomata (Zamski et al., 2006). However, not every lenticel or stomata on infected apple fruit or shoots ooze (Slack et al., unpublished). Soft tissue is more easily torn allowing build up and movement of cells (Fisher, 1959; Schouten 1991). The force behind the buildup of exopolysaccharides and bacterial cells could potentially rupture the epidermis (Schouten, 1989).

The multiplication hypothesis, in which the bacteria seep or burst out of the plant since their populations become so great that there simply isn't enough room for all of the cells internally (Schouten, 1990). This theory was historically popular, but due to the large amounts of exopolysaccharides produced by the bacterial cells this may only happen in some instances (Gooden et al., 1974; Bennett and Billings, 1980; Schouten, 1989).

4.2 The "Pressure Hypothesis"

There is also a theory known as the "pressure hypothesis" that attempts to explain how the bacteria moves through the host tissue without necessarily relying on the xylem or phloem (Schouten, 1989; Vanneste, 2000; Zamski et al., 2006). The "pressure hypothesis" states that bacterial multiplication or the absorption of water (by exopolysaccharides, possibly amylovoran but not named in all citations) increases the physical pressure in the intracellular spaces (Schouten, 1989; Vanneste, 2000; Zamski et al., 2006). Since no cell wall degrading enzymes are produced by *E. amylovora*, the breaking through cell walls is probably caused by the pressure of

the expanding exopolysaccharides (Seemuller and Beer, 1976; Schouten, 1989). There is direct and model evidence that exopolysaccharides absorb water and cause swelling (Schouten, 1989; Schouten, 1990; Schouten, 1991). This swelling would lead to the bacteria moving through spaces without the host pushing or pulling the bacteria along the vascular system. It is thought that this pressure could be responsible for the exudation of ooze droplets in asymptomatic tissue (Schouten, 1989; Zamski et al., 2006). The pressure hypothesis is dependent on certain weather conditions, and is discussed more in the weather section of this review. This hypothesis might only be true for apples, since instead of oozing, Hawthorne trees form blisters and only when physically ruptured the ooze emerges (Schouten, 1989).

4.3 External forces acting on *E. amylovora* in the host to produce ooze drops

Besides internal forces, the application of external substances, such as pesticide oils can also cause bacterial cells to rupture from the epidermis. However they are usually seen in the form of aerial stands and not ooze droplets (Bauske, 1968; Eden-Green and Billings, 1972; Keil and van der Zwet, 1972). The coating of the oils on the epidermis may block air flow, causing pressure to build faster, causing the strains to spew out in the thin ruptures that do occur.

5. *E. amylovora* Exits Host Tissue as Aerial Strands besides Ooze Droplets

Aerial stands are thought to indicate internal pressure and usually appear during initial stages of infection. *E. amylovora* strand formation may correspond to long internal, longitudinally oriented strands in healthy tissue (Hockenhull, 1974; Wilson et al, 1987). Aerial strands were 80% matrix and 20% cells (Keil and van der Zwet, 1972). Keil and van der Zwet (1972) found that *E. amylovora* isolated from aerial strands were more virulent on wounded trees than unwounded trees, however it is known that wounded trees are more susceptible (Crosse et

al, 1972; Keil and van der Zwet, 1972). The aerial stands can be colorless, amber, or brick red from apple (Eden-Green and Billing, 1972). Eden-Green and Billing (1972) Counted *E. amylovora* populations in strands that were between $1.6x10^7$- $3.2x10^8$ cells/mm^3 in dry strands, and fresh aerial strand populations were not counted.

6. *E. amylovora* Ooze Drops Range in Color

Hildebrand (1939) states colors of ooze that are not white or dark brown are thought to be contaminants of other organisms on pears (Hildebrand, 1939). The colors could possibly be due to contamination of the drops by another bacteria or yeast. Ooze has been observed in shades of yellow, brown, and red as well as black, green, and other shades of various colors (van der Zwet, 2012). Aerial stands are thought to be amber to dark brick red (Keil and van der Zwet, 1972).

7. Weather Factors that may have an Effect on *E. amylovora* Ooze Drops

According to Schouten's model, ooze is more likely to be produced during evening, late night, or early morning before sunrise when water potential is increasing. If the day time temperature is high (20-30^oC) oozing could increase according to this model. However oozing is rarely observed in the day time or after the plants harden off (Slack, personal observations). At night time there is very high water potential and the pressure and potential for ooze is the highest. (Schouten, 1991.)

Other known weather conditions important for disease incidence are in temperatures under 19^oC (66^oF) for shoot blight and even 13^oC (55^oF) during the critical blossom period. It can also become widespread in conditions with 70% relative humidity, heavy fog, heavy dew, and high wind speeds (McManus and Jones, 1994; van der Zwet and Beer, 1995).

7.1 Fire Blight Forecasting Systems

There is no model that directly uses the presence of ooze or a quantitative analysis to determine epidemic severity. However, there are many programs that monitor certain weather conditions and predict when an outbreak of fire blight may occur.

The oldest system is the Mills system, circa 1955. This forecasting system is based on temperature and moisture during bloom (Mills, 1955; Parker et al., 1956; van

der Zwet and Beer, 1995).

MARYBLYT, developed in Maryland, uses some forecasting events other than weather to determine whether or not there could potentially be a fire blight outbreak. These factors are: blossom or canker symptoms develop, if insect vectors are available, and the daily temperatures (15.6^{O}C or above) (Steiner and Lightner, 1992; van der Zwet and Beer, 1995.)

8. Exopolysaccharides produced by *E. amylovora*

E. amylovora produces an exopolysaccharide (EPS) capsule, formed from amylovoran and levan, around the bacteria cell (Koczan et al., 2009). These two components are found in ooze and are essential to the pathogenicity or virulence of *E. amylovora* (Oh and Beer, 2005; Koczan et al., 2009). Mutants without the capability to make any EPS are non-virulent (Koczan et al., 2009). Besides being important in virulence or pathogenicity, the EPS can shield the bacterium from host defenses and possibly even antibiotics (Geider et al, 1993).

These EPS compounds have been known, albeit not named, for many years as the source of the shepherd's crook symptom. A compound isolated from external ooze was noted as the fire blight toxin (Hildebrand, 1939). Many studies and papers have noted the toxin, (van der Zwet, 1966; Keil and van der Zwet, 1972; Beer and Norelli, 1974), however the mystery compound

wasn't solved until Goodman et al. (1974) published research in Science that had identified the mysterious compound known as "amylovorin" (Goodman et al., 1974). The name has since been changed to amylovoran.

8.1 Amylovoran

Amylovoran is a heterogeneous, acidic polysaccharide made up of repeating subunits of galactose molecules all linked to a glucuronic acid residue (Goodman et al, 1974; Bennett and Billing, 1978). This EPS is essential for pathogenicity and the formation of biofilm (Koczan et al, 2009; 2011). Without amylovoran, there is no biofilm produced (Koczan et al., 2011).

8.2 Levansucrase

E. amylovora also produces a fructose homopolymer called levan (Gross e al., 1992). Sucrose is needed for the levansucrase to make levan (Gross et al., 1992). This levan EPS is an important virulence factor as knockout mutants have dramatically decrease virulence (Koczan et al, 2011).

Of the main components of apple and pear blossom nectar is sucrose, suggesting that the production of levansucrase by the bacteria would intensely aide in procuring enough sugar assimilation (Gross et al., 1992). The levan also aides in expansion as part of the biofilm (Schouten, 1989).

8.3 The role of exopolysaccharides in ooze

Besides bacteria, the rest of the ooze droplet is formed from EPS (Bennett and Billing, 1978). Thus, the exopolysaccharide (EPS) capsule plays a huge role in ooze production. Without EPS, no bacteria would be able to bind together, as EPS is thought to be the basis for biofilms

(Koczan et al., 2011). The other major functions of EPS, such as water and nutrient retention, would be vital for ooze production and bacteria viability inside the droplet (Goodman et al., 1974; Sutherland, 1988; Koczan et al., 2011). The binding of water could be important for the internal swelling of the ooze, allowing for the dramatic burst exiting. The nutrient retention could explain the phenomenon of bacteria being viable in ooze droplets for at least a year as well (Hildebrand, 1939).

9. Questions that Merit Further Investigation

Research focusing on ooze is minimal, and many questions have not been answered. Though Eden-Green and Billing (1972) counted *E. amylovora* populations in dry strands, they did not look at ooze drops or fresh aerial strands (Eden-Green and Billing, 1972). From the literature, no papers have published the quantities of *E. amylovora* present in ooze. There have only been notes of the color differences, no experiments on quantification of the ooze colors or if the colors have anything to do with disease progression have been completed. Do the ooze droplets vary in volume, population, and color? Are there any significant interactions between these three variables, and could they tell us more about *E. amylovora*?

There is evidence that *E. amylovora* can build enough pressure to rupture xylem vessels (Schouten, 1989; Geider et al, 1993), so why can ooze not rupture out of the epidermis? More evidence is needed to determine how the ooze is escaping from the host.

There has been work done on the internal population of *E. amylovora* in the host,

but the populations were not quantified, just tracked or confirmed. What are the internal populations in regards to the ooze drops? How does the disease allocate population for dispersal

and subsequent new infection of hosts?

With new genetic capabilities, advances in microscopy techniques, bacterial tracking, and biochemical analysis further study on ooze should be conducted to answer the questions the literature review raised as well as look into genetic expression of pathogenicity and virulence factors to see if the ooze-dwelling *E. amylovora* are primed for invasion of new host tissues.

CHAPTER 1
A MICROBIOLOGICAL EXAMINATION OF EXOPOLYSACCHARIDE OOZE

1. Abstract

Fire blight, caused by the pathogen *Erwinia amylovora* (Burrill) Winslow et al, is the most devastating bacterial disease of pome fruits in North America and around the world. The primary dispersal method of *E. amylovora* is through ooze, a mass of exopolysaccharides and bacterial cells that is exuded from infected host tissue. Over the 2013 and 2014 field season, 631 ooze droplets (201 in 2013 and 435 in 2014) were collected from field inoculated trees. Populations of *E. amylovora* in ooze drops range from 10^7 to 10^{11} colony forming units per micro liter (cfu/µl). The droplets that had higher populations were typically smaller in total volume and had darker coloring, such as orange, red, or dark red hues. These darker colors may be more attractive to insects that disperse ooze. When the examining host tissue for the origin of the ooze droplets using scanning electron microscopy, no natural openings were discovered in the vicinity of the droplets; erumpent mounds and small (10 µm) tears were the only bacterial sources observed. Even though large amounts of bacteria and exopolysaccharide ooze are forcing out of the host parenchyma and epidermis, even larger populations reside in the host. The ooze droplet is at most one third of the population in the surrounding 1 cm of tissue, meaning that the pathogen is allotting the most population resources to further infection of the current host instead of spread or survival. Three distinct cultivars with different levels of fire blight resistance were infected with four native Michigan *E. amylovora* strains, which indicated that ooze production can vary between host and the virulence of the strain. Genetic expression analysis indicated that *E. amylovora* cells in stem sections located above ooze drops and in ooze drops were actively expressing virulence genes suggesting that these cells would be primed for infection if disseminated to susceptible host tissue. The current study suggests the following: high populations of *E. amylovora* are present in ooze droplets which larger populations found in darker pigmented, smaller volume droplets. These droplets are rupturing out from the

parenchyma and epidermis of the host, with evidence of immense pressure being involved from SEM observations. Ooze droplet volume and population can vary between host cultivar and the virulence of a specific *E. amylovora* strain. Genetic expression analysis of virulent factors in *E. amylovora* indicated that the bacteria in ooze were primed and ready to infect a new susceptible host.

2. Introduction

Erwinia amylovora (Burrill) Winslow et al., a rod-shaped, gram negative bacterium that is a member of the *Enterbacteriaceae* family, is the causal agent of fire blight, the most serious bacterial disease present in apples and pears. *E. amylovora* is thought to be native to North America, infecting hawthorns and other native Rosaceae species. Since commercial apples and pears are usually susceptible to fire blight, which can ultimately result in the death of the host (van der Zwet and Keil, 1979; Vanneste, 2000). The disease can spread quite rapidly and cause large amounts of destruction, hence the name fire blight. The main reason *E. amylovora* can be dispersed so quickly between trees and within or between orchards is because of the ability of cells to extrude to the surface of infected tissue in droplets. Bacterial ooze droplets form outside of the host and can serve as a source for a large amount of bacteria which can be spread through an orchard. Even though ooze droplets are an important epidemiological trait, no major studies have been conducted on ooze droplets since the 1930s. The last fully dedicated paper to ooze was published in 1939 by E.M. Hildebrand (Hildebrand, 1939). Since then, most work involving ooze has been based more on the exopolysaccharide identification (Bennett and Billings, 1979) and aerial stands (Eden-Green and Billing, 1972). Ooze has been mentioned as a side note in *E. amylovora* dispersal (Eden-Green and Knee, 1974; Bennett and Billing, 1978; Vanneste, 2000)

and in movement (Schouten, 1989; 1990; 1991). Ooze was also mentioned in Blachinsky et al, 2006, however they defined ooze being inside the plant, as opposed to ooze droplets.

Ooze is both the primary and a secondary form of inoculum for the transmission of fire blight. The fire blight primary inoculum stage consists of ooze that forms on the surface of over wintering cankers (Schroth et al., 1974; Vanneste, 2000). The ooze from viable cankers is thought to be dispersed via rain, wind, and insects until enough cells build on flowers to infect, causing blossom blight (Schroth et al., 1974). This does not bode well as a minimum population of $1.04x10^2$ CFU/ml of *E. amylovora* present on the flowers is needed for blight to occur (Schroth et al., 1974). For an epidemic to occur there only needs to be a few flowers infected initially (Billing, 2011). After the initial infection, ooze is readily produced from flower pedicels or shoots. Bacterial cells in the ooze then function as secondary inoculum that can easily spread from shoot to shoot and quickly over take an orchard with blight (Schroth et al., 1974; Vanneste, 2000). This is due to the abundance of fast growing, succulent shoots, large quantities of ooze, and early summer rains. Shoot blight can be more devastating to the orchard than blossom blight since it can spread faster through a planting (van der Zwet and Beer, 1995; Vanneste, 2000). Mass outbreaks of shoot blight are thought to occur from insect or weather-mediated events.

Ooze consists of bacterial cells, EPS, and possibly plant materials as well, which is discussed later in this chapter. Thus, the EPS capsule plays a main role in ooze production. The main exopolysaccharides present in ooze are amylovoran and levan (Oh and Beer, 2005; Koczan et al., 2009). Without EPS, there wouldn't be a way for bacteria to bind together, as EPS is thought to be the basis for biofilm (Koczan et al., 2011). The other major functions of EPS, such as water and nutrient retention, would be vital for ooze production and bacterial viability inside the droplet (Goodman et al., 1974; Sutherland, 1988; Koczan et al., 2011). The binding of water

could be important for the internal swelling of the ooze, allowing for the dramatic burst exiting. The nutrient retention could also help to explain the phenomenon of bacteria being viable in ooze droplets for at least one year (Hildebrand, 1939). Not much is known about the color of ooze from the literature, other than it can be colorful (Hildebrand, 1939; Keil and van der Zwet, 1972; van der Zwet et al., 2012).

The method of escape of ooze from the host tissue has been widely debated in the phytopathology literature for decades (Hildebrand, 1939; Fisher, 1959; Seemuller and Beer, 1976; Billings, 1981; Schouten, 1991; Zamski et al., 2006). There does not seem to be a consensus on whether ooze passively seeps from the plant or bursts out from the parenchyma cell layer. Since there are no cell wall degrading enzymes produced by *E. amylovora*, the breaking through compartmental walls is probably caused by the pressure of the expanding exopolysaccharides (EPS) (Seemuller and Beer, 1976; Schouten, 1989). There is direct and model evidence that EPS absorb water and cause swelling (Schouten, 1989; Schouten, 1990; Schouten, 1991). This swelling would lead to the bacteria moving through spaces without the host pushing or pulling the bacteria along the vascular system (Schouten, 1988).

Weather is always cited as being a major contributing factor to the spread of fire blight, thus it would seem that weather and ooze production must somehow be tied together. According to Schouten (1991), when water potential is high, typically around 20-30^{o}C, oozing could increase. There is also evidence that epidemics occur in conditions with 70% relative humidity, heavy fog, heavy dew, and high wind speeds (McManus and Jones, 1994; van der Zwet and Beer, 1995).

Based from the literature, we proposed a microbiological study on *E. amylovora* ooze to fill some of the gaps about the dissemination mechanism. We created a list of seven objectives

and with them seven hypotheses based on previous literature. This chapter seeks to answer these questions:

Objectives:

1. Determine the size and population of *E. amylovora* in ooze droplets.
2. Determine if there is a correlation between droplet color, size, and bacterial population.
3. Determine the cause of the differences in ooze droplet color.
4. Determine if there is a correlation between ooze droplets and internal shoot *E. amylovora* populations.
5. Determine the mechanism of ooze escape from the host tissue.
6. Determine if host cultivar or *E. amylovora* strain is a factor in ooze population or size.
7. Determine what, if any, weather factors influence the development of ooze.

Hypotheses:

1. There is a relationship between size and population of ooze droplets.
2. There is a correlation between droplet color, size, and population.
3. The color differences could be an indicator of internal damage.
4. There is a correlation between ooze droplets and internal *E. amylovora* populations.
5. The mechanism of ooze escape from the host tissue is not mainly through natural openings.
6. Host cultivar and *E. amylovora* strain are factors in ooze population or size.
7. Weather factors influence the development of ooze, especially the temperature and relative humidity.

3. Materials and Methods

3.1 Shoot blight ooze collection methods

3.1.1 Ea110 ooze study during the 2013 and 2014 field seasons

Erwinia amylovora Ea110, a spontaneous rifampicin resistant mutant isolated in Michigan (Zhao et al., 2005), was manually inoculated in an orchard located in East Lansing, MI. The host apple trees were 'Kit Jonathan' (*Malus* x *domestica*) grafted onto M9 rootstock. The inoculum was prepared using cell suspensions grown in Luria-Bertani (LB) broth at 28^{o}C for 12 hrs. Prior to inoculation populations were adjusted using a Tecan Safire spectrometer to 1x10^{6} CFU/ml in 0.5x phosphate buffered saline (PBS). These adjusted cell suspensions were kept on ice until inoculation, occurring less than an hour after the suspension was prepared. Using sterile scissors dipped into the cell suspension, the growing tips of leaves on healthy shoots were cut horizontally across the midvein, removing a quarter to a third of the tip of the leaf (McGhee et al., 2011). Inoculation occurred weekly throughout the growing season, starting in late May and ending in early July, resulting in seven inoculations in both 2013 and 2014 (Table 1-1).

Table 1-1: Inoculation dates for 2013 and 2014 field seasons along with the 'Kit Jonathan' number of shoots inoculated with E. amylovora strain Ea110. In 2014, three additional *E. amylovora* strains, EL01, GH9, and K2, were inoculated on the same day as Ea110 on cultivars 'Kit Jonathan,' 'Linda Mac,' and 'September Wonder Fuji.'. *For 30 June 2014, five additional inoculations were performed on 'Linda Mac' trees as not enough ooze had been collected from this cultivar.

Inoculation	1	2	3	4	5	6	7
2013 Date	24 May	28 May	4 June	7 June	15 June	20 June	25 June
2013 Shoot No.	1-20	21-40	41-45	46-55	46-65	66-75	76-80
2014 Date	22 May	29 May	5 June	10 June	19 June	26 June	30 June
2014 Shoot No.	1-20	21-40	41-60	61-70	71-80	81-90	91-95*

When present in the early morning, bacterial ooze was collected using sterile .6 ml tubes by scraping the ooze off the branch using the inner lip of the lid cylinder. This scraping insured

that the entire ooze drop was in the microcentrifuge tube. Only ooze deemed fresh, not hardened and less than 24 hrs old, was collected. 100 µl of 0.5x PBS was added to the 0.6 ml tube in a sterile lab hood. The ooze drop was vortexted until completely dissolved. Next, 100 µl of the solution was removed from the 0.6 ml tube and added to a 1.5 ml microcentrifuge tube containing 900 µl of 0.5x PBS to be further diluted. The remaining solution in the original .6 ml tube was removed using a 10 µl pipette 1ul at a time until all liquid was removed. This amount was determined to be the original volume of the ooze droplet. The 100 µl of the original solution was then diluted and the appropriate dilutions were drop plated onto LB medium amended with cycloheximide at 50 µg ml^{-1} and rifampicin at 100 µg ml^{-1} to inhibit fungal and unwanted bacterial growth respectively. The colony counts were used to determine the original population of the ooze drop. With each ooze drop, the color (Figure 1-1), physical location, date, current weather conditions, and shoot number were collected. In 2014, the distance from the ooze drop to the bud scar was also collected.

Figure 1-1: A color scale of E. amylovora ooze droplets seen in the field. From left to right the colors are white, yellow, orange, red, and dark red. This figure represents the scale used to define what color category each ooze drop was assigned.

3.1.2 Stem inoculation of three distinct apple cultivars with four *E. amylovora* strains during the 2014 field season

In 2014, three additional *E. amylovora* strains (K2, GH9, and EL01) isolated from Michigan orchards (McGhee and Sundin, 2012) were tested on three cultivars of apple, *M. x*

domestica, (‘Linda Mac’ on M9 rootstock, ‘Kit Jonathan’ on M9 rootstock, and ‘September Wonder Fuji’ on M7 or MM106) to determine if there were differences in ooze production compared to the Ea110 strain used previously in 2013. Each strain, including Ea110, was inoculated, collected, and processed using the methods listed above with the exception that the LB media plates were not amended with rifampicin for the new strains. Also, only orange droplets were collected for this study instead of every ooze drop produced to allow for a higher population sample with one less variable.

3.1.3 Internal population samples from apple stems

In addition to collecting the ooze drop, 1 cm sections of the tissue surrounding the exuded ooze (labeled A, B, and C) were collected and evaluated for internal populations. The inoculation was the same as for the Ea110 ooze drop collection methods described previously. When the ooze was collected using the same method listed as above, the surrounding tissue was also collected and brought back to the laboratory immediately for making the sections. The middle section (B) consisted of 0.5 cm on either side of the ooze drop, resulting in the 1 cm section. The top section (A), was the 1 cm piece above section B and the bottom (C) was directly under section B (Figure 1-2). Each piece was weighed and then chopped up using a sterile razor blade. The chopped stem pieces were placed into glass sonication blanks containing 9 ml of 0.5x PBS. The solution was then homogenized using a PT 10-35 polytron (Brinkmann, Westbury, NY) for 3-5 seconds (McGhee et al, 2011). The solution was then plated using methods previously listed for ooze drops to determine population levels. Final population counts were standardized for weight based on the original stem piece. This was repeated twice in a growth chamber, once in 2013 then again in 2014 using isolate Ea110 on ‘Buckeye Gala on EMLA 7 rootstock.’ In spring/summer 2014 this was again repeated in the field using Ea110 on Kit Jonathans.

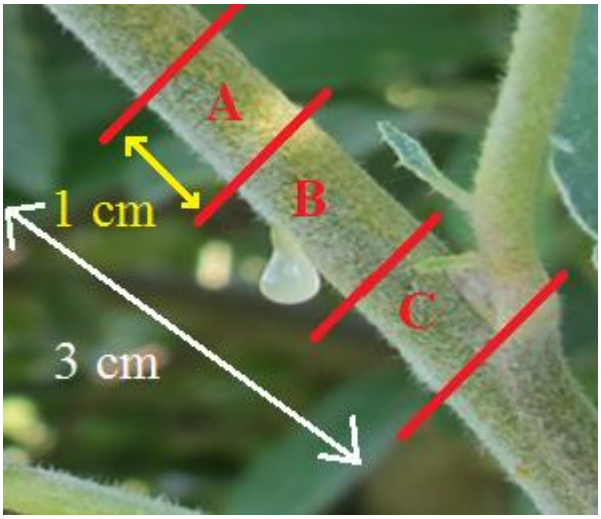

Figure 1-2: Diagram of sections A, B. and C and the 1 cm cuts used in sampling for internal stem populations surrounding the ooze droplets. This method was used in both the growth chamber and in the 2014 field season.

3.2 Blossom Blight

In Spring 2014, *E. amylovora* strains Ea110, K2, GH9, and EL01 (isolated from various Michigan orchards) were tested on different cultivars of apple, *M. x domestica*, ('Linda Mac', 'Kit Jonathan', and 'September Wonder Fuji') to determine if there were any differences in ooze production from blossom infection. Populations were adjusted to $1x10^6$ cell density in 0.5x PBS as described for shoot inoculum. The suspensions were kept chilled en route to the orchard and until inoculation. Fifty blossoms per cultivar and strain were inoculated by hand pipetting 5 µl of inoculum directly onto the stigma of king bloom flowers. A total of 600 king bloom flowers were inoculated. After inoculation, ooze was harvested and processed as previously described in the shoot blight ooze section. Besides ooze; percent infection, severity of infection, and weather conditions were also recorded.

3.3. Scanning Electron Microscopy

All scanning electron microscopy (SEM) samples were processed in the Center for Advance Microscopy at MSU. Samples were fixed in paraformaldehyde/glutaraldehyde (2.5% of each compound in 0.1 M sodium cacodylate buffer) (Electron Microscopy Sciences, 151 Hatfield, PA). The tissue was ethanol dehydrated, then critical point dried (Balzers CPD, Lichtenstein). Tissue was sliced after critical point drying to reduce potential artifacts from the fixation process and, then mounted on aluminum mounting stubs using carbon tabs (Electron Microscopy Sciences).

3.4 Analysis of virulence gene expression of *E. amylovora* in apple stem tissue and ooze

E. amylovora Ea110 was cultured in LB and inoculated on apple shoots using a previously described dipping scissors method. Seventeen ooze drops were collected from 17 individual apple shoots from four different cultivars (‘Linda Mac’, 5; ‘Kit Jonathan’, 5; ‘Gala’, 3; ‘September Wonder Fuji’, 4), Ooze drops were collected 4 to 6 days post inoculation, on five sampling dates (3 June, 10 June, 17 June, 23 June, and 30 June 2014). Early stage infected shoots were collected at 48 hrs post inoculation on 14 June. Section A samples were collected from shoots 1 cm above the ooze drops on the same sampling date of the ooze drops (23 June and 30 June). Ea110 was cultured in LB and induced in Hrp-inducing minimal medium (Hrp MM) (Guttman et al., 2002) for 12 hrs. Total RNA was isolated from the LB culture and the Hrp MM culture as negative and positive controls for T3SS gene expression. Total RNA was isolated on the same sampling dates using an E.Z.N.A.Plant RNA Kit (Omega Biotek) and a miRNeasy kit (Qiagen). The quality and quantity of RNA isolated was tested using a Nanodrop1000 (Thermo Scientific) and a bioanalyzer (Agilent Technologies). Reverse transcription was performed using TaqMan reverse transcription reagents (Applied biosystems). qRTPCR was

performed by a StepOnePlus Real-Time PCR System (Life technologies), using a SYBR Green PCR master mix (Applied biosystems). The PCR amplification specificity was confirmed using a melting curve method. Housekeeping gene *recA* was used as an endogenous control. The expression of target genes was quantified by a comparative CT (ΔΔCT) method, normalized by the endogenous control. Gene expression levels in ooze drops and plant samples were presented as the expression fold in comparison to the expression in Ea110 LB culture. Primers used in real time PCR are *recA* (F:5'-ATCATTGTTGACTCCGTTGC, R: 5'-CATTGCCTGGCTCATCATAC), *hrpL* (F:5'- GATCTGGAGCAAATGACCTG, R: 5'-TTTAAGGCAATGCCAAACAC), *dspE* (F: 5'-CGCAACATCGGAACCATTAA, R: 5'-TGCGACCTGCGGATTAGC), *lsc* (F: 5'-ACCAGACGGAAGAGCAGAAC, R: 5'-CACGTTTCCTTCAAACAGCA), and *amsK* (F: 5'-CGGCACGCTGAAATCATTC, R: 5'-TGCCGCAAAGGGCTTTT).

3.5 Spectra analysis of ooze and apple tissue pigment colors

Samples of red, orange, and yellow ooze collected from the field in spring 2014, orange-tipped fire blight strikes, healthy apple tissue, and cultured *E. amylovora* were prepared in a 10% diH_2O 90% methanol solution and scanned with a Cary 50 UV-Visible spectrometer (Varian, Walnut creek, CA) to gauge the chemical composition of pigments.

4. Results

4.1 Ea110 ooze study during the 2013 and 2014 field seasons

In the fire blight field seasons of 2013 and 2014, which lasted from late May to early July, a total of 201 and 116 ooze droplets respectively were collected from *E. amylovora* strain Ea110-inoculated 'Kit Jonathan' apple trees. By color, the number of ooze droplets collected in 2013 ooze droplets: 15 dark red, 39 red, 91 orange, 29 yellow, and 28 white (Table 1-2). The 116

collected droplets in 2014 droplet colors were: 9 dark red, 13 red, 44 orange, 22 yellow, and 22 white (Table 1-2). These ooze droplets were examined for *E. amylovora* population, droplet volume, weather event significance, and the internal population remaining in host tissue related to ooze droplet location.

Ooze droplets were also observed over time in field to determine if they changed color when drying. There were no observances of the droplets changing color.

Table 1-2: Ooze droplets totals collected both in 2013 and 2014 broken down by color.

Year/Color	Dark Red	Red	Orange	Yellow	White	Total for year
2013	15	39	91	44	22	201
2014	9	13	44	22	22	116
Total	24	52	150	66	44	317

4.1.1 Ea110 Population size in ooze droplets

E. amylovora populations recovered red ooze droplets were the highest in the 2013 collection year with an average population $6.17x10^{10}$ CFU/µl with a range $2.51x10^{9}$-$3.72x10^{11}$ CFU/µl. However the dark red ooze droplets were not significantly different than the dark red (average population was $5.75x10^{10}$ CFU/ µl; range was $2.88x10^{9}$-$1.95x10^{11}$ CFU/ µl) and orange colored droplets (average population of $5.50x10^{10}$ CFU/ µl and range $2.24x10^{8}$-$3.31x10^{11}$ CFU/ µl) (Table 1-3; Figure 1-1). In 2014 the red droplets with an average population of $1.86x10^{10}$ CFU/ µl (range of $9.55x10^{8}$-$7.08x10^{10}$ CFU/ µl) were the largest in population size (Table 1-2; Figure 1-2). However, the red ooze droplets were not significantly different than the orange (average population was $1.66x10^{10}$ CFU/ µl, range was $3.55x10^{7}$-$1.95x10^{11}$ CFU/ µl) and dark red ($9.77x10^{9}$ CFU/ µl range of $1.48x10^{8}$-$3.80x10^{10}$ CFU/ µl) (Table 1-2; Figure 1-2). Even

though in both sample years had red, orange and dark red colored droplets had the highest populations, there was a significant difference between the two field seasons (Figures 4.1.1). White and yellow pigmented ooze droplets harbored the lowest populations both years (Figures 1-1 and 1-2). In 2013 white droplets had an average cell population of $3.24x10^{10}$ CFU/µl and a range from $4.27x10^{8}$-$1.05x10^{11}$ CFU µl and in 2014 had a population average of $4.37x10^{9}$ CFU/µl and ranged $8.51x10^{6}$-$5.37x10^{10}$ CFU/µl (Table 1-2). Yellow ooze droplets were also on average lower in population than the other dark shades; in 2013 the population averaged $2.88x10^{10}$ CFU/µl (range $5.37x10^{8}$-$3.31x10^{11}$ CFU/ µl) and in 2014 averaged $1.92x10^{10}$ CFU/ µl (ranged $1.17x10^{7}$-$7.08x10^{10}$ CFU/µl) (Table 1-2; Figures 1-3 and 1-4). In 2013 the lighter colors of yellow and white were statistically different than the darker colors. However in 2014 only white was significantly less than red and orange droplets. (Table 1-3; Figures 1-3 and 1-4).

When comparing populations of *E. amylovora* on a day to day basis during the 2013 and 2014 field seasons there were two statistically occurring groups in 2013 and three groups in 2014 (Figures 1-5 and 1-6). Not every color of ooze was observed on each sampling date. The populations were different between colors on the same sampling date as well (Figures 1-5 and 1-6). There was not an observed trend in *E. amylovora* population size in either 2013 or 2014 as the season progressed (Figure 1-5 and 1-6).

Weather data was also collected from the Enviro-weather station in East Lansing, MI (MSUHORT). In 2013 the only corresponding significance between any specific date and weather event was minimum air temperature (Table 1-4). There was no significant interaction of any weather factor and population size from 2014 (Table 1-5). When comparing the average population of the same size ooze droplets between 2013 and 2014, droplet sizes of 1 and 2 microliters had statistically different population sizes (Figure 1-7).

Table 1-3: E. amylovora Population averages and ranges by color for each the 2013 and 2014 field season. All samples were collected from 'Kit Jonathan' trees inoculated with *E. amylovora* strain Ea110.

Field Season	CFU	Color of Ooze Droplets				
		Dark Red	Red	Orange	Yellow	White
2013	Average	$5.75x10^{10}$	$6.17x10^{10}$	$5.50x10^{10}$	$2.88x10^{10}$	$3.24x10^{10}$
	Range	$2.88x10^{9}$- $1.95x10^{11}$	$2.51x10^{9}$- $3.72x10^{11}$	$2.24x10^{8}$- $3.31x10^{11}$	$5.37x10^{8}$- $3.31x10^{11}$	$4.27x10^{8}$- $1.05x10^{11}$
2014	Average	$9.77x10^{9}$	$1.86x10^{10}$	$1.66x10^{10}$	$1.92x10^{10}$	$4.37x10^{9}$
	Range	$1.48x10^{8}$- $3.80x10^{10}$	$9.55x10^{8}$- $7.08x10^{10}$	$3.55x10^{7}$- $1.95x10^{11}$	$1.17x10^{7}$- $7.08x10^{10}$	$8.51x10^{6}$- $5.37x10^{10}$

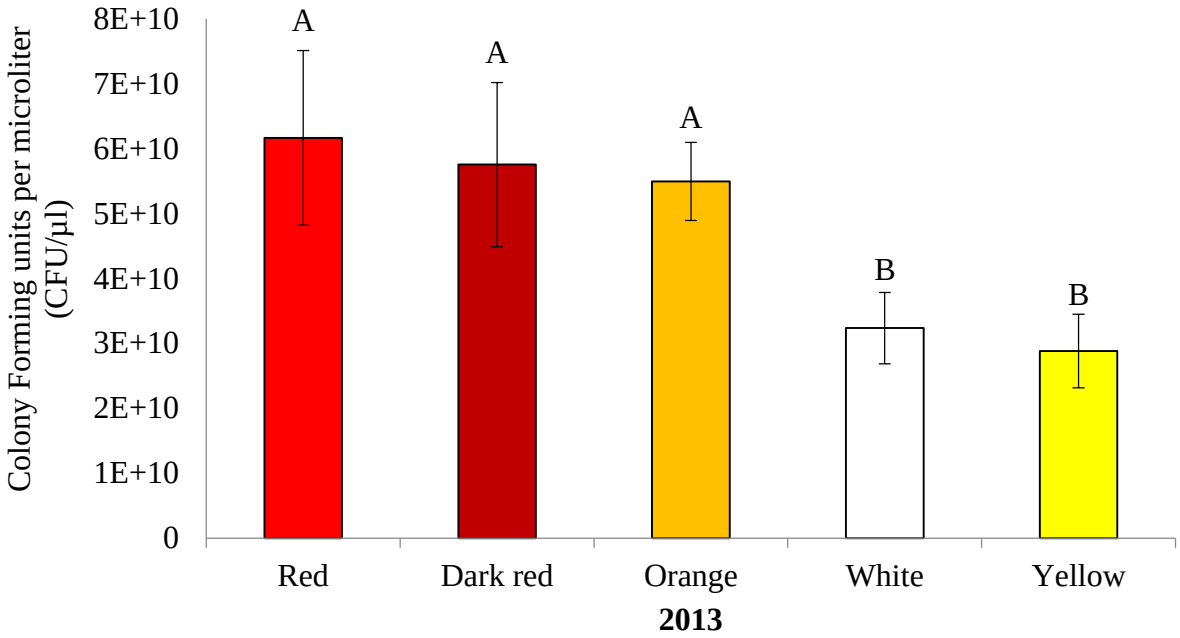

Figure 1-3: E. amylovora log_{10} population in ooze droplets sorted by color for the 2013 field season. All samples were collected from 'Kit Jonathan' trees inoculated with *E. amylovora* strain Ea110. Error bars represent standard error, $P < .0001$

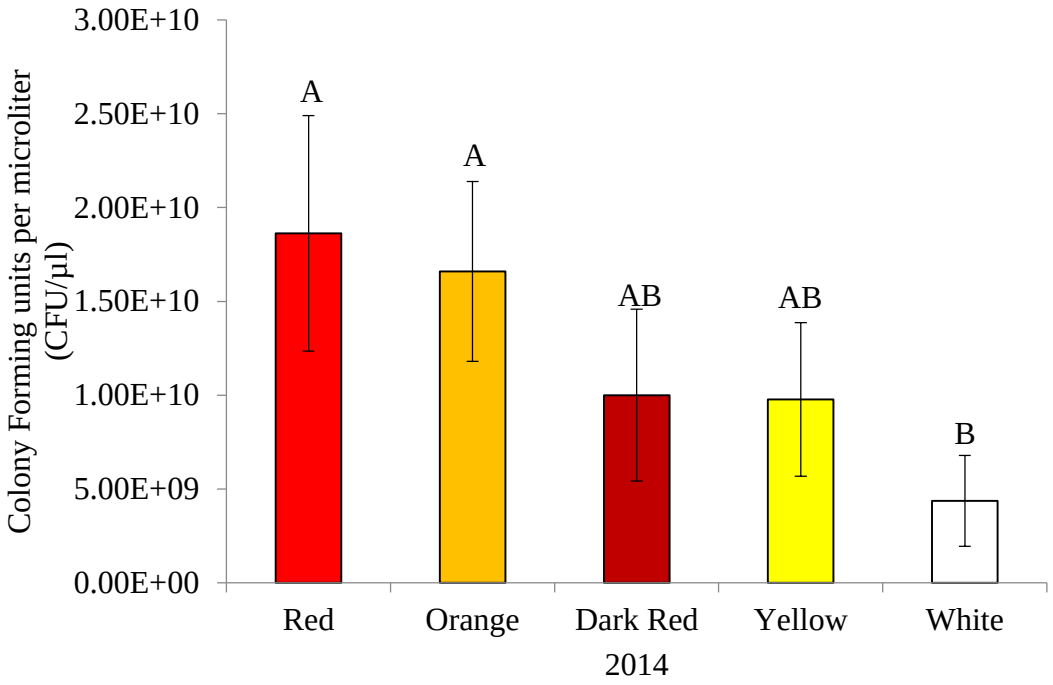

Figure 1- 4: E. amylovora log$_{10}$ population in ooze droplets sorted by color for the 2014 field season. All samples were collected from 'Kit Jonathan' trees inoculated with *E. amylovora* strain Ea110. Error bars represent standard error, *P* <.0001

Table 1-4: 2013 Weather data comparing different weather variables (maximum and minimum relative humidity, maximum and minimum air temperature, maximum wind speed, and precipitation) with ooze droplet population.

Variables	P-value
Bacteria population in ooze and date sampled in relation to max. relative humidity	.4051
Bacteria population in ooze and date sampled in relation to min. relative humidity	.8326
Bacteria population in ooze and date sampled in relation to maximum air temp.	.1423
Bacteria population in ooze and date sampled in relation to minimum air temp.	**<.0001**
Bacteria population in ooze and date sampled in relation to maximum wind speed	.8448
Bacteria population in ooze and date sampled in relation to precipitation	.5250

Table 1-5: 2014 Weather data comparing different weather variables (maximum and minimum relative humidity, maximum and minimum air temperature, maximum wind speed, and precipitation) with ooze population.

Variables	*P*-value
Bacteria population in ooze and date sampled in relation to max. relative humidity	.4493
Bacteria population in ooze and date sampled in relation to maximum air temperature	.5730
Bacteria population in ooze and date sampled in relation to minimum air temperature	.7134
Bacteria population in ooze and date sampled in relation to maximum wind speed	.1700
Bacteria population in ooze and date sampled in relation to precipitation	.2913

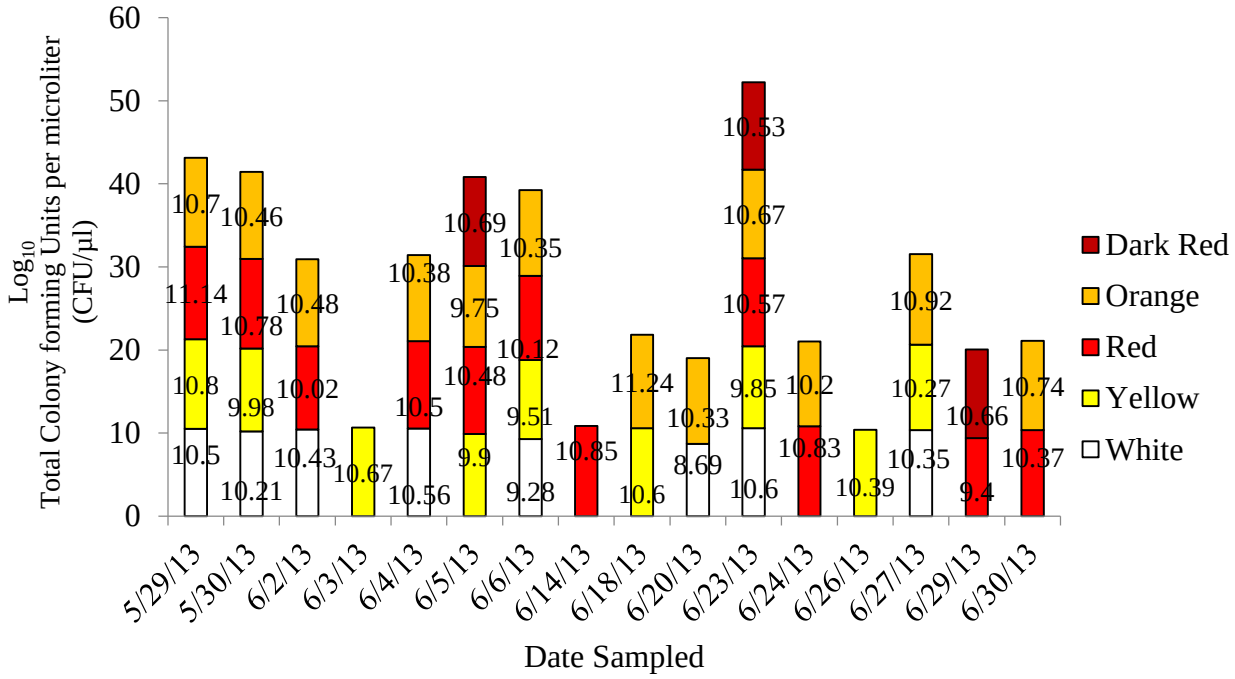

Figure 1-5: E. amylovora total ooze droplet log$_{10}$ population per date sampled in 2013 divided by color of the droplet. There was no observable trend in *E. amylovora* population averages throughout the apple shoot growing period. All samples were collected on sixteen dates from 'Kit Jonathan' trees inoculated with *E. amylovora* strain Ea110.

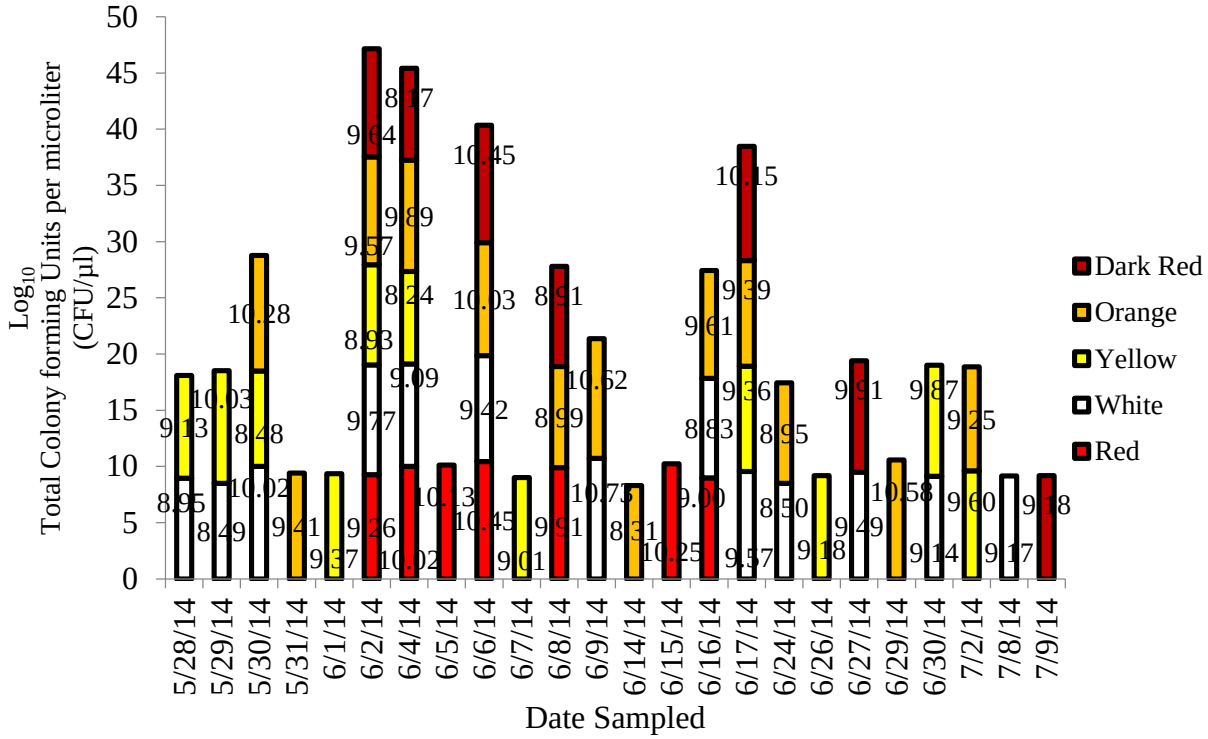

Figure 1-6: E. amylovora total ooze droplet log$_{10}$ population by date sampled in 2014 divided by color of droplet. There was no observable trend in *E. amylovora* population averages throughout the apple shoot growing period. All samples were collected on twenty-eight dates from 'Kit Jonathan' trees inoculated with *E. amylovora* strain Ea110.

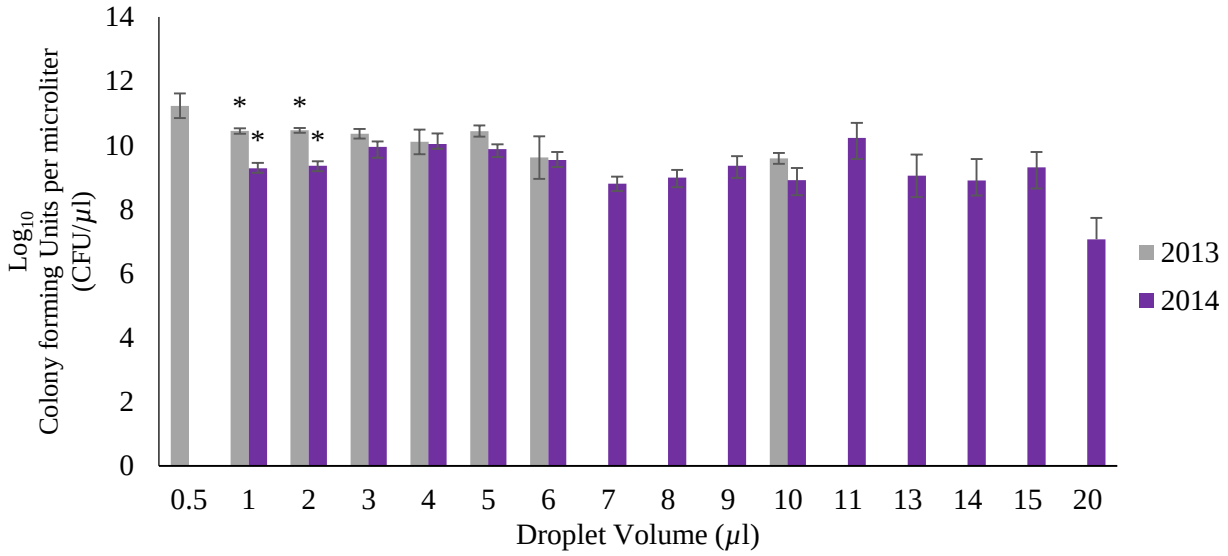

Figure 1-7: Average Log$_{10}$ population of E. amylovora by droplet volume for the 2013 and 2014 field seasons. Over the 2013 and 2014 seasons 317 ooze droplets were collected from field-inoculated 'Kit Jonathan' trees that ranged in sizes from 0.5 µl to 20 µl. Error bars represent standard error, P <.0001. Asterisks (*) represent significant difference in populations between field seasons.

4.1.2 Ea110 Ooze Droplet Volume

Droplet volumes collected from 2013 and 2014 revealed that yellow ooze droplets were the highest in volume both years with an average volume of 4.9 µl in 2013 and 6.11 in 2014. In 2014 the ooze droplets were larger overall than in 2013, however statistically only white and orange droplets were different from each other in each year. In 2013, yellow droplets (averaging 4.9 µl, range 1-20 µl) were significantly larger than white (average 2.25 µl, range 1-10ul) and orange (average 1.9 µl, range 0.5-10 µl) and equivalent to red (3.46 µl average, 1-10 µl range) and dark red (averaging 2.5 µl, range of 1-5 µl) (Table 1-7; Figure 1-8). For 2014 the volume of the droplets was different than in 2013; The yellow droplets ooze droplets (6.11 µl, range 1-20 µl) was only significantly equivalent to dark red droplets (average 5.22 µl, range 1-9 µl). the yellow droplets were significantly larger than dark red (average 5.22 µl, range 1-9 µl) orange (average 5.5 µl, range 1-11 µl), white (average 4.27 µl, range 1-15 µl), and red(average 3.92 µl, range 1-9 µl) (Table 1-6; Figure 1-9).

When comparing volume of ooze droplets on a day to day basis during the 2013 and 2014 field seasons there were three statistically occurring groups in 2013 and 2014 (Figures 1-10 and 1-11). The ooze droplet sizes were different between colors on the same sampling date as well (Figures 1-10 and 1-11). There was not an observable trend in *E. amylovora* population size in either 2013 or 2014 as the season progressed (Figure 1-4 and 1-5).

Weather data was also collected from the Enviro-weather station in East Lansing, MI (MSUHORT). In 2013 the only corresponding significance between droplet size and weather events was minimum air temperature, which also corresponded to *E. amylovora* population size

(Table 1-4 and 1-7). For 2014 there was a significant interaction between droplet size and maximum wind speed; however this was not the case population size (Tables 1-5 and 1-8).

Table 1-6: Ooze droplet size averages and ranges by color for each the 2013 and 2014 field season. All samples were collected from 'Kit Jonathan' trees inoculated with *E. amylovora* strain Ea110.

Field Season	Microliter (µl)	Color of Ooze Droplet				
		Dark Red	Red	Orange	Yellow	White
2013	Average	2.5	3.46	1.9	4.9	2.25
	Range	1-5	1-10	0.5-10 µl	1-20	1-10
2014	Average	5.22	3.92	5.5	6.11	4.27
	Range	1-9	1-9	1-11	1-20	1-15

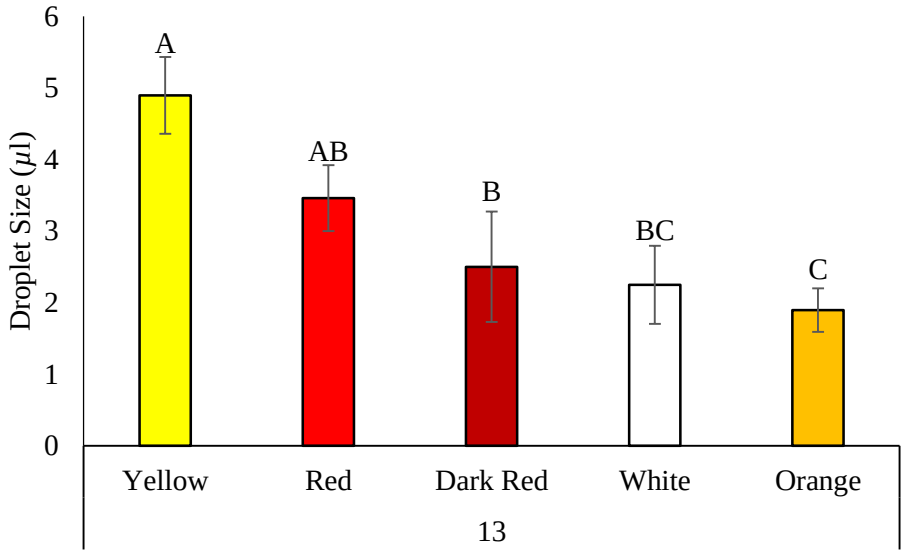

Figure 1-8: E. amylovora ooze droplet volume sorted by color for the 2013 field season. All samples were collected from 'Kit Jonathan' trees inoculated with *E. amylovora* strain Ea110. Error bars represent standard error, $P < .0001$.

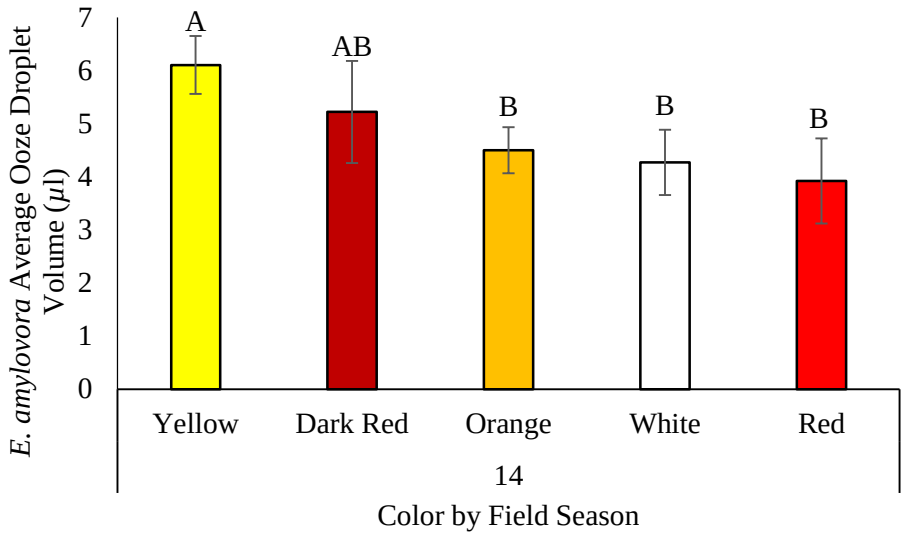

Figure 1-9: E. amylovora ooze droplet volume sorted by color for the 2014 field season. All samples were collected from 'Kit Jonathan' trees inoculated with *E. amylovora* strain Ea110. Error bars represent standard error *P* <.0001.

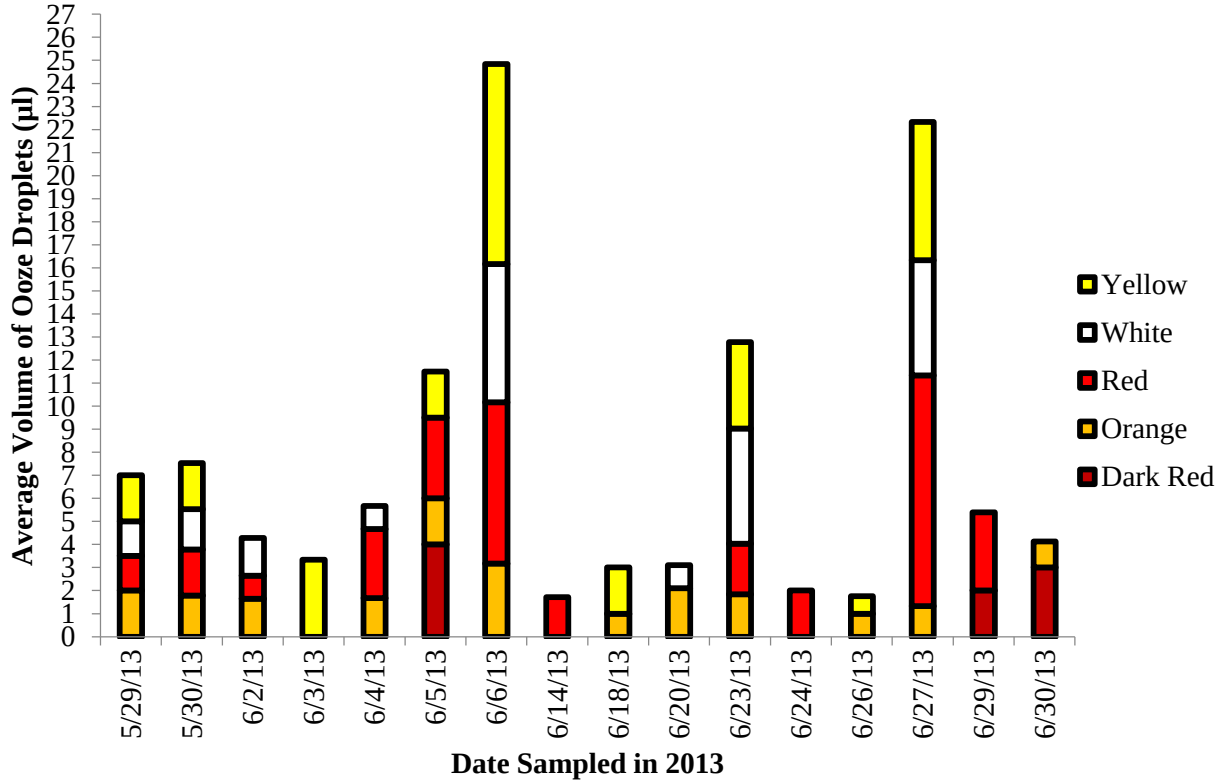

Figure 1-10: E. amylovora average ooze droplet volume per date by color in 2013. Ooze droplets were collected from field-inoculated 'Kit Jonathan' trees that ranged in sizes from 0.5 µl to 10 µl with overall daily averages falling in between 1.5 µl and 5.6 µl from sixteen sample dates.

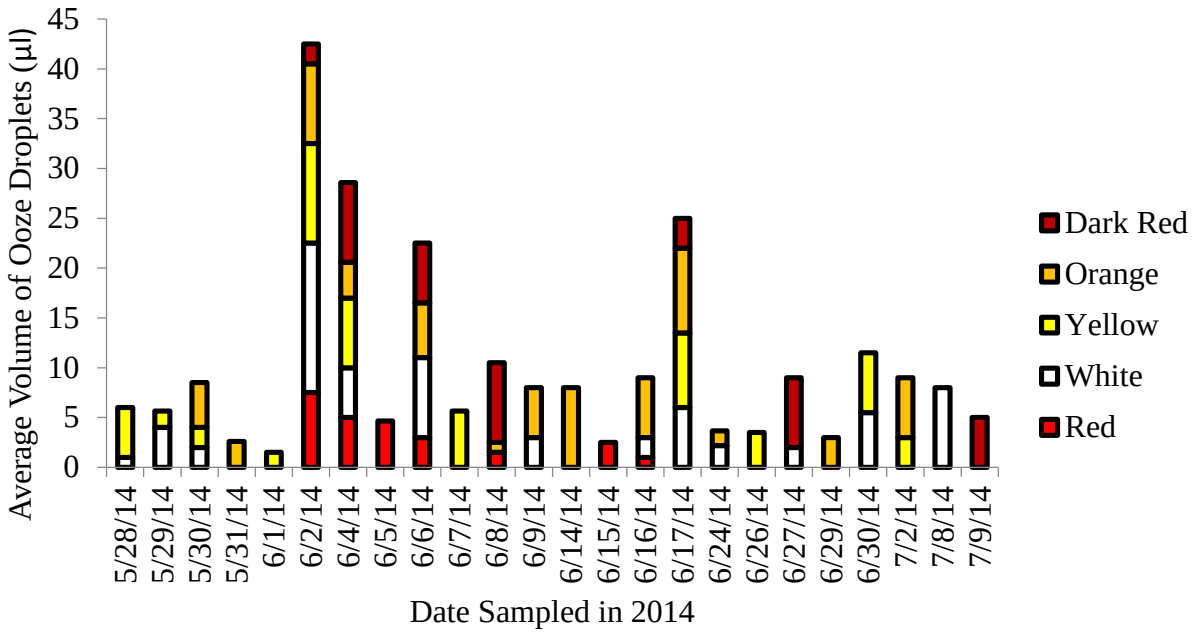

Figure 1-11: E. amylovora average ooze droplet volume per sample date divided by color in 2014. Ooze droplets were collected from field-inoculated 'Kit Jonathan' trees that ranged in sizes from 1 µl to 20 µl with daily averages between 1.5 µl and 8.8 µl from twenty-eight sample dates.

Table 1-7: 2013 Weather data comparing different weather variables (maximum and minimum relative humidity, maximum and minimum air temperature, maximum wind speed, and precipitation) with ooze droplet volume.

Variables	*P*-value
Ooze droplet volume and date sampled in relation to maximum relative humidity	.5878
Ooze droplet volume and date sampled in relation to minimum relative humidity	.7020
Ooze droplet volume and date sampled in relation to maximum air temp.	.7953
Ooze droplet volume and date sampled in relation to minimum air temp.	**.0137**
Ooze droplet volume and date sampled in relation to maximum wind speed	.6128
Ooze droplet volume and date sampled in relation to precipitation	.6451

Table 1-8: 2014 Weather data comparing different weather variables (maximum and minimum relative humidity, maximum and minimum air temperature, maximum wind speed, and precipitation) to ooze droplet volume.

Variables	*P*-value
Ooze droplet volume and date sampled in relation to maximum relative humidity	.9054
Ooze droplet volume and date sampled in relation to maximum air temperature	.7432
Ooze droplet volume and date sampled in relation to minimum air temperature	.9509
Ooze droplet volume and date sampled in relation to maximum wind speed	**.0005**
Ooze droplet volume and date sampled in relation to precipitation	.0743

4.2 Internal population samples from apple stems

In 2014, the internal populations from field inoculated trees were not statistically different between the three sections; however the ooze droplet population was significantly lower than all the sections (Figure 1-12). While the growth chamber experiments from 2013 and 2014 also show that there was no significant difference between the internal population sections (Figure 1-13), the ooze droplet population was only significantly different than Section B (Figures 1-13). The overall percentage of bacteria found in and on section B, including the ooze droplet, showed that the *E. amylovora* population in ooze comprises a relatively small percentage of the total bacteria present compared to the internal population, ranging between 7% and 36% of the total *E. amylovora* population (Figures 1-15 and 1-14).

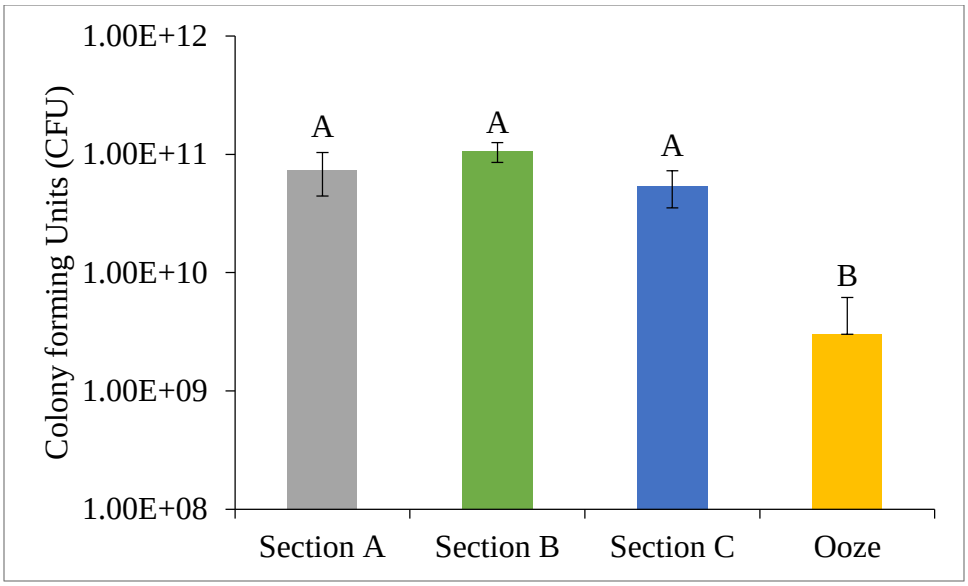

Figure 1-12: E. amylovora internal populations sampled from the field. 1 cm sections of 'Kit Jonathan' apple shoots and an ooze drop from section B recovered from the field in 2014. Error bars represent standard error; *P*<.0001.

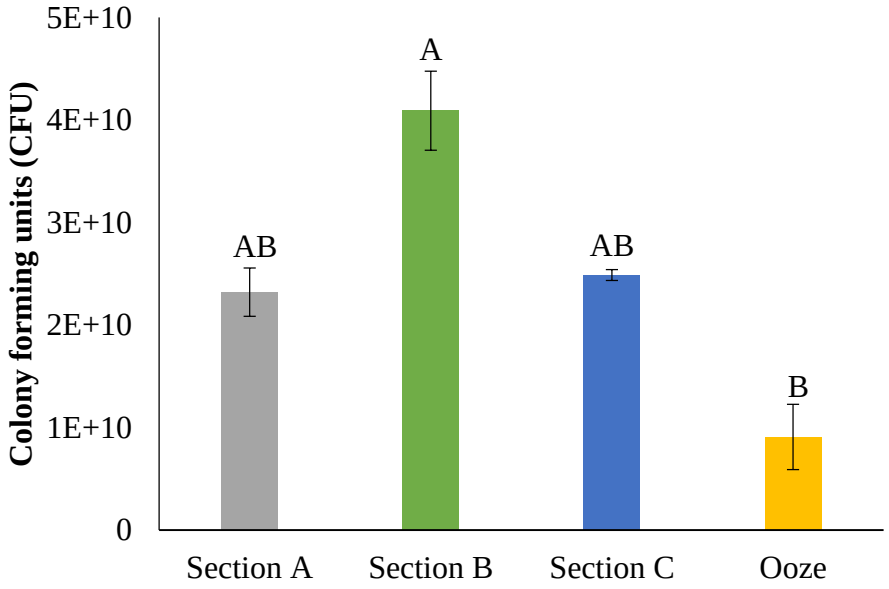

Figure 1-13: E. amylovora internal populations sampled from growth chamber. 1 cm sections of 'Gala' apple shoots and an ooze drop from section B from growth chamber experiments in 2013 and 2014. Error bars represent standard error; *P*= <.05.

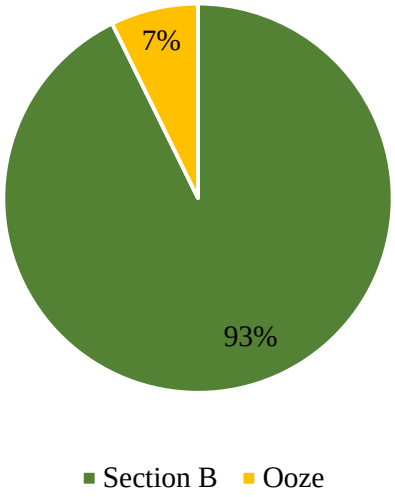

Figure 1-14: E. amylovora average internal population of section B compared to droplets from field. E. amylovora average internal population present in the 1 cm section B of 'Kit Jonathan' apple shoots and average ooze drop population from the 2014 field season. The percentage indicates how much bacteria was found in each area, ooze or internal, of section B.

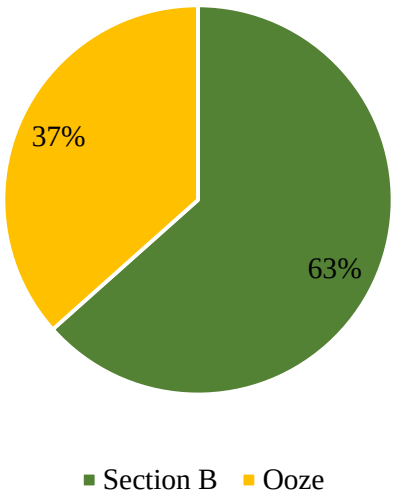

Figure 1-15: E. amylovora average internal population of section B compared to droplets from growth chamber. *E. amylovora* average internal populations present in the 1 cm section B of 'Gala' apple shoots and average ooze drop population from growth chamber experiments in 2013 and 2014. The percentage indicates how much bacteria was found in each area, ooze or internal, of section B.

4.3 Stem inoculation of three distinct apple cultivars with four *E. amylovora* strains during the 2014 field season

In 2014, 364 ooze droplets were collected from late May to early July from three cultivars of apple ('September Wonder Fuji,' 'Kit Jonathan,' and 'Linda Mac') that were inoculated with four strains of *E. amylovora* (Ea110, EL01, GH9, and K2) (Table 1-9). At least thirty ooze droplets were collected over the season from each cultivar and strain combination except for 'Kit Jonathan' and strain Ea110, as the orange droplets were also part of the main ooze study, and 'Linda Mac' and strain Gh9, which did not produce ooze droplets as readily as the other cultivar and strain combinations (Table 1-9).

Table 1-9: Ooze droplet totals collected from 3 cultivars and 4 strains in 2014.

Cultivar	Strain	Number of droplets
'September Wonder Fuji'	Ea110	30
	EL01	30
	GH9	30
	K2	30
'Kit Jonathan'	Ea110	44
	EL01	30
	GH9	30
	K2	30
'Linda Mac'	Ea110	30
	EL01	30
	GH9	21
	K2	30

4.3.1 Differences in population between apple cultivars and *E. amylovora* strains

Populations of strain Ea110 were statistically significant between each cultivar (Figure 1-16). The populations of the three other strains (EL01, GH9, and K2) were significantly different between cultivars; all three of the strains exhibited a significant reduction in population in the

cultivar ‘Linda Mac’ (Figure 1-16). There was no significant interaction between strain, cultivar, and date in relation to population (Table 1-10).

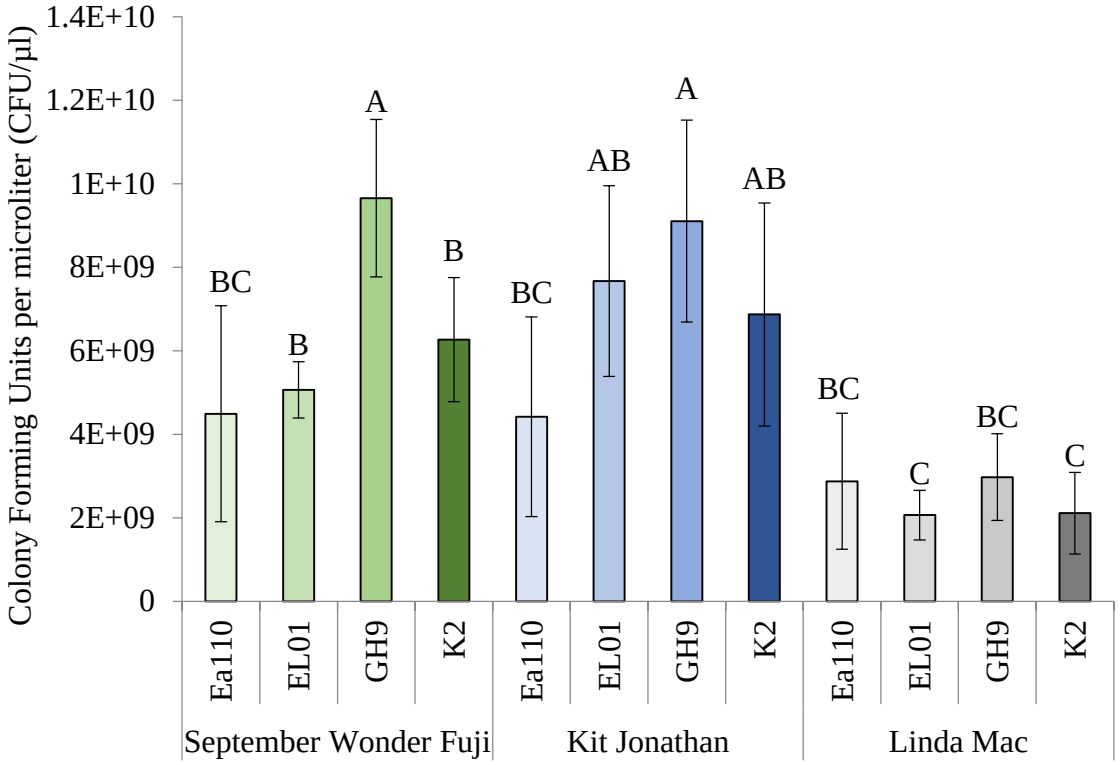

Figure 1-16: E. amylovora population in ooze droplets compared between strains and cultivars. Strains listed are Ea110, EL01, GH9, and K2 and cultivars ‘September Wonder Fuji,’ ‘Kit Jonathan,’ and ‘Linda Mac.’ Droplets were collected from the MSU Plant Pathology farm from the field-inoculated trees Error bars represent standard error; *P* <.0001.

4.3.2 Differences in ooze droplet volume between apple cultivars and *E. amylovora* strains

Strain K2 produced statistically larger drops in ‘September Wonder Fuji’ than the other two cultivars, as well as in comparison with the other strains in ‘September Wonder Fuji’ (Figure 17). In ‘Linda Mac,’ strain EL01 had larger droplets than the other strains in that cultivar, however the ‘Kit Jonathan’ EL01 was not statistically different than any of the other strains, but when compared to the ‘Linda Mac’ EL01 it was statistically different (Figure 17). All four

strains in ‘Kit Jonathan’ were statistically the same with no variation, while the other two cultivars, ‘September Wonder Fuji’ and ‘Linda Mac’ each had an outlier strain, K2 and EL01, respectively (Figure 17). Comparisons between the strains Ea110 and GH9 were statistically the same, whereas again EL01 and K2 had larger volumes in different cultivars (Figure 1-17).

When the strains and cultivar ooze droplet volumes were combined by date sampled, the 17 June 2014 was significantly different than the rest of the sampling dates (Figure 1-18). When further explored however, there was no significance between volume, cultivar, and strain (*P*=.3314) or for the time of inoculation (*P*=.3560). The only significant weather variable was max wind speed, which was 37.8 miles per hour (*P* <.0001). When the population, cultivar, and droplet volume were statistically examined as a whole, there was no significant difference between the cultivars (Table 1-10). However the strains were all significantly different when examined against droplet population and volume (Table 1-11).

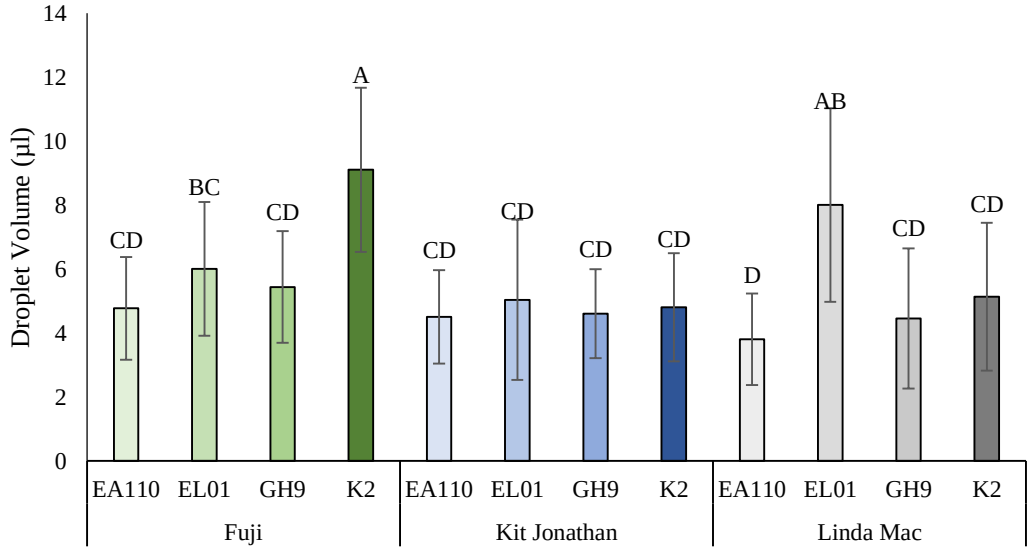

Figure 1-17: E. amylovora droplet volume compared between strains and cultivars. Strains listed are Ea110, EL01, GH9, and K2 as well as cultivars ‘September Wonder Fuji,’ ‘Kit Jonathan,’ and ‘Linda Mac.’ Droplets were collected from the MSU Plant Pathology farm from the field-inoculated trees. Error bars represent standard error, *P* <.0001.

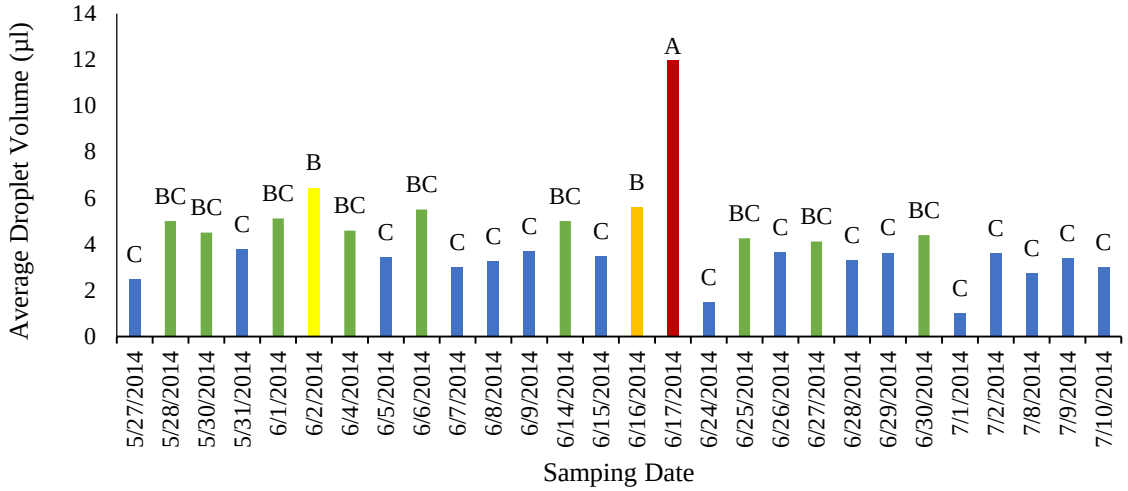

Figure 1-18: Average E. amylovora ooze drop volume over the twenty-eight day sampling period in 2014 combined for all cultivars and strains. Error bars represent standard error, *P*<.0001.

Table 1-10: 2014 Chart of all insignificant interactions between variables for the 2014 inoculation of four strains onto three cultivars. The variables shown are: Strain, cultivar, date collected, distance from bud scar, and time of inoculation along with the p-value of significance.

Insignificant interaction	*P*-value	
Strain, cultivar, and date in relation to population volume.	.2631	
Strain, cultivar, and date in relation to droplet volume.	.5128	
Distance from bud scar, date, and time of inoculation in relation to population volume.	.5178	
Population and cultivar in relation to ooze volume.	p- value for interaction	0.1278
	‘Linda Mac’	0.0549
	‘Kit Jonathan’	0.4314
	‘September Wonder Fuji’	0.1058

Table 1-11: Chart of the four strains from the 2014 field season showing that there was significant difference between strains in regards to droplet volume and population.

Strain	*P*-value
GH9	.0032
EA110	.0131
K2	.0001
EL01	<.0001

4.4 Scanning Electron Microscopy

In SEM micrographs of collapsed ooze droplets, bacterial cells can clearly be observed surrounding what could possibly be the remains of exopolysaccharides (Figure 1-19). Another notable observation is the masses of bacteria which appear to emerge from the exit point for the ooze droplet from the fruit. There were no natural openings observed around the collapsed ooze droplets; the tearing in the skin of the apple was an artifact from the SEM fixation, as it was also seen in uninfected fruit from the same variety and age (Figure 1-19 B; Figure 1-20).

When the ooze dried before fixation, the ooze would be more rigid than the waspy-looking edges of the collapsed droplets (Figure 1-19 A; Figure 1-20). Again, no natural opening was observed. This circular shaped structure from which the ooze emerged does not appear to be a natural opening, as it is only 10 µm in diameter (Figure 1-21). When submerged in ethanol, ooze droplets did not dissolve when shaken (personal observations). Only when 10% water was added and the droplets shaken vigorously did the dried ooze dissolve, meaning that the early stages of critical point drying (50% ethanol 50% diH20) could have possibly affected the shape and texture of the ooze droplets as they were processed. However the SEM critical point drying

protocol does not result in samples being vigorously shaken, if anything the opposite is desired as not to harm the specimens in this fragile form.

Erumpent mounds, large convex shaped mounds with a central tear, were found underneath large ooze droplets (Figure 1-22). These mounds were also visible with the naked eye after the drop was removed (personal observations). These mounds appear to have been formed from internal pressure based on the shape of the stem or petiole tissue (Figure 1-22). Bacteria was also found around the erumpent mounds where the ooze drop had been removed. Dried bacterial ooze is prevalent even when ooze droplets are physically removed before drying (Figure 1-23). The dried ooze could also stick to trichomes in the surrounding area of the erumpent mound. Along the edges of the erumpent mounds bacterial cells and exopolysaccharides could be found attached to the surface of the plant tissue (Figure 1-23 C). There were observances of ooze emerging from the same wound underneath an older, dried droplet in the field.

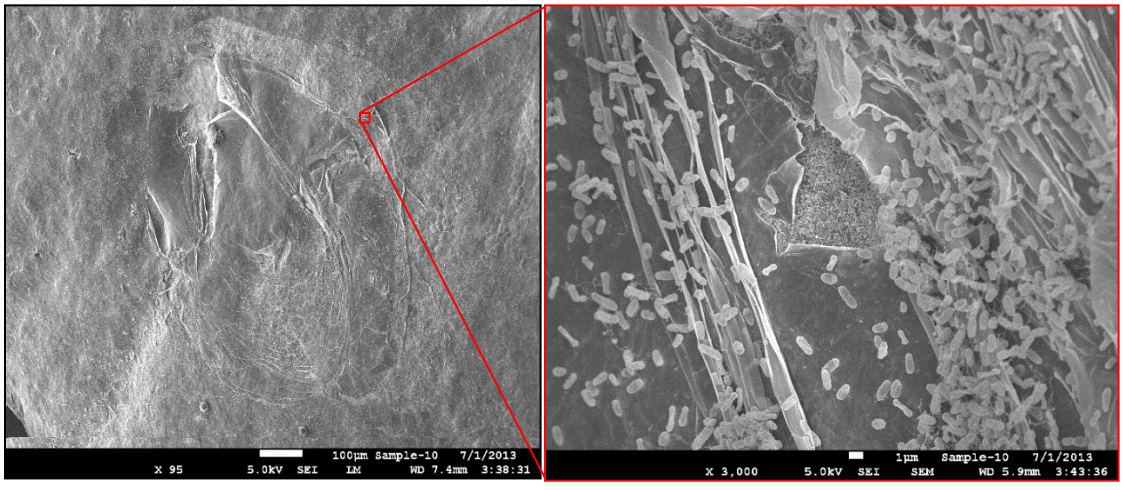

Figure 1-19: SEM micrographs of a collapsed ooze droplet that formed on a naturally occurring E. amylovora infected immature apple fruit. The ooze droplet was not removed before SEM critical point drying of the fruit. A: Collapsed ooze droplet on an infected immature apple fruit. B: Close up of the edge of the collapsed ooze droplet with bacteria still attached to the fruit and exopolysaccharides.

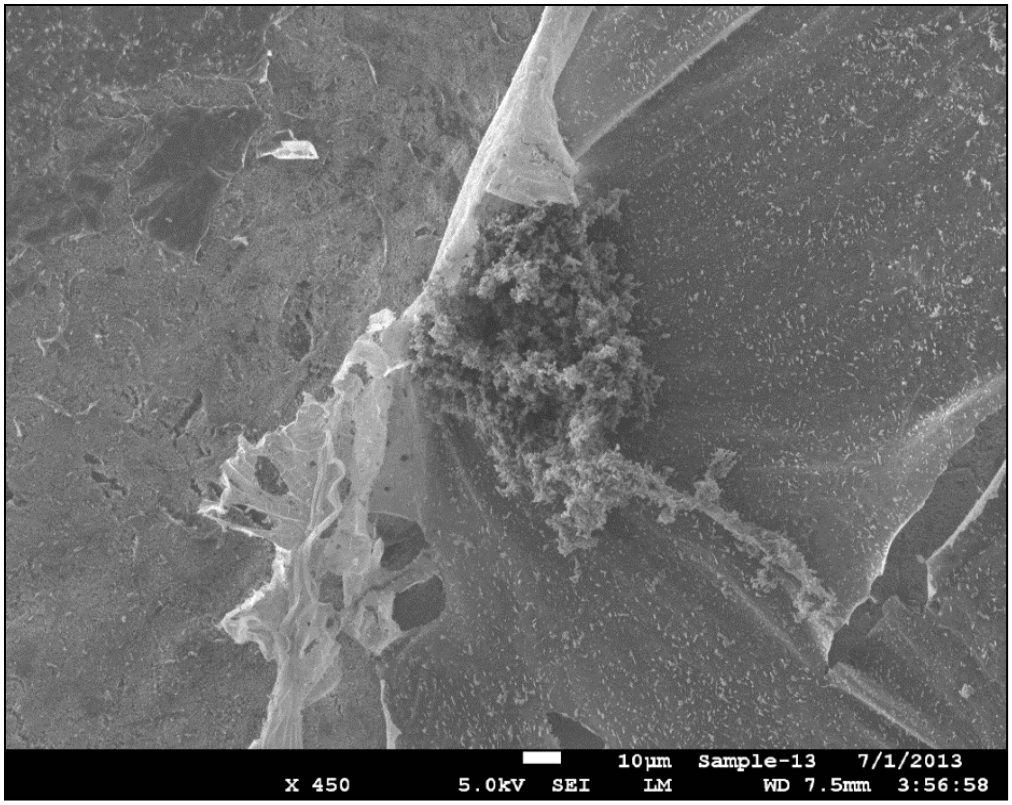

Figure 1-20: SEM micrograph zoomed to 450x of a collapsed ooze droplet that formed on a naturally occurring E. amylovora infected immature apple fruit. The ooze droplet was fresh and not removed before SEM critical point drying of the fruit. The mound of bacteria pictured is thought to be the exit point of the *E. amylovora* from the internal apple fruit tissue.

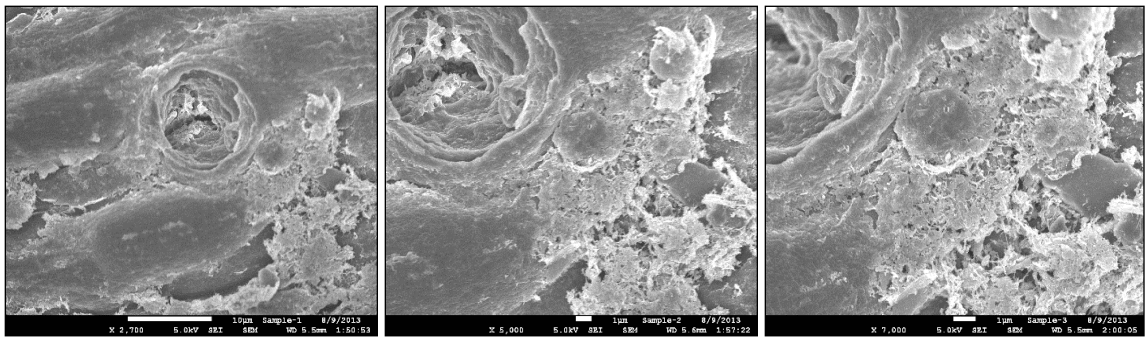

Figure 1-21: SEM micrographs displaying a gradual close up of an exit point by an E. amylovora ooze drop on an inoculated stem. The ooze droplet was allowed to dry before SEM critical point fixation processing. The close up fixates on the dried mass of exopolysaccharides and bacteria present near the circular exit hole.

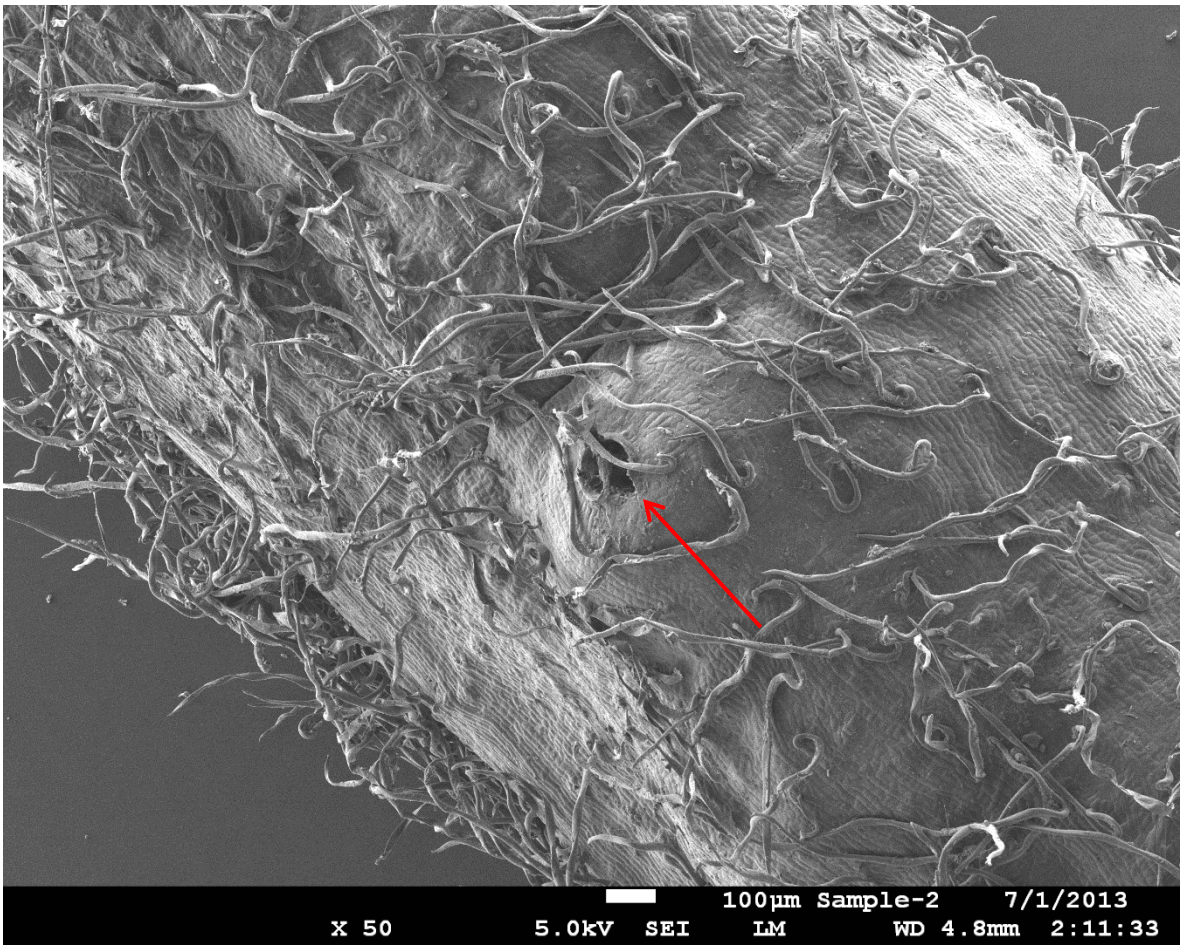

Figure 1-22: An erumpent mound found underneath a large ooze droplet on a leaf petiole from an inoculated stem. The ooze droplet was removed prior to critical point drying for SEM fixation. The red arrow points to exit point of ooze droplet.

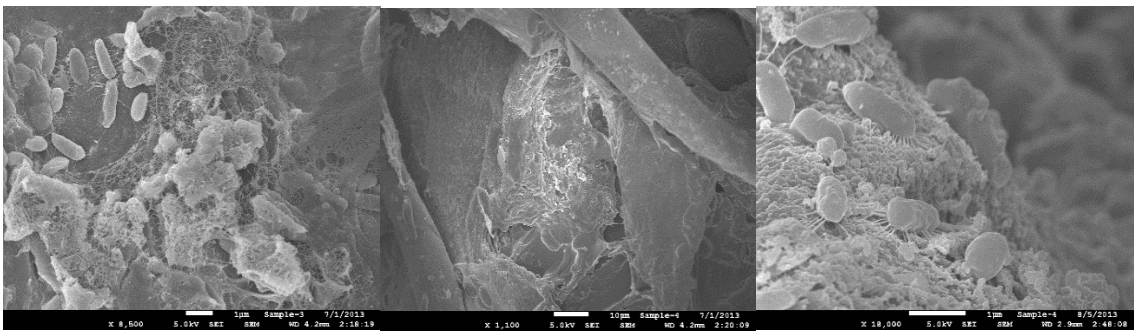

Figure 1-23: Various images of dried ooze formed around erumpent mounds. The fresh ooze was removed prior to SEM critical point fixation, however dried ooze was still seen around area near exiting wound. A: dried ooze on surface nearby an erumpent mound found on an apple stem. B: Dried ooze nearby the erumpent mound, ooze is also present on nearby trichomes. C: Area of an erumpent mound at the edge of the exit point.

4.5 Analysis of virulence gene expression of *E. amylovora* in apple stem tissue and ooze

Expression of *hrpL* is an indicator for activation of the type III secretion system which is required for infection of host plant cells. Gene expression of *hrpL* was different between the cultivars; Ea110 infecting 'Linda Mac' had higher *hrpL* expression in the 48 hour (early stage) infected shoots while bacteria isolated from stem Section A dwelling bacteria had less *hrpL* expression compared to the 'Kit Jonathan' EA110 bacteria (Figure 1-24). Ea110 *E. amylovora* in ooze expressed *hrpL* similar to the Hrp MM control for both cultivars (Figure 1-24).

The expression of the *dspE* gene is also an indication of the type III secretion system and is required for pathogenicity of *E. amylovora*. *dspE* gene expression was reduced for all Ea110 from the field compared to the HRP minimal medium, however it was still being expressed at a higher level in the infected plant tissue compared to the LB grown *E. amylovora* (Figure 1-25).

The gene *lsc* encodes levansucrase, which is an important virulence factor in *E. amylovora* that allows for sugar acquisition and biofilm formation. For both cultivars, bacteria isolated from section A had the largest expression of *lsc*, around 6-7 fold higher expression than the Hrp MM bacteria (Figure 1-26). For the 'Linda Mac' isolated bacteria, the expression was also elevated at 4 fold higher than Hrp MM. Bacteria in ooze for both cultivars and in 48 hr 'Kit Jonathan' shoots were only around 1.5x higher than the Hrp MM (Figure 1-26).

Amylovoran is an important pathogenicity factor in *E. amylovora* and production is measured by the expression of gene *amsK*. Without actively producing some level of amylovoran, *E. amylovora* is not able to infect a host. The *E. amylovora* in ooze were lower expressing of *amsK* than the bacteria in the Hrp MM, however they are probably not significantly different. The fold of expression for *amsK* are low, and there is probably not any significant difference between the cultivars or tissues sampled (Figure 1-27).

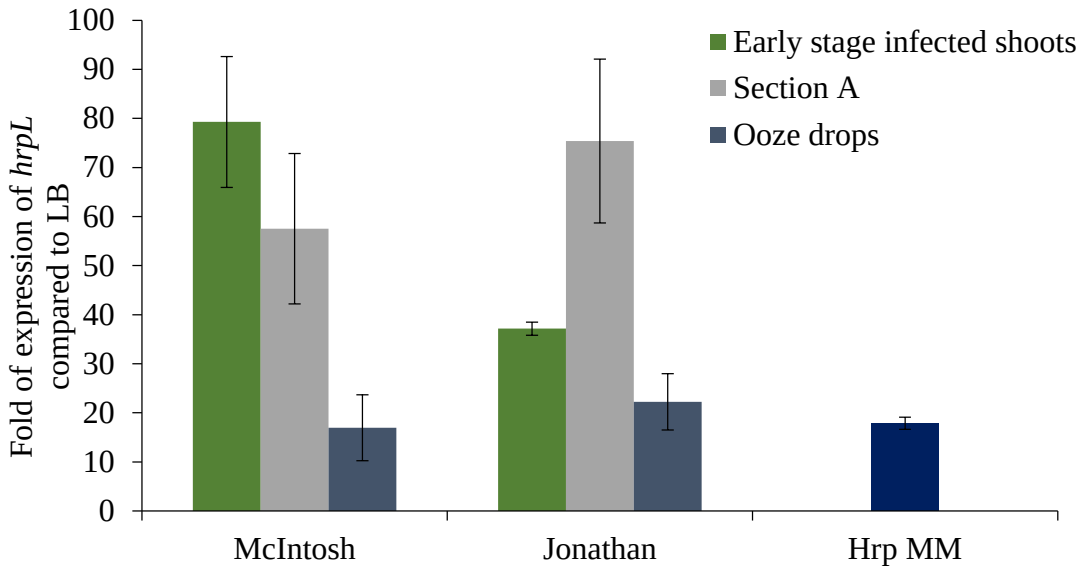

Figure 1-24: Relative expression of hrpL in different infected plant tissues, ooze drops, and Hrp inducing minimal medium in comparison to expression levels in LB medium. The expression levels of target genes were quantified by a comparative CT (ΔΔCT) method, normalized by an endogenous control *recA.*

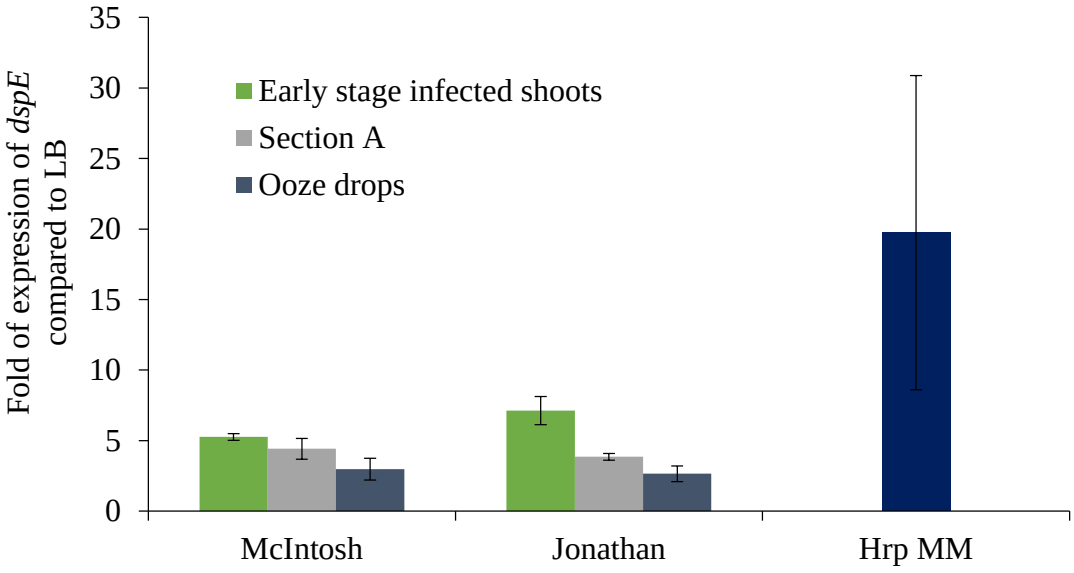

Figure 1-25: Relative expression of dspE in different infected plant tissues, ooze drops, and Hrp inducing minimal medium in comparison to expression levels in LB medium. The expression levels of target genes were quantified by a comparative CT (ΔΔCT) method, normalized by an endogenous control *recA.*

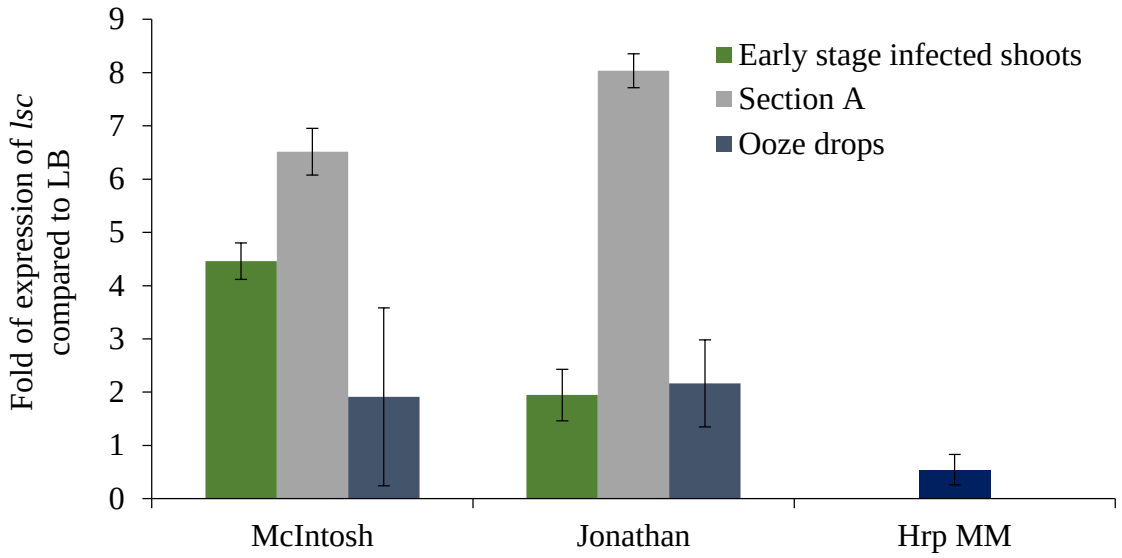

Figure 1-26: Relative expression of lsc in different infected plant tissues, ooze drops, and Hrp inducing minimal medium in comparison to expression levels in LB medium. The expression levels of target genes were quantified by a comparative CT (ΔΔCT) method, normalized by an endogenous control *recA.*

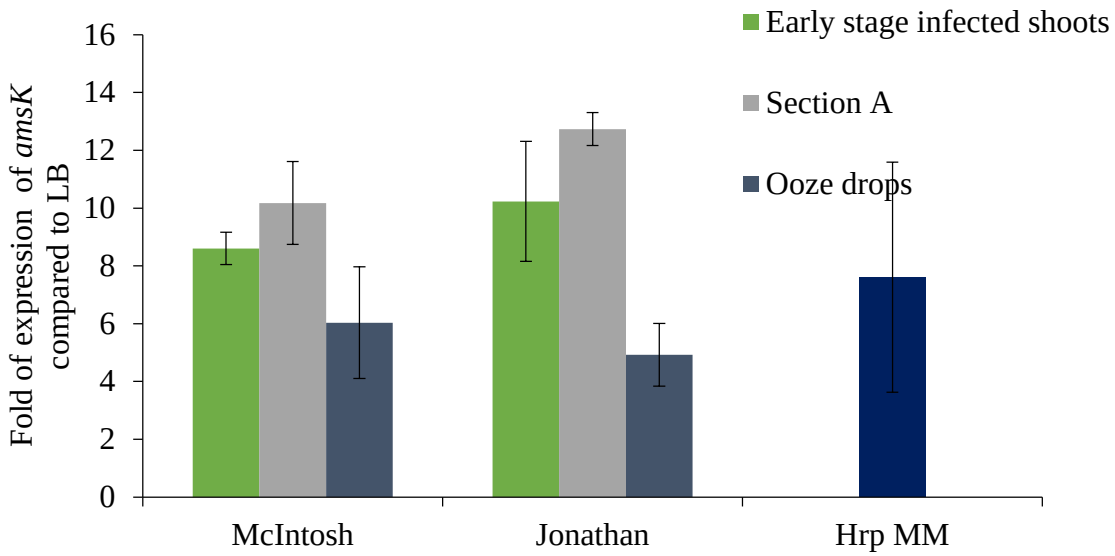

Figure 1-27: Relative expression of amsK in different infected plant tissues, ooze drops, and Hrp inducing minimal medium in comparison to expression levels in LB medium. The expression levels of target genes were quantified by a comparative CT (ΔΔCT) method, normalized by an endogenous control *recA.*

4.6 Spectra analysis of ooze and apple tissue pigment colors

When ran in the spectrometer, there was a consistent peak and shoulder at different concentrations present for every sample that contained pigment (dark red, red, orange, yellow ooze droplets and orange-tipped fire blight shoots) (Figure 1-28). The peak was slightly visible for the control of a non-infected apple stem; however the stem tissue had been wounded in preparation for the spectrometer. The *E. amylovora* grown in culture did not contain the peak or shoulder present in the other samples (Figure 1-28). The peak and shoulder is consistent with flavanones, which are known defense chemicals produced against *E. amylovora.*

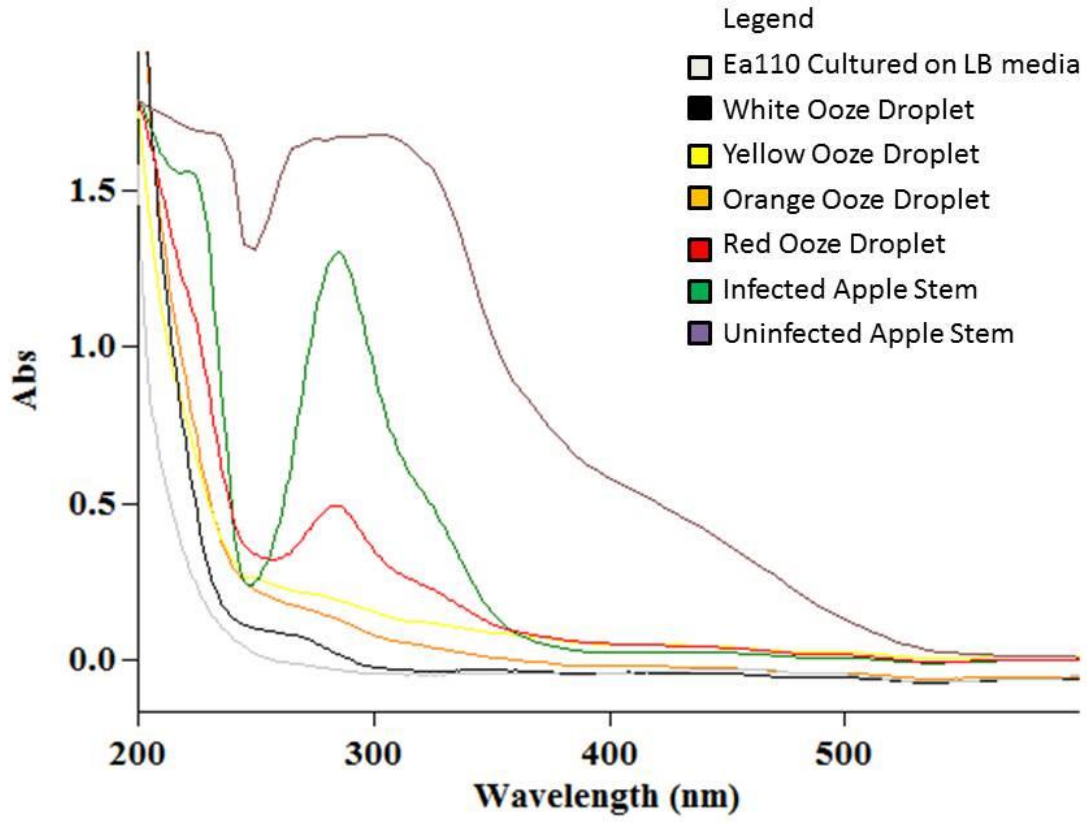

Figure 1-28: Spectra of various tissues containing pigment (ooze droplets and orange-tipped fire blight shoots) against controls (uninfected apple stem tissue).

5. Discussion

Our field and laboratory data indicated that *E. amylovora* populations within ooze droplets were extremely large, that populations were consistently higher in darker-colored ooze drops, and that the cells present in ooze drops were expressing virulence genes suggesting that these cells would be primed for infection if disseminated to susceptible host tissue. When considering the average volume of ooze droplets and the population size determined per microliter, overall populations per individual ooze droplet ranged from 10^8 to 10^{11} cells. *E. amylovora* ooze functions in the epidemiology of fire blight through both direct dissemination of virulent bacterial cells via rain-splash, wind, or insect activity (Schroth et al., 1974) or in long-term survival, as *E. amylovora* cells in dried ooze drops can remain viable and pathogenic for periods of 1-2 years (Hildebrand, 1939; van der Zwet et al, 2000). Ooze populations are many orders of magnitude larger than those necessary to establish infections in inoculated, wounded apple shoots. For example, Crosse et al (1972) determined a median inoculum dose of only 38 bacterial cells following pipetting of a droplet of cells onto the cut end of the main leaf veins of an apple leaf at the shoot tip, and Ruz et al (2008) reported a median effective pathogen dose of 2.01×10^5 cells using the most effective inoculation method in a comparative study: cutting an apple leaf with scissors dipped in cell inoculum.

Why then might *E. amylovora* ooze populations be so large? In nature, ooze from cankers supplies primary inoculum for flower infection, and is primarily disseminated by insects such as flies. When considering dissemination of *E. amylovora* from cankers among native Rosaceae trees, these trees were likely scattered randomly in the landscape, with potentially large distances between them. Flies contacting ooze on a canker would likely only pick up fractions of a microliter of ooze, necessitating dense bacterial populations to ensure successful carriage and

cell delivery between canker and flower. There would also be a time component between acquisition and delivery; it is possible that the exopolysaccharide matrix of ooze would enhance survival during this dissemination phase. For shoot infection in orchards, freshly-injured tissue at shoot tips is not always available; thus, larger cell populations are probably required to initiate shoot infections than those reported previously in wound inoculation studies (Crosse et al. 1972; Ruz et al. 2008). This again would signify a requirement for excessively large *E. amylovora* populations in ooze. In addition, during the long-term period of cell survival in dried ooze, large amounts of the population may die and a high initial population would be needed to allow for the survival of the pathogen.

While the *E. amylovora* populations in ooze drops are very large, our analyses indicated that these populations represent at most 36% of the total *E. amylovora* population within the infected shoot. Thus, ooze represents an allocation of infecting cells to dissemination, meaning that a larger proportion of cells are available for continued systemic infection of the host. Based on our SEM imaging studies, ooze drops appear to have been formed after a rupturing of the parenchyma and epidermis layers of the cell. Since *E. amylovora* does not secrete cell wall degrading enzymes, the pressure hypothesis formed by Schouten (1989) that enables cells in exopolysaccharide to move through the host could also explain ooze drop formation (Seemuller and Beer, 1976; Schouten, 1989).

When ooze drops were examined in the field, they ranged in color from white to dark red; the color of the drops did not change if the ooze drops were allowed to dry. The *E. amylovora* populations were found to be consistently higher in the darker shades of ooze than white and yellow. When ooze is released from cankers, it is typically orange to dark red. Our field observations confirm those made by others in observing a high frequency of visits by flies to

oozing cankers, with the flies walking through and appearing to consume the ooze droplets (Ark and Thomas, 1936; Hildebrand 1939; Van der Zwet and Keil, 1979; Hildebrand et al., 2001). Hildebrand et al (2001) conducted a large field study of insect dispersal of *E. amylovora*, however he did not address cell counts (Hildebrand et al., 2001). These flies may be attracted to the darker red pigment, however, whether the pigment is directly involved or the source of the pigment is the attractant is still unknown. The pigment is likely a flavanone, particularly flavanone-3-hydroxlase or 3-dexpyflavonoid derived compounds. Besides being a potential defense response to *E. amylovora*, the role of these compounds in host response is unknown or circumstantial at best (Flachowsky et al., 2012).

The *E. amylovora* present in ooze droplets had expression levels of virulence genes that were reduced compared to actively invading cells, however they were still high enough to be considered primed compared to the HRP MM or LB grown bacteria. *E. amylovora* could possibly have regulators that when exposed to oxygen, may contribute to the oppression of gene expression. Since the ooze droplets are external and exposed to more oxygen than in intracellular spaces, these regulators may play a role in the suppression compared to the bacterial cells that remain inside the host.

CHAPTER 2
A FIRST-YEAR REPORT ON THE BIOLOGICIAL CONTROL COMPOUND BLOSSOM-PROTECT IN MICHIGAN ORCHARDS

1. Abstract

As the National Organic Program's allowance of antibiotic synthetic compounds for fire blight comes to a close, along with streptomycin resistance, there needs to be new ways to protect pome orchards from *Erwinia amylovora,* the causal agent of fire blight. One way this could be achieved is by finding biological control agents with a lower variability in effectiveness. In Michigan, trials are being run of a product called Blossom-Protect, which is a yeast-like fungus *Aureobasidium pullulans*. *A. pullulans* strains 10 and 40 have had success in suppressing other plant pathogens. Due to biological control agents in Michigan lack of reliability year to year, other organic compounds were added to the trials (Sundin et al., 2009). Blossom blight control was significantly greater for Blossom-Protect + Cueva at 3 sprays (BP+C3), Blossom-Protect + Oxidate at 2 sprays (BP+O2), Blossom-Protect at 2 late sprays (BP+2L) and Blossom-Protect + Nordox + Umbrella at 3 sprays (BP+N+U3) compared to the unsprayed control (USC); Blossom-Protect at 4 sprays (BP+4) was the same as USC in percent infection. BP+O2 also resulted in no significant russeting and had significantly decreased apple scab presence. In year one of the study, there were no significant differences between pure Blossom-protect sprays in regards to russeting.

2. Introduction

Fire Blight, caused by the gram negative bacterium *Erwinia amylovora,* is a devastating disease of pome fruit that occurs in orchards around the world. Traditionally, antibiotics such as copper and streptomycin have been used to combat the primary stage of infection which occurs at flower bloom (Sundin et al, 2009). The need to use a chemical defense against *E. amylovora* is so crucial even organic apple and pear growers could use antibiotics; the National Organic Program allowed the use of antibiotic synthetic compounds from 2002 to 21 Oct 2014 on tree

fruit for fire blight control (Granatstein, 2014). As 2014 was the last year that organic growers had antibiotics available in their disease combat tool box, new methods of chemical control for fire blight are needed. The other concern about traditional antibiotics is the level of streptomycin resistance occurring in *E. amylovora* (Jones and Schnabel, 2000; Sundin et al, 2009; Vanneste, 2011). Since the mid 1990's research on alternative chemical controls, such as biological control, have been gaining momentum and are continuing to promise new methods of disease control (Vanneste, 2011).

Biological control is the use of another organism or the products produced by an organism as an antagonizer against an unwanted population of organisms. Since flowers are the primary infection site, specifically the stigma (Thomson, 1986), many biological controls have tried to target that ecological niche in the flower (Vanneste, 2011). This biological control mode of action is known as competition (Pal and McSpadden Gardener, 2006). The other mode of action taken advantage of by fire blight antagonists are antibiotic-mediated suppression (Pal and McSpadden Gardener, 2006). There are currently a few biological controls available for apple and pear growers against fire blight (Sundin et al, 2009; Vanneste, 2011). The majority of control agents for fire blight are bacterial antagonists, including *Pseudomonas fluorescens* strain A506 (BlightBan A506), *Pantoea agglomerans* strain E235 (Bloomtime FD Biopesticide), *Pantoea vagans* strain C9-1 (Blightban C9-1), and *Bacillus subtilis* strain 713 (Serenade) (Sundin et al., 2009; Vanneste, 2011).

The challenge with biological control is the variability in effectiveness (Sundin et al, 2009; Vanneste, 2011). In a study conducted by Sundin et al. (2009) that spanned over three states and six years, there were many inconsistencies with performance of the biologicals they tested: *Pseudomonas fluorescens* strain A506 (BlightBan A506), *Pantoea agglomerans* strain

E235 (Bloomtime FD Biopesticide), *Pantoea vagans* strain C9-1 (Blightban C9-1), and a mixture of *Pseudomonas fluorescens* strain A506 (BlightBan A506) and *Pantoea vagans* strain C9-1 (Blightban C9-1). Ultimately, they found that streptomycin was still the best control for fire blight over the four biological control treatments (Sundin et al., 2009).

Besides bacteria, yeasts have been screened for potential biological control agents as well (Pusey et al., 2009). However, like bacteria, yeasts have shown mixed results as biological control agents. A yeast success as a biological control was for postharvest apple diseases grey mold (*Botris cinerea*) and blue mold (*Penicillum expansum*) by *Cryptococcus laurentii* LS28 and *Aureobasidium pullulans* LS30 (Lima et al., 2003). An example of a failed commercial product was Aspire, containing *Canidida oleophilas*, which could not control postharvest diseases (Droby et al. 1998).

Aureobasidium pullulans is a black yeast-like fungus that has tested to have antagonistic properties against many plant pathogens (Lima et al., 2003; Kunz, 2004; Duffy et al., 2006; Pusey et al., 2009). One way that the yeast is currently being marketed is as Blossom-Protect, a mixture of two strains, *A. pullulans* 10 and 40 (*Ap* CF10 and *Ap* CF40 respectively) (Kunz, 2004). Developed in the early 2000s in Germany, Blossom-Protect could be a successful biological control against *E. amylovora* (Kunz, 2004; Duffy et al., 2006; Pusey et al., 2009). The mode of action of *A. pullulans* is thought to be antibiosis of nutrient acquisition and niche competition (Duffy et al., 2006). However in another study involving control of molds, *A. pullulans* produced antifungal compounds to inhibit mold growth and no antibiosis was observed (Castoria et al., 2001).

There have been many studies on the efficacy of Blossom-Protect on controlling fire blight before the biological was released. Duffy et al. (2006) found that Blossom-Protect had

significant fire blight control on seven cultivars, with variation, and found that 24-48 hour prior to bacterial inoculation reduced the bacterial population by 3 log of colony forming units (log CFU) (Duffy et al., 2006). Both Kunz (2004) and Pusey et al. (2009) also report that *A. pullulans* colonized detached apple blossoms better than other antagonistic yeasts and reduced *E. amylovora* populations as well (Kunz, 2004; Pusey et al., 2009). Other field trials in Germany have reported that two to four applications of yeasts as biological controls as can result in fruit russeting (Pusey et al., 2009). Kunz (2004) however states that no increase in russeting was found in a two year organic study on various cultivars (Kunz, 2004). The label for Blossom-Protect however indicates that russeting may occur in susceptible fruit in late blossom.

In year one of this study, Blossom-Protect was evaluated for use in Michigan. Different overall rates of the product were tested in the field as with a laboratory component observing the *A. pullulans, E. amylovora,* fungal, and total bacterial viability on the blossoms. Incidences of russeting, blossom and shoot blight, as well as apple scab were also reported later in the season.

3. Materials and Methods

In 2014 Blossom-Protect (Westbridge Agricultural Products, Vista, CA) was applied to McIntosh trees at different bloom intervals on the MSU Plant Pathology Research Farm in East Lansing, MI. Along with Blossom-Protect, Cueva (Certis, USA) a copper compound, Nordox 75 WG (Nordox AS, Oslo, Norway) another copper bactericide, and Umbrella (Agrian, Fresno, CA USA) which contains terpene resins, tall oil fatty acids and alkyl phenol ethoxylate. Each treatment had four single-tree replicates

(Table 2-1).These treatments and replicates were arranged in a complete randomized block design. In each treatment, *E. amylovora* was inoculated at 80-100% bloom. The *E. amylovora*

strain Ea110, a spontaneous rifampicin mutant native to Michigan, was used in this trial. Prior to use, Ea110 was stored at -80°C in 15% glycerol. Prior to inoculation, cells were grown in Luria-Burtani broth (LB) and adjusted to $1x10^6$ CFU/ml in 0.5x PBS. The suspension was kept on ice to the field then sprayed on the trees with an 11.4-liter pump mist sprayer (Solo, Newport News, VA).

Table 2-1: 2014 field season Blossom-Protect treatment list along with product rates used and the percent bloom timing of each treatment. Treatment abbreviation is used to denote which compounds are used in each treatment and how many times the treatment was applied.

Treatment Abbreviation	Treatment and product per acre	Treatment Timings (Percent bloom)
1 BP-4	Blossom-Protect 1.34 lb. Buffer A 9.35 lb.	10%, 40%, 70-80%, 100%
2 BP+C3	Blossom-Protect 1.34 lb. Buffer A 9.35 lb. Cueva .5gal/100gal	40%, 70-80%, 100%
4 BP+O2	Oxidate Blossom-Protect 1.34 lb. Buffer A 9.35 lb.	70-80% (before and after), 100%
5 BP+L2	Blossom-Protect 1.34 lb. Buffer A 9.35 lb.	70-80%, 100%
8 BP+N+U3	Blossom-Protect 1.34 lb. Buffer A 9.35 lb. Nordox 75WG 1.25lbs/Acre Umbrella 16 FL oz./100gal	40%, 70-80%, 100%
10 USC	Unsprayed Control	

3.1 Laboratory Studies of Blossom-Protect Methods

For each time point, eight blossoms were randomly collected from four trees (one each rep). See Table 2-1 for percent bloom time point that treatment was applied and sampled. Each rep was separately added to a glass sonication tube with 20 mL .5x PBS and sonicated for seven minutes (McGhee et al., 2011) The resulting solution was then diluted and spread plated onto Potato Dextrose Agar (PDA), NAG agar (NAG), and King's B agar (KB). These plates were

non-amended unless noted to see the full scope of microorganisms present. Counts were taken two days after plating for yeast, bacteria (with no differentiating *E. amylovora* from other colonies unless noted), and non-yeast-like fungal colonies. For BP+O2, an oxidate spray was applied four hours before the other 70-80% sprays. Sampling occurred prior to this spray and again four hours later. *E. amylovora* was inoculated on 17 May 2014 between 80%-100% bloom as described above. On the 100% bloom date, *E. amylovora* was also directly counted using KB plates amended with rifampicin 100 µg ml^{-1} and cycloheximide at 50 µg ml^{-1} to inhibit fungal growth. When the blossoms were sampled at each time point, 28 other flowers were pressed to NAG media to observe yeast colonies.

Table 2-2: 2014 field study dates indicating the percent bloom sampled for Blossom-Protect. The X indicates that no spray was applied.

Date 2014	13 May	14 May	15 May	16 May	17 May	18 May
Percent Bloom Sampled	10%	40%	X	80%	Inoculation	100%

3.2 2014 Field Aspect of Blossom-Protect Methods

On 25 July 2014, ratings were taken for prevalence of blossom and shoot blight. Blossom and shoot rating were percent of infected blossoms/shoots out of a hundred randomly selected shoots. Ratings for apple scab were also taken for this experiment on 8 September 2014, as crucial apple scab fungicide sprays were not applied as to not kill the yeast. Apple scab was rated by counting all the leaves on a randomly selected shoot and giving a percentage of infected leaves. This was repeated twenty times per rep. For fruit, 100 randomly selected fruit per rep were observed for scab. Fruit russeting was also rated, as noted by Pusey et al (2009) that is could be a concern. The rating system for this was 60 random fruit were rated by the USDA

grade fancy protocol (USDA, 2002). Any fruit with over 10% russet was determined unmarketable. The calyx and stem flesh of the apples were also rated on russeting according to the USDA standards for fancy fruit (USDA, 2002).

3.3 Statistical analysis

All statistical analysis was conducted using SAS 9.4, PROC GLM and PROC Mixed using complete randomized blocking.

4. Results

4.1 Laboratory Studies of Blossom-Protect Results

The oxidate spray along with the Nordox and Umbrella treatment were the only ones that significantly lowered bacterial populations; comparing the 40% and 80% populations were significantly less at 100% bloom for both treatments (Figure 2-1). In oxidate treatment, there were no bacteria present at the 80% sampling which was after the oxidate spray. Populations did recover at the 100% sampling and but was statistically less than before the oxidate spray (Figure 2-1). There were significantly less bacteria at 100% bloom at the recommended sprays (BP+4) compared to 80%, but was statistically at the 40% treatment. The other two treatments, Cueva and the late Blossom Protect, were statistically similar throughout the time points. (Figure 2-1). The 2 late sprays were statistically the same as the 4 recommended sprays.

The fungal population was significantly reduced after the oxidate spray (Figure 2-2). The reduction in fungal growth however, was similar to populations at 100% bloom of Cueva. Cueva (BP+C3) had a significant raise in fungal populations in 80%, but dropped back to levels at 40% at 100% bloom (Figure 2-2). The recommended Blossom Protect rate had higher fungal populations as bloom went on.

For *A. pullulans* populations, 10% bloom was sampled after the first spray of Blossom-Protect was applied. For the recommended spray (BP-4) there was a significant sharp population decline from 10% to 40% bloom (Figure 2-3). However the *A. pullulans* populations significantly recovered at 80% but failed to maintain population at 100% (Figure 2-3). The Cueva (BP+C3) gained a jump in population from 40% bloom to 80% bloom, but reverted back down to the lowest population level at 100% bloom (Figure 2-3). The Oxidate treatment (BP+O2), where Blossom-Protect was applied four hours after the oxidate burst, saw high levels of *A. pullulans* populations for both 80% and 100% bloom (Figure 2-3). BP+2L showed a significant increase in *A. pullulans* populations from 80% to 100% bloom, whereas BP+N+U3 stayed consistent through 40%, 80% and 100% bloom (Figure 2-3). The recommended spray (BP-4) had the highest yeast population levels.

For each treatment, *E. amylovora* populations were sampled at 100% bloom. All of the treatments have significantly less *E. amylovora* present than USC (Figure 2-4). There were some differences in the treatments as well; the Cueva treatment had no *E. amylovora* present (Figure 2-4).The Oxidate treatment had significantly more *A. amylovora* present than the other three treatments with populations present (Figure 2-4).

For the blossom presses indicating where or not yeast is present inside the blossom, BP+C3 shows a loss of *A. pullulans* positive ratings as bloom goes on, however the trend isn't statistically significant (Figure 2-5). For BP+4, there is also no significant difference in the screening for blossom protect (Figure 2-5). Besides the pre oxidate spray of BP+O2, the only other significant drop off is in BP+N+U3, where 100% bloom is statistically lower than any other of the blossom prints (Figure 2-5).

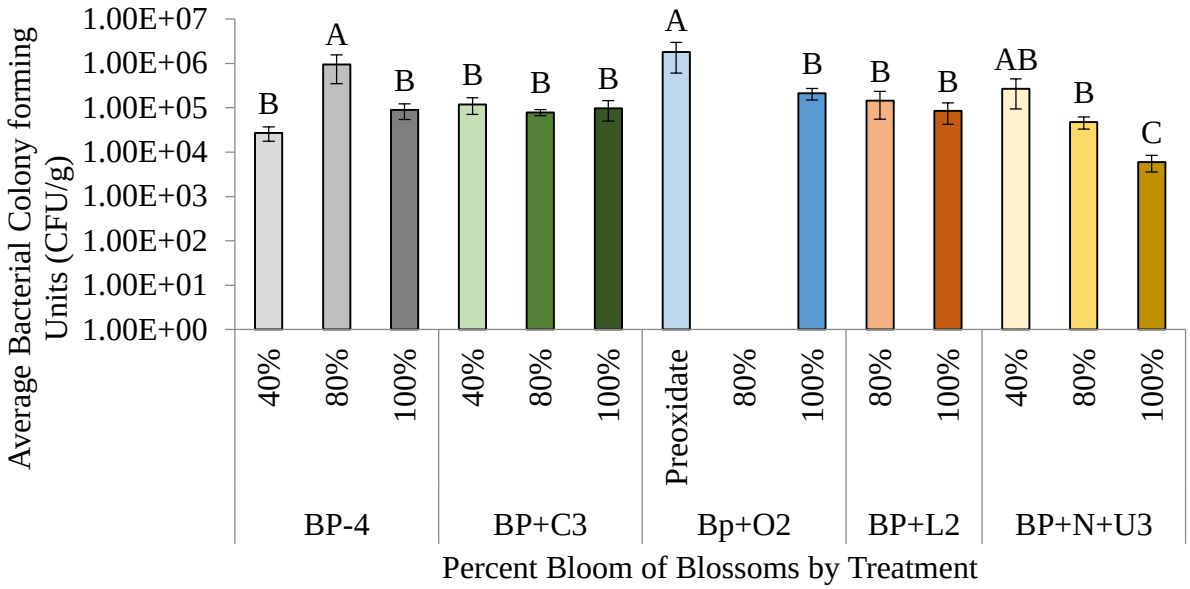

Figure 2-1: Total bacterial populations from flowers sampled in spring 2014 which were collected at the percent bloom indicated in the horizontal axis. Error bars represent standard error, *P* = >.05. Axis legend: Blossom-Protect + Cueva at 3 sprays (BP+C3), Blossom-Protect + Oxidate at 2 sprays (BP+O2), Blossom-Protect at 2 late sprays (BP+2L), Blossom-Protect + Nordox + Umbrella at 3 sprays (BP+N+U3), Blossom-Protect at 4 sprays (BP+4).

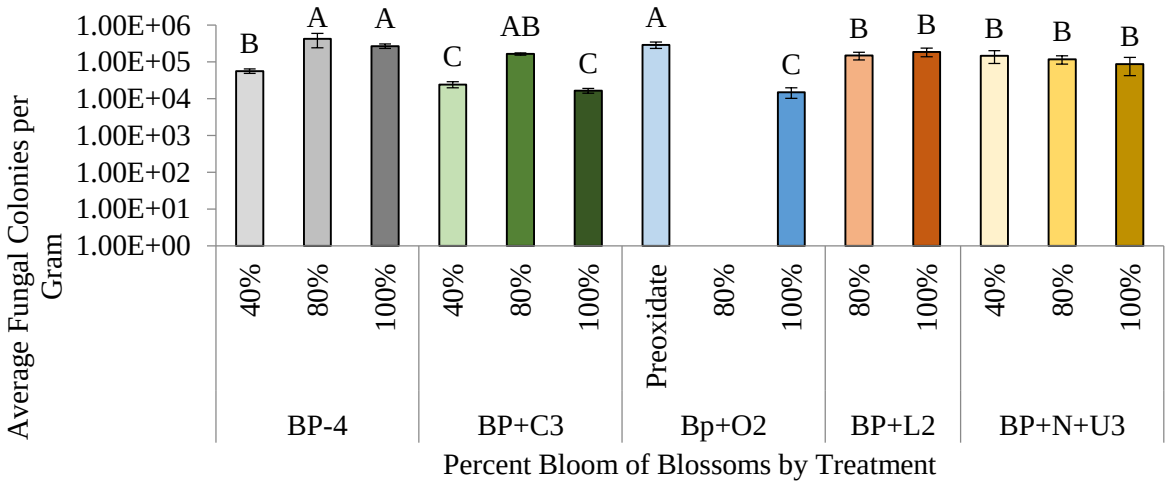

Figure 2-2: Fungal populations from flowers sampled in spring 2014 which were collected at the percent bloom indicated in the horizontal axis. Error bars represent standard error, *P* = >.05. Axis legend: Blossom-Protect + Cueva at 3 sprays (BP+C3), Blossom-Protect + Oxidate at 2 sprays (BP+O2), Blossom-Protect at 2 late sprays (BP+2L), Blossom-Protect + Nordox + Umbrella at 3 sprays (BP+N+U3), Blossom-Protect at 4 sprays (BP+4).

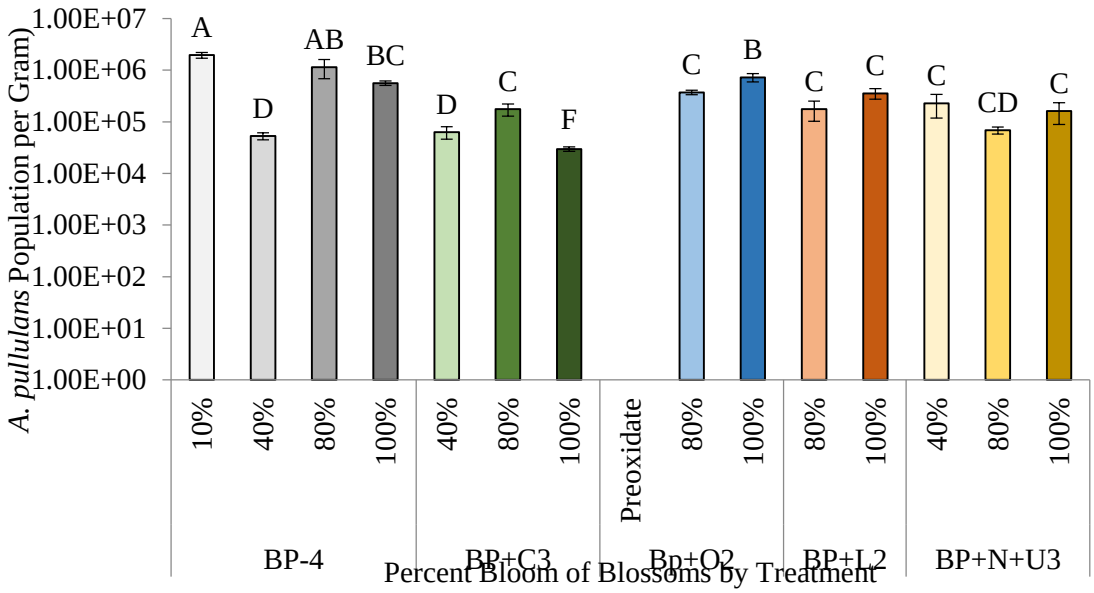

Figure 2-3: A. pullulans populations from flowers sampled in spring 2014 which were collected at the percent bloom indicated in the horizontal axis. Error bars represent standard error, *P* = >.05. Axis legend: Blossom-Protect + Cueva at 3 sprays (BP+C3), Blossom-Protect + Oxidate at 2 sprays (BP+O2), Blossom-Protect at 2 late sprays (BP+2L), Blossom-Protect + Nordox + Umbrella at 3 sprays (BP+N+U3), Blossom-Protect at 4 sprays (BP+4)

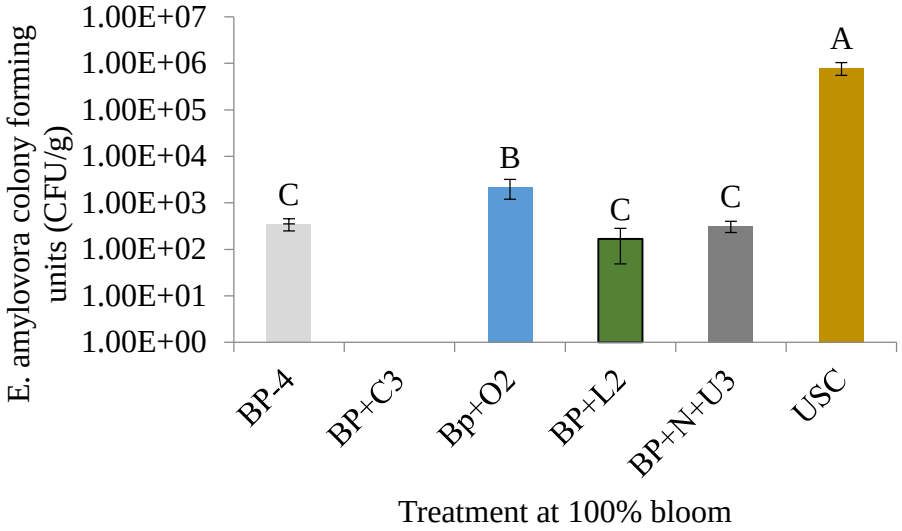

Figure 2-4: E. amylovora populations at 100% bloom for each treatment. Error bars represent standard error, *P* = >.05. Axis legend: Blossom-Protect + Cueva at 3 sprays (BP+C3), Blossom-Protect + Oxidate at 2 sprays (BP+O2), Blossom-Protect at 2 late sprays (BP+2L), Blossom-Protect + Nordox + Umbrella at 3 sprays (BP+N+U3), Blossom-Protect at 4 sprays (BP+4).

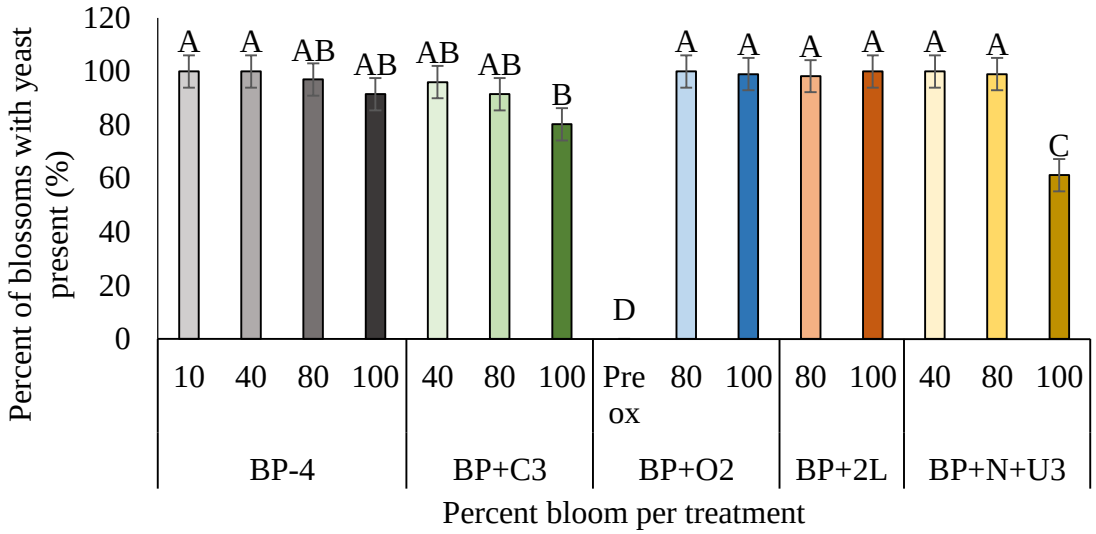

Figure 2-5: Average percent of blossom prints taken in the field that tested positive for A. pullulans at each sampling percentage. Error bars represent standard error, *P* = >.05. Axis legend: Blossom-Protect + Cueva at 3 sprays (BP+C3), Blossom-Protect + Oxidate at 2 sprays (BP+O2), Blossom-Protect at 2 late sprays (BP+2L), Blossom-Protect + Nordox + Umbrella at 3 sprays (BP+N+U3), Blossom-Protect at 4 sprays (BP+4).

4.2 2014 Field Results

On average, each treatment, though not always significant, was lower than USC for overall scab prevalence (Figure 2-6). Surprisingly, a few of the treatments were significantly lower than USC; for leaf and fruit scab BP+O2 and BP+N+U3 were significantly lower (Figure 2-6). Also lower than the USC was BP+C3 for fruit scab, however BP+4 and BP+2L were significantly the same as the USC (Figure 2-6).

In regards to shoot blight, all but one treatment had significantly lower incidence of blight; BP-4 actually had a higher prevalence of shoot blight than USC, but statistically lower blossom blight (Figure 2-7). The other treatments; BP+C3, BP+O2, BP+2L, and BP+N+U3 all had significantly lower incidence of blossom and shoot blight than USC, but there was no

significant difference between them (Figure 2-7). Between shoot and blossom blight however there are some differences; BP-4, BP+O2 and the USC had significantly lower incidence of shoot blight than blossom blight (Figure 2-7).

For fruit russeting, there was no significant difference in russeting between the stem (*P* =.1424) or calyx (*P* =1540), however there was a difference in over ten percent total fruit russeting (Figure 2-8). BP+N+U3 had 11% of the fruit having more than 10% russet, which was significantly different than the rest of the treatments. BP+4, BP+O2, and BP+2L had statistically the same amount of russet as the USC; while BP+C3 had more than the other treatments, the average was still lower than BP+N+U3 (Figure 2-8).

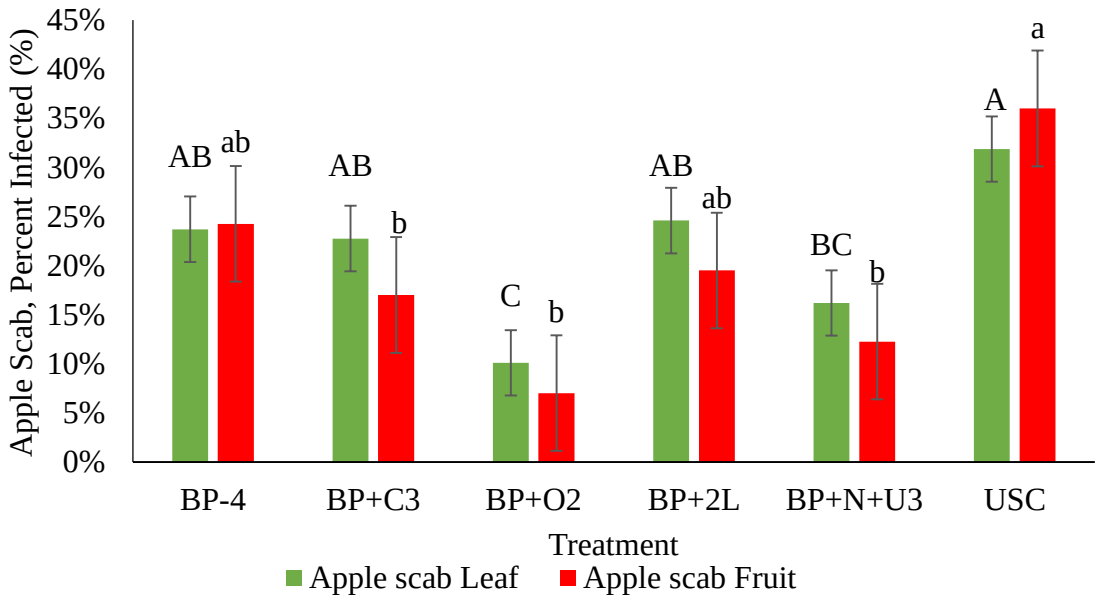

Figure 2-6: 2014 field data for Blossom-Protect treatment trials indicating the percent of infected apple leaves and fruit with apple scab. There were no significant differences between the leaf and fruit percent infected per treatment (*P*= .5532). Error bars represent standard error, *P* = >.05. Axis legend: Blossom-Protect + Cueva at 3 sprays (BP+C3), Blossom-Protect + Oxidate at 2 sprays (BP+O2), Blossom-Protect at 2 late sprays (BP+2L), Blossom-Protect + Nordox + Umbrella at 3 sprays (BP+N+U3), Blossom-Protect at 4 sprays (BP+4).

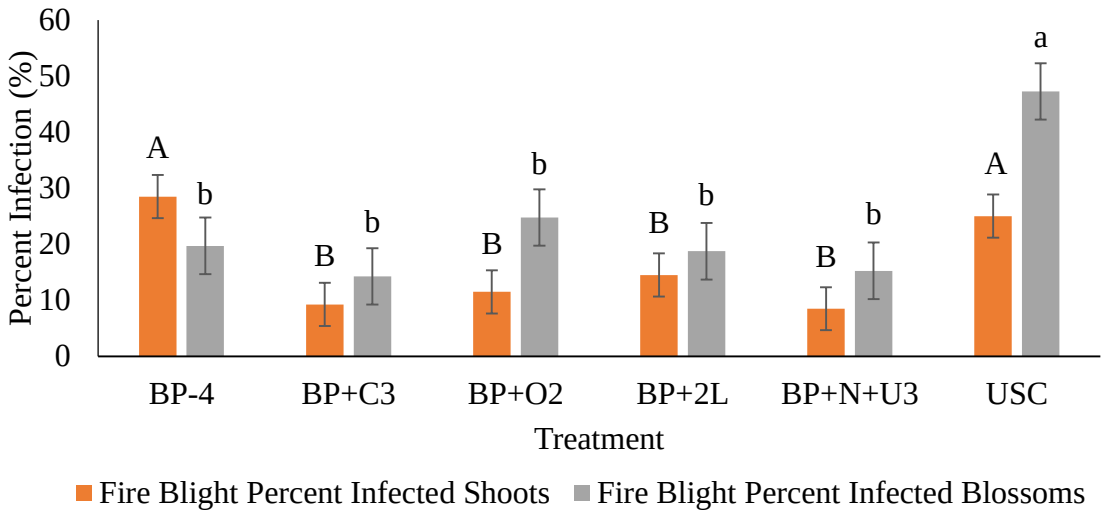

Figure 2-7: 2014 field data for Blossom-Protect treatment trials indicating the percent of infected apple shoots and blossoms with fire blight. There were significant differences (not noted in figure) between BP-4, BP-O2, and the USC shoot and blossom percent infected per treatment (*P* = >.05). Error bars represent standard error, *P* = >.05. Axis legend: Blossom-Protect + Cueva at 3 sprays (BP+C3), Blossom-Protect + Oxidate at 2 sprays (BP+O2), Blossom-Protect at 2 late sprays (BP+2L), Blossom-Protect + Nordox + Umbrella at 3 sprays (BP+N+U3), Blossom-Protect at 4 sprays (BP+4).

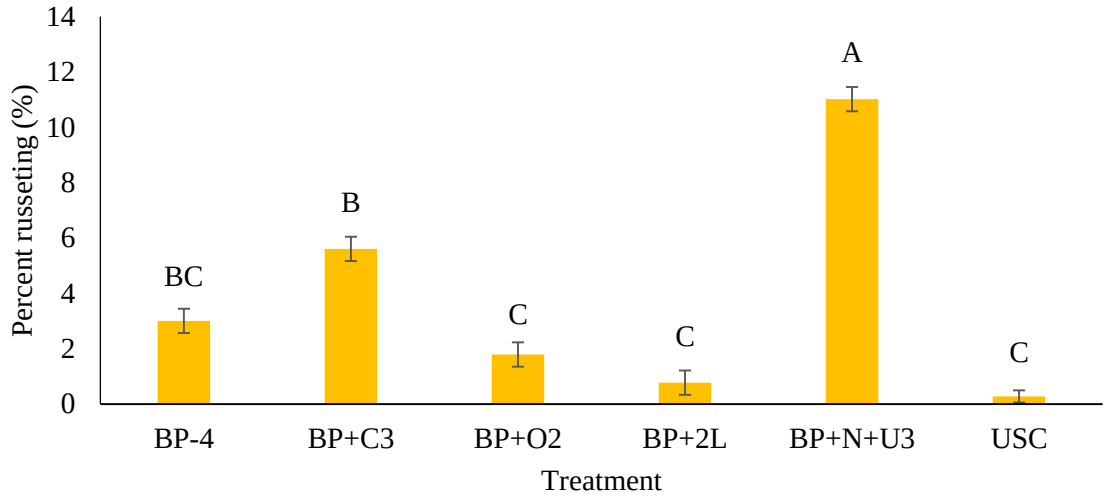

Figure 2-8: 2014 field data for Blossom-Protect treatment trials indicating the percent of fruit having more than 10% russeting. Error bars represent standard error, *P*= >.05. Axis legend: Blossom-Protect + Cueva at 3 sprays (BP+C3), Blossom-Protect + Oxidate at 2 sprays (BP+O2), Blossom-Protect at 2 late sprays (BP+2L), Blossom-Protect + Nordox + Umbrella at 3 sprays (BP+N+U3), Blossom-Protect at 4 sprays (BP+4).

4. Discussion

Overall there did seem to be some differences in the treatments for Blossom-Protect. One misleading treatment was BP+O2. BP+O2 may have done worse over all in the field, there seemed to be a decrease in fruit set for this treatment (personal observations). The flowers had a severe phytotoxic reaction to the oxidate spray, causing lower fruit yield. The oxidate spray may have had an effect on scab presence however, as the treatment had lower fungal populations present (Figure 2-2) and the incidence of scab was significantly lower (Figure 2-6). It might be advantageous to use the oxidate spray, if in fact that no significant decrease in yield is observed, simply to try to replace the fungicides that cannot be applied.

Surprisingly, the treatment with the most russeting was not BP+O2 with the oxidate application but BP+N+U3, which had the Nordox and Umbrella components. Nordox was observed to have higher incidences of russeting in other treatments in the orchard (personal observations). The treatment that should have shown the highest amount of russeting according to other trials was BP+4, as it had four applications of Blossom-Protect. However though this treatment was not significantly different the control, the treatment did have higher incidence of russeting on average (Figure 2-8). The Cueva component to BP+C3 could have resulted in higher russeting as well. Over all, the treatments that were purely Blossom-Protect did not show any increase in russet from the untreated control.

Even though BP+C3 had no *E. amylovora* present on the plates from the lab (Figure 2-7), the incidence of fire blight was still significantly the same as other treatments that contained higher populations of *E. amylovora* on plates (Figure 2-7). It is possible that uneven blossoming could have allowed for blossoms to not come in contact with Blossom-protect and allow

infection to occur. It is also possible that the blight that occurred was not from the applied Ea110, but from *E. amylovora* previously sprayed in that particular orchard.

The Blossom-Protect treatment did reduce incidence of blossom blight in spring 2014. Laboratory results showed that the yeast is able to compete and survive epiphytically on the flowers. More field trials in different weather seasons are needed to confirm that this product would be good for Michigan growers.

CHAPTER 3: AN ATTEMPT AT UV-C MUTAGENESIS OF FUNGAL PATHOGENS OF TREE FRUIT TO CONFER RESISTANCE TO SUCCINATE DEHYDROGENASE INHIBITORS FUNGICIDES

1. Abstract

New chemistries of SDHIs have been introduced for Michigan fruit trees to help combat fungal pathogens already resistant to other fungicide chemistries. With the advent of these new chemistries, cross-resistance may become a problem and render the new SDHIs useless. By using UV-C mutagenesis, new mutations can be selected in vitro that could allow for laboratory studies of cross-resistance before isolating resistant fungal pathogens in the field becomes a reality. Unfortunately, the UV-C mutagenesis conducted failed to induce mutations in *Monilinia fructicola* conferring 100% resistance to boscalid or fluxapyroxad. UV-C mutagenesis also failed to produce any level of SDHI fungicide resistance in *Blumeriella jaapii* or *Venturia inaequalis.* As a result of the failed mutagenesis, no cross-resistance was detected as well.

2. Introduction

2.1 Background on tree fruit fungicides

2.1.1 Cherry and Other Stone Fruit

In stone fruit, there are three board spectrum chemicals used for cherry leaf spot control: captan, chlorothalonil and copper. Cholorothanlonil cannot be used after chuck spilt, and copper can cause phytotoxicity to trees if applied under drought or low water conditions ((McManus et al., 2007). There are two major fungal diseases of cherry: American brown rot (*Monilinia fructicola*) and Cherry Leaf Spot (*Blumeriella jaapii*). Powdery mildew on cherries (*Podosphaera clandestina*) is also controlled by the fungicides used for these other two diseases.

Besides broad spectrum fungicides, single-site fungicides are also used in stone fruit production. For cherry leaf spot, demethylation inhibitors (DMIs) were traditionally used all season for control and were effective against American brown rot and powdery mildew

(McManus & Weidman, 2001). Due to consistent spraying of DMIs Cherry leaf spot resistance, especially in Michigan, has been observed and there have been notable failures in control (Sundin et al., 2005; Proffer et al., 2006). There has also been documented cross resistance to multiple fungicides that use DMIs as the mode of action (Proffer et al., 2006). A possibly reason for the resistance is the overexpression of *CYP51*, which is known to cause DMI resistance, has been found in DMI resistant *B. jaapii* isolates (Ma et al., 2006). The other major used class of fungicides are strobilurins (QoIs), which are used on American brown rot, powdery mildew and cherry leaf spot, and this class of fungicide also has resistance concerns (McManus et al., 2007).

The newest mode of action released for fungal disease of stone fruit are succinate dehydrogenase inhibitors (SDHIs) and were released in 2004. There is a chance that this chemistry is the last 'new' (the chemistry has been in use in other cropping systems longer) mode of action that tree fruit may see in a while, given current fungicide development trends.

2.1.2 Apple

In apple, two major diseases are controlled with fungicides: apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*). There are other diseases, such as Black rot (*Botryosphaeria obtusa*) or Flyspeck (*Schizothyrium pomi*) that fungicides can also control in the field. Typically in a commercial orchard, broad spectrum fungicides are applied to trees first to protect against initial infection. For apples, these broad spectrums include captan, which has a seasonal use limit, and ethylenebisdithiocarbamates (EBDCs) which also have a seasonal use limit and a 77 day pre-harvest interval (PHI), meaning that the fungicide cannot be used however many days indicated before fruit harvest.

After the broad spectrum fungicides, single-site fungicides are also used in commercial orchards for disease control. There is already evidence for resistance for QoIs in *V. inaequalis* (Steinfeld et al., 2002), as well as DMIs (Gao et all, 2009). The SDHIs, as like stone fruit, are the newest and possibly the only new chemistry that growers can utilize in their programs for years to come.

2.2 Background of SDHIs

The target enzyme for SDHI chemistries is succinate dehydrogenase which is part of the tricarboxylic cycle in the mitochondrial transport chain. This enzyme has four subunits which form a binding site by B, C, and D which usually bind ubiquinone; this binding site is the target for SDHI fungicides (Kuhn, 1984; Sierotzki and Scalliet, 2009; Avenot and Michaildies, 2010; Proffer et al., 2013; Sierotzski and Scalliet, 2013).

There are four SDHI compounds available for tree fruit: penthiopyrad, boscalid, fluopyram, and fluxapyroxad. Boscalid was introduced in Michigan for the 2004 growing season and is mostly used on cherry for cherry leaf spot and powdery mildew (Proffer et al., 2013). However, boscalid resistance has already been reported in other fungal diseases (Proffer et al., 2013; Sierotzski and Scalliet, 2013). Penthiopyrad was introduced in Michigan in 2012 and fluxapyroxad and fluopyram were both introduced in Michigan in 2013. Some of these compounds share chemical groups according to the FRAC (Table 3-1). As of 2010, there was evidence of no cross-resistance reported between SDHIs and other classes of fungicides, like QoIs (Avenot and Michaildies, 2010). However, cross-resistance between SDHI compounds have been reported, especially between boscalid and penthiopyrad (Fraaije et al., 2012; Proffer, 2013). However, fluopyram has a different binding chemistry and cross-resistance may take longer to develop (Fraaije et al., 2012).

Single-site fungicides typically have high risk of resistance development and one way FRAC and chemical companies are trying to preserve chemistries from resistance is by selling compounds as premixes. Most new fungicides, including the SDHIs, are sold as these premixes. Unfortunately for tree fruit, there is already resistance present for the second chemistry added to the SDHIs as mentioned above; premixes are meant for broad use of fungal diseases and crops and thus added selection pressure is placed on SDHI site. In general, the two fungicides in premixes are typically unequal in efficacy, length of use, and overall risk of resistance development.

Table 3-1: SDHI chemistries available and sorted into chemical group and common name. Common name in bold are for use on tree fruit. Taken from the FRAC website

CODE	TARGET SITE OF ACTION	CHEMICAL GROUP	COMMON NAME
7	Complex II; succinate-dehydrogenase	Phenyl-benzamides	Benodanil Flutolanil Mepronil
		Pyridinyl-ethyl-benzamide	**Fluopyram**
		Furan-carboxamides	Fenfuram
		Oxathiin-carboxamides	Carboxin Oxycarboxin
		Thiazole-carboxamides	Thifluzamide
		Pyrazole-carboxamides	Bixafen **Fluxapyroxad** Furametpyr Isopyrazam Penflufen **Penthiopyrad** Sedaxane
		Pyridine-carboxamides	**Boscalid**

With the emergence of more SDHI chemistries in Michigan, more studies involved in detecting cross-resistance between the compounds is needed to ensure proper rotations and to

avoid resistance in general. By using UV-C generated mutants, we can observe if any cross-resistance is even possible before finding isolates from the field.

3. Materials and Methods

3.1 *Monilinia fructicola*

3.1.1 UV-C dose determination for *M. fructicola* for a 80-90% conidial kill curve

M. fructicola conidia were collected from 3-4 day old cultures grown on V8 agar and transferred to 1.7 ml microcentrifuge tubes containing 500 µl of sterile diH_20. Three to four glass beads were also placed in the tubes. The tubes were then vortexed for 30 to 60 seconds to break up the conidial chains. The solution was poured into a 14 ml tube that had three layers of sterile cheesecloth plugged into the opening of the tube; this was completed using sterilized forceps. The filtered solution in the bottom of the 14 ml tube after being strained by the cheesecloth was then free of mycelium and large chains of conidia that were not broken by the vortex and beads. This new suspension was quantified using a hemocytometer with a light microscope then adjusted to $1x10^5$ conidia /ml by diluting the suspension with additional sterile diH_20 if needed. The suspension was then placed into a glass petri dish (Pyrex). The bottom of the dish was wrapped in a layer of parafilm to prevent slippage in the subsequent steps of the experiment before the conidia suspension was added. To determine the correct dose for a conidia survival rate of 10-20%, these cultures were exposed to either ~100, 250, 500, 750, or 1000 J m^{-2} of UVC (254 nm) radiation from an XX-15 UV lamp (UVP Products, San Gabriel, CA) placed horizontally at a fixed height above the conidia suspension. The lamp was turned on 15 minutes prior to use to allow for stabilization of the UV output. The XX-15 UV lamp was monitored with a UV-X radiometer fitted with a UV-25 sensor (UVP Products) and determined to be 1.3 J m^{-2} s^{-1} (Weigand and Sundin, 2009). The spore solutions were mixed during the UVC dosage by protected hand. After the UV-C dose was given to the conidia, the dishes were sealed and

wrapped in heavy grade aluminum foil and placed on a shaker for 24 hrs to prevent photoreactivation. A control glass plate was also used in each round of UV-C exposure and treated exactly the same just not subjected to the UV-C. After the dark incubation period was over, the conidia were recounted using the same method listed above. The spores were then adjusted if needed to $1x10^3$/µl using sterile diH_2O and 100 µl of the diluted suspension was transferred to water agar and spread plated. Conidia germination was counted 12 hrs after plating by using a plate grid and a dissection microscope to ensure accuracy. The control plates were counted first and the percent germination was determined based off the control plates.

3.1.2 Creating UV-C Mutant Isolates

Isolates were subjected to UV-C as described in the dose determination section, however the dose used was ~750 J m^{-2} of UVC (254 nm) to get 10-20% conidia survival rate. After treatment, the conidia suspension was plated onto V8-S media (V8 strained with cheese cloth then centrifuged and the supernatant was used instead of pure V8). V8-S media was ultimately used for *M. fructicola* as the conidia germinated more uniformly and the color of the media was easier to use in the subsequent steps. After the dark period for the reduced risk of phytoremediation mentioned in Section 3.1.1, 100 µl of treated or control conidia suspension was spread onto 10 ml V8-S medium and allowed to dry for 30 minutes. The plates were then treated with a 10 ml overlay (1/5 V8-S and .7% bacto agar) dosed with 10 µg/ml boscalid. Plates were then sealed and wrapped in aluminum foil and left for 12 hours. Plates were then examined for mycelium protruding out of the overlay. Any mycelium found growing through the overlay were harvested with sterile forceps and transferred to fresh PDAY (Potato dextrose agar with .5g yeast) amended with 10 µg/ml boscalid. These plates were monitored for growth, and if growth occurred, transferred to long term storage (PDAY slants stored at 4^oC).

3.1.3 Screening Mutant Isolates for Fungicide Resistance

After 25 isolates were harvested from the amended 10 µl/ml boscalid PDAY media, more rigorous fungicide testing began. These isolates were plug-plated onto 10, 15, 25, and 35 µg/ml boscalid or fluxapyroxad amended PDAY in triplicate. Growth was monitored daily and diameter measurements were taken each day until the control plates (un-amended PDAY) filled the plates. The original isolate that the conidia was originally harvested from was also used as a control in these screenings. This screen was repeated twice.

3.2 *V. inaequalis and B. jaapii* Mutant Isolation and Screening

The procedures used in the *M. fructicola* methods section were also subjugated upon *V. inaequalis* and *B. jaapii* with the UV-C dose determined to be 1000 and 750 J m^{-2} of UV-C (254 nm) respectively (results not shown). The spore counts dosed were also lower, averaging around $1x10^4$ conidia/µl due to the slow growth of these fungi in culture. When plating to count percent survival, Malt Dextrose agar (MMEA) and PDAY were used. Even though spores germinated and a dose was found, 10-15 runs, producing 10-20 plates a run, of UV-C followed with the 10 µg/ml boscalid overlay failed to produce any resistant mutants. Plates were even stored and checked for at least two months with no sign of germination.

4. Featured *M. fructicola* Results

The optimal dose for *M. fructicola* isolate SCHM13 was determined to be 750 J/M^2 as the results on average gave a conidial survival rate between 10-20% (Figure 3-1). After the first isolate kill curve was obtained, the other isolates were screened in a smaller window of doses closer to the doses that were effective with *M. fructicola* isolate SCHM13 (Figure 3-2). Another example of finding this kill curve was for isolate *M. fructicola* isolate BR36, where there was not

as many repeats carried out to determine the optimal dose (Figure 3-2). For all *M. fructicola* isolates, the dose of 750 J/M^2 delivered the 10-20% conidia survival rate.

An example of the lack of finding mutants for *M. fructicola* is featured in Table 3-1. From ten rounds of UV-C completed between 8/1/13 and 9/25/13, 25 mutants were discovered (Table 3-1). After the first round of UV-C, only two *M. fructicola* isolates were treated a round due to time constraints. The rates of finding mutants were consistently low, as anywhere from 400 to 800 spores were screened at a time per isolate (Table 3-1).

In the preliminary screening of boscalid and fluxapyroxad resistance in *M. fructicola* isolate SCHM13 and generated SCHM13 mutants there were differences comparing the mutants to the original SCHM13 isolate, however none of the mutants were at the same growth level as the un-amended media control. (Figure 3-3). Two of the mutants featured in this figure, UV 3 and UV 4, were closer achieving the same colony diameter as the un-amended control, but still were statistically reduced in size (Figure 3-3). These same mutants were screening at higher rates of fungicide, 15, 25, and 35 µg/ml boscalid and 15, 25, 35 µg/ml fluxapyroxad (Figure 3-4). These results however, indicate that while all of the mutants grew significantly larger than the original SCHM13 isolate on boscalid amended media, they were statistically lower in diameter than the un-amended control plate (Figure 3-4). The high fluxapyroxad doses were even less exciting, with many of the UV mutants having statistically similar growth as the SCHM13 isolate (Figure 3-4).

Though more resistant than the original SCHM13 isolate, which some mutants reaching over 200% more growth than the SCHM13 isolate on boscalid amended media, the mutants did not have the same relative growth levels as themselves or SCHM13 on un-amended media (Table 3-2). The higher rates of boscalid and fluxapyroxad had similar or even less drastic

relative growth levels (Tables 3-3 and 3-4). After this screening, *M. fructicola* isolates BROM72 and BR36 were dropped from this study as their mutants had even less growth than the SCHM13 mutants in relative growth differences (Data not shown).

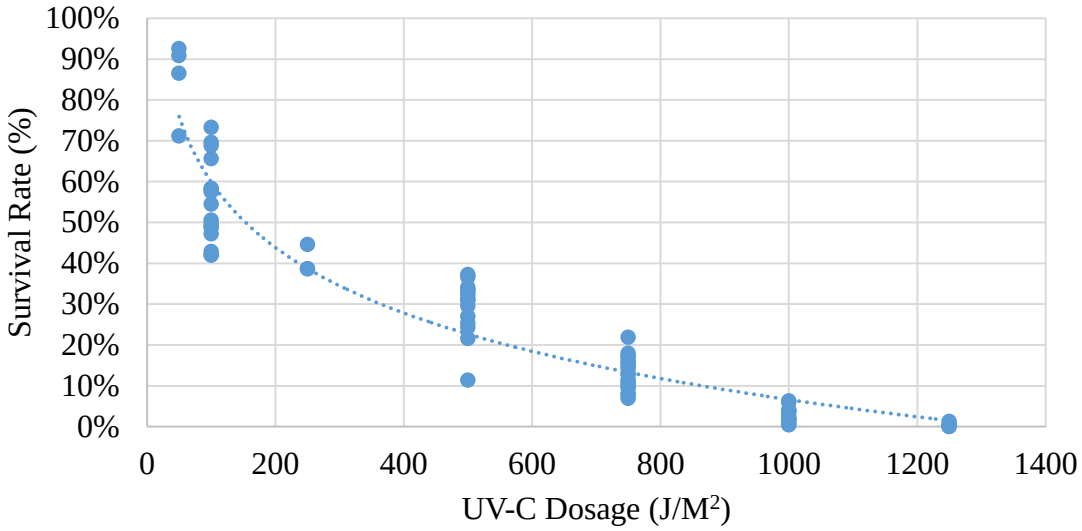

Figure 3-1: Survival rates of M. fructicola isolate SCHM13 conidia exposed to various doses of UV-C to determine a kill curve.

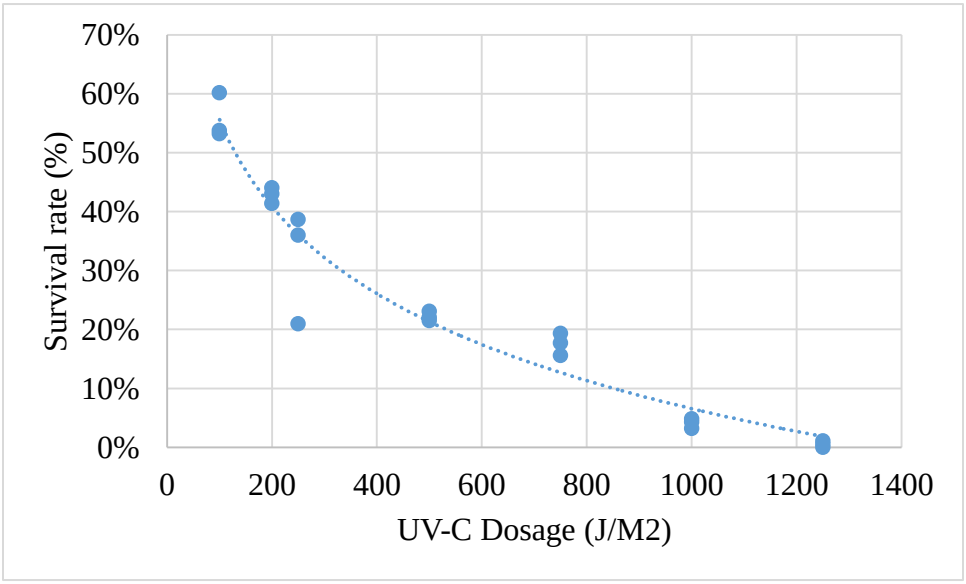

Figure 3-2: Survival rates of M. fructicola isolate BR36 conidia exposed to various doses of UV-C to determine a kill curve.

Table 3-2: The number of mutants isolated from three M. fructicola isolates (RBOM72, SCHM13, and BR36) from various rounds of UV-C treatments. Each round of UV-C used a fresh conidial suspension of two or three strains. When the total number of screened mutants got to 25, further fungicide resistance screening was conducted on the mutants.

Round of UV-C	RBOM72	SCHM13	BR36
1 (8/1/13)	2	1	3
2 (8/15/13)	-	1	1
3 (8/29/13)	1	1	-
4 (9/1/13)	-	1	1
5 (9/14/13)	2	1	-
6 (9/15/13)	1	2	-
7 (9/21/13)	-	1	0
8 (9/22/13)	-	2	0
9 (9/24/13)	1	1	-
10 (9/25/13)	0	2	-
Total	7	13	5

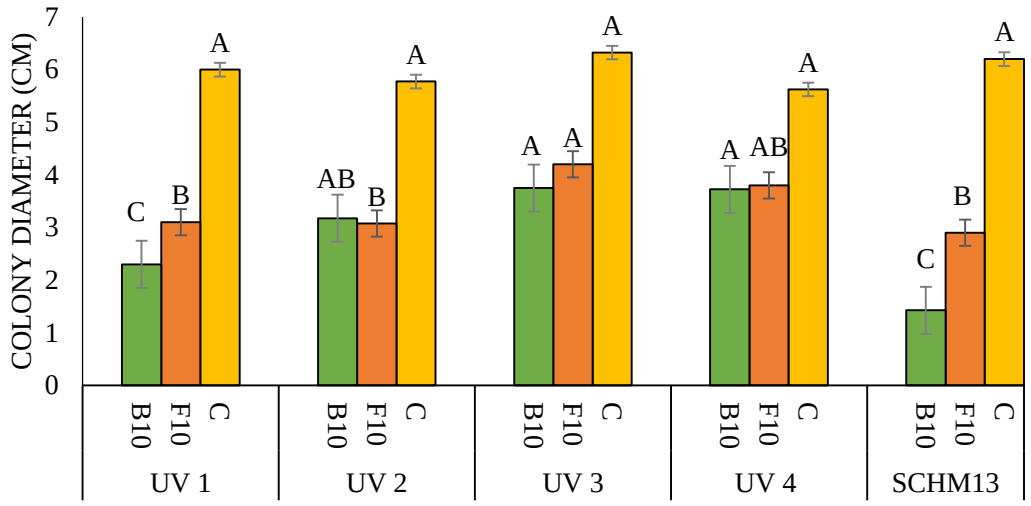

Figure 3-3: Preliminary screening of boscalid and fluxapyroxad resistance in M. fructicola isolate SCHM13 and generated SCHM13 mutants. B10 represents a dose of 10 µg/ml boscalid; F10 represents a dose of 10 µg/ml fluxapyroxad; C represents an un-amended control. Error bars represent standard error, *P*=<.05, same letters on the same color bar represents no significant difference.

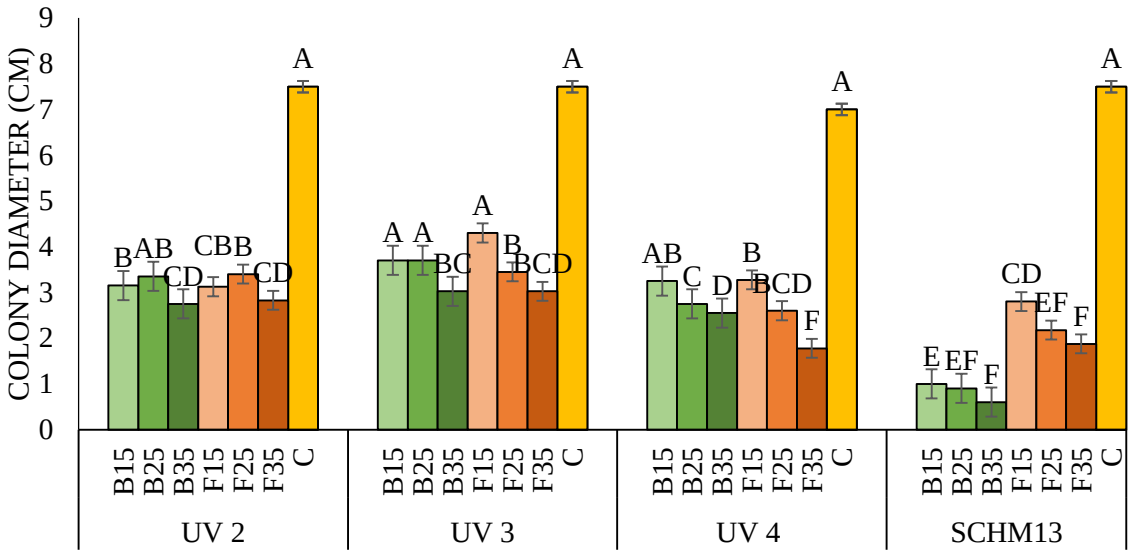

Figure 3-4: Higher doses of boscalid and fluxapyroxad resistance in M. fructicola isolate SCHM13 and generated SCHM13 mutants. B15, B25, B35 represents a dose of 15, 25, and 35 µg/ml boscalid; F15, 25, 305 represents a dose of 15, 25, 35 µg/ml fluxapyroxad; C represents an un-amended control. Error bars represent standard error, *P*=<.05, same letters on the same color bar represents no significant difference.

Table 3-3: Preliminary screening doses of boscalid and fluxapyroxad resistance in M. fructicola isolate SCHM13 and generated SCHM13 mutants. B10 represents a dose of 10 µg/ml boscalid; F10 represents a dose of 10 µg/ml fluxapyroxad; C represents an un-amended control. 'RG% to control dose' indicates the percent of relative growth to the control isolate (SCHM13) whereas 'RG% to control dose' indicates the percent of relative growth to the same mutant isolate grown on un-amended agar.

Isolate	Dose	RG% to Control Isolate	RG% to control dose	Isolate	Dose	RG% to Control Isolate	RG% to control dose	Isolate	Dose	RG% to Control Isolate	RG% to control dose
S101	B10	161.40	38.33	S101	C	96.77	100.00	S101	F10	106.90	51.67
S102	B10	182.46	47.27	S102	C	88.71	100.00	S102	F10	85.34	45.00
S11	B10	226.32	50.79	S11	C	102.42	100.00	S11	F10	131.90	60.24
S21	B10	171.93	43.75	S21	C	90.32	100.00	S21	F10	101.72	52.68
S31	B10	142.11	80.96	S31	C	108.06	100.00	S31	F10	70.69	75.6
S41	B10	222.81	54.98	S41	C	93.15	100.00	S41	F10	106.03	53.25
S51	B10	185.96	44.92	S51	C	95.16	100.00	S51	F10	98.28	48.31
S61	B10	187.72	46.12	S61	C	93.55	100.00	S61	F10	106.03	53.02
S62	B10	133.33	33.93	S62	C	90.32	100.00	S62	F10	98.28	50.89
S71	B10	263.16	59.29	S71	C	102.02	100.00	S71	F10	144.83	66.40
S81	B10	175.44	42.74	S81	C	94.35	100.00	S81	F10	105.17	52.14
S82	B10	261.40	66.22	S82	C	90.73	100.00	S82	F10	131.03	67.56
S91	B10	238.60	54.84	S91	C	100.00	100.00	S91	F10	141.38	66.13
SCHM13	B10	100.00	22.98	SCHM13	C	100.00	100.00	SCHM13	F10	100.00	46.77

Table 3-4: Higher screening doses of boscalid for resistance in M. fructicola isolate SCHM13 and generated SCHM13 mutants. B15, B25, B35 represents a dose of 15, 25, and 35 µg/ml boscalid; C represents an un-amended control. 'RG% to control dose' indicates the percent of relative growth to the control isolate (SCHM13) whereas 'RG% to control dose' indicates the percent of relative growth to the same mutant isolate grown on un-amended agar.

Isolate	Dose	RG% to Control Isolate	RG% to control dose	Isolate	Dose	RG% to Control Isolate	RG% to control dose	Isolate	Dose	RG% to Control Isolate	RG% to control dose
S11	B15	325	43.33	S11	B25	344.44	41.33	S11	B35	450	36
S21	B15	315	42	S21	B25	372.22	44.67	S21	B35	458.33	36.67
S31	B15	227.5	75.83	S31	B25	227.78	68.33	S31	B35	308.33	61.67
S61	B15	325	46.423	S61	B25	305.56	39.29	S61	B35	425	36.43
S71	B15	370	49.33	S71	B25	411.11	49.33	S71	B35	504.17	40.33
S81	B15	360	51.064	S81	B25	397.22	50.71	S81	B35	441.67	37.59
S82	B15	250	44.64	S82	B25	313.89	50.45	S82	B35	312.5	33.48
SCHM13	B15	100	13.33	SCHM13	B25	100	12	SCHM13	B35	100	8

Table 3-5: Higher screening doses of fluxapyroxad for resistance in M. fructicola isolate SCHM13 and generated SCHM13 mutants. F15, 25, 305 represents a dose of 15, 25, 35 µg/ml fluxapyroxad; C represents an un-amended control. 'RG% to control dose' indicates the percent of relative growth to the control isolate (SCHM13) whereas 'RG% to control dose' indicates the percent of relative growth to the same mutant isolate grown on un-amended agar.

Isolate	Dose	RG% to Control Isolate	RG% to control dose	Isolate	Dose	RG% to Control Isolate	RG% to control dose	Isolate	Dose	RG% to Control Isolate	RG% to control dose
S11	F15	126.79	47.33	S11	F25	159.77	46.33	S11	F35	88	22
S21	F15	111.61	41.67	S21	F25	156.3	45.33	S21	F35	150.67	37.67
S31	F15	78.57	73.33	S31	F25	77.01	55.83	S31	F35	65.33	40.83
S61	F15	116.96	46.79	S61	F25	119.54	37.14	S61	F35	94.67	25.36
S71	F15	153.57	57.33	S71	F25	158.6	46	S71	F35	161.33	40.33
S81	F15	133.93	53.19	S81	F25	106.9	32.98	S81	F35	85.33	22.7
S82	F15	66.07	33.036	S82	F25	22.99	8.93	S82	F35	26.67	8.93
SCHM13	F15	100	37.33	SCHM13	F25	100	29	SCHM13	F35	100	25

5. Discussion

None of the mutant isolates generated by UV-C of *M. fructicola* had comparable growth to control isolates or plates, and therefore did not express full resistance to the fungicides. There was also no evidence of cross-resistance in the mutant isolates screened. Though kill curves were established, no mutants were obtained that had any level of resistance to boscalid or fluxapyroxad from *B. jaapii* or *V. inaequalis.* The UV-C treatments given were ineffective at creating 100% resistant isolates to any of the SDHI compounds tested.

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