ANALYSIS OF *ERWINIA AMYLOVORA* POPULATION DYNAMICS AT FLOWER BLOOM AND FURTHER SYSTEMIC MOVEMENT OF THE PATHOGEN THROUGH HOST TISSUE

By

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ABSTRACT

ANALYSIS OF *ERWINIA AMYLOVORA* POPULATION DYNAMICS AT FLOWER BLOOM AND FURTHER SYSTEMIC MOVEMENT OF THE PATHOGEN THROUGH HOST TISSUE

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Population dynamics of *Erwinia amylovora* have been utilized for many applications over the past 50 years. In this work, populations were tracked over the course of apple bloom under naturally occurring environmental conditions. Flower stigmas inoculated on the 1st day of being open can harbor large (10^7) populations after 3 or 5 days post inoculation, with 100-fold increases in *E. amylovora* stigma populations observed in atmospheric conditions with daily average temperatures near 14^{o}C. These large 100-fold increases seem to occur at night, indicating that *Erwinia amylovora* is able to infect flowers in colder field temperatures than previously reported. In tandem, timing of antibiotic application relative to *E. amylovora* presence on flower stigmas had little impact on population dynamics, with streptomycin and kasugamycin consistently reducing populations while oxytetracycline was more variable. This study also led to the identification that Kasumin is prone to photodegradation. Culturable bacteria, yeast, and fungal populations were assessed over the course of bloom in relation to application of a biological control agent yeast (*Aureobasidium pullulans*) and a contact sterilant. Though populations fell rapidly directly after application, by 24 hours all populations returned to pre-spray levels. In regards to further systemic spread, a type three secretion system effector was indicted in blocking the abscission of infected flowers. The use of prohexadione calcium (Pro-ca) and acibenzolar-*S*-methyl (ASM) reduced internal *E. amylovora* spread in a seemingly synergistic manner on young trees, with rates of 28.3 g Pro-Ca + 28.3 g ASM or 56.6 g Pro-Ca + 28.3 g ASM. The sum of this

work gives us a better understanding of *E. amylovora* population dynamics in field conditions as well as the population response to current treatment options.

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KEY TO ABBREVIATIONS

ANOVA	Analysis of variance
ASM	Acibenzolar-*S*-methyl
Pro-Ca	Prohexadione calcium
Tukey's HSD	Tukey's honest significant difference test
WT	*Erwinia amylovora* strain Ea1189

CHAPTER 1

LITERATURE REVIEW OF POPULATION DYNAMICS IN *ERWINIA AMYLOVORA*

Introduction

Population dynamics in *Erwinia amylovora* has historically focused on a single strain's population size and reaction, either waxing or waning, in response to external stimuli. External stimuli examined for effect on populations includes environmental factors (Billing, 1974; Pusey, 1986; Schouten, 1987). Billing (1974) used temperature to show that *E. amylovora* growth rates were comparable between 21 and 30°C, and below 18°C the bacterial growth was reduced. Many studies have utilized *E. amylovora* population dynamics in some capacity for developing disease forecasting models (Steiner, 1990; Smith 1996; Billing, 2011; Smith and Pusey 2011; Turechek and Biggs 2015). Other researchers have further built on these forecasting models based on population dynamics, though mainly for the improvement of timing biological control applications (Thomson and Gouk, 2003; Pusey and Curry, 2004; Johnson et al., 2009). Population dynamics have also been utilized to directly gauge the potential effectiveness of various compounds produced by biological control agents (Pusey, 1997; Giddens et al., 2003; Pusey et al., 2009), and recently, Slack et al. (2021) published the effects of three antibiotics on *E. amylovora* stigma population dynamics.

The other definition of population dynamics focuses on the diversity and spatial distribution of individuals within that population. With the advent of genetic advances in strain characterization, many studies have introduced methods for differentiating *E. amylovora* strains from a genetic basis (McManus and Jones, 1995; Rezzonico et al., 2011; McGhee and Sundin, 2012; Zeng et al., 2018; Singh and Khan, 2019). In a review published by Puławska and

Sobiczewski in 2012, the authors compared the current and historical findings of using techniques such as Restriction fragment length polymorphism (RFLP), Random amplification of polymorphic DNA (RAPD), Amplified fragment length polymorphism (ALFP), Multiple Locus Variable-number Tandem Repeat Analysis (MLVA), and clustered regularly interspaced short palindromic repeats (CRISPR) arrays and found that only MVLA and CRISPR array typing was able to show a high degree of separation between isolates (Puławska and Sobiczewski, 2012). Recently, sequencing whole genomes has become both cheaper and easier. Hence, more studies have been published identifying other repeat regions potentially useful for differentiating strains (Smits et al., 2017)

In this mini review, I will examine topics regarding population dynamics of *E. amylovora*. Specifically, impacts of the environment, biological control agents and microbiomes, strain dynamics and distribution, and possible prospective directions will be discussed in this text.

Impacts of Environment

The role of environment in fire blight severity has been known since the 1930's (Rosen, 1933; Thomas and Ark, 1934). Over the past hundred years of fire blight research, there have been many models proposed for predicting blossom blight severity and best timing of management intervention, many utilizing population dynamics. As for which weather factors are the most important for population growth, the only true consensus is that temperature plays a crucial role in growth (Billings, 1974; Taylor et al., 2003; Pusey and Curry, 2004; Pusey and Smith, 2008; Farkas et al., 2012). Though it is known that temperature in general is important, the critical component seems to be the average temperature over the course of bloom and not the

minimum or maximum daily temperatures (Pusey and Curry, 2004). In this study, Pusey and Curry tracked the population of *E. amylovora* over multiple days in with different temperature fluctuations and found no effect of wild temperature swings on the growth trends. Under ideal atmospheric conditions of 24°C with 100% relative humidity, less than 24 hr is needed for *E. amylovora* populations to build up to 10^7 on detached flower stigmas (Pusey and Curry, 2004). However, in general, relative humidity is thought to be less crucial, with multiple studies concluding that relative humidity directly is not a major factor in the rapid growth of *E. amylovora* (Thomson, 1986; Taylor et al., 2003).

The other environmental factor heavily studied with population dynamics is flower phenology, mainly through flower age (Thompson and Gouk, 2003; Pusey and Curry, 2004; Dewdney et al., 2007). Younger flowers, open 1-3 days can support large populations of E. amylovora (Thomson and Gouk, 2003). Flowers that are 4-5 days old can also harbor large amounts of bacteria, however not as high as the freshly opened flowers (Thomson and Gouk, 2003; Pusey and Curry, 2004). Thomson and Gouk (2003) also found no difference in saprophytic bacteria populations between 3- to 6-day-old pistils, which led to the conclusion that flower age is more significant than the presence of other microbes. Thomson and Gouk observed flowers in the field and used weather inputs into MaryBlyt, however they did not use any statistical models to correlate weather to populations. For the entire study, EIPs from MaryBlyt indicated that conditions were ideal for infection. The study also rated for disease incidence, however there was no significant difference between flowers with different ages in the United States portion of the study (Thomson and Gouk, 2003). Different floral organs have been inoculated to determine the best growth sites of *E. amylovora*, with a consensus that the stigma is where the population mainly surges (Thomson, 1986; Rundle and Beer, 1997; Hasler and

Mamming, 2001). Not just apple flowers have been evaluated for differences in population dynamics, in 2006 Johnson et al observed *E. amylovora* populations on stigmas from 11 different plant genera. Using a constant temperature of 15°C, they found all but one genus from the rose family supported epiphytic populations of *E. amylovora* at rates comparable to apple stigmas (Johnson et al., 2006).

Impact of Biological Control Agents and Microbiomes

Bacterial antagonists

The use of other microbes as a possible biocontrol for fire blight has been examined since at least the 1970s (Riggle and Klos, 1970; Riggle and Klos, 1972; Erskine, 1973). Part of confirming a potential biological control agent is to evaluate if the addition of these candidate microbes changes the population dynamics of the pathogen. Starting in 1970, Riggle and Klos (1970) began evaluating *Pantoea agglomerans* (previously referred to as *Erwinia herbicola*) as a potential biological control agent. Using differences in population, they were able to ascertain that *P. agglomeran*s is a potential biological control agent for fire blight. Rundle and Beer (1987) further characterized the population dynamics of *E. amylovora* and *P. agglomerans* by observing populations on different organs over the whole flowers. They observed population shifts mainly on stigmas, and found that the growth was not as rapid with the addition of *P. agglomerans* (Rundle and Beer, 1987). Wilson and Sigee (1992) examined a strain of *P. agglomerans* isolated from hawthorn leaves against *E. amylovora* on hawthorn flower stigmas resulting in observing a decrease of growth rate of *E. amylovora* (Wilson and Sigee, 1992). Other strains of *Pantoea agglomerans*, such as Eh1087 can compete with *E. amylovora* if Eh1087 can establish before the pathogen arrives (Giddens et al., 2003). Other bacterial antagonists have been evaluated on the

basis of shifts in population dynamics, include *Pseudomonas fluorescens* and multiple *Bacillus* spp. (Wilson and Lindow, 1993, Pusey, 1997; Sundin et al., 2009).

In 1997, Pusey developed a protocol for using crab apple flowers in growth chambers to evaluate population dynamics between *E. amylovora* and potential biological controls. This work is widely used to compare the potential efficacy of potential biological control agents (Pusey, 1997). The use of bacterial antagonists in tandem with antibiotics have also been studied, with limited effect. The consensus is still that conventional biocides, such as streptomycin, are superior to antagonistic bacteria at reducing *E. amylovora* populations, either directly though observing population dynamics or through fire blight incidence (Stockwell et al., 1996; Sundin et al., 2009)

With the advent of high throughput sequencing what scientists know about the flower microbiome has changed rapidly over the past decade (Shade et al., 2013; Steven et al., 2018). The diversity of the microflora is immense, and simply applying a bacterial antagonist to flowers that are newly opened may not be fast enough to out complete the native bacteria in the system. A recent study by Cui et al. (2020) analyzed changes in the flower microbiome with the addition of *E. amylovora* and found that once inoculated *E. amylovora* grows rapidly on stigmas, outcompeting other species.

Bacteriophages

Another biocontrol agent that works by manipulating population dynamics are bacteriophages. Eve Billing first demonstrated that exopolysaccharides produced by *E. amylovora* could impact the ability of bacteriophage to infect cells (Billing, 1960). Other studies have touched on using bacteriophages as a biological control for fire blight (Hendry et al., 1967; Erskine, 1973; Richie and Klos, 1977; Schnabel et al., 1998).

In vitro analysis found that a phage cocktail was able to effectively reduce *E. amylovora* populations in a liquid broth (Schnabel et al., 1998). However, when the scientists took their cocktail to the field, the disease incidence was not as reduced as they had hoped suggesting that developing methodologies to enhance bacteriophage longevity in the field was needed (Schnabel et al., 1998). Roach et al. (2013) further quantified the impact of exopolysaccharides on bacteriophage pathogenesis and found that it was a factor effective for control.

A recent study examined the interactions between E. *amylovora, P. agglomerans*, and bacteriophages that infect and kill both species. The group has been studying the use of *P. agglomerans* strains as the phage carrier to overcome some of the problems identified with phage application. This study used quantitative real-time PCR to quantify population shifts instead of playting. Using a novel cocktail of multiple bacteriophages, they were able to successfully infect *P. agglomerans* that was then able to reduce *E. amylovora* populations by over four log units in 24 hours (Gayder et al., 2020). This study lays the ground work for the group to work on flowers in field or studying population dynamics on stigmas.

Antagonistic Yeasts

Besides bacterial antagonists and bacteriophages, yeasts have also been screened as potential biological control agents (Pusey et al., 2009). One of most promising candidates is *Aureobasidium pullulans*, a black yeast-like fungus, a common epiphyte of plants, and has been shown to have antagonistic properties (Lima et al., 2003, Kunz, 2004, Duffy et al., 2006, Pusey et al., 2009). In 2004, a Kunz observed that this yeast reduced incidence of fire blight in apple orchards. This observation was also noted by Duffy et al. (2006), who found that *A. pullulans* exhibited significant fire blight control on seven cultivars. The study also evaluated application timing, and determined that applying *A. pullulans* 24–48 hrs prior to *E. amylovora* inoculation

reduced pathogen population growth on flowers (Duffy et al., 2006). Pusey et al., 2009 evaluated the protocol developed in 1997 for testing potential antagonists and found that the yeast colonized detached apple flowers better than other antagonistic yeasts while also reducing *E. amylovora* populations.

Future Prospects

Population dynamics related to dissemination and internal movement

Slack et al., 2017 found that ooze droplets only contain between 9-14% of the population of internal tissues surrounding the ooze droplet immersion site, suggesting that more cells stay within the host and continue systemic spread rather than disseminate to potential new hosts (Slack et al., 2017). Future studies could be done expanding current knowledge of the internal dynamics of *E. amylovora* by identifying factors that interact with population movement.

Tracking strains through spatial dynamics

A study conducted by Wallis et al. (2020), also tracked the distribution and movement of *E. amylovora* through New York state. They utilized similar techniques presented in this work, however they also suggest developing an online platform plant pathogens in real time, modeled after the GenomeTrakr system developed by NCBI and FDA for tracking human pathogens. This would be a useful tool for tracking disease populations in real time. If strains can be associated with aggressiveness, streptomycin and other antibiotic resistance in real time, this could mean differences in orchard management of fire blight.

Conclusions

Studies on population dynamics have contributed to our basic understanding of the underlying conditions that lead to pathogen grown on flowers. These studies have developed forecasting models, identified potential biological control agents (some have been commercialized), and other applied applications. Even in the past few years, the hunt for new potential biological control agents is still underway, and with advances in microbiome analysis these potential biologicals may be screened more thoroughly than ever before. The use of molecular techniques for differentiating genomes will enhance knowledge on strain spread, virulence, and potentially change the way we recommend growers treat their orchards.

CHAPTER 2

IN-ORCHARD POPULATION DYNAMICS OF *ERWINIA AMYLOVORA* ON APPLE FLOWER STIGMAS

Abstract

Populations of the fire blight pathogen *Erwinia amylovora* on apple flower stigmas were tracked over the course of apple bloom in field studies conducted between 2016 and 2020. Flower stigmas inoculated on the 1st day of opening can harbor large (10^7 cells / flower) populations of *E. amylovora* three to five days post-inoculation. However, populations inoculated on stigmas of flowers that were already open for three days only exceeded 10^7 cells / flower in one of five experiments, and populations inoculated on stigmas of flowers that were already open for five days never exceeded 10^4 cfu / flower. The incidence of fire blight disease symptoms was also significantly higher for flowers inoculated when open for 1 day. During this study, ≥10-fold increases in *E. amylovora* stigma populations in a 24-hr time period (population surges) were observed on 36.2%, 20.0%, and 8.0% of possible days on 1-day, 3-day, and 5-day open flowers, respectively. Population surges were also observed in atmospheric conditions with daily average temperatures below 14ºC. The relative growth rate of *E. amylovora* on 1-day open flowers was as high as 12.8% per hour. Frequent sampling during days and at night revealed that many population surges occurred between 2200 and 0200 during the night. The incidence of fire blight on inoculated flowers that experienced low daily atmospheric temperatures (below an average of 14°C) was between 45-89%. This study refines our knowledge of *E. amylovora* population dynamics and further indicates that *E. amylovora* is able to infect flowers during exposure to colder field temperatures than previously reported.

Introduction

Fire blight, a disease of rosaceous plants caused by *Erwinia amylovora* (Burrill), first appears in spring in the form of blossom blight. Blossom blight can be quite devastating, as both yield and tree health are directly impacted at this stage; infected flowers are killed and the pathogen cells have a direct route into the host tissue. Once systemic, *E. amylovora* cells move through the cortical parenchyma or via the vascular system into the trunk of the tree, and migrate to the rootstock:scion junction where cankers are formed that girdle trees and eventually cause host death (Norelli et al., 2003; Billing, 2011). The initial infection period of bloom is thought to be the best time for disease management, as flowers are only open and susceptible to bacterial infection for a short period of time (Norelli et al., 2003; Pusey and Curry, 2004; Pusey and Smith, 2008; Farkas et al., 2012), and *E. amylovora* cells are located on the external stigma surface.

On apple, blossom blight disease symptoms (flower death) of fire blight are typically initiated in the spring on flowers as overwintering inoculum from cankers is disseminated to the surface of stigmas predominantly by insects (Norelli et al., 2003). The surface of the tips of stigmas particularly favors exponential growth and the massive buildup of *E. amylovora* in the intercellular spaces between columnar papillae cells as observed via scanning electron microscopy (Thomson and Gouk, 2003; Malnoy et al., 2012). Analysis of stigma exudates from apple flowers has shown the presence of approximately 2-3 μg of sugars per flower (mostly fructose and glucose) that are exuded from papillae cells (Pusey et al., 2008). Under conducive weather conditions, *E. amylovora* populations can double rapidly, and populations of 10^6 to 10^7 cells per flower are common (Thomson, 1986; Pusey, 2000; Taylor et al., 2003). The availability of free moisture through rain or heavy dew is then required to facilitate the migration of motile

E. amylovora cells down the style where these cells infect flowers through natural openings present in the nectaries (Bubán and Orosz-Kovács, 2003; Farkas et al., 2012; Thomson, 1986).

The role of environmental conditions in affecting blossom blight severity has been described since the 1930s (Rosen, 1933; Thomas and Ark, 1934). In an assessment of the growth rate of *E. amylovora* strains measured in vitro as a function of temperature, Billing (1974) showed that the relative growth rate was essentially the same between 21 and 30°C, and a considerable reduction in growth rate was observed below 18°C. Calculation of potential cell doublings per day by Billing (1978), with correction by Schouten (1987), indicated that *E. amylovora* cell populations could double approximately 1.9, 11.3, and 16.2 times during days with an average temperature of 8, 18, and 24°C, respectively. These calculations resulted in a slightly increased number of doublings for days with the same average temperature but higher maximum temperature, up to 28°C (Schouten, 1987). Cooler temperature effects on *E. amylovora* growth and infection potential have been less studied, but it was recently shown that *E. amylovora* can cause infection in vitro at temperatures as low as 4°C (Santander and Biosca, 2017). In a growth chamber experiment, *E. amylovora* cell populations inoculated to apple flower stigmas increased greater than 100-fold over a three-day period on trees incubated at 10°C, although the population increase was ca. 10,000-fold on trees incubated at 24°C (Dewdney et al., 2008b).

Another critical component affecting *E. amylovora* growth on flower stigmas is flower age. A field inoculation experiment of apple flowers, aged 1-8 days, conducted for one year at two locations, showed that 1-3 day-old flowers could support growth of the inoculated *E. amylovora* strain, but 5-8 day-old flowers did not support an increase in *E. amylovora* population (Thomson and Gouk, 2003). Likewise, in a greenhouse experiment, Dewdney et al. (2008a)

observed a 3-log increase in the inoculated *E. amylovora* strain Ea273 on 1 day-old apple flower stigmas and observed a highly variable response of Ea273 populations on 5 day-old flower stigmas. Thomson and Gouk (2003), using culture-based methods, found no difference in saprophytic bacterial populations between 3- to 6-day-old pistils, which led to their conclusion that flower age is more significant than the presence of other microbes in affecting *E. amylovora* population size.

Over the past century, many models have been proposed for predicting blossom blight severity and for identifying the best timings for management practices. Currently, the two mostly commonly-used predictive models for calculating fire blight risk at bloom in the United States are MARYBLYT and CougarBlight (Steiner, 1990; Smith, 1996; Smith and Pusey, 2011; Turechek and Biggs, 2015). Both models input weather data and flower age as factors for determining risk levels for fire blight infection, which, in MARYBLYT, is referred to as Epiphytic Infection Potential (EIP). MARYBLYT is modeled to predict both infection events and symptom development, whereas CougarBlight only predicts flower infection (Dewdney et al., 2007).

Historically, threshold maximum temperatures of 18°C and 21-27°C during bloom were suggested to be critical boundaries with the potential for severe infection when these daily maximum temperatures were reached (Mills, 1955; Leupschen et al., 1961; Billing, 1980). Embedded within these studies has been the long-standing assumption that higher daily maximum temperatures (up to 28-30°C) during bloom provide the conditions enabling maximal growth of *E. amylovora*. Mean daily temperatures have been used in predictive models; for example, Thomson et al. (1982) used critical mean temperatures of 16.7°C for early bloom (early March) infection and 14.4°C for infections occurring during secondary (rattail) bloom (1 May)

on pear in California. This seasonally-dependent reduction in threshold temperature was later suggested by Johnson et al. (2006) to reflect the epiphytic buildup and abundance of *E. amylovora* inoculum increasing over time during bloom. Experimental results from growth chamber experiments have shown that the average daily temperature over the course of bloom and not the minimum or maximum daily temperatures is most important for disease occurrence (Pusey and Curry, 2004).

Though there have been field studies focusing on stigma populations in the past, none have spanned the entire bloom event and considered various weather factors over multiple years and how these factors impact *E. amylovora* growth. The population dynamics of *E. amylovora* on stigmas under cooler temperatures in the field has also not been documented. We hypothesized that a multi-year field study of *E. amylovora* population dynamics on apple flower stigmas would enable us to more precisely understand the epiphytic behavior of this pathogen, refine disease prediction, and improve blossom blight disease management. In this study, we examined *E. amylovora* population dynamics on apple flowers of different ages from multiple cultivars. This enabled the monitoring of *E. amylovora* populations over time, as the flowers aged, and as weather conditions changed. We also assessed population dynamics at different times of day and night, revealing that the epiphytic growth of *E. amylovora* on flower stigmas occurs predominantly during the night.

Materials and methods

Population dynamics of *E. amylovora* on apple flower stigmas. All experiments were conducted on the apple cultivars ‘Fuji’, ‘Gala’, ‘Ginger Gold’, ‘Jonathan’, and ‘McIntosh’. The trees used for these field experiments are located at the Michigan State University Plant

Pathology Research Farm (42.689280, -84.484750) in East Lansing, MI or at the Northwest Michigan Horticultural Research Center (NWMHRC) (44.881996, -85.675251) in Traverse City, MI. Experiments were conducted between 2016 and 2019. Individual flowers were tagged at the pink phenological stage and inoculated with *E. amylovora* strain Ea110 (Zhao et al., 2005) on either the first day, third day, or fifth day after opening. Ea110 is a spontaneous rifampicin-resistant strain, facilitating re-isolation from field samples. Prior to field inoculation, strain Ea110 was cultured in LB broth overnight at 28°C, and populations were adjusted turbidimetrically (optical density at 600 nm) to $1x10^{3-4}$ CFU μl^{-1} using a Tecan Spark plate reader (Männedorf, Switzerland). The prepared inoculum was maintained on ice and inoculated within 1 hr onto flower stigmas at a rate of 1 μl per flower.

For analysis of stigmatic populations, at each sampling time point, 5 flowers were harvested from each replicate for a total of four replicates. With this there were 25 stigmas per replicate (5 stigmas per flower) and 100 per timepoint. Stigmas were harvested in the field with a sterilized razor blade and forceps, and then placed into 5 ml culture tubes containing 1 ml of 0.5 x phosphate-buffered saline solution. An initial re-isolation of inoculated flowers was conducted for a 0 hr time point, and the sampling was conducted every 24 hrs for 5 days. Stigma samples were kept on ice prior to arrival to the lab, then sonicated for 5 min (model 250T; VWR Scientific, Houston, TX), and placed back on ice. Plating methods were as described (Slack et al., 2021) with serial dilutions drop-plated onto LB agar plates amended with 100 μg ml^{-1} rifampicin and 50 μg ml^{-1} cycloheximide. The addition of both rifampicin and cycloheximide aided in the re-isolation of *E. amylovora* Ea110 and in reduction of fungal contaminants. Plates were wrapped in aluminum foil and incubated at 24°C for 48 hr before colonies were counted.

The resulting colony counts were used to determine the population size of *E. amylovora* Ea110 over the course of the 5 day experimental time periods.

Additional experiments were conducted with more frequent samplings. Stigmas of apple cv. Gala flowers that were open for 1 day were inoculated with *E. amylovora* Ea110 as described above. In one set of experiments, flower stigmas (four replicates) were sampled as described above at 2-3-hr intervals from 0800 to 1900 on the day after inoculation, and then at 48 hr after inoculation. This experiment was conducted in both 2017 and 2018. A second set of experiments were also conducted on flowers that were open for 1 day. In these experiments, flower stigmas were sampled overnight at 4-hr intervals starting on the second evening after inoculation (1800, 2200, 0200 (+1), 0600 (+1), 1000 (+1)). This experiment was conducted four times in 2019.

Incidence of blossom blight infection was rated approximately 2-3 weeks after petal fall for all experiments conducted in 2017 to 2019. During the initial inoculation of trees, 20 additional flowers were inoculated and used for the disease incidence rating to determine rates of infection for each treatment.

Data calculations and statistical analyses. We used the formula from Johnson et al. (2006) to calculate relative growth rates (*r*) of *E. amylovora* from 24-hr periods when population surges of at least 10 fold were observed. Additionally, equations formulated from Billing (1974) were used to determine the number of generations and the generation time of *E. amylovora* during population surges.

All statistical analyses were performed with the program RStudio for statistical computing (R Core Team, 2013). Hourly weather data were retrieved from the Enviroweather monitoring system stationed at the Michigan State University Horticulture farm (HORT) or the Traverse City weather station (NWMHRS). The HORT weather station was located within 2 km

of the test orchards; the NWMHRS station was less than 1 km from the inoculated trees. Weather factors measured hourly were downloaded from Enviroweather for temperature, relative humidity, precipitation, and windspeed. The weather variables were analyzed using the R package "Corrplot" to calculate Pearson correlations between the weather variables and *E. amylovora* populations for 24-hr periods when 10-fold increases in populations were observed (Wei et al., 2017). While calculating Pearson correlations, P-values were adjusted using Bonferroni correction (Napierala, 2012).

Results

Population dynamics of *E. amylovora* on apple flower stigmas. Population dynamics series experiments on 1-day, 3-day, and 5-day open flowers were conducted five times over three years, and an additional 21 experiments were conducted on 1-day open flowers over five years. *Erwinia amylovora* populations on 1-day open flowers reached the carrying capacity of apple flower stigmas (> 10^7 cfu / flower) in 10 of 25 experiments, and were greater than 10^6 cfu / flower in 18 of 25 experiments conducted over a five year period (Table 1). In contrast, *E. amylovora* populations on 3-day open flowers only exceeded 10^6 cfu / flower in 1 of 5 experiments, and populations only exceeded 10^4 cfu / flower in 1 of 5 experiments on 5-day open flowers (Table 1).

Data from two representative sets of experiments conducted in 2017 on 1-, 3-, and 5-day open flowers are shown in Figure. 1. The 1-day open flowers from cultivars 'Jonathan' and 'Gala' were inoculated on 26 April and 27 April 2017, respectively. Populations surged quickly on 'Jonathan" flower stigmas (~ 100-fold increase within 24 hr) and reached the carrying capacity by the fifth day of sampling (Figure 1). On 3-day open 'Jonathan' stigmas, the *E.*

amylovora Ea110 population increased about 10-fold over the first two days, and then decreased after that, never reaching the flower carrying capacity, and the population remained constant over the 5-day sampling period on 5-day open flowers (Figure 1). On 1-day open 'Gala' flowers, populations rose ca. 100-fold over the first two days of sampling, then were reduced and rebounded, but did not reach the flower carrying capacity. Populations on 3- and 5-day open 'Gala' flowers remained constant or were reduced over the five day sampling period (Figure 1). Weather parameters were compiled for the days during which all flower population data sets were generated, and selected weather parameters associated with one specific sampling period are shown in Figure 2. Air temperature was warmest on the first day of the experiment, when only 1-day open 'Jonathan' flowers were inoculated. A rain event in the middle of the experiment was associated with declines in populations in several of the treatments. The relative humidity was > 60% throughout the entire sampling period, and wind speeds dropped below 2 m s^{-1} during each overnight period (Figure 2).

Analysis of the 25 experiments conducted on 1-day open flowers revealed that inoculation of 10^3 to 10^4 cells of *E. amylovora* on 1-day open flowers was typically followed by a lag period, as populations similar to the inoculated total were recovered on the next sampling day (24 hr after inoculation) in 21 of these experiments. The exceptions to this observation where growth was observed on the first day were on warm days (24-hr avg. air temperature > 18ºC) or days with a precipitation event. Following this lag period, growth occurred over days 2-5 of the sampling period. We defined surges in *E. amylovora* growth as 24-hr periods in which increases in population size of greater than 10-fold were observed. We observed population surges on at least one day in 24 of the 25 experiments, and in 5 experiments, population surges were observed on three of the five days of the experiment (Table 1). Decreases in population size in a 24-hr

period were also observed in 19 of the 25 experiments (27 total events) conducted in 1-day open flowers; these decreases in population were associated with rain events or average daily relative humidity of under 40% in 25 of the 27 events (Table 1).

As defined by the relationships from Johnson et al. (2006) and Billing (1974), growth surges and also calculated the number of generations and generation time in hours (Billing, 1974). Growth rates during population surges ranged from 3.8 to 12.8% hr^{-1}, and the number of generations occurring during a 24-hr population surge was as high as 10.2 (generation time of 2.4 hr) (Table 2).

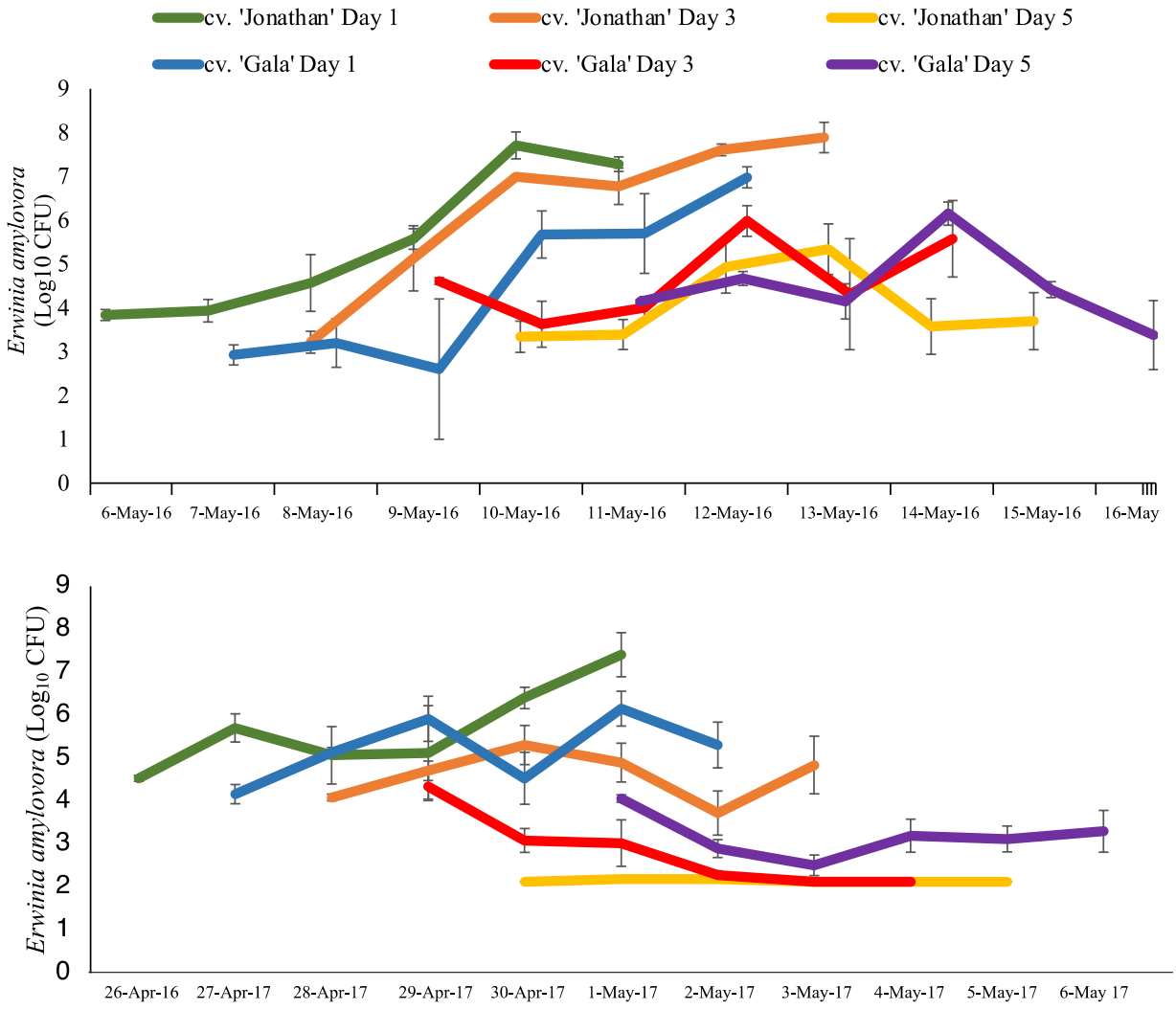

Figure 2-1. Population dynamics of *Erwinia amylovora* Ea110 from four representative experiments on 1-day, 3-day, and 5-day open flowers of apple cultivars ‘Gala’ and ‘Jonathan’ over a 5-day experimental period. Mean $\log_{10}$ populations ± the standard error of the mean are shown. Daily samplings were conducted at 1000.

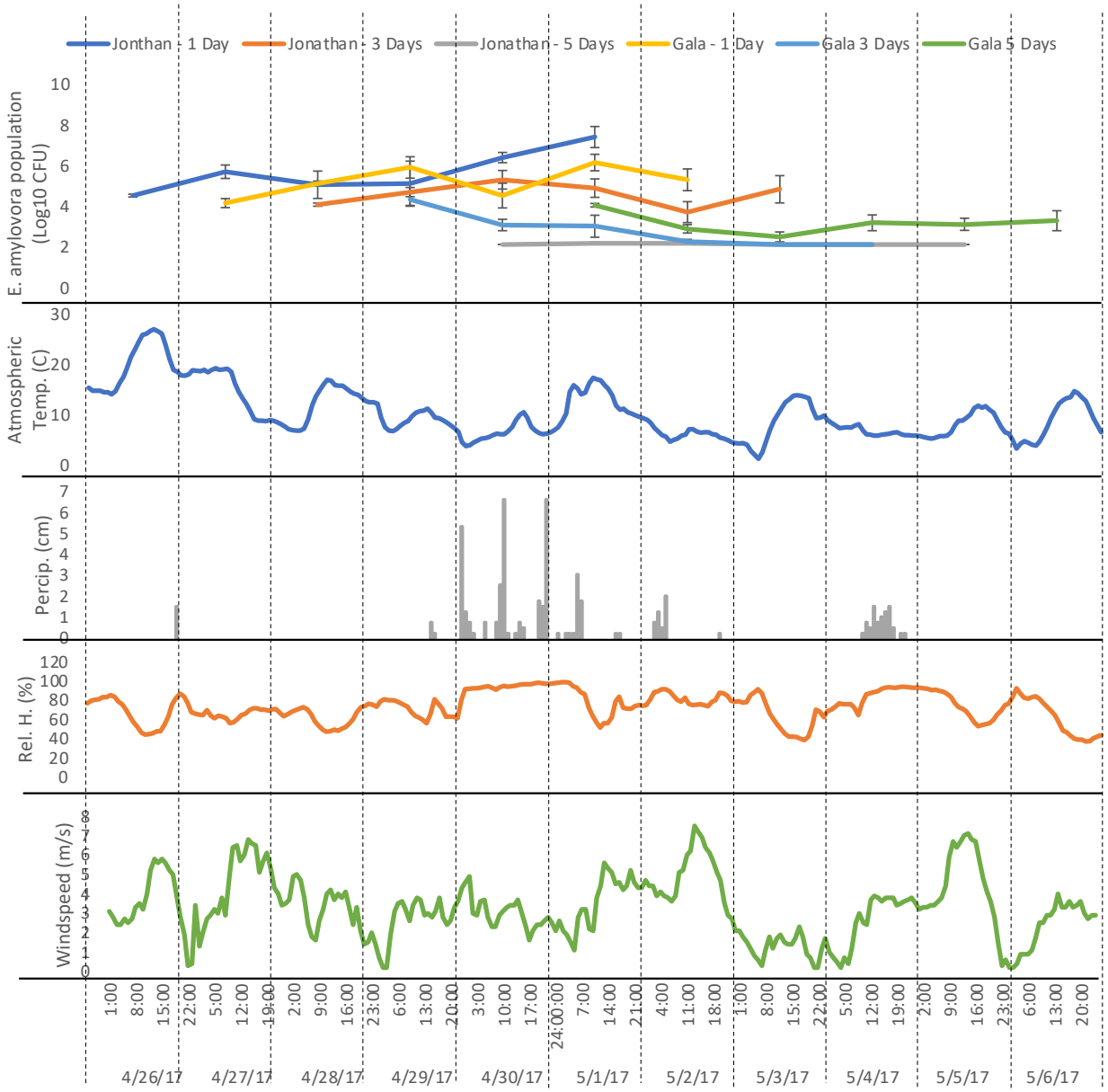

Figure 2-2. Population dynamics of *Erwinia amylovora* Ea110 from two representative experiments on 1-day, 3-day, and 5-day open flowers of apple cultivars 'Gala' and 'Jonathan' over a 5-day experimental period. Mean $\log_{10}$ populations ± the standard error of the mean are shown. Daily samplings were conducted at 1000. Weather parameters, temperature, precipitation, relative humidity, and windspeed are also shown.

Table 2-1. Results from field experiments tracking the population dynamics of *Erwinia amylovora* on apple flower stigmas where experiments were initiated on different-aged flowers.

Expt.[a]	Flower Age[b]	Initial population[c]	Final population	Population surges[d] (no. of days)	Decreasing populations in 24-hr period[e] (no. of days)	Rain days	RH < 40% days
16-F	1	2.9 ± 0.1	7.2 ± 0.1	3	1	1	0
16-F	1	3.5 ± 0.2	7.4 ± 0.2	2	1	0	0
16-G	1	2.9 ± 0.5	7.0 ± 0.6	2	1	0	1
16-G	1	2.9 ± 0.3	7.5 ± 1.0	3	0	0	0
16-G	1	3.2 ± 0.2	6.9 ± 0.3	2	1	0	0
16-G	1	4.2 ± 0.1	7.3 ± 0.2	2	0	0	0
16-J	1	3.8 ± 0.1	7.3 ± 0.2	3	1	1	0
17-G	1	5.7 ± 0.1	6.0 ± 0.2	2	2	2	0
17-G	1	4.3 ± 0.2	6.0 ± 0.5	2	2	2	0
17-G	1	4.2 ± 0.1	7.3 ± 0.2	2	1	0	1
17-G	1	4.2 ± 0.2	6.0 ± 0.5	1	1	1	0
17-G	1	3.0 ± 0.6	7.1 ± 0.1	1	0	0	0
17-G	1	5.9 ± 0.2	7.2 ± 0.2	1	2	2	0
17-J	1	4.6 ± 0.1	8.0 ± 0.5	2	2	2	0
17-J	1	2.2 ± 0.2	6.4 ± 0.5	3	1	1	0
17-J	1	2.3 ± 0.2	7.1 ± 0.1	3	0	0	0
17-M	1	4.3 ± 0.1	5.4 ± 0.3	2	2	2	0
17-M	1	3.8 ± 0.1	5.7 ± 0.4	2	2	2	0
17-M	1	3.8 ± 0.1	5.5 ± 0.2	2	2	2	0
17-M	1	4.3 ± 0.1	4.5 ± 0.4	2	2	2	0
18-G	1	2.8 ± 0.1	5.7 ± 0.5	1	0	0	0
18-G	1	4.0 ± 0.2	6.1 ± 0.1	1	0	0	0
18-G	1	4.1 ± 0.1	6.1 ± 0.3	1	0	0	0
19-GG	1	4.4 ± 0.1	4.3 ± 0.3	0	1	1	0
19-GG	1	3.6 ± 0.1	5.0 ± 0.2	1	0	0	0
19-J	1	4.1 ± 0.1	3.5 ± 0.4	0	1	1	0
19-M	1	4.3 ± 0.1	6.4 ± 0.1	2	0	0	0
20-F	1	1.8 ± 0.6	5.7 ± 0.3	2	1	0	1
16-F	3	3.4 ± 0.1	5.8 ± 0.2	1	1	1	0
16-G	3	3.6 ± 0.2	5.6 ± 0.8	2	2	2	0
16-J	3	3.2 ± 0.2	7.9 ± 0.3	2	0	0	0
17-G	3	4.6 ± 0.3	2.1 ± 0.1	0	2	2	0
17-J	3	4.1 ± 0.1	4.8 ± 0.6	0	2	2	0
16-F	5	3.8 ± 0.2	4.0 ± 0.8	0	1	2	0
16-G	5	4.1 ± 0.1	3.4 ± 0.8	1	4	2	0
16-J	5	3.3 ± 0.4	3.7 ± 0.6	1	2	1	0

Table 2-1 (cont'd).

17-G	5	4.1 ± 0.1	3.3 ± 0.5	0	3	2	0
17-J	5	2.1 ± 0.1	2.1 ± 0.1	0	0	0	0

[a] Experiment. 16-20 refer to years the specific experiment was conducted (2016-2020). F, 'Fuji'; G, 'Gala'; GG, 'Ginger Gold'; J, 'Jonathan'; M, 'McIntosh'.

[b] Flower age refers to the age of flowers when experiments were initiated. 1, 1-day open; 3, 3-day open; 5, 5-day open.

[c] Populations are in $\log_{10}$ colony-forming units per flower, and were isolated from five stigmas per flower.

[d] Population surges tabulates the number of days where the *E. amylovora* population increased by 10-fold or more.

[e] Decreasing populations tabulates the number of days in which a reduction in population was observed compared to the previous day, and if these decreases were associated with days with measurable precipitation or days where the average relative humidity was less than 40%.

Table 2-2. Analysis of population surge events (daily increase of $\log_{10}$ 0.9 or greater) observed in the field for *Erwinia amylovora* on 1-day and 3-day open flowers including the number of generations and relative growth rates observed in the respective 24-hr periods.

		Population surges				
Expt.[a]	Flower Age[b]	Initial population[c]	Final population	Number of generations[d]	Generation time (hr)	Relative growth rate (% / hr)[e]
16-F	1	3.3 ± 0.4	4.7 ± 0.4	4.7 ± 0.4	5.2 ± 0.2	5.8 ± 0.2
16-F	1	4.7 ± 0.4	6.1 ± 0.4	4.7 ± 0.4	5.1 ± 0.2	5.9 ± 0.2
16-F	1	5.7 ± 0.3	7.2 ± 0.1	5.0 ± 0.2	4.8 ± 0.1	6.2 ± 0.1
16-F	1	3.1 ± 0.2	5.1 ± 0.3	6.6 ± 0.2	3.6 ± 0.1	8.3 ± 0.1
16-G	1	2.6 ± 1.6	5.7 ± 0.5	10.2 ± 1.1	2.4 ± 0.5	12.8 ± 0.5
16-G	1	5.6 ± 0.9	7.0 ± 0.2	4.6 ± 0.6	5.2 ± 0.3	5.7 ± 0.1
16-G	1	6.5 ± 0.6	7.5 ± 0.2	3.3 ± 0.4	7.3 ± 0.2	4.1 ± 0.1
16-G	1	3.8 ± 0.3	6.0 ± 0.1	7.2 ± 0.2	3.3 ± 0.1	9.0 ± 0.1
16-J	1	4.6 ± 0.6	5.6 ± 0.2	3.2 ± 0.4	7.4 ± 0.2	4.1 ± 0.1
16-J	1	5.6 ± 0.2	7.7 ± 0.3	7.1 ± 0.2	3.4 ± 0.1	8.9 ± 0.1
17-F	1	3.0 ± 0.6	4.2 ± 0.2	4.2 ± 0.4	5.7 ± 0.2	5.3 ± 0.1
17-G	1	4.2 ± 0.2	5.1 ± 0.1	3.2 ± 0.1	7.5 ± 0.1	4.0 ± 0.1
17-G	1	3.5 ± 0.6	6.2 ± 0.4	5.4 ± 0.5	4.5 ± 0.2	6.8 ± 0.2
17-J	1	4.5 ± 0.1	5.7 ± 0.3	3.9 ± 0.2	6.2 ± 0.1	4.9 ± 0.1
17-J	1	5.1 ± 1.1	6.4 ± 0.2	4.3 ± 0.7	5.6 ± 0.3	5.3 ± 0.1
17-J	1	6.4 ± 0.2	7.4 ± 0.5	3.3 ± 0.3	7.2 ± 0.1	4.2 ± 0.2
17-J	1	2.2 ± 0.2	3.2 ± 0.3	3.4 ± 0.2	7.1 ± 0.1	4.2 ± 0.1
17-J	1	3.2 ± 0.3	4.7 ± 0.6	5.1 ± 0.4	4.7 ± 0.2	6.4 ± 0.3
17-J	1	3.7 ± 0.6	7.0 ± 0.2	7.4 ± 0.4	3.2 ± 0.2	9.3 ± 0.1
17-J	1	2.3 ± 0.2	3.4 ± 0.1	3.6 ± 0.1	6.8 ± 0.1	4.5 ± 0.1
17-J	1	3.4 ± 0.1	5.3 ± 0.3	6.3 ± 0.2	3.8 ± 0.1	8.0 ± 0.1
17-J	1	5.3 ± 0.3	6.8 ± 0.1	4.8 ± 0.2	5.0 ± 0.1	6.0 ± 0.1
17-M	1	3.8 ± 0.1	4.8 ± 0.1	3.3 ± 0.1	7.2 ± 0.1	4.2 ± 0.1
17-M	1	4.2 ± 0.4	5.5 ± 0.2	4.5 ± 0.3	5.3 ± 0.1	5.6 ± 0.1
17-M	1	4.1 ± 0.2	5.7 ± 0.4	5.2 ± 0.2	4.6 ± 0.1	6.5 ± 0.1
17-M	1	3.8 ± 0.3	5.8 ± 0.1	6.5 ± 0.2	3.7 ± 0.1	8.2 ± 0.1
18-F	1	4.2 ± 0.2	7.1 ± 0.1	9.3 ± 0.1	2.6 ± 0.1	11.7 ± 0.1
18-G	1	2.9 ± 0.1	3.8 ± 0.2	3.0 ± 0.1	8.0 ± 0.1	3.8 ± 0.1
18-G	1	3.8 ± 0.2	5.7 ± 0.5	6.3 ± 0.3	3.8 ± 0.1	7.9 ± 0.2
19-GG	1	5.4 ± 0.1	6.4 ± 0.1	3.3 ± 0.1	7.2 ± 0.1	4.2 ± 0.1
19-GG	1	3.7 ± 0.2	5.0 ± 0.2	4.1 ± 0.2	5.8 ± 0.1	5.2 ± 0.1
19-M	1	4.3 ± 0.1	5.4 ± 0.1	3.5 ± 0.1	6.8 ± 0.1	4.4 ± 0.1
20-F	1	4.4 ± 0.9	6.3 ± 0.2	6.4 ± 0.6	3.7 ± 0.3	8.0 ± 0.1
16-F	3	3.9 ± 0.2	6.2 ± 0.2	7.6 ± 0.2	3.1 ± 0.1	9.6 ± 0.1
16-G	3	4.0 ± 0.3	6.0 ± 0.3	6.6 ± 0.3	3.6 ± 0.1	8.3 ± 0.1

Table 2-2 (cont'd).

16-G	3	4.3 ± 1.3	5.6 ± 0.9	4.2 ± 1.0	5.7 ± 0.4	5.3 ± 1.0
16-J	3	3.2 ± 0.2	5.1 ± 0.7	6.4 ± 0.3	3.8 ± 0.1	8.0 ± 0.3
16-J	3	5.1 ± 0.7	7.0 ± 0.1	6.2 ± 0.4	3.9 ± 0.2	7.8 ± 0.1
17-J	3	3.7 ± 0.3	4.8 ± 0.7	3.7 ± 0.4	6.2 ± 0.2	4.7 ± 0.4

[a] Experiment. 16-20 refer to years the specific experiment was conducted (2016-2020). F, 'Fuji'; G, 'Gala'; GG, 'Ginger Gold'; J, 'Jonathan'; M, 'McIntosh'.

[b] Flower age refers to the age of flowers when experiments were initiated. 1, 1-day open; 3, 3-day open; 5, 5-day open.

[c] Populations are in $\log_{10}$ colony-forming units per flower, and were isolated from five stigmas per flower.

[d] The number of generations per day for each population surge event was calculated using the formula

[e] The relative growth rate per hour (r) was calculated from Johnson et al. (2006) using the formula: $r = 1/24 \bullet [\ln(y_{24}) - \ln(y_{zero})]$ where y_{zero} and y_{24} are observed population means at the beginning and end of a 24-hr period where a surge in population was observed.

Identification of weather parameters most associated with effects on *E. amylovora* growth on apple flower stigmas and blossom blight disease incidence. We selected 15 (out of 25) representative 24-hr population surge events on 1-day open flowers, and tabulated daily averages and maximum and minimum values for temperature, relative humidity, and windspeed occurring during these time periods (Table 3). The average temperature recorded during 24-hr periods with population surges ranged from 8.7 to 23.0°C, and average daily relative humidity ranged from 41.0 to 88.2% (Table 3). The minimum wind speed recorded during the 24-hr periods ranged between 0.0 and 1.7 m s^{-1} (Table 3). The average daily temperature was not consistently high during days when the *E. amylovora* growth rate was high. For example, we observed a growth rate of 10.3% per hr on 'McIntosh' flowers when the average daily temperature was 8.7°C which exceeded the growth rates of 6.4% and 8.0% per hr on 'Jonathan' flowers when the average daily temperature was 23.0°C (Table 3). The daily maximum temperature exceeded the reported threshold maximum temperatures of 18°C and 21-27°C associated with the potential for severe infection (Mills 1955; Leupschen et al. 1961; Billing 1980) in only 6 of 15 datasets, and, in 5 of 8 instances, daily growth rates above 5.9% per hr occurred on days with maximum temperatures below 14.8°C (Table 3).

A calculated Pearson's correlation coefficient value was used to numerically discern the relationship between population surges and specific weather variables (Table 4). Coefficient values for weather variables with a P-value < 0.05 were considered significant. Using this calculation, we determined that increases in atmospheric temperature and relative humidity along with decreases in wind speed were the three weather variables that significantly affected *E. amylovora* population surges (Table 4). Other weather variables assessed, leaf wetness and solar radiation, did not significantly affect population surges.

Blossom blight disease incidence was determined for 12 experiments conducted between 2017 and 2019 on 1-day open flowers, and ranged from 30% to 100% of inoculated flowers (Table 5). For these experiments, we also tabulated the ending *E. amylovora* population five days after inoculation, and then for days 5 to 14 after inoculation, we quantified the number of days with: maximum air temperature > 18ºC, average temperature > 16.7°C [critical mean temperature for early bloom infection (Thomson et al. 1982)], rainfall ≥ 0.25 mm, average RH > 85%, and overnight air temperature falling within 2.0ºC of the dewpoint (Table 5). We observed 64% blossom blight disease incidence in one experiment where there were 0 days (5-14 days after inoculation) with a maximum air temperature > 18°C and average temperature > 16.7°C, and observed blossom blight incidence ranging from 45 to 89% in three other studies in which only one day occurred (5-14 days after inoculation) with a maximum air temperature > 18ºC and average temperature > 16.7°C (Table 5). Similarly, days with average RH above 85% did not occur 5-14 days after inoculation in six of the experiments with blossom blight incidence ranging from 40 to 89% (Table 5). We did observe 3-5 days with rainfall greater than 0.25 mm in the period from 5 to 14 days after inoculation in all of the experiments, and also observed at least six overnight conditions that were likely suitable for dew formation (Table 5).

Table 2-3. Weather parameters associated with population surges of *Erwinia amylovora* during growth on 1-day open apple flower stigmas.

Cultivar	Growth rate in 24 hours	Total Pcpn (mm)	% RH high	% RH low	% RH Avg	Average temp (C° hourly average)	High temp C°	Low temp C°	Average Windspeed (m/s)	Windspeed High (m/s)	Windspeed Low (m/s)
Jonathan	2.6	0	48	33.2	41.0	10.8	13.6	8.7	1.9	4.6	0.2
Fuji	5.9	0	82	37.4	57.4	9.6	14	5.4	3.1	4.8	1.7
Gala	12.9	0	57.4	47.7	53.7	12.2	11.6	11.6	1.8	3.5	0.4
Gala	5.2	0	61.9	34.2	47.5	13.4	15.7	11.3	1.4	2.3	0.2
Fuji	12.2	0	54.1	33.8	46.4	14.8	17.7	12.2	1.6	3.8	0.2
McIntosh	10.3	0	70.9	68.9	70.1	8.7	8.9	8.5	2.2	3.8	0.2
Gala	3.3	0	76.2	60.5	70.4	13.3	14.2	12.5	3.25	4.9	1.6
McIntosh	8.2	0	76.2	60.5	70.4	13.3	14.2	12.5	3.25	4.9	1.6
Jonathan	6.4	0	60	43.5	53.3	23.0	25.4	21.7	3.3	5.6	1.5
Jonathan	8.0	0	60	43.5	53.3	23.0	25.4	21.7	3.3	5.6	1.5
Gala	12.0	0	81.5	48	62.3	20.3	22.6	22.6	3.8	6.0	1.5
G. Gold	4.2	0	55.4	74.7	80.5	8.3	12.1	6.5	2.5	3.1	1.2
McIntosh	4.2	0	89.1	68.3	80.9	17.7	19.8	17	1.9	2.7	0.3
Jonathan	3.1	8.1	95.6	80	88.2	18.2	20.5	15.7	2.4	4.4	0
Fuji	4.5	0	96.3	61.1	76.4	19.6	23.3	15.7	2.2	3.5	0.2

Table 2-4. Calculated Pearson correlations of weather variables with overnight growth of *Erwinia amylovora* during days with population surges on apple flower stigmas. Correlations shown are statistically significant ($P < 0.05$). Positive correlations are depicted with positive value, and negative correlations have a negative value. Values range between -1 and 1; the stronger the correlation, the closer the value will be to 1 or -1, depending on the type of relationship. For this table, if a correlation is positive, then growth increased as the weather variable value increased.

	Pearson correlations		
	Average atmospheric Temperature (^{o}C)	Average windspeed (m s^{-1})	Average %RH
Population surges	0.38	-0.71	0.22

Table 2-5. Incidence of blossom blight infection on apple flowers previously inoculated with *Erwinia amylovora* at the 1-day open age. Weather parameters with the potential to affect growth and infection are also listed.

			Number of days (from 5 to 14 days after inoculation) with the following conditions[c]				
Expt.[a]	*E. amylovora* population on day 5[b]	Blossom blight incidence (%)	Max. temp. > 18°C	Avg. temp. > 16.7°C	Rainfall ≥ 0.25 mm	Avg. RH > 85%	Overnight air temp. within 2.0 °C of dewpoint
M	5.4 ± 0.3	89	1	1	4	0	7
G	5.3 ± 0.5	45	1	1	4	0	7
M	4.5 ± 0.4	77	1	1	4	0	7
J	7.4 ± 0.5	64	0	0	4	0	8
J	7.1 ± 0.1	75	4	4	5	2	11
GG	4.3 ± 0.3	30	3	3	4	2	14
GG	5.0 ± 0.2	35	5	5	4	2	14
M	6.4 ± 0.1	65	6	6	5	5	14
J	3.5 ± 0.4	100	6	6	4	5	14
F	5.7 ± 0.3	57	11	11	5	1	12
M	5.7 ± 0.4	40	3	3	3	0	6
M	5.5 ± 0.2	50	3	3	3	0	6

[a] F, ‘Fuji’; G, ‘Gala’; GG, ‘Ginger Gold’; J, ‘Jonathan’; M, ‘McIntosh’.

[b] *E. amylovora* populations recorded on day 5 after inoculation are in $\log_{10}$ colony-forming units per flower, and were isolated from five stigmas per flower.

Abbreviations: Max. temp. is the maximum daily temperature; Avg. temp. is the average daily temperature from hourly measurements; Avg. RH is the average daily relative humidity from hourly measurements.

Population dynamics assessment of *E. amylovora* on apple flower stigmas with frequent samplings. To better understand the growth dynamics of *E. amylovora* on apple flower stigmas, we conducted experiments on 1-day open 'Gala' flowers in 2017 and 2018 at the NWMHRC, using a sampling frequency of every 2-3 hr during the day with a 13-14 hr gap between the last evening sample and the next sample the following morning. In 2017, we observed a ca. 10-fold increase in population between 1500 and 1900, with an additional increase of over 100-fold between 1900 and 0800 the next morning (Figure 3). In 2018, we observed a ~ 10-fold increase in population between 1400 and 2000, with an additional increase of slightly greater than 10-fold occurring between 2000 and 1000 the next morning (data not shown).

These experimental results led us to conduct a further series of four experiments in 2019 using overnight sampling to identify timings during the night in which *E. amylovora* population size was increasing. For these experiments, 1-day open flowers were utilized, and intensive sampling was initiated on the second overnight period after inoculation to account for the lag phase in growth which was typically observed on the first day after inoculation. In experiment 1, a 10-fold increase in population size was observed in samplings between 1800 and 0200 (Figure 3A), and in the other three experiments, increases in population size of 10-fold or greater were observed in samplings between 2200 and 0600 (Figure 4B - 4D). In experiment 3, a 10-fold increase was observed between 2200 and 0200 (Figure 4C). We also graphed the air temperature and dew point during the overnight hours of these experiments, which indicated that the air temperature was close to the dewpoint during the overnight hours in two of the four experiments (Figure 5A and 5C), and that the air temperature was within 1°C of the dewpoint between 2200 and 0200 in the other two experiments (Figure 5B and 5D).

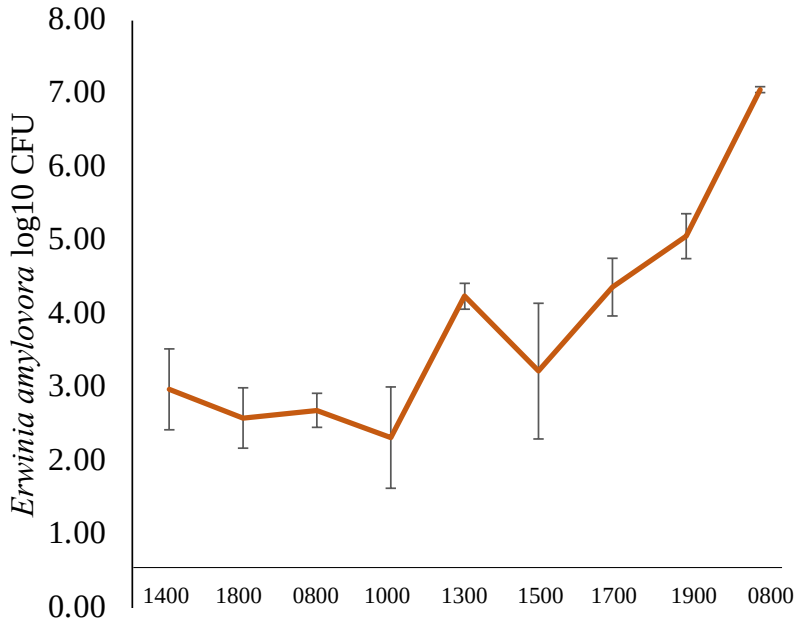

Figure 2-3. Population dynamics of *Erwinia amylovora* Ea110 on 1-day open flowers of apple cultivar 'Gala' over a 42-hr experimental period with frequent samplings during daylight hours of the second day of the experiment. Mean $\log_{10}$ populations ± the standard error of the mean are shown.

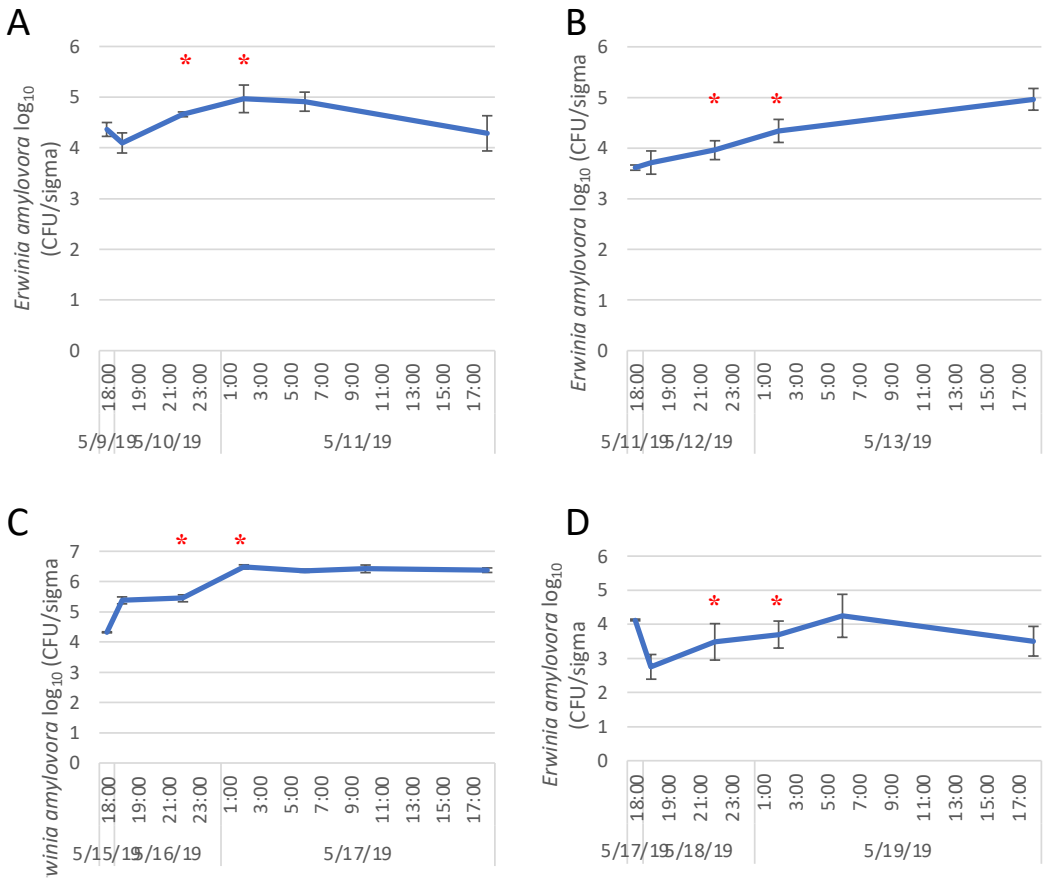

Figure 2-4. Population dynamics of *Erwinia amylovora* Ea110 on 1-day open flowers of apple cultivar 'Gala' over a 47-hr experimental period with frequent samplings during overnight hours of the second day of the experiment. Mean $\log_{10}$ populations ± the standard error of the mean are shown. Red asterisks highlight the 2200 and 0200 (+1) sampling time points.

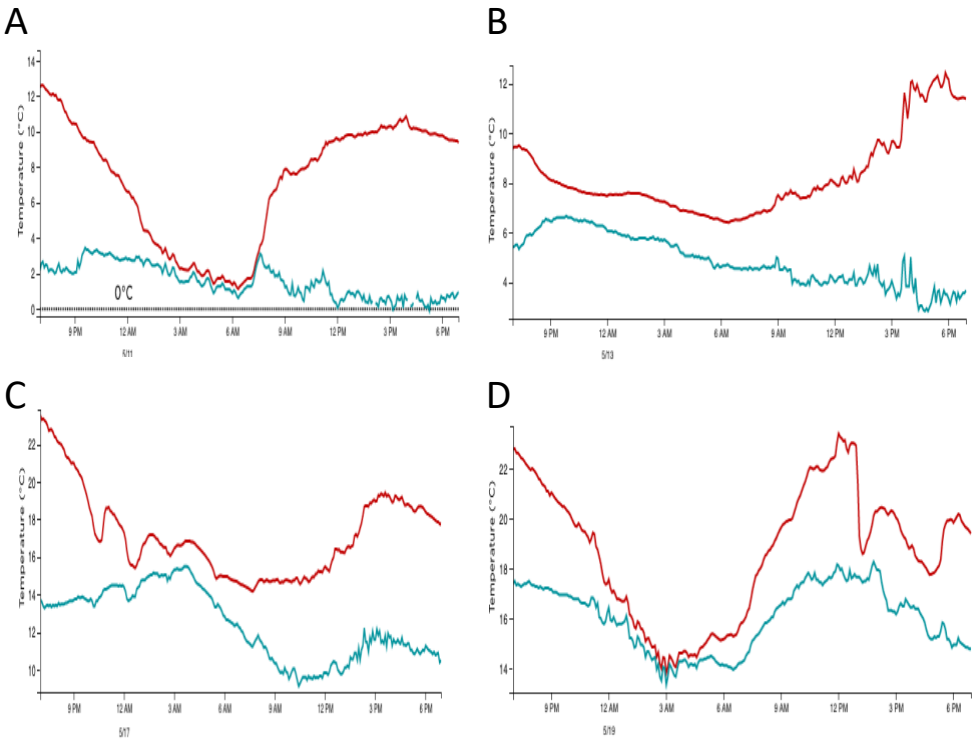

Figure 2-5. Temperature and dewpoint readings for the overnight periods of four experiments monitoring the population dynamics of *Erwinia amylovora* on 1-day open apple flower stigmas with frequent overnight sampling time points.

Discussion

Our analysis of the population dynamics of *E. amylovora* on apple flower stigmas under field conditions has shown that: (i) growth is most robust on younger flowers; (ii) the most important weather parameters affecting growth on stigmas are average daily temperature, average daily relative humidity, and average windspeed; (iii) short-term large increases (> 10-fold) in population size can occur when average daily temperatures are considered suboptimal by widely-used current fire blight disease prediction models; and (iv) surges in population growth occur at night. The factor that most favored population growth of *E. amylovora* to the flower carrying capacity was flower age and, as expected, the more favorable growth conditions on 1-day open flowers were correlated with an increased chance of infection.

We generated a total of 25 datasets tracking *E. amylovora* population dynamics on apple flower stigmas from 1-day open flowers over 5-day periods, and noted that surges, increases of greater than 10-fold in population over a 24-hr period, and growth to carrying capacity of the flower occurred in 92% and 38.5% of the experiments, respectively. In contrast, growth to carrying capacity was only observed in 1 of 5 experiments using 3-day open flowers and 0 of 5 experiments using 5-day open flowers. Our field results of population dynamics of *E. amylovora* on apple flower stigmas of different aged flowers conducted over five years are similar to previous field results from Thomson and Gouk (2003), combining one year of results from Utah with one year of results from New Zealand, and greenhouse results from Dewdney et al. (2008a). As flowers age, the quantity of free sugars, mostly glucose and fructose, increased from 1.9 μg / flower at early anthesis (1 day open) to 3.2 μg / flower at late anthesis (4-5 days open), and that polysaccharide (mostly arabinose and galactose), ranged from 17 to 33 μg / flower at late anthesis (Pusey et al. 2008). However, flower aging is also accompanied by the collapse of

papillae cells accompanied by extensive stigmatic secretions (Thomson and Gouk 2003). These modifications to the stigma surface appear to affect new colonization by *E. amylovora*, and may block access to preferred sites for growth. However, our data do show that *E. amylovora* populations that were established on 1-day open flowers can continue to grow between the fourth and fifth day after inoculation. A recent study by Cui et al. (2020) has shown that the diversity of the apple flower stigma microbiome decreases as flowers age, and predominantly consists of Enterobacteriaceae and Pseudomonadaceae in 5 day open flowers. These results might also indicate that immigrant bacteria in general have difficulty in colonizing older flowers, and that Enterobacteriaceae and Pseudomonadaceae are the most competitive bacteria in the preferred growth sites of the apple flower stigma.

Our work tracking the population dynamics of *E. amylovora* on 1-day open flowers over four years encompassed a wide range of environmental conditions and allowed for identification of optimal timings for growth and the critical environmental parameters that significantly affect growth when the physical status of the stigma habitat was optimal. Historically, most studies of *E. amylovora* populations on flowers have focused on temperature as the most important environmental factor affecting growth and/or the potential for blossom blight infection (Mills, 1955; Leupschen et al., 1961; Billing, 1980; Thomson et al., 1982; Thomson, 1986; Thomson and Gouk, 2003). While these previous studies focused on the importance of warmer temperatures driving blossom blight infection, there have been a few studies indicating that *E. amylovora* growth (Dewdney et al., 2008b) and infection (Santander and Biosca, 2017) can occur at colder temperatures (ex. 4°C and 10°C) under controlled conditions. While we did identify temperature as a significant factor driving *E. amylovora* growth, particularly affecting 24-hr

surges in population size, these surges occurred many times when the average daily temperatures were below previously-identified thresholds.

In most situations where population surges occurred under colder conditions, blossom blight disease was also observed. This result suggests that critical temperature minimums or average daily temperature thresholds utilized by fire blight disease prediction programs may be too high and therefore underestimate the disease risk at lower temperatures. It should be noted, however, that we were studying flowers that were inoculated with 10^{3-4} cfu of *E. amylovora* cells. Normally, under cool to cold conditions (average daily temperature < 10-14°C), ooze droplets may not be present on overwintered cankers, and insect activity would be much reduced, significantly decreasing the chances for delivery of the pathogen to flowers. Thus, the important consideration from the stand point of disease prediction would be growth potential on cool to cold days that follow a warmer period such that *E. amylovora* populations on flowers would already be established.

We found that rain events were mostly associated with 24-hr decreases in *E. amylovora* populations on apple flower stigmas. This is not surprising as rain splash can disperse bacterial cells to other flowers, and also because rain facilitates the movement of *E. amylovora* cells down the style to the hypanthium. Thus, even though reductions in population were observed, we recorded a comparable level of blossom blight incidence in experiments impacted by rain. In a small number of cases, we also observed reductions in *E. amylovora* population size occurring on days where the average RH was below 40%. Pusey (2000) has shown the relationship between RH and *E. amylovora* population size on apple flowers maintained in a growth chamber with results indicating that *E. amylovora* reached the highest population densities when the RH was 80-100%.

Our observation of *E. amylovora* growth on flower stigmas occurring at night, specifically between 10 PM and 2 AM is interesting because the optimal growth temperature for this organism observed in vitro is close to 28°C (Billing, 1974). Growth at night does validate the finding of Pusey and Curry (2004) from growth chamber studies that showed the importance of the contribution of the average daily temperature to *E. amylovora* growth rather than the daily temperature range. But how do environmental conditions positively impact *E. amylovora* growth, at times of day when the atmospheric temperature is declining?

A major clue could come from our determination that declining average wind speeds and rising relative humidity are significantly associated with increasing populations of *E. amylovora* (Table 4). We hypothesize that dew formation on apple flower stigmas occurring during the night provides free moisture that stimulates bacterial growth. Rosaceae family stigmas are characterized as "wet", with "abundant, free-flowing surface secretions" that contain polysaccharide slimes and mucilages along with lipids and lipoproteins that have evolved to function in pollen adhesion (Clarke et al., 1979; Heslop-Harrison and Heslop-Harrison, 1985). The intercellular spaces between the columnar papillae cells of apple flower stigmas have also been postulated to provide a "stigmatic hydration factor", functioning to rehydrate pollen grains (Ferrari et al., 1981); this factor would ensure a high humidity microenvironment on the stigma (Wilson et al., 1989). It is possible that the "wet" apple stigma surface does not provide enough free moisture during daylight hours in the absence of dew, such that moisture is limiting for *E. amylovora* growth during the day, unless it is provided by atmospheric precipitation. However, in the context of *E. amylovora* growth, an optimal growth rate may only be achieved in the presence of additional free moisture.

Dew consists of water droplets formed by the condensation of water vapor on a cold surface. Dew forms when the temperature of the surface of an object falls below the dew point temperature. Plant surfaces, for example, exhibit radiative cooling during the night, and can become colder than the ambient air. Dew will form under conditions with relative humidity > 85% and light winds (0.5 m s^{-1} to no greater than 4.5 m s^{-1}), and dew forms optimally under clear skies (Beysens et al., 2005; Muselli et al., 2002; Shaw, 1973; Xiao et al., 2013). The formation of dew requires condensation nuclei, usually small particles that water droplets will form around; these nuclei simultaneously increase the size of the water droplet and decrease the probability of evaporation of the droplet (Hussein et al., 2018). Recently, Steiner et al. (2015) showed that subpollen particles (SPP), pollen grains that have ruptured under high humidity conditions, can function as atmospheric cloud condensation nuclei. Since the "wet" surface of the apple stigma is a nonselective adhesive surface for pollen, it is possible that the occurrence of SPPs on stigmas could serve as condensation nuclei for dew formation. Dew formation on apple flower stigmas would serve as a source of free moisture to stimulate *E. amylovora* growth.

It should also be noted that during the process of dew formation, the condensation of water from the atmosphere into the liquid phase generates heat or the latent heat flux (Xiao et al., 2013). The importance of this heat generation to *E. amylovora* growth is currently unknown, although the perception of heat input into the microenvironment of the stigma may also stimulate *E. amylovora* growth. Recently, Rougerie-Durocher et al. (2020) used thermocouples to measure apple flower stigma temperatures, and to document the difference between these temperatures and the ambient air temperature. Their comparative measurements of both temperatures during the overnight hours indicated that stigma temperatures were consistently lower than the ambient air, by sometimes as much as 1-2°C (Rougerie-Durocher et al., 2020). These observations are

consistent with the knowledge that plant surfaces exhibit radiative cooling, and suggest that stigmas would serve as suitable locations for dew formation.

The growth of *E. amylovora* on apple flower stigmas is critical for pathogenesis, as establishment of large populations of 10^5 to > 10^7 per flower are associated with an increased frequency of blossom blight. Our data have shown that population surges of greater than 10-fold increases per day can occur when temperatures are below previously reported thresholds and well below known optimal growth temperatures in vitro. Our demonstration of growth occurring at night also suggests that evening applications of antibiotics during bloom might enhance population reduction since orchards should harbor net smaller populations of *E. amylovora* on flowers in evenings compared to the following morning. Evening applications of antibiotics, such as kasugamycin and oxytetracycline that are sensitive to photodegradation (Slack et al., 2021), may also increase efficacy against blossom blight.

CHAPTER 3

EFFECT OF KASUGAMYCIN, OXYTETRACYCLINE, AND STREPTOMYCIN ON IN-ORCHARD POPULATION DYNAMICS OF *ERWINIA AMYLOVORA* ON APPLE FLOWER STIGMAS

Abstract

We assessed the effect of three antibiotics (streptomycin, oxytetracycline, and kasugamycin) on populations of the fire blight pathogen *Erwinia amylovora* on apple flower stigmas during three field seasons. Timing of application relative to *E. amylovora* presence on flower stigmas had little impact on both population dynamics and subsequent disease incidence. While *E. amylovora* populations on water-treated flowers increased to 106-7 cfu flower-1 after 4-5 days in each experiment, the antibiotics streptomycin and kasugamycin caused statistically-significant reductions of stigma populations by as many as 4-5 logs over a 4-5 day period in two of the three experiments. In contrast, the effect of oxytetracycline on *E. amylovora* populations on stigmas was more variable, with reductions in *E. amylovora* populations only observed in one of the three experiments. In agreement with the population data, disease incidence was significantly higher on oxytetracycline-treated flowers compared to the other antibiotic treatments in two of three years. Statistical analyses of effects of weather parameters on antibiotic activity revealed that solar radiation and temperature negatively impacted the activity of both kasugamycin and oxytetracycline. We further assessed the potential for photodegradation of formulated kasugamycin (Kasumin 2L, Arysta LifeSciences), and found that Kasumin 2L was susceptible to degradation in vitro after exposure to a 16-hr photoperiod of daily light integrals (DLIs) varying from 6 to 35 mol·m–2·d–1. We further determined that exposure to three consecutive 16-hr photoperiods of DLIs of 23 or 35 mol·m–2·d–1 reduced the available concentration of Kasumin

2L (assessed using a bioassay) from 100 □g ml-1 to 10-20 □g ml-1. Our results correlate the superior blossom blight control efficacy of kasugamycin and streptomycin to significant population reductions of *E. amylovora* on apple flower stigmas but indicate that, similar to oxytetracycline, kasugamycin is vulnerable to photodegradation which would suggest that further considerations should be made when applying this antibiotic.

Source

For a full text of this work go to: Slack, S.M., Walters, K. J., Outwater, C., & Sundin, G. W. (2020). Effect of kasugamycin, oxytetracycline, and streptomycin on in-orchard population dynamics of *Erwinia amylovora* on apple flower stigmas. *Plant Disease*. Accepted . https://apsjournals.apsnet.org/doi/10.1094/PDIS-07-20-1469-RE

CHAPTER 4

EVALUATION OF A CONTACT STERILANT AS A NICHE-CLEARING METHOD TO ENHANCE THE COLONIZATION OF APPLE FLOWERS AND EFFICACY OF *AUREOBASIDIUM PULLULANS* IN THE BIOLOGICAL CONTROL OF FIRE BLIGHT

Abstract

Due to rapid expansion of organic apple production and issues with antibiotic resistance in conventional production, there is a need for novel strategies to protect orchards from outbreaks of fire blight caused by the bacterial pathogen *Erwinia amylovora*. The biological control material, Blossom Protect (consisting of two strains of the fungus *Aureobasidium pullulans*), has shown promising results in some apple growing regions but has shown reduced efficacy in humid apple-growing regions such as the Midwestern and Eastern United States. In an attempt to increase both flower colonization by *A. pullulans* and disease control efficacy, we applied a contact sterilant to clear the flower niche of native microbial populations 4 hr prior to application of Blossom Protect. Results from four years of field testing showed that application of the contact sterilant resulted in a significant reduction in populations of bacteria, fungi, and native yeast populations; however, all of these microbial populations recovered after 24 h. Examination of fire blight incidence revealed that use of the contact sterilant did not significantly impact disease control. Two applications of Blossom Protect, at 70–80% bloom and full bloom, exhibited the best efficacy in disease control over four years. In addition, we observed marginal differences in the marketability of the final fruit between treatments, each with less than 8% deemed unmarketable due to russet. Our results indicate that niche-clearing of apple flowers did not enhance flower colonization by *A. pullulans*, nor produce an increase in biological control efficacy. However, our current experimental results (2014–2018) do indicate consistency and an increase in efficacy of Blossom Protect compared to results from previous years.

Source

For a full text of this work go to: Slack, S.M., Outwater, C. A. Grieshop, M. J., & Sundin, G. W. (2019). Evaluation of a contact sterilant as a niche-clearing method to enhance the colonization of apple flowers and efficacy of *Aureobasidium pullulans* in the biological control of fire blight. *Biological Control, 139*, 104073. https://doi.org/10.1016/j.biocontrol.2019.104073

CHAPTER 5

DECIPHERING THE ROLE OF A TYPE THREE SECRETION SYSTEM EFFECTOR IN THE PATHOGENICITY OF *ERWINIA AMYLOVORA*

Abstract

Flowers are ephemeral in nature, may only be open for 1-2 weeks, and if a flower is not pollinated, it may quickly abscise. When apple flowers are infected with *Erwinia amylovora*, it can take weeks for the necrotic symptom development to occur. However, these infected flowers showing blossom blight symptoms do not abscise throughout the entire growing season. We conducted a field study showing that flowers excluded from pollinators and inoculated with *E. amylovora* did not abscise, whereas flowers inoculated with nonpathogenic bacteria abscised at the same rate as an untreated control. The *E. amylovora* HopPtoC$_{Ea}$ (HopC) effector, which is part of the Hrp Type III secretion system (T3SS), was implicated as functioning to delay flower abscission, as a mutant (Ea1189Δ*HopC*) without this effector elicited a response of early flower abscission under growth chamber conditions. Further investigation into the peduncle abscission zone indicated that zone separation was occurring at 3 days post inoculation of the effector mutant. This study provides the foundation for more in-depth investigations into the functional role of *E. amylovora* HopC in host-pathogen interactions.

Introduction

Abscission occurs in plants as the shedding of unwanted organs, including leaves, flowers, individual floral organs, and fruits (Patharkar & Walker, 2018). Abscission takes place at anatomically distinct cell layers termed abscission zones (AZ) and is mediated by several phytohormones, such as ethylene, jasmonic acid, abscisic acid, auxins, gibberellins, and brassinosteroids (Roberts *et al.*, 2002; Estornell *et al.*, 2013). Separation at the AZ is a natural process typically triggered by developmental or environmental cues. For example, shortening photoperiods and cooler temperatures trigger leaf abscission in the fall and pollination or fertilization, or the lack thereof, modulates floral organ or whole flower abscission in a wide range of plant species (van Doorn & Stead, 1997). Abscission has been heavily studied in apple, particularly at the AZ located at the base of the flower pedicel (Berüter and Droz, 1991; Kolarič, 2000; Sun et al., 2009; Botton et al., 2011). Flowers that are not pollinated abscise within a week after petal fall (Wertheim, 1999).

There have been many studies attempting to decipher the role of plant hormones in apple abscission. The overall consensus is that auxins block abscission while ethylene stimulates it, though their relationship is not that straightforward. During apple anthesis, the timing where flowers are most susceptible to *E. amylovora* infection, the flower ovaries have little growth and low auxin levels (Gruber and Bangerth, 1990). The low auxin levels create an opportunity for flowers to abscise, and ethylene (ethephon) has been used in commercial orchards as a chemical thinner at this stage with ease (Byers, 1993; Meland and Kaiser, 2011). After pollination however, the seeds create their own source of auxin leading to a rapid decrease in natural abscission rates (Wertheim, 1999).

Other than a natural response to environmental and internally-controlled hormone regulation, abscission of plant organs also can occur following the infection of plants by pathogens, as an active plant immune response. Bacterial pathogens have been long documented to cause infected host organ abscission as a plant defense response (Ben-David et al., 1986; Patharkar *et al.*, 2017). Though there have many studies demonstrating this abscission phenotype, a more recent study utilizing *Pseudomonas syringae* pv. tomato DC3000 demonstrated a correlation between bacterial infection and the abscission of Arabidopsis cauline leaves (Patharkar *et al.*, 2017). This pathogen-triggered leaf abscission required a functional type III secretion system (T3SS), a well-studied protein secretion/translocation system in bacteria that triggers effector-triggered immunity (ETI) in plants (Hueck, 1998; Deng *et al.*, 2017; Patharkar et al., 2017).

The T3SS enables bacterial pathogens to translocate effector proteins directly into the host cell cytoplasm through a needle-like pilus. These effectors are thought to interfere in the regulation of host cell functions that ultimately benefit the pathogen's survival and multiplication (Coburn *et al.*, 2007; Büttner & He, 2009). In *E. amylovora*, the T3SS is a pathogenicity factor; as T3SS-mutant strains are unable to cause disease symptoms (Oh *et al.*, 2005, Oh & Beer, 2005). In *E. amylovora*, genomic and secretomic studies have revealed that there are at least five effectors (DspA/E, Eop1, Eop3, Eop4 (AvrRpt2$_{Ea}$), and HopPtoC$_{Ea}$) that are secreted by the T3SS (Bogdanove *et al.*, 1998a; Zhao *et al.*, 2005; Zhao *et al.*, 2006; Nissinen *et al.*, 2007). DspA/E is currently the only of the five effectors that is a pathogenicity factor; it contributes to blocking defense from the host that would otherwise cause callose deposition (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998b; DebRoy *et al.*, 2004; Yuan *et al.*, 2020). The other effectors, Eop1, Eop3, AvrRpt2$_{Ea,}$ and HopPtoC$_{Ea}$ are considered virulence or avirulence factors (Yuan et al., 2020). Eop1, Eop3, and HopPtoC$_{Ea}$ are relatively understudied in the literature, partially

because deletion of these effectors does not affect virulence (Zhao et al., 2005; Asselin *et al.*, 2006; Bocsanczy *et al.*, 2012). Of relevance to abscission, there has been recent evidence that the T3SS is active during the first stages of flower infection (Cui et al., 2020).

The potential role of flower abscission in apple in response to pathogen infections have not been documented. However, many studies have utilized tomato and pepper as a model for abscission, and recent studies have determined that many of the host genes required in those systems are present in apple (Nakano et al., 2019). Even with gene homology, we observed a lack of abscission in the *Erwinia*-apple phytosystem, suggesting that *E. amylovora* is using a T3SS effector to manipulate the apple host response to block infected flowers from abscising.

Materials and Methods

Field Studies

In 2017 and 2018, 600 apple flower clusters at pink stage from the cultivar 'Gala' were covered with translucent silk bags to exclude pollinators (Figure 5-1A; Native Seeds, Tucson, AZ). Upon opening, king bloom flowers were treated with either *E. amylovora* strain Ea110 (Zhao et al., 2005), *Pantoea agglomerans* strain E325 (Pusey et al., 2011), *Pseudomonas fluorescens* strain A506 (Stockwell et al., 2010), a phosphorus buffer, or a water control. *E. amylovora* strain Ea110 has been widely utilized in pathogenicity field trials at Michigan State University (Slack et al., 2017), and both *P. agglomerans* strain E325 (Pusey et al., 2011) and *P. fluorescens* strain A506 (Stockwell et al., 2010) have been used as biological controls for fire blight and are nonpathogenic on apple flowers. Prior to inoculation, all bacteria were grown overnight, and populations were adjusted turbidimetrically to $1x10^6$ CFU/ml; a total of 5 µl of the cell suspensions was directly pipetted into the hypanthium of each flower. Each experimental

treatment consisted of 4 replicates of 25 flowers each, for a total of 100 flowers per treatment. Every few days, flowers were checked for abscission after inoculation on 27 April 2017 and 1 May 2018, respectively.

Figure 5-1. Flowers with pollinator excluder bags attached. A: Image of a recently opened apple flower cluster covered by a pollinator excluder bag. B: Image of an apple flower cluster that was inoculated with *Pantoea agglomerans* E235. Observation was 20 days after inoculation from a field experiment conducted in 2018.

In 2020, two rates of ethephon (375 and 600 ppm; Meland and Kaiser, 2011) were used to determine if treatment with this compound would cause flower abscission on flowers inoculated with *E. amylovora* strain Ea110. Unlike in the previous field experiments, whole 'Gala' trees were sprayed with strain Ea110 at a rate of $1x10^6$ CFU/ml, not inoculated directly, and pollinator excluders were not used as well. Four replicate trees per treatment were used, and this experiment was repeated twice. Immediately prior to ethephon application, all open flowers were tagged, and only flowers that had been open during inoculation and treatment were used to rate the results.

Growth chamber experiments

Feathered 'Gala' trees on EMLA-26 rootstock were planted into 18.9 L plastic pots, and trees were moved into a growth chamber where they broke bud and began to flower. Flower cluster development was relatively uniform among the trees. Treatments were either bacterial suspensions or a water check: *E. amylovora* strain Ea1189 (Ea1189 WT), effector mutant 1 (Ea1189Δ*HopC*), effector mutant 2 (Ea1189Δ*DspE*), effector mutant 3 (Ea1189Δ*AvrPpt2*), a non-T3SS virulence factor mutant (Ea1189ΔV1), *P. agglomerans* strain E325, and a non-bacterial water control. All *E. amylovora* mutants were previously characterized (Zhao et al., 2005). All bacterial strains were directly pipette inoculated into the flower hypanthium at a rate of $1x10^4$ CFU/µl with 5 µl per hypanthium; we also applied 5µl sterile distilled water for the control treatment. Flowers were checked daily for abscission and trials were conducted twice in June and July 2018.

Similar to the field experiments, 'Gala' flowers from 24 trees were inoculated with either WT, Ea1189Δ*HopC*, or Ea1189Δ*DspE* (8 trees each) at rates of $1x10^7$ CFU/µl per hypanthium. Populations of *E. amylovora* were tracked over a time course in separate flower structures: the

flower base, the pedicel, and the flower cluster base. Due to the destructive nature of this sampling and limited availability of flowers, populations were sampled at 0, 12, and 48 hours.

Histology of abscission

In 2019, 'Gala' flowers from 24 trees were inoculated with either WT, Ea1189Δ*HopC*, or a water control (8 trees each) at rates of $1x10^4$ CFU/µl with 5 µl per hypanthium. At days 0, 3, and 5, whole flower clusters were carefully collected to avoid causing premature flower detachment and taken to the Michigan State University Histopathology Services Center (https://humanpathology.natsci.msu.edu/). Samples were carefully cut to size and inserted into small embedding cassettes (40.1 x 28.5 mm; Fisher Scientific, Waltham, MA). Samples were then processed with 3% glutaraldehyde and in 0.02 M phosphate buffer and mounted in paraffin wax. Slides were constructed and dyed with Toluidine Blue O as previously described (Sakai, 1973).

Statistical analysis

All statistical analyses were completed using the open access program "R" (R Core Team, 2013) utilizing a one way ANOVA to check for statistical differences, and then separating means with Tukey's HSD test.

Results

In 2017 and 2018 in the field, flowers treated with any bacterial strain or compound other than *E. amylovora* abscised at similar rates, albeit significantly slower in 2018 compared to 2017 (Table 5-1). Flowers abscised at the AZ located at the base of the pedicel, and there was no other observed AZ or artificial breakage caused by the bags (Figure 5-1B). By 15 or 20 days after inoculation irrespective of year, all flowers abscised besides flowers inoculated with *E.*

amylovora. The first sign of infection was ooze production, and this typically occurred between 5-10 days after inoculation, with necrosis on floral organs starting to occur at day 10 in both years; flower tissue was completely dead with systemic spread of the *E. amylovora* pathogen apparent on some flowers by Day 20 for each year (Table 5-1).

Table 5-1. Results of the 2017 and 2018 field trial observing abscission on flowers inoculated with either *Erwinia amylovora* Ea110, *Pantoea agglomerans* E325. *Pseudomonas fluorescens* A506, a phosphorus buffer, or a water control with 4 replicates and a total of 100 flowers per treatment. Flowers were inoculated on 27 April 2017 and 1 May 2018 respectively. Statistical differences between treatments were compared by a one way ANOVA with means separation by Tukey HSD. * indicates statistical difference between treatments.

Year	Days after inoculation	Percent flower abscission and standard error				
		Erwinia amylovora strain 110	*Pantoea agglomerans* strain E325	*Pseudomonas fluorescens* strain A506	Phosphorus Buffer	Water Control
2017	5 days	0.0 ± 0	0.0 ± 0	0.0 ± 0.0	0.03 ± 0.01	0.0 ± 0.0
	10 days	0.0 ± 0*	0.17 ± 0.04	0.13 ± 0.05	0.11 ± 0.02	0.11 ± 0.03
	15 days	0.0 ± 0*	1.0 ± 0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
2018	5 days	0.0 ± 0	0.0 ± 0	0.0 ± 0.0	0.02 ± 0.02	0.0 ± 0.0.0
	10 days	0.0 ± 0*	0.07 ± 0.01	0.07 ± 0.01	0.05 ± 0.1	0.06 ± 0.02
	15 days	0.0 ± 0*	0.86 ± 0.04	0.90 ± 0.04	0.82 ± 0.07	0.79 ± 0.1
	20 days	0.0 ± 0.0*	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0

For the growth chamber experiments, early abscission elicited by Ea1189Δ*HopC* was observed on day 3 of the experiment for an average of 29.5% of flowers; the incidence of abscission in all other treatments was below 5% abscission. This trend continued until day 5, when abscission observed for other virulence factor mutant strains as well as the uninoculated and *P. agglomerans*-inoculated flowers reached levels similar to Ea1189Δ*HopC* (Figure 5-2). Flowers from day 3 show the differences in flower response to the missing effector (Figure 5-3A and 5-3B), and that the host cells are still alive in the wild type at day 3, and dead by day 7 in growth chamber conditions (Figure 5-3C). We observed that 1 out of 6 flower clusters that pathogen cells were still able to systemically spread from the flower base to the flower cluster base before abscission (Figure 5-3D).

Populations of *E. amylovora* (WT, Ea1189Δ*HopC*, and Ea1189Δ*DspE*) were observed in multiple flower organs 24 hr after inoculation into the hypanthium (Figure 5-4). The mutant strains were able to spread internally to the cluster base, though with not as a large population as the WT. A smaller number of cells of the WT was also observed in the inoculation site, indicating that there may be an advantage for the WT strain for systemic spread (Figure 5-4).

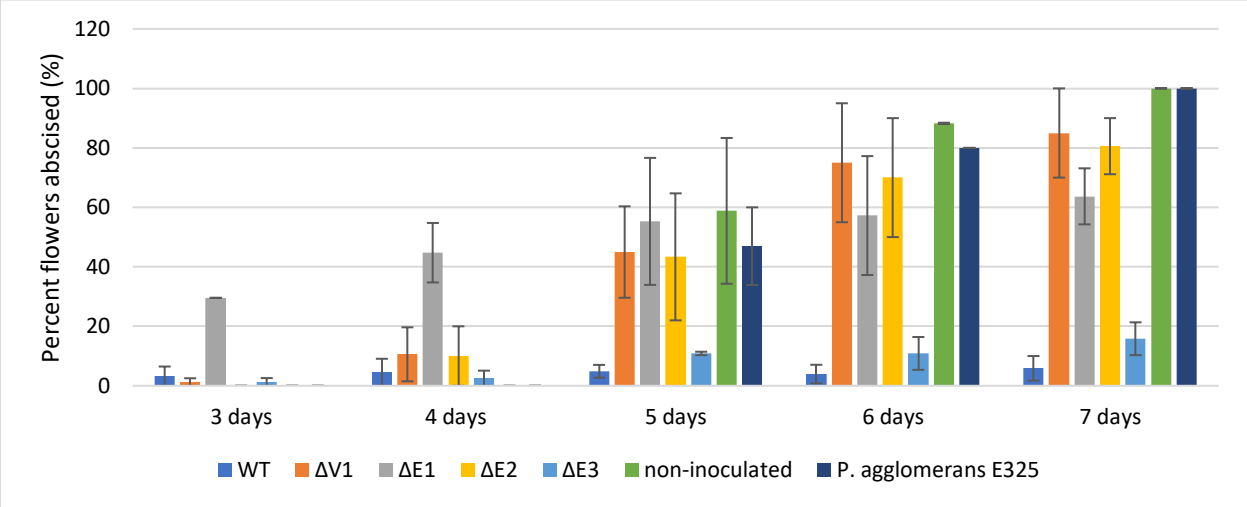

Figure 5-2: Rate of abscission elicited by *E. amylovora* WT and effector and virulence factor mutant strains from growth chamber experiments. Results of two experimental replicates with a total of 6 trees per treatment averaging 120 flowers per treatment total. Error bars represent standard error of the mean percentages.

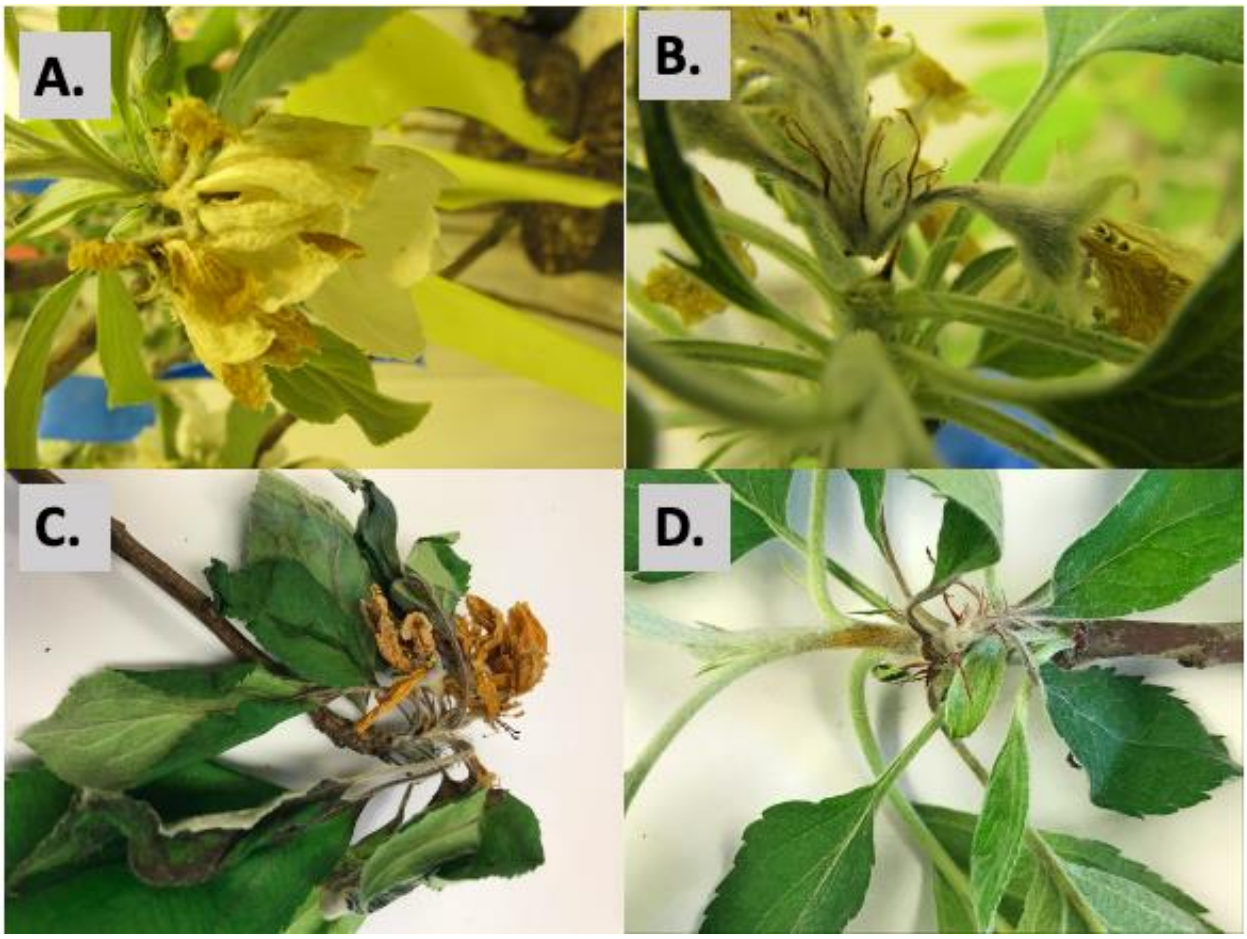

Figure 5-3. Comparison from growth chamber experiments of selected flower clusters at three days and seven days after inoculation wither either *E. amylovora* strain 1189 (WT) or the effector mutant 1 (Ea1189Δ*HopC*). WT at 3 days (A), Ea1189Δ*HopC* at 3 days (B), WT at 7 days (C), and Ea1189Δ*HopC* at 7 days (D).

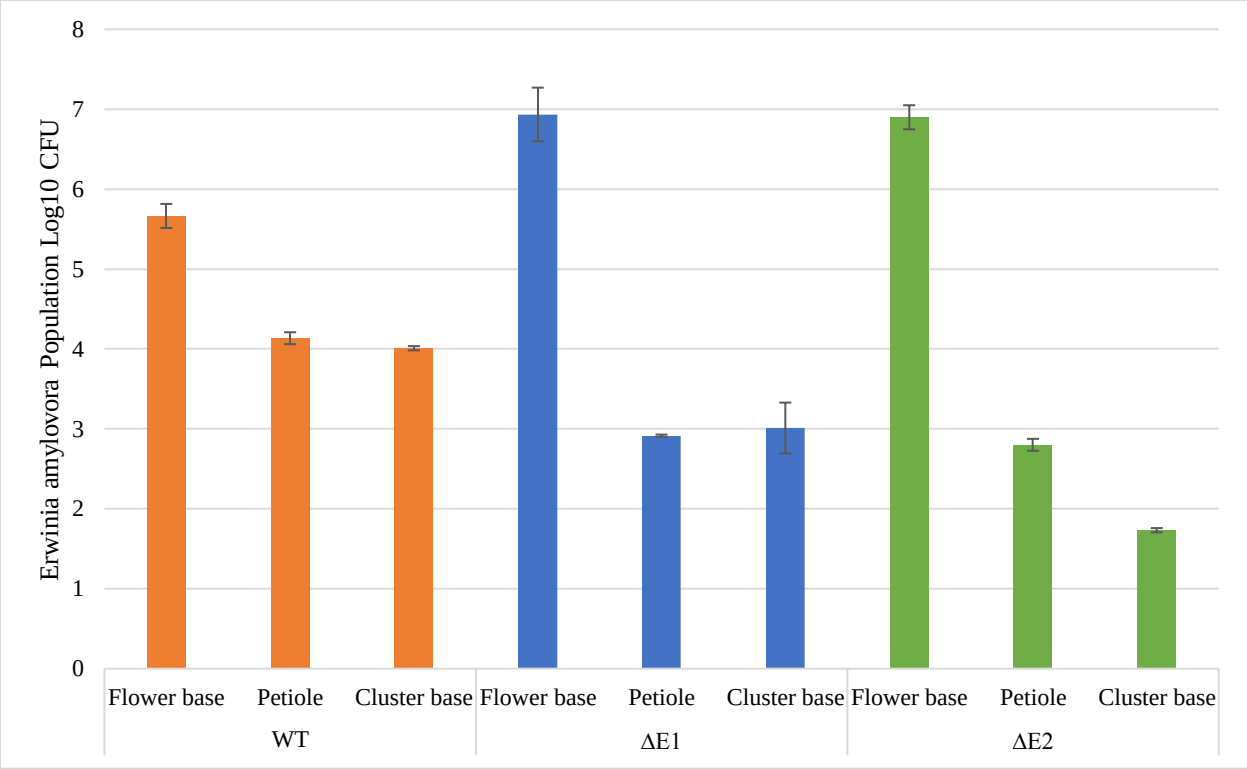

Figure 5-4. *Erwinia amylovora* strain populations 24 hr after inoculation into the hypanthium. Flowers were inoculated with 1ul of $1x10^7$ CFU. Error bars indicate the standard error of the population.

Observations at the AZ shows distinct separation on flowers inoculated with Ea1189Δ*HopC* after three days (Figure 5-5C) while the WT and uninoculated control flowers do not show that phenotype at the AZ (Figure 5-5A and 5-5B). By 5 days post flower inoculation, the AZ on the uninoculated control flowers has spilt, where the WT remind attached (Figure 5D and 5E).

Ethephon-treated uninoculated flowers abscised at rates comparable to flowers not treated with the plant hormone (Figure 6). When comparing inoculated to uninoculated trees, there was significant differences in the rates of abscission. However inoculated flowers treated with 600 ppm Ethephon was statistically the same as uninoculated flowers treated with either 375 ppm Ethephon or no ethephon treatment. A higher rate of flower abscission in inoculated trees was observed in 2020; however a frost event damaged many flowers in Michigan in this year (Figure 6).

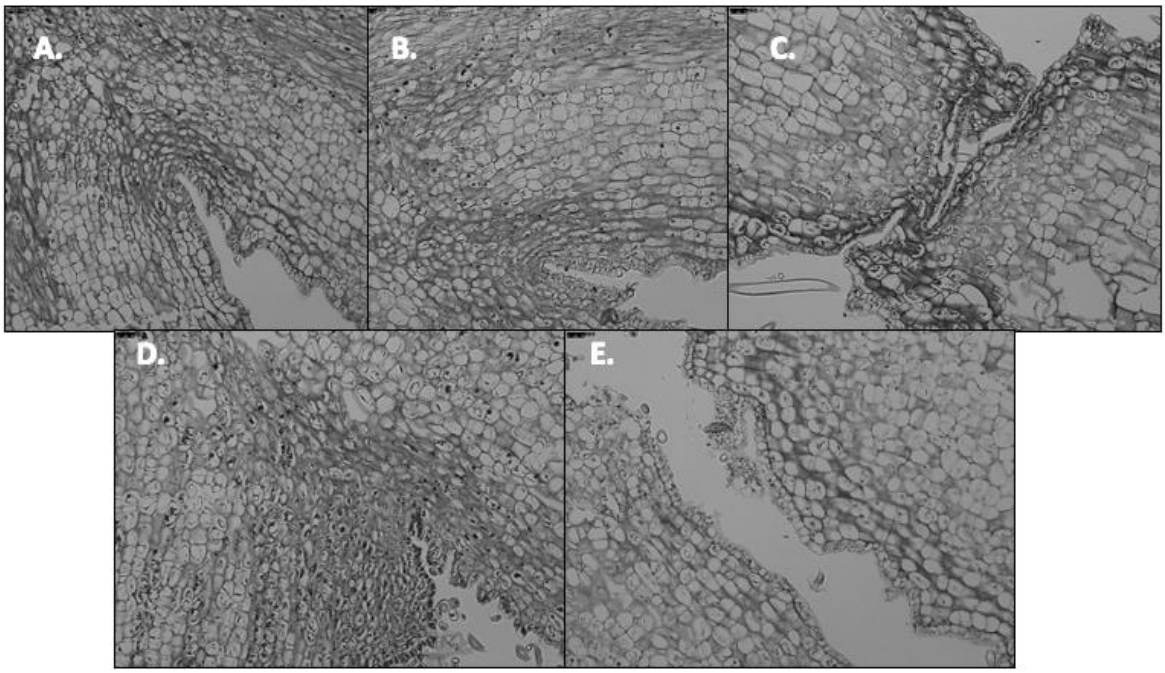

Figure 5-5. Histology sides observing the abscission zone in apple. A. WT at 3 days post inoculation, B. Un-inoculated control at 3 days post inoculation, C. Ea1189Δ*HopC* at 3 days post inoculation, D. WT at 5 days post inoculation, and E. Un-inoculated control at 5 days post inoculation.

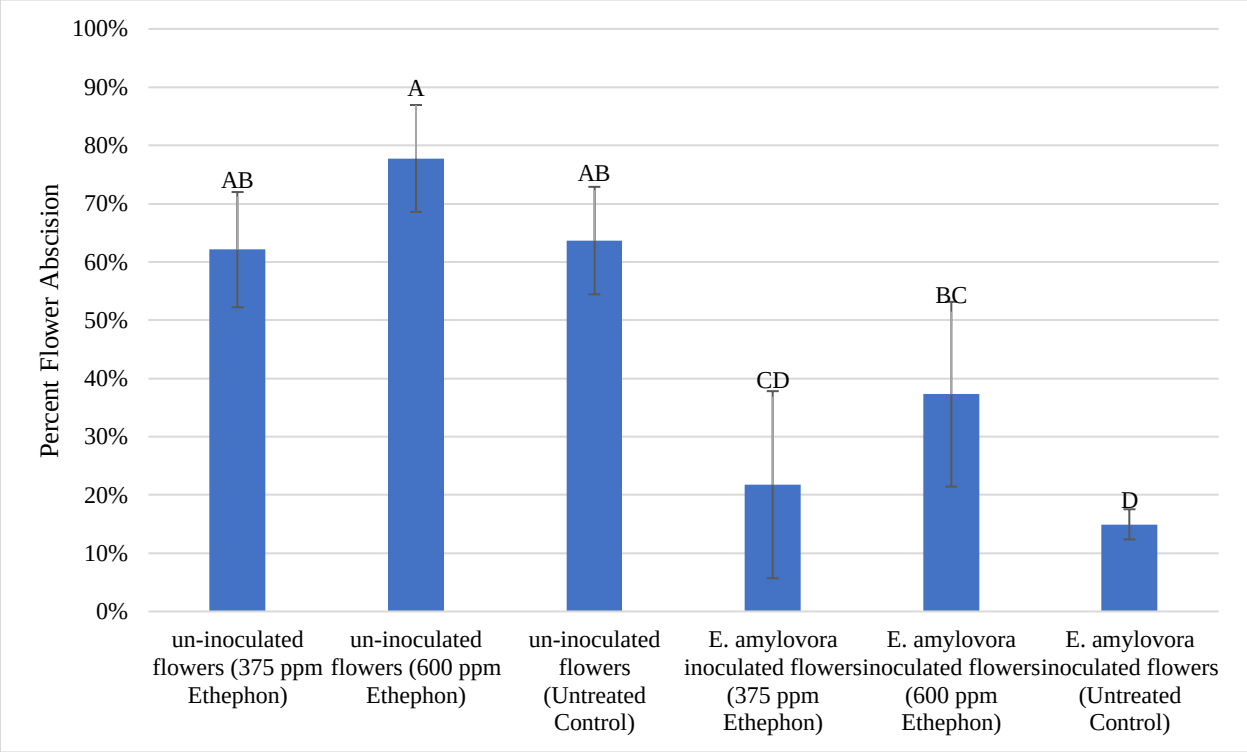

Figure 5-6: Effect of Ethephon on flowers inoculated with *Erwinia amylovora*. Error bars indicate standard error of mean percentages. Treatments that are significantly different have a different letter, means separated one way ANOVA followed by Tukey HSD. Use Non-inoculated instead of un-inoculated; italicize E. amylovora

Discussion

Abscission, potentially as a defense response, was observed in apple flowers inoculated with an effector mutant of *E. amylovora*. This study found that flower abscission was not rapid enough to inhibit the bacteria from internally spreading past the flower AZ. However, it may be that artificial inoculation was partially responsible for this slowed abscission. In orchard settings, it typically takes 2-3 days for *E. amylovora* populations to develop on flower stigmas, and for cells to migrate to the hypanthium (Bubán and Orosz-Kovács, 2003); we inoculated the bacteria directly into the hypanthium, therefore bypassing this step. Also, it has been noted that *E. amylovora* is translocating effectors into cells on the stigma (Cui et al., 2020), meaning that the effectors used in this study are functionally utilized by the pathogen before the bacteria begin to spread systemically, but in this instance we created a situation where the bacteria and effectors are moving and translocating concurrently.

While may *E. amylovora* spread too quickly for abscission to inhibit spread completely, the use of an effector to disable abscission in hosts where spread is slower would be advantageous for a disease-causing organism.

In this study, we have verified that abscission in apple flowers is delayed by an effector protein secreted by the T3SS system in *E. amylovora*. The Ea1189Δ*HopC* strain elicited an early abscission response in flowers, and, when complemented (not shown in this chapter), the lack of flower abscission was restored. The phenotype of abscission was not present on leaves (data not shown), suggesting that the target of the effector is flower specific. The lack of abscission caused by application of ethephon suggests that the ethylene response is potentially being blocked by *E. amylovora*. Thus, in commercial orchards where ethephon is applied for flower thinning, growers may be inadvertently selecting for infected flowers.

Aside from the findings demonstrated here, it would be important to determine the host pathways that impacted by the effector as well as locating the effector targets. In addition, the identification of potential genes and the effector target could help with breeding resistant apple lines. If there were breading lines that could recognize effector at the initiation of infection, i.e. during the buildup of populations on flower stigmas, there would be enough time for flowers to abscise prior to infection by *E. amylovora*. Such abscission at the early stages of flowering could also be advantageous for crop thinning as well, since growers already induce abscission in flowers.

CHAPTER 6

POSSIBLE SYNERGISTIC EFFECT OF PROHEXADIONE-CALCIUM AND ACIBENZOLAR-S-METHYL IN MANAGING SHOOT BLIGHT OF APPLE

Abstract

The secondary cycle of fire blight is evident in pomaceous shoots, with symptoms of necrosis, shepherd's crooks dieback, and development of dark sunken lesions. With limited management options, low rates of prohexadione calcium and acibenzolar-*S*-methyl were evaluated for three years on 'Gala' apple trees of various ages, including newly planted trees. Following inoculation of trees with *Erwinia amylovora*, lesions developed more slowly in shoots treated with both prohexadione calcium (Pro-Ca) and acibenzolar-*S*-methyl (ASM) as a tandem treatment compared to these materials used alone. We found the rates of 28.3 g Pro-Ca + 28.3 g ASM and 56.6 g Pro-Ca + 28.3 g ASM per hectare significantly ($P < 0.5$) reduced shoot blight lesion incidence without significantly inhibiting growth on trees less than 5 years old. This rate did not impact tree yield, caused minimal winter injury defined as dead wood or injured shoots, and had statistically less incidence of *E. amylovora* systemic spread than control trees. On apple trees between 5 to 10 years old, these same rates of Pro-Ca + ASM significantly reduced lesion size in 2018-2020, while only reducing shoot length in 2018. Comparable rates of either treatment alone were not as effective in reducing lesion length as compared with the combination of Pro-Ca + ASM.

Introduction

Fire blight is a polycyclic disease of rosaceous plants include pome fruit, and is caused by the bacterium *Erwinia amylovora* (Burill). This disease can cause sporadic but devastating losses for both pear and apple production (Vanneste, 2000). Recently, concerns over losses due to fire blight have increased, both as a result of climate change and modern orchard design. Increased severity and frequently of epidemics possibly resulting from climate change and new planting systems have led to increased concerns from apple stakeholders. The interest in high density, 'spindle tree trellised" plantings has greatly increased the risk of fire blight, as trees are grown close together, and rapid growth is greatly encouraged (Robinson et al., 2004). One of the major features of high-density plantings is the lack of permanent scaffolding branches as most growth is less than 5 years old and directly off the central leader of the tree. Previously, the cultivation of long-lived scaffolding branches was a pivotal strategy for traditional stand-alone trees (Robinson et al., 2004), and such longer-lived scaffolds were more recalcitrant to fire blight infection.

The first line of defense against fire blight is cultural control methods including pruning cankers that formed the previous season, and sanitation via removal of pruned wood from the orchard (van der Zwet et al., 2012). The goal of removing cankers is to reduce inoculum in the orchard, as *E. amylovora* cells emerge from the cankers as ooze droplets in the early spring (Slack et al., 2017). However, many disease management tactics focus on the site primary infection which are flowers(Wise, 2021). Flowers have a short window of susceptibility for *E. amylovora* colonization and subsequent infection, making them prime for precise chemical management (Pusey, 2000; Dewdney et al., 2007, van der Zwet et al., 2012; Slack et al., 2021). Currently, three antibiotics and numerous biopesticides and copper products egistered in the

United States for control at bloom (Wise, 2021). There are disease forecasting programs freely available for grower ~~commercial enterprises~~ to precisely time applications antibiotics (Steiner, 1990; Smith 1996; Dewdney et al 2007; Billing, 2011; Smith and Pusey 2011; Turechek and Biggs 2015).

Fire blight is a polycyclic disease and secondary infections occur in the form of shoot blight. New shoot growth is highly susceptible to infection, and ooze emerging from cankers, infected flowers, or other infected shoots is easily spread by wind, rain, and insects through the orchard leading to new infections (Slack et al., 2017; Boucher et al., 2019). Although there have been some efforts have been made to select for resistant cultivars, the most popular commercial cultivars are susceptible to fire blight being principally bred for color, taste and storability as breeding for these traits comes first, which is why there few fire blight resistant cultivars (Kellerhals, et al., 2017).

Unlike blossom blight, both curative and preventive options are limited for shoot blight control (Wise, 2021). One of the limited options for control is prohexadione calcium (Pro-Ca), which is plant growth regulator registered for use in apple since the late 1990s (Evans et al., 1999). Pro-Ca is a gibberellin inhibitor that decreases the length of internodes on woody shoots. Though originally marketed as a product to reduce shoot growth for pruning and fruit color management, researchers quickly realized the potential of Pro-Ca for shoot blight management (Breth et al., 1998; Jones et al., 1999; Yoder et al., 1999). These early studies recommended a shoot blight management program consisting of two applications one at 125 mg/liter or one application of 250 mg/liter before shoots reached 10 cm in length, as this compound acts. Pro-Ca is used as a preventative measure as there is little evidence of curative action (McGrath et al 2009; Adwinkle et al., 2002; Yoder et al., 1999). The mode of action of Pro-Ca is thought to be

by thickening the cell wall of host cells, preventing infection by *E. amylovora*, as the width of the cell walls was greater than the length of the type three secretion pilus, which is required to translocate effector proteins that drive disease occurrence (McGrath et al., 2009).

Another promising option for shoot blight management is acibenzolar-*S*-methyl (ASM). In 2000, Brisset et al., found that ASM applied to apple trees at rates of 100 and 200 mg/L active ingredient reduced incidence of fire blight. Recommendations rates of ASM have ranged from biweekly applications of 75 mg/L to once every 7-10 days at 200 mg/L (Brisset et al., 2000; Maxon-Stein et al., 2002; Johnson and Temple 2016; Johnson et al., 2016). ASM elicits a systemic acquired resistance (SAR) response by the plant through the salicylic acid pathway and leads to the expression of pathogenesis-related (PR) genes (Maxon-Stein et al., 2002). Specifically, defense-related enzymes, peroxidases, and β-1,3-glucanases, which last up to 17 days after application were found important for fire blight symptom reduction (Brisset et al., 2000). Salicylic acid is key to the SAR response and can be elicited by both endogenous production and exogenous application of ASM. While Maxson-Stein et al., (2002) found that ASM alone was not enough to control fire blight in the field, they did note that, when integrated weekly with applications of streptomycin at flower bloom, the incidence of fire blight was reduced (Maxon-Stein et al., 2002). Similar findings were later reported by Johnson et al., (2016), where the addition of ASM to bloom-applied antibiotics reduced fire blight incidence by 6-11%.

Both Pro-Ca and ASM also have drawbacks that could limit their use in commercial apple production. Pro-Ca reduces shoot growth which may be important in establishment years, and ASM is cost prohibitive at amounts recommended. Recently, Wallis and Cox (2020) found that applications of Pro-Ca at bloom reduced both blossom and shoot blight with a minimal

growth reduction on 15 year old apple trees, which were well into maturity. Presently, little is known about the impacts of Pro-Ca on young (1-3 years) high density plantings were trees encouraged to grow rapidly and are most susceptible to fire blight. Since young high-density trees are smaller in size than traditional trees, testing lower rates of Pro-Ca for efficacy is needed, as an increase in rate causes greater reductions in shoot growth (McGrath et al., 2009). Low ~~volume~~ rate Pro-Ca recommendations are needed for growers that may not necessarily want to stop shoot growth, but need to protect trees during a severe infection year. Similarly, updated standard recommended application rates are needed for ASM to maximize cost effective-ness.

We hypothesized that we could use lower rates of Pro-Ca with ASM to promote shoot blight defense without negatively impacting rapid shoot growth in young apple trees. On both apple trees less than 5 years old and 6-10 years old, applications of low rates of Pro-Ca and ASM, both alone and combined, were applied on calendar schedules with 7-14 day intervals depending on treatment over a three year period. We found that rates of 28.3 g Pro-Ca + 28.3 g ASM and 56.6 g Pro-Ca + 28.3 g ASM ha^{-1} significantly reduced shoot blight lesion severity without inhibiting growth. These rates had little impact on yield, caused minimal winter damage, and had less incidence of *E. amylovora* systemic spread.

Materials and Methods

Established Gala Planting

In 2018, 8-year old apple cv. ‘Gala’ trees on semi-dwarfing rootstocks were assigned treatments in a randomized block design with repeated measures and four single-tree replicates. Treatments were repeated per year on these same trees from 2018-2020. There were a total of 10 treatments (ha^{-1}): 28.3 g Pro-Ca, 56.6 g Pro-Ca, 84.9g Pro-Ca, 113.4 g Pro-Ca, 226.4 g Pro-Ca,

28.3 g ASM, 56.6 g ASM, 28.3 g Pro-Ca + 28.3 g ASM, 28.3 g Pro-Ca + 56.6 g ASM, 56.6 Pro-Ca+ 28.3 g ASM, and an untreated control. Treatments were applied using a 15 L backpack sprayer (Solo, Newport News, VA). Treatments were either applied biweekly applications (28.3 g Pro-Ca, 56.6 g Pro-Ca, 84.9g Pro-Ca, 113.4 g Pro-Ca, 226.4 g Pro-Ca, 28.3 g ASM, 56.6 g) or weekly (28.3 g ASM, 56.6 g ASM, 28.3 g Pro-Ca + 28.3 g ASM, 28.3 g Pro-Ca + 56.6 g ASM, 56.6 Pro-Ca+ 28.3 g ASM) for a total of 3 to 6 applications based on timing schedule (Table 1).

After the third application, 5 shoots per tree were inoculated with *Erwinia amylovora* strain Ea110 (Zhao et al., 2005) for a total of 20 inoculated shoots per treatment. Strain Ea110 was cultured in LB broth overnight at 28^{o}C in a shaking incubator, and populations were adjusted turbidometrically ($OD_{600\ nm}$) to 1 x 10^{8} CFU ml^{-1} using a Tecan Spark plate reader (Männedorf, Switzerland). The prepared inoculum was maintained on ice while taken to the field, and inoculations were done within 1 hr of inoculum preparation. Inoculation of apple shoots was done by cutting leaves located at the tip of rapidly growing shoots with sterilized scissors that had been dipped in the *E. amylovora* inoculum (Slack et al., 2017). Total shoot length (new growth) and total lesion length on tagged shoots were measured weekly starting within 48 hrs of the first treatment applications. Weekly measurements ceased when either lesions reached the bud scar from the previous year or shoots had formed terminal buds on untreated trees.

Table 6-1. Application rates and times of Pro-Ca and ASM for High Density and Young Gala trees. The following treatments were applied at time points 1, 3 and 5: 128.3 g Pro-Ca, 56.6 g Pro-Ca, 84.9g Pro-Ca, 113.4 g Pro-Ca, 226.4 g Pro-Ca, 28.3 g ASM, 56.6 g ASM, 28.3 g Pro-Ca + 28.3 g ASM, 28.3 g Pro-Ca + 56.6 g ASM, 56.6 Pro-Ca+ 28.3 g ASM; and at time points 2, 4 and 6: 28.3 g ASM, 56.6 g ASM, 28.3 g Pro-Ca + 28.3 g ASM, 28.3 g Pro-Ca + 56.6 g ASM, 56.6 Pro-Ca+ 28.3 g ASM.

Time points	Application schedule
1	Pro-Ca treatments Pro-Ca + ASM treatments
2	Pro-Ca + ASM treatments
3	Pro-Ca treatments Pro-Ca + ASM treatments
4	Pro-Ca + ASM treatments
5	Pro-Ca treatments Pro-Ca + ASM treatments
6	Pro-Ca + ASM treatments

Young high-density planting.

In 2017, ‘Gala’ apple trees were planted with 3 ft spacing utilizing a trellis system, with 36 ft spacing between poles. Trees were planted to, ‘B.9’ and ‘EMLA-26’, were utilized in this planting alternating every 6 trees, allowing for sets of 12 trees with 6 trees per rootstock between poles as replicates (Figure 6-1). Flowers were removed from trees in years 2017-2019 on cool days to ensure that no blossom blight developed and confounded shoot blight inoculations.

From 2018 and 2020, treatments were assigned to 12 trees, 6 of each rootstock with four replicates using a randomized block design with repeated measures, as treatments were applied to the same trees each year (Figure 6-1). A total of seven rates of varying levels of fertilizer, ASM (Actigard 50 WG, Syngenta: Basel, Switzerland), and Pro-Ca (Apogee, BASF: Ludwigshafen, Germany) were applied weekly or biweekly starting at petal fall. Treatments were as follows (ha^{-1}): untreated control with no fertilizer, 28.3 g ASM, 113.4 g Pro-Ca, 56.6 g Pro-Ca + 28.3 ASM, 28.3 Pro-Ca + 28.3 ASM, 113.4 g Pro-Ca + 28.3 ASM and a untreated control + fertilizer. In 2019 and 2020, three of the six trees per treatment were inoculated with *E. amylovora* Ea110 as described above with the modification of two branches were inoculated per tree, with six inoculated shoots per rootstock, per treatment replicate resulting in a total of 24 shoots per treatment. The same trees inoculated in 2019 were used in 2020. Hence, only half of the treatment replicates were inoculated . Shoot length was measured on from 2018-2020, and lesion growth was recorded weekly until terminal bud set or shoot blight extended from the tip to the bud scar.

					South					
	2		3		7		4		6	
Row 1	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9
	1		6		7		1		5	
Row 2	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26
	3		2		4		X		5	
Row 3	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9
	2		7		5		3		6	
Row 4	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26
	5		3		7		1		4	
Row 5	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9
	1		4		6		2		X	
Row 6	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26	Bud-9	Emla-26
					North					

Figure 6-1. Lay out of the high density orchard block utilized for the experiment.

High Density Horticultural Measurements:

Tree productivity was determined by measuring the height from the top of the rootstock junction to the top of the central leader at end of the growing season from 2017-2020. In spring 2019 and summer 2020, both the severity of winter injury and systemic lesion development were qualitatively evaluated. Winter injury was categorized as blind wood and evidence of shoots not hardening off before winter and subsequent tip dieback. Systemic spread was defined as occurring if the lesions for six shoots spread beyond the bud scar and nearby shoots started to display symptoms. In 2020, on the fourth year, apples were harvested and weighed; apples from both inoculated and control trees were harvested to determine if the inoculated trees had reduced yields.

Statistical analysis

All statistical analyses were performed with the program RStudio for statistical computing (R Core Team, 2013). Differences between treatments were compared using ratios of shoot growth to lesion length. Total rainfall from first to last measurement was retrieved from the Enviroweather monitoring system stationed at the Michigan State University Horticulture farm (HORT) located within 1 km of the test orchards.

Results

Established Gala Planting

In 2018, seven of ten treatments exhibited significantly less growth once the onset of treatments began. However, there were no significant differences in shoot length between treatments in both 2019 and 2020 (Figure 6-2). Again in 2018, seven of the ten treatments had statistically smaller fire blight lesion sizes than the control by the end of the growing season

(Figure 6-3). 2019, the only treatment that did not have significantly smaller lesion size was 28.3 g ASM, indicating that even though there was no difference in shoot length size, lesion size was reduced in nine of the ten treatments. In 2020, lesion size was less than that of the untreated control for the following treatments: 56.6 g ASM, 28.3 g Pro-Ca + 28.3 ASM, 28.3 g Pro-Ca + 56.6 g ASM, and 56.6 g Pro-Ca + 28.3 ASM (Figure 6-3). These 4 treatments had consistently lower lesion lengths than the control each year, however in 2018, 56.6 g ASM and 56.6 g Pro-Ca + 28.3 ASM exhibited a growth reduction (Figure 6-2). Overall shoot growth in the orchard was highest in years receiving more precipitation. 2018 was the driest of the test years, followed by 2019 and 2020 recorded 117 mm of precipitation over the 6 weeks of treatment applications (Table 6-2).

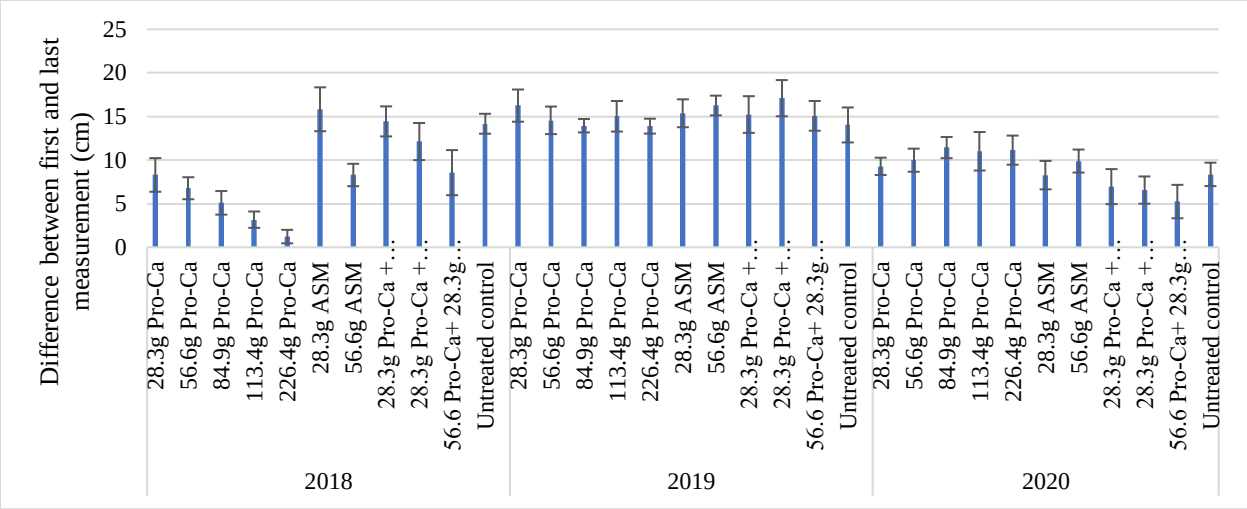

Figure 6-2. Average difference in shoot length of treatments in 8-10 year old Galas from 2018 to 2020. Error bars represent standard error of the mean.

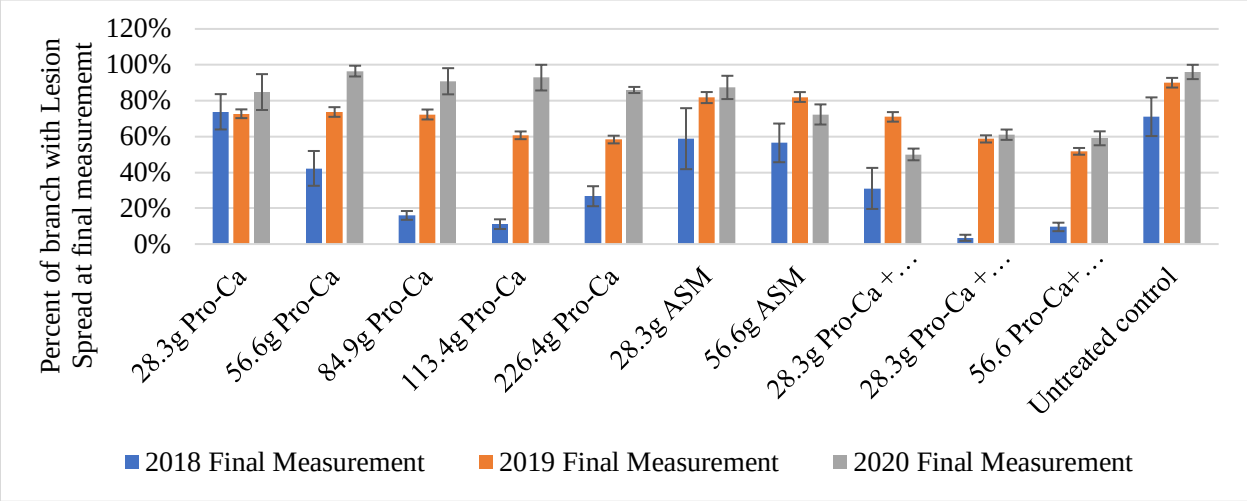

Figure 6-3. Average Percent lesion length of treatments in 8-10 year old Galas from 2018, 2019, and 2020. Error bars represent standard error of the mean.

Table 6-2. Total precipitation (mm) during application period for each year.

Year	2018	2019	2020
Precipitation (mm)	57.7	117	73.7

Young high-density planting: B.9 Rootstock

In 2018, the first year of treatment applications and tree establishment, the only difference in growth was in the control with no fertilizer application compared to the fertilizer control. None of the Pro-Ca and ASM treatments had reductions in tree growth (Table 3). With the exception of the control with no fertilizer treatment, trees in the 113.4 g Pro-Ca + 28.3 ASM and 113.4 g Pro-Ca treatments had reduced growth from 2019 and 2020 compared to other treatments . By the end of the study in 2020, 113.4 g Pro-Ca + 28.3 ASM and 113.4 g Pro-Ca treated trees had only gained an average of 67.3 ± 18.4 and 70.4 ± 21.4 cm in height respectively, compared to 117.1 ± 22.5 by the fertilized control. The other 3 treatments were not significantly different than the fertilized control (Table 6-3). Trees treated with 113.4 g Pro-Ca + 28.3 g ASM and 113.4 g Pro-Ca also had statistically higher than average incidence of winter injury compared to the other treatments in 2019 and 2020 (Table 6-3). In both years, all treatments had lower systemic lesion development than the fertilized control with 28.3 Pro-Ca + 28.3 g ASM having the shortest lesion development in 2019 and 56.6 g Pro-Ca + 28.3 g ASM having the shortest lesion development in 2020 (Table 6-3).

In 2018 and 2020, there were no differences between treatments in shoot length. However, in 2019 all treatments excepted for the 56.6 g Pro-Ca + 28.3 g ASM treatment had statistically lower growth than the control (Figure 6-4). Comparing percent of shoots with lesion development, all treatments had significantly reduced lesion elongation than the fertilizer control. In 2020, where the same trees had been inoculated as prior, only the 56.6 g + 28.3 g ASM and 113.4 g Pro-Ca treatments had shorter lesions (Figure 6-5). Fire blight symptom development only hindered apple production on 113.4 g Pro-Ca treated trees; however,

regardless of *E. amylovora* inoculation, trees treated with 113.4 g Pro-Ca and 113.4 g Pro-Ca +28.3 g ASM had fewer apples at the end of year 4 (Figure 6-6).

EMLA-26 rootstock

The trends in treatment impacts on growth of trees on EMLA-26 were similar to those of apples on B.9, with 113.4 g Pro-Ca + 28.3 g ASM and 113.4 g Pro-Ca treated trees having the least amount of growth. Tree uniformity was unfortunately not as similar, with the treatment of 28.3 g Pro-Ca + 28.3 g ASM having greatest variability in tree heights, making it difficult to resolve significant differences between treatments in the experience (Table 6-3). Winter injury was more pronounced in scions grafted to EMLA-26, with only 28.3 g Pro-Ca + 28.3 g ASM having statistically less injury than the other treatments in 2019 and 2020. Systemic lesion development was also higher than B9 in EMLA-26, with 28.3 Pro-Ca + 28.3 g ASM, 56.6 Pro-Ca + 28.3 g ASM, and 113.4 g Pro-Ca consistently having statistically less symptom development (Table 6-3).

In 2018, 28.3 Pro-Ca + 28.3 g ASM and 113.4 g Pro-Ca + 28.3 g ASM treated trees had reduced height compared to all other treatments (Figure 6-4). In 2019, only 28.3 Pro-Ca + 28.3 g ASM treatment had reduced height and in 2020 no treatments had any reductions in height compared to the control (Figure 6-4). All treatments had considerably lower lesion development than the fertilized control in 2019 (Figure 6-5). In 2020 however, there was no differences in lesion ratios between any treatments (Figure 6-5). Unlike B9, trees inoculated with *E. amylovora* had numerically fewer marketable fruit, but no statistical differences among treatments. However, the effect of treatment compounded with inoculation decreased marketable fruit production in all treatments except 28.3 g ASM with only 133.4 g Pro-Ca having less marketable fruit when not inoculated (Figure 6-6).

Table 6-3 Summary of horticultural measurements from 2018-2020 on High Density trees including: average tree height treatment per rootstock; average incidence of winter damage per treatment per root stock; average incidence of systemic spread by *E amylovora* per treatment per rootstock.

Rs	Average Horticultural measurements		Treatment						
		Year	28.3 g ASM	28.3 Pro-Ca + 28.3 g ASM	56.6 g Pro-Ca + 28.3 g ASM	113.4 g Pro-Ca + 28.3 g ASM	113.4 g Pro-Ca	Control + Fertilizer	Control - no fertilizer
B9	Tree Height Growth (cm)	2018	34.1 ± 7.2	29.1 ± 4.6	24.3 ± 6.6	31.2 ± 7.2	34.1 ± 12.9	36.4 ± 3.4*	22.9 ± 6.8
		2019 - 2020	49.5 ± 16.5	38.8 ± 24.3	47.5 ± 29.3	9.1 ± 3.3	20.2 ± 14.6	71.2 ± 23.4	12.5 ± 13.7
		3 year Growth	117.7 ± 17.1	101.3 ± 23.9	95.9 ± 10.7	67.3 ± 18.4	70.4 ± 21.4	117.1 ± 22.5	85.4 ± 21.0
	Incidence of winter damage (Percent of trees)	2019	12.4 ± 2.2 *	6.6 ± 0.6	12.0 ± 1.4 *	11.0 ± 4.0 *	16.8 ± 7.6 *	6.0 ± 1.6	6.0 ± 0.3
		2020	29.2 ± 7.9	27.2 ± 5.8	20.8 ± 12.5	46.7 ± 11.8 *	50.0 ± 15.2 *	33.3 ± 5.8	29.6 ± 14.2
	Incidence of systemic spread (Percent of trees)	2019	37.5 ± 4.1	25.0 ± 1.6	41.7 ± 2.8 *	29.2 ± 1.4	58.3 ± 4.8 *	75.0 ± 3.6 *	33.3 ± 3.9
		2020	54.2 ± 10.5	55.0 ± 8.2 *	25.0 ± 14.4 *	45.8 ± 7.9 *	47.5 ± 10.4 *	95.8 ± 4.0 *	79.2 ± 7.9
EMLA 26	Tree Height Growth (cm)	2018	30.4 ± 12.2	26.8 ± 8.7	40.8 ± 10.4	21.3 ±5.8	19.4 ± 5.9	28.3 ± 9.4	29.7 ± 12.9
		2019 - 2020	43.8 ± 11.6	30.9 ± 15.5	55.8 ± 34.2	25.6 ± 22.7	10.0 ± 7.1	46.4 ± 21.8	16.2 ± 12.07
		3 year Growth	91.8 ± 33.1	54.6 ± 47.9	107.1 ± 11.2	86.5 ± 13.9	88.2 ± 15.3	103.1 ± 16.5	71.2 ± 27.3
	Incidence of winter damage (Percent of trees)	2019	12.7 ± 1.7 *	3.2 ± 0.5	11.6 ± 1.3 *	9.3 ± 2.1 *	12.5 ± 1.7 *	11.7 ± 0.8 *	2.5 ± 0.5
		2020	33.3 ± 9.6	20.8 ± 4.0	41.7 ± 11.8 *	62.5 ± 10.5 *	60.0 ± 9.5 *	25.0 ± 8.3	20.8 ± 12.5
	Incidence of systemic spread (Percent of trees)	2019	50.0 ± 3.2 *	29.2 ± 1.4	29.2 ± 3.5	45.8 ± 4.7 *	33.3 ± 3.2	66.7 ± 3.9 *	29.2 ± 4.2
		2020	79.2 ± 15.7	45.8 ± 14.2	26.7 ± 11.3	20.8 ± 7.9	50.0 ± 15.2	100.0 ± 0.0	83.3 ± 6.8

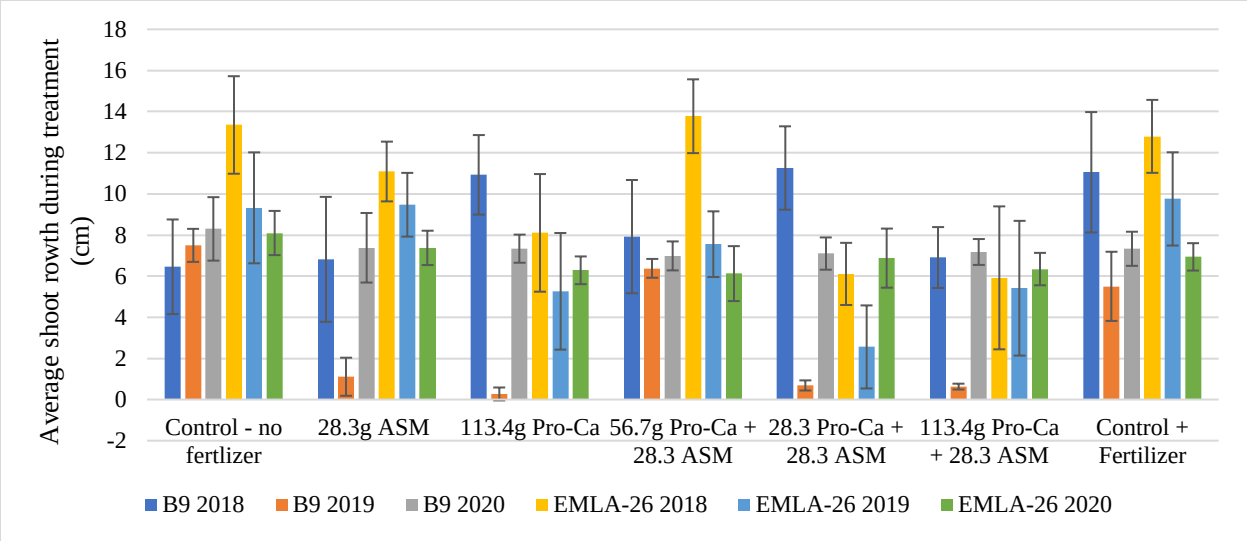

Figure 6-4. Average shoot length growth in high density treatments from 2018, 2019, and 2020 per rootstock.

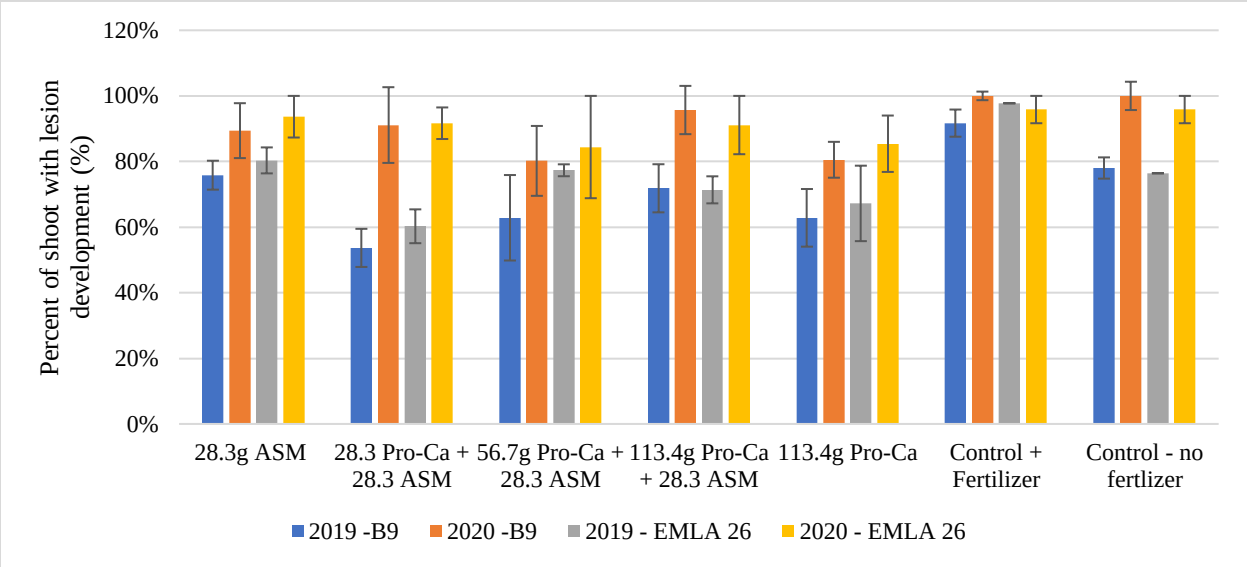

Figure 6-5. Average percent branch lesion in the high density Gala orchard in 2019 and 2020 per rootstocks. Error bars represent standard error of the treatment mean.

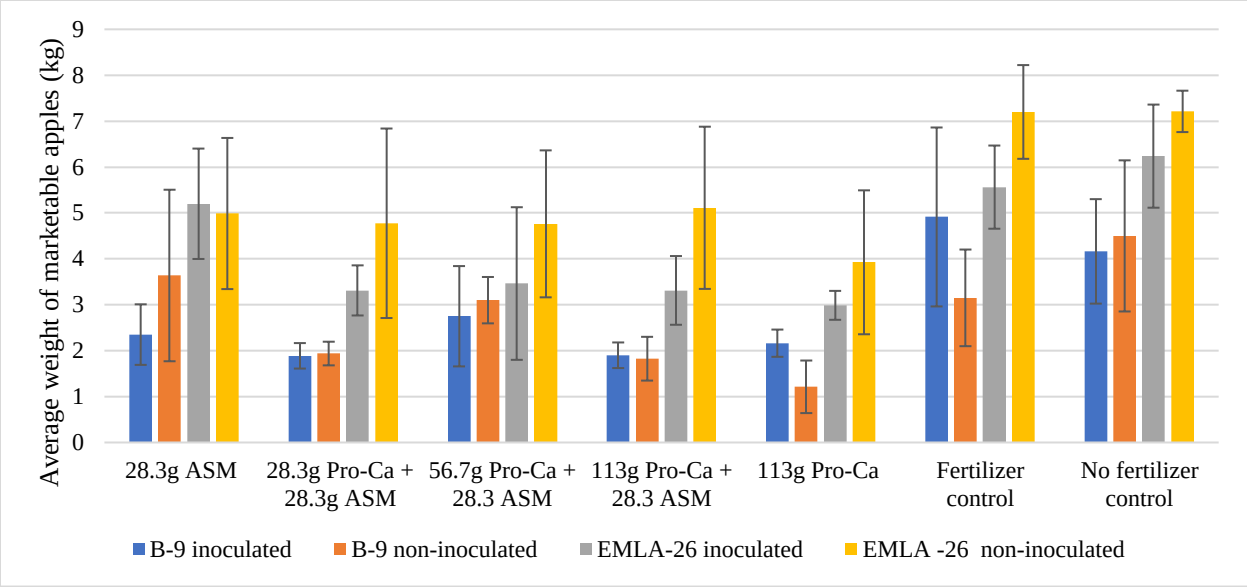

Figure 6-6. Average weight of marketable apples in the high density Gala orchard from 2020 per rootstock, comparing inoculated and uninoculated trees within treatment. Error bars represent standard error of the treatment mean.

Discussion

Overall, trees treated with a combination of Pro-Ca and ASM had slower lesion development. Though no treatment was able to completely prevent lesion development, the slowing of pathogen systemic spread as evidence by lesion elongation would enable the implementation of cultural management before the pathogen is able to spread to the point where the tree would be lost. The slower lesion spread in Pro-Ca and ASM treatments was observed in years with high rates of shoot growth, either from high precipitation (2019) or a small crop load from frost (2020). Trees beginning to come into their profitable years (Young Gala trees) benefited from the Pro-Ca and ASM combined treatments with little growth reduction rates. Trees under 5 years old would stand to benefit from yearly repeated applications of low volumes of Pro-Ca and ASM combined, with either 28.3 g Pro-Ca + 28.3 g ASM or 56.6 g Pro-Ca + 28.3 g ASM having little effect on horticultural characteristics, such as tree height and yield.

EMLA-26 is more susceptible to fire blight infection than B9, and the Gala scions had fewer marketable apples in year 4 on trees that had been inoculated compared to trees that had never had fire blight (Ferree et al., 2002; Figure 6-6). Whereas trees grafted on to B9, a more resistant root stock, only had fewer marketable apples on trees continuously treated with the highest amounts of Pro-ca (Ferree et al., 2002; Figure 6-6). The first year of harvest is important for growers to make back the initial investment in the trees as well as the trellis system, and any treatment that reduced profitability will not be adopted by growers~~adapted~~. Rootstocks are selected by growers not only for disease resistance, but dwarfing (tree size), soil quality, irrigation capabilities, and other topographical considerations. From this experiment, if the trees are exposed to *E. amylovora* on EMLA26, regardless of treatment, they will have fewer marketable fruit than trees exposed to fire blight on B9. If a grower in a high fire blight pressure

area had to choose between the two, the results from this study suggest that a resistant rootstock will help with initial returns in yield.

The higher application rates of Pro-ca had high incidence of winter damage, mainly through buds breaking dormancy late in the season after applications had stopped. These tender shoots were subjected to harsh Michigan winter cold, frost, and freezes, not surprisingly dying in the process. Another option would be to apply these low rates at the timing suggestions from Wallis and Cox (2020) to protect early shoots from injury. These low rates may be able to prevent flower infection as well as protectshoots under 5 cm in young trees. Although the goal was to find ways of reducing systemic spread later in the growing season, typically when growers start to notice shoot infection, the use of these products earlier in the season may be advantageous on newly planted or young non-bearing trees.

In summary, treatments of Pro-ca combined with ASM reduced shoot blight over three years in trees between 5-10 years old and over 3 years with trees 0-5 years old. When applied alone at the same rates, the same level of control was not consistently observed. The possibility of a synergism with both products potentially thickening cell walls either more consistently or having higher callous deposition without shortening shoots is ideal for young orchards in high fire blight risk areas.

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