EFFECT OF KASUGAMYCIN APPLICATION ON ANTIBIOTIC RESISTANCE AND THE ROLE OF LEVAN IN OOZE EMERGENCE IN *ERWINIA AMYLOVORA*

By

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ABSTRACT

EFFECT OF KASUGAMYCIN APPLICATION ON ANTIBIOTIC RESISTANCE AND THE ROLE OF LEVAN IN OOZE EMERGENCE IN *ERWINIA AMYLOVORA*

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Kasugamycin (Ks) is an alternative to the use of streptomycin for the control of bacterial plant diseases. However, there is a concern that Ks application in orchards will select for Ks resistance that could be linked with other resistance genes that are active against antibiotics used in human medicine. To monitor for this, we assessed the effect of the use of Ks in orchard systems on the level of resistance to Ks and to five other antibiotics used in human medicine. Two sets of leaf and soil samples were collected (treated and non-treated with Ks) from 41 fruit tree orchards throughout the United States in 2015 and 2016. Samples were processed in the laboratory and dilution plated onto King's B medium with or without Ks amendment. Bacterial population sizes were determined per sample and Ks-insensitive gram-negative isolates were further evaluated for multi-drug resistance. No differences were observed in Ks-insensitive populations or levels of resistance to the five other antibiotics tested between Ks treated vs. non-treated sites.

Erwinia amylovora, the causal agent of fire blight of apple, is disseminated in ooze droplets, which consist of bacterial cells embedded in at least two exopolysaccharides, levan and amylovoran. Based on previous observations, we hypothesized that a reduction in levan production would result in a decrease in ooze droplets. To study this, *E. amylovora* strains with variation in levan production were evaluated for ooze production in growth chamber and field trials. Strains with a reduction in levan showed reduced ooze production in shoots in growth chamber studies. However, no differences in ooze production were observed in field trials.

To My Mom, Dad, Sister, and Brother

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History of Antibiotic use in Agriculture

Antibiotics were originally isolated from fungi and bacteria in the environment (Waksman, Bugie, & Schatz, 1944). For example, both streptomycin and oxytetracycline resistant bacteria were isolated from soil; streptomycin from *Streptomyces griseus* and oxytetracycline from *Streptomyces rimosus* (Dille, 1951; V.O Stockwell & Duffy, 2012). Penicillin, a commonly used antibiotic in human medicine, was isolated from a soil fungus (Fleming, 1929).

Antibiotics vary in chemical structure, mode of action, in intrinsic ability to inhibit bacterial growth, and their ability to inhibit or kill bacteria. Although many inhibit protein synthesis, the mechanisms for doing so differ. For example, streptomycin inhibits protein synthesis by binding to the S12 protein of the 30S ribosomal unit, whereas kasugamycin alters the methylation of the 16S RNA changing the shape of the 30S subunit (Table 1.1.1). Antibiotics also vary in the dosage required to inhibit bacterial growth. For example, there is a 10 fold difference between the final working concentration of streptomycin and kasugamycin (Table 1.1) (Raleigh, Elbing, & Brent, 2002). Some antibiotics are bacteriostatic while others are bactericidal. “Bacteriostatic” refers to an antibiotic that prevents the growth of bacterium by keeping the bacteria in the stationary phase of growth, whereas “bactericidal” refers to an antibiotic that kills the bacterium (Pankey & Sabath, 2004). From the standpoint of disease control, bactericidal antibiotics provide the best control as they kill bacteria, reducing or eliminating the pathogen on the plant surface and reducing the chance of disease development. Bacteriostatic, on the other hand, only inhibits bacteria while the antibiotic is present, which prolongs the onset of disease development. Bacteriostatic antibiotics, such as tetracycline, are

beneficial for diseases that occur at specific stages in plant growth, such as with fire blight of apple, in which disease is most likely to occur during bloom (Raleigh et al., 2002).

The ecological function of antibiotics has been speculated to be to inhibit the growth of other microorganisms in order for the antibiotic-producing organism to outcompete others in the environment (Patricia S. McManus, Stockwell, Sunding, & Jones, 2002). However, evidence also exists indicating that this may not be the case. Antibiotics may instead serve as signaling molecules when produced at low concentrations (Martinez, 2008). These conflicting ideas about the role of antibiotic production in nature indicate the need for further research on the functional role of antibiotics in the natural environment.

Current use of Antibiotics

Antibiotics have multiple uses in several areas, such as in clinical and veterinary medicine, animal agriculture, and plant agriculture. In clinical medicine, veterinary medicine, and plant agriculture antibiotics are used to treat bacterial diseases of humans, animals (Witte, 1998), and plants (Vidaver, 2002). They are also used in animal agriculture as growth promoters, prophylaxis, and chemotherapy (Witte, 1998). Several researchers found that the addition of antibiotics to animal feed at low levels increased the weight of the animals (Gustafson & Bowen, 1997) up to five percent compared to those not fed antibiotics (Witte, 1998). This was seen in calves (Bartley, Fountaine, & Atkeson, 1950), pigs (Cunha, 1950), chickens, and turkeys (McGinnis, Berg, Stern, Wilcox, & Bearse, 1950). Antibiotics are also used in animal agriculture as prophylaxis to prevent the development of infections in large groups, especially when a small percentage of them have been diagnosed with an illness. This is allows the farmer to protect the other animals in close contact with an infected individual and reducing the chance of economic losses (Gustafson & Bowen, 1997).

Table 1.1 Antibiotics, their modes of action, and modes of bacterial resistance.

Antibiotic[b]	Stock conc. (mg/ml)	Final conc. (µg/ml)	Mode of action	Mode of resistance
Ampicillin[c]	4	50	Bacteriocidal: only kills growing *E. coli*; inhibits cell wall synthesis by inhibiting formation of the peptidoglycan cross-link	B-lactamase hydrolyzes ampicillin before it enters the cell
Chloramphenicol in methanol	10	20	Bacteriostatic: inhibits protein synthesis by interacting with the 50S ribosomal subunit and inhibiting the peptidyltransferase reaction	Chloramphenicol acetyltransferase inactivates chloramphenicol
Gentamycin	10	15	Bacteriocidal: inhibits the protein synthesis by binding to the L6 protein of the 50S ribosomal subunit	Aminoglycoside acetyltransferase and aminoglycosidenucl-eotidyltransferase inactivates gentamycin; mutations in *rplF* (encodes the L6 protein) prevents gentamycin from binding
Kanamycin	10	30	Bacteriocidal: inhibits the protein synthesis; inhibits translocation and elicits miscoding	Aminoglycoside phosphotransferase, also known as neomycin phosphotransferase, and aminoglycoside nucleotidyltransferase; inactivates kanamycin
Kasugamycin	10	1000	Bacteriocidal: inhibits protein synthesis by altering the methylation of the 16S RNA and thus an altered 30S ribosomal subunit	Mutations prevent kasugamycin from binding to the ribosome; mutations decrease the uptake of kasugamycin
Rifampicin[d] in methanol	34	150	Bacteriostatic: inhibits RNS synthesis by binding to and inhibiting the β subunit of RNA polymerase; rifampicin sensitivity is dominant	Mutation in the β subunit of RNA polymerase prevents rifampicin from complexing; rifampicin resistance is recessive
Spectinomycin	10	100	Bacteriostatic: inhibits translocation of peptidyl tRNA from the A site to the P site	Mutations in *rpsE* (encodes the S5 protein) prevent spectinomycin from binding; spectinomycin sensitivity is dominant and resistance is recessive
Streptomycin	50	30	Bacteriocidal: inhibits protein synthesis by binding to the S12 protein of the 30S ribosomal subunit and inhibiting proper translation; streptomycin sensitivity is dominant	Aminoglycoside phosphotransferase inactivates streptomycin; mutations in *rpsL* (encodes the S12 protein) prevent streptomycin from binding; streptomycin resistance is recessive
Tetracycline[d] in 70% ethanol	12	12	Bacteriostatic: inhibits protein synthesis by preventing binding of aminoacyl tRNA to the ribosome A site	Active efflux of drug from cell

[a]Data assembled from Raleigh *et al.*, 2002
[b]All antibiotics should be stored at 4°C, except tetracyclie, which should be stored at -20°C. All antibiotics should be dissolved in sterile distilled water unless otherwise indicated. Antibiotics dissolved in methanol often can be dissolved in the less hazardous ethanol.
[c]Carbenicillin, at the same concentration, can be used in place of ampicillin. Carbenicillin can be stored in 50% ethanol/50% water at -20C.
[d]Light-sensitive; store stock solution and plates in the dark.

*Information from Raleigh *et al*, 2002

It is estimated that 40 million pounds of antibiotics are used annually in the U.S., with approximately 0.1% of that being used in plant agriculture (Levy, 1992). Although a small percentage is used in plant agriculture, approximately 80% of antibiotics are used in animal agriculture (Figure 1.1) (Hollis & Ahmed, 2013). The remaining 20% of antibiotics are used for human and animal medicine. The quantity of antibiotics used in human medicine has been increasing, especially in developing countries (Figure 1.2) (Laxminarayan et al., 2013). This rise in antibiotic use increases the risk of developing antibiotic resistance.

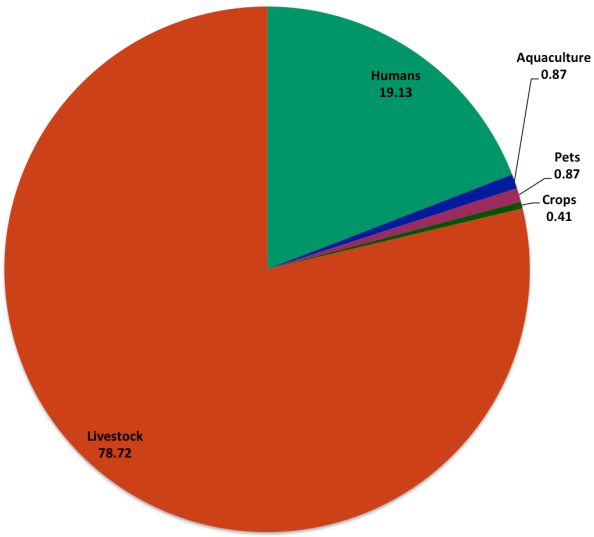


Figure 1.1 Estimated percentage of annual antibiotic use in the United States. Data is shown as approximate percentages based on kilograms of antibiotics used per year. (Figure information from Hollis and Ahmed, 2013).

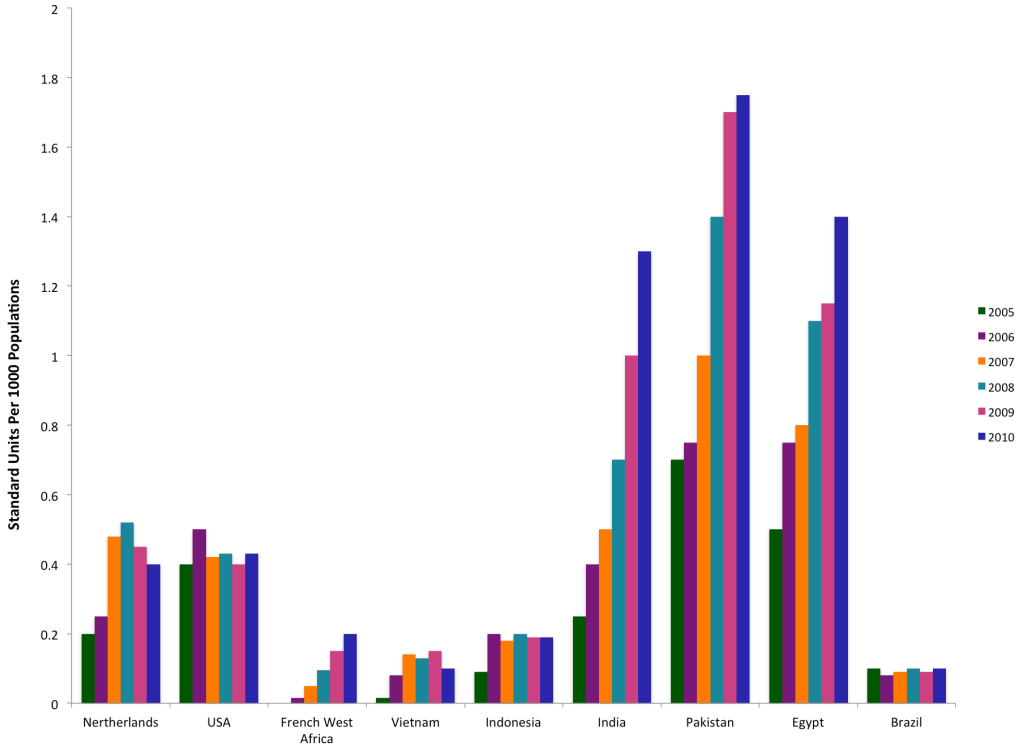


Figure 1.2. **Trends in retail sales of carbapenem antibiotics for gram-negative bacteria** between 2005 and 2010 based on data obtained from IMS Health's MIDAS™ database. (Figure information from Laxminarayan *et al*, 2013).

There are two antibiotics, streptomycin and oxytetracycline, that are registered for use on agricultural crops. Streptomycin was identified as a tool for the control of plant diseases in the 1950s (Goodman, 1954) and is registered in the U.S. for use on apple, peach, pear, tomato, celery, pepper, potato, and tobacco (United States Environmental Protection Agency, 2011), although it is used primarily for the control of fire blight of apples and pears (Nufarm, 2016). Oxytetracycline is registered for use on food apple, nectarine, peach, and pear (Nufarm, 2016). These antibiotics can be sprayed one or two times a week for control of bacterial disease of crops (K. Johnson & Stockwell, 1998). Streptomycin, labeled as Agri-Mycin 17, can be sprayed at four to five day intervals for celery, tomatoes, and peppers, at three to four day intervals for apple and pear, and at five to seven day intervals for tobacco (United States Environmental

Protection Agency, 2011). Oxyetetracycline, labeled as FireLine 45 WP, can be sprayed on apple and pear trees at three to four day intervals and on peach and nectarine trees at five to seven day intervals (United States Environmental Protection Agency, 2016).

Fire blight is a devastating disease of apple and pears. The pathogen, *Erwinia amylovora*, multiplies on the stigma of flowers, migrates down to and enters the nectaries through natural openings where the bacteria can then multiply and spread systemically, increasing the inoculum for subsequent shoot infections (Gowda & Goodman, 1970; P. W. Miller, 1929). Due to the surface exposure of the pathogen on stigmas, the flowers are the best method for targeted control of fire blight (McGhee et al., 2011). Since the 1950s, the use of streptomycin during bloom has been the most effective control method for fire blight (T. D. Miller & Schroth, 1972). However, the increase in the prevalence of streptomycin resistance in orchards since the late 1900s has reduced the efficacy for the control of fire blight and has heightened the need for alternative control methods (P.S. McManus & Jones, 1994). Kasugamycin, an antibiotic in the same class as streptomycin, is one alternative for fire blight control (McGhee et al., 2011).

Kasugamycin, an aminoglycoside antibiotic, was isolated from *Streptomyces kasugaensis* and was originally utilized as a fungicide for the control of rice blast, caused by *Piricularia oryzae* (Ikeno, Tsuji, Higashide, & Kinoshita, 1998; Suhara et al., 1972). After its use as a fungicide, kasugamycin was identified as a bactericide for the control of fire blight (Adaskaveg et al., 2008; Adaskaveg, Wade, & Forster, 2007; Aldwinckle & Norelli, 1990). As a bactericide, kasugamycin, like other aminoglycosides, such as streptomycin, inhibits protein synthesis. Unlike streptomycin, kasugamycin inhibits protein synthesis by binding to the 30S ribosomal subunit and interacts with protein S2 (Okuyama, Tanaka, & Komai, 1975; Tanaka, Yamaguchi, & Umezawa, 1966). Kasugamycin is a good alternative to streptomycin for agricultural use as it

has been shown to be as effective as streptomycin at controlling fire blight (McGhee & Sundin, 2011) and is not used in clinical medicine (Duffin & Seifert, 2009). Although it has been shown to inhibit some bacteria of clinical importance, such as *Psuedomonas* spp, *Escherichia coli* strains, and *Neisseria gonorrhoeae*, kasugamycin is not used to treat clinical infections (Duffin & Seifert, 2009). The lack of use in clinical medicine is due to its poor ability to inhibit many bacteria of clinical importance, the high minimal inhibitory concentration needed (125µg/ml or higher), and the high frequency of kasugamycin resistance observed in clinical isolates in which it is capable of inhibiting (Duffin & Seifert, 2009; Levitan, 1967). In its agricultural use, as with any antibiotic, levels of resistance have been and must continue to be monitored in order to understand the impacts that the use of kasugamycin will have on management practices in the future.

Antibiotic Resistant Genes and Antibiotic Resistant Bacteria

Antibiotic use is known to directly lead to the development of Antibiotic Resistant Bacteria (ARB) carrying antibiotic resistant genes (ARGs) (Williams-Nguyen et al., 2016). This resistance allows the bacterium to survive in the presence of the antibiotic, therefore providing a fitness advantage when in the presence of the specific antibiotic. However, in the absence of the given antibiotic, the persistence of antibiotic resistance is dependent on its fitness cost to the bacterium. For example, if the ARG, whether mutational or located on a plasmid, reduces the fitness of the bacterium, the ARB will be outcompeted by the susceptible strains when the given antibiotic in absent (D.I. Andersson, 2003). Other ARG, such as chromosomal streptomycin resistance, allows the ARB carrying these genes to persist, even in the absence of the antibiotic (D.I. Andersson, 2003).

ARGs can occur in non-pathogenic environmental bacterial populations, such as in soil, lakes, rivers, wastewater, and drinking water (Knapp, Dolfing, Ehlert, & Graham, 2010; Leonard, Zhang, Balfour, Garside, & Gaze, 2015; Martinez, 2008; Storteboom, Arabi, Davis, Crimi, & Pruden, 2010). These ARB can increase the prevalence of antibiotic resistance in the environment through cell division or through the transfer of the ARG to other bacteria in the environment (Williams-Nguyen et al., 2016). In situations where the fitness cost to the ARB is minimal, the ARG can persist in the environment when the antibiotic is not present (D.I. Andersson, 2003). In these situations, the ARG increases in occurrence and the risk of being transferred into clinical pathogens becomes higher. Once in clinical pathogen populations, the treatment of clinical infections becomes increasingly more difficult (Sharma, Johnson, Cizmas, McDonald, & Kim, 2016).

History of Antibiotic Resistance in Plant Pathogens

Antibiotic resistance in target plant-pathogenic bacteria was observed in the early 1960s, shortly after the introduction of streptomycin (A. Jones, 1982). Streptomycin resistance determinants have been discovered to be chromosomal or located on plasmids or transposable elements. All resistance determinant types have been detected in human pathogens, plant pathogens, and environmental bacterial strains (G.W. Sundin & Bender, 1996). Bacteria harboring a specific ARG have an enhanced survival advantage in the presence of that antibiotic.

In terms of streptomycin and plant pathogens, resistance has been primarily observed in *E. amylovora*, the fire blight pathogen (Patricia S. McManus et al., 2002; G.W. Sundin & Bender, 1996). Two types of resistance to streptomycin have been observed in *E. amylovora*; spontaneous mutations of the chromosomal target gene and acquired resistance genes (Patricia S. McManus et al., 2002). The spontaneous mutation is in the *rpsL* gene, which encodes ribosomal

protein S12 (C.S. Chiou & Jones, 1995b). The acquired resistance to streptomycin involves two genes, *strA* and *strB*, that are encoded on the Tn*5393* transposon (C.S Chiou & Jones, 1993) and have been detected on plasmids, such as pEa34 (C.S. Chiou & Jones, 1995a) and pEa29 (McGhee et al., 2011) in *E. amylovora*. The genes *strA* and *strB* encode the enzymes aminoglycoside-3''-phosphotransferase (APH(3'')-Ib) and aminoglycoside-6''-phosphotransferase (APH(6'')-Ib), respectively, that work together to inhibit streptomycin (C.S. Chiou & Jones, 1995a).

The *strA-strB* genes are not the only genes that are known to confer resistance to streptomycin (G.W. Sundin & Bender, 1996). For example, the gene *aadA,* which encodes an adenylyltransferase, was detected on two transposable elements, Tn*7* and Tn*21* (Fling, Kopf, & Richards, 1985; Grinsted, de la Cruz, & Schmitt, 1990). However, the *strA-strB* genes have been the most widely distributed Streptomycin resistance (Sm^R) genes and reside on broad host range plasmids, such as Tn*TP2*, RS*F1010*, and Tn*5393*. These genes were nearly identical in some plant pathogenic bacteria, including *E. amylovora*, *Xanthomonas campestris* pv. *vesicatoria*, and *Pseudomonas syringae* pvs. *papulans* and *syringae* (G.W. Sundin & Bender, 1996). The *strA-strB* genes in soil and phylloplane samples from pear nurseries and tomato fields were associated with Tn*5393* and were located on plasmids 30kb or larger in gram-negative bacteria (George W. Sundin, Monks, & Bender, 1995). In some cases, Sm^R and tetracycline resistance (Tc^R) were observed in the same isolate (George W. Sundin et al., 1995). The presence of both resistance genes in gram-negative bacteria was more common in soil than phylloplane isolates (George W. Sundin et al., 1995). Clinical isolates were not observed to harbor the *strA-strB* on Tn*5393*, whereas plant and soil bacterial isolates did. This difference in the location of the *strA-strB* genes indicates that multiple sequence divergent events have occurred in the evolution of the *strA-strB*

genes, leading to differences in Sm^R between clinical and environmental bacterial isolates (George W. Sundin, 2002). Therefore, the use of streptomycin in agriculture may not be responsible for Sm^R in clinical pathogens, indicating that agricultural use of antibiotics may not be the primary factor for resistance in clinical isolates.

Regardless of the mechanism of resistance, Sm^R poses a threat to plant agriculture since resistance has been detected in orchards (Loper et al., 1991; Moller, Schroth, & Thomson, 1981) and celery fields (Pohronezny, Sommerfeld, & Raid, 1994) that have not used streptomycin in over ten years (Patricia S. McManus et al., 2002). Similarly, Sm^R clinical isolates were detected several decades after streptomycin use was significantly reduced (O'Brian & *al.*, 1987; G.W. Sundin & Bender, 1996). These findings indicate that Sm^R is a stable trait and is unlikely to decrease in the population over time. Detecting antibiotic resistance decades after stopping antibiotic use is expected as many resistance genes do not pose a large fitness cost to the bacterium and, therefore, can be maintained even when the selection pressure is gone (Pruden et al., 2013).

Multi-Antibiotic Resistance

From a clinical medicine perspective, multi-antibiotic resistance is of even greater concern (Piras et al., 2012). This linkage is a result of the physical association of multiple antibiotic resistance determinants, either on the bacterial chromosome or on transferable elements (Chapman, 2003). These linked genes can be transferred to other bacteria in the environment through horizontal gene transfer (de la Cruz & Davies, 2000). These plasmids can be selected for in bacterial populations when exposed to any one of these antibiotics. Therefore, it may not be necessary for the antibiotic to be used in order for selection for that antibiotic to occur (Baker-Austin, Wright, Stepanauskas, & McArthur, 2006). If resistance to a new antibiotic

occurs on a plasmid or transposon that is carrying resistance genes to older antibiotics, then the use of the new antibiotic will continue to select for resistance to the other antibiotics in which it is genetically linked to, even if the other antibiotics are not being used (H.-H. Chang et al., 2015). Therefore, the use of one antibiotic can select for resistance to multiple antibiotics, including those that are no longer used or are not commonly utilized in a specific environment. This leads to the current concern that the application of Ks, a new antibiotic, in orchards will result in resistant to Ks in a genetic background with resistance to clinically important antibiotics. Evidence to support this concern is the resistance observed in clinical strains of *Staphylococcus aureus* and *E. coli* in which multi-antibiotic resistance has been identified with linkage to drugs such as streptomycin, vancomycin, and gentamycin even though their usage has significantly decreased (Brumfitt & Hamilton-Miller, 1989a; Dennesen, Bonten, & Weinstein, 1998; Piras et al., 2012).

Evidence of Antibiotic Resistance

Antibiotic resistance can evolve through multiple mechanisms. Two common means for antibiotic resistance is through mutations in the bacterial genome or by acquisition of resistance genes from other species through horizontal gene transfer (Finley et al., 2013; Witte, 1998). The evolution of antibiotic resistance is facilitated by a combination of the presence of resistance genes in the bacterial population and the use of antibiotics that aide in the selection for antibiotic resistance within bacterial populations, both in pathogenic and non-pathogenic species (Figure 1.3) (Finley et al., 2013; Singer & Williams-Nguyen, 2014; Witte, 1998). Antibiotic resistance genes are always present in the environment, as the antibiotic producers also encode relevant resistance genes in order to prevent harming itself (Finley et al., 2013). In order to better understand the role that agricultural antibiotic use plays in the level of resistance in human

pathogens, models that incorporate the complex relationship between bacterial exposure to resistance genes and the observation of resistance need to be developed (Singer & Williams-Nguyen, 2014; Smith, Dushoff, & Morris, 2005). For example, these methods would need to take into account the rate of horizontal gene transfer from a non-pathogen to a human pathogen, the complex nature of bacterial population structures, and the natural environment (Smith et al., 2005).

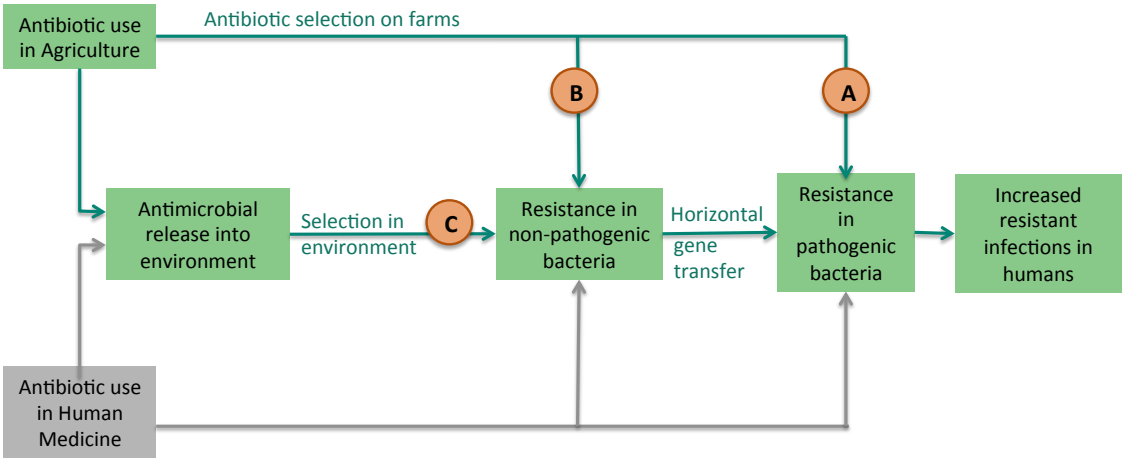


Figure 1.3. Conceptual model of the ways in which agriculture antibiotic use (AAU) can cause increased resistant infections in humans. In Scenario **A**, AAU leads to the increase in resistant pathogens, which are then transmitted to humans via the food chain or the environment. In Scenario **B**, AAU selects for resistance in non-pathogens, which then transfer resistance genes to pathogens leading to more resistant infections in humans. In Scenario **C**, active antimicrobial compounds are released into the environment, where resistance selection occurs predominantly in non-pathogens, and is then transferred horizontally to pathogens as in B. Human antibiotic use is shown for reference but not discussed. (Figure information from Singer and Williams-Nguyen, 2014).

The *strA-strB* genes occur on transposons in non-pathogenic gram-negative plant epiphytes and in soil-borne bacteria from orchards and nurseries that were both treated and non-treated with streptomycin. Since streptomycin resistance has been observed in locations treated and non-treated with the antibiotic, it indicates that streptomycin resistance occurs naturally in the environment rather than as a result to exposure. As streptomycin has been observed in pathogenic strains as well as non-pathogenic environmental isolates, it is likely that the

streptomycin resistance gene was acquired in plant pathogenic bacteria through horizontal gene transfer from non-pathogenic environmental strains (Patricia S. McManus et al., 2002). Further research has identified *Pantoea agglomerans* as the most likely source of the transfer of streptomycin resistance to *E. amylovora* as both were found to carry Tn*5393* containing IS*1133* and the plasmid pEa34 was transferred from *P. agglomerans* to *E. amylovora* with high frequency in *vitro* (C.S Chiou & Jones, 1993). Since both bacterial species occupy the same niche, apple flowers, it is reasonable that this transfer would occur in nature (Riggle & Klos, 1970). However, there have yet to be studies done to prove that the *strA-strB* genes have been transferred from plant pathogens and human pathogens (Patricia S. McManus et al., 2002).

Although antibiotics and antibiotic resistance genes have been around prior to their use in clinical medicine and agriculture, antibiotic use by humans has increased the abundance of antibiotic resistance in the environment. For example, tetracycline (*tet*(M) *tet*(O) *tet*(Q), and *tet*(W)), erythromycin (e*m*(B), *em*(C), *em*(E), and *em(F)*), and β-Lactameases ($bla_{\text{TEM-1}}$ and $bla_{\text{SHV-1}}$) resistance genes were found to be up to fifteen times higher in the Netherlands in 2008 compared to the 1970s (Knapp et al., 2010). These antibiotics have been used in the Netherlands for nearly 80 years (Knapp et al., 2010) and overall antibiotic use has been increasing (Fig. 1.1.2).

The Risk of Antibiotic use in Plant Agriculture to Human Medicine

Antibiotic resistance causes serious problems in human medicine. Bacterial infections are becoming harder, and in some cases impossible, to treat. This causes a longer period of time with illness and a larger mortality rate from infections that were previously treatable with antibiotics (Lipsitch, Singer, & Levin, 2002). Antibiotic resistance also increases the cost of treating antibiotic resistant infections as well as decreasing the number of medical procedures that rely on

antibiotics to prevent post operational complications (Laxminarayan et al., 2013). The increasing amount of resistance has led to the need for alternative methods for control of human diseases and the need to limit the use of antibiotics in all avenues of its use.

There is an increased concern that the use of antibiotics in plant agriculture will increase the prevalence of antibiotic resistance genes in bacterial populations, despite the low level of antibiotics used in this area in comparison to animal husbandry and human medicine (P.S. McManus, 2014; Williams-Nguyen et al., 2016). However, there is limited literature evaluating the level of natural occurring antibiotic resistance. This lack of knowledge hinders the ability to draw a correlation between antimicrobial use and an increase in resistance, especially in human pathogenic bacteria (Vidaver, 2002). Also, the link between antibiotic resistance from environmental bacteria and in human pathogenic bacteria is not well understood (Finley et al., 2013).

It is unlikely that human pathogens would share a niche with plant pathogenic bacteria, as most human pathogenic bacteria do not survive well on plants. This reduces the probability of the transfer of resistance genes from plant pathogens, such as *E. amylovora*, to human pathogens (V.O Stockwell & Duffy, 2012). Despite this, there are a few human pathogenic bacteria, such as E. coli species and *Salmonella*, that can be on food crops due to contamination events and then ingested by humans (Q. Chang, Wang, Regev-Yochay, Lipsitch, & Hanage, 2014). Since antibiotics likely land on the soil surface in the process of spraying crops, it has been speculated by many researchers that the antibiotics that end up in the soil can select for antibiotic resistance in soil populations and this ARG can then be transferred to human pathogenic bacteria (V.O Stockwell & Duffy, 2012). Despite this, no evidence has been found to support this hypothesis (V.O Stockwell & Duffy, 2012). The antibiotic resistance crisis in clinically important bacteria

poses the need to reconsider the use of antibiotics and the role their utilization plays in the development of antibiotic resistance. However, antibiotics, such as streptomycin and oxytetracycline, have been used in plant agriculture for over 50 years with no reported direct impact on humans. Despite this, the agricultural practice of spraying crops with antibiotics for the control of plant diseases and its effect on human medicine has been a topic of great debate (V.O Stockwell & Duffy, 2012).

The concern about antibiotic use in agriculture is legitimized when considering animal agriculture. One example is the discovery of fluoroquinolone resistant *Campylobacter jejuni,* a bacterium that causes gastroenteritis, food poisoning, in humans through contamination of chicken in supermarkets (Lipsitch et al., 2002). The consumption of this contaminated chicken could lead to food poisoning in humans if the chicken is not prepared properly and would result in complications in medical treatment (Lipsitch et al., 2002). Although this appears to be proof of the link between antibiotic use and antibiotic resistance observed in human diseases, there are complications in verifying this as it is nearly impossible to prove that the antibiotic resistant bacterium occurred as a result of exposure to the antibiotic (Lipsitch et al., 2002).

The concern about antibiotic use in agriculture and the implication to human medicine has initiated research looking at the effects of the use of antibiotics in agriculture on the level of antibiotic resistant and the level of multi-antibiotic resistance. McGhee and Sundin evaluated the effect of kasugamycin use in orchards on levels of resistance to kasugamycin in orchard bacterial populations (McGhee & Sundin, 2011). The appearance of Sm^R and its increasing prevalence has drastically complicated the control of plant bacterial diseases (Patricia S. McManus et al., 2002), including the control of fire blight of apples. There was a variation in the levels of kasugamycin resistance between plant and soil isolates, with higher levels of resistance from soil isolates. Of

the isolates evaluated, 49% were resistant to both kasugamycin and streptomycin (McGhee et al., 2011). However, since kasugamycin is not used in human medicine, resistance to kasugamycin alone in agricultural systems poses little to no threat to human medicine. The high level of co-resistance to kasugamycin and streptomycin observed by McGhee and Sundin raises the concern for transferring multi-antibiotic resistance to human pathogens as streptomycin is used in clinical medicine and would select for the linkage of the kasugamycin and streptomycin resistance genes in clinical pathogens.

Reducing the Risk of Further Antibiotic Resistance Development

Antibiotic resistance, specifically in human and animal pathogens, limits the options available to veterinarians and physicians to treat infections, such as those caused by *E. coli*, *Salmonella*, and *Staphylococcus* species (Angulo, Baker, Olsen, Anderson, & Barrett, 2004; V.O Stockwell & Duffy, 2012; Witte, 1998). This increases the cost of treatment, length of the illness, and mortality rate (Laxminarayan et al., 2013). It is becoming evident that our use and regulation of antibiotics needs to change.

It will be necessary to refrain from unnecessary use of antibiotics in order to reduce the chance of antibiotic resistance development and the transfer of this resistance into human pathogens. Antibiotic use should be limited to the treatment of human and animal diseases and used only when no alternative options are available in agricultural systems. Other options for control of plant diseases need to be further investigated, such as biological control agents, transgenic plant use, and other chemical control agents. It may be beneficial to initiate either a ban of antibiotic use in specific avenues, such as animal and plant agriculture, or an added fee to the use of all antibiotics. A ban may be too expensive, as it would require supervision of antibiotic use in all areas. An added fee, on the other hand, is much more practical. The fee will

increase the cost of antibiotics to the user, which will decrease the use of antibiotics in situations where other options are available and will remain in use in areas where alternative options do not exist.

In human medicine, antibiotics need to be prescribed only when necessary and taken as directed. In order to prevent the over use of antibiotics by the public and health practitioners, programs need to be implemented that work to educate the public on the proper use of antibiotics and the risk of their over use. Lastly, further research needs to be done to provide a more rapid identification of antibiotic resistant strains. Having better and more reliable diagnostic tools will allow for a more rapid change in treatment of human, animal, and plant diseases and will increase the effectiveness of antibiotics in all areas.

SECTION 2: *Erwinia amylovora* and Levan Production

Introduction

Fire blight, caused by *Erwinia amylovora,* was first discovered in New York on pear (Denning, 1794). This disease later spread throughout the pome fruit growing regions of North America, England, and in Northern parts of the European continent (Peil et al., 2009). The disease affects all plants in the Rosaceae family, including apple, pear, and raspberry (Rosen & Grovs, 1928; Thomas & Ark, 1934a).

Erwinia amylovora overwinters in cankers formed in woody tissue infected during the previous season (P. W. Miller, 1929). In the spring, the pathogen multiplies at the margin of a small percentage of these overwintering cankers, exudes from the host in ooze droplets, and are disseminated from cankers to growing shoot tips or flowers by insects, wind, or rain (Steven V. Beer & Norelli, 1977; P. W. Miller, 1929). The pathogen enters the host tissue through wounds or natural openings, multiplies intercellularly, and spreads throughout the host causing cell death

(Gowda & Goodman, 1970). Primary infections initially occur on the stigma of flowers, where the environment is optimal for the rapid growth and spread of the pathogen (T. D. Miller & Schroth, 1972; Thomson, Wagner, & Gouk, 1999). Secondary infections can occur when the pathogen emerges from shoot and blossom tissue as ooze and is disseminated to growing shoot tips or wounds (S.V. Beer, 1979). The damage of fire blight to the tree depends on the location of disease and the distance it spreads within the host. If the disease spreads to the main limbs, the yield of the current year is affected and future growth and production may also be affected. In severe cases, the disease will spread to the trunk of the tree, ultimately resulting its death (Momol et al., 1998).

The pathogenicity of *E. amylovora* depends on the *hrp* (hypersensitive reaction and pathogenicity) genes, which encode the formation of the type three secretion system. This system functions in the transfer of effector proteins into the host, which allows the pathogen to surpass the host defense system (McNally et al., 2011). Along with these proteins, *E. amylovora* secretes two exopolysaccharides, levan and amylovoran, which also play an important role in the pathogenticity of *E. amylovora* (Robert A. Bennett & Billing, 1980).

The dissemination of *E. amylovora* depends on the formation and spread of ooze droplets from infected tissue. These ooze droplets consists of a matrix of exopolysaccharides (EPS), both levan and amylovoran, and bacterial cells (Schroth, Thomson, & Hildebrand, 1974). The ooze droplet may be one of a variety of colors, ranging from white to dark red (Van der Zwet, 1994). *E. amylovora* cells in ooze are disseminated by insects to new blossoms or shoots mechanically. Temperature, margin type, and age of the tissue affects the ability of *E. amylovora* to be exuded from cankers. *E. amylovora* was recovered from 50% of cankers produced by artificial

inoculation of two year old trees when grown at 21°C, but only 10% and 5% from trees grown at 28°C and 17°C, respectively (Steven V. Beer & Norelli, 1977).

Disease Control

Fire blight is a devastating disease that can rapidly spread within and between orchards. This disease is difficult to control due to the lack of effective bactericides and the fact that many popular apple cultivars are susceptible to fire blight (Norelli, Jones, & Aldwinckle, 2003). Current control options include cultural practices, chemical application, (Schroth et al., 1974), and biological control agents (K. B. Johnson et al., 1993).

Cultural Practices

General control methods for fire blight include cultural based practices and have been in use since the early 1900s. These practices include the use of sterilized tools to prevent the spread of the pathogen, inspecting hold over cankers for signs of activity, minimal use of nitrogen based fertilizers, and the removal of infected host tissue 20-30cm bellow visible disease during the dormant season (Schroth et al., 1974). Although cultural practices can help to prevent the spread of the disease, it is not the most effective method.

Biological Control

The use of biological control agents have been shown to be a useful tool for organic apple production where the use of effective chemical control agents are not available (K. B. Johnson & Temple, 2013). For example, *Pantoea agglomerans*, *Pseudomonas fluorescens*, or a mixture of these bacterial antagonists sprayed onto blossoms has been shown to reduce the occurrence of fire blight (K. Johnson & Stockwell, 1998; K. B. Johnson et al., 1993; Lindow, McGouty, & Elkins, 1996). In the western U.S., application of flowers with *Aureobasidium pullulans* (Blossom Protect) after treatment with 2% lime sulfur and 2% fish oil has been shown to reduce

the incidence of fire blight up to 90% (K. B. Johnson & Temple, 2013). Although biological control has been effective at controlling fire blight, it is not effective in all environments. Field trials were conducted in Michigan, New York, and Virginia to evaluate the efficacy of the biological control agent's *P. fluorescens* A506, *P. agglomerancs* C9-1, and *P. agglomerans* E325 for the control of fire blight. Sundin *et. al* (2009) showed that in the warmer, humid climate of the Eastern U.S., these biological control agents were inconsistent in the level of control from year-to-year and were always less effective than streptomycin. When the biological control agents were implemented into programs with streptomycin, the control level was consistent and reduced the number of streptomycin sprays needed to maintain equivalent control (George W. Sundin, Werner, Yoder, & Aldwinckle, 2009). Despite the reduction in streptomycin application, the use of biological control agents in the Eastern U.S. is currently unlikely as it is not a cost effective method for the growers.

Bloom Sprays

The first report of successful use of antibiotics for the control of fire blight occurred in 1952 and was confirmed by multiple research groups (Ark, 1953; Goodman, 1954; Heuberger & Paulos, 1952; Luepschen, Parker, & Mills, 1960; Murneek, 1952; Schroth et al., 1974). Since the 1950s, streptomycin has been the most effective control method for fire blight. However, the first signs of streptomycin resistance in *E. amylovora* was observed in 1971 in the Sacramento Valley in California (Schroth et al., 1974). Kasumin 2L is an alternative control option for apple and pear orchards that have streptomycin resistance as it has been shown to be as effective as streptomycin for the control of fire blight (McGhee & Sundin, 2011).

EPS and their Role in the Virulence of *E. amylovora*

E. amylovora produces three types of exopolysaccharides (EPS), amylovoran, levan, and cellulose, which all play an important role in the virulence of *E. amylovora* (Ayers, Ayers, & Goodman, 1979; R.A. Bennett & Billing, 1978; Castiblanco & Sundin, 2018; Koczan, McGrath, Zhao, & Sundin, 2009). Amylovoran and levan are involved in biofilm formation, which protects the bacterial cells from harsh environmental conditions, increases the ability of the pathogen to acquire nutrients and water, and aides in the systemic spread within the host (Bellemann & Geider, 1992; Castiblanco & Sundin, 2018; Koczan et al., 2009). They are also major components of ooze (Robert A. Bennett & Billing, 1980; Eden-Green & Knee, 1974).

Amylovoran

Amylovoran, which produces a capsule around the bacterium, is an acidic EPS and is an important virulent factor of *E. amylovora* (Nimtz et al., 1996; Steinberger & Beer, 1988). It is composed of repeating units of one glucuronic acid and four galactose residues (Nimtz et al., 1996) and is synthesized in the presence of sorbitol (Robert A. Bennett & Billing, 1980). Amylovoran is thought to aide in bacterial protection by shielding the cells from host defense mechanisms (Bellemann & Geider, 1992). The synthesis of amylovoran by *E. amylovora* involves a cluster of 12 *ams* genes (Bellemann & Geider, 1992; Bugert & Geider, 1995) and is regulated by the regulatory proteins RcsA and RcsB (Stefan Bereswill & Geider, 1997; Kelm, Kiecker, Geider, & Bernhad, 1997). Amylovoran plays a vital role in biofilm formation as well as the virulence of the pathogen. A deletion in the *ams* operon resulted in a lack of biofilm formation in vitro (Koczan et al., 2009). Strains with a deletion in the *ams* operon were avirulent, indicating that it is a necessary component for disease development (Steinberger & Beer, 1988).

The synthesis of amylovoran was also shown to be positively regulated by c-di-GMP (Edmunds, Castiblanco, Sundin, & Waters, 2013a).

Cellulose

Castiblanco and Sundin (2017) showed that cellulose is also a major component of biofilms, both *in vitro* and *in vivo*. Strains with a deletion in *bcsA*, a component of the cellular membrane integrated cellulose synthesis multi-protein complex, showed a reduction in biofilm formation on both glass coverslips ant TEM grids. The *bcsA* mutant also showed a reduction in the fibrilar material of biofilms. Similar to amylovoran, cellulose production was shown to be positively regulated by c-di-GMP (Castiblanco & Sundin, 2018).

Levan

Levan is a neutral EPS (Michael Gross, Geier, Rudolph, & Geider, 1992) that is involved in biofilm formation and the virulence of *E. amylovora (Geier & Geider, 1993; Koczan et al., 2009)*. Levan is synthesized by the secreted enzyme levansucrase when in the presence of sucrose by cleavage of the sugar followed by the plolymerization of fructose into a polyfructan (Geider, Aldridge, Bereswill, Bugert, & C., 1996; Geier & Geider, 1993; Michael Gross et al., 1992). The levansucrase enzyme is involved in the catalysis of two reactions, the hydrolysis of sucrose resulting in fructose and glucose products and the transfructosylation in which a fructosyl unit is transformed from the enzyme-frucosyl intermediate to an acceptor, either an oligomer or a polymer of fructose or sucrose (Chambert, Treboul, & Dedonder, 1974). In vitro, strains with a deletion in the lsc gene showed a reduction in biofilm formation and overall virulence, indicating that this gene, as well as levan, is important in the virulence of *E. amylovora* (Koczan et al., 2009). However, the exact function that levan and the *lsc* gene plays in pathogenesis are not well known.

The *lsc* gene is positively controlled by the regulatory protein RlsA and is located upstream of the *hrp/dsp* cluster (Du & Geider, 2002; Oh & Beer, 2005). Two other activators, RlsB and RlsC, have also been described. RlsB is located adjacent to the start of the *lsc* gene in the opposite orientation and may be a limiting factor for the activation of the *lsc* promoter (Du & Geider, 2002). Levan deficient strains were found to be restored to levels higher than wild type when complemented with either *rlsA*, *rlsB*, or *rlsC* (Du & Geider, 2002; Du, Jakovljevic, Salm, & Geider, 2004).

Examination of the sequence of the l*sc* gene from *E. amylovora* by Geier and Geider indicated that there was not a high degree of similarity to levansucrase genes of other species. The closest similarity was found to the levansucrase gene (*sacB*) of *Bacillus subtilis* (Geier & Geider, 1993). A levansucrase gene is also present in *Pseudomonas phaseolicola* (M. Gross & Rudolph, 1987), *P. syringae pv. tomato* (Visnapuu et al., 2011), *P. chlororaphis* subsp. *aurantiaca* (Visnapuu et al., 2011), *Zymomonas mobilis (Lyness & Doelle, 1983)*, and *Erwinia herbicola* (Cote & Jmam, 1989), although their similarity to that of *E. amylovora lsc* was not reported. The levansucrases of different bacteria have specificity in forming either fructooligosaccharides (FOS), common in gram-negative bacteria, or high molecular weight polymers, common in gram-positive bacteria. *E. amylovora* Lsc produces short-chain FOS of three to six units (Caputi, Cianci, & Benini, 2013).

E. amylovora Lsc is composed of eight molecules, which are grouped into four crystallographic dimers. The catalytic active triad (Asp46, Asp203, and Glu287) is conserved among levansucrases. A switch from Arg to His at Arg 360 in *B. subtilis* SacB (His305 in *E. amylovora* Lsc) was suggested to act as a switch between production of polymers and the shorter fructoologosaccharides. There a nine loop structures at the rim of the active site funnel of

levansucrase. Loop 8 in *E. amylovora* Lsc is smaller than that in *B. subtilis* SacB and in *Gluconacetobacter diazotrophicus* LsdA. This is hypothesized to be a result of the need for a faster substrate/product turnover in the presence of a lower concentration of sucrose in the nectar during infection (Wuerges et al., 2015).

It was shown that the phloem of apple trees as well as the nectar of apple flowers contain high concentrations of sucrose (Geier & Geider, 1993; Michael Gross et al., 1992). *E. amylovora* is capable of growing in medium containing up to 60 percent sucrose (T. D. Miller & Schroth, 1972; Thomas & Ark, 1934b), which is significantly higher than the 40 percent that is considered a toxic level (Schaad, 1988). It was shown that *E. amylovora* produces 12.5g of levan per liter in two days when grown in medium containing high levels of sucrose (100g per liter) (Michael Gross et al., 1992). This high level of levan produced in environments with high concentrations of sucrose provides *E. amylovora* a protective shield that allows the bacterium to retain moisture and prevent desiccation as well as utilizing a large portion of the sucrose in the environment that would otherwise be detrimental to its survival (Michael Gross et al., 1992).

EnvZ/OmpR System and its Effect on Levan Formation

Two-component signal transduction systems (TCSTs) are a common occurrence for signal transduction in prokaryotes (Stock, Ninfa, & Sock, 1989). Two such systems, EnvZ/OmpR and GrrS/GrrA, are global and dual regulators in gamma-Proteobacteria (K. Brzostek, K. Skorek, & A. Raczkowska, 2012). The EnvZ/OmpR system was first identified as regulators of outer membrane porins, OmpF and OmpC. EnvZ/OmpR system is now known to also play a role in the regulation of various cellular components, including EPS production, motility, fatty acid transport, curli fiber formation, cell division, and virulence (Berry, DeVault, & Chakrabarty, 1989; K. Brzostek et al., 2012; Pickard et al., 1994). The *ompR* gene is involved

in the regulation of the synthesis of Vi, a capsular exopolysaccharide in *Salmonella typhi* (Pickard et al., 1994). Type three secretion system (T3SS) genes are regulated by EnvZ/OmpR for several pathogenic bacteria, including *Salmonella typhimurium, Yersinia enterocolitica,* and *Pseudomonas syringae* (Brzostek, Brzostkowska, Bukowska, Karwicka, & Raczkowska, 2007; Feng, Oropeza, & Kenney, 2003; Feng, Walthers, Oropeza, & Kenney, 2004).

It was found that the EnvZ/OmpR system negatively regulates amylovoran synthesis, plays a role in the regulation of motility, and negatively regulates *hrp*-T3SS gene expression in *Erwinia amylovora*. Strains with a deletion in *envZ*, *ompR*, or both genes showed a reduction in levan production compared to the wild type strain (Ea1189), but were not statistically different from each other. These strains did not shown significant differences in virulence compared to Ea1189 in immature pears or apple shoots. It was found that strains with a deletion in *grrS*, *grrA*, or both showed nearly no levan production compared to Ea1189 (Li, Ancona, & Zhao, 2014).

Production of Ooze in *E. amylovora*

The mechanism of ooze emergence from host tissue has been widely debated (E. Billing, 1981; Fisher, Parker, Luepschen, & Kwong, 1959; E. M. Hilderbrand, 1939; Henk J. Schouten, 1991; Seemuller & Beer, 1976; Slack, Zeng, Outwater, & Sunding, 2017; Zamski, Shtienberg, & Blachinsky, 2006). There has not been an agreement on whether ooze emergence is a result of the seepage of EPS and bacterial cells from natural openings or a result of bursting out of host tissue. A larger number of *E. amylovora* cells were observed in the intercellular space than in the xylem or phloem of infected host tissue along with ooze (Zamski et al., 2006). Slack *et. al* (2017) observed bacterial cells emerging from wounded tissue when collapsed ooze droplets were examined using SEM. When the ooze was removed, they observed wounds and erumpent mounds underneath the ooze droplets; however, no natural opening was observed near the ooze

emergent site (Slack et al., 2017). Schouten and Hawthorn also observed these erumpent mounds, which they found to contain bacterial cells (Henk J. Schouten, 1991). It was hypothesized that *E. amylovora* cells growing in the intercellular space of the host would create a change in pressure and lead to ooze emergence (Eden-Green & Billing, 1972). Schouten *et. al* evaluated the role of water potential and pressure change within the intercellular spaces of the host as a result of *E. amylovora* cells and EPS on pathogenesis (H.J. Schouten, 1988, 1989a; Henk J. Schouten, 1991). Biomass, consisting of bacterial cells and EPS, was observed to change in response to changes in water potential (H.J. Schouten, 1989a). In the intercellular space, the biomass can absorb water and swell as water potential changes, such as after a rain event (H.J. Schouten, 1988). The expanding mass of bacterial cells and EPS in the confinement of the intercellular space may result in a change in pressure large enough to cause tearing of the surrounding host tissue, especially soft tissue (Henk J. Schouten, 1991). These findings indicate that EPS plays a primary role in the formation of ooze by *E. amylovora*.

Conclusion

Antibiotics originated from environmental microorganisms and have been utilized for the control of bacterial diseases in plant agriculture, animal agriculture, and human medicine. The use of antibiotics in all avenues has led to the increased prevalence of antibiotic resistance. This antibiotic resistance complicates the control of all bacterial diseases. However, the difficulty of controlling bacterial diseases of humans due to antibiotic resistance is of significant importance to society as it increases the rate of mortality to diseases that were once treatable. The concern about the risk of increasing antibiotic resistance in human medicine has led to the concern with the use of antibiotics in plant agriculture. This concern has been increasing over the years, even though only a small percentage of total antibiotic use is used in plant agriculture.

Antibiotics, such as streptomycin, have been used in both human medicine and plant agriculture for over sixty years. The occurrence of streptomycin resistance became a concern shortly after the introduction of streptomycin use in plant agriculture. The occurrence of streptomycin resistance has caused serious problems for both plant agriculture and human medicine. One problem streptomycin resistance has caused is the decreased ability to control bacterial diseases, such as fire blight of apple. A second problem is the development of multi-antibiotic resistance, making the control of such diseases increasingly more difficult.

Fire blight of apples is one bacterial disease in which streptomycin has been used in plant agriculture. The severity of this disease relies on the development of colonization of stigmas. Therefore, antibiotic utilization occurs solely during bloom when the development of fire blight is most severe. With the increasing occurrence of streptomycin resistance in populations of *E. amylovora*, streptomycin has become significantly less effective. In order to prevent fire blight outbreaks, alternative control methods have become necessary.

One of these alternative methods is the use of a new antibiotic, kasugamycin, which is not used in human medicine or animal medicine. Kasugamycin has been shown to be as effective as streptomycin at controlling fire blight and may be useful in the control of bacterial diseases in other plant systems as well. Although the use of this new antibiotic is the best alternative to the use of streptomycin, there remains a strong concern about the use of antibiotics in plant agriculture. The fear is that the use of kasugamycin in plant agriculture will result in an increase in the prevalence of multi-antibiotic resistance and that this multi-drug resistance will move into human bacterial pathogens, making the treatment of these diseases significantly more complicated, if not impossible, to treat.

A second alternative to streptomycin is to develop management strategies based on the mechanism of dispersal of *E. amylovora*. In order to do this, we first need to better understand the role that EPS plays in its dispersal. It is known that the EPS amylovoran plays a major role in the virulence of *E. amylovora* as strains without the *ams* operon are avirulent. Levan is also known to play an important role in the virulence of *E. amylovora* as a deletion in the *lsc* gene significantly reduces its virulence. It is also a major EPS in ooze droplets, which is the means for dispersal of the pathogen. However, the role that levan plays in the formation of these ooze droplets and the ultimate spread of the bacterium is not understood.

The objectives of this study is to understand 1) the effect of the use of kasugamycin in plant agriculture has on the level of resistance to kasugamycin and on the level of multi-antibiotic resistance and 2) the role that levan plays in the formation of ooze droplets and the dispersal of *E. amylovora*.

CHAPTER 2: Effect of Kasugamycin use on Levels of Resistance to Kasugamycin and to other Antibiotics

Abstract

Kasugamycin (Ks) is a an alternative antibiotic to the use of streptomycin for the control of bacterial plant diseases and it has been shown to be as effective as streptomycin in controlling fire blight, caused by *Erwinia amylovora*. However, there remains a concern that Ks application in orchards will select for Ks resistance that could be linked with other resistance genes that are active against antibiotics used in human medicine. To monitor for this possibility, we assessed the effect of the use of Ks (Kasumin 2L) in orchard systems on the level of resistance to Ks and five other antibiotics (streptomycin, ampicillin, gentamicin, cefotaxime, and tetracycline). Two sets of leaf and soil samples were collected (treated and non-treated with Ks) from a total of 25 orchards in 2015 (5 different tree hosts from 7 states) and 16 orchards in 2016 (3 different tree hosts from 5 states). Samples were processed in the laboratory and dilution plated onto King's B medium with or without Ks amendment. Bacterial population sizes were determined per sample and up to 15 gram-negative colonies growing on Ks-amended medium per sample (a total of 1,038 and 603 isolates in 2015 and 2016, respectively) were used in antibiotic resistance screening. Although the bacterial population sizes were larger on Ks-amended medium from soil compared to leaf samples, there were no differences in populations from Ks treated vs. non-treated sites. Similarly, there was no difference in levels of resistance to the five antibiotics tested between Ks-treated and non-treated sites.

Introduction

Bacterial diseases of plants pose a severe threat to plant agriculture worldwide. For example, losses due to fire blight of pome fruit can be greater than $100 million (Norelli et al.,

2003). Traditional management of these diseases involves a combination of cultural practices and chemical treatment with preventative copper or antibiotic sprays (Acimovic, Zeng, McGhee, Sundin, & Wise, 2015). Despite the level of importance of these diseases, there are only a few antibiotics that are available and effective at controlling them (V.O Stockwell & Duffy, 2012). In the U.S., streptomycin, oxytetracycline, and kasugamycin are the only antibiotics available for agricultural use (V.O Stockwell & Duffy, 2012).

Streptomycin is an aminoglycoside antibiotic that was utilized in plant agriculture in the 1950s (Goodman, 1954) and has been the most effective at managing bacterial plant diseases (T. D. Miller & Schroth, 1972). Oxytetracycline has been registered for the control of disease of four fruit crops, including fire blight (Vidaver, 2002). Streptomycin and oxytetracycline have the longest history in the management of fire blight. Streptomycin has been the most effective against fire blight due to its ability to kill the pathogen on the flower surface by binding irreversibly to the bacterial ribosome, blocking the synthesis of proteins (C.S. Chiou & Jones, 1995b). Oxytetracycline has been less effective as it inhibits the growth of bacterial cells, rather than killing them (Patricia S. McManus et al., 2002).

The newest antibiotic in plant agriculture is kasugamycin (Ks), which was originally isolated from *Streptomyces kasugaensis*, a soil bacterium, and was first identified as a fungicide to treat rice blast, caused by *Pyricularia oryzae* (Copping & Duke, 2007). McGhee and Sundin showed that Ks, labeled as Kasumin 2L (Arysta Lifescience, Cary, NC), was equivalent to streptomycin for the control of fire blight of apple (McGhee & Sundin, 2011).

For fire blight control, antibiotics are applied as a spray to blossoms every 3-7 days until petal drop (Vidaver, 2002). This timing of antibiotic spray is a result of the life cycle of the fire bight pathogen, *Erwinia amylovora* (*Ea*). Fire blight begins with the growth of the pathogen on

flower stigmas followed by movement into the nectaries and systemic migration (Gowda & Goodman, 1970; T. D. Miller & Schroth, 1972).

With its high level of efficacy and low toxicity, streptomycin has been the primary choice for control of several diseases, including fire blight (T. D. Miller & Schroth, 1972). This has resulted in its excessive usage and has lead to the development of resistance in target pathogen populations (C.S Chiou & Jones, 1993). Antibiotic resistance was detected as early as the 1960s, shortly after the commercial use of streptomycin in plant agriculture (A. Jones, 1982). Two types of streptomycin resistance (Sm^R) have been found in *Ea* populations, including spontaneous mutation and acquired resistance. Acquired Sm^R is the most prevalent form in *Ea* strains and is a result of the acquisition of a plasmid carrying the transposon Tn5393 with the gene pair *strA* and *strB* (C.S Chiou & Jones, 1993).

The *strA-strB* genes have been found to be nearly identical in many plant pathogenic bacteria, including *Erwinia amylovora*, *Xanthomonas campestris* pv. *vesicatoria*, and *Pseudomonas syringae* pv. *papulans* and *syringae* (G.W. Sundin & Bender, 1996). Sundin et. al. found that in some cases, Sm^R and tetracycline resistance (Tc^R) were observed in the same isolate. The presence of both resistance genes in gram-negative bacteria was more common in soil isolates (George W. Sundin et al., 1995).

With the high prevalence of Sm^R in target pathogen populations, there is a severe need for new control options. Despite the large number of known antibiotics, most of these are used in clinical medicine and are not used in agriculture (McGhee & Sundin, 2011). The restriction on the use of antibiotics in agriculture is mainly due to the presence of transferable antibiotic resistance in native bacterial populations and the severe impact that resistance has on the control of human and animal infections (McGhee & Sundin, 2011). It was originally hypothesized that

antibiotic use first selects for antibiotic resistance in non-target bacteria and can then be transferred to target bacteria (McGhee & Sundin, 2011) through horizontal gene transfer.

In plant agriculture, antibiotics are mixed with water and sprayed onto the plant surfaces. In this process, some of the antibiotic can run-off into the soil (McGhee & Sundin, 2011). Antibiotics on both plant surfaces and in soil have the potential to create a selection pressure for resistance in bacterial populations (V.O Stockwell & Duffy, 2012). Antibiotic resistance in soil populations poses a higher risk to clinical medicine as soil harbors higher populations of gram-negative bacteria and a higher number of bacterial species that contain human pathogens (Cruz, Cazacu, & Allen, 2007; McGhee & Sundin, 2011). The abundance of bacteria with potential as human pathogens and their ability to acquire antibiotic resistance is of great concern.

From a clinical medical perspective, multi-antibiotic resistance is of greater concern as it makes clinical infections more difficult to treat (G.W. Sundin & Bender, 1996). This multi-antibiotic resistance is a result of the physical association of multiple antibiotic resistance determinants on transferable elements, such as plasmids and transposons, and can be transferred to other bacteria (Dan I. Andersson & Hughes, 2011). These plasmids are selected for in bacterial populations when exposed to any one of the genetically linked antibiotics. Therefore, exposure to only one antibiotic can lead to the selection of resistance to multiple antibiotics (Baker-Austin et al., 2006; H.-H. Chang et al., 2015). This leads to the current concern that the application of Ks, a new antibiotic, in orchards will result in resistance to Ks in a genetic background with resistance to clinically important antibiotics.

It is clear that antibiotic resistance in environmental strains needs to be evaluated in order to preserve their use in clinical medicine. The loss of antibiotic effectiveness would severely complicate the treatment of clinical infections. Therefore, in this study, we evaluated the effect

of the application of Ks in orchards on non-target bacteria, surveyed leaf and soil for reduced sensitivity to Ks, and evaluated the role of Ks application on the selection for Ks resistance linked to resistance of important antibiotics used in clinical medicine.

Material and Methods

Bacterial Isolations and Kasugamycin Resistance Testing:

In the summers of 2015 and 2016, leaf and soil samples from orchards of five different crops located across the Unites States were collected (Table 1). For each location, there were a total of three replications consisting of both trees treated with kasugamycin and trees not treated with kasugamycin, resulting in six samples for both leaf and soil. Samples were collected, placed in a zip lock bag, placed in a Styrofoam cooler with ice packs and shipped next day air to East Lansing, Michigan for sample processing. Samples were placed in a 4°C fridge once received. Leaf samples were processed the day they were received and soil samples were processed with in several weeks.

Individual leaf samples (consisting of 25 leaves each) for all crops, except walnut, were cut into 2.5cm strips using sterile scissors, placed back into the plastic bag and shaken to evenly mix the leaf strips. Two grams of each leaf sample was weighed out and placed into a sonication tube containing 20ml of chilled 0.5x potassium phosphate buffer saline (PBS) and sonicated for seven minutes in an ultra sonic bath (model 250T and model 97043-972, VWR Scientific, Houston, TX) (McGhee & Sundin, 2011). For walnut leaf samples, leaves were weighed out to approximately 2g, the weight was recorded, and the leaves were placed in a flask consisting of 20ml of chilled 0.5x PBS and placed in a shaking incubator for 30 minutes. Tubes and flasks were kept on ice when not being used. Samples were serial diluted and 0.1ml of appropriate dilutions were plated onto both King's B medium amended with 100μg/ml cycloheximide

(KBC) and KBC amended with 100μg/ml kasugamycin (KBC 100). Bacterial population size was calculated and was averaged for each site and each media type.

Two grams of each soil sample (consisting of 5 cores each) was weighed out after mixing by shaking the soil in a plastic bag. The soil was placed into a sonication tube containing chilled 0.5x PBS and sonicated for seven minutes in an ultra sonic bath. Tubes were kept on ice when not being used. Samples were serial diluted and 0.1ml of appropriate dilutions were spread plated onto KBC and KBC amended with 250μg/ml kasugamycin (KBC 250). Bacterial population size was calculated and was averaged for each site and media type.

Storing of Bacterial Isolates:

Following enumeration of bacterial colonies on plates, colonies were tooth-picked onto either KBC 100 and KBC 250 for leaf samples or KBC 250 and KBC amended with 500μg/ml kasugamycin (KBC 500) for soil samples. A sample of colonies was selected from KBC 250 (for both leaf and soil samples) for collection and storage. Prior to selection, bacterial colonies were screened using the gram-reaction test using 3% KOH to detect gram-negative colonies. From the pool of gram-negative colonies, fifteen colonies per site were randomly chosen for storage; however, we attempted to collect and store colonies with variation in colony morphology. A total of 1,137 and 605 gram-negative colonies were chosen for further analysis in 2015 and 2016, respectively, purified by mass streaking, and stored in 15% glycerol at -72°C (Table 1) (McGhee & Sundin, 2011).

Table 2.1. List of the number of sites per crop 2014, 2015 and 2016 sampling times.

Year	Crop	Number of Sites
2014	Apple	13
2015	Almond	5
	Cherry	3
	Olive	4
	Walnut	3
	Peach	10
2016	Cherry	4
	Walnut	4
	Apple	4

Antibiotic Screening:

Of these 1,137, and 605 isolates, 99 and 2 isolates did not grow when taken from the -80°C stock in 2015 and 2016, respectively. For 2014, 350 isolates out of 781 were used for further screening due to lack of growth from -80°C and contamination. The 1,038 and 603 isolates, in 2015 and 2016 respectively, that were viable along with the 350 from 2014 were further screened for antibiotic resistance using a standardized disc diffusion agar dilution protocol (R. N. Jones et al., 1989). BD BBL Sensi-disc antimicrobial susceptibility test discs (Becton-Dickinson, and Co., Sparks, MD) amended with the following antibiotics were used: gentamycin 10µg, cefotaxime 30µg, streptomycin 10µg, ampicillin 10µg, and tetracycline 5µg. Selected isolates were taken directly from the -70°C stock and placed into Luira broth (LB) and grown at 28°C overnight in a shaking incubator at 200rpm. 100µl of the turbid culture was spread plated onto Muller Hinton II agar (Becto-Dickinson) and allowed to dry for 30 minutes. Once the plates were dry, antibiotic discs were placed onto the medium surface using the BD BBL sensi-disc 6-place self-tamping dispenser (Becto-Dickinson, and Co., Sparks, MD). Prior to loading the antibiotic discs into the dispenser, the dispenser was sterilized following the

recommended procedure. This consisted of placing the dispenser in a 3% Lysol solution for 30 seconds without releasing the lever, followed in a similar fashion in 85% ethanol, and twice in diH_20. The antibiotic discs were placed in the dispenser in a specified order (Figure 2.1) with the random antibiotic placed in spot six as a place holder to prevent the spread of bacteria from one plate to another. Following incubation for 48 hours, the diameter of the zones of inhibition was measured to the nearest mm. The diameter of the disc, 6.5mm, was included in this measurement. The number of strains that were resistant, intermediate, and sensitive was recorded for each antibiotic based on the diameter of the inhibition zone (mm) listed by BD BBL (Becto-Dickinson). The number of isolates resistant to each antibiotic was compared between treated and non-treated sides using the Fisher's Test.

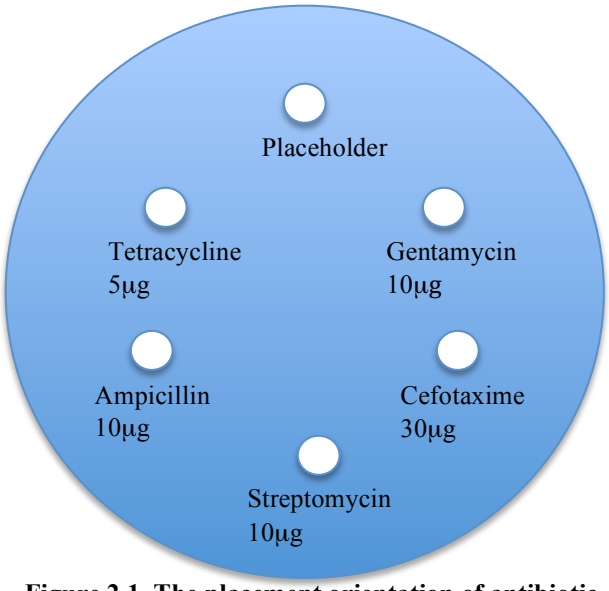


Figure 2.1. The placement orientation of antibiotic discs on petri plates spread plated with bacteria. The placeholder was one of the five antibiotic discs used and was used to fill the empty slot in the sensi-disc dispenser to prevent contamination of plates.

Results

Bacterial Isolations and Kasugamycin Resistance Testing:

Two grams of leaf and soil samples from each orchard was serial diluted and plated onto Kings B agar or Kings B amended with 100μg/ml or 250μg/ml of Kasugamycin (Ks), for leaf and soil samples, respectively. Population levels were calculated for each media type for all samples and sites (Table 2.2). There were higher populations levels from soil samples compared to leaf samples (Figure 2.1-2.3). In most cases there was either significantly lower populations insensitive to Kasugamycin when compared to total population levels or no significant difference between the two (Figure 2.2- 2.4). In a few cases there were higher populations insensitive to kasugamycin (Figure 2.4B; Figure 2.3G). There was either no difference between kasugamycin treated vs. non-treated sites or significantly lower populations from treated sites (Figure 2.2-2.4). However, several sites had higher insensitive kasugamycin population levels, such as NW sites (Figure 2.2A), SW sites (Figure 2.2B), CA-3 (Figure 2.3A), CA-1 and CA-4 (Figure 2.3F), CA-2 (Figure 2.3G), MI-1, NC-1, and NJ-1 (Figure 2.3I), and Va-1 and MI-1 (Figure 2.4A).

Table 2.2. List of the number of sites and isolates per crop from 2014, 2015, and 2016 sampling times.

Year	Crop	Number of Sites	Number of Soil Isolates	Number of Leaf Isolates	Total Number of Strains Stored per Crop	Total Number of Strains Stored per year	Number Evaluated in Antibiotic Screening
2014	Apple	13	437	344	781	781	350
2015	Almond	5	102	80	182	1,137	1,038
	Cherry	3	85	89	174		
	Olive	4	96	50	146		
	Walnut	3	90	79	169		
	Peach	10	242	224	466		
2016	Cherry	4	105	100	205	605	603
	Walnut	4	119	61	180		
	Apple	4	118	102	220		

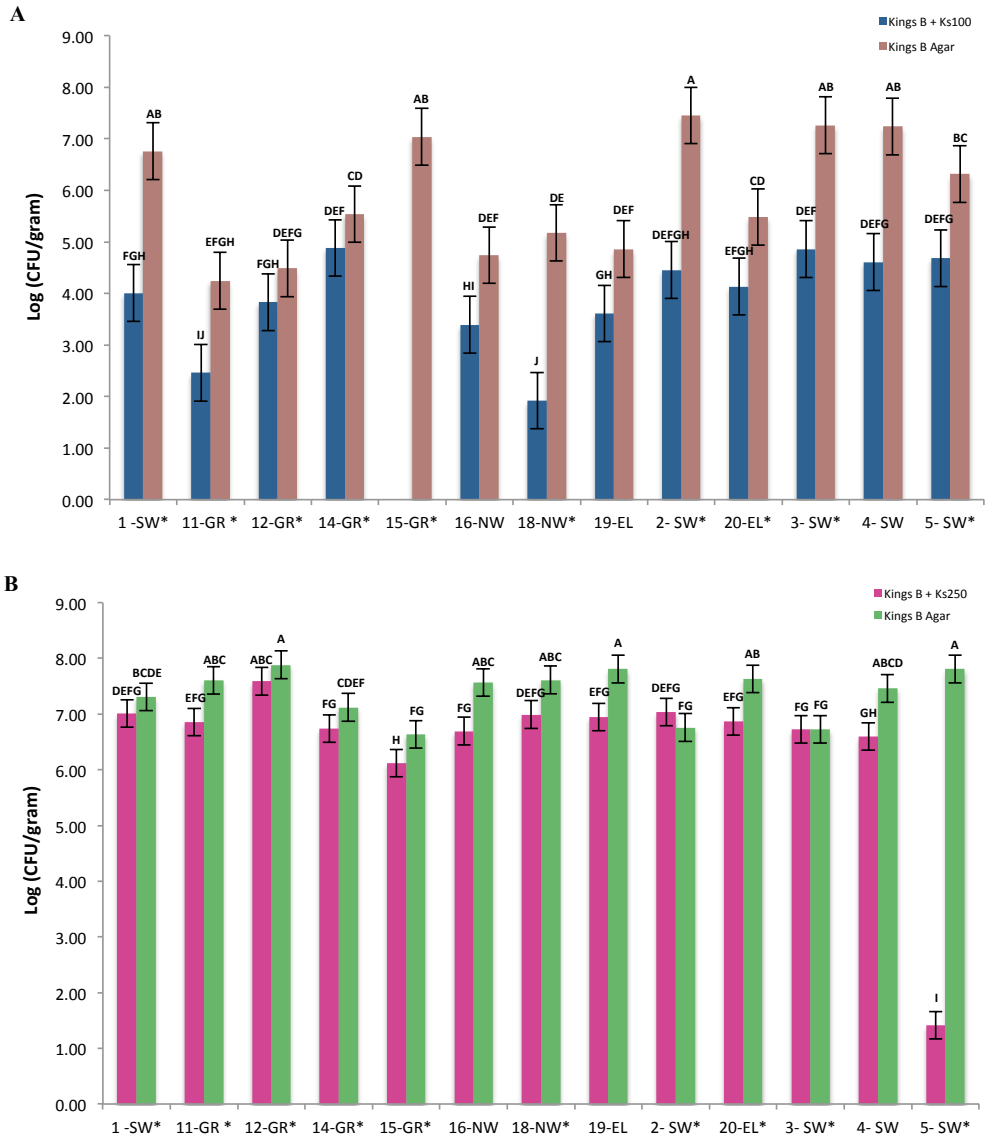


Figure 2.2. Populations recovered in 2014 on King's B Agar and King's B amended with either 100µg/ml or 250µg/ml Kasugamycin (Ks100 or Ks250) from Apple leaf (A) and Apple soil (B). Sites with (*) indicated sites that were treated with Kasugamycin.

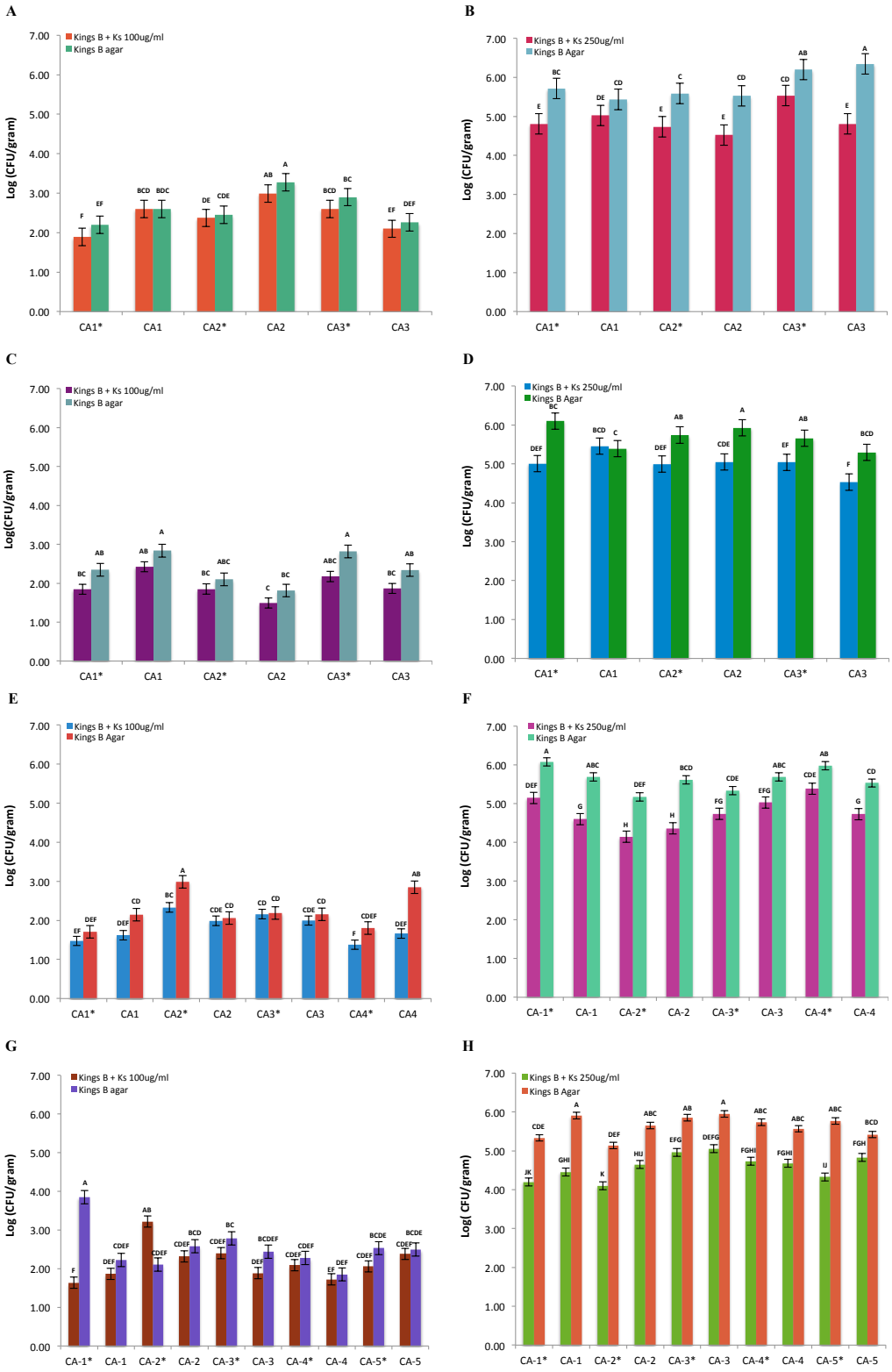
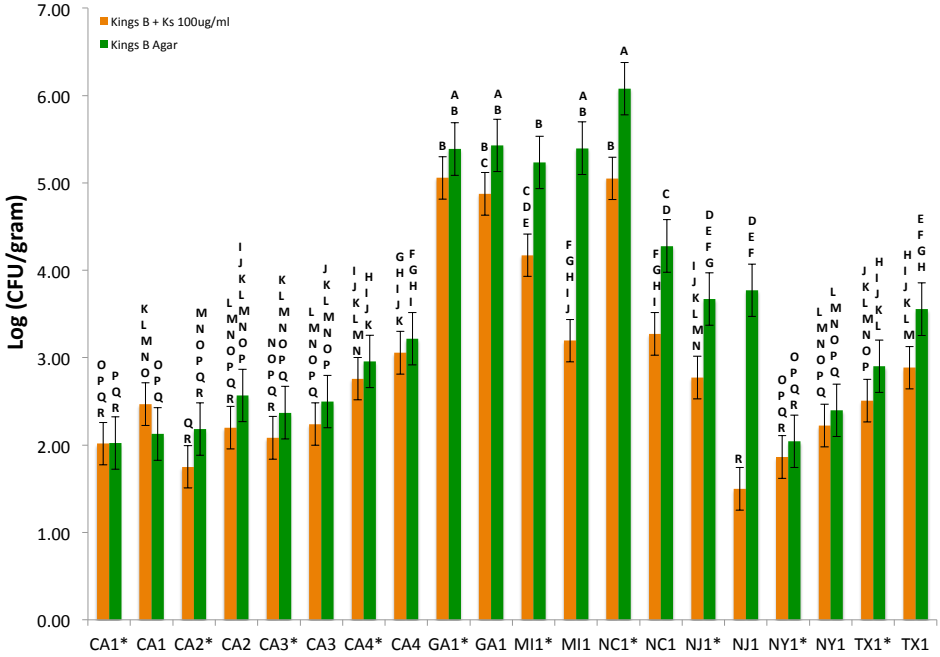


Figure 2.3 Populations recovered in 2015 on King's B Agar and King's B amended with either 100µg/ml or 250µg/ml Kasugamycin (Ks100 or Ks250) from Cherry leaf (A), Cherry soil (B), Walnut leaf (C), Walnut soil (D), Olive leaf (E), Olive soil (F), Almond leaf (G), Almond soil (H), Peach leaf (I) and Peach soil (J). Sites with (*) indicated sites that were

Figure 2.3. (cont'd)

I



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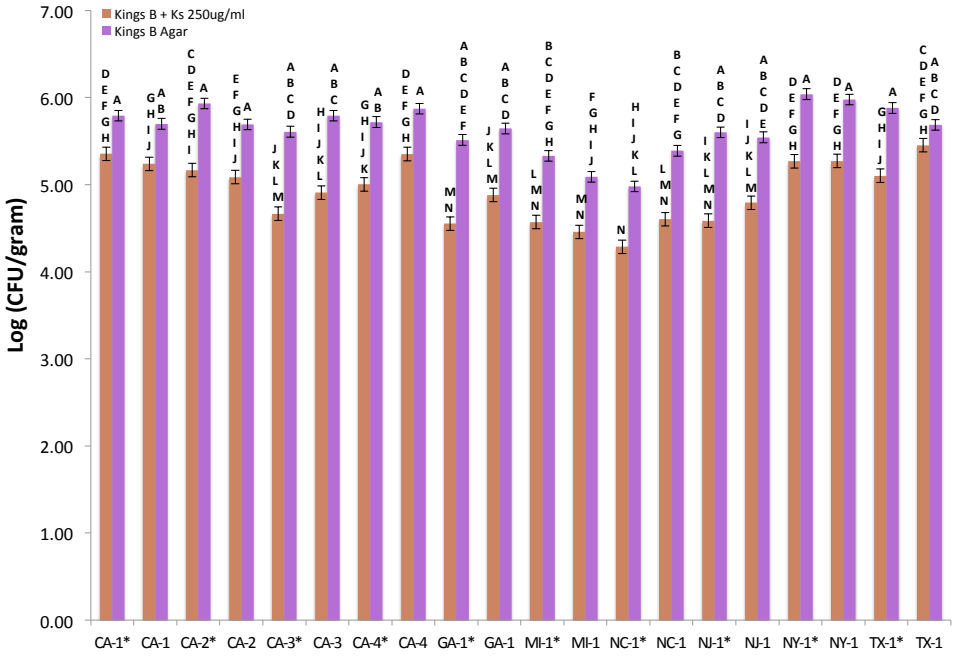


Antibiotic Screening:

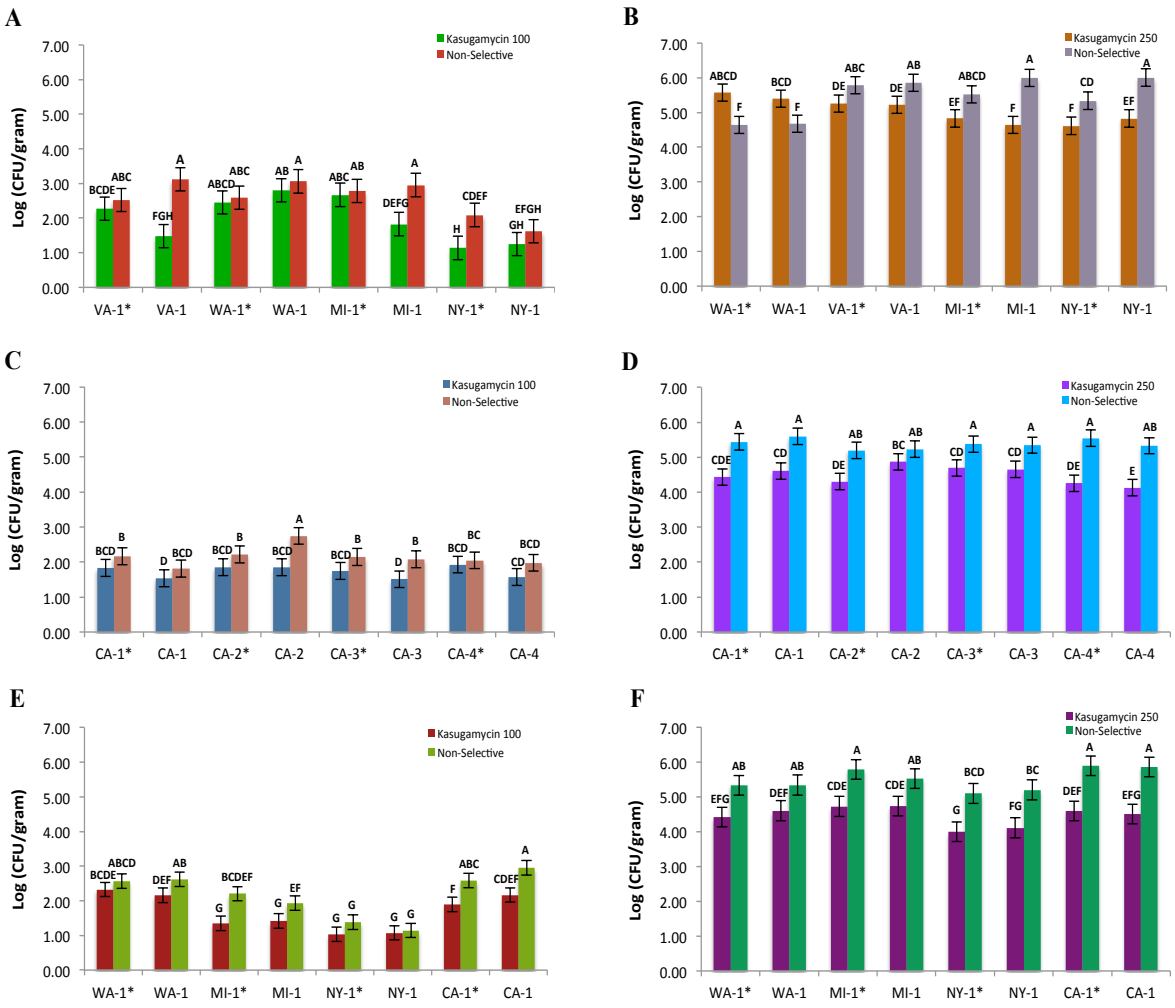


Figure 2.4 Populations recovered in 2016 on King's B Agar and King's B amended with either 100µg/ml or 250µg/ml Kasugamycin (Ks100 or Ks250) from Apple leaf (A), Apple soil (B), Walnut leaf (C), Walnut soil (D), Cherry leaf (E), and Cherry soil (F). Sites with (*) indicated sites that were treated with Kasugamycin.

A total of 350, 1,028, and 603 gram-negative colonies underwent antibiotic sensitivity screening in 2014, 2015, and 2016, respectively, (Table 2.2) to ampicillin, cefotaxime, gentamycin, streptomycin, and tetracycline. Isolates were recorded as sensitive, intermediate, or resistant to each antibiotic using the measured diameter of the inhibition zone and the ranges for each category as provided by BD BBL (Becto-Dickinson) (Figure 2.5). Overall, there was no significant difference between treated and non-treated sites for percentage of resistant isolates to

the antibiotics tested. Of the 18 population groups evaluated, six, eight, and ten showed significant differences in resistant, intermediate, and sensitive populations, respectively (Figure 2.6-2.8). In the cases in which significant differences were seen between treated and non-treated sites, the majority had either a higher percentage in the non-treated site or a mixture between treated and non-treated sites. In the resistant populations, there were three instances where a higher percentage was seen in treated sites (Figure 2.6B; Figure 2.7E, F) and three sites with a higher population in the untreated site (Figure 2.6A; Figure 2.7B, G). For Intermediate isolates, the majority of crops with significant differences had a mixture (Figure 2.6A and B; Figure 2.7B, I; Figure 2.8B, E) and only two had a higher percentage in non-treated sites (Figure 2.7E, F). There was one instance for the intermediate isolates in which there as a significant difference between treated and non-treated sites, with a higher percentage in the treated site (Figure 2.7E). The crops that showed significant differences in sensitive isolate percentages either had a higher amount in the non-treated site (Figure 2.6A; Figure 2.7E, F; Figure 2.8E, F) or a mixture (Figure 2.7B, D, J; Figure 2.8A). Overall, more differences were found in the number of sensitive isolates than resistant, with few sites showing higher numbers in the treated sites.

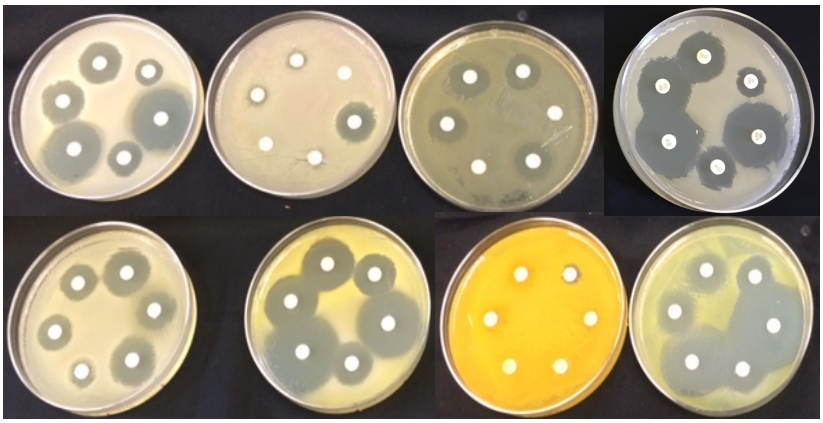


Figure 2.5 Zones of inhibition on Muller Hinton II Agar.

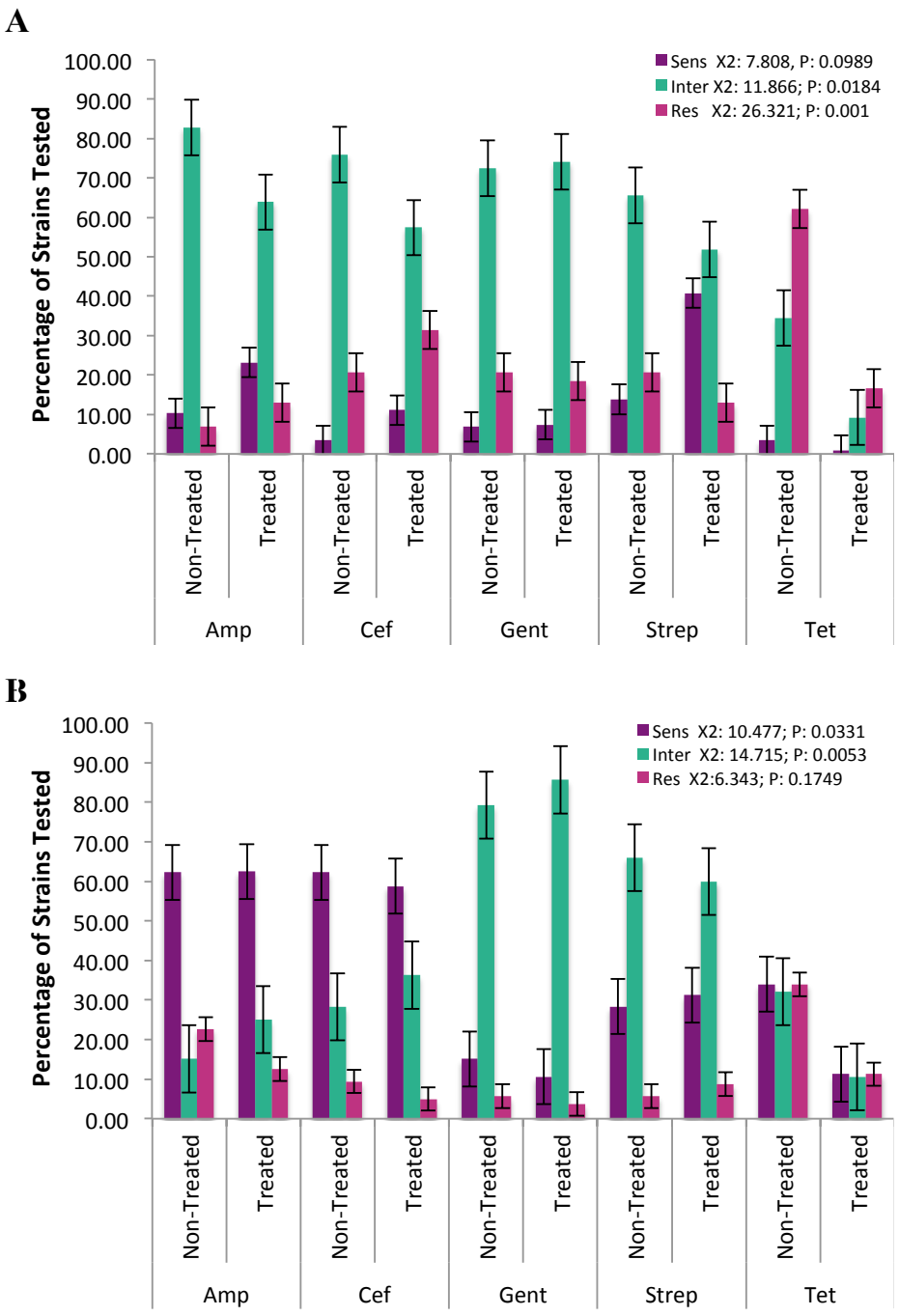


Figure 2.6 2014 Kasugamycin resistant bacterial isolates sensitivity to five antibiotics listed as critical by the CDC for isolates from Apple (A) leaves (B) soil. Purple bars represent sensitive isolate, Blue bars represent isolates with intermediate levels of resistance, and pink bars represent resistant isolates.

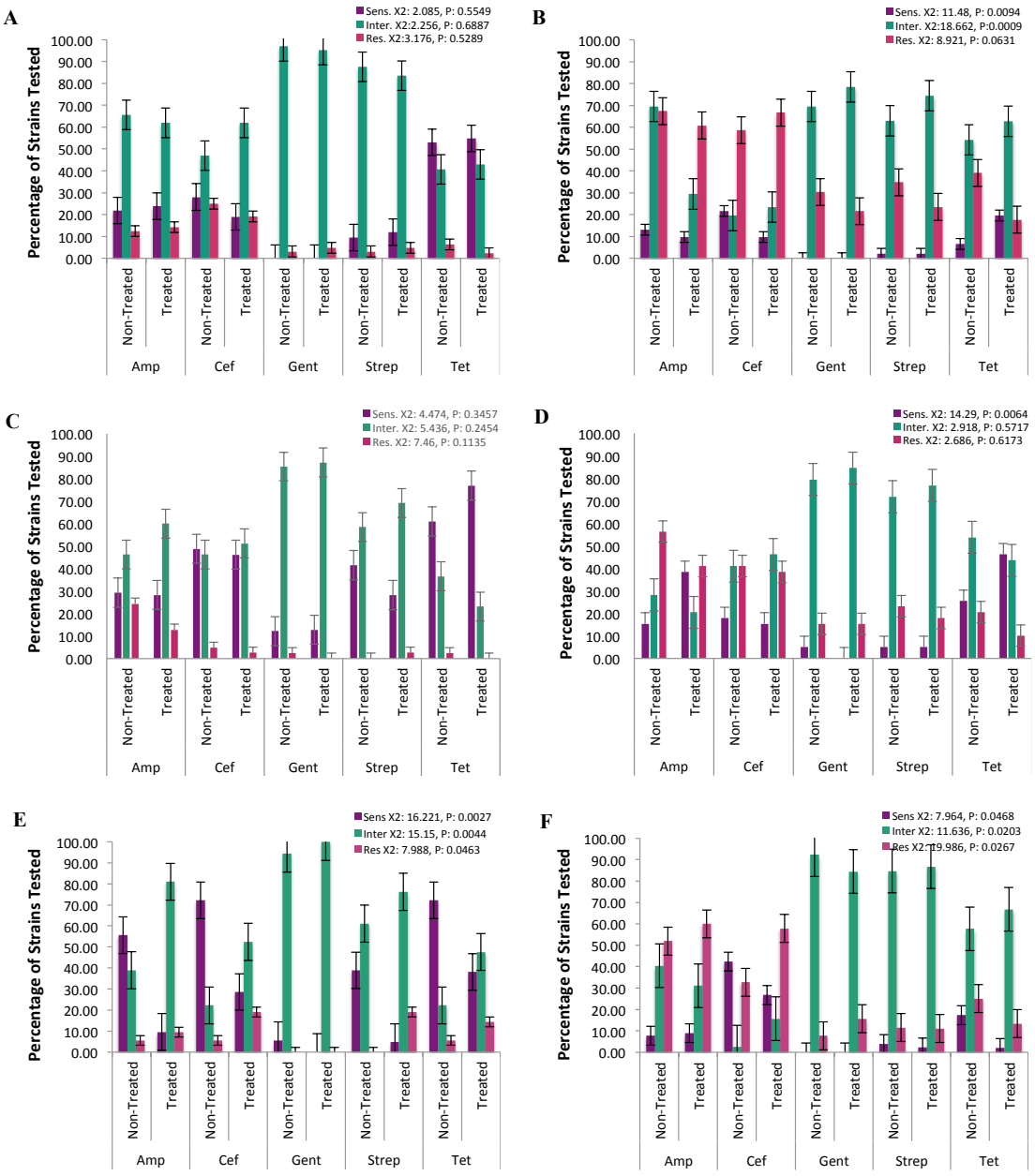
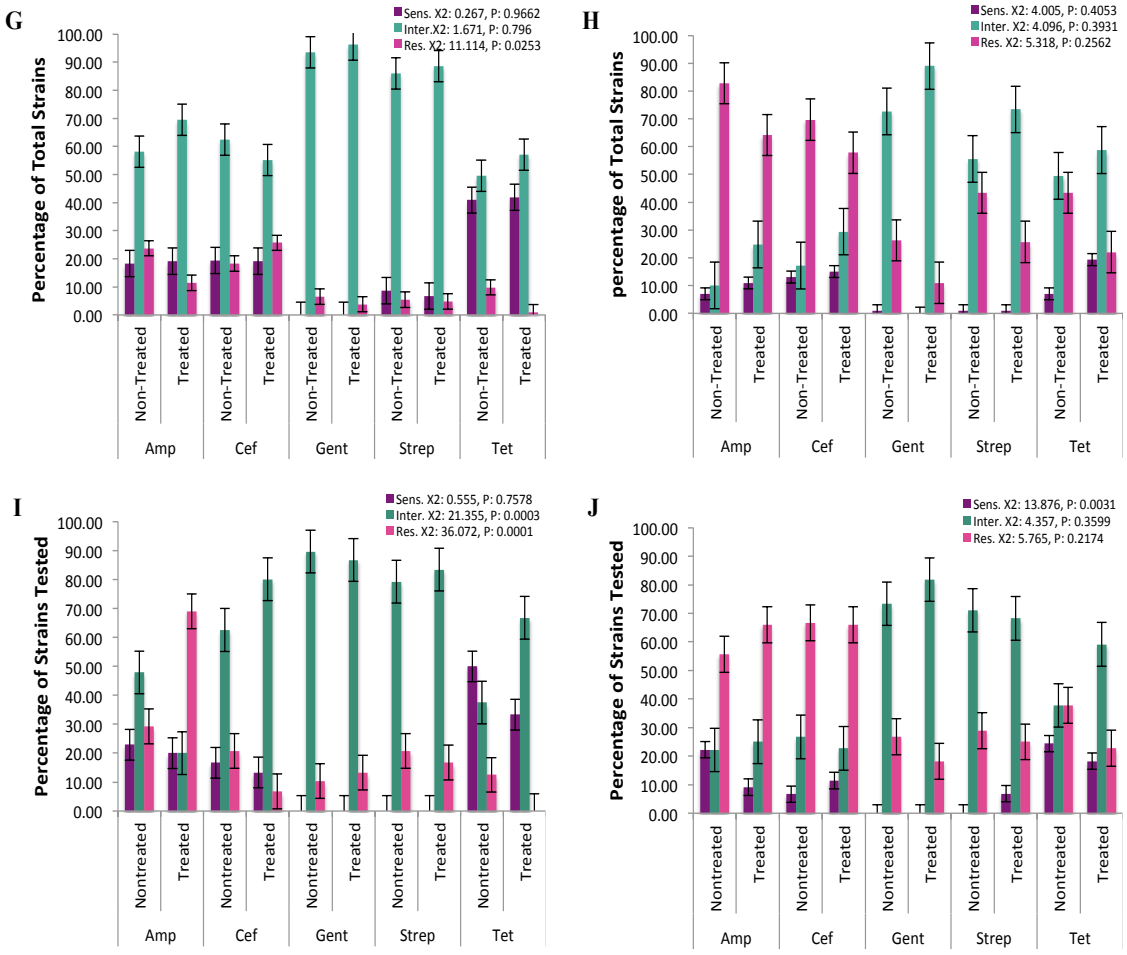


Figure 2.7 2015 Kasugamycin resistant bacterial isolates sensitivity to five antibiotics listed as critical by the CDC for (A) Almond leaves (B) Almond soil (C) Cherry leaves (D) Cherry soil (E) Olive leaves, (F) Olive soil, (G) Peach leaf, (H) Peach soil, (I) Walnut leaf, (J) Walnut soil. Purple bars represent sensitive isolate, Blue bars represent isolates with intermediate levels of resistance, and

Figure 2.7 (cont'd)



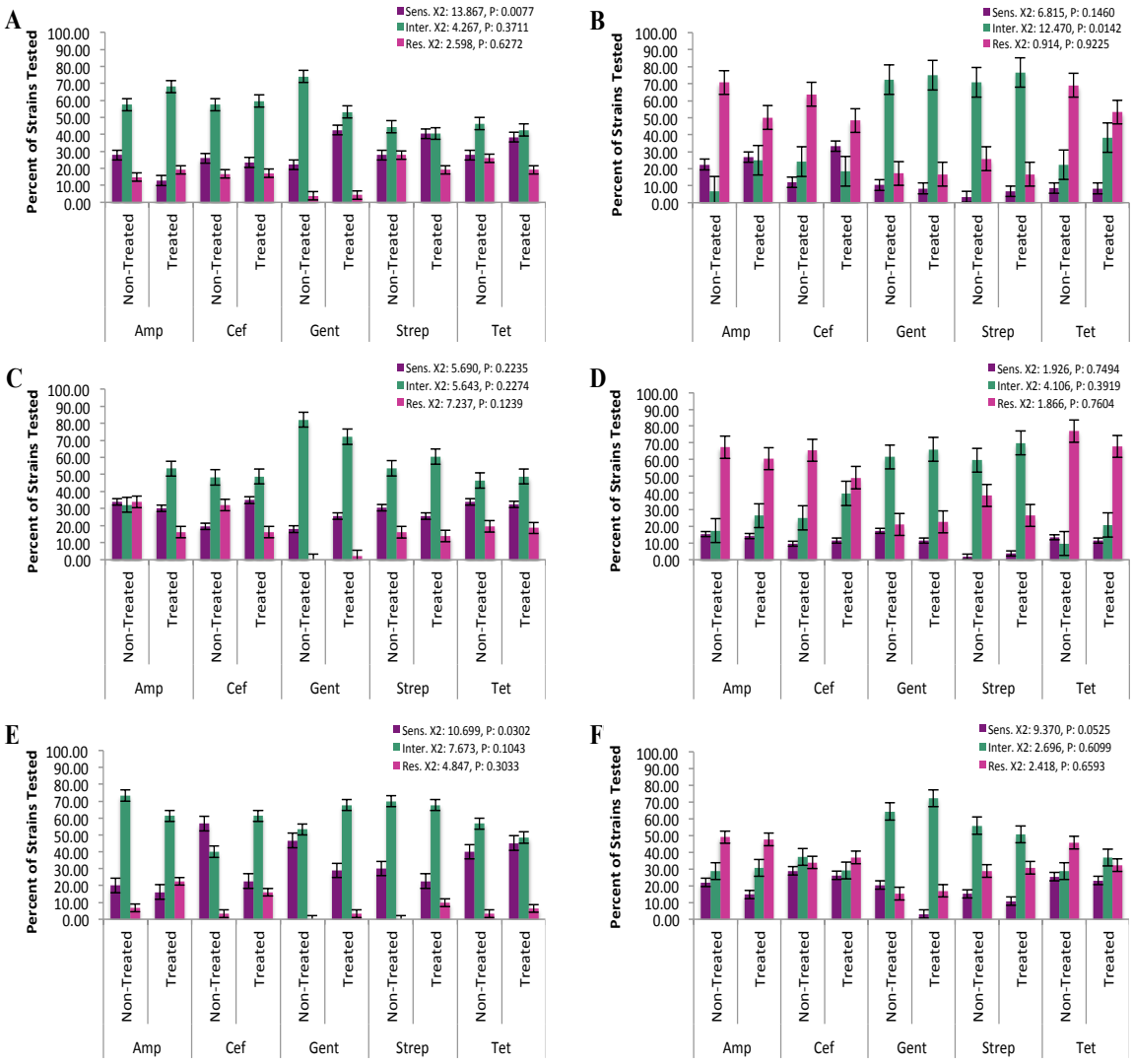


Figure 2.8 2016 Kasugamycin resistant bacteria isolates sensitivity to five antibiotics listed as critical by the CDC for (A) Apple leaves (B) Apple soil (C) Cherry leaves (D) Cherry soil (E) Walnut leaves, (F) Walnut soil. Purple bars represent sensitive isolates, Blue bars represent isolates with intermediate levels of resistance, and pink bars represent resistant isolates.

The distribution of cefotaxime sensitivity was evaluated for each crop and sample type and compared between sites treated and non-treated with Ks. The distribution of leaf isolates fell among the intermediate and sensitive range, whereas soil isolates fell more in the resistant and intermediate range (Figure 2.9-2.17), with the exception of Cherry leaf isolates from 2015 that

had a more even distribution across all three categories (Figure2.16 A, B). Walnut cefotaxime distributions were compared between 2015 and 2016, where all samples were taken from sites in California. For soil samples, distributions for non-treated sites did not change much between the two years; however, the treated sites became more evenly distributed with more isolates falling into the intermediate category (Figure 2.12C, D; Figure 2.17 C, D). In treated leaf samples there was a slight increase in the amount of resistant isolates (Figure 2.12 A; Figure 2.17A) whereas there was a slight decrease in the number of resistant isolates in non-treated leaf samples from 2015 to 2016 (Figure 2.12B; Figure 2.17B).

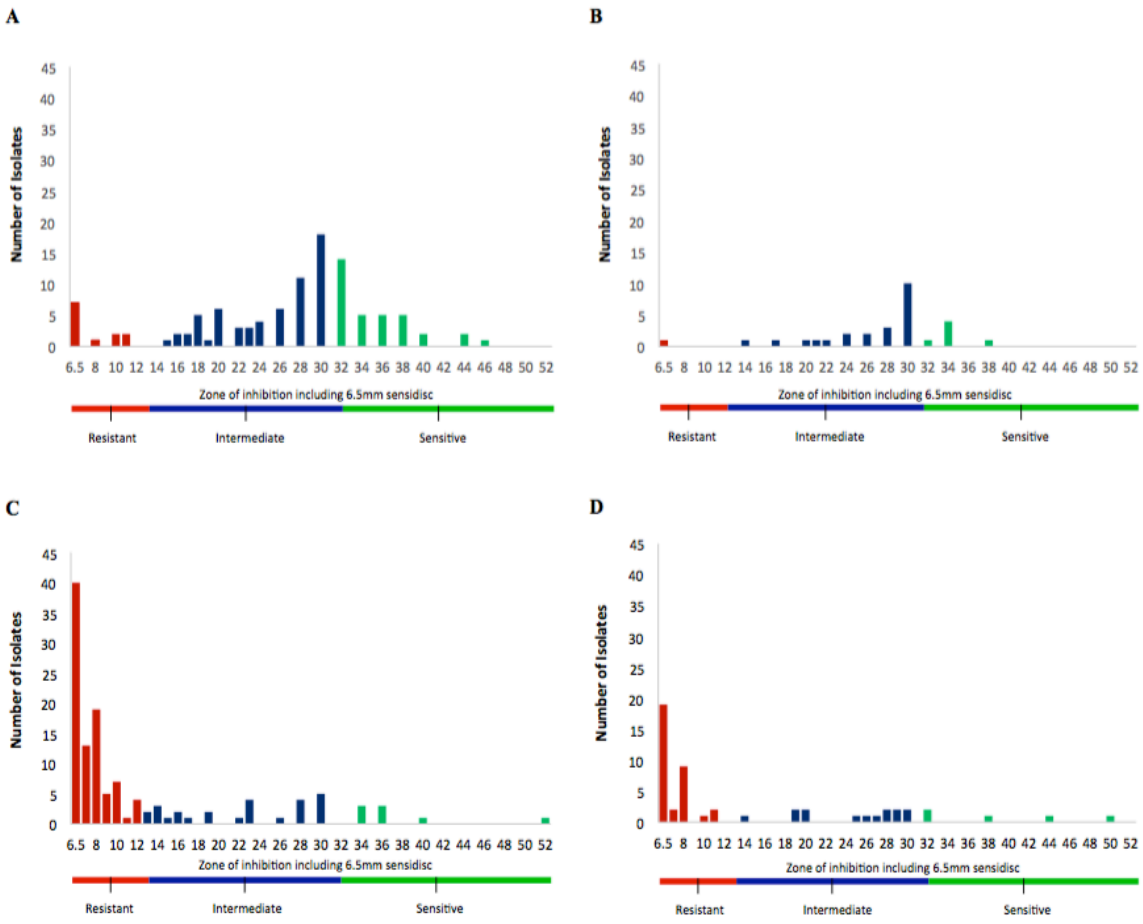


Figure 2.9 Histograms showing the number of 2014 apple bacterial isolates with specific zones of inhibition surrounding cefotaxime disks. Apple leaf isolates recovered in 2014 from trees treated with Kasumin (A), apple leaf isolates recovered from soil beneath untreated trees (B), soil isolates recovered bellow apple trees treated with Kasumin (C), and soil isolates recovered bellow apple trees untreated with Kasumin . All isolates were resistant to Kasugamycin > 100 ppm. Ranges of inhibition zones from isolates termed resistant, intermediate and sensitive are shown.

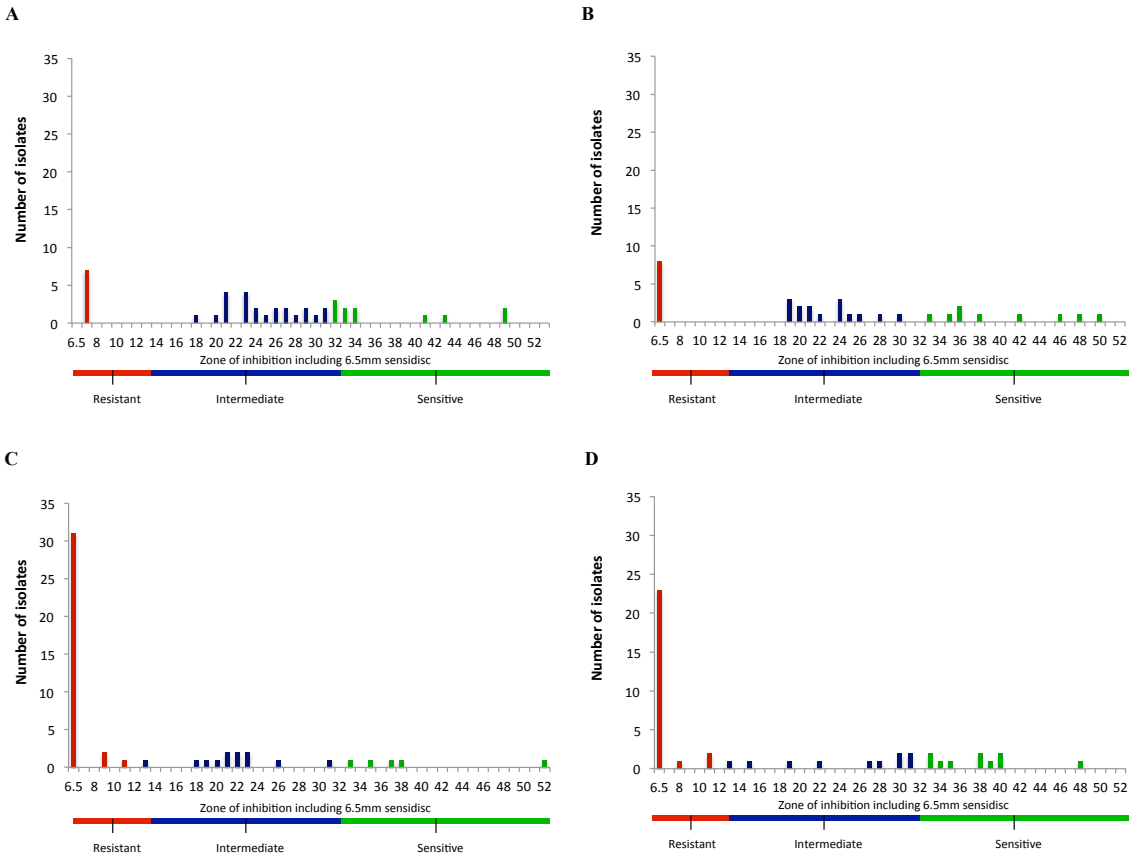


Figure 2.10 Histograms showing the number of 2015 almond bacterial isolates with specific zones of inhibition surrounding cefotaxime disks. Almond leaf isolates recovered in 2015 from trees treated with Kasumin (A), almond leaf isolates recovered from soil beneath untreated trees (B), soil isolates recovered bellow almond trees treated with Kasumin (C), and soil isolates recovered from almond trees untreated with Kasumin . All isolates were resistant to Kasugamycin > 100 ppm. Ranges of inhibition zones from isolates termed resistant, intermediate and sensitive are shown.

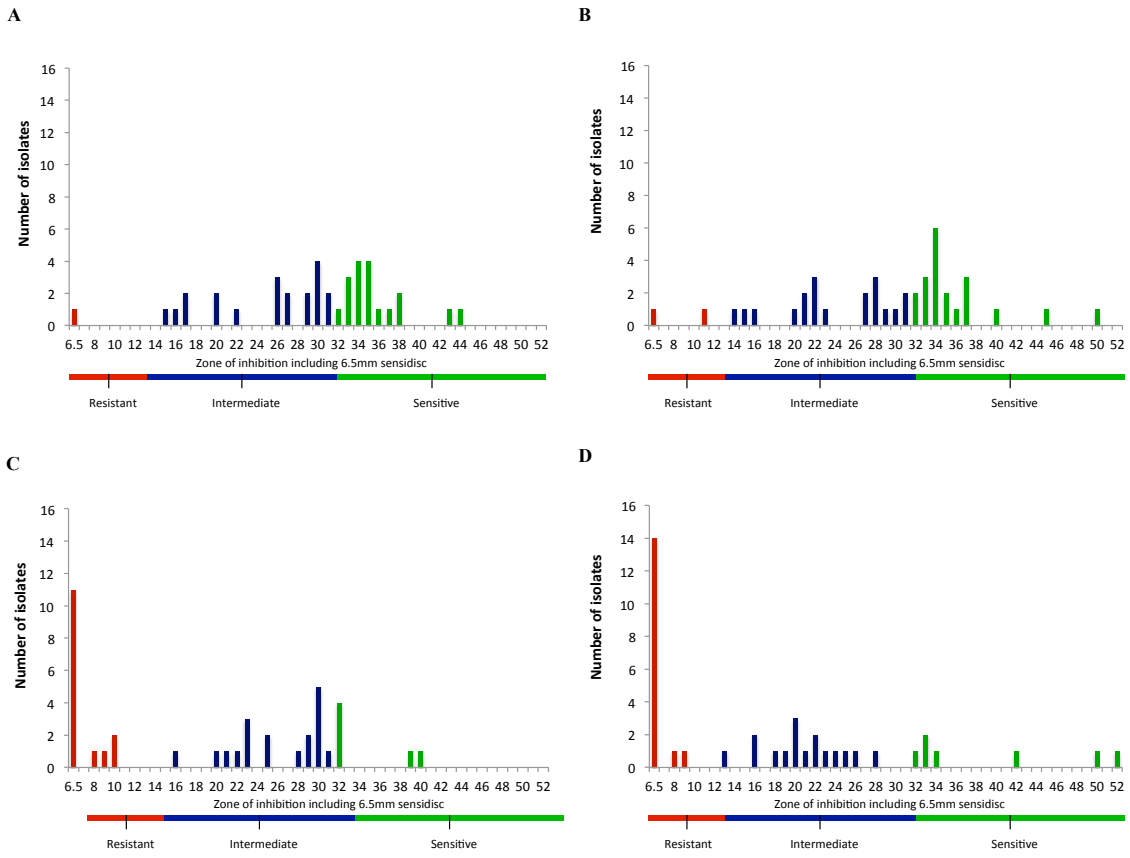


Figure 2.11 Histograms showing the number of 2015 cherry bacterial isolates with specific zones of inhibition surrounding cefotaxime disks. Cherry leaf isolates recovered in 2015 from trees treated with Kasumin (A), leaf isolates recovered from soil beneath untreated trees (B), soil isolates recovered bellow trees treated with Kasumin (C), and soil isolates recovered from trees untreated with Kasumin . All isolates were resistant to Kasugamycin > 100 ppm. Ranges of inhibition zones from isolates termed resistant, intermediate

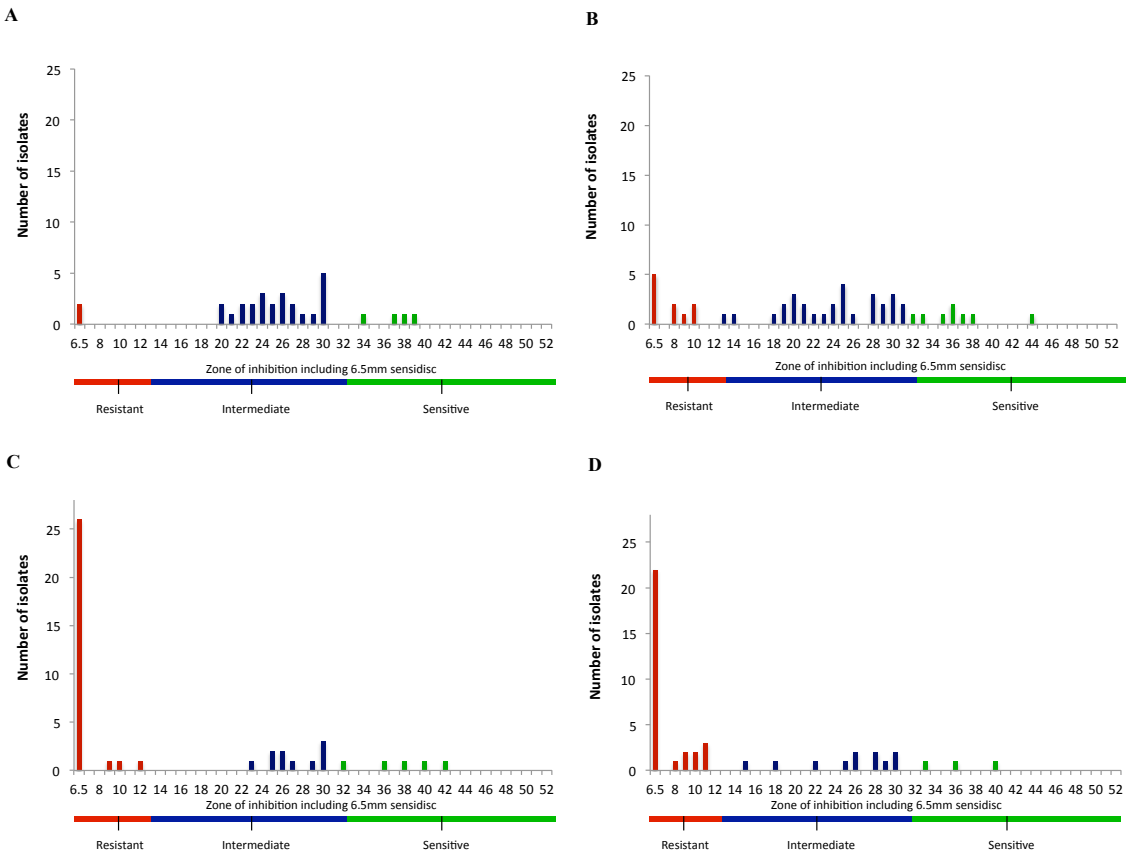


Figure 2.12 Histograms showing the number of 2015 walnut bacterial isolates with specific zones of inhibition surrounding cefotaxime disks. Walnut leaf isolates recovered in 2015 from trees treated with Kasumin (A), leaf isolates recovered from soil beneath untreated trees (B), soil isolates recovered under trees treated with Kasumin (C), and soil isolates recovered from trees untreated with Kasumin . All isolates were resistant to Kasugamycin > 100 ppm. Ranges of inhibition zones from isolates termed resistant, intermediate and sensitive are shown.

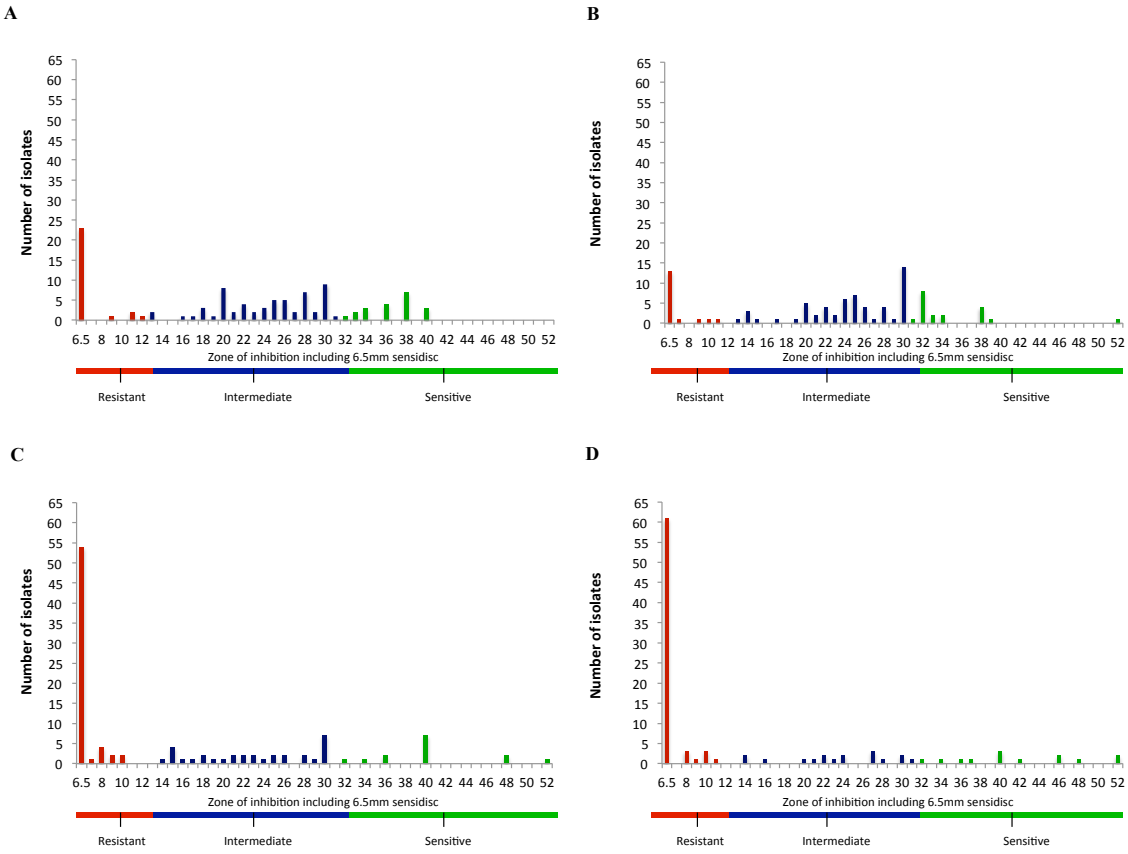


Figure 2.13 Histograms showing the number of 2015 peach bacterial isolates with specific zones of inhibition surrounding cefotaxime disks. Peach leaf isolates recovered in 2015 from trees treated with Kasumin (A), leaf isolates recovered from soil beneath untreated trees (B), soil isolates recovered below trees treated with Kasumin (C), and soil isolates recovered from trees untreated with Kasumin . All isolates were resistant to Kasugamycin > 100 ppm. Ranges of inhibition zones from isolates termed resistant, intermediate and sensitive are shown.

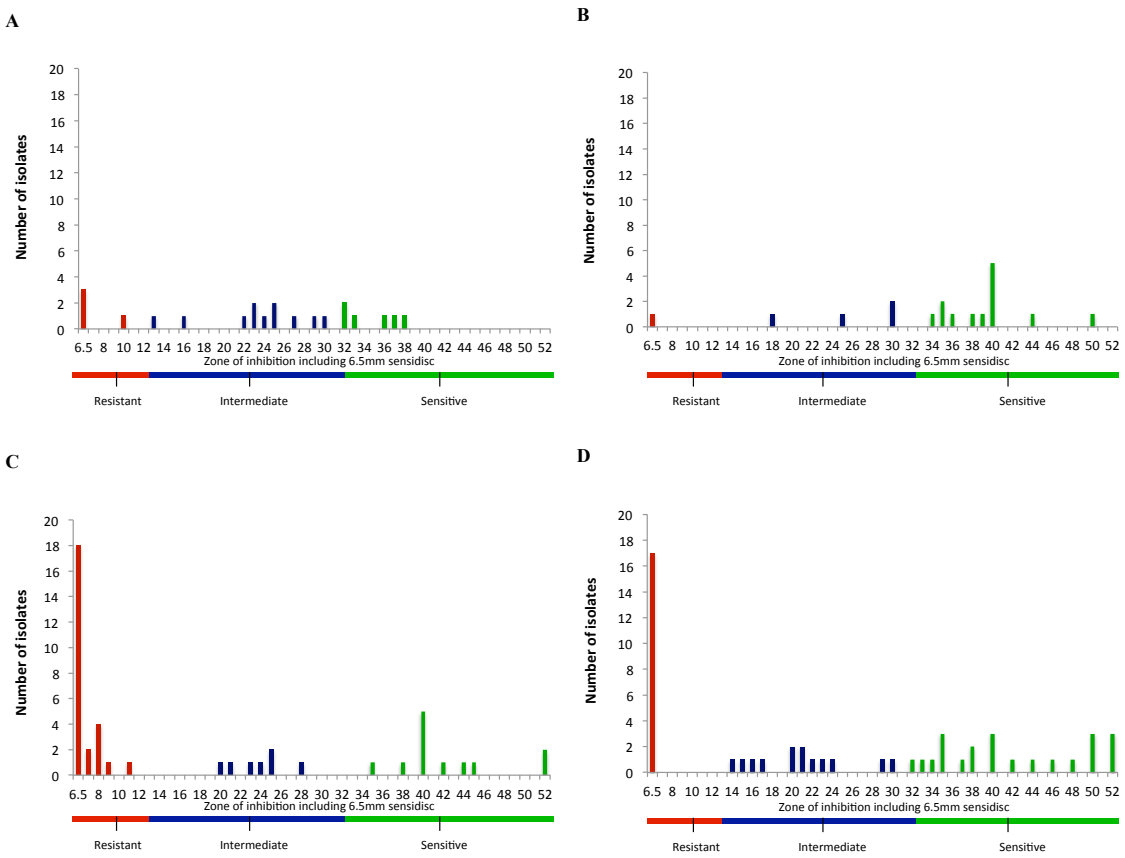


Figure 2.14 Histograms showing the number of 2015 olive bacterial isolates with specific zones of inhibition surrounding cefotaxime disks. Olive leaf isolates recovered in 2015 from trees treated with Kasumin (A), leaf isolates recovered from soil beneath untreated trees (B), soil isolates recovered bellow trees treated with Kasumin (C), and soil isolates recovered from trees untreated with Kasumin . All isolates were resistant to Kasugamycin > 100 ppm. Ranges of inhibition zones from isolates termed resistant, intermediate and sensitive are shown.

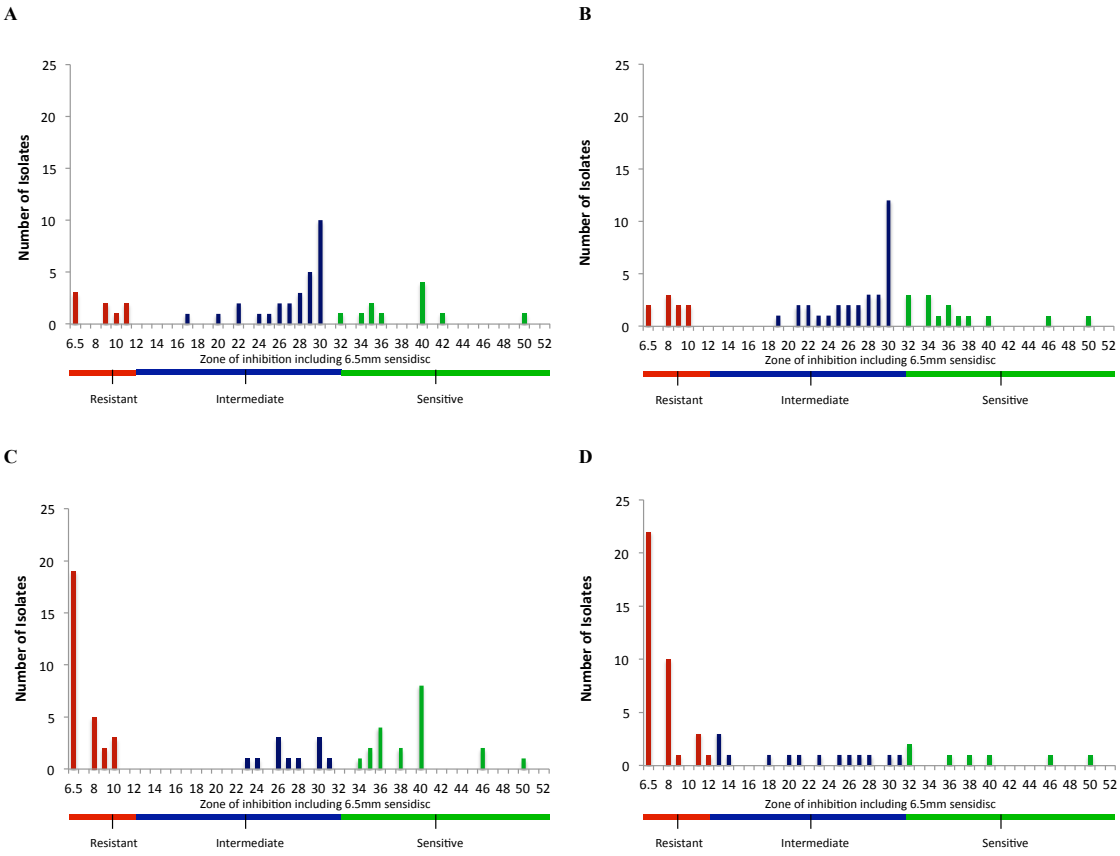


Figure 2.15 Histograms showing the number of 2016 apple bacterial isolates with specific zones of inhibition surrounding cefotaxime disks. Apple leaf isolates recovered in 2016 from trees treated with Kasumin (A), leaf isolates recovered from soil beneath untreated trees (B), soil isolates recovered bellow trees treated with Kasumin (C), and soil isolates recovered from trees untreated with Kasumin . All isolates were resistant to Kasugamycin > 100 ppm. Ranges of inhibition zones from isolates termed resistant, intermediate and sensitive are shown.

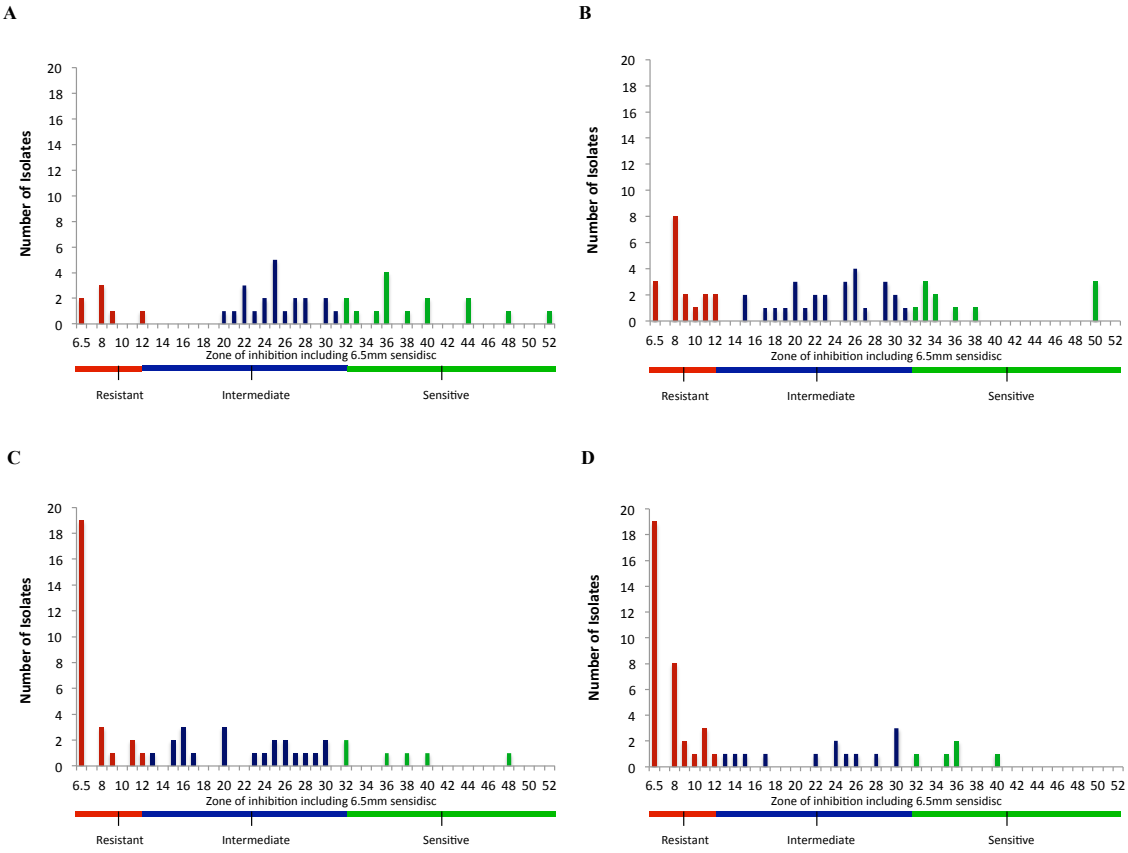


Figure 2.16 Histograms showing the number of 2016 cherry bacterial isolates with specific zones of inhibition surrounding cefotaxime disks. Cherry leaf isolates recovered in 2016 from trees treated with Kasumin (A), leaf isolates recovered from soil beneath untreated trees (B), soil isolates recovered bellow trees treated with Kasumin (C), and soil isolates recovered from trees untreated with Kasumin . All isolates were resistant to Kasugamycin > 100 ppm. Ranges of inhibition zones from isolates termed resistant, intermediate and sensitive are shown.

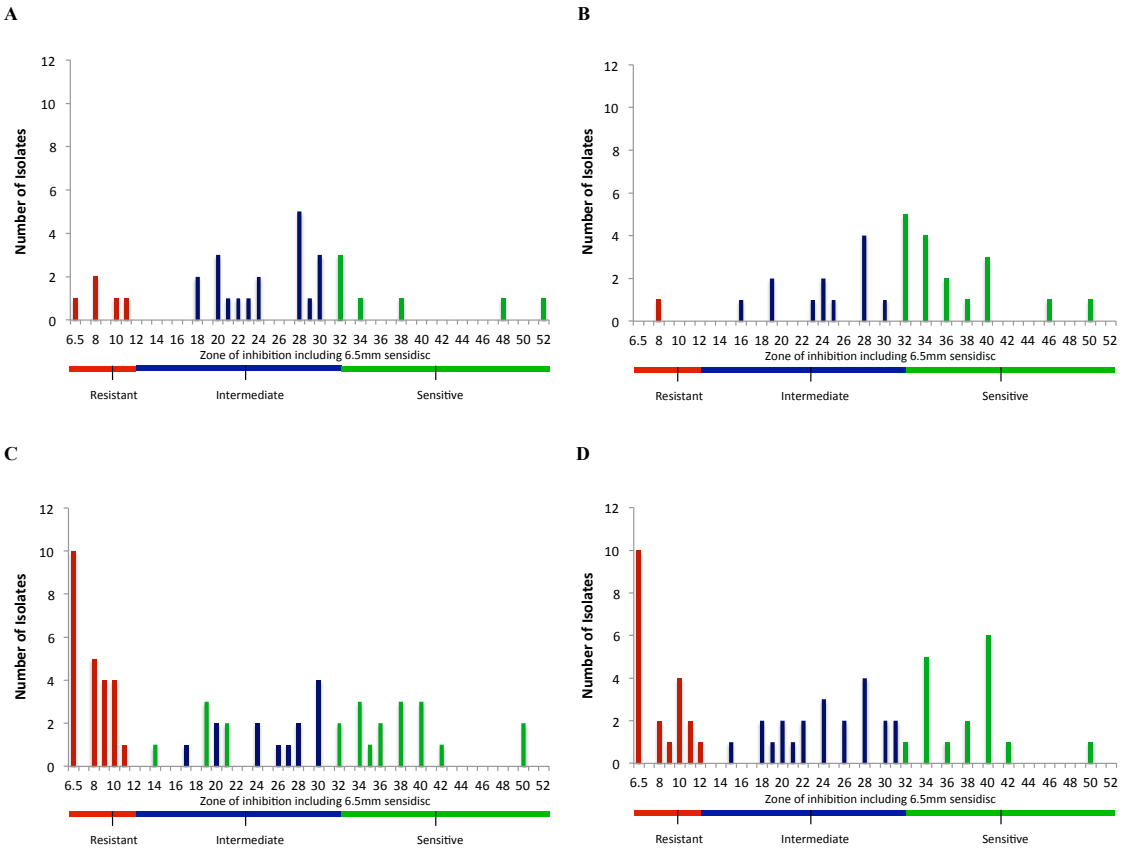


Figure 2.17 Histograms showing the number of 2016 walnut bacterial isolates with specific zones of inhibition surrounding cefotaxime disks. Walnut leaf isolates recovered in 2016 from trees treated with Kasumin (A), leaf isolates recovered from soil beneath untreated trees (B), soil isolates recovered below trees treated with Kasumin (C), and soil isolates recovered from trees untreated with Kasumin . All isolates were resistant to Kasugamycin > 100 ppm. Ranges of inhibition zones from isolates termed resistant, intermediate and sensitive are shown.

Discussion

Our field experiments indicate that Ks application in orchard settings does not select for linked antibiotic resistance genes to five antibiotics important in clinical medicine; streptomycin, tetracycline, gentamycin, ampicillin, and cefotaxime. These experiments did not indicate a linkage between Ks and either streptomycin or tetracycline, clinical antibiotics that are also used in specific cropping systems in plant agriculture. Its application also did not impact the distribution of cefotaxime resistance in either soil or leaf populations. In field studies conducted

by other researchers, the efficacy of Ks in controlling fire blight was equal to that of streptomycin and oxytetracycline (McGhee & Sundin, 2011). Taken together, these findings indicate that the application of Ks in orchard settings is beneficial for controlling fire blight and does not pose a risk to clinical medicine or plant agriculture.

The long-term utilization of Ks will depend heavily on the development of resistance genes on either plasmids or transposons in bacterial populations (de la Cruz & Davies, 2000). Bacteria can transfer resistance genes that are located on plasmids or transposons to other bacteria that share a similar niche, including bacteria in the same species as well as different species or different genera. The presence of transferable antibiotic resistance genes in both plant and human pathogens makes preventing or treating diseases extremely difficult and expensive. In plant agriculture this ultimately decreases the profit to the growers, increases the cost of food, and can decrease the overall supply of food (Patricia S. McManus et al., 2002). The effect of antibiotic resistance in clinical medicine is not merely a loss of money, but an increased probability of mortality to diseases that were once easily treated (Lipsitch et al., 2002).

Ks is a relatively new antibiotic that was register for the control of fire blight in New York in 2015 (Tancos & Cox, 2016) and in Michigan in 2014 as an emergency exception in places where streptomycin resistance is high (Kasumin 2L, Michican.gov). Historically, the introduction of antibiotics into both commercial agriculture and clinical medicine has resulted in the quick development of resistance. In plant agriculture, resistance to streptomycin was observed within ten years of its commercial use (Vidaver, 2002). In clinical medicine, antibiotic resistance was first seen in *Staphylococcus aureus* to penicillin within ten years of its use and resistance to other antibiotic was discovered in other clinical pathogens over the next twenty years (Dennesen et al., 1998). Therefore, it is of concern that Ks application will select for Ks

resistance in orchard bacterial populations and transfer into *Ea* populations or into human pathogens. Despite this concern, our findings from our 2014, 2015, and 2016 resistance screening indicated that the application of Ks in orchards did not have a significant impact on the level of Ks insensitivity in native bacterial leaf and soil populations.

Since Ks is not used in either human or animal medicine, the potential for the selection of Ks resistance with linkage to other antibiotic resistance genes poses an even greater concern to clinical medicine (Adaskaveg, Forset, & Wade, 2011). Multi-drug resistance significantly increases the difficulty in treated clinical infections and the prevalence of such isolates has been increasing. Currently, the most significant cases of multi-drug resistance are with MARSA (methicillin-aminoglycoside-resistant *S. aureus*) and foodborne strains of *E. coli* (Brumfitt & Hamilton-Miller, 1989b; Piras et al., 2012). Strains of MARSA were found to be resistant to combinations of methicillin, aminoglycosides (including gentamycin and streptomycin), tetracycline, and many others (Brumfitt & Hamilton-Miller, 1989b) and many foodborne strains of *E. coli* were resistant to vencomycin, penicillin, tetracycline, and neomycin (Dennesen et al., 1998; Piras et al., 2012). Variations in resistance to other antibiotics were found in *E. coli*, with the majority of the strains being resistant to more than five different antibiotics (Piras et al., 2012). These examples increase the concern that antibiotic use in plant agriculture will select for non-target bacteria with multi-drug resistance that could then transfer these multi-drug resistant complexes into human pathogens.

Our findings from 2014, 2015, and 2016 indicate that application of Ks in orchards does not select for multi-drug resistance. No evidence was found that linked Ks resistance to antibiotics important in clinical medicine. The distribution of cefotaxime resistance did not differ between sites treated and non-treated with Ks. Cefotaxime is a relatively new broad-spectrum

antibiotic in clinical medicine and is relied on for treating bacterial diseases, especially those with resistance to other antibiotics (Rao, Patrudu, Rao, Kumar, & Rao, 2016). It is important to note the Ks application in orchards did not select for resistance gene linkage with cefotaxime and does not pose an immediate risk to its use in clinical medicine.

In terms of plant agriculture, we did not find evidence for linkage of Ks with either streptomycin or tetracycline. This is an important finding as both of these antibiotics are used to control bacterial plant diseases. Streptomycin has been used regularly in apple and pear orchards and tetracycline, used in the form of oxytetracycline, has been regularly used in peach and pear orchards and occasionally in apple orchards where streptomycin resistance was high (P.S. McManus & Jones, 1994; Patricia S. McManus et al., 2002). These findings indicate that the use of these antibiotics in orchard disease management strategies is not likely to be affected by the efficacy of Ks.

There were higher population levels, both total populations and those insensitive to Ks, in soil compared to epiphytic populations for all crops and all sampling years. This corresponds to previous foundings in population levels in soil compared to leaves (Franke-Whittle, Manici, Insam, & Stres, 2015; McGhee & Sundin, 2011; G.W. Sundin & Bender, 1996; Yashiro & McManus, 2012). Populations on apple leaves consist mainly of Protobacteria (Alpha, Gamma, and Beta), Bacteroidetes, and Actinomyces. The most common phylogenetic groups observed on apple leaves are *Sphingomonas*, *Pseudomonas*, *Pantoea*, and *Methylobacterium* (Yashiro & McManus, 2012). The most common phylogenetic groups observed in orchard soil are Proteobacteria, Actinomyces, and Acidobacteria. The most common phyla in soil were Phenylobacterium, Lysobacter, and Sphingomonas (Franke-Whittle et al., 2015). In terms of gram-negative bacteria, there were a higher number on flowers and leaves combined compared

to soil (McGhee & Sundin, 2011; G.W. Sundin & Bender, 1996). These gram-negative bacteria included *Dickeya dadantii*, *Enterobacter* sp., *Erwinia amylvora*, *Pantoea* sp., and *Pseudomonas* sp. (McGhee & Sundin, 2011). These common phyla and species found in these studies include bacteria that are known to cause diseases in plants as well as humans. For those species that are not known to be pathogenic, including some species of *Enterobacter* and *Pseudomonas*, are closely related to human and plant pathogens and therefore pose a great chance of transferring antibiotic resistance genes.

Since it is common to find gram-negative bacteria, specifically those in Bacteroidetes and Proteobacteria, which include *E. coli* and *Pseudomonas* species, on both leaves and soil in orchards, it is important to discuss the persistence of antibiotics in these environments. The persistence of antibiotics in the environment are impacted by weather (rainfall, sunlight, temperature), limited rate of absorption by the plant, and water solubility (Acimovic et al., 2015). In soils, the persistence also depends on ability to bind to soil, soil type, soil pH, and amount of UV light exposure (Kumar, Gupta, Chander, & Singh, 2005). On plants, antibiotics remain effective for less than five days as exposure to light can degrade them and rainfall reduces the concentration on plant surfaces (Rao et al., 2016; V.O. Stockwell, 2014). It is important to note that antibiotics used in plant agriculture are suspended in water and applied as a spray to the plant and excess liquid, containing the antibiotic, likely runs off into the soil (Acimovic et al., 2015). This runoff during antibiotic application along with rain splashing allows for accumulation of antibiotics in orchard soil. However, particles in the soil are capable of absorbing antibiotics, such as tetracycline, and degrading them over time. This can reduce the amount of active antibiotics in the soil, rather than continuously building up (Kumar et al., 2005;

V.O. Stockwell, 2014). These findings together indicate that, from a medical standpoint, antibiotic resistance in soil is of a greater importance and concern.

It is necessary to stress the fact that the Ks sensitive strains isolated in our studies are not necessarily resistant to Ks as it is unknown whether they contain a transferable resistance gene. However, the possibility of the existence of such a gene is clear in orchard settings in which either Ks is regularly applied or in soil where bacteria are exposed to naturally produced Ks, resulting in a selection pressure for resistance. Another possibility is the presence of bacteria intrinsically resistant to Ks (McGhee & Sundin, 2011). This intrinsic resistance may be due to functional or structural characteristics such as the permeability of the cell membrane and the presence of a heightened efflux system. Both of these mechanisms work to keep the antibiotic outside of the bacteria cell, where it is unable to interact with its target and affect the bacterium (Blair, Webber, Baylay, Ogbolu, & Piddock, 2015; McGhee & Sundin, 2011). Bacteria that have this type of resistance will not have an effect on the development of antibiotic resistance in the environment, as no transferable resistance genes are present. If the Ks insensitive bacteria isolated in our studies are primarily intrinsically resistance, then the threat to clinical medicine is significantly reduced, as these strains will not have an impact on medically important bacteria.

In summary, we have determined that Ks application in orchards does not appear to select for Ks insensitivity or for multi-drug resistance in Ks insensitive strains. This indicates that Ks application in orchards does not pose a sever risk to clinical medicine. We also determined higher bacterial population levels in soil compared to leaves, both for total population levels and Ks insensitive populations. Although Ks application in orchards does not currently select for Ks or multi-drug resistance, the potential for Ks resistance development in orchard populations remains a concern for the future. It will be necessary to continue to monitor orchard bacterial

populations for increased Ks insensitivity, multi-drug resistance, and development of Ks resistance genes in order to be better equipped at managing bacterial disease of humans as well as plants.

Chapter 3: Role of Levan in Ooze Production of *Erwinia amylovora*

Abstract

Erwinia amylovora, the causal agent of fire blight of apple, is disseminated in ooze droplets, which consists of bacterial cells embedded in at least two exopolysaccharides, levan and amylovoran. Levan is a fructose polymer that is synthesized by the enzyme levansucrase, encoded by the *lsc* gene, in the presence of sucrose. Levan production results in prominent domed colonies when grown on medium containing 5% sucrose. We hypothesized that levan production in vivo results in an increase in pressure inside the plant leading to damage of host tissue, ultimately resulting in the emergence of ooze droplets. To study this, an *E. amylovora* 1189Δ*lsc* mutant was generated and was evaluated, along with a wild type strain and other strains previously shown to have a reduction in levan production, for levan production *in vitro* and ooze production in shoots. The *E. amylovora* 1189Δ*lsc* strain was reduced in levan production on both agar plates and in broth containing sucrose and showed a reduction in ooze during shoot infection. In growth chamber studies, strains with a reduction in levan showed a reduction in ooze droplet numbers. For field studies, we screened Michigan strains of *E. amylovora* that were reduced in levan production compared to the virulent strain Ea110. These strains did not show a significant reduction in ooze droplet size, numbers, or population levels in field trials, although many other factors besides levan production could account for these differences.

Introduction

Fire blight, caused by *Erwinia amylovora*, is a devastating disease of Rosaceae plants and can result in losses as high as 100 million dollars (Norelli et al., 2003). The pathogen overwinters in holdover cankers formed by the previous year's infection. In the spring, the pathogen

multiplies at the margins of these cankers and emerges in ooze droplets (M. Hilderbrand, Dicler, & Geider, 2000; Schroth et al., 1974). Blossoms are the location for initial infections. Once the pathogen reaches the blossoms it rapidly multiplies on the stigmas, migrates down to the nectaries and then systemically through the host (Schroth et al., 1974). Secondary infections occur by the spread of bacteria from ooze on shoots and blossom petioles to growing shoot tips, where they enter through wounds or natural openings (S.V. Beer, 1979). The ability of *E. amylovora* to cause infection depends on temperature and moisture, with an increased infection rate at temperatures above 18°C (65°F) and humidity above 70 percent (Eve Billing, 1980).

Erwinia amylovora produces two exopolysaccharides (EPS), amylovoran and levan, which both contribute to biofilm formation and virulence (Robert A. Bennett & Billing, 1980; Koczan et al., 2009). Amylovoran is known to play a vital role in biofilm formation and virulence as a deletion in the *ams* operon, which includes the genes responsible for the synthesis of amylovoran, results in no biofilm formation and renders the bacteria avirulent (Koczan et al., 2009). Levan is a neutral EPS that is synthesized by the enzyme levansucrase in the presence of sucrose. In *E. amylovora,* the enzyme is encoded by the *lsc* gene and its expression is not dependent on sucrose in the environment (Geier & Geider, 1993). Levan plays a similar role as amylovoran; however, it is less efficient as strains with a deletion in *lsc* showed a reduction in biofilm formation as well as virulence. These strains also showed a delay in symptom development in both shoot and immature pear (Geier & Geider, 1993; Koczan et al., 2009).

Li *et al.* found that strains with a deletion in *envZ, ompR,* and *envZ*/*ompR* had a reduction in levan production (Li et al., 2014). The EnvZ/OmpR system is known to regulate various components of bacterial cellular function, including EPS production and biofilm formation (Katarzyna Brzostek, Karolina Skorek, & Adrianna Raczkowska, 2012; Pickard et al., 1994). In

E. amylovora, the EnvZ/OmpR system negatively regulates amylovoran synthesis, plays a role in motility regulation, and negatively regulates *hrp*-type three secretion system (T3SS) gene expression. The GrrS/GrrA system has been shown to positively regulate the synthesis of levan in *E. amylovora* (Li et al., 2014).

The primary role of levan may be in the dispersal of the pathogen. *E. amylovora* is dispersed within and among orchards through the spread of ooze droplets, a matrix of bacterial cells and EPS, both levan and amylovoran (Schroth et al., 1974). High numbers of *E. amylovora* and ooze were found in the intercellular space of infected host tissue (Zamski et al., 2006). Slack *et. al* observed bacterial cells emerging from wounded tissue under SEM. When the ooze was removed, they observed wounds and erumpent mounds underneath ooze droplets with no natural openings, indicating the ooze emerged as a result of internal pressure (Slack et al., 2017). Bacterial biomass was found to increase or decrease with change in water potential (H.J. Schouten, 1989b). In the intercellular space, the bacterial biomass can swell as water potential changes, such as after a rain event (H.J. Schouten, 1988). The expanding mass of bacterial cells and EPS may result in a change in pressure leading to the tearing of the surrounding host tissue and the emergence of ooze droplets (Henk J. Schouten, 1991).

On agar plates containing sucrose, *E. amylovora* colonies have a dome shaped appearance. This domed appearance is a result of levan secreted by the bacterial cells and has low viscosity (Du & Geider, 2002). Therefore, levan may be the primary EPS responsible for the increased pressure in the intercellular space and the emergence of ooze droplets. In this study we evaluated the effect of levan on ooze production using *E. amylovora* strains with a reduction in levan production, both natural and mutational, and its role in other virulence characteristics.

Materials and Methods

Bacterial strains, plasmids, and growth conditions:

The bacterial strains and plasmids used in this study are listed in Table 3.1. All bacterial strains were grown in Luria Broth (LB) medium at 28°C, unless otherwise noted. Growth medium was supplemented with the antibiotics ampicillin (Amp) (50μg/ml), kanamycin (Km) (30μg/ml), chloramphenicol (Cm) (20μg/ml), Gentamycin (Gm) (15μg/ml), or rifampicin (Rif) (100μg/ml) as necessary.

Table 3.1 Bacterial strains and plasmids used in this study and their relevant characteristics.

Strain or Plasmid	Relevant Characteristic(s)	Source or reference
Strain		
Ea1189	Wild Type	GSPB; M. Ullrich
Ea110	Rif^R	Datsenko and Wanner 2000; McGhee and Sundin 2012
Ea(T3)2		McGhee and Sundin 2012
EaGH9		McManus and Jones 1995
EaVH7-9		This Study
EaK2		McManus and Jones 1995
EaGH9 R3	Rif	This Study
Ea(T3)2 R5	Rif	This Study
EaK2 R3	Rif	This Study
EaVH7-9 R3	Rif	This Study
Ea1189ΔenvZ	Km^R-insertional mutant of *envZ* of Ea1189; Km^R	Li et al. 2014
Ea1189ΔompR	Km^R-insertional mutant of *ompR* of Ea1189; Km^R	Li et al. 2014
Ea1189Δams	Deletion mutant of the *ams* operon; Cm^RAmp^R	Zhao et al. 2009
Ea1189Δlsc	Cm^R-insertional mutant of *lsc* of Ea1189; CM^RAmp^R	This Study; Koczan et al. 2009
Plasmids		
pKD46	Expresses recombinases red, β, λ, and exo for construction of deletion mutants; Amp^r	Zhao et al. 2009
pSJG1	pBBR1MCS-5 backbone; *lsc* gene inserted as Kpn1-SacI fragment; Gm^r	This Study

Insertion and deletion mutagenesis and complementation:

A deletion mutant of the levansucrase gene, *lsc,* of *E. amylovora* was generated using the λ phage recombinases as described for *Escherichia coli* (Datsenko & Wanner, 2000). Briefly, we transformed *E. amylovora* strain Ea1189 with plasmid pKD46 encoding recombinases *red β, Y, exo*. The transformed Ea1189 (pKD46) was grown overnight in LB broth at 28°C, transferred into fresh LB broth medium containing 0.01% arabinose, and grown to exponential phase. The cells were then made electrocompetent and stored at -80°C. Recombination fragments consisting of a Cm resistance gene (Cm^R) with its own promoter, flanked by 50-nucleotide homology arms of the *lsc* gene, were generated by polymerase chain reaction (PCR) using the plasmid pKD3 as a template. The primers Lsc-Deletion F and Lsc-Deletion R were used for construction of an *lsc* deletion mutant (Table 3.2). Another primer pair, Cm1 and Cm2, of the Cm^R resistance gene was used to confirm the mutant by PCR. The PCR products were purified using a PCR purification kit (Qiagen, Valencia, CA). Following electroporation, transformants were spread onto LB medium amended with Amp and Cm. In the resulting mutant, the *lsc* gene was replaced with the Cm^R. The Ea1189Δl*sc* mutant was complemented with plasmid PSJG1, a clone containing the *lsc* gene from Ea1189 without its native promoter ligated into plasmid pBBR1MCS-5 (Table 3.1).

Table 3.2 Primers used in this study

Primer designation	Sequence
Lsc-Mutation ID F	5'-TGCTGCCCCCTTATCAGTAGCTTCTCAAACCTGTTA-3'
Lsc-Mutation ID R	5'-GGAACTTCCCCGCGCCACCATGATTTTATA-3'
Lsc-Deletion F	5'ATGTCAGATTATAATTATAAACCAACGCTGTGGACTCGTGCCGATGCAT TGTGTAGGCTGGAGCTGCTTC-3'
Lsc-Deletion R	5'TTATTTTAAAGTAATGACTTTCATTGCCGGAATATATCCATAATCGAAG CCATATGAATATCCTCCTTA-3'

Levan Plate Assay:

Strains were grown in LB broth overnight and cultures were adjusted to an OD_{600nm} of 0.1 using a Safire micro-plate reader (Tecan, Research Triangle Park, NC), serial diluted, and spread plated onto LB +5% sucrose and amended with antibiotics as needed. Plates were grown at 28°C for 72 hours. Single colonies were evaluated for dome and colony size (Zhang & Geider, 1999).

Levan Liquid Assay:

Strains were grown in LB broth +5% sucrose at 28°C with shaking for 48 hours. The culture turbidity was measured using an OD_{600nm}. The culture was centrifuged at 6000rpm for ten minutes and the supernatant was mixed with an equal volume of LS buffer (100mM sodium phosphate pH7.0, 2M sucrose, and 0.05% NaN_3) and incubated for 24 hours at 28°C with shaking. Levan was quantified using a turbidity measurement at OD_{410nm} and was standardized using the turbidity at OD_{600nm} of the culture in LB broth + 5% sucrose (S. Bereswill, Jock, Aldridge, Janse, & Geider, 1997; Caputi et al., 2013; Roach, Sjaarda, Castle, & Sviroev, 2013; Zhang & Geider, 1999).

Amylovoran Assay:

Strains were grown overnight in LB broth with necessary antibiotics. The cultures were centrifuged, washed with 0.5x PBS three times, the pellet was re-suspended in 200µl of 0.5x PBS, and 100µl of the bacterial suspension was inoculated into MBMA medium (3g KH_2PO_4, 7g K_2HPO_4, 1g $(NH_4)_2SO_4$, 2ml glycerol, 0.5g citric acid and 0.3g $MgSO_4$ per liter) with 1% sorbitol. The culture was grown for 48 hours at 28°C with shaking. The concentration of the culture was checked using an OD_{600nm}. After centrifuging 1ml of the culture, 40µl of 50mg/ml cetylpyridinium chloride (CPC; Sigma, Carlsbad, CA, USA) was added to 0.8ml of the culture

supernatant and incubated for ten minutes at room temperature. The amylovoran concentration was determined by measuring the turbidity at OD_{595nm} and was standardized using the turbidity at OD_{600nm} of the culture in MBMA medium (Geier & Geider, 1993; Roach et al., 2013).

Biofilm Assay

Bacteria were grown overnight in LB medium and 20µl of the culture was inoculated into 2ml of 0.5x LB broth in a well of a 24 well plate containing a glass cover slip. The plates were incubated at 28°C for 48 hours after which the glass cover slips were placed in a new 24 well plate and 10% crystal violet was added to the rim of the well and allowed to stain for one hour. The crystal violet was removed from the well followed by rinsing the glass cover slip in diH_2O. After air-drying, the crystal violet was solubilized using 200µl of a solution of 40% methanol and 10% glacial acetic acid. The biofilm was quantified by measuring the turbidity at OD_{600nm} (Koczan et al., 2009).

Immature Pear Assay:

Immature pears (*Pyrus communis L. Cv. Bartlett*) were sterilized using 10% bleach and placed in a sterile laminar flow hood to dry. One ml of an overnight culture was centrifuged and washed with 0.5x PBS three times, after which the solution was adjusted to an OD_{600nm} of 0.1 and diluted 100 fold. Each immature pear was inoculated by pricking the center of the pear with a sterile needle and placing 2µl of either the inoculum or 0.5x PBS on top of the wound. A total of 10 pears were inoculated per strain. The pears were incubated at 28°C in covered bins on top of moist paper towels. The lesion diameter was measured 2, 4, and 6 days post inoculation (Koczan et al., 2009).

Field Shoot Assay:

Blossom Inoculations

The stigmas of Fuji apple blossoms were inoculated with 2µl of a $1x10^8$ CFU/ml bacterial suspension (McGhee et al., 2011). A total of three blossoms per clusters were inoculated, with a total of 50 clusters per strain. The non-inoculated blossoms were removed from the cluster. Ooze droplets were counted and recorded each morning until petal fall (~2 weeks). A total of ten ooze drops per strain was collected in 0.5ml Eppendorf tubes for measuring the volume of the ooze droplet and population counts per ooze droplet. The ooze droplet was suspended in 100µl of 0.5x PBS and the volume of the ooze droplet was determined by taking the difference in the initial and final volumes. To determine the population level within the ooze droplet, serial dilutions of the remainder of the ooze droplet in 0.5x PBS was made and drop plated onto LB medium with rifampicin and grown at 28°C for three days. Disease severity was evaluated 1-2 weeks post inoculation by recording the number of infected blossoms per inoculated shoot cluster.

Shoot Inoculations

Kit John apple shoots were inoculated by cutting perpendicularly to the mid-vein approximately 2cm from the tip of the leaf using sterile scissors dipped into a bacterial suspension of $1x10^8$ CFU/ml (Koczan et al., 2009). A total of 50 shoots were inoculated per strain. The experiment was repeated a total of 6 times during the 2015 field season. Ooze droplets were counted and recorded each morning for 3-4 weeks post inoculation. A total of ten ooze droplets per strain per experiment were collected in 0.5ml Eppendorf tubes for measuring the volume of the ooze droplet and population counts per ooze droplet as previously described. Disease severity ratings were taken 4-6 weeks post inoculation by taking a percentage of the shoot infected.

Growth Chamber Assay:

The youngest leaf on a growing shoot of a two-year-old potted apple tree (cv Gala on M9 rootstock) was cut perpendicularly to the mid-vein approximately 2cm from the tip of the leaf using sterile scissors dipped into a bacterial suspension of $1x10^8$ CFU/ml (Koczan et al., 2009). Eleven, five, and five shoots were inoculated per strain in experiments one, two, and three respectively. Shoots were bagged overnight beginning 36 hours post inoculation to increase humidity and encourage ooze production. Ooze droplets were counted and recorded each morning after removing bags from the shoots for 2 weeks (experiments 1 and 2) and one week (experiment 3). Ooze droplets were collected and the volume and populations were measured per ooze droplet as previously described. Disease severity ratings were taken 7 (for experiments 1, 2, and 3), 14 (for experiments 1 and 2), and 21 (for experiment 2) days post inoculation.

Results

Insertion and deletion mutagenesis and complementation:

Ea1189Δ*lsc* strain was reduced in levan production on both agar plates (Figure 3.1) and in broth (Figure 3.2) containing 5% sucrose. The deletion of the levansucrase gene was confirmed by sequencing.

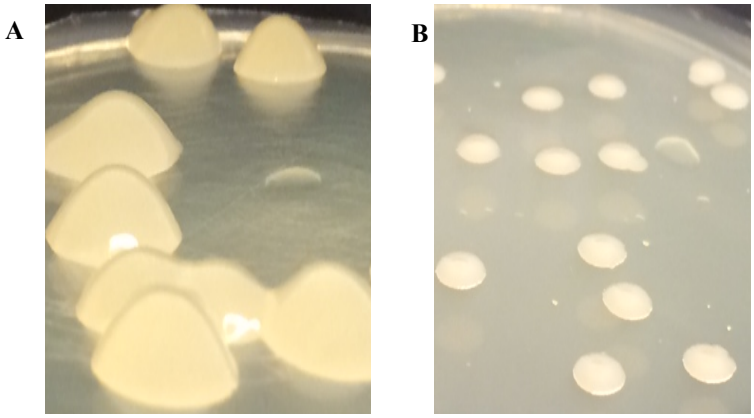


Figure 3.1. Confirrmation of the deletion of the *lsc* gene in Ea1189 on agar plates. Visualization of levan production by Ea1189 (A) and Ea1189Δ*lsc* (B) after growth for four days on LB agar containing 5% sucrose.

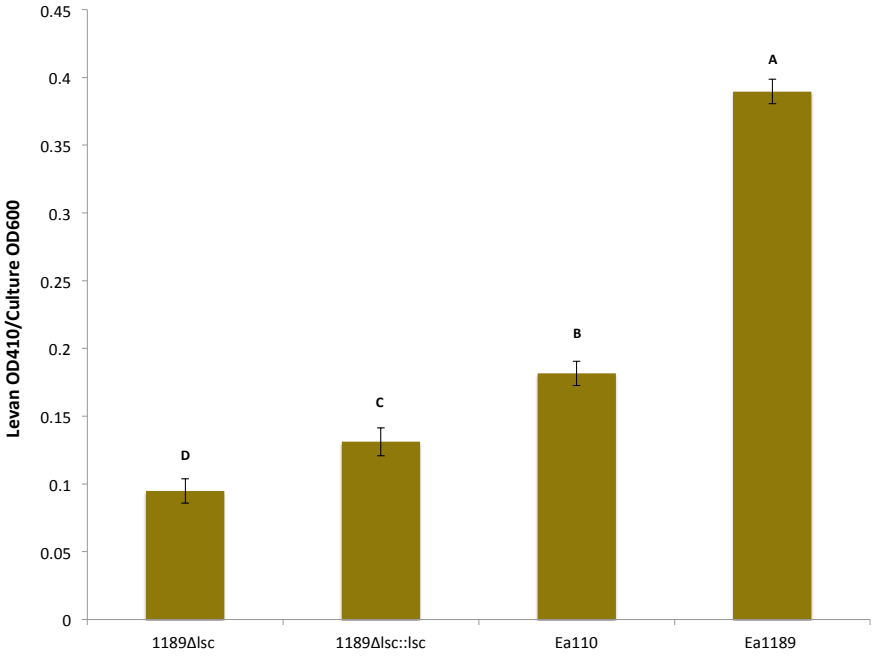


Figure 3.2. Quantification of levan production by Ea1189, Ea110, 1189Δlsc, and 1189Δlsc::lsc in LB medium containing 5% sucrose after 48 hours of growth. Sample means were compared by an analysis of variance and separated using the student's t test. The presence of different letters above sample mean value indicates that the means were significantly different at $P<0.005$.

Michigan *E. amylovora* Isolates with Variation in Levan Production

Four Michigan isolates of *E. amylovora* were identified as having variation in levan production when compared to Ea110. Isolates EaGH9 and EaK2 were significantly reduced in levan production compared to both Ea110 and Ea1189. Isolates EaT3(2) and EaVH7-9 were also reduced in levan production compared to Ea110, but had higher levan production than Ea1189, EaGH9, and EaK2. All four isolates had significantly higher levels of levan production compared to Ea1189Δlsc (Figure 3.3).

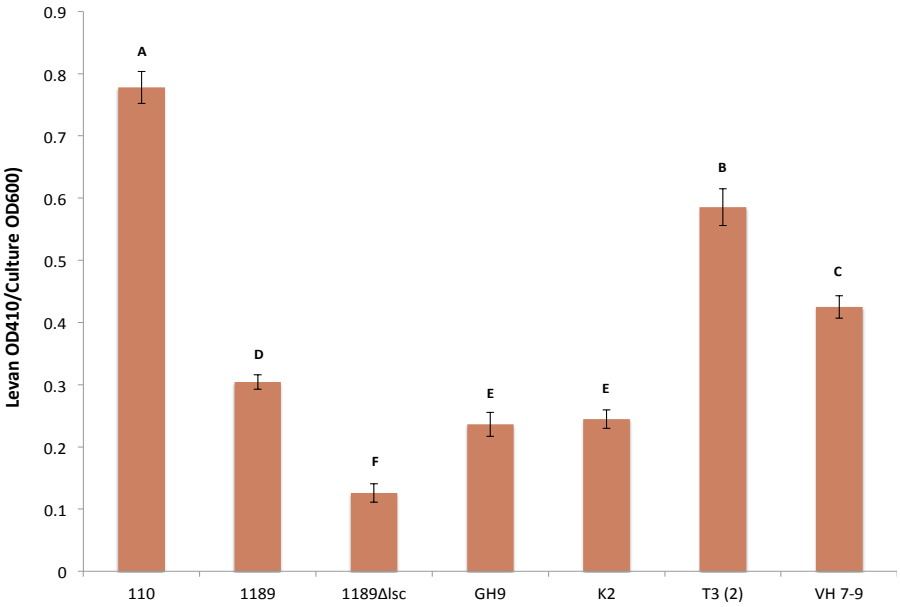


Figure 3.3. Quantification of levan production by strains Ea1189, Ea110, Ea1189Δlsc, EaGH9, EaK2, EaT3(2), and EaVH7-9 in LB medium containing 5% sucrose after 48 hours of growth. Sample means were compared by an analysis of variance and separated using the student's t test. The presence of different letters above sample mean value indicates that the means were significantly different at P<0.005.

The four Michigan *E. amylovoran* isolates with variation in levan production were evaluated for virulence characteristics, including amylovoran levels, biofilm formation, and disease development in immature pears. All four strains produced significantly less amylovoran than both Ea110 and Ea1189. Isolates EaK2, EaVH7-9, and EaT3(2) were not significantly

different from Ea1189Δams; however, EaK2 and EaVH7-9 were also not significantly different from EaGH9 (Figure 3.4). All four Michigan isolates were not significantly different from Ea1189 for biofilm formation, but were significantly different from Ea1189Δams (Figure 3.5). Isolates EaT3(2) and EaVH7-9 showed a slower rate of disease development in immature pears compared to the other isolates. However, by six days post inoculation (DPI), only strain EaT3(2) had a significant reduction in lesion diameter compared to Ea110 (Figure 3.6).

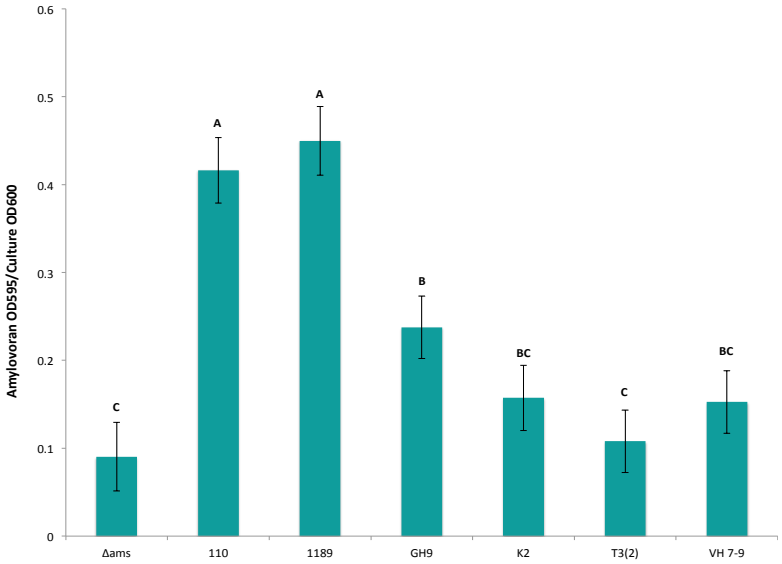


Figure 3.4. Quantification of amylovoran production using the CPC-binding assay of Michigan *E. amylovora* isolates compared to Ea1189Δams, a mutant deficient in amylovoran production. Means represent three biological replicates and the error bars indicate the standard error of the mean. Sample means were compared using an analysis of variance and separated using student's t test. The presence of different leters above the sample mean indicates that the means were significantly different at $P<0.005$).

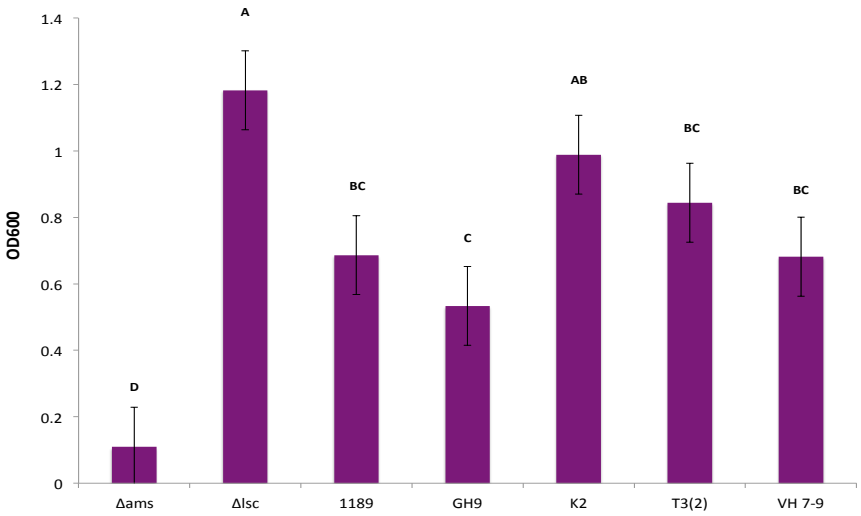


Figure 3.5. Quantification of biofilm formation of Michigan *E. amylovoran* isolates compared to Ea1189Δams, a mutant strain deficient in biofilm formation. The means consist of three biological replicates and the error bars represent the standard error of the means. Sample means were compared using an analysis of variance and separated using student's t test. The presence of different letters above the sample means indicates that the means were significantly different at P<0.005.

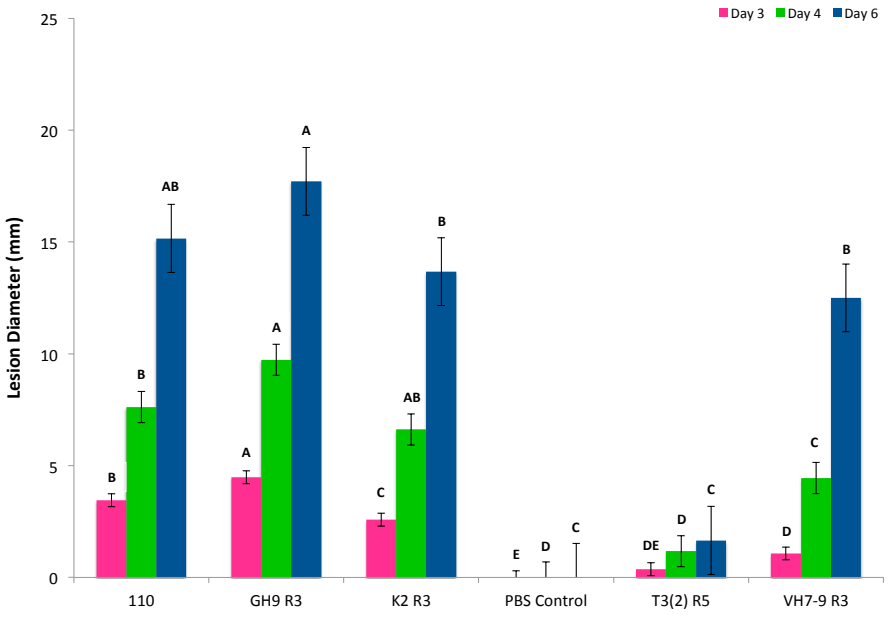


Figure 3.6. Virulence of *E. amylovora* strains Ea110, EaGH9, EaK2, EaT3(2), and EaVH7-9 in immature pears at 2DPI, 4DPI, and 6DPI. Values represent the mean of 10 samples from one representative experiment. Sample means on each day were compared by an analysis of variance and separated using student's t test. The presence of different letters above the sample mean values indicates that the means are significantly different at P<0.05.

In field trials, we evaluated population levels in ooze droplets and disease severity in apple shoot and blossom studies for Ea110 and rifampicin resistant variants of the four Michigan isolates of *E. amylovora* that were reduced in levan production for ooze droplet production. Isolate EaT3(2) had significantly less disease in both shoot and blossom studies compared to all other strains. In blossom studies, EaK2 had approximately twenty percent reduced disease severity compared to Ea110 and EaVH7-9. All other strains did not show a significant difference in disease severity in apple shoots (Figure 3.7). Isolate EaT3(2) produced significantly less ooze in both blossom and shoot inoculations compared to Ea110 and isolate EaK2 had a significant reduction in ooze production in shoots only. All other isolates were not significantly different from Ea110 in ooze production (Figure 3.8). For blossom inoculations, all isolates except EaT3(2), which did not produce ooze, had significantly higher populations per microliter of ooze than Ea110. For shoot inoculations, EaK2 had lower populations per microliter of ooze compared to Ea110 and EaGH9; however, this difference was small (Figure 3.9).

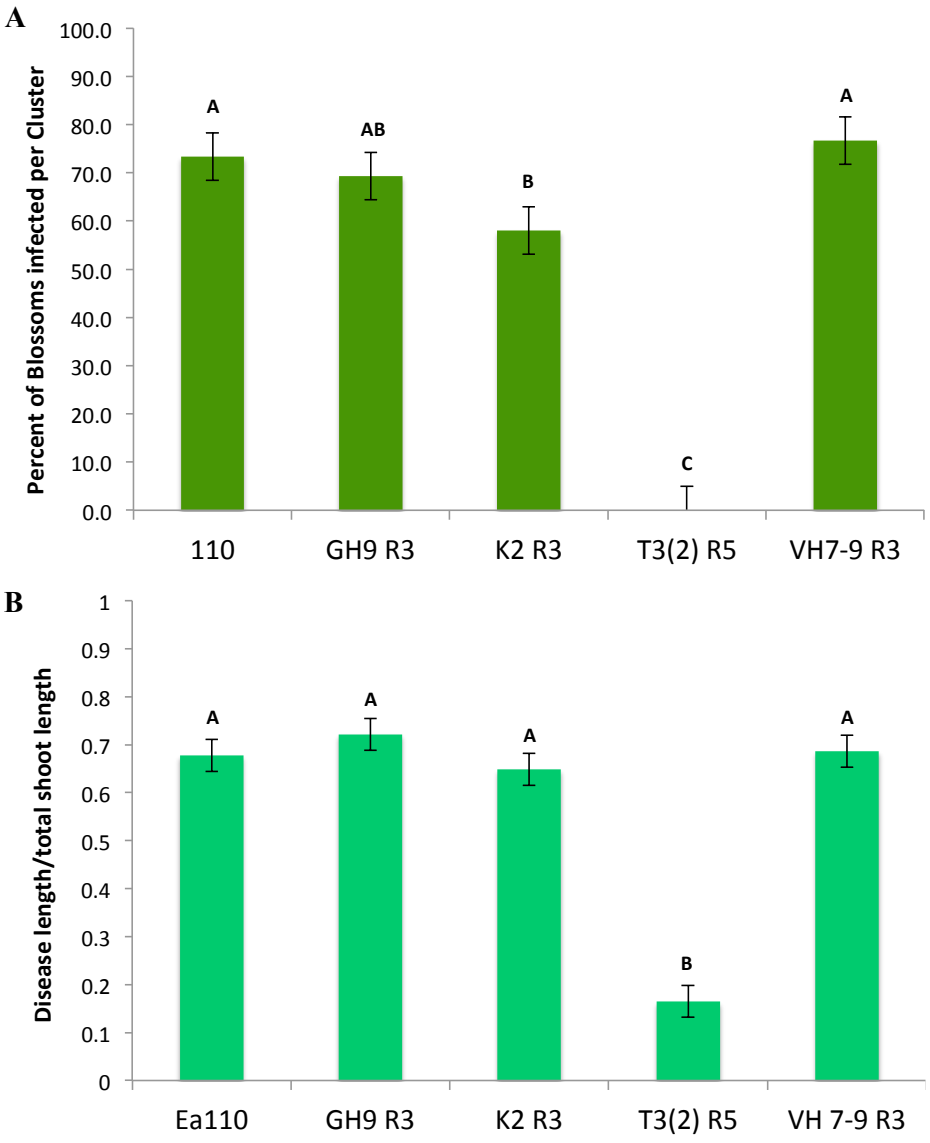


Figure 3.7. Disease incidence of Michigan *E. amylovora* isolates on (A) apple blossoms using the percentage of infected blossoms per cluster, with the means representing 50 samples and (B) the standardized disease length of apple shoots, with the means representing three biological replicates consisting of 50 samples each. Sample means were compared using an analysis of variance and separated using the student's t test. The presence of different letters about the sample means indicates that the means were significantly different at $P<0.05$.

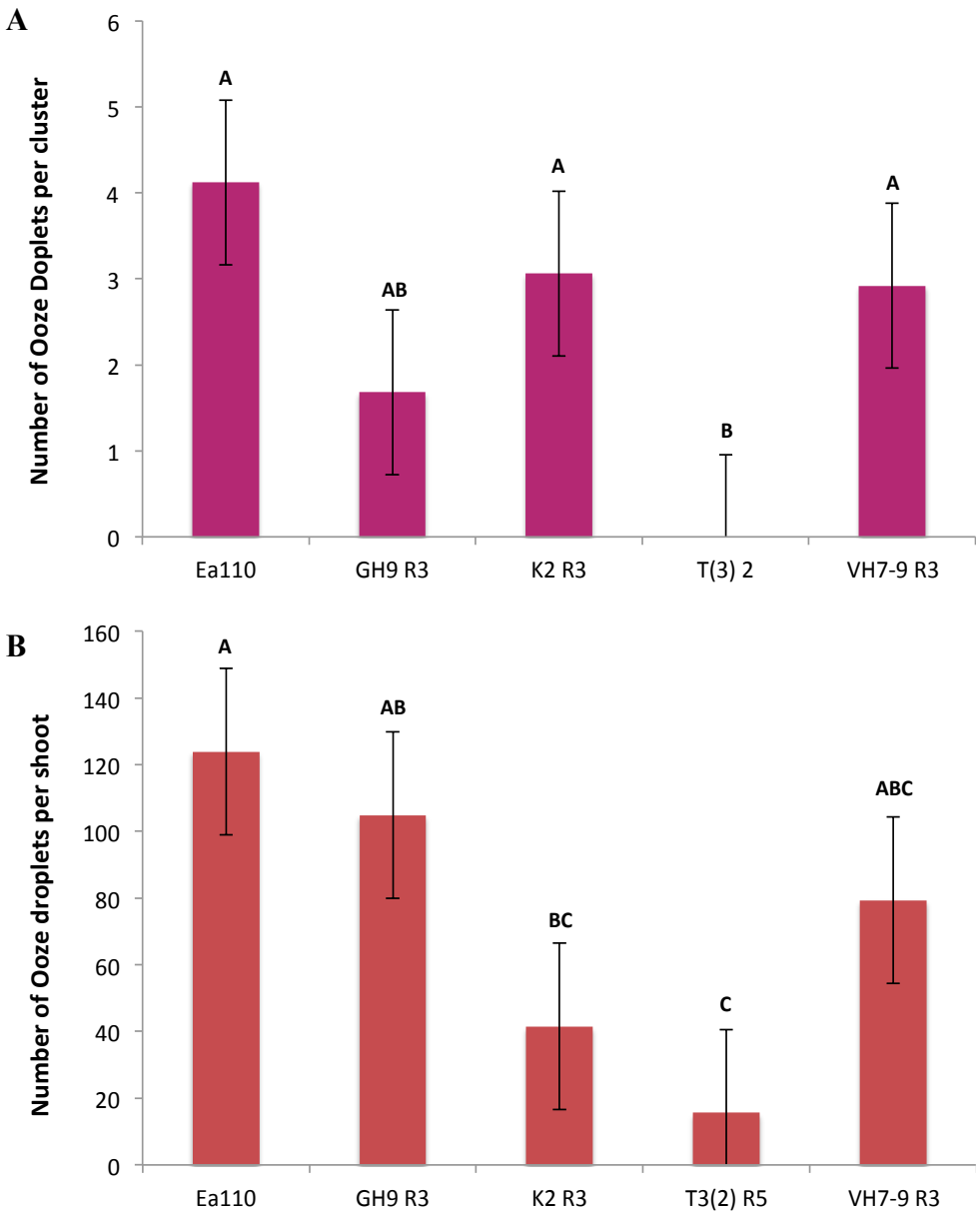


Figure 3.8. Ooze production by Michigan isolates of *E. amylovora* with variation in levan production on orchard inoculated apple blossoms, with the means representing 50 samples (A) and orchard inoculated apple shoots, with means representing three biological replicates consisting of 50 samples each (B). Sample means were compared using an analysis of variance and separated using the student's t test. The presence of different letters above the sample means indicates that the means were significantly different at $P<0.05$.

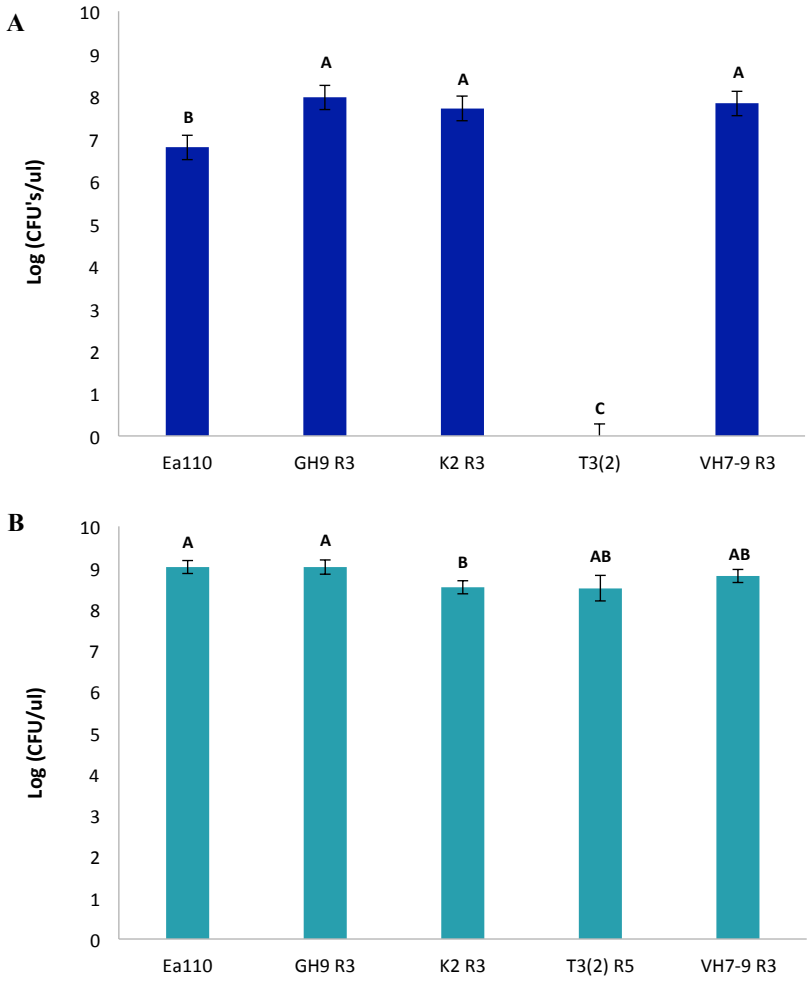


Figure 3.9. Quantification of population levels of Michigan *E. amylovora* in ooze droplets on apple blossoms (A) and apple shoots (B) in orchard inoculations. Values represent the average from ten ooze droplets. Sample means were compared using an analysis of variance and separated using the student's t test. The presence of different letters above the sample means indicates that the means were significantly different at $P<0.05$.

E. amylovoran Mutants with a Reduction in Levan Production:

We confirmed the findings of Zhao *et al* that Ea1189ΔgrrA, Ea1189ΔgrrS, and Ea1189ΔgrrA/grrS had little to no levan production and were not significantly different from Ea1189Δlsc. Mutants Ea1189ΔompR and Ea1189ΔenvZ had a reduction in levan compared to Ea1189 and were significantly higher that Ea1189Δlsc. (Figure 3.10) (Youfu Zhao, Wang,

Nakka, Sundin, & Korban, 2009). Mutants Ea1189ΔompR and 1189ΔenvZ were chosen for further analysis.

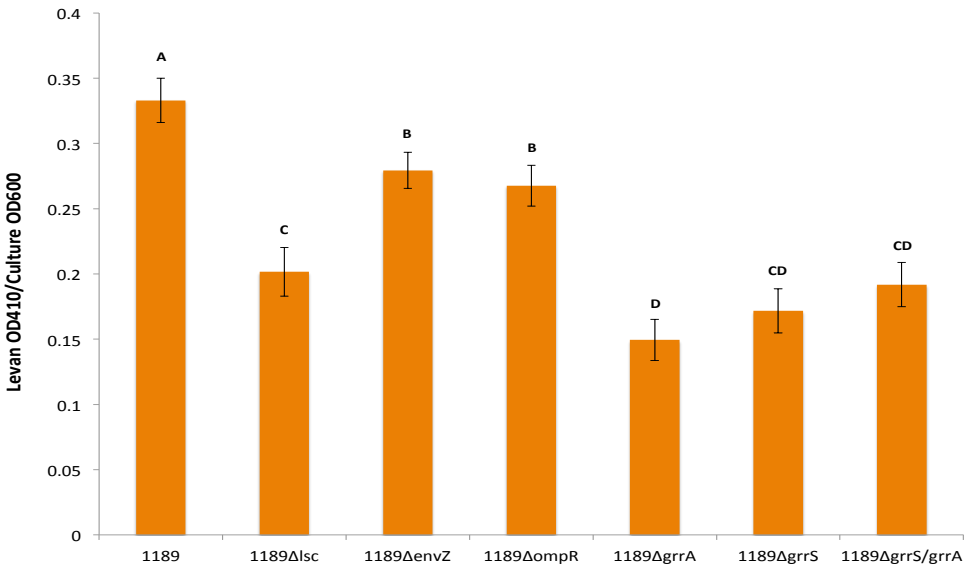


Figure 3.10. Quantification of levan production by Ea1189, Ea1189Δlsc, Ea1189ΔenvZ, Ea1189ΔompR, Ea1189ΔgrrA, Ea1189ΔgrrS, and Ea1189ΔgrrS/grrA in LB medium containing 5% sucrose after 48 hours of growth. Sample means were compared by an analysis of variance and separated using the student's t test. The presence of different letters above sample mean value indicates that the means were significantly different at P<0.005.

Ea mutant strains Ea1189ΔompR and Ea1189ΔenvZ had a reduction in virulence in immature pears and apple shoots, ooze production, and population levels in ooze droplets when compared to the wildtype strain. In immature pears, both Ea1189ΔompR and Ea1189ΔenvZ had slower progression of necrosis compared to Ea1189. However, by 4DPI all strains were not significantly different from Ea1189 (Figure 3.11). In apple shoots, Ea1189ΔenvZ had significantly lower disease severity compared to Ea1189; howerver, there was only a 10% difference. Neither Ea1189ΔompR or Ea1189ΔenvZ were significantly different from each other in their disease severity in apple shoots (Figure 3.12). Mutants Ea1189Δlsc, Ea1189ΔenvZ, and Ea1189ΔompR showed a reduction in ooze droplet numbers on apple shoots compared to

Ea1189 and were not significanlty different from each other (Figure 3.13). There was no difference between Ea1189ΔenvZ, Ea1189ΔompR, or Ea1189 in population levels per microliter of ooze. There were significantly lower population levels per microliter of ooze for both Ea1189Δlsc and Ea1189Δlsc::lsc compared to all other strains tested (Figure 3.14).

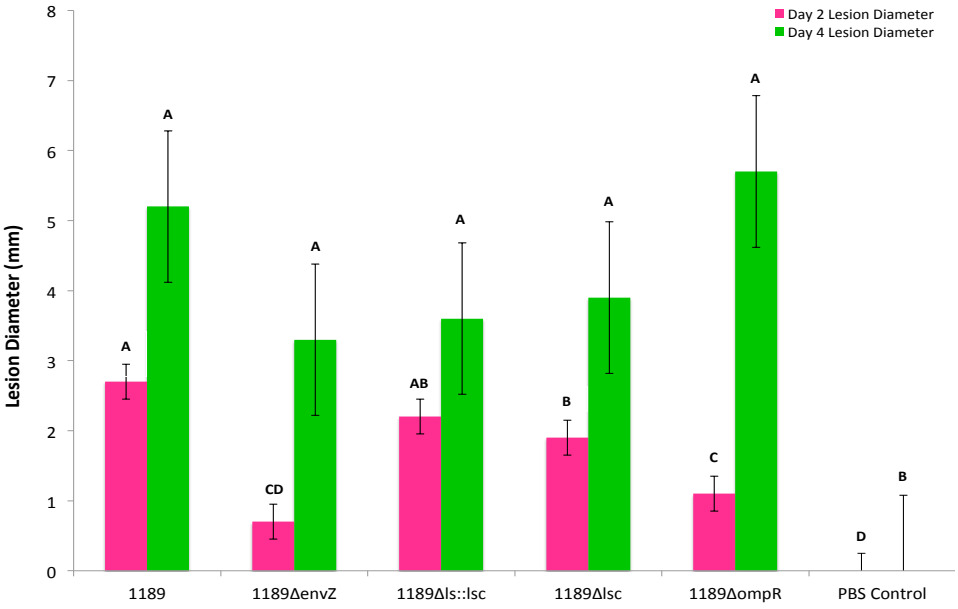


Figure 3.11. Virulence of strains Ea1189, Ea1189ΔenvZ, Ea1189Δlsc::lsc, Ea1189Δlsc, and Ea1189ΔompR in immature pears at 2DPI and 4DPI. Values represent the mean of 6 samples. Sample means on each day were compared by an analysis of variance and separated using student's t test. The presence of different letters above the sample mean values indicates that the means are significantly different at $P<0.05$.

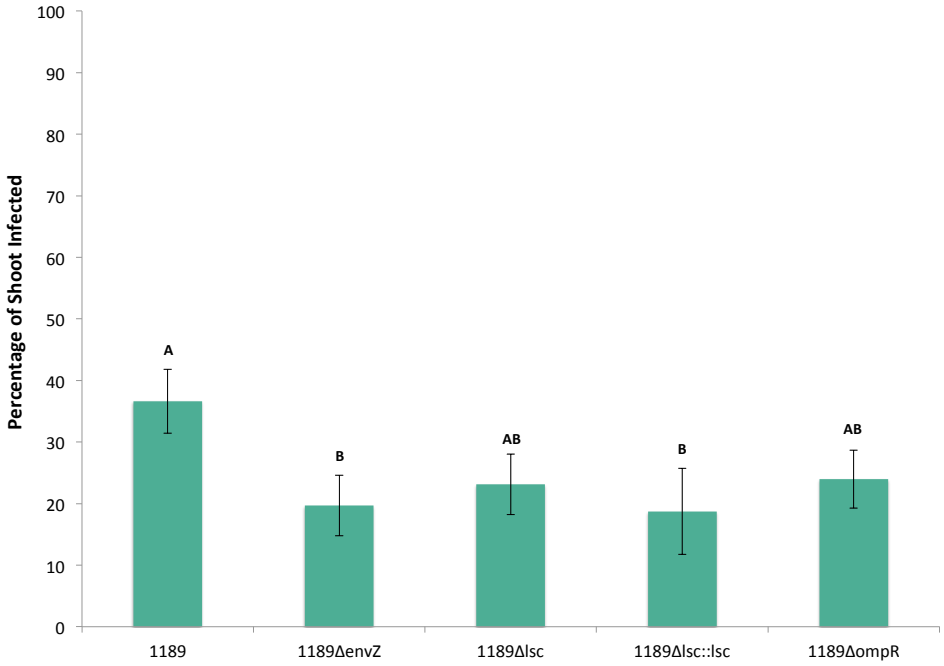


Figure 3.12. Disease incidence of *E. amylovora* mutant strains in growth chamber inoculated apple shoots. The sample means represent two biological replications. The sample means were compared using an analysis of variance and separated using the student's t test. The presence of different letters above the sample means indicates that the means were significantly different at P<0.05.

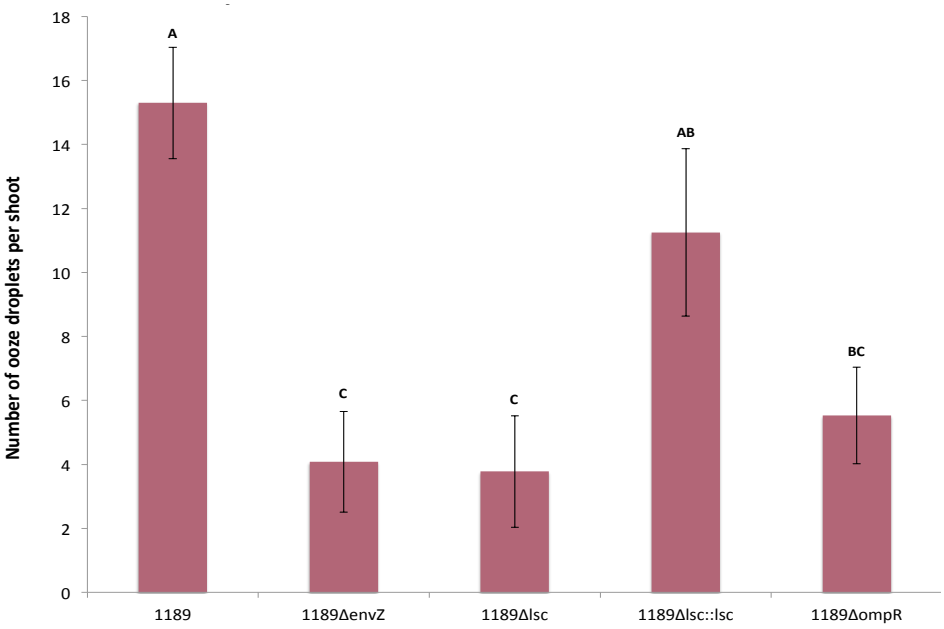


Figure 3.13. Number of ooze droplets per shoot produced by *E. amylovora* mutant strains in growth chamber inoculated apple shoots. The sample means represents two biological replicates. The sample means were compared using an analysis of variance and separated using the student's t test. The presence of different letters above the sample means indicates that the means were significantly different at P<0.05.

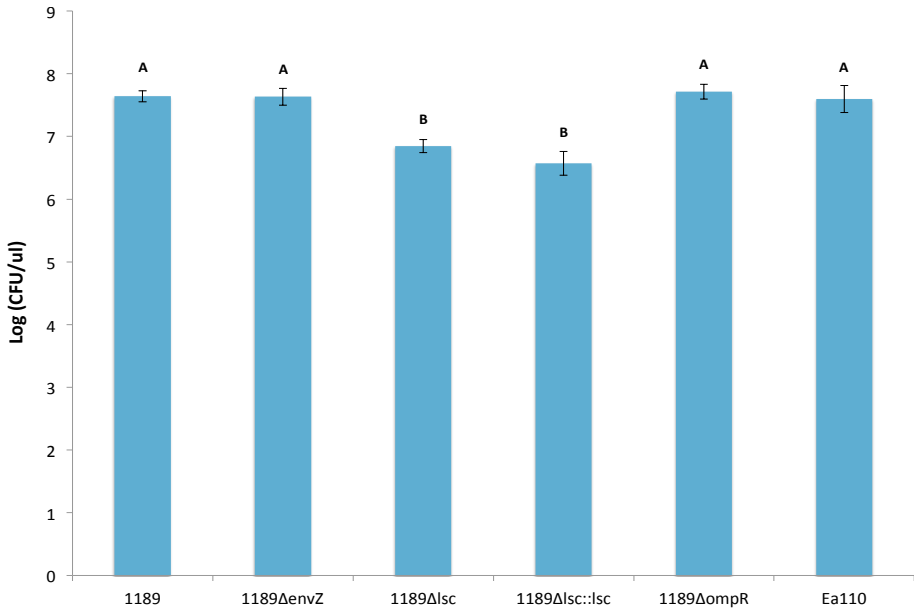


Figure 3.14. Quantification of population levels of *E. amylovora* mutants in ooze droplets from growth chamber inoculated apple shoots. The sample means represents two biological replicates. The sample means were compared using an analysis of variance and separated using the student's t test. The presence of different letters above the sample means indicates that the means were significantly different at $P<0.05$.

Discussion

Our growth chamber studies showed that a reduction in levan significantly reduced the amount of ooze droplets produced during apple shoot infections. We also confirmed previous findings that low levan production results in reduced disease severity in both apple shoots and immature pears (Koczan et al., 2009; Y. Zhao, Sundin, & Wang, 2009). These findings indicate that levan plays an important role in the dispersal and overall virulence of *E. amylovora*.

The ability of *E. amylovora* strains to produce ooze during plant infection has been shown to be correlated to virulence in apple and pear as avirulent strains did not produce ooze (Robert A. Bennett & Billing, 1980). Once an ooze droplet escapes from the infected host tissue, the bacteria can be spread to new trees by either wind, rain, or mechanically by insects (M.

Hilderbrand et al., 2000; Nadarasah & Stavrinides, 2011; Schroth et al., 1974; Thomson et al., 1999). The ability to produce ooze is therefore necessary for the virulence of *E. amylovora*.

Although it is well known that ooze plays an important role in the virulence of *E. amylovora*, the mechanism underlying its production is not well understood. Several microscopy studies have led to the identification of EPS as an important component in the development of ooze. These studies showed that both *E. amylovora* cells and EPS are found in the intercellular space near parenchyma cells of infected tissue (Zamski et al., 2006) and in xylem parenchyma cells (R.N. Goodman & J.A. White, 1981). In SEM studies, bacterial cells were observed emerging from wounds and both wounds and erumpent mounds were observed beneath ooze droplets (Slack et al., 2017). It was found that the presence of EPS resulted in distortion and damage of the xylem parenchyma cells and lead to the release of bacterial cells into the intercellular space (R.N. Goodman & J.A. White, 1981). It was hypothesized that the presence of EPS results in a buildup of pressure that causes damage to the parenchyma cells (Eden-Green & Billing, 1972). The weight of EPS changed in response to water and it was speculated that the expanding of EPS in the intercellular space of the host could result in enough pressure to damage host tissue, especially soft tissue (H.J. Schouten, 1988, 1989b). These studies indicate that EPS is the cause for the emergence of ooze droplets. While evaluating gene regulation during immature par infection, Zhao *et al.* identified the up-regulation of the *lsc* gene (Youfu Zhao, Blumer, & Sundin, 2005). This, along with the observations of levan producing a thick, gummy domed appearance to colonies grown on sucrose containing agar plates, suggests that levan, rather than amylovoran, is responsible for this pressure build-up.

In growth chamber studies of apple shoot infection, we showed that Ea1189Δlsc as well as two mutant strains that showed a reduction in levan, Ea1189ΔenvZ and Ea1189ΔompR, had a

significant reduction in ooze droplet numbers compared to the wild type strain. Reduction in levan did not have an effect on the population levels within ooze droplets, but did result in a slight reduction in disease development in apple shoots and immature pears. This correlates with previous findings that the elimination of levan reduced the rate of disease development (Koczan et al., 2009; Li et al., 2014). Our findings reiterate previous observations that there is a correlation between EPS and ooze formation within infected tissue (R. N. Goodman & J.A. White, 1981; Zamski et al., 2006) and indicates that levan is the major EPS involved.

In our 2015 field studies, Michigan *E. amylovora* isolates with variation in levan production did not have a reduction in ooze droplet numbers and there were no differences between any of the strains, other than EaT3(2), in terms of disease severity. This is not an indication that levan does not play a role in ooze formation, as there are several other factors that can account for this in orchards. One of these factors is weather, including temperature and moisture levels. It was shown that cankers that had been kept moist and were incubated at 21°C resulted in a higher percentage of *E. amylovora* recovery than those that were kept dry or incubated at either 17°C or 28°C (Steven V. Beer & Norelli, 1977).

In the summer of 2015, the weather conditions were conducive for fire blight development and ooze production. Fire blight occurs at a higher rate at temperatures between 21 and 28C and in moist or humid conditions (Steven V. Beer & Norelli, 1977; Eve Billing, 1980). The average daily minimum and maximum temperatures during our field trials were 14C and 25C, respectively, and the average relative humidity and amount of rainfall were 78% and 0.58cm per day, respectively. The frequent rainfall and high humidity would cause a rapid rise in water potential in the xylem and intercellular space. Since ooze has been shown to shrink or swell with changes in water pressure, the high rainfall and humidity would cause the EPS in the

host tissue to swell and would likely be forced out of the host as ooze droplets (H.J. Schouten, 1989b). Therefore, it is not surprising that large numbers of ooze droplets were seen on shoots and blossoms inoculated with all strains and did not result in significant differences. The amount of rainfall not only increased the amount of ooze droplets produced, but also caused the ooze to become runny and difficult to accurately count. This difficulty in counting the number of ooze droplets was a major factor in the inability to identify statistical differences in ooze production among the Michigan isolates.

A second factor contributing to lack of a correlation between levan production and ooze formation in our field trials is the role that other genes play in the formation of ooze. The expression of important virulence genes is unknown for the Michigan isolates used in these field trials. Although we showed that these isolates had a reduction in biofilm and amylovoran production, the expression of the genes involved in these processes are unknown. Genes that regulate the production of EPS may be increased in these isolates, such as the regulator genes of amylovoran, *rcsA* and *rcsB*, (Koczan et al., 2009), the levansucrase gene regulators, *rlsA*, *rlsB*, and *rlsC* (Du & Geider, 2002; Du et al., 2004; Zhang & Geider, 1999) and *grrS* and *grrA* (Li et al., 2014). Also, changes in c-di-GMP levels, regulated by diguanylate cyclase (DGCs) and phosphodiesterases (PDEs) affect the level of amylovoran and cellulose (Castiblanco & Sundin, 2018; Edmunds, Castiblanco, Sundin, & Waters, 2013b). However, the role of c-di-GMP in levan synthesis has not been evaluated. The expression of EPS regulatory genes in each of the Michigan isolates along with the role of c-di-GMP in levan synthesis will need to be further investigated in order to better understand how they impact ooze production both *in vitro* and *in vivo*.

In summary, our findings indicate that levan plays a role in ooze formation in *E. amylovora*. However, the inability to show a correlation between levan and ooze formation in field trials with natural levan deficient Michigan isolates reiterates the importance of weather and other virulence genes in the formation of ooze and the spread of *E. amylovora*. Future research will need to generate mutant strains in the *lsc* gene within the same genetic background for use in ooze studies in order to overcome these obstacles.

REFERENCES

REFERENCES

Acimovic, S. G., Zeng, Q., McGhee, G. C., Sundin, G. W., & Wise, J. C. (2015). Control of fire blight (*Erwinia amlovora)* on apple trees with trunk-injected plant resistance inducers and antibiotics and assessment of induction of pathogenesis-related protein genes. *Frontiers in Plant Science, 6*, 16.

Adaskaveg, J. E., Driever, G. F., Forster, H., Gubler, W. D., Wade, L. M., & Holtz, B. (2008). Detection of *Erwinia amylovora* strains with reduced sensitivity to currently registered antibiotics and management of fire blight of pome fruits using kasugamycin in integrated strategies. *Acta Hort, 793*, 375-382.

Adaskaveg, J. E., Forset, H., & Wade, M. L. (2011). Effectiveness of kasugamycin against *Erwinia amylovora* and its potential use for managing fire blight of pear. *Plant Disease, 95*, 448-454.

Adaskaveg, J. E., Wade, L., & Forster, H. (2007). Streptomycin resistance in *Erwinia amylovora* in California and a new bactericide for management of fire blight of pears. *Phytopathology, 97*(S2).

Aldwinckle, H. S., & Norelli, J. L. (1990). Evaluation of Kasumin for control of fire blight. *Acta Hort, 273*, 391.

Andersson, D. I. (2003). Persistence of antibiotic resistant bacteria. *Current Opinion in Microbiology, 6*, 452-456.

Andersson, D. I., & Hughes, D. (2011). Persistence of antibiotic resistance in bacterial populations. *FEMS Microbiology Reviews, 35*.

Angulo, F. J., Baker, N. L., Olsen, S. J., Anderson, A., & Barrett, T. J. (2004). Antimicrobial use in agriculuture: controlling the transfer of antimicrobial resistant to humans. *Seminars in Pediatric Infectious Diseases, 15*(2), 78-85.

Ark, P. A. (1953). Use of streptomycin dust to control fireblight. *Plant Disease Reporters, 37*(404-406).

Ayers, A. R., Ayers, S. B., & Goodman, R. N. (1979). Extracellular polysaccharide of *Erwinia amylovora*: a coorelation with virulence. *Applied and Environmental Microbiology, 38*(4), 659-666.

Baker-Austin, C., Wright, M. S., Stepanauskas, R., & McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends in Microbiology, 14*(4), 176-182.

Bartley, E. E., Fountaine, F. C., & Atkeson, F. W. (1950). The effects of an APF concentrate containing aureomycin on the growth and well-being of young dairy calves. *Journal of Animal Science, 9*, 646-647.

Beer, S. V. (1979). Fireblight inoculum: sources and dissemination. *European and Mediterranean Plant Protection Organization, 9*(1), 13-25.

Beer, S. V., & Norelli, J. L. (1977). Fire blight epidemiology: factors affecting release of *Erwinia amylovora* by cankers. *Phytopathology, 67*, 1119-1125.

Bellemann, P., & Geider, K. (1992). Localization of transposon insertions in pathogenicity mutants of *Erwinia amylovora* and their biochemical characterization. *General Microbiology, 138*, 931-940.

Bennett, R. A., & Billing, E. (1978). Capsulation and virulence in *Erwinia amylovora*. *Annals of Applied Biology, 89*, 41-45.

Bennett, R. A., & Billing, E. (1980). Origin of the polysaccharide component of ooze from plants. *General Microbiology, 116*, 341-349.

Bereswill, S., & Geider, K. (1997). Characterization fo the rcsB gene from *Erwinia amyloora* and its influence on exopolysaccharide synthesis and virulence fo the fire blight pathogen. *American Society for Microbiology, 179*(4), 1354-1361.

Bereswill, S., Jock, S., Aldridge, P., Janse, J. D., & Geider, K. (1997). Molecular characterization of natural *Erwinia amylovora* strains deficient in levan synthesis. *Physiological and Molecular Plant Pathology, 51*, 215-225.

Berry, A., DeVault, J. D., & Chakrabarty, A. M. (1989). High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. *J. Bacteriol., 171*, 2312-2317.

Billing, E. (1980). Fireblight (*Erwinia amylovora*) and weather: a comparison of warning systems. *Annals Applied Biology, 95*, 365-377.

Billing, E. (1981). Weather analysis and fireblight in different climates. *Acta Horticulture, 177*, 45-48.

Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O., & Piddock, L. J. V. (2015). Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology, 13*, 42-51.

Brumfitt, W., & Hamilton-Miller, J. (1989a). Methicillin-resistanct *Staphylococcocus aureus*. *The New England Journal of Medicine, 320*(18), 1188-1196.

Brumfitt, W., & Hamilton-Miller, J. (1989b). Methicillin-Resistanct *Staphylococcocus aureus*Methicillin-resistanct Staphylococcocus aureus. *The New England Journal of Medicine, 320*(18), 1188-1196.

Brzostek, K., Brzostkowska, M., Bukowska, I., Karwicka, E., & Raczkowska, A. (2007). OmpR negatively regulates expression of invasin in *Yersinia enterocolitica*. *Microbiology, 153*, 2416-2425.

Brzostek, K., Skorek, K., & Raczkowska, A. (2012). OmpR, a central integrator of several cellular responses in *Yersinia enterocolitica*. *Adv. Exp. Med. Biol., 954*, 325-334.

Brzostek, K., Skorek, K., & Raczkowska, A. (2012). OmpR, a central integrator of several cellular responses in *Yersinia enterocolitica*. *Advances in Experimental Medicine and Biology, 954*, 325-334.

Bugert, P., & Geider, K. (1995). Molecular analysis of the *ams* operon required for exopolysaccharide synthesis of *Erwinia amylovoran*. *Molecular Microbiology, 15*, 917-933.

Caputi, L., Cianci, M., & Benini, S. (2013). Cloning, expression, purification, crystallization and preliminary X-ray analysis of EaLsc, a levansucrase from *Erwinia amylovoran*. *Structural Biology and Crystallization Communications, 69*(5), 570-573.

Castiblanco, L. F., & Sundin, G. W. (2018). Cellulose production, activated by cyclid di-GMP through BcsA and BcsZ, is a virulence factor and an essential determinant of the three-dimensional architectures of biofilms formed by *Erwinia amylovora* Ea1189. *Molecular Plant Pathology, 19*, 90-103.

Chambert, R., Treboul, G., & Dedonder, R. (1974). Kinetic studies of levansucrase of *Bacillus subtilis*. *European Journal of of Biochemistry, 41*, 285-300.

Chang, H.-H., Cohen, T., Grad, Y. H., Hanage, W., O'Brien, T. F., & Lipsitch, M. (2015). Origin and proliferation of multiple-drug resistance in bacterial pathogens. *Microbiology and Molecular Biology Reviews, 79*(101-116).

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M., & Hanage, W. P. (2014). Antibiotics in agriculture and the risk to human health: how worried should we be? *Evolutionary Applications, 8*, 240-245.

Chapman, J. S. (2003). Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *International Biodeterioration and Biodegradation, 51*, 271-276.

Chiou, C. S., & Jones, A. L. (1993). Nucleotide sequence analysis of a transposon (Tn5393) carrying streptomycin resistance genes in *Erwinia amylovora* and other gram-negative bacteria. *Bacteriology, 175*(3), 732-740.

Chiou, C. S., & Jones, A. L. (1995a). Expression and identification of the *strA-strB* gene pair from streptomycin-resistant *Erwinia amylovora*. *Gene, 152*, 47-51.

Chiou, C. S., & Jones, A. L. (1995b). Molecular analysis of high level streptomycin resistance in *Erwinia amylovora*. *Phytopathology, 85*, 324-328.

Copping, L. G., & Duke, S. O. (2007). Natural products that have been used commercially as crop protection agents. *Pest Management Science, 63*, 524-554.

Cote, L. G., & Jmam, S. H. (1989). Purification and properties of an extracellular levansucrase from *Erwinia herbicola* NRRL B-1678. *Carbohydrate Research, 190*, 299-307.

Cruz, A. T., Cazacu, A. C., & Allen, C. H. (2007). *Pantoea agglomerans*, a plant pathogen causing human disease. *Journal of Clinical Microbiology, 45*(6), 1989-1992.

Cunha, T. J. (1950). Effect of aureomycin and other antibiotics on the pig. *Journal of Animal Science, 9*, 653-654.

Datsenko, K. A., & Wanner, B. L. (2000). One step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences, 97*, 6640-6645.

de la Cruz, F., & Davies, J. (2000). Horizontal gene transfer and the origin of species: lessons from bacteria. *Trends in Microbiology, 8*(3).

Dennesen, P. J. W., Bonten, M. J. M., & Weinstein, R. A. (1998). Multiresistant bacteria as a hospital epidemic problem. *Annals of Medicine, 30*(2), 176-185.

Denning, W. (1794). On the decay of apple trees. *New York Society for the Promotion of Agricultural Arts and Manufacturers Transaction, 2*, 219-222.

Dille, J. M. (1951). Antibiotics-their mechanism of action and uses as anti-infective agents. *Oral Surgery, Oral Medicine, Oral Pathology, 4*(2), 251-267.

Du, Z., & Geider, K. (2002). Characterization of an activator gene upstream of lsc, invovled in levan synthesis of *Erwinia amylovora*. *Physiological and Molecular Plant Pathology, 60*, 9-17.

Du, Z., Jakovljevic, V., Salm, H., & Geider, K. (2004). Creation and genetic restoration of *Erwinia amylovora* strains with low levan synthesis. *Physiological and Molecular Plant Pathology, 65*, 115-122.

Duffin, P. M., & Seifert, H. S. (2009). ksgA mutations confer resistance to kasugamycin in *Neisseria gonorrhoea*. *International Journal of Antimicrobial Agents, 33*(4), 321-327.

Eden-Green, S. J., & Billing, E. (1972). Fireblight: occurence of bacterial strands on various hosts under glasshouse conditions. *Plant Pathology, 21*, 121-123.

Eden-Green, S. J., & Knee, M. (1974). Bacterial polysaccharide and sorbitol in fireblight exudate. *General Microbiology, 81*, 509-512.

Edmunds, A. C., Castiblanco, L. F., Sundin, G. W., & Waters, C. M. (2013a). Cyclic di-GMP modulates the disease progression of *Erwinia amylovora*. *Bacteriology, 195*(10), 2155-2165.

Edmunds, A. C., Castiblanco, L. F., Sundin, G. W., & Waters, C. M. (2013b). Cyclic Di-GMP Modulates the Disease Progression of *Erwinia amylovora*Cyclic di-GMP modulates the disease progression of Erwinia amylovora. *Bacteriology, 195*(10), 2155-2165.

Feng, X., Oropeza, R., & Kenney, L. J. (2003). Dual regulation by phospho-OmpR of *ssrA/B* gene expression in *Salmonella* pathogenicity island 2. *Mol. Microbiol., 48*, 1131-1143.

Feng, X., Walthers, D., Oropeza, R., & Kenney, L. J. (2004). The response regulator SsrB activates transcription and binds to a region overlapping OmpR binding site at *Salmonella* pathogenicity island 2. *Mol. Microbiol., 54*, 823-835.

Finley, R. L., Collignon, P., Larsson, D. G. J., McEwen, S. A., Li, X.-Z., Gaze, W. H., . . . Topp, E. (2013). The scourage of antibiotic resistance: The important role of the environment. *Clinical Infectious Diseases, 57*(5), 704-710.

Fisher, E. G., Parker, K. G., Luepschen, N. S., & Kwong, S. S. (1959). The influence of phosphorus, potassium, mulch, and soil drainage on fruit size, yield, and firmness of the bartlett pear and on development of the fire blight disease. *In Proceedings of the American Society for Horticultural Science, 73*, 78-90.

Fleming, A. (1929). On antibacterial action of cultures of Penicillium with special reference to their use in the isolation of B. influenzae. *British Journal of Experimental Pathology, 10*, 226.

Fling, M. E., Kopf, J., & Richards, C. (1985). Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltranferase. *Nucleic Acids Research, 13*, 7095-7106.

Franke-Whittle, I. H., Manici, L. M., Insam, H., & Stres, B. (2015). Rhizosphere bacteria and fungi associated with plant growth in soils of three replanted apple orchards. *Plant Soil, 395*, 317-333.

Geider, K., Aldridge, P., Bereswill, S., Bugert, P., & C., L. (1996). Characterization of exoplysaccharide synthesis by *Erwinia amylovora*. *Acta Horticulture, 411*, 259-263.

Geier, G., & Geider, K. (1993). Characterization and influence on virulence of the levansucrase gene from the fireblight pathogen *Erwinia amylovora*. *Physiological and Molecular Plant Pathology, 42*, 387-404.

Goodman, R. N. (1954). Development of methods for use in antibiotics to control fireblight (*Erwinia amylovora*). *Missouri Agricultural Experiment Station Research Bulletin, 540*(1-16).

Goodman, R. N., & White, J. A. (1981). Xylem Parenchyma Plasmolysis and Vessel Wall Disorientation Caused by *Erwinia amylovora*. In (Vol. 71, pp. 844-852): Phytopathology.

Goodman, R. N., & White, J. A. (1981). Xylem parenchyma plasmolysis and vessel wall disorientation caused by *Erwinia amylovora*. *Phytopathology, 71*(8), 844-852.

Gowda, S. S., & Goodman, R. N. (1970). Movement and persistence of *Erwinia amylovora* in shoot, stem, and root of apple. *Plant Disease Reporter, 54*, 576-580.

Grinsted, J., de la Cruz, F., & Schmitt, R. (1990). The Tn21 subgroup of bacterial transposable elements. *Plasmids, 24*, 163-189.

Gross, M., Geier, G., Rudolph, K., & Geider, K. (1992). Levan and levansucrase synthesized by the fireblight pathogen *Erwinia amylovora*. *Physiological and Molecular Plant Pathology, 40*, 371-381.

Gross, M., & Rudolph, K. (1987). Studies on the extracellular polysaccharides (EPS) produced in vitro by *Pseudomonas phaseolicola*. *Phytopathology, 119*, 289-297.

Gustafson, R. H., & Bowen, R. E. (1997). Antibiotic use in animal agriculture. *Journal of Applied Microbiology, 83*, 531-541.

Heuberger, J. W., & Paulos, P. L. (1952). Control of fireblight and frog-eye leaf spot (black rot) disease of apples in Delaware. *Plant Disease Reporter, 37*(81-83).

Hilderbrand, E. M. (1939). Studies on the fire-blight ooze. *Phytopathology, 29*, 142-156.

Hilderbrand, M., Dicler, E., & Geider, K. (2000). Occurrence of *Erwinia amylovora* on insects in a fire blight orchard. *Phytopathology, 148*, 251-256.

Hollis, A., & Ahmed, Z. (2013). Preserving antibiotics rationally. *New England Journal of Medicine, 369*(26), 2474-2476.

Ikeno, S., Tsuji, T., Higashide, K., & Kinoshita, N. (1998). A 7.6kb DNA region of *Streptomyces kasugaensis* M338-M1 includes some genes responsible for kasugamycin biosynthesis. *The Journal of Antibiotics, 51*(3), 341-352.

Johnson, K., & Stockwell, V. (1998). Management of fire blight: a case study in microbial ecology. *Annual Review of Phytopathology, 36*, 227-248.

Johnson, K. B., Stockwell, V. O., McLaughlin, M. J., Sugar, D., Loper, J. E., & Roberts, R. G. (1993). Effect of bacterial antagonists on establishment of honey bee-dispersed *Erwinia amylovora* in pear blossoms and on fire blight control. *Phytopathology, 83*, 995-1002.

Johnson, K. B., & Temple, T. N. (2013). Evalation of strategies for fire blight control in organic pome fruit without antibiotics. *Plant Disease, 97*(3), 402-409.

Jones, A. (1982). Chemical control of phytopathogenic prokaryotes. In M. Mount & G. Lacy (Eds.), *Phytopathogenic prokaryotes* New York: Academic Press.

Jones, R. N., Gavan, T. L., Thornsberry, C., Fuchs, P. C., Gerlach, E. H., Knapp, J. S., . . . Washington II, J. A. (1989). Standardization of disk disffusion and agar dilution susceptibility test for *Neisseria gonorrhoeae: i*nterpretive criteria and quality control guidelines for ceftriaxone, penicillin, spectinomycin, and tetracycline. *Journal of Clinical Microbiology, 27*(12), 2758-2766.

Kelm, O., Kiecker, C., Geider, K., & Bernhad, F. (1997). Interaction of the regulator proteins RcsA and RcsB with the promoter of the operon for amylovoran biosynthesis in *Erwinia amylovora Molecular Genetics and Genomics, 256*, 72-83.

Knapp, C. W., Dolfing, J., Ehlert, P. A. I., & Graham, D. W. (2010). Evidence of increasing antibiotic resistance gene abundance in archived soils since 1940. *Environmental Science and Technology, 44*, 580-587.

Koczan, J. M., McGrath, M. J., Zhao, Y., & Sundin, G. W. (2009). Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: implications in pathogenicity. *Bacteriology, 99*(1), 1237-1244.

Kumar, K., Gupta, S. C., Chander, Y., & Singh, A. K. (2005). Antibiotic use in agriculture and its impact on the terrestrial environment. *Advances in Agronomy, 87*, 1-54.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K. M., Werheim, H. F. L., Sumpradit, N., . . . Cars, O. (2013). Antibiotic resistance-the need for global solutions. *The Lancet Infectious Diseases Commission, 13*, 1057-1098.

Leonard, A. F. C., Zhang, L., Balfour, A. J., Garside, R., & Gaze, W. H. (2015). Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. *Environmental International, 82*, 92-100.

Levitan, A. A. (1967). In vitro antibacterial activity of kasugamcyin. *Appled Microbiology, 15*(4), 750-753.

Levy, S. (1992). Further ecological considerations: antibiotic use in agriculture, aquaculture, pets, and minor animal species. In *The Antibiotic Paradox: How Miracle Drugs are Destroying the Miracle* (pp. 157-181). New York: Plenum Press.

Li, W., Ancona, B., & Zhao, Y. (2014). Co-regulation of polysaccharide production, motility, and expression of type III secretion genes by EnvZ/OmpR and GrrS/GrrA systems in *Erwinia amylovora*. *Molecular Genetics and Genomics, 289*, 63-75.

Lindow, S. E., McGouty, G., & Elkins, R. (1996). Interactions of antibiotics with *Pseudomonas fluorescens* A506 in the control of fire blight and frost injury of pear. *Phytopathology, 86*, 841-848.

Lipsitch, M., Singer, R. S., & Levin, B. R. (2002). Antibiotics in agriculture: when is it time to close the barn door? *Proceedings of the National Academy of Sciences, 99*(9), 5752-5754.

Loper, J. E., Henkels, M. D., Roberts, R. G., G.G., G., Willett, M. J., & Smith, T. J. (1991). Evaluation of streptomycin, oxytetracycline, and copper resistance in *Erwinia amylovora* isolated from pear orchards in Washington State. *Plant Disease, 75*, 287-290.

Luepschen, N. S., Parker, K. G., & Mills, W. D. (1960). Fire blight control with streptomycin, as influenced by temperature and other environmental factors and by adjuvants added to sprays. *New York Agricultural Experiment Station Memoirs, 375*, 1-39.

Lyness, E. W., & Doelle, H. W. (1983). Levansucrase from *Zymomonas mobilis*. *Biotechnology letters, 5*, 345-350.

Martinez, J. L. (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science, 321*(5887), 365-367.

McGhee, G. C., Guasco, J., Bellomo, L. M., Blumer-Schuette, S., Shange, W. W., Irish-Brown, A., & Sundin, G. W. (2011). Genetic analysis of streptomycin-resistant (Sm^R) strains of *Erwinia amylovora s*uggests that dissemination of two genotypes is responsible for the current distribution of Sm^R *E. amylovora* in Michigan. *Phytopathology, 101*, 182-191.

McGhee, G. C., & Sundin, G. W. (2011). Evaluation of kasugamycin for fire blight management, effect on nontarget bacteria, and assessment of kasugamycin resistance potential in *Erwinia amylovora*. *Phytopathology, 101*(2), 192-204.

McGinnis, J., Berg, L. R., Stern, J. R., Wilcox, R. A., & Bearse, G. E. (1950). The effect of aureomycin and streptomycin on the growth of chicks and turkeys. *Poultry Science, 29*, 771.

McManus, P. S. (2014). Does a drop in the bucket make a splash? Assessing the impact of antibiotic use on plants.

Current Opinion in Microbiology, 19, 76-82.

McManus, P. S., & Jones, A. L. (1994). Epidemiology and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. *Phytopathology, 84*, 627-633.

McManus, P. S., Stockwell, V. O., Sunding, G. W., & Jones, A. L. (2002). Antibiotic use in plant agriculture. *Annual Review of Phytopahtology, 40*, 443-465.

McNally, R. R., Toth, I. K., Cock, P. J. A., Pritchard, L., Hedley, P. E., Morris, J. A., . . . Sundin, G. W. (2011). Genetic characterization of the HrpL Regulo of the fire blight pathogen *Erwinia amylovora* reveals novel virulence factors. *Molecular Plant Pathology, 13*(2), 160-173.

Miller, P. W. (1929). Studies of fire blight of apple in Wisconsin. *Agricultural Research, 39*, 579-621.

Miller, T. D., & Schroth, M. N. (1972). Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. *Phytopathology, 62*, 1175-1182.

Moller, W. J., Schroth, M. N., & Thomson, S. V. (1981). The scenario of fire blight and streptomycin resistance. *Plant Disease, 65*, 563-568.

Momol, M. T., Norelli, J. L., Piccioni, D. E., Momol, E. A., Gutafson, H. L., Cummins, J. N., & Aldwinckel, H. S. (1998). Internal movement of *Erwinia amylovora* through symptomless apple scion tissue in the rootstock. *Plant Disease, 82*, 646-650.

Murneek, A. E. (1952). Thiolutin as a possible inhibitor of fire blight. *Phytopathology, 42*, 57.

Nadarasah, G., & Stavrinides, J. (2011). Insects as alternative hosts for phytopathogenic bacteria. *Federation of European Microbiology Society, 35*, 555-575.

Nimtz, M., Mort, A., Domke, T., Wray, V., Zhang, Y., Qui, F., . . . Geider, K. (1996). Structure of amylovoran, the capsular exopolysaccharide from the fire blight pathogen *Erwinia amylovora*. *Carbohydrate Research, 287*, 59-76.

Norelli, J. L., Jones, A. L., & Aldwinckle, H. S. (2003). Fire blight management in the twenty-first century: using new technologies that enhance host resistance in apple. *Plant Disease, 87*, 756-765.

Nufarm. (2016). Crop Product Reference Guide.

O'Brian, T. F., & *al., e.* (1987). Resistance of bacteria to antibacterial agents: report of task force 2. *Reviews of Infectious Diseases, 9*(3), S244-S260.

Oh, C.-S., & Beer, S. V. (2005). Molecular genetics of *Erwinia amylovora* involved in the development of fire blight. *Federation of European Microbiology Association Letters, 253*, 185-192.

Okuyama, A., Tanaka, N., & Komai, T. (1975). The binding of kasugamycin to the *Escherichia coli* ribosomes. *The Journal of Antibiotics, 28*(11), 903-904.

Pankey, G. A., & Sabath, L. D. (2004). Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of gram-negative bacterial infections. *Clinical Infectious Diseases, 38*, 864-870.

Peil, A., Bus, V. G. M., Geider, K., Richter, K., Flachowsky, H., & Hanke, M. V. (2009). Improvement of fire blight resistance in apple and pear. *International Journal of Plant Breeding, 3*, 1-27.

Pickard, D., Li, J., Roberts, M., Maskell, D., Hone, D., Levine, M., . . . Chatfield, S. (1994). Characterization of defined *ompR* mutants of *Salmonella typhi: ompR* is involved in the regulation of Vi polysaccharide expression. *Infection and Immunity, 62*(9), 3984-3993.

Piras, C., Soggiu, A., Bonizzi, L., Gaviraghi, A., Deriu, F., de Martino, L., . . . Roncada, P. (2012). Comparative proteomics to evaluate multi drug resistance in *Escherichia coli*. *Molecular Biosystems, 8*, 1060-1067.

Pohronezny, K., Sommerfeld, M. L., & Raid, R. N. (1994). Streptomycin resistance and copper tolerance among strains of *Pseudomonas cichorii* in celery seedbeds. *Plant Disease, 78*, 150-153.

Pruden, A., Larsson, D. G. J., Amezquita, A., Collignon, P., Brandt, K. K., Graham, D. W., . . . Zhu, Y.-G. (2013). Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environmental Health Perspectives, 121*(8), 878-885.

Raleigh, E. A., Elbing, K., & Brent, R. (2002). Current protocols in molecular biology. *Wiley Online Library, Supplement 59*, 1.4.1-1.4.14.

Rao, T. N., Patrudu, T. B., Rao, N. K., Kumar, G., & Rao, M. V. B. (2016). Dissipation kinetics of copper oxychloride and kasugamycin fungicide residues in different pH waters. *Analytical Chemistry, 16*(10), 407-416.

Riggle, J. H., & Klos, E. J. (1970). Relationship of *Erwinia herbicola* to *Erwinia amylovora*. *Canadian Journal of Botany, 50*, 1077-1083.

Roach, D. R., Sjaarda, D. R., Castle, A. J., & Sviroev, A. M. (2013). Host exopolysaccharide quality and composition impact *Erwinia amylovora* bacteriophage pathogenesis. *Applied and Environmental Microbiology, 79*(10), 3249-3256.

Rosen, H. R., & Grovs, A. B. (1928). Studies on fire blight: host range. *Agricultural Research, 37*, 493-505.

Schaad, N. W. (1988). *Laboratory guide for identification of plant pathogenic bacteria* (Vol. 2nd edn.). St. Paul, Minnesota: American Phytopathology Society.

Schouten, H. J. (1988). Notes on the role of water potential in the pathogenesis of fire blight, caused by *Erwinia amylovora Netherland Journal of Plant Pathology, 94*, 213-220.

Schouten, H. J. (1989a). A possible role in pathogenesis for the swelling of extracellular slime of *Erwinia amylovora* at increasing water potential. *Netherlands Journal of Plant Pathology, 95*(Supplement 1).

Schouten, H. J. (1989b). A possible role in pathogenesis for the swelling of extracellular slime of *Erwinia amylovora* at increasing water potential. *Netherland Journal of Plant Pathology, 95*, 169-174.

Schouten, H. J. (1991). Simulation of pressure caused by multiplication and swelling of *Erwinia amylovora* in intercellular space of host tissue. *Netherland Journal of Plant Pathology, 97*, 139-141.

Schroth, M. N., Thomson, S. V., & Hildebrand, D. C. (1974). Epidemiology and control of fireblight. *Phytopathology, 12*, 389-412.

Seemuller, E. A., & Beer, S. V. (1976). Absence of cell wall polysaccharide degradation of *Erwinia amylovora*. *Phytopathology, 66*, 433-436.

Sharma, V. K., Johnson, N., Cizmas, L., McDonald, T. J., & Kim, H. (2016). A review of the influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes. *Chemosphere, 150*, 702-714.

Singer, R. S., & Williams-Nguyen, J. (2014). Human health impacts of antibiotic use in agriculture: a push for improved casual inference. *Current Opinion in Microbiology, 19*, 1-8.

Slack, S. M., Zeng, Q., Outwater, C. A., & Sunding, G. W. (2017). Microbiological examination of *Erwinia amylovora* exopolysaccharide ooze. *Phytopathology, In Review*.

Smith, D. L., Dushoff, J., & Morris, G. J. (2005). Agricultural antibiotics and human health. *PLoS Medicine, 2*(8), 731-735.

Steinberger, E. M., & Beer, S. V. (1988). Creation and complementation of pathogenicity mutants of *Erwinia amylovora*. *Molecular Plant-Microbe Interactions, 1*, 135-144.

Stock, J. B., Ninfa, A. J., & Sock, A. M. (1989). Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol Rev., 53*, 459-490.

Stockwell, V. O. (2014). Overview of concerns surrounding antibiotic use for control of fire blight. *Acta Horticulture, 1056*, 39-42.

Stockwell, V. O., & Duffy, B. (2012). Use of antibiotics in plant agriculture. *Scientific and Technical Review of the Office International des Epizooties (Paris), 31*(1), 199-210.

Storteboom, H., Arabi, M., Davis, J. G., Crimi, B., & Pruden, A. (2010). Identification of antibiotic-resistance-gene molecular signatures suitable as tracers of pristine river, urban, and agricultural sources. *Environmental Science Technology, 44*, 1947-1953.

Suhara, Y., Sasaki, F., Koyama, G., Maeda, K., Umezawa, H., & Ohno, M. (1972). The total synthesis of kasugamycin. *Journal of the American Chemical Society, 94*(18), 6501-6507.

Sundin, G. W. (2002). Distinct recent lineages of the strA-strB streptomycin-resistance genes in clinical and environmental bacteria. *Current Microbiology, 45*, 63-69.

Sundin, G. W., & Bender, C. L. (1996). Dissemination of the strA-strB streptomycin-resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. *Molecular Ecology, 5*, 133-143.

Sundin, G. W., Monks, D. E., & Bender, C. L. (1995). Distribution of the streptomycin-resistance transposon Tn5393 among phylloplane and soil bacteria from managed agricultural habitats. *Canadian Journal of Microbiology, 41*, 792-799.

Sundin, G. W., Werner, N. A., Yoder, K. S., & Aldwinckle, H. S. (2009). Field evaluation of biological control of fire blight in the eastern United States. *Plant Disease, 93*, 386-394.

Tanaka, N., Yamaguchi, H., & Umezawa, H. (1966). Mechanism of kasugamycin action on polypeptide synthesis. *The Journal of Biochemistry, 60*(4), 429-434.

Tancos, K. A., & Cox, K. D. (2016). Effects of consecutive streptomycin and kasugamycin applications on epiphytic bacteria in the apple phyllosphere. *Plant Disease, PDIS-06-16-0794-RE*.

Thomas, H. E., & Ark, P. A. (1934a). Fire blight of pears and related plants. *California Agricultural Experiment Station Circulation, 586*, 1-43.

Thomas, H. E., & Ark, P. A. (1934b). Nectar and rain in relation to fire blight. *Phytopathology, 24*, 682-685.

Thomson, S. V., Wagner, A. C., & Gouk, S. C. (1999). Rapid epiphytic colonization of apple flowers and the role of insects and rain. *Acta Horticultural, Proc. of the 8th Int. Workshop of Fire Blight*, 459-464.

Agri-Mycin 17, (2011).

FireLine 45 WP, (2016).

Van der Zwet, T. (1994). The various means of dissemination of the fire blight bacterium *Erwinia amylovora*. *Office of Executive Policy and Programs Bulletin, Bulletin 24*, 209-214.

Vidaver, A. K. (2002). Uses of antimicrobials in plant agriculture. *Clinical Infectious Diseases, 34*(3), S107-S110.

Visnapuu, T., Mardo, K., Mosoarca, C., Zamfir, A. D., Vigants, A., & Alamae, T. (2011). Levansucrase from *Pseudomonas syringae* pv. *tomato* and *P. chlororaphis* subsp. *aurantiaca*: substrate specificity, polymerizating porperties and usage of different acceptors for fructosylation. *Biotechnology, 155*, 338-349.

Waksman, S. A., Bugie, E., & Schatz, A. (1944). Isolation of antibiotic substances from soil micro-organisms. *Proceedings of the Staff Meetings, Mayo Clinic, 19*, 537.

Williams-Nguyen, J., Sallach, J. B., Bartelt-Hunt, S., Boxall, A. B., Durso, L. M., McLain, J. E., . . . Zilles, J. L. (2016). Antibiotics and antibiotic resistance in agroecosystems: state of the science. *Environmental Quality, 45*, 394-406.

Witte, W. (1998). Medical consequences of antibiotic use in agriculture. *Science, 279*, 996-997.

Wuerges, J., Caputi, L., Cianci, M., Biovin, S., Meijers, R., & Benini, S. (2015). The crystal structure of *Erwinia amylovora* levansucrase provides a snapshot of the products of sucrose hydrolysis trapped into the active site. *Structural Biology, 191*(3), 290-298.

Yashiro, E., & McManus, P. S. (2012). Effect of streptomycin treatment on bacterial community structure in the apple phyllosphere. *PLoS ONE, 7*(5), e37131.

Zamski, E., Shtienberg, D., & Blachinsky, D. (2006). The role of ooze exudation in the migration of *Erwinia amylovora* cells in pear trees infected by fire blight. *Israel Journal of Plant Sciences, 54*, 301-307.

Zhang, Y., & Geider, K. (1999). Molecular analysis of the rlsA gene regulating levan production by the fireblight pathogen *Erwinia amylovora*. *Physiological and Molecular Plant Pathology, 54*, 187-201.

Zhao, Y., Blumer, S. E., & Sundin, G. W. (2005). Identification of *Erwinia amylovora g*enes induced during infection of immature pear tissue. *Journal of Bacteriology, 187*(23), 8088-8103.

Zhao, Y., Sundin, G. W., & Wang, D. (2009). Construction and analysis of pathogenicity island deletion mutants of *Erwinia amylovora*. *Canadian Journal of Microbiology, 55*, 457-464.

Zhao, Y., Wang, D., Nakka, S., Sundin, G. W., & Korban, S. S. (2009). Systems level analysis of two-component signal transduction systems in *Erwinia amylovora:* role in virulence,

regulation of amylovoran biosynthesis and swarming motility. *BMC Genomics, 245*(10), 1-16.