STATUS OF STREPTOMYCIN-RESISTANT *ERWINIA AMYLOVORA* IN ILLINOIS APPLE ORCHARDS AND EVALUATION OF ALTERNATIVE COMPOUNDS TO MANAGE FIRE BLIGHT DISEASE

BY

ANDREW G. JURGENS

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Crop Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

Advisor:

Professor Mohammad Babadoost

ABSTRACT

Illinois apple growers produce approximately 17,963 metric tons of apples annually. Fire blight disease, caused by the bacterium *Erwinia amylovora*, is a serious threat to apple production in Illinois and other Midwestern states. Streptomycin has been the most effective chemical for control of fire blight of pome fruits in Illinois, as well as nationwide. In 2008 and 2009, severe fire blight occurred in Illinois apple orchards, leading to speculation that streptomycin-resistant strains of *E. amylovora* might be present in some orchards. Statewide surveys were conducted in 2010, 2011, and 2012, and 117, 129, and 170, *E. amylovora* isolates were collected each year, respectively, from 19 counties. None of the 416 *E. amylovora* isolates tested were streptomycin-resistant at 50 mg/L. However, nine non-*E. amylovora* isolates contained both a *str*A-*str*B streptomycin-resistance gene and IS1133 on transposon Tn5393, which could be a potential source of streptomycin-resistance for Illinois *E. amylovora* in the future. *E. amylovora* isolates collected in this survey also were tested for copper resistance. All 84 isolates evaluated were sensitive to 0.16 mM copper sulfate, indicating copper compounds are still effective for management of fire blight in Illinois. During 2011-2012, laboratory and field trials were conducted to evaluate efficacy of streptomycin-alternatives (oxytetracycline, kasugamycin, *Bacillus subtilis*, *Pseudomonas fluorescens*, and prohexadione calcium) for control of fire blight. Kasugamycin compounds (Kasumin 2L and ARY-4016-06) significantly ($P<0.10$) reduced blossom infection in the orchard. In 2012, effectiveness of growth regulator prohexadione calcium (Apogee 27.5DF) in combination with streptomycin-alternatives was also evaluated. An Apogee x Kasumin 2L interaction significantly ($P<0.0009$) reduced shoot blight infection. This interaction was not observed with ARY-4016-06 or any other treatment.

ACKNOWLEDGEMENTS

The author would like to acknowledge his advisor Dr. M Babadoost for the introduction to applied plant pathology and the opportunity to refine many skills. I am grateful for the diverse trials on multiple crops and the experience gained. Also thanks to Dr. Carl Bradley, Dr. Joanne Chee-Sanford, and Dr. Frank Zhao for serving on the graduate committee and sharing advice and expertise. In addition, the author also appreciates support from Dr. George W. Sundin and Gayle C. McGhee from Michigan State University for sharing research techniques and providing control isolates.

The author thanks many colleagues for support: Abbasali ‘Ali’ Ravanlou, for sharing techniques and suggestions; Sita Thapa, for assistance during “crunch time” and her cheerful attitude; John Fleischmann, for shouldering responsibility; Yussef Nickpeyma, for reminders on life’s priorities; Jeremy Shaffer, Bryan Warsaw, and Ken Horn who provided moral and material support in numerous field trials; Ruth Green and Heather Lash, who made greenhouse research enjoyable; Dr. Terry Niblack, Dr. Leslie Domier, Ursula Reuter-Carlson, and Nancy McCoppin, for graciously sharing laboratory equipment; and Dianne Carson, Julie Price, Sandy Osterbur, and other staff members of the Department of Crop Sciences.

The author also appreciates participating Illinois apple growers for permitting us to roam their orchards searching for fire blight. The author particularly acknowledges Randy Graham (Curtis Orchard) for writing a letter of support that aided in receiving a graduate student grant, Tom Swartz for towing my truck on a Sunday morning, and Kurt Christ (Christ Orchards) for

graciously donating pears for the pathogenicity tests. This research was in part financially supported by NCR-SARE. Any other support not specifically mentioned was equally appreciated.

TABLE OF CONTENTS

CHAPTER 1 ……1

INTRODUCTION ……1

THESIS OBJECTIVES ……8

TABLES AND FIGURES ……9

LITERATURE CITED ……12

CHAPTER 2 ……18

STATUS OF STREPTOMYCIN-RESISTANT *ERWINIA AMYLOVORA* IN ILLINOIS APPLE ORCHARDS……18

MATERIALS AND METHODS ……19

RESULTS ……25

DISCUSSION ……28

TABLES AND FIGURES ……32

LITERATURE CITED ……45

CHAPTER 3 ……47

EVALUATION OF STREPTOMYCIN-ALTERNATIVE COMPOUNDS FOR MANAGEMENT OF FIRE BLIGHT DISEASE OF APPPLE……47

MATERIALS AND METHODS ……48

RESULTS ……54

DISCUSSION ……57

TABLES AND FIGURES ……61

LITERATURE CITED ……70

CHAPTER 1

INTRODUCTION

Apple (*Malus* x *domestica* Borkh.) and Pear (*Pyrus* spp.) are major pome crops worldwide. In 2009, commercial apple production in the United States (US) was valued at 2.2 billion dollars (USDA/NASS, 2010). Illinois producers marketed 17,963 metric tons (39.6 million pounds) of apples in both 2008 and 2009 (USDA/NASS, 2010). Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al., is a serious disease of apple worldwide and particularly in the midwestern US. Yield losses to *E. amylovora* and control costs in the US are estimated at 100 million dollars per year (Norelli et al., 2009). Fire blight has become more destructive over the past twenty years, due to an increase of high density plantings and preference shifting to more susceptible cultivars (Cooley et al., 2008). In 2009, twenty apple cultivars accounted for 90% of US apple production (Economic Research Service, 2010). Of the top twenty cultivars in 2009, ten were highly susceptible to fire blight (representing 35.6% of total US apple tonnage), eight susceptible (representing 30.7% of total US apple tonnage), and two were moderately resistant (representing 24.4% of total US apple tonnage).

Erwinia amylovora

Erwinia amylovora, a member of the Enterobacteriaceae family, is a gram-negative, rod-shaped bacterium (3 x 0.5-1.0 μm) (Beer, 1997). Colonies of *E. amylovora* grow at 5-30ºC, with an optimum temperature of 27ºC (Beer, 1997). Moist conditions with humidity greater than 60%

favor reproduction of the pathogen (Kado, 2010). Species in 39 genera of the Rosaceae family have been reported susceptible to *E. amylovora* (van der Zwet et al., 2012b).

History of fire blight

In 1780, fire blight was observed on pears and quince in New York's Hudson River Valley (Coxe, 1817; Denning, 1794; Kado, 2010). Subsequently, the disease was identified in other states and after 1826 it was considered a destructive disease (Kado, 2010). Fire blight occurred widely in Illinois by the 1840s and reported in California in 1887 (Kado, 2010; van der Zwet et al., 2012a). Three Illinois scientists conducted initial research to understand the development of fire blight. In 1870, E.S. Hull transmitted the disease by tissue grafts. In 1879, J.B. Turner reported transmission of fire blight by grafting knife. In 1880, T.J. Burill presented an "epoch-making contribution in the field of plant pathology" by referring to the disease as "fire blight" and the "anthrax of fruit" (van der Zwet et al., 2012c). By 1883, T.J. Burrill believed the disease was caused by a bacterium, which was confirmed by J.C. Arthur in 1885 by completing Koch's postulate. A series of fire blight epidemics from 1910 to 1930 shifted commercial apple and pear production from New York to the Midwest and then settling to the drier climate of the west coast (Kado, 2010). *E. amylovora* was reported in British Columbia in 1911, New Zealand in 1919, throughout Northern Europe in the 1960s, and was considered distributed worldwide by 1997 (Kado, 2010).

Symptoms and signs of fire blight (*Erwinia amylovora*)

The disease caused by *E. amylovora* is named fire bight because symptoms on hosts have a burnt or scorched appearance. Oozing of bacterial cells, the sign of the pathogen, is commonly

observed on infected tissues under humid, moist conditions. In apples and pears, six phases of infection occur, which are known as: blossom blight, shoot blight, fruit blight, trauma blight, rootstock blight, and cankers. Shoot infection (Fig. 1.2), usually originating from blossom infection (Fig. 1.1), results in the Sheppard's crook symptom followed by gray-green discoloration of the shoot (Beer, 1997). Infected fruits (Fig. 1.3) usually remain small, shriveled, and dark when infected at early stages. Fruit infections following hail or insect damage develop red, brown, or black lesions; and ooze clear, milky, red to brown, and glassy when dry (Beer, 1997). Cankers (Fig. 1.4) form on the truck, scaffold branches, and limbs. Cankers become the overwintering location for the bacterium and can expand to girdle portions of the tree. Rootstock blight (Fig. 1.5) occurs when the rootstock become infected and rapidly leads to death of the entire tree.

Disease cycle of fire blight

E. amylovora overwinters at the edges of cankers and in spring, when temperatures exceed 18.3ºC, the bacterium rapidly multiply and becomes the source of primary inoculum (Cooley et al., 2008). Rain splash, wind, and insects spread the bacteria onto the blossoms, leaves, and shoots (Fig. 1.6). Infection of plant tissues takes place through natural openings and wounds. Bacteria multiply, invade, and kill host tissue. Secondary cycles of the disease occur as the bacteria are disseminated by rain splash, wind, and insects. Three factors determine the severity of fire blight each season: (i) presence of virulent pathogen in the orchard, (ii) conducive weather conditions (e.g., warm temperatures with moisture), and (iii) host cultivar susceptibility.

Management of fire blight

Control of fire blight requires year-round comprehensive management practices. The most effective control of the disease is avoiding susceptible cultivars (e.g., Jonathan, Gala, Idared, Rome Beauty) and susceptible rootstocks (e.g., M.9 and M.26). Newly infected shoots should be removed throughout the growing season by cutting 20 cm below the visible margin of infection (Beer, 1997). Piercing and sucking insects, such as aphids and leafhoppers, should be controlled to prevent wounding and possible pathogen movement. A higher degree of insect control is required for fire blight control than necessary for preventing insect damage to the tree (Beer, 1997). Following trauma events, such as wind or hail storms, applications of a bactericide may be warranted. During winter pruning, cankers harboring the pathogen should be removed. In early spring, at the 'silver tip' growth stage of apple, it is important to apply copper at high concentrations to all trees in the orchard. Copper reduces the bacterial population potential to build up on buds and bark before bloom (Cooley et al., 2008). As bloom approaches, the potential for *E. amylovora* infections increases when certain temperature and moisture requirements are met. A bactericide, traditionally streptomycin, is applied during bloom to prevent the bacterium from entering through the stigma and infecting the plant.

Disease prediction models of fire blight

Weather monitoring sensors, such as Watch Dog 1000 Micro Station (Spectrum Technologies; Plainfield, IL), can be placed in the orchard and used in conjunction with computer programs such as MARYBLYT or Cougarblight. These forecasting systems monitor weather conditions, assesses fire blight infection risk, and help growers determine the appropriate timing of bactericide applications. For instance, the MARYBLYT model predicts blossom infection when

all of the following conditions are met: (1) open flowers with petals intact; (2) accumulation of at least 110 degree hours > 18.3ºC from full pink; (3) dew, rain ≥ 0.25 mm current day or ≥ 2.5 mm rain the previous day; and (4) an average daily temperature of ≥ 15.6ºC (Steiner, 1990).

Streptomycin resistance of *Erwinia amylovora*

Since the 1950s, streptomycin has been the primary bactericide used for fire blight control and has been highly effective. However, in 1971, the first detection of streptomycin-resistant (SmR) *E. amylovora* occurred in California (Miller and Schroth, 1972). The first case of streptomycin-resistance occurred in the western US and is believed to have been caused by growers applying streptomycin throughout the growing season when infection was unlikely to occur (Jones and Schnabel, 2000). Currently, SmR *E. amylovora* are widespread throughout the western US, Michigan, Canada, and Israel and have been isolated in New York (Bartels, 2012; Kleitman et al., 2004; Loper et al., 1991; McGhee et al., 2011; Russo et al., 2008; Sholberg et al., 2001).

In 1983 and 1984, SmR (>500 mg/L) *E. amylovora* was reported to be localized in three neighboring orchards in west-central Missouri (Shaffer and Goodman, 1985). In 1989 in Indiana, three *E. amylovora* isolates from Morgan and Daviess counties were classified as "SmR" (5-10 mg/L on 5% sucrose nutrient agar) (Shoeib and Pecknold, 1991). However, there has been no further reports of SmR in any of these regions.

During 2003-2009, in Michigan, two genotypes of SmR isolates were collected: (i) *strA-strB* gene, encoding phosphtranserase enzymes modifying streptomycin to a non-bactericidal form, and (ii) a point mutation of ribosomal S12 protein, RspL. (McGhee et al., 2011). McGhee et al.

(2011) discovered that *strA-strB* genes harbored on transposon Tn5393 made up 98.7% SmR isolates in Michigan; where as, RspL mutations dominate other streptomycin resistant regions. This indicates that in Michigan, SmR likely moved from SmR non-target orchard bacteria via horizontal gene transfer on Tn5393 into *E. amylovora*, but apparently this transfer does not occur readily because only two different insertion sites were detected (McGhee et al., 2011).

Streptomycin-alternative antibiotics and biocontrol agents

Although alternatives to streptomycin for fire blight control have been researched for some time, available agricultural bactericides are generally considered less effective, unless SmR populations are present. Oxytetracycline (e.g., Mycoshield 17WP) is bacteriostatic compound inhibiting *E. amylovora* growth (McManus and Jones, 1994; Norelli and Gilpatrick, 1982). Kasugamycin (e.g., Kasumin 2L) is an antibiotic in the same class as streptomycin, but has a different mode of action (Copping and Duke, 2007; McGhee and Sundin, 2011). Recent field data suggest Kasumin 2L is suitable for use in regions with SmR (Adaskaveg et al. 2011; McGhee and Sundin, 2011); however, its use currently is restricted.

In the eastern US (Michigan, New York, Virginia), biocontrol agents (e.g., *Pseudomonas fluorescens* A506, *Pantoea agglomerans* C9-1, *P. agglomerans* E325, and *Bacillus subtilis* Serenade) have been evaluated for control of fire blight, but results have been inconsistent and less effective than streptomycin when used as the sole control agent (Sundin et al., 2009). However, integrating biocontrol agents with streptomycin allowed a reduction in streptomycin applications, which may help reduce selection pressure for SmR development (Sundin et al., 2009).

Growth regulators

Growth regulator, prohexadione-calcium (e.g. Apogee 27.5WG), has been utilized to help reduce shoot blight severity and is an important tool in areas with SmR resistant areas (McGrath et al., 2009; Norelli et al., 2003). In multiple studies, prohexadione-calcium applications have decreased shoot blight, formation of overwintering cankers, and in some cases even blossom blight (Miller and Yoder, 2012). McGrath et al. (2009) reported that bacterial populations are not reduced in Apogee treated strikes and hypothesized the growth regulator induces a physical barrier in shoot-cell tissue stopping the systemic spread of the pathogen.

Cultivar and rootstock resistance to fire blight

After many years of research and breeding there is still no commercial apple cultivar with complete-resistance to *E. amylovora*. However, there are marked differences among cultivars and how they respond to fire blight infection as shown in Table 1.1 (van der Zwet et al., 2012c). Furthermore, cultivars (i.e., scions) have been shown to vary in susceptibility depending on the rootstock to which grafted (van der Zwet et al., 2012c). The scion-rootstock interaction has been attributed to earlier flower production and may also alter scion physiology making the scion more susceptible (van der Zwet et al., 2012c). In the future, development of resistant cultivars and rootstocks may be greatly enhanced with genome and QTL data generated by the Genome Database for Rosacae project.

Important aspects for Illinois apple growers

Fire blight historically has been important in Illinois. In 1909, there were 9.9 million apple trees of bearing age in Illinois (Pickett, 1916). A fire blight outbreak in 1914 was responsible for

apple and pear crop losses estimated at 1.5 million dollars (Pickett, 1914). Another blight epidemic was reported in southern Illinois in 1930, reducing the crop by at least 30% (Anderson, 1924-1936). In northern Illinois, fire blight was reported to be severe in 1938 (Anderson, 1939).

THESIS OBJECTIVES

In 2008 and 2009, fire blight was severe and widespread throughout Illinois, particularly in western parts of the state. Some growers hypothesized SmR strains of *E. amylovora* might be present in Illinois. Russo et al. (2008) concluded that "streptomycin is the most effective antibiotic for use on apple and is likely to remain as such; therefore, it is imperative to identify cases of antibiotic-resistance early, before the bacterial populations become established." The main goal of this study was to determine if streptomycin resistant populations could be detected in Illinois. The specific objectives of this project were:

(i) To evaluate Illinois *E. amylovora* populations for streptomycin resistance

(ii) To evaluate Illinois *E. amylovora* response to copper and streptomycin-alternatives

TABLES AND FIGURES

Table 1.1. Popular commercial apple cultivars, listed by relative fire blight susceptibility ratings[a].

Least susceptible	Moderately susceptible	Most susceptible
Arkansas Black	Alkmene	Abbondanaza
Boskoop	Baldwin	Beacon
Delicious (Red)	Ben Davis	Berlepsch
Glockenapfel	Cortland	Burgundy
Haralson	Elstar	Cox's Orange Pippin
Jamba	Empire	Fuji
Jugol	Fiesta	Gala
Liberty	Golden Delcious	Gloster
Maigold	Granny Smith	Goldparmane
Mantet	Gravenstein	Idared
Nova	Grimes Golden	Ingrid Marie
Ontario	Jerseymac	James Greive
Priam	Jonafree	Jonagold
Prima	Jonamac	Jonathan
Priscilla	Macoun	Klarapfel
Quinte	McIntosh	Lodi
Redfree	Melrose	Mollies Delicious
Sir Prize	Monroe	Morgenduft
Splendor	Mutsu	Niagara
Winesap	Northwestern Greening	Nittany
	Oldenburg	Northern Spy
	Royal Gala	Paulared
	Spartan	Rhode Island Greening
	Stayman	Rome Beauty
	Summer Rambo	Twenty Ounce
		Tydeman's Early
		Wayne
		Wealthy
		Winter Banana
		Yellow Newton
		Yellow Transparent
		York Imperial

[a] Adapted from "Utilizing Host Resistance to Fire Blight." Table 19. (van der Zwat et al., 2012b)

Fig. 1.1. Blossom blight

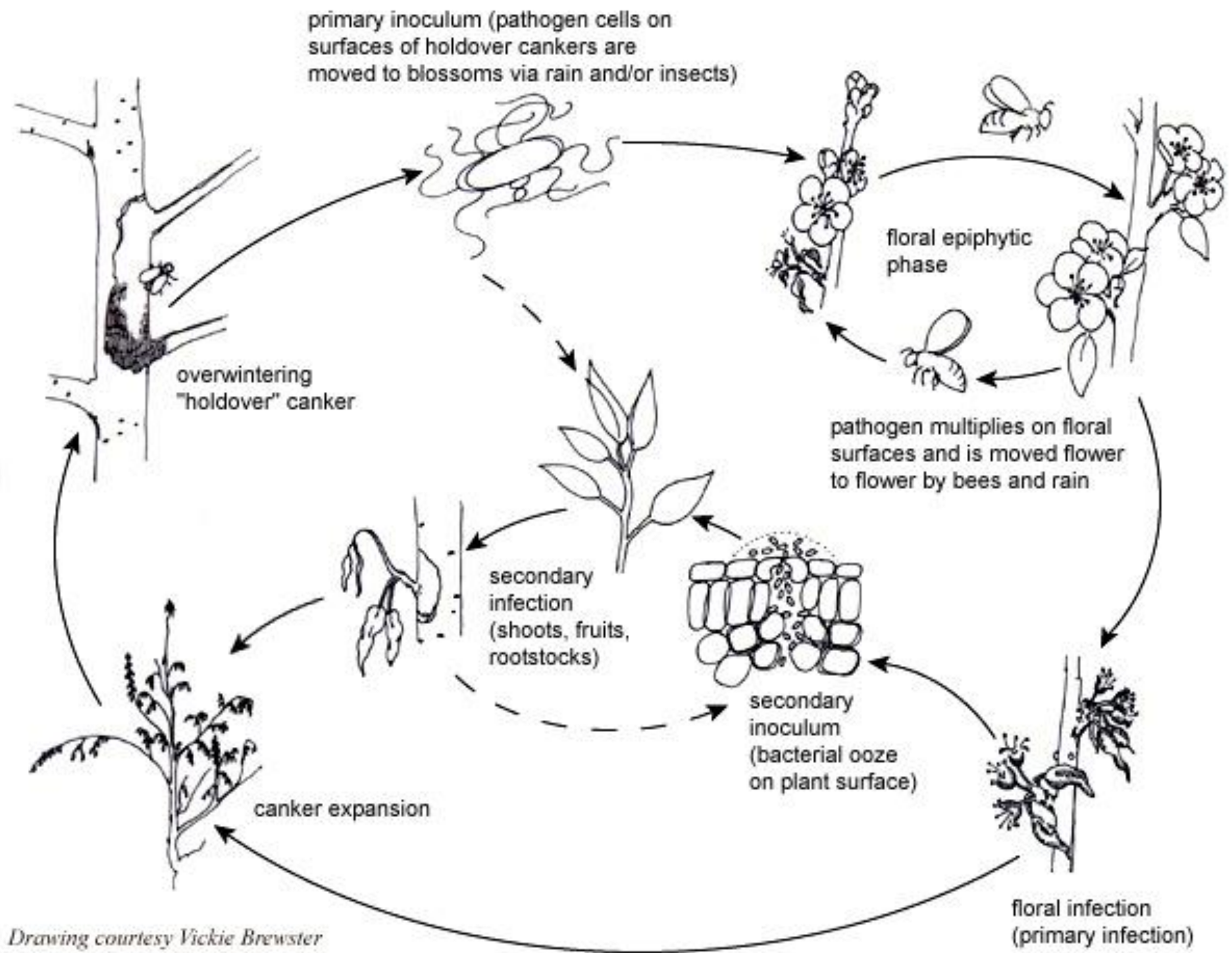
Fig. 1.2. Shoot blight (Courtesy of M. Babadoost)

Fig. 1.3. Fruit blight (Courtesy of R.L. Jones)

Fig. 1.4. Cankers (Courtesy of J.L. Norelli)

Fig. 1.5. Rootstock blight (Courtesy of M. Babadoost)

Fig. 1.6. Disease cycle of fire blight.



Reproduced from Johnson, K.B. 2000. Fire blight of apple and pear. *The Plant Health Instructor*. DOI: 10.1094/PHI-I-2000-0726-01 *Updated 2005.*

LITERATURE CITED

Adaskaveg, J.E., Forster, H., and Wade, M.L. 2011. Effectiveness of kasugamycin against *Erwinia amylovora* and is potential use for managing fire blight of pear. Plant Dis. 95:448-454.

Anderson, H.W. 1924-1936. Seek pear resistant to destructive fire blight. Ill. Agric. Exp. Stn. Annu. Rep. 37:134-135; 38:152-153; 40:230-231; 41:263-264; 43:234-236; 44:238-239; 45:199-200; 4:214-215: 48:251-252.

Anderson, H.W. 1939. Fire blight in 1938: fire blight in Illinois. Plant Dis. Rep. 23:34.

Bartels, Rebecca. 2012. Strep-Resistant Fire Blight Found in New York. American Fruit Grower. 132 (2). Pp. 21.

Beer, S.V. 1997. Fire Blight. Pp 61-63 in: Compendium of Apple and Pear Diseases, 3rd ed. A.L Jones and H.S. Aldwinckle, eds. APS Press, St. Paul, MN.

Bereswill, S., Jock, S., Bellemann, P., and Geider, K. 1998. Identification of *Erwinia amylovora* by growth morphology on agar containing copper sulfate and by capsule staining with lectin. Plant Dis. 82:158-164.

Billing, E., Crosse, J.E., and Garrett, C.U.E. 1960. Laboratory diagnosis of fire blight and bacterial blossom blight of pear. Plant Pathol. 9:19-25.

Chiou, C.S., and Jones, A.L. 1993. Nucleotide sequence analysis of a transposon (Tn*5393*) carrying streptomycin resistance genes in *Erwinia amylovora* and other gram negative bacteria. J. Bacteriol. 175:732-740.

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

Cooley D.R., Autio W.R., Clements J.M., Cowgill W.P., and Spitko R. 2008. Annual fire blight management programs for apples. University of Massachusetts Extension.
url: http://www.umass.edu/fruitadvisor/factsheets/F-133.pdf

Copping, L.G., and Duke, S.O. 2007. Natural products that have been used commercially as crop protection agents. Pest Manag. Sci. 63:524-554.

Coxe, W. 1817. Pears. Pp 175-176 in: A View of the Cultivation of Fruit Trees. M. Carey & Son, Philadelphia.

Denning, W. 1794. On the decay of apple trees. Trans. N.Y. Soc. Prom. Agric. Arts Manuf. 2:219-222.

Economic Research Service. 2010."U.S. Apple Statistics." USDA, accessed 3/2012, http://usda.mannlib.cornell.edu.

Jock, S., Rondoni, B., Gillings, M., Kim., W.-S., Copes, C., Merriman, P., and Geider, K. 2000. Screening of ornamental plants from the botanic gardens of Melbourne and Adelaide for the occurrence of *Erwinia amylovora*. Australas. Plant Pathol. 29:251-258.

Jones, A.L., and Schnabel, E.L. 2000. The development of streptomycin-resistant strains. Pp 235-251 in: Fire Blight: The Disease and Its Causative Agent, *Erwinia amylovora*. J.L. Vanneste ed. CABI Publishing, New York, NY.

Kado, C.I. 2010. Rapid Necrotizing Diseases. Pp 79-84 in: Plant Bacteriology. APS Press, St. Paul, MN. 336 pp.

Kleitman, F., Manulis, S., Kritzman, G., Oppenheim, D., Zilberstaine, M., and Shtienber, D. 2004. Use of a diagnostic medium for in situ determination of the response of *Erwinia amylovora* strains to bactericides. Phytoparastica 32:127-131.

Lindow, S.E., McGourty, G., and Elkins, R. 1996. Interactions of antibiotics with *Pseudomonas fluorescens* strain A506 in the control of fire blight and frost injury to pear. Phytopathology 86:841-848.

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

McGhee, G., Guasco, J., Bellomo, L., Blumer-Schuette, S., Shane, W., Irish-Brown, A., and Sundin, G.W. 2011. Genetic analysis of streptomycin-resistant (SmR) strains of *Erwinia amylovora* suggests that dissemination of two genotypes is responsible for the current distribution of SmR *E. amylovora* in Michigan. Phytopathology 101:182-191.

McGhee, G., and 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 101:192-204.

McGrath, M. J., Koczan, J. M., Kennelly, M. M., and Sundin, G. W. 2009. Evidence that prohexadione-calcium induces structural resistance to fire blight infection. Phytopathology 99:591-596.

McManus, P.S., and Jones, A.L. 1995. Detection of *Erwinia amylovora* by nested PCR and PCR-dot-blot and reverse-blot hybridizations. Phytopathology 85:618-623.

McManus P.S. and Jones, AL. 1994. Epidemiology and genetic analysis of streptomycin resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. Phytopathology 84:627–33.

McManus, P.S., Stockwell, V.O., Sundin G.W., and Jones A.L. 2002. Antibiotic use in plant agriculture. Ann. Rev. Phytopath. 40:443–65.

Miller, S.S., and Yoder, K.S. 2012. Plant Growth Regulators. Pp 249-255 in: Fire Blight: History, Biology, and Management. van der Zwet, T., Orolaza-Halbrendt, and N. Zeller, W. eds. APS Press, St. Paul, MN. 421pp.

Miller, T.D., and Schroth, M.N. 1972. Monitoring the epiphytic population of *Erwinia amylovora* on pear with selective medium. Phytopathology 62:1175-82.

Norelli, J.L., and Gilpatrick, J.D. 1982. Techniques for screening chemicals for fire blight control. Plant Dis. 66:1162-65.

Norelli J.L., Farrell R.E., Bassett C.L., Baldo A.M., Lalli D.A., Aldwinckle H.S., and Wisniewski M.E. 2009. Rapid transcriptional response of apple to fire blight disease revealed by cDNA suppression and subtractive hybridization analysis. Tree Genetics & Genomes. 5:27-40.

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

Pickett, B.S. 1914. The blight of apples, pears, and quinces. Ill. Agric. Exp. Stn. Circ. 172.

Pickett, B.S. 1916. Field Experiments in Spraying Apple Orchards. Ill. Agric. Exp. Stn. Circ. 185.

Russo, N.L., Burr, T.J., Breth, D.I., and Aldwinckle, H.S. 2008. Isolation of streptomycin resistant isolates of *Erwinia amylovora* in New York. Plant Dis. 92:714-718.

Schaad, N.W., Jones B.J., and Chun, W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria, 3rd ed. APS, St. Paul, MN. 373 pp.

Shaffer, W.H., and Goodman, R.N. 1985. Appearance of streptomycin-resistant *Erwinia amylovora* in Missouri apple orchards. Phytopathology 75:1281-1281.

Shoeib, A.A., and Pecknold, P.C. 1991. Streptomycin resistance in *Erwinia amylovora* and *Pseudomonas syringae* pv. *Papulans* in pear and apple orchards of Indiana. Alexandria J. Agric. Res. 36:61-75.

Sholberg, P.L., Bedford, K.E., Haag, P., and Randall, P. 2001. Survey of *Erwinia amylovora* isolates from British Columbia for resistance to bactericides and virulence on apple. Can. J. Plant Pathol. 23:60-67.

Steiner, P.W. 1990. Predicting apple blossom infections by *Erwinia amylovora* using the MARYBLYT model. Acta Hort. 273:139-148.

Sundin, G.W., Werner, N.A., Yoder, K.S., and Aldwinckle, H.S. 2009. Field evaluation of biological control of fire blight in the eastern United States. Plant Dis. 93:386-394.

United States Department of Agriculture, National Agricultural Statistical Service. 2010. Crop Values 2009 Summary. url:http://usda.mannlib.cornell.edu/usda/current/CropValuSu/CropValuSu-02-19-2010_new_format.pdf

United States Department of Agriculture, National Agricultural Statistical Service. 2010. Illinois Farm Report. Vol 31, No. 2. url:http://www.nass.usda.gov/Statistics_by_State/Illinois/Publications/Farm_Reports/2010/ifr1002.pdf

van der Zwet, T., Orolaza-Halbrendt, N., and Zeller, W. 2012a. Early Theories and Discoveries Regarding Fire Blight. Pp 3- 14 in: Fire Blight: History, Biology, and Management. APS Press, St. Paul, MN. 421pp.

van der Zwet, T., Orolaza-Halbrendt, N., and Zeller, W. 2012b. Symptomatology of Fire Blight and Host Range of *Erwinia amylovora*. Pp 45-64 in: Fire Blight: History, Biology, and Management. APS Press, St. Paul, MN. 421pp.

van der Zwet, T., Orolaza-Halbrendt, N., and Zeller, W. 2012c. Utilizing Host Resistance to Fire Blight. Pp 227-247 in: Fire Blight: History, Biology, and Management. APS Press, St. Paul, MN. 421pp.

CHAPTER 2

STATUS OF STREPTOMYCIN-RESISTANT *ERWINIA AMYLOVORA* IN ILLINOIS APPLE ORCHARDS

This study was conducted to determine if streptomycin-resistant strains of *Erwinia amylovora* could be detected in apple orchards in Illinois. Statewide surveys were conducted in 2010, 2011, and 2012, and 117, 129, and 170, *E. amylovora* isolates were collected, respectively, from 19 counties. None of the 416 *E. amylovora* isolates tested were streptomycin-resistant at 50 mg/L. However, nine non-*E. amylovora* isolates contained both a *str*A-*str*B streptomycin-resistance gene and IS1133 on transposon Tn5393, which could be a potential source of streptomycin-resistance for Illinois *E. amylovora* in the future. Differences in the streptomycin-sensitivity of 133 *E. amylovora* isolates were evaluated. Boone ($P<0.0014$) and Champaign ($P<0.0028$) county isolates were less sensitive to streptomycin than the state mean. In contrast, Calhoun ($P<0.0143$), Madison ($P<0.0002$), and Union ($P<0.0056$) county isolates were more sensitive to streptomycin than the state mean.

MATERIALS AND METHODS

Field survey and sample collection

During 2010-2012, fire blight incidence and severity were assessed in Illinois apple orchards (Fig 2.1). In 2010, 2011, and 2012, 24, 35, and 39 apple orchards, respectively, were surveyed. At each orchard, 20 symptomatic shoots and 50 asymptomatic blossoms were collected for isolation of the bacterium (*Erwinia amylovora*). No symptomatic blossoms were observed. Each infected shoot was cut 20 cm below visible necrotic tissue and pruners were dipped in 95% ethanol between cuts. Collected blossoms and shoots were placed in plastic bags, kept on ice during transportation, and stored at 4ºC in the laboratory within 12 h of collection. In 2010, shoots were collected during 3 June and 22 July. In 2011, blossoms were collected during 10 April and 16 May; and shoots collected during 7 June and 22 June. In 2012, blossoms were collected during 23 March and 9 April; and shoots collected during 25 May and 8 June.

Isolation, purification, and maintenance of *E. amylovora*

Luria-Bertani (LB) medium was used for isolation and maintenance of *E. amylovora* (McGhee et al., 2011). LB medium was amended with cycloheximde (50 mg/L) to make LBch. LBch was amended with 50 mg/L of streptomycin (Agrimycin 17 WP, Nufarm Americas Inc., Burr Ridge, IL) to make LBcham. All collected sample tissues were processed within 36 h from collection.

Five blossoms were placed in a sample mesh bag (Agida, Inc., Elkhart, IN) with 5 ml of sterilized 0.5x PBS buffer, on ice. Blossom tissue was macerated for approximately 20 seconds or until tissue was well ground using a tissue homogenizer (Agdia, Inc., Elkhart, IN) attached to

a drill press. For isolation of the bacterium from shoots, a 10-15 mm piece of shoot tissue was cut immediately below the necrotic lesion and bark was removed using sterile scalpel and tweezers. Leaf petioles adjacent to a lesion were also used for isolation. The shoot tissues were macerated as described for blossoms. Then underneath a sterile hood, extract from ground tissue was diluted (3:1) in sterilized 0.5x PBS buffer. Using a bacterial loop, the suspension was streaked to single colonies on LBch and LBcham. The plates were incubated at 27ºC for 48 h (12 h light/12 h dark). White colonies characteristic of *E. amylovora* on LBch and all white colonies growing on LBcham were sub-cultured, transferred to cryogenic vials containing 15% glycerol, and stored at -20ºC and -80ºC for further studies.

PCR identification of *E. amylovora*

The identity of each *E. amylovora* isolate was confirmed by using polymerase chain reaction (PCR). PCR was conducted using identification primers AJ75 (5'CGC ATT CAC GGC TTC GCA GAT 3') and AJ76 (5'AAC CGC CAG GAT AGT CGC ATA 3') targeting ubiquitous plasmid pEA29 in *E. amylovora* (McManus and Jones, 1995). Using a sterile pipette tip, a colony was suspended in 100-µl of sterile-distilled water (SDW). Then, 0.5-µl of the colony suspension was added to 10-µl DNA-free water, 1-µl of each primer AJ75 and AJ76 (10 pmol/ 1-µl) and 12.5-µl Gotag Green Master Mix 2x (Promega Corporation, Madison, WI) for a final reaction volume of 25-µl. Cycling conditions for PCR (Model PCT-200, MJ Research Inc., Waltham, MA) included initial denaturation at 94ºC for 5 min; 37 cycles of denaturation for 1 min, annealing at 52ºC for 2 min, and extension at 72ºC for 2 min; followed by 15 min final extension at 72ºC and kept at 4ºC until electrophoresis. The PCR product was run on 1% agarose gel containing ethidium bromide at 100 volts for 60 minutes, and photographed under UV light.

The bands were compared to known streptomycin-sensitive *E. amylovora* isolate, MK1, and streptomycin-resistant *E. amylovora* isolate, Ea88, supplied by George Sundin (Michigan State University). The process was repeated at least once for each isolate.

16S rRNA sequencing of bacterial isolates

Some bacterial isolates were selected for 16S rRNA sequencing. PCR was performed to amplify the 16S rRNA genes from the extracted DNA using the primers 27F (5'AGA GTT TGA TCM GGC TCA G 3') and 1492R (5' GGT TAC CTT GTT ACG ACT T 3') (Lane, 1991). Cycling conditioning included initial denaturing at 95°C for 2 min; 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min; followed by a final extension at 72°C for 5 min. PCR products were purified with the Wizard SV gel and PCR Clean-Up system (Promega, Madison, WI). Purified 16S rRNAs were single-end sequenced from 27F at the University of Illinois Core DNA Sequencing Facility. Edited sequences were compared using the BLASTn database (http://www.ncbi.nlm.nih.gov).

Virulence of *E. amylovora* isolates

An immature pear fruit assay (Billing, et al., 1960) was used to determine isolate pathogenicity and virulence of 48 *E. amylovora* isolates from Illinois. Immature 'Seckel' pear fruit were collected from Peoria County in early August and stored at 4ºC. Pears were surface disinfested in a 75% ethanol solution, rinsed in SDW, and dried on a sterile lab bench. Using a sterile pipette, a 15-μl suspension (10^7 CFU/ml) of each isolate was inoculated into each pear fruit (approximately 2 mm deep). *E. amylovora* isolates MK1 and Ea88 (a less virulent isolate (McGhee et al., 2011)) and SDW were used as positive and negative controls. Inoculated and

control pears were placed in a humidity box and stored at 27ºC in darkness. Necrotic lesions were considered a positive reaction of *E. amylovora* pathogenicity and lesion diameter was measured perpendicularly 4 and 7 days post inoculation. To re-isolate the bacterium from pear fruit, tissue from the edge of the necrotic lesion was removed with a sterile scalpel, suspended in SDW, and shaken vigorously. The suspension was serially diluted onto LB medium and colony development was observed after 48 h and compared to original cultures. Each virulence experiment had five replications (e.g., five pears per isolate). All experiments were repeated once.

The virulence tests were complimented with the tobacco leaf assay (Kado, 2010) to determine the presence of hrp genes. Leaves, on actively growing *Nicotiana benthamiana* in a greenhouse, were used to evaluate isolates for a hypersensitive response. Using a sterilized needle-less syringe, 1 ml of *E. amylovora* suspension (10^7 CFU/ml) of each isolate was injected into the sub-axial surface between secondary leaf veins. SDW was used as a negative control. Plants were examined five days post-injection for hypersensitive response (rapid death of cells in the local region of injection). Each isolate was tested twice, with three replications (three sub-leaf areas on three separate leaves).

Screening *E. amylovora* isolates for streptomycin-resistance

Each *E. amylovora* isolate was recovered from glycerol storage by culturing on LB medium for 48 h LB and LB amended with 50 mg/L of streptomycin (Agrimycin 17) were prepared and bacterial colonies were streaked onto replicate Petri plates. A sub-set of 85 isolates was selected and additionally screened at 25 mg/L streptomycin (Agrimycin 17). Streptomycin-susceptible isolate, MK1, and streptomycin-resistant isolate, Ea88, were used as negative and positive

controls. Colony development was rated at 24 and 48 h. Each isolate was evaluated in two experiments with three replications per experiment.

Determining streptomycin sensitivity by inhibition zone

One hundred thirty-three *E. amylovora* isolates, collected during 2010-2012, were recovered from glycerol storage and tested for streptomycin sensitivity. Each isolate was grown separately on LB medium in Petri plates for 48 h. Colonies were washed in SDW and the suspension was adjusted to 10^7 CFU/ml using a spectrophotometer (Smart Spec 3000; Bio-Rad, Philadelphia, PA). Using a sterilized bent glass rod, a 50-µl bacterial suspension was spread onto LB agar in a Petri plate. Using methods described by Loper et al. (1991) and Russo et al. (2008), a 12-mm filter-disc paper was soaked in a 100 mg/L solution of streptomycin (Agrimycin 17), briefly dried, and placed onto the agar surface. Streptomycin-susceptible isolate (MK1) and streptomycin-resistant isolate (Ea88) were used as positive and negative controls. Clear zones of inhibition were measured at 24 and 48 h. Width of zone with no bacterial colony was considered a measure of sensitivity of *E. amylovora* to streptomycin. In our studies, an inhibition zone 1mm or greater on LB medium was considered a sensitive isolate response. Each isolate was tested twice. Each test had three replications and each replication had four measured zones (e.g., separate filter discs).

Streptomycin-sensitivity of *E. amylovora* in amended liquid medium

Ten Illinois *E. amylovora* isolates were compared in LB broth, Miller (Arcos Organics, Geel, Belgium) amended with 0, 0.5, 1, 2, 3, 4 and 5 mg/L streptomycin (Agrimycin 17). Additionally, the same isolates were compared in nutrient broth (BD, Sparks, MD) amended with 0, 0.25, 0.5,

and 1 mg/L streptomycin (Agrimycin 17). The test was conducted using sterile 24-well plates (351147; BD Falcon, Franklin Lakes, NJ). Twenty microliters from each bacterial suspension (10^8 CFU/ml) was added to 2 ml of broth per well and incubated on a shaker at 28ºC for 18 h. Bacterial cell density was assessed using a spectrophotometer (Smart Spec 3000; Bio-Rad, Philadelphia, PA) at OD 600. The bacterial cell density from the streptomycin amended cultures were compared to un-amended cultures and expressed as a percent. Each isolate was tested twice with four replications.

Non-*Erwinia amylovora* bacteria in Illinois orchards

While testing blossom and shoot samples for the presence of *E. amylovora*, other bacteria were collected, such as *Pseudomonas* spp., *Pantoea* spp. *Curtobacterium* spp., and *Bacillus* ssp., that were capable of growing at 50 mg/L streptomycin. One hundred nineteen non-*E. amylovora* isolates were screened for the presence of IS1133 (associated with Tn5393) using primer set IS1133-F ('GCG TGA TGC AGT TCG CAT AGC') and IS1133-R ('CAT ACG CGG CCT ACC ATA GCT') (McGhee et al., 2011).

The IS1133 region of nine isolates was sequenced using primer IS1133-F, as described previously for 16S rRNA, except the cycling parameters were modified as described below. These nine non-*E. amylovora* isolates were also screened with primers strab01-F ('TGG TGT CCC GCA ATG CCG TC') and strab01-R ('CCC GGA TCG GGA GAA GGG CA') to amplify a portion of *str*B region on Tn5393. PCR cycling conditioning included initial denaturing for 4 min at 94°C; 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C (IS1133 primer set) or 60°C (strab01 primer set) for 30 s, and extension at 72°C for 2.5 min; followed by a final

extension at 72°C for 7 min. The PCR product was run on 1% agarose gel containing ethidium bromide at 100 volts for 60 min, and photographed under UV light. The bands were compared *E. amylovora* isolates MI 5-1 and W4 which are known to contain Tn5393 (McGhee et al, 2011). The process was repeated twice for each isolate.

Data analysis

All statistical analysis was performed using SAS 9.3 (SAS Institute Inc. Cary, NC). Homogeneity of variances was tested using the Brown-Forsyth test and normality was observed. Percent cell multiplication values from liquid amended media data were analyzed using ANOVA and least significant difference (LSD) mean separation procedures in PROC GLM at alpha=0.05. Pear pathogenicity data was square root transformed [$\sqrt{(x + 1/6)}$] before analysis. Data were analyzed using analysis of variance (ANOVA) in PROC MIXED and macro pdmix800 (Saxton 1998) was used to indicate mean letter separation (similar to an LSD in PROC GLM). Pear pathogenicity data were separated at alpha=0.01 and zone of inhibition data at alpha=0.05.

RESULTS

Occurrence of fire blight in Illinois apple orchards

No blossom blight was observed during this study. *E. amylovora* was isolated from two of 214 and one of 221 blossom samples in 2011 and 2012, respectively. In 2010, 2011, and 2012, percentage of orchards with shoot blight was 91.7, 74.3, and 82.0%, respectively. Central Illinois and northern Illinois had the highest and lowest incidence of shoot blight infection, respectively (Table 2.1.a). Calhoun County had the highest shoot blight incidence and severity in all three

years. The percentage of infected trees (within a fire blight occurrence) ranged from <1 to 100% (average 25.7%) in 2010; from <1 to 100% (average 24.6%) in 2011; and from <1 to 100% (average 28.4%) in 2012. Percentage of infected shoots (within individual trees with fire blight) ranged from <1 to 80% (average 6.0%) in 2010; from <1 to 60% (average 5.7%) in 2011; and from <1 to 70% (average 4.9%) in 2012. Apple cultivar 'Jonathan' had the highest incidence/severity of shoot blight in Illinois, while 'Red Delicious' had the lowest incidence/severity (Table 2.1.b).

Streptomycin-resistant *Erwinia amylovora*

E. amylovora was isolated from 117 of 218, 127 of 274, and 169 of 227 symptomatic shoots in 2010, 2011, and 2012, respectively (Tables 2.2, 2.3, and 2.4). Overall, *E. amylovora* isolates were collected in 39 individual orchards in 19 counties during 2010-2012 (Fig 2.1). None of the 416 *E. amylovora* isolates tested were streptomycin-resistant at 50 mg/L. Additionally, none of 85 isolates developed colonies on culture medium with 25 mg/L streptomycin.

Virulence of *E. amylovora* isolates

All 48 isolates of *E. amylovora* tested produced lesions on immature 'Seckel' pear fruit and produced a hypersensitive reaction on tobacco leaves. *E. amylovora* was recovered from the necrotic lesions of fruit. Previously determined less virulent isolate Ea88 (McGhee et al., 2011) did not produce lesions on pear fruit. Isolate 7711 from Woodford County produced the largest lesion (Table 2.5). Overall, isolates from all counties were similar in virulence ($P<0.1928$). However, isolates from St. Clair county were more virulent than the state mean on immature pear fruit ($P<0.0091$).

Differences in streptomycin sensitivity

Orchards within a county had similar streptomycin-sensitivity, with a few exceptions (Calhoun 2011, 2012; Jackson 2012; St. Clair 2012) (Table 2.6). However, differences in streptomycin sensitivity at the county level were detected (Table 2.7). When zone of inhibition data from 2010, 2011, and 2012 were combined for analysis, Boone ($P<0.0014$) and Champaign ($P<0.0028$) county isolates were less sensitive to streptomycin than the state mean. In contrast, Calhoun ($P<0.0143$), Madison ($P<0.0002$), and Union ($P<0.0056$) county isolates were more sensitive to streptomycin than the state mean. Results from ten individual isolates in liquid medium tests also supported these results (Table 2.8). Calhoun isolate 6081 was the most sensitive isolate with 0% cell multiplication at 3 mg/L streptomycin; while, Boone isolate 7101 was the least sensitive isolate with 16.1% cell multiplication at 5 mg/L streptomycin. The remaining nine isolates were capable of cell multiplication between 0 and 4 mg/L streptomycin. However, at 1 mg/L streptomycin, rate of cell multiplication of all Illinois isolates was significantly ($P<0.05$) reduced when compared to the streptomycin-resistant control (Ea88). In amended nutrient broth, none of the 10 selected Illinois *E. amylovora* isolates were capable of cell multiplication at 0.25, 0.5, or 1 mg/L streptomycin.

Occurrence of non-*Erwinia amylovora* bacteria with Tn5393 with IS1133

Nine of the 119 non-*E. amylovora* streptomycin-resistant bacteria tested produced a band similar to the positive controls containing IS1133 (Fig. 2.2). Sequencing of the IS1133 region for six of six (100%) isolates produced a 400 bp sequence nearly identical to the portion of the streptomycin-resistant controls. Seven of the nine non-*E. amylovora* isolates developed bands similar to the control isolates using primers strab01-F and strab01-R amplifying the *str*B region

of Tn5393 (Fig. 2.3). In summary, seven non-*E. amylovora* isolates developed on streptomycin (50 mg/L) amended LB medium; and molecular analysis confirmed these isolates to contain both IS1133 and *str*B on Tn5393. Three isolates (5897, 7234, and 5174) were identified as *Pantoea agglomerans* and one isolate (6114) as *Psuedomonas graminis* based on 16s rRNA sequences (450 to 600 bp) when compared in BLASTn.

DISCUSSION

Erwinia amylovora was isolated from only three of 435 blossom samples tested during 2011 and 2012. Although 2011 and 2012 seasons were not highly conducive for blossom infection, the results support our observations in the past 13 years that blossom blight is not common in apple orchards in Illinois. The reasons for not having blossom blight in Illinois are: (i) streptomycin is still an effective bactericide in Illinois, (ii) almost all apple growers apply streptomycin during bloom; (iii) and unfavorable temperature/moisture conditions for production and dispersal of *E amylovora* population.

Using the MARYBLYT disease prediction model, it was observed in warm seasons individual flowers can be open for a few days, but without sufficient moisture escapes infection (Steiner, 1990). Steiner (1990) also observed, that in cool seasons when average temperatures exceed 4.4ºC, but rarely exceeds 18.3ºC, flowers may mature before sufficient inoculum has built up. Both of these conditions were observed in Illinois in 2011 and may explain the low *E. amylovora* recovery from blossoms. In 2011, the low recovery could be explained in southern Illinois due to sudden extremely warm conditions, the bloom period was unusually short and dry from 9-11

April. In central and northern Illinois, temperatures were unseasonably warm then rapidly became cool at bloom. In Champaign, bloom period was unusually extended under cool conditions from 20 April to 11 May.

Since blossom infection is not a common phase of fire blight in Illinois apple orchards, how fruit blight, shoot blight, and root-stock blight occurs widely in this state is not clearly known. One possible explanation for higher incidence of shoot blight is that rain storms are common during April and May in Illinois. We believe that injuries caused by windstorms on new shoots is the main factor in initiating fruit and shoot blight, as streptomycin applications have ceased by the end of the bloom period. Further epidemiological study is required to determine what, if any, role blossom blight infection plays in Illinois apple orchards.

All *E. amylovora* isolates tested were virulent on immature pear. Overall, virulence of isolates from different counties were not significantly different than the state mean. However, a single isolate from Woodford and isolates from St. Clair county produced larger lesions in the immature fruit assay. Although *E. amylovora* infection is most common in apple orchards in Calhoun and Jersey counties, isolates from these counties were not more virulent than the state mean at $P<0.6998$ and $P<0.5585$, respectively. Thus, the higher incidence and severity of *E. amylovora* infection in Calhoun and Jersey counties is not due to increased virulence in *E. amylovora* populations. The wide occurrence of fire blight in these counties may be due to irregularity in timely-applications of copper and streptomycin.

While no streptomycin-resistance *E. amylovora* isolate was detected in Illinois during 2010-2012, it was observed that isolates collected from Boone and Champaign counties were less sensitive to streptomycin than the state average. In contrast, *E. amylovora* isolates from Calhoun, Madison, and Union counties were more sensitive to streptomycin than the state average. While, slight streptomycin-sensitivity differences were determined in some Illinois counties, they are not expected to affect the effectivness of streptomycin applications in the field. In our studies, no bacterial multiplication was observed in nutrient broth amended with 0.25 mg/L streptomycin. In comparison, *E. amylovora* isolates in California were reported to have reduced bacterial multiplication rate beginning at 0.30 mg/L streptomycin in nutrient agar (Adaskaveg, et al., 2011).

The detection of Tn5393 is common in bacteria in orchards that have received streptomycin applications (McGhee et al., 2011; Sundin and Bender, 1995; Sundin and Bender, 1996). However, this study is the first confirmation of bacterial isolates containing *str*A-*str*B paired with IS1133 in the Illinois orchards. We detected both the *str*B gene and the insertion sequence IS1133 on Tn5393 on nine non-*E. amylovora* isolates in Illinois (Calhoun, Jersey, McHenry, St. Clair, and Union counties). The insertion sequence IS1133 is required for expression of *str*A-*str*B resistant genes via promoting transcription of streptomycin-resistance genes (Chiou and Jones, 1993; McGhee et al., 2011; Sundin and Bender, 1995; Sundin and Bender, 1996). The finding in this study indicates that all the pieces are in place for the chance of acquisition of Tn5393 streptomycin-resistance into Illinois *E. amylovora* populations.

Results from the 2010-2012 surveys indicated that no streptomycin-resistant *E. amylovora* was detected in Illinois. However, it is very possible a streptomycin-resistant strain will be introduced or develop in the state. We encourage growers to be vigilant when ordering nursery stock from states with streptomycin-resistant populations, since the streptomycin-resistance occurrence in New York was likely related to imported nursery stock (Russo et al., 2008). It is also believed that the original occurrence of streptomycin-resistance was due to repeated unnecessary streptomycin applications (Jones and Schnabel, 2000); therefore, we encourage growers to use less frequent, but high rate antibiotic applications. A key component of this strategy is applying antibiotics only when disease monitoring models, such as MARYBLYT, indicate infection is likely. We also must emphasize the continued importance of integrated disease management approaches, such as planting less susceptible cultivars/rootstocks, proper pruning, and timely applications of copper in early spring. Since streptomycin has been the sole antibiotic used in Illinois orchards for decades, further research is warranted on integrating oxytetracycline, kasugamycin, and biocontrol agents into the spray rotation to prevent or delay streptomycin-resistance.

TABLES AND FIGURES

Table 2.1.a. Occurrence of shoot blight (*Erwinia amylovora*) in Illinois apple orchards during 2010-2012.

	Fire blight in orchards and infected orchard blocks											
	2010				2011				2012			
	Orchards		Blocks [a]		Orchards		Blocks		Orchards		Blocks	
Region	Total (no.)	With blight [no.(%)[b]]	Incidence (%)[c]	Severity (%)[d]	Total (no.)	With blight [no. (%)]	Incidence (%)	Severity (%)	Total (no.)	With blight [no. (%)]	Incidence (%)	Severity (%)
Northern	4	2(50.0)	1.0	< 0.1	6	5(83.3)	3.0	0.6	6	1(16.7)	< 0.1	< 0.1
Central	16	16(100)	32.6	7.9	19	14(73.7)	35.7	7.0	19	16(84.2)	33.3	4.5
Southern	4	4(100)	10.5	1.4	10	7(70.0)	19.3	6.9	15	15(100)	23.4	5.4
Illinois	24	22(91.7)	25.7	6.0	35	26(74.3)	24.6	5.7	39	32(82.0)	28.4	4.9

[a] Incidence and severity data were collected in a non-random fashion. Surveying was focused at blocks (50 trees) within orchards with a history of fire blight, susceptible cultivars, and/or localized disease.
[b] Percentage of orchards where shoot blight symptoms were observed.
[c] If shoot blight was observed in an orchard, percent trees with shoot blight symptoms within a diseased area.
[d] If shoot blight was observed in an orchard, percent infected shoots within each tree (evaluated as percent of tree canopy).

Table 2.1.b. Occurrence of shoot blight (*Erwinia amylovora*) on apple cultivars when disease is present in Illinois in 2012.

Cultivar [a]	Locations [b] (no.)	Fire blight occurrence Incidence [c] (%)	Severity [d] (%)
Jonathan (no streptomycin application)	1	100.0	42.50
Jonathan (with streptomycin application)	14	36.4	1.50
Golden (no streptomycin application)	1	60.0	0.60
Golden (with streptomycin application)	10	27.8	0.82
Red Delicious (with streptomycin application)	8	6.25	0.09

[a] Cultivars were identified by growers or by tree phenotype.
[b] Locations were selected as diseased areas within an orchard and adjacent cultivars were assessed for shoot blight incidence and severity.
[c] Percent trees with fire blight symptoms; an average of 10 trees per location.
[d] Percent infected shoots within each tree.

Table 2.2. Occurrence of streptomycin-resistant *Erwinia amylovora* in Illinois apple orchards in 2010.

County	Shoot samples tested (no.)	Tested *Erwinia amylovora* isolates [a] (no.)	Streptomycin-resistant [b] *Erwinia amylovora* isolates (no.)
Boone	15	5	0
Calhoun	36	35	0
Champaign	6	6	0
Jersey	29	16	0
Kane	11	0	-
Macoupin	9	8	0
Madison	10	0	-
Marshall	16	15	0
McHenry	6	0	-
Peoria	11	0	-
Putnam	10	10	0
Sangamon	6	4	0
St. Clair	42	15	0
Winnebago	4	0	-
Woodford	7	3	0
Total	218	117	0

[a] *E. amylovora* isolates were identified using primers AJ75 (5'CGC ATT CAC GGC TTC GCA GAT 3') and AJ76 (5'AAC CGC CAG GAT AGT CGC ATA 3').

[b] Colony development was assessed on Luria-Bertani agar amended with 50 mg/L streptomycin (Agrimycin 17) after 48 h.

Table 2.3. Occurrence of streptomycin-resistant *Erwinia amylovora* in Illinois apple orchards in 2011.

County	Shoot samples tested (no.)	Tested *Erwinia amylovora* isolates[a] (no.)	Streptomycin-resistant[b] *Erwinia amylovora* isolates (no.)
Boone	14	9	0
Calhoun	53	25	0
Champaign	17	18	0
Dekalb	0	-	-
Jackson	22	8	0
Jefferson	0	-	-
Jersey	30	11	0
Kane	5	2	0
Macoupin	0	-	-
Madison	11	5	0
Marshall	17	8	0
McHenry	8	0	-
Peoria	17	7	0
Piatt	1	0	-
Pope	0	-	-
Putnam	7	3	0
Randolph	0	-	-
St. Clair	34	16	0
Union	18	12	0
Winnebago	5	0	-
Woodford	15	3	0
Total	274	127	0

[a] *E. amylovora* isolates were identified using primers AJ75 (5'CGC ATT CAC GGC TTC GCA GAT 3') and AJ76 (5'AAC CGC CAG GAT AGT CGC ATA 3').

[b] Colony development was assessed on Luria-Bertani agar amended with 50 mg/L streptomycin (Agrimycin 17) after 48 h.

Table 2.4. Occurrence of streptomycin-resistant *Erwinia amylovora* in Illinois apple orchards in 2012.

County	Shoot samples tested (no.)	Tested *Erwinia amylovora* isolates[a] (no.)	Streptomycin-resistant[b] *Erwinia amylovora* isolates (no.)
Boone	1	0	-
Calhoun	31	27	0
Champaign	13	8	0
Clinton	8	8	0
Dekalb	0	-	-
Jackson	23	14	0
Jefferson	5	6	0
Jersey	32	18	0
Kane	1	0	-
Macoupin	10	7	0
Madison	8	8	0
Marion	5	4	0
Marshall	8	5	0
McHenry	0	-	-
Peoria	4	3	0
Piatt	4	3	0
Pope	5	1	0
Putnam	2	2	0
Sangamon	8	7	0
St. Clair	36	33	0
Union	15	12	0
Winnebago	1	0	-
Woodford	7	3	0
Total	227	169	0

[a] *E. amylovora* isolates were identified using primers AJ75 (5'CGC ATT CAC GGC TTC GCA GAT 3') and AJ76 (5'AAC CGC CAG GAT AGT CGC ATA 3').

[b] Colony development was assessed on Luria-Bertani agar amended with 50 mg/L streptomycin (Agrimycin 17) after 48 h.

Table 2.5. Virulence assessment of Illinois *Erwinia amylovora* isolates on immature pear fruit.

	2010		2011	
County	Isolate[a]	Lesion diameter (mm)[b]	Isolate[a]	Lesion diameter (mm)[b]
Boone	193b	0.3 hi[c]	6951	5.7 a-c[c]
	196b	0.3 hi	7101	5.7 a-d
	-	-	7071	5.2 a-d
Calhoun	49c	3.8 a-f	7651	5.5 a-d
	45d	2.2 a-h	7621	5.0 a-e
	15a	1.7 c-i	6081	4.4 b-g
	68d	1.7 a-i	7571	3.9 b-g
	26d	1.3 d-i	7751	3.2 d-g
	38c	1.3 f-i	-	-
Champaign	2a	1.7 a-i	4802	1.8 g
Jackson	-	-	5341	3.9 b-g
Jersey	75a	4.3 ab	6002	4.3 b-g
	30b	3.0 a-e	7671	3.1 c-g
	28b	2.6 a-f	7531	3.0 fg
Kane	-	-	4951	2.5 e-g
Madison	-	-	5472	4.9 a-f
Macoupin	10c	4.5 a-c	-	-
Marshall	108d	3.7 a-c	7841	5.3 b-f
	118b	2.6 a-g	7501	4.1 b-g
	111c	0.1 g-i	-	-
Peoria	-	-	7461	4.9 b-f
Putnam	170c	1.2 f-i	7691	4.8 b-f
Sangamon	84d	1.9 b-i	-	-
St. Clair	93c	4.6 a	5461	6.6 ab
	89a	4.1 a-c	5752	5.3 a-d
	128c	3.7 a-d	-	-
	127b	3.0 a-g	-	-
Union	-	-	5152	3.2 c-g
	-	-	5232	3.2 c-g
Woodford	165e	2.5 a-g	7711	8.9 a
	-	-	7731	5.0 b-f
	MK1[d]	0.9 e-i	MK1	2.6 g

Table 2.5. Continued.

[a] Isolates were evaluated in two separate experiments.
[b] Using a sterile pipette, a 15-µl suspension (10^7 CFU/ml) of each isolate was inoculated into each immature 'Seckel' pear fruit (approximately 2 mm deep). Pears were placed in a humidity box and stored at 27ºC. Necrotic lesions were measured perpendicularly 7 days post inoculation.
[c] A larger lesion was considered more virulent. Data were transformed [$\sqrt{(x + 1/6)}$] and analyzed using PROC MIXED with macro pdmix800; in each column values with a letter in common are not significantly different from each other (P<0.01).
[d] MK1: a pathogenic *Erwinia amylovora* isolate.

Table 2.6. Streptomycin-sensitivity of *Erwinia amylovora* isolates collected from Illinois apple orchards during 2010-2012.

County	2010[a]			2011			2012		
	Isolates[b] tested (no.)	Inhibition zone radius (mm)[c] Range	Inhibition zone radius (mm)[c] Orchard mean	Isolates tested (no.)	Inhibition zone radius (mm) Range	Inhibition zone radius (mm) Orchard mean	Isolates tested (no.)	Inhibition zone radius (mm) Range	Inhibition zone radius (mm) Orchard mean
Boone	3	3.2-3.5	3.3 d[d]	2	1.6-2.6	2.1 e-h	-	-	-
	-	-	-	2	1.7-2.0	1.8 h	-	-	-
Calhoun	2	4.2-4.3	4.3 ab	1	-	3.6 a-e	1	-	4.6 a
	3	4.2-4.3	4.3 a	3	3.1-3.6	3.3 a-d	1	-	3.7 a-g
	1	-	4.6 a	1	-	1.7 gh	1	-	3.0 c-j
	4	4.0-4.4	4.3 a	3	3.1-4.8	3.6 ab	1	-	3.0 d-j
	2	3.5-4.0	3.7 a-d	-	-	-	1	-	3.9 a-f
Champaign	-	-	-	2	2.7-3.1	2.9 a-h	1	-	3.3 a-j
	1	-	3.8 a-d	2	1.8-3.0	2.4 c-h	1	-	2.1 ij
	-	-	-	-	-	-	1	-	2.5 g-j
	-	-	-	-	-	-	1	-	2.0 j
Clinton	-	-	-	-	-	-	1	-	3.0 c-j
Jackson	-	-	-	2	2.1-2.7	2.4 c-h	1	-	3.1 c-j
	-	-	-	-	-	-	2	2.5-2.9	2.7 g-j
	-	-	-	-	-	-	2	3.9-4.1	4.0 a-d
Jefferson	-	-	-	-	-	-	2	2.1-3.4	2.9 f-j
Jersey	2	3.2-3.5	3.3 cd	2	3.6-3.6	3.6 a-c	2	2.5-3.1	2.8 f-j
	2	4.1-4.1	4.1 a-c	3	2.6-3.1	2.9 a-h	2	3.0-3.6	3.3 b-i
	-	-	-	-	-	-	1	-	3.4 a-i
Kane	-	-	-	1	-	2.7 a-h	-	-	-
Macoupin	3	3.8-4.9	4.4 a	-	-	-	2	2.3-3.0	2.6 h-j
Madison	-	-	-	1	-	4.4 a	2	4.0-4.5	4.2 ab
Marion	-	-	-	-	-	-	2	2.8-3.3	3.0 f-j
Marshall	2	3.4-4.3	3.8 a-d	-	-	-	-	-	-
	6	3.1-4.4	3.7 b-d	3	2.7-3.2	3.0 a-h	2	2.7-3.5	3.1 d-j
Peoria	-	-	-	2	2.6-2.8	2.7 b-h	2	2.8-3.2	3.0 f-j
Piatt	-	-	-	-	-	-	2	3.5-3.9	3.7 a-f
Putnam	2	3.9-4.1	4.0 a-d	2	1.7-2.7	2.2 d-h	1	-	2.0 j

Table 2.6. Continued.

County	2010[a] Isolates[b] tested (no.)	2010 Inhibition zone radius (mm)[c] Range	2010 Orchard mean	2011 Isolates tested (no.)	2011 Inhibition zone radius (mm) Range	2011 Orchard mean	2012 Isolates tested (no.)	2012 Inhibition zone radius (mm) Range	2012 Orchard mean
Sangamon	2	3.2-3.6	3.4 cd [d]	-	-	-	2	3.2-3.8	3.5 a-h
St. Clair	5	3.4-4.7	4.2 ab	3	2.4-3.4	3.1 a-g	1	-	3.4 a-j
	-	-	-	2	2.4-4.6	3.5 a-e	2	2.3-2.6	2.5 ij
	4	4.1-4.3	4.2 a	-	-	-	2	3.4-3.5	3.5 b-h
Union	-	-	-	2	3.0-3.6	3.3 a-e	1	-	4.4 a-c
	-	-	-	-	-	-	2	3.6-4.3	4.0 a-e
Woodford	-	-	-	1	-	2.0 d-h	-	-	-
	2	3.7-4.1	3.9 a-d	1	-	1.9 d-h	1	-	2.3 ij
MK1 (Streptomycin-sensitive)			4.4 -			2.6 -			2.9 -
Ea88 (Streptomycin-resistant)			0.0 -			0.0 -			0.0 -

[a] Isolates were tested in three separate experiments.
[b] Each group of the isolates represents a separate orchard.
[c] A 50-µl of bacterial suspension (10^7 CFU/ml) was spread on Luria-Bertani agar, then a 12 mm filter disk was soaked in 100 mg/L solution of streptomycin (Agrimycin 17), briefly dried and placed on the agar surface. Zone of inhibition was measured after 48 h.
[d] Data were analyzed using PROC MIXED with macro pdmix800, in each column values with a letter in common are not significantly different from each other ($P<0.05$).

Table 2.7. Streptomycin-sensitivity of *Erwinia amylovora* populations in Illinois apple orchards during 2010-2012.

County	Isolates [a] tested (no.)	Inhibition zone radius (mm) [b]	*P* value compared to state mean [c]
Boone	7	2.4^{-} [d]	*P*<0.0014
Calhoun	25	3.5^{+}	*P*<0.0143
Champaign	9	2.6^{-}	*P*<0.0028
Clinton	1	3.0	*P*<0.8560
Jackson	7	3.1	*P*<0.9626
Jefferson	2	2.9	*P*<0.4742
Jersey	14	3.2	*P*<0.6900
Kane	1	3.2	*P*<0.9031
Macoupin	5	3.2	*P*<0.8728
Madison	3	4.4^{+}	*P*<0.0002
Marion	2	3.1	*P*<0.9806
Marshall	13	3.1	*P*<0.6079
Peoria	4	3.2	*P*<0.9780
Piatt	2	3.8	*P*<0.0964
Putnam	5	2.6	*P*<0.0256
Sangamon	4	3.4	*P*<0.5290
St. Clair	19	3.3	*P*<0.3828
Union	5	3.9^{+}	*P*<0.0056
Woodford	5	2.6	*P*<0.0442
Illinois	133	3.3	-

[a] Isolates were tested in three separate years and combined for analysis.

[b] A 50-µl of bacterial suspension (10^7 CFU/ml) was spread on Luria-Bertani agar, then a 12 mm filter disk was soaked in 100 mg/L solution of streptomycin (Agrimycin 17), briefly dried and placed on the agar surface. Zone of inhibition was measured after 48 h.

[c] Data were analyzed using PROC MIXED, the lsmean of each county was compared to the lsmean of all counties combined.

[d] $^{-}$ = less sensitive to streptomycin than the state mean at *P*<0.02, $^{+}$ = more sensitive to streptomycin than the state mean at *P*<0.02.

Table 2.8. Cell multiplication of *Erwinia amylovora* in streptomycin-amended Luria-Bertani (LB) Broth.

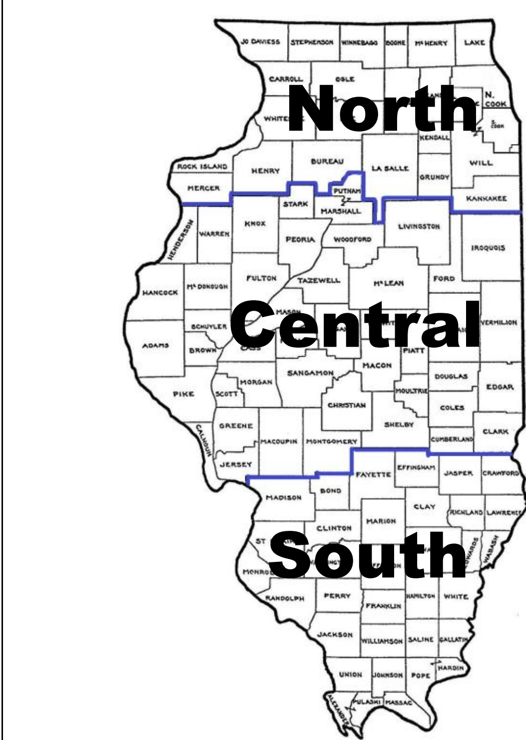
County	Isolate[a]	mg/L streptomycin (Agrimycin 17) in LB Broth												
		0	0.5		1		2		3		4		5	
	Ea88[b]	*100%*[c]	*94.6%*	*b-d*[d]	*100.2%*	*a*	*115.7%*	*a*	*105.6%*	*a*	*104.7%*	*a*	*99.7%*	*a*
Boone	7101	100	96.6	b-d	88.9	b	81.7	bc	70.8	b	44.6	b	16.1	b
Boone	6951	100	86.6	d	87.3	b	90.2	bc	28.4	e	0.8	g	0.0	c
Calhoun	68d	100	105.8	a-c	86.0	b	87.9	bc	41.1	d	10.1	de	0.0	c
Calhoun	6081	100	92.7	cd	88.7	b	27.8	d	0.0	g	0.0	g	0.0	c
Jersey	6002	100	100.5	a-d	89.3	b	77.2	bc	60.6	c	15.6	c	0.8	c
Madison	5471	100	99.2	a-d	88.6	b	48.6	d	10.1	f	1.3	fg	0.0	c
Marshall	118b	100	95.8	b-c	85.8	b	80.7	bc	27.6	e	6.1	ef	0.0	c
St. Clair	89a	100	104.6	a-c	88.4	b	73.4	c	30.0	e	7.5	de	0.0	c
Union	5232	100	110.1	ab	89.4	b	80.6	bc	37.2	d	8.1	de	0.0	c
Woodford	165e	100	113.8	a	88.3	b	98.4	ab	40.0	d	12.7	dc	0.0	c
LSD (P<0.05)			17.1		11.0		21.8		5.7		5.2		9.9	
Illinois mean			**100.6**		**88.1**		**74.6**		**34.6**		**10.7**		**1.7**	

[a] Isolates in streptomycin-amended Luria-Bertani Broth were compared to the un-amended culture at 18 h using OD 600 values measured by a spectrophotometer and expressed as a percent.

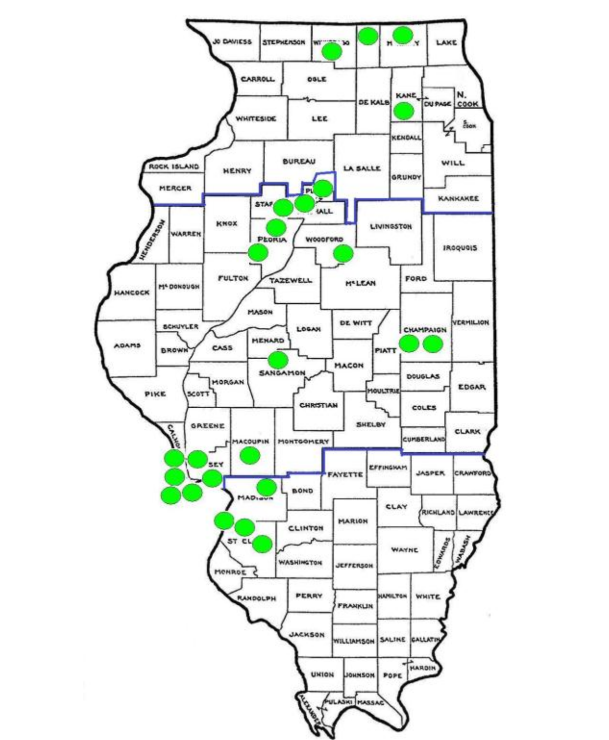
[b] Streptomycin-resistant control.

[c] A value of 100% indicates colony developmend was not reduced compared to the control.

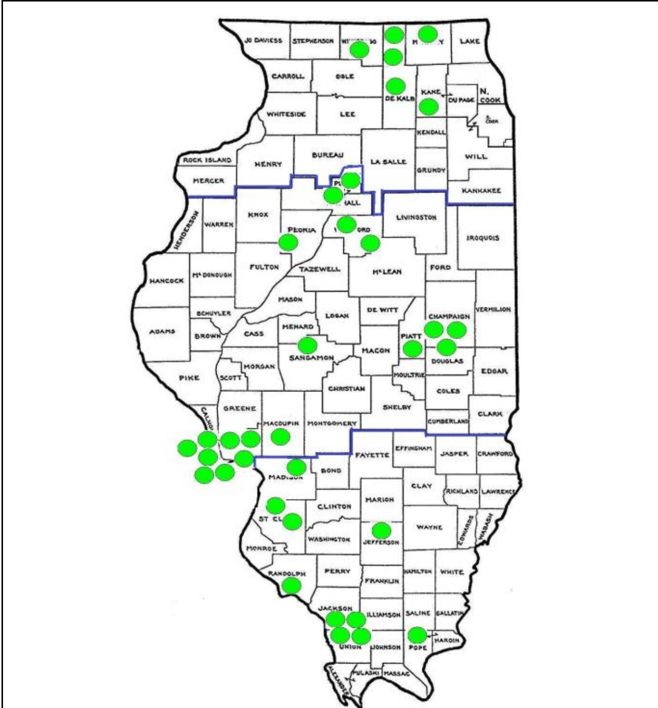
[d] According to Fishers Protected LSD ($P<0.05$) values in each column with a letter in common are not significantly different from each other.



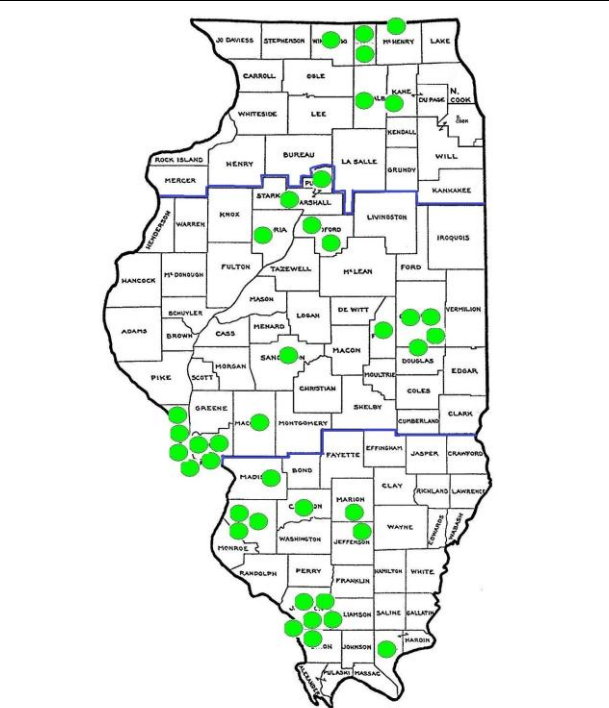
Illinois apple growing regions



24 orchards surveyed in 2010.



35 orchards surveyed in 2011.



39 orchards surveyed in 2012.

Fig. 2.1. Illinois apple orchards for surveyed for streptomycin-resistant *Erwinia amylovora*.

Fig. 2.2. Streptomycin-resistant non-*Erwinia amylovora* in Illinois apple orchards. Bacteria were screened with primers IS1133-F ('GCG TGA TGC AGT TCG CAT AGC') and IS1133-R ('CAT ACG CGG CCT ACC ATA GCT') to detect IS1133 on Tn5393. *E. amylovora* isolate 'W4' containing Tn5393 was used as positive control. Nine of 119 isolates screened produced similar bands to the positive control.

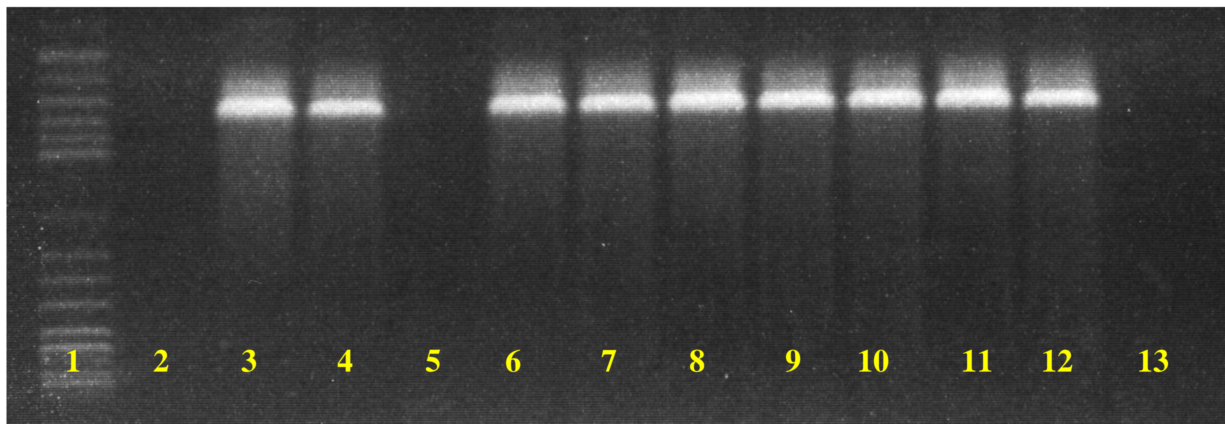


Fig. 2.3. Positive identification of strA-B genes in seven non-*Erwinia amylovora* bacterial colonies from Illinois apple orchards with primers strab01-F ('TGG TGT CCC GCA ATG CCG TC') and strab01-R ('CCC GGA TCG GGA GAA GGG CA'). Lane: (1) DNA ladder; (2) negative control [Mk1]; (3) 5897 [Jersey], (4) 7234 [McHenry], (5) 5374 (no band) [Jackson], (6) 6114 [Calhoun], (7) 5814 [St. Clair], (8) 6004 [Jersey], (9) 4264 [Calhoun], (10) 5174 [Union]; (11) and (12) positive controls [W4 and MI 5-1]; and (13) water negative control.

LITERATURE CITED

Adaskaveg, J.E., Forster, H., and Wade, M.L. 2011. Effectiveness of kasugamycin against *Erwinia amylovora* and its potential use for managing fire blight of pear. Plant Dis. 95:448-454.

Billing, E., Crosse, J. E., and Garrett, C. U. E. 1960. Laboratory diagnosis of fire blight and bacterial blossom blight of pear. Plant Pathol. 9:19-25.

Chiou, C.S., and Jones, A.L. 1993. Nucleotide sequence analysis of a transposon (Tn*5393*) carrying streptomycin resistance genes in *Erwinia amylovora* and other gram negative bacteria. J. Bacteriol. 175:732-740.

Jones, A.L and Schnabel, E.L. 2000. The development of streptomycin-resistant strains. Pp 235-251. In: Fire blight: the disease and its causative agent, *Erwinia amylovora*. J.L. Vanneste ed. CABI Publishing, New York, NY. 400 pp.

Lane, D.J. 1991. 16S/23S rRNA sequencing. Pp 115-175. In: Nucleic Acid Techniques in Bacterial Systematics. E. Stackebrandt and M. Goodfellow (eds). J.Wiley and Sons Ltd., Chichester, UK. 329 pp.

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

McGhee, G., Guasco, J., Bellomo, L., Blumer-Schuette, S., Shane, W., Irish-Brown, A., and Sundin, G.W. 2011. Genetic analysis of streptomycin-resistant (SmR) strains of *Erwinia amylovora* suggests that dissemination of two genotypes is responsible for the current distribution of SmR *E. amylovora* in Michigan. Phytopathology 101:182-191.

McManus, P.S., and Jones, A.L. 1995. Detection of *Erwinia amylovora* by nested PCR and PCR-dot-blot and reverse-blot hybridizations. Phytopathology 85:618-623.

Russo, N.L., Burr, T. J., Breth, D. I., and Aldwinckle, H. S. 2008. Isolation of streptomycin resistant isolates of *Erwinia amylovora* in New York. Plant Dis. 92:714-718.

Saxton, A.M. 1998. A macro for converting mean separation output to letter groupings in Proc Mixed. In Proc. 23rd SAS Users Group Intl., SAS Institute, Cary, NC, pp1243-1246.

Steiner, P.W. 1990. Predicting apple blossom infections by *Erwinia amylovora* using the MARYBLYT model. Acta Hort. 273:139-148.

Sundin, G.W., and Bender, C.L. 1995. Expression of the strA-strB streptomycin resistance genes in *Pseudomonas syringae* and *Xanthamonas campestris* and characterization of IS6100 in X. *campestris*. Appl. Environ. Microbiol. 61:2891-2897.

Sundin, G.W., and Bender, C.L. 1996. Dissemination of the strA-strB streptomycin resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. Mol. Ecol. 5:133-143.

CHAPTER 3

EVALUATION OF STREPTOMYCIN-ALTERNATIVE COMPOUNDS FOR MANAGEMENT OF FIRE BLIGHT DISEASE OF APPLE

During 2011-2012, laboratory and field trials were conducted to evaluate the efficacy of streptomycin-alternatives, including oxytetracycline, kasugamycin, *Bacillus subtilis*, *Pseudomonas fluorescens*, and prohexadione calcium for control of fire blight disease of apple (*Erwinia amylovora*). Kasugamycin products (Kasumin 2L and ARY-4016-06) significantly ($P<0.10$) reduced blossom infection in the orchard. In 2012, effectiveness of growth regulator prohexadione calcium (Apogee 27.5DF) in combination with streptomycin-alternatives were also evaluated. In our one-year experiment an Apogee x Kasumin 2L interaction significantly ($P<0.0009$) reduced shoot blight infection. All 84 *E. amylovora* isolates evaluated were sensitive to 0.16 mM copper sulfate, indicating copper compounds are still effective for management of fire blight disease in Illinois.

MATERIALS AND METHODS

Bacterial strains and culture preparation

Eighty-four *Erwinia amylovora* isolates collected from Illinois were selected for *in-vitro* evaluation of streptomycin-alternative compounds. Bacterial isolates were routinely cultured on Luria-Bertani (LB) medium (McGhee et al., 2011) for three days. Bacterial suspensions were prepared by washing Petri plates with sterile-distilled water (SDW) and the suspension was adjusted to 10^7 CFU/ml using a spectrophotometer (Smart Spec 3000; Bio-Rad, Philadelphia, PA). Also, Casitone-Yeast Extract (CYE) medium (1.7 g of casitone, 0.35g yeast extract, 2 g glucose, and 15 g agar per liter) was used for evaluation of copper compounds (Loper et al., 1991).

In-vitro evaluation of copper sulfate

Eighty-four *Erwinia amylovora* isolates collected from Illinois were selected for *in-vitro* evaluation of copper sulfate. CYE medium was amended with 0.08, 0.16, and 1.1 mM copper sulfate (Cuprofix Ultra 40DF; United Phosphorus, King of Prussia, PA). A 10-µl aliquot of bacterial suspension from each isolate was spotted onto the agar surface with a pipet tip. All experiments were designed as a randomized complete block design and included non-amended agar plates for controls. Colony development was assessed after 48 h and 72 h on CYE medium.

In-vitro comparison of copper compound formulations

Eleven *E. amylovora* isolates were used to evaluate effects of different copper formulations on colony development. CYE medium was amended with copper sulfate (Cuprofix Ultra 40);

copper hydroxide (Kocide 3000 40.1DF; Dupont, Wilmington, DE); cuprous oxide (Nordox 75 WG; Nordox Industrier AS, Oslo Norway); and a mixture of copper hydroxide/ copper oxychloride (Badge X2 45DF; Isagro USA, Morrisville, NC). All copper compounds were evaluated at 0 and 14 mg/L of metallic copper. Using a bacterial loop, colony suspensions (10^7 CFU/ml) were streaked onto agar on each Petri plate and colony development was assessed after four days. All tests were designed as a randomized complete block design and included non-amended agar for controls. Each isolate was tested twice with three replications (plates) per test.

Cuprofix Ultra 40, Kocide 3000 40.1DF, Nordox 75WG, and Badge X2 45DF were additionally evaluated in CYE broth (CYE medium without agar), with Illinois *E. amylovora* isolate 26D, at 0, 7, and 14 mg/L metallic copper. The test was conducted using sterile 24-well plates. Twenty microliters from a bacterial suspension (10^8 CFU/ml) was added to 2 ml of broth per well and incubated on a shaker at 28ºC for 18 h. Bacterial cell density was assessed using a spectrophotometer at OD600 and expressed as percent compared with the non-amended control. CYE medium only, and CYE medium with compounds only, and CYE medium with isolate only served as the negative and positive controls. Each compound was tested twice with four replications (individual wells) per test in a randomized complete block design.

***In-vitro* evaluation of oxytetracycline and kasugamycin**

Eighty-four *E. amylovora* isolates collected from Illinois were selected for *in-vitro* evaluation of antibiotics. LB medium (pH 7) was amended with oxytetracycline (Mycoshield 17 WP; NuFarm Americas Inc., Burr Ridge, IL) at 0, 50, 100, and 200 mg/L. Also, two formulations of kasugamycin (Kasumin 2L and ARY-4016-06; Arysta Life Science, Cary,

NC) were evaluated at 0, 25, 50, and 100 mg/L on amended LB medium with bacterial suspensions streaked on the agar surface. All experiments were designed as a randomized complete block design and included non-amended agar for controls. Colony development was assessed after 24 and 48 h.

***In-vitro* comparison of kasugamycin formulations in amended liquid medium**

Eleven Illinois *E. amylovora* isolates were compared in nutrient broth (pH 7) amended with kasugamycin (Kasumin 2L) and kasugamycin (ARY-4016-06) at 0, 3, 5, 10 mg/L. The test was conducted using sterile 24-well plates. Twenty microliters from each bacterial suspension (10^8 CFU/ml) was added to 2 ml of broth per well and incubated on a shaker at 28ºC for 18 h. Bacterial cell density was assessed using a spectrophotometer at OD600 and expressed as percent compared to the non-amended control. Each isolate was tested twice with four replications (individual wells) per test in a randomized complete block design.

***In-vitro* evaluation of biocontrol agents**

Biocontrol agents: *Bacillus pumilus* QST 2808 (Sonota ASO; Agraquest, Davis, CA), *Bacillus subtilis* QST 713 (Serenade Max; Agraquest, Davis, CA), *Pseudomonas fluorescens* A506 (BlightBan A506; Nufarm Americas Inc., Burr Ridge, IL), and the negative control, *Xanthamonas cucurbitae* 621, were evaluated *in-vitro* as follows. On day 0, 10-µl aliquots of biocontrol suspensions were spotted down the center of a 110-mm diameter Petri plate containing LB agar. On day 3, 10-µl aliquots of *E. amylovora* 2A suspensions were spotted adjacent to the established biocontrol colonies. On day 5, zones of inhibition were measured from the edge of the biocontrol colony to the closest *E. amylovora* colony. Each biocontrol agent

was tested twice with at least four replications (plates) per test in a randomized complete block design.

Field trials

Field experiments were conducted on ‘Jonathan’ apple at the University of Illinois Fruit Research Farm, Urbana, IL. *E. amylovora* inoculum was prepared from freshly growing colonies of native orchard isolate 2A and suspended in 0.5x PBS buffer at 10^8 CFU/ml (in 2011) and $5x10^6$ CFU/ml (in 2012). The bacterial suspension was transported on ice and applied when MARYBLYT disease forecasting logger (Spectrum Technologies, Plainfield, IL) indicated high risk of fire blight infection. All treatments and inoculations were applied using a gas powered backpack mist sprayer (SR400; Stihl, Virginia Beach, VA) at 1,407 liters/ha (150 gallons/acre). Data were collected on four sides of each tree from the upper, middle, and lower canopy. A total of 120 blossoms and 120 shoots were observed in each tree when possible.

Field evaluation in 2011

The 2011 experiment was designed as randomized complete block design with three blocks, eight treatments, and two spray-application times (24 h pre- and 24 h post-inoculation). Treatments included: control (no inoculum applied), control (inoculum applied), streptomycin (Agrimycin 17 Nufarm, Burr Ridge, IL) at 100 mg/L, oxytetracycline (Mycoshield) at 200 mg/L, and kausgamycin (Kasumin 2L and ARY-4016-06) at 100 mg/L. Biocontrol agents *Pseudomonas florescences* A506 (BlightBan A506) at 369 mg/L, and *Bacillus subtillus* QST713 (Serendade Max) at 525 mg/L were also included.

Cuprofix Ultra 40D (7.8 kg/ha) and dormant oil were applied to all trees on 31 Mar 2011. Apple tree growth development stages were full pink (18 April), 1% bloom (20 April), 40-60% bloom (25 April), and petal fall (11 May). The trees were inoculated with the pathogen on 26 April and 8 May. Pre-inoculation spray treatments were applied on 25 April and 7 May and post-inoculation sprays were applied on 27 April and 9 May. Treatments were also applied on 29 April. Blossom infection was evaluated on 12 May and 23 May; and shoot infection was evaluated on 10 June, 30 June, and 27 July.

Field evaluation in 2012

The 2012 experiment was designed as a split-plot in a randomized complete block design with four blocks, 11 main-plot treatments (antibiotics and biocontrol agents) and a split-plot, with or without growth regulator prohexadione calcium (Apogee 27.5DF; BASF Corporation, Research Triangle Park, NC) at 600 mg/L. The main plot treatments included: (i) control (inoculum applied); (ii) copper hydroxide (Kocide 3000) at 240 mg/L + mancozeb at 2,700 mg/L (Dithane 75DF; Dow AgroSciences LLC, Indianapolis, IN); (iii) streptomycin (Agrimycin 17) at 100 mg/L; (iv) oxytetracycline (Mycoshield) at 200 mg/L; (v) oxytetracycline (Mycoshield) at 200 mg/L+ copper hydroxide (Kocide 3000) at 240 mg/L; (vi) kausgamycin (ARY-4016-06) at 100 mg/L; (vii) kausgamycin (Kasumin 2L) at 100 mg/L; (viii) *Pseudomonas florescences* A506 (BlightBan A506 71) at 369 mg/L; (ix) *Bacillus subtillus* QST713 (Serenade Max 14.6) 525 at mg/L; (x) kausgamycin (Kasumin 2L) at 100 mg/L and (xi) streptomycin (Agrimycin 17) at 100 mg/L. Treatments i, ii, iii, iv, v, vi, and vii, were applied 24 h pre- and 24 h post-inoculation; biocontrol agent treatments viii and ix were applied 24 h pre- and 48 h pre-inoculation (to help encourage biological control agent establishment on the apple blossoms); and treatments x and xi

were applied 24 h post-inoculation only. All eleven treatment applications included a nonionic surfactant, Regulaid (Kalo, Inc., Overland Park, KS), at 125ml/100 liters.

Cuprofix Ultra 40D (7.8 kg/ha) and dormant oil were applied to all trees on 13 March 2012. Apple tree growth development stages were full pink (23 March), 1% bloom (26 March), 40-60% bloom (28 March), and petal fall (12 April). Split applications of Apogee were applied on 29 March and 12 April. The trees were inoculated on 30 March and 3 April. Main-plot treatments were applied on 29 March, 31 March, 2 April, and 4 April, respectively. Additional applications of Serenade Max and BlightBan A506 were applied on 28 March to help encourage biocontrol agent establishment. Blossom infection was evaluated on 17 April, 24 April, and 1 May; and shoot infection was evaluated on 8 May, 22 May, 5 June, and 3 July.

Data analysis

All statistical analysis was performed using SAS 9.3 (SAS Institute Inc. Cary, NC). Homogeneity of variances was tested using the Brown-Forsyth test and data were checked for normality. Data were square root transformed [$\sqrt{(x + 1/6)}$], when necessary, to meet valid assumptions. Data were analyzed using ANOVA in PROC MIXED and macro pdmix800 (Saxton 1998) was used to indicate mean letter separation (alpha=0.10 for field and alpha=0.05 for laboratory data). Blossom data in 2012 were analyzed as a covariate to account for uneven blossom distribution in each tree.

RESULTS

In-vitro evaluation of copper compounds

All 84 isolates tested developed colonies on CYE medium amended with 0.08 mM copper sulfate. At 0.16 mM and 1.1 mM copper sulfate, none of the isolates developed colonies (Table 3.1). Moreover, all copper formulations (e.g., copper sulfate, copper hydroxide, cuprous oxide, and copper hydroxide/copper oxychloride) at 14 mg/L metallic copper inhibited colony development on CYE medium and bacteria cell multiplication in CYE broth. At 7 mg/L metallic copper in CYE broth, copper hydroxide, copper sulfate, copper hydroxide/copper oxychloride, and cuprous oxide reduced cell multiplication of *E. amylovora* by 94.0, 92.8, 85.0, and 83.0%, respectively, compared to the un-amended control.

In-vitro evaluation of oxytetracycline and kasugamycin

Development of colonies of all 84 *E. amylovora* isolates were inhibited on LB medium amended with oxytetracycline at 50, 100, and 200 mg/L. Also, colony development of all *E. amylovora* isolates were inhibited at 100 mg/L kasugamycin (Kasumin 2L). However, the same concentration of active ingredient (100 mg/L kasugamycin) in ARY-4016-06 inhibited colony development of only 35 of 84 (42%) of the Illinois isolates tested (Table 3.2). Similarly, at 50 mg/L kasugamycin, Kausmin 2L inhibited 29 of 84 (35%) of isolates, while ARY-4016-06 at the same active ingredient rate, inhibited only 1 (<1%) of the isolates. At 25 mg/L kasugamycin neither formulation inhibited colony development; however, colony development was visually slower compared to the non-amended control.

A comparison of both kasugamycin formulations by measuring the optical density in amended liquid medium showed similar results as the solid media (Table 3.3). Compared to the un-amended controls at 18 h, mean colony growth was 7.1% and 31.1% with Kasumin 2L (10 mg/L kasugamycin) and ARY- 4016-06 (10 mg/L kasugamycin), respectively. Two *E. amylovora* isolates tested from Calhoun County were among the most kasugamycin-sensitive isolates. One isolate (7711) from Woodford County was the least-sensitive; but another isolate, (165E) also from Woodford county was more sensitive and comparable to other Illinois isolates.

In-vitro **evaluation of biocontrol agents**

The negative control, *Xanthamonas cucurbitae*, did not produce zones of inhibition in any test. *Pseudomonas fluorescens* (BlightBan A506), *Bacillus pumilus* (Sonota ASO), and *Bacillus subtilis* (Serenade Max), produced 8.2, 10.9 and 12.1 mm zones of inhibition, respectively. Statistically, the two *Bacillus* species performed the same (P<0.2076); and both *Bacillus* species produced significantly (P< 0.0023) larger zones of inhibition than *Pseudomonas fluorescens*.

Field evaluation in 2011

Due to high inoculum density applied, disease pressure was high in the 2011 trial; however, some treatments reduced blossom infection (Table 3.4). The industry-standard streptomycin (Agrimycin 17) applied pre-infection was not significantly different (P<0.20) from the control treatments in 2011. However, three antibiotics treatments (Kasumin 2L pre-, Agrimycin 17 post- and ARY-4016-06 post-inoculation) did significantly (P<0.10) reduce blossom infection compared to the control. Shoot infection was not significantly (P<0.50) reduced by bloom-time applications (Table 3.5).

Field evaluation in 2012

Apple trees in Urbana, IL bloomed about four weeks earlier in 2012 than in normal years. Nearing petal fall, a freezing event occurred on 11 April with a nightly low temperature reaching -3.9°C, which resulted in a 100% crop loss; however, blossom infection still occurred in inoculated trials. Four antibiotic treatments significantly reduced blossom infection compared to the control which included, Agrimcyin 17, pre/post-inoculation ($P<0.0485$); Agrimycin 17, post-inoculation only ($P<0.0951$); ARY 0416-06, pre/post-inoculation ($P<0.0698$); and Kasumin 2L, pre/post-inoculation ($P<0.1081$), (Table 3.6). Applications of Serenade Max also reduced infection but to a lesser extent (Table 3.6).

In this experiment, growth regulator Apogee (split-application at 5% bloom and petal fall), did not significantly ($P<99.19$) affect blossom infection and no significant ($P<0.7174$) Apogee x treatment interactions were observed. Similarly, no significant ($P<0.2161$) difference in shoot infection was observed between Apogee and non-Apogee treated plots on 8 May. However, two weeks later (22 May), averaged over all bactericide and biocontrol treatments, the application of Apogee significantly ($P<0.0004$) reduced shoot infection by 26.6%. Though not statistically significant, it was also observed following the 28 May hail storm, bloom-time Apogee applications reduced trauma shoot infections by 28.0% (Table 3.9).

Plots that received both Apogee and Kasumin 2L (pre/post- and post-inoculation) had significantly less ($P<0.03$ and $P<0.0009$, respectively) shoot infection than Kasumin 2L alone (Table 3.8). This interaction was not observed in plots treated with ARY-0416-06 ($P<0.45$), another formulation of kasugamycin, or any other treatment.

DISCUSSION

All 84 Illinois *Erwinia amylovora* isolates tested were sensitive to copper sulfate at 0.16 mM. Sholberg et al. (2001) reported that 18 of 42 (43%) *E. amylovora* isolates tested in British Columbia developed colonies in the presence of 0.16 mM copper sulfate on CYE medium. They also reported that two of 42 isolates (5%) developed at 0.32 mM copper sulfate on CYE. However, Sholberg et al. (2001) concluded, these levels were not a cause for concern in copper sprayed orchards because all isolates were controlled at 1.1 mM. In our study, various copper compounds evaluated in CYE broth showed *in-vitro* differences in effectiveness at 7 mg/L metallic copper. However, at 14 mg/L metallic copper, all copper compounds tested inhibited *in-vitro* cell multiplication of *E. amylovora*. Since all Illinois *E. amylovora* isolates were sensitive to both 0.16 mM copper sulfate and 14 mg/L metallic copper, thus copper is still an effective compound in managing fire blight of apple in Illinois.

Our study showed that kasugamycin in Kasumin 2L was more effective in prohibiting cell multiplication of *E. amylovora in-vitro* than kasugamycin in ARY-406-06. The results agree with the report of McGhee and Sundin (2011) that the carrier in Kasumin 2L does increase the efficacy of the compound. We think the higher concentration of kausgamycin in ARY-4016-06 (e.g., lower concentration of carrier per volume) explains the different efficacy of the kasugamycin products *in-vitro*. However, both kasugamycin formulations provide effective field control. Adaskavey et al. (2011) reported kausgamycin was more effective at pH 5 than pH 7 *in-vitro* and also reported that flower blossoms have approximately pH 5. This may explain why kasuagmycin is more effective in the orchard than laboratory assays. The carrier in Kasumin 2L

may serve as another “mode of action” to help prevent/delay kasugamycin-resistance development in *E. amylovora* in the future if it is used for control of fire blight. Two *E. amylovora* isolates tested from Calhoun were among the most kasugamycin-sensitive isolates. This is especially interesting as Calhoun isolates were also among the most sensitive to streptomycin.

In the 2011 field trial, disease pressure was too high. We inoculated the pathogen at 10^8 CFU/ml, while other investigators commonly use 10^6 CFU/ml. Additionally, rainfall was more frequent at the experimental site during bloom and after applying treatments. The combination of these factors may explain why the industry-standard pre-inoculation streptomycin application was not effective at reducing blossom infection. However, application of post-inoculation streptomycin and kasugamycin products provided blossom blight control under such conducive conditions for blossom blight development. Although some treatments in 2011 reduced blossom infection, none of the treatments significantly affected shoot infection by *E. amylovora*. These data may partially explain why severe shoot blight can be observed in orchards even though streptomycin is still effective against blossom blight and applied at the recommend time (during bloom). The high inoculum density used in this study, may explain the high disease incidence and severity that occurs in Calhoun county each year, as Calhoun orchards are in closer proximity and likely inoculum sources and density are abundant compared to most other apple orchards in Illinois.

In the 2012 field trial, disease pressure was moderate. Similar to 2011, streptomycin (pre/post- and post-inoculation) and kasugamycin products (pre/post-inoculation) reduced blossom blight infection. The kasugamycin post-inoculation treatment did not provide the same blossom blight

control observed in the 2011 trial. Further studies comparing effectiveness of kasugamycin post-blossom infection may be warranted. Similar to the 2011 trial, bloom-time treatments did not reduce shoot blight occurrence in the summer.

In 2011 and 2012 trials, oxytetracycline (Mycoshield) did not significantly reduce blossom blight infection. Similarly, biocontrol agents Serenade Max and BlightBan A506 did not effectively reduce blossom blight infection. Although, Serenade Max did reduce blossom infection more than BlightBan A506. These results are similar to the multi-year, multi-location observations reported by Sundin, et al. (2009). Our data indicates that in the Illinois climate (also in other Midwestern states), applications of biocontrol agents alone can not provide effective control of fire blight of apple, and may not be considered a streptomycin alternative.

Our 2012 field trial showed reductions in shoot blight incidence when Apogee was applied to Kasumin 2L (kasugamycin) treated trees. Application of Apogee with ARY-4016-06, which also contains kasugamycin, did not significantly affect shoot blight incidence. Although based on one-year of field data, to our knowledge, this is the first documentation of a synergistic interaction between Kasumin 2L and Apogee significantly reducing shoot blight in apple. Additional field trials are needed to evaluate interactions between kasugamycin, the carrier in Kasumin 2L, and Apogee in controlling fire blight in pome fruit.

This study evaluated various compounds for fire blight control in apple orchards and established Illinois' *E. amylovora* population's base line sensitivities to kasugamycin. In this study, we did not detect copper-resistance in Illinois populations, indicating copper remains an effective

management tool. Streptomycin remains the best available antibiotic to protect apple trees from *E. amylovora* infection in Illinois. Every effort should be made to avoid the development/ introduction of streptomycin-resistant strains in Illinois. Also, our data indicates kasugamycin compounds, if registered in Illinois, would be a suitable compliment/replacement to streptomycin. If kasugamycin is available for use in Illinois prior to the appearance of streptomycin-resistant *E. amylovora*, it would provide an effective chemical alternation using a different mode of action than streptomycin.

TABLES AND FIGURES

Table 3.1. Effect of oxytetracycline and copper on inhibition of colony development of Illinois *Erwinia amylovora* isolates.

	Isolates	Colony development							
	tested	oxytetracycline mg/L[a]				copper sulfate mM[b]			
County	(no.)	0	50	100	200	0	0.08	0.16	1.1
Boone	6	6[c]	0	0	0	6	6	0	0
Calhoun	20	20	0	0	0	20	20	0	0
Champaign	6	6	0	0	0	6	6	0	0
Jackson	2	2	0	0	0	2	2	0	0
Jersey	9	9	0	0	0	9	9	0	0
Kane	2	2	0	0	0	2	2	0	0
Macoupin	3	3	0	0	0	3	3	0	0
Madison	2	2	0	0	0	2	2	0	0
Marshall	9	9	0	0	0	9	9	0	0
Peoria	2	2	0	0	0	2	2	0	0
Putnam	4	4	0	0	0	4	4	0	0
Sangamon	2	2	0	0	0	2	2	0	0
St. Clair	11	11	0	0	0	11	11	0	0
Union	2	2	0	0	0	2	2	0	0
Woodford	4	4	0	0	0	4	4	0	0
Illinois	**84**	84	0	0	0	84	84	0	0

[a] Colony development was evaluated on Luria-Bertani medium amended with oxytetracyline (Mycoshield 17WP) after 48 h.
[b] Colony development was evaluated on casitone-yeast extract (CYE) medium amended with copper sulfate (Cuprofix Ultra 40DF) after 72 h.
[c] Number of isolates tested that developed colonies.

Table 3.2. *In-vitro* evaluation of colony development of *Erwinia amylovora* on kasugamycin amended Luria-Bertani (LB) medium.

		Colony development											
	Isolates	Kasumin 2L						ARY-4016-06					
	tested	50 mg/L a.i.[a]			100 mg/L a.i.			50 mg/L a.i.			100 mg/L a.i.		
County	(no.)	-[b]	-/+	+	-	-/+	+	-	-/+	+	-	-/+	+
Boone	6	3[c]	2	1	6	0	0	0	0	6	2	4	0
Calhoun	20	9	11	0	20	0	0	0	3	17	12	8	0
Champaign	6	4	2	0	6	0	0	1	1	4	5	1	0
Jackson	2	1	1	0	2	0	0	0	1	1	2	0	0
Jersey	9	4	5	0	9	0	0	0	0	9	5	4	0
Kane	2	1	1	0	2	0	0	0	1	1	1	1	0
Macoupin	3	0	2	1	3	0	0	0	0	3	1	1	1
Madison	2	0	1	1	2	0	0	0	0	2	0	2	0
Marshall	9	2	4	3	9	0	0	0	2	7	2	6	1
Peoria	2	1	1	0	2	0	0	0	0	2	0	2	0
Putnam	4	1	1	2	4	0	0	0	1	3	2	2	0
Sangamon	2	0	2	0	2	0	0	0	0	2	0	1	1
St. Clair	11	2	8	1	11	0	0	0	0	11	2	8	1
Union	2	0	1	1	2	0	0	0	0	2	0	0	2
Woodford	4	1	2	1	4	0	0	0	1	3	1	2	1
Total	84	29	44	11	84	0	0	1	10	73	35	42	7
(Percent)	(100)	(35)	(52)	(13)	(100)	(0)	(0)	(<1)	(12)	(87)	(42)	(50)	(8)

[a] a.i.=active ingredient (kasugamycin).

[b] LB medium (pH 7) was amended with each concentration of kasugamycin and bacterial colonies were streaked onto the agar surface with a bacterial loop. Colony development was assessed after 48 h.
(–) indicates no colony development, (-/+) indicates colony development was sometimes observed,
(+) indicates colony development was consistently observed.

[c] Number of isolates tested that developed colonies.

Table 3.3. *In-vitro* evaluation of the effects of kasugamycin formulations on *Erwinia amylovora* multiplication in nutrient broth[a] .

		mg/L kasugamycin (Kasumin 2L)			mg/L kasugamycin (ARY 4016-06)		
County	Isolate	3	5	10	3	5	10
Boone	6951	98.9%[b] bc[c]	55.8%c	0.0% e	78.7% b-d	37.5% cd	11.3%d
Calhoun	6081	100.7 a-c	1.0 e	0.0 e	60.7 b-e	21.1 de	0.0 e
Calhoun	7651	52.4 e	1.1 e	0.0 e	46.4 de	10.1 e	0.0 e
Champaign	4802	72.0 d	9.4 d	0.0 e	38.0 e	6.9 e	0.0 e
Jersey	6002	95.6 b	67.9 bc	0.0 e	83.9 b	51.6 bc	20.7 c
Sangamon	84d	97.8 b	85.9 a	37.7 a	82.0 b	69.5 b	59.9 a
St. Clair	89a	87.6 c	73.4 b	12.7 c	73.2 cd	69.2 b	58.5 a
Union	5232	94.9 b	70.3 b	0.0 e	81.7 b	70.8 b	55.4 a
Woodford	7711	116.6 a	102.1 a	24.0 b	117.7 a	98.4 a	56.9 a
Woodford	165e	94.6 b	73.5 b	3.7 d	79.4 bc	51.1 c	37.7 b
	Mean	**96.1**	**61.1**	**7.1**	**78.8**	**51.5**	**31.1**

[a] Nutrient Broth was adjusted to pH 7.

[b] Bacterial cell densities were measured with a spectrophotometer at OD600 at 18 h and expressed as a percent compared to un-amended control. A value of 100% indicates colony development was not reduced compared to the control; 0% indicates no colony development detected.

[c] Data were analyzed using PROC MIXED with macro pdmix800. In each column values with a letter in common are not significantly different from each other ($P<0.05$).

Table 3.4. Field evaluation of antibiotics and biocontrol agents for control of blossom blight (*Erwinia amylovora*) on 'Jonathan' apple trees in Urbana, IL in 2011.

Treatment[a]	Active ingredient (rate)	Application time[b]	Infected blossom clusters (%)	
			12 May	23 May
Control (un-inoculated)	-	-	4.4 bc[c]	31.1 a-c
Control (inoculated)	-	-	6.9 abc	29.1 a-c
Agrimycin 17	streptomycin (100 mg/L)	pre-inoculation	6.4 bc	22.7 b-e
		post-inoculation	2.5 c	15.7 de
Mycoshield	oxytetracycline (200 mg/L)	pre-inoculation	5.8 bc	33.1 a-c
		post-inoculation	4.2 bc	24.4 b-d
Serenade Max	*Bacillus subtilis* QST713 (525 mg/L)	pre-inoculation	7.7 abc	23.9 b-e
		post-inoculation	10.8 a	38.5 a
Blight Ban A506	*Pseudomonas syringae* A506 (369 mg/L)	pre-inoculation	5.8 bc	31.4 a-c
		post-inoculation	7.5 ab	34.4 ab
Kasumin 2L	kasugamycin (100 mg/L)	pre-inoculation	3.1 bc	15.7 de
		post-inoculation	2.5 c	17.5 de
ARY-0416-06	kasugamycin (100 mg/L)	pre-inoculation	2.5 c	21.7 c-e
		post-inoculation	2.5 c	13.6 e

[a] All treatments were applied at maximum label rates using gas powered backpack sprayer at 1,407 liters/ha.

[b] Pre-inoculation treatments applied 24 h before and post-inoculation treatments applied 24 h after inoculation. All trees were inoculated with *E. amylovora* isolate 2A at 10^8 CFU/ml on 26 April and 8 May.

[c] On each tree, 120 blossom clusters were evaluated per tree. Data were transformed [$\sqrt{(x + 1/6)}$] and analyzed in PROC MIXED. Values within each column with a letter in common are not significantly different from each other (P<0.10) using macro pdmix800 to indicate mean letter separation.

Table 3.5. Severity of shoot blight (*Erwinia amylovora*) on 'Jonathan' apple trees following applications of antibiotics and biocontrol agents at bloom in Urbana, IL in 2011.

Treatment[a]	Active ingredient (rate)	Application time[b]	Tree canopy infection (%)		
			10 June	30 June	27 July
Control (un-inoculated)	-	-	35.0	59.3	52.0
Control (inoculated)	-	-	38.0	48.7	47.7
Agrimycin 17	streptomycin	Pre	34.3	44.7	44.7
	(100 mg/L)	Post	24.3	37.7	28.3
Mycoshield	oxytetracycline	Pre	40.3	46.3	39.3
	(200 mg/L)	Post	33.0	43.3	39.0
Serenade Max	*Bacillus subtilis* QST713	Pre	38.3	57.7	47.7
	(525 mg/L)	Post	41.7	41.0	35.7
Blight Ban A506	*Pseudomonas syringae* A506	Pre	37.3	49.0	41.3
	(369 mg/L)	Post	40.3	49.3	45.7
Kasumin 2L	kasugamycin	Pre	33.3	48.3	34.7
	(100 mg/L)	Post	35.7	51.3	46.3
ARY-0416-06	kasugamycin	Pre	41.7	53.3	44.0
	(100 mg/L)	Post	32.7	51.7	50.0
LSD (P<0.10)			NS[c]	NS	NS

[a] All bloom-time treatments were applied at maximum label rates using gas powered backpack sprayer at 1,407 liters/ha. (150 gallons/acre).

[b] Pre, indicates applied 24 h before inoculation and post, indicates applied 24 h after inoculation. All trees were inoculated with *E. amylovora* isolate 2A at 10^8 CFU/ml on 26 April and 8 May.

[c] Data were analyzed using PROC GLM, values within each column were not significantly different (NS) from each other according to Fisher's protected LSD (P<0.10).

Table 3.6. Field evaluation of antibiotics and biocontrol agents for control of blossom blight (*Erwinia amylovora*) on 'Jonathan' apple trees in Urbana, IL in 2012.

Treatment [b]	Active ingredient (rate)	Application time[c]	Infected blossom clusters (%)[a]		
			17 April	24 April	1 May
Control (inoculated)	-	-	3.3 a[d]	9.5	18.7 a
Agrimycin 17	streptomycin (100 mg/L)	Pre/Post	0.2 cd	2.1	1.9 d
		Post only	0.0 d	1.1	2.5 cd
Mycoshield	oxytetracycline (200 mg/L)	Pre/Post	1.3 ab	3.0	5.7 a-d
Mycoshield + Kocide	oxytetracycline (200 mg/L)+ copper hydroxide (240 mg/L)	Pre/Post	1.7 a-c	1.9	5.6 a-d
Serenade Max	*Bacillus subtilis* QST713 (525 mg/L)	Pre only	2.3 b-d	3.6	4.0 b-d
Blight Ban A506	*Pseudomonas syringae* A506 (369 mg/L)	Pre only	1.3 a-d	3.3	4.8 ab
Dithane + Kocide	mancozeb (2,700 mg/L) + copper hydroxide (240 mg/L)	Pre/Post	0.9 a-c	2.4	5.9 a-d
Kasumin 2L	kasugamycin (100 mg/L)	Pre/Post	0.0 d	1.2	2.4 cd
		Post only	2.0 ab	4.5	7.3 a-c
ARY 0416-06	kasugamycin (100 mg/L)	Pre/Post	0.2 b-d	1.1	1.8 cd
				NS	

[a] The number of clusters present varied among trees, (on average 70 clusters were present per tree). Total and infected clusters were counted and expressed as a percent for comparison.

[b] All treatments included Regulaid (125 ml/100 liters) and were applied at 1,407 liters/ha. All trees were inoculated with *E. amylovora* isolate 2A at 5 x 10^6 CFU/ml on 30 March and 3 April.

[c] Pre, indicates treatment application 24 h before inoculation; Post, indicates treatment application 24 h after inoculation.

[d] Data is presented as percent blossom cluster infection. Original counted cluster data were transformed [$\sqrt{(x + 1/6)}$] and analyzed as a covariate in PROC MIXED. Values within each column with a letter in common are not significantly different from each other (P<0.10) using macro pdmix800 to indicate mean letter separation. Blossom cluster infection on 24 April was not significantly different (NS).

Table 3.7. Severity of shoot blight (*Erwinia amylovora*) on 'Jonathan' apple trees following applications of antibiotics and biocontrol agents at bloom in Urbana, IL in 2012.

Treatment[a]	Active ingredient (rate)	Application time[b]	Tree canopy infection (%) 8 May	5 June	3 July
Control (inoculated)	-	-	9.0 ab[c]	15.7 b-e	20.1 a-e
Agrimycin 17	streptomycin (100 mg/L)	Pre/post	7.0 de	9.0 de	12.8 e
		Post only	5.0 e	9.6 c-e	17.8 c-e
Mycoshield	oxytetracycline (200 mg/L)	Pre/post	13.9 a-c	18.6 a-c	27.5 a-c
Mycoshield + Kocide	oxytetracycline (200 mg/L)+ copper hydroxide (240 mg/L)	Pre/post	5.9 b-d	22.4 ab	27.6 a-c
Serenade Max	*Bacillus subtilis* QST713 (525 mg/L)	Pre only	4.0 b-e	20.0 ab	28.4 ab
Blight Ban A506	*Pseudomonas syringae* A506 (369 mg/L)	Pre only	12.3 a	26.9 a	32.0 a
Dithane + Kocide	mancozeb (2,700 mg/L) + copper hydroxide (240 mg/L)	Pre/post	4.6 b-e	19.5 ab	26.5 a-c
Kasumin 2L	kasugamycin (100 mg/L)	Pre/post	3.6 c-e	18.6 a-c	23.3 b-e
		Post only	8.2 a-c	18.3 a-d	22.5 a-d
ARY 0416-06	kasugamycin (100 mg/L)	Pre/post	2.5 e	8.3 e	15.8 de

[a] All treatments included Regulaid (125 ml/100 liters) and were applied at 1,403 liters/ha. All trees were inoculated with *E. amylovora* isolate 2A at 5 x 10^6 CFU/ml on 30 March and 3 April.

[b] Pre, indicates treatment application 24 h before inoculation; Post, indicates treatment application 24 h after inoculation.

[c] Data were collected and averaged over all plots (e.g., Apogee and non-Apogee treatments were combined). Data were transformed [$\sqrt{(x)}$] and analyzed in PROC MIXED using macro pdmix800. Values within a column with a letter in common are not significantly different from each other ($P<0.10$).

Table 3.8. Severity of shoot blight (*Erwinia amylovora*) on 'Jonathan' apple trees following application of antibiotics/biocontrol agents and prohexadione calcium (Apogee) and in Urbana, IL in 2012.

Treatment[a]	Active ingredient (rate)	Apogee application[b]	Infected shoots (no.)[c]	Difference	*P* value[d]
Control	-	-[e]	14.3	1.2	0.9285
		Apogee	15.5		
Agrimycin 17	streptomycin (100 mg/L)	-	8.5	-3	0.6368
		Apogee	5.5		
Agrimycin 17 (Post only)	streptomycin (100 mg/L)	-	7	-4	0.2216
		Apogee	3		
Mycoshield	oxytetracycline (200 mg/L)	-	17	-6.2	0.2992
		Apogee	10.8		
Mycoshield+ Kocide	oxytetracycline (200 mg/L) + copper hydroxide (240 mg/L)	-	18.5	-8.7	0.2733
		Apogee	9.8		
Serenade Max (Pre only)	*Bacillus subtilis* QST713 (525 mg/L)	-	13.9	-5.9	0.2870
		Apogee	8		
Blight Ban A 506 (Pre only)	*Pseudomonas syringae* A506 (369 mg/L)	-	31.3	-12.8	0.1882
		Apogee	18.5		
Dithane + Kocide	mancozeb (2,700 mg/L) + copper hydroxide (240 mg/L)	-	9.3	1	0.7899
		Apogee	10.3		
Kasumin 2L	kasugamycin (100 mg/L)	-	23.3	-19.8	0.0009
		Apogee	3.5		
Kasumin 2L (Post only)	kasugamycin (100 mg/L)	-	23.5	-15	0.0388
		Apogee	8.5		
ARY-0416-06	kasugamycin (100 mg/L)	-	5.5	-1	0.4526
		Apogee	4.5		

[a] Treatments were applied 24 h pre- and 24 h post-inoculation, unless otherwise indicated. Treatments included Regulaid (125 ml/100 liters) and were applied at 1,407 liters/ha. All trees were inoculated with *E. amylovora* isolate 2A at 5 x 10^6 CFU/ml on 30 March and 3 April.

[b] A split-application of Apogee (600 mg/L prohexadione calcium) plus Regulaid (125 ml/100 liters) was applied on 29 March (40-60% bloom) and 12 April (petal fall).

[c] On 22 May, 120 shoots were evaluated in each tree.

[d] Data were transformed [$\sqrt{(x)}$] and analyzed in PROC MIXED. Treatment x Apogee *P* value is shown.

[e] - =No prohexadione calcium (Apogee) application.

Table 3.9. Severity of trauma shoot blight (*Erwinia amylovora*) on 'Jonathan' apple trees, following applications of antibiotics/biocontrol agents and prohexadione calcium (Apogee), in Urbana, IL in 2012.

Treatment [a]	Apogee application [b]	Trauma shoot infection (no.) [c]	Reduction in shoot infection(%) [d]
Control	- [e]	4.0	5.0
	Apogee	3.8	
Agrimycin 17	-	5.8	69.0
	Apogee	1.8	
Agrimycin 17 (Post only)	-	9.3	59.1
	Apogee	3.8	
Mycoshield	-	10.8	44.4
	Apogee	6.0	
Mycoshield+ Kocide	-	14.5	17.2
	Apogee	12.0	
Serenade Max (Pre only)	-	12.6	4.8
	Apogee	12.0	
Blight Ban A 506 (Pre only)	-	9.5	44.2
	Apogee	5.3	
Dithane + Kocide	-	16.3	33.0
	Apogee	11.0	
Kasumin 2L	-	8.5	-8.6
	Apogee	9.3	
Kasumin 2L (Post only)	-	6.0	3.4
	Apogee	5.8	
ARY-0416-06	-	5.3	56.6
	Apogee	3.3	
Overall Mean	-	9.3	28.0
	Apogee	6.7	

[a] Treatments were applied 24 h pre- and 24 h post-inoculation unless otherwise indicated. All treatments included Regulaid (125 ml/100 liters) and were applied at 1,407 liters/ha. All trees were inoculated with *E. amylovora* isolate 2A at 5 x 10^6 CFU/ml on 30 March and 3 April.

[b] A split-application of Apogee (600 mg/L prohexadione calcium) plus Regulaid (125 ml/100 liters) was applied on 29 March (40-60% bloom) and 12 April (petal fall).

[c] A hail storm occurred on 28 May. On 5 June, 120 shoots were evaluated in each tree for new shoot infection.

[d] Data were analyzed in PROC MIXED. Overall, bloom-time Apogee applications did not statistically reduce (P< 0.1551) trauma shoot infection following the hail storm, but a 28.0% reduction was observed.

[e] - =No prohexadione calcium (Apogee) application.

LITERATURE CITED

Adaskaveg, J.E., Forster, H., and Wade, M.L. 2011. Effectiveness of kasugamycin against *Erwinia amylovora* and is potential use for managing fire blight of pear. Plant Dis. 95:448-454.

Copping, L.G., and Duke, S.O. 2007. Natural products that have been used commercially as crop protection agents. Pest Manag. Sci. 63:524-554.

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

McGhee, G., Guasco, J., Bellomo, L., Blumer-Schuette, S., Shane, W., Irish-Brown, A., and Sundin, G.W. 2011. Genetic analysis of streptomycin-resistant (SmR) strains of *Erwinia amylovora* suggests that dissemination of two genotypes is responsible for the current distribution of SmR *E. amylovora* in Michigan. Phytopathology 101:182-191.

McGhee, G. and 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 101:192-204.

McGrath, M. J., Koczan, J. M., Kennelly, M. M., and Sundin, G. W. 2009. Evidence that prohexadione-calcium induces structural resistance to fire blight infection. Phytopathology 99:591-596.

Saxton, A.M. 1998. A macro for converting mean separation output to letter groupings in Proc Mixed. In Proc. 23rd SAS Users Group Intl., SAS Institute, Cary, NC, pp1243-1246.

Sholberg, P.L., Bedford, K.E., Haag, P., and Randall, P. 2001. Survey of *Erwinia amylovora* isolates from British Columbia for resistance to bactericides and virulence on apple. Can. J. Plant Pathol. 23:60-67.

Sundin, G. W., Werner, N. A., Yoder, K. S., and Aldwinckle, H. S. 2009. Field evaluation of biological control of fire blight in the eastern United States. Plant Dis. 93:386-394.