Annual Report - 2013

Prepared for the California Pear Board

Project Title:	Evaluation of new bactericides for control of fire blight of pears caused by *Erwinia amylovora*
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SUMMARY

1. In antibiotic resistance surveys, all 44 isolates from Lake Co. and three isolates from an experimental orchard at UC Davis were found to be sensitive against streptomycin, oxytetracycline, and kasugamycin. Among 105 isolates from 13 locations in Sacramento Co., 6.7% were streptomycin-resistant, but all were sensitive to oxytetracycline and kasugamycin. The seven isolates with moderate resistance to streptomycin came from two orchard locations with mixed sensitive/resistant populations. The low level of streptomycin resistance in Sacramento Co. in 2013 is of interest because in previous surveys, the majority of isolates from this county were resistant.
2. In toxicity studies with three biocontrol agents against chemicals used for fire blight control in our studies, streptomycin, oxytetracycline, kasugamycin, captan, and mancozeb at 40 ppm were all inhibitory against *Streptomyces lydicus* (Actinovate) and *Bacillus amyloliquifaciens* (Double Nickel 55) (Table 2). In contrast, *Aureobasidium pullulans* (Blossom Protect) was not inhibited in growth by the three antibiotics at 40 ppm, but was inhibited by captan and mancozeb. These data indicate that in field applications only Blossom Protect could be safely used in combination with the three antibiotics.
3. Two air-blast field trials under low disease pressure were conducted on the management of fire blight.
   a. Kasugamycin continued to be highly effective in reducing the incidence of fire blight. Registration of the product for California is expected for 2014.
   b. In the trial on cv. Bartlett pear, numerically the lowest amount of disease was observed with the Kasumin-Firewall mixture, followed by Kasumin-Actigard, Fireline-Firewall, and Fireline-Actigard mixtures. Badge also significantly reduced the incidence of disease.
   c. In trial on cv. Bartlett pear, Actigard improved the performance of Kasumin but not of Fireline. Actigard did not improve the effectiveness of Kasumin in the trial on cv. Comice pear where trees were inoculated with the pathogen.
   d. K-Phite was effective in the cv. Comice trial, but not in the cv. Bartlett trial.

INTRODUCTION

Fire blight, caused by the bacterium *Erwinia amylovora,* is a very destructive disease of pome fruit trees worldwide, especially pears. It is one of the most difficult diseases to manage. The infection period is long, and moreover, very few effective chemicals are available. Integrated programs that combine sanitation and orchard management with chemical and biological controls are the best approaches. If the disease is in its early stage and only a few twigs are blighted, it often can be eliminated by pruning. Thus, aggressive and regular scheduled pruning of diseased tissue is essential for keeping inoculum levels low in an orchard.

Current chemical control programs for fire blight control are based on protective schedules, because available compounds are contact treatments and are not systemic. Control with copper compounds is only satisfactory when disease severity is low to moderate. On Bartlett (summer) pears, copper treatments are widely used only during dormant and bloom periods because phytotoxicity commonly occurs on fruit as russeting. New formulations of copper, however, allow for reduced rates based on the metallic copper equivalent (MCE) and thus, extended usage past the bloom period may provide an effective rotational treatment or mix-partner without causing russeting. The antibiotics streptomycin and the less effective oxytetracycline (terramycin) have also been used for many years. Because of lack of alternative control materials, resistance developed against streptomycin at many locations in California, mostly in Sacramento Co. growing areas. In our antibiotic resistance surveys, we also detected isolates of *E. amylovora* with reduced sensitivity to oxytetracycline at several locations over the years. At one of these locations field treatments with Mycoshield were reported to be ineffective in controlling the disease. In inoculation experiments using these strains we demonstrated that the disease could not be effectively controlled using oxytetracycline. Thus, field resistance has occurred in some locations. Surveys on antibiotic resistance monitoring were continued in 2013 in collaboration with farm advisors.

In the past years, in our evaluations of new materials for fire blight control, kasugamycin (Kasumin) was identified as the most effective alternative treatment with an efficacy equal or higher to streptomycin and oxytetracycline. This compound also showed very good efficacy in controlling fire blight in field trials in other pome fruit growing areas of the country. Although concerns have been expressed by regulatory agencies regarding the use of antibiotics in agriculture, kasugamycin is not used in human and animal medicine and has a different mode of action from streptomycin or oxytetracycline (no cross-resistance). Through our efforts, registration of Kasumin in California is pending in 2014. Kasugamycin was again effectively used in our field trials in 2013. It was applied by itself or in mixtures with selected other materials, including other antibiotics, copper, mancozeb, and Actigard that is enhancing host defense mechanisms in some plants. These evaluations were done to identify effective mixture treatments that would reduce the potential for resistance development. In the past, we also successfully evaluated rotation programs with Kasumin. In 2013, we also tested the reduced MCE copper compound Badge in a program with four consecutive sprays and K-Phite, a mixture of monopotassium phosphate and dipotassium phosphite that potentially also could increase plant host defense mechanisms.

In another objective of our project we have been investigating the molecular mechanism of streptomycin resistance in California isolates of *E. amylovora*. Several mechanisms have been described for isolates of the pathogen from various locations. The two major groups are: i) a point mutation in the chromosomal *rps*L gene; and ii) resistance genes *StrA* and *StrB* that are associated with a transposon (i.e., Tn*5393*) and that are most commonly located on one of several plasmids. Strains with a high level of streptomycin resistance are associated with the chromosomal gene; whereas, moderate streptomycin resistance is associated with the *StrA* and *StrB* genes in California. We have determined that the majority of recent streptomycin-resistant isolates in California have the *StrA* and *StrB* genes. These are, however, located on a plasmid that previously has not been found to carry resistance genes. This novel mode of resistance is currently summarized in a manuscript.

Several inhibitors of bacterial biofilms have been recently found to be effective by others in inhibiting in vitro growth of *E. amylovora* and reducing disease on inoculated, immature pear fruit. Biofilms are produced by the pathogen and are thought to help protect bacteria from harsh environments. By inhibiting biofilms and by keeping bacteria in a planktonic state (single-cell state), they may be more sensitive to chemical treatments. Thus, we evaluated the potential of 2-aminoimidazole (Worthington et al., Plant Dis. 96:1638-1644, 2012) to inhibit biofilm formation and possibly overcome streptomycin resistance. Another biofilm inhibitor, dehydroproline (De Zoysa et al., Plant Pathology 62:767-776, 2013), will be evaluated in the future.

OBJECTIVES

1. Evaluate and optimize the performance of the antibiotic kasugamycin (Kasumin) and other antibiotics such as streptomycin (e.g., Firewall) and oxytetracycline (e.g., Mycoshield, Fireline) for fire blight control in cooperation with UCCE.
    a. Laboratory in vitro tests to evaluate the bactericidal activity of antibiotics with and without biofilm inhibitors such as 2-aminoimidazole using spiral gradient dilution assays.
    b. Small-scale hand-sprayer tests using different treatment-inoculation schedules to evaluate bio-film inhibitors in combination with antibiotics and/or low MCE copper products.
    c. Field trials with protective air-blast spray treatments at several locations:
        i. New formulations of copper (e.g., Kocide 3000, Badge X2) with and without antibiotics.
        ii. Plant defense activators (e.g, ProAlexin, Actigard, PM-1) with and without antibiotics.
        iii. Bio-film inhibitors (e.g., 2-aminoimidazole) with and without antibiotics or low MCE copper (*pending lab and small-scale trials*).

d. Evaluate the efficacy of biological controls (e.g., Actinovate, Blossom Protect, Double Nickel 55), and natural products (e.g., Cerebrocide) in integrated programs using antibiotics and low MCE copper products.
    i. Efficacy in field trials
    ii. Compatibility of new biocontrol agents with antibiotics in in vitro studies
e. Efficacy of sanitizing agents (Deccosan, Citrox) and other treatments (titanium dioxide – AgriTitan)

2. Determine the distribution of streptomycin- or oxytetracycline -sensitive and -resistant strains of *E. amylovora* in pear orchards in California (continuation of surveys).
    a. Laboratory in vitro tests to evaluate the bactericidal activity with and without additives in amended agar assays.
    b. Characterization of streptomycin- and oxytetracycline-resistant strains using molecular approaches: characterize plasmids that harbor the resistance genes and compare to *E. amylovora* populations from other parts of the country.

MATERIALS AND METHODS

Isolation of* E. amylovora*, bacterial culturing, and verification of species identity. Pear samples with fire blight symptoms were obtained in the spring and early summer of 2013 from orchards in Sacramento, Solano, and Lake Co. Infected plant material (fruit, stems, and pedicels) was surface-disinfested for 1 min using 400 mg/L sodium hypochlorite, rinsed with sterile water, cut into small sections, and incubated in 1 ml of sterile water for 15 to 30 min to allow bacteria to stream out of the tissue. Suspensions were streaked onto yeast extract-dextrose-$CaCO_3$ agar (YDC). Single colonies were transferred and the identity of the isolates as *E. amylovora* was verified by colony morphology and by PCR using primers specific for the ubiquitous *E. amylovora* plasmid pEA29 described by Bereswill et al. (Appl. Environ. Microbiol. 58:3522-2536). The presence of a 1-kb DNA fragment after gel electrophoresis confirmed a positive identification. A total of 152 isolates of *E. amylovora* from 35 orchard locations were obtained in 2013.

***Laboratory studies on the toxicity of bactericides against* E. amylovora.** Kasugamycin (Kasumin 2L, Arysta Life Sciences, Cary NC), streptomycin (Sigma, St. Louis, MO), and oxytetracycline (Sigma) were evaluated for their in vitro toxicity using the spiral gradient dilution method. For this, a radial bactericidal concentration gradient was established in nutrient agar media in Petri dishes by spirally plating out a stock concentration of each antimicrobial using a spiral plater (Autoplate 4000; Spiral Biotech, Inc., Norwood MA). After radially streaking out suspensions of the test bacteria (10 µl of 10^8 cfu/ml as determined by measurement of optical density at 600 nm) along the concentration gradient, plates were incubated for 2 days at 25ºC. Measurements were visually taken for two inhibitory concentrations: i) the lowest inhibitory concentration (LIC; the lowest concentration where inhibition of bacterial growth was observed, i.e., where the bacterial streak became less dense visually), and ii) the minimal concentration that inhibited growth by >95% (MIC). The actual antibiotic concentrations were obtained by entering the radial distances of inhibition (measured from the center of the plate) into the Spiral Gradient Endpoint computer program (Spiral Biotech, Inc.).

For evaluation of the biofilm inhibitor 2-aminoimidazole, streptomycin was spiral plated onto nutrient agar plates amended with 2-aminoimidazole. Streptomycin-sensitive and -resistant isolates of *E. amylovora* were then streaked along the gradient and growth was evaluated after two days as described above.

Toxicity of chemicals used for fire blight control in our studies against three biocontrol agents. The spiral gradient dilution method was used to evaluate the toxicity of streptomycin, oxytetracycline, kasugamycin, captan, and mancozeb against *Streptomyces lydicus* (Actinovate), *Aureobasidium pullulans* (Blossom Protect), and *Bacillus amyloliquifaciens* (Double Nickel 55). Stock concentrations of the chemical were used that resulted in maximum concentrations in the agar medium of approximately 40 ppm. The biocontrol agents were radially streaked along the concentration gradients, and plates were evaluated after two days. Inhibition of growth by a chemical was rated as the chemical is active against the biocontrol agent.

Field studies using protective treatments during the growing season. In field studies in a commercial cv. Bartlett orchard in Live Oak, and an experimental cv. Comice orchard at UC Davis four or three applications, respectively, of selected treatments (see Results) were done using a back-pack airblast sprayer at 100 gal/A. Disease evaluation in the commercial orchard was based on natural incidence, whereas at the UC Davis trial site trees were inoculated with *E. amylovora* between the second and third bactericide application. Disease was evaluated in mid-April 2013 and for this, the number of infected spurs was determined for each single-tree replication. Data were analyzed using analysis of variance and LSD mean separation procedures of SAS 9.1.

RESULTS AND DISCUSSION

***Survey of antibiotic sensitivity among* E. amylovora *strains collected in California*.** Isolates of *E. amylovora* were confirmed for species identity by PCR amplification of a 1-kb DNA fragment using specific primers for plasmid pEa29 that is ubiquitously found in this bacterium. A total of 152 isolates from 35 pear orchard locations were obtained and tested for their sensitivity against streptomycin, oxytetracycline, and kasugamycin. Thirteen of the locations were in Sacramento Co., 21 were in Lake Co., and one was in Solano Co. There were 4 to 10 isolates per location, 1 to 5 isolates per location, and 3 isolates, respectively, for each of the three counties.

Table 1: Incidence of streptomycin resistance in isolates of *Erwinia amylovora* collected in surveys in 2013

County	Number of orchards	Number of isolates	Incidence of Streptomycin resistance (%)
Sacramento	13	105	6.7 (2 locations)
Lake	21	44	0
Solano	1	3	0
Total	35	152	

All 44 isolates from Lake Co. were found to be sensitive against the three antibiotics (Table 1). This is in agreement with surveys from previous years where streptomycin-resistance was found only once in this county. Isolates from the experimental orchard at UC Davis were also all sensitive. Among 105 isolates from 13 locations in Sacramento Co., 6.7% were streptomycin-resistant, but all were sensitive to oxytetracycline and kasugamycin. The seven isolates with moderate resistance to streptomycin came from two orchard locations that both had mixed sensitive/resistant populations. The low level of streptomycin resistance in Sacramento Co. in 2013 is of interest because in previous surveys, the majority of isolates from this county were resistant. Although there was an overlap between grower identifiers among years of sampling, it needs to be determined if exactly the same locations were sampled. This would give an indication as to the stability of streptomycin resistance. Spray records that were included with the submitted plant samples indicated that both orchards with streptomycin-resistance were treated with copper and Mycoshield in 2013; thus, the pathogen had not been under streptomycin selection pressure in 2013.

The biofilm inhibitor 2-aminoimidazole was evaluated in spiral gradient assays to determine if when mixed with streptomycin, streptomycin-resistant isolates of *E. amylovora* could be inhibited. No inhibition of growth was observed. When examining the publication by Worthington et al., we found that the authors had provided some inaccurate information and they actually did not use 2-aminoimidazole in their assays, but derivatives of the compound that are not commercially available. We will continue our studies on biofilm inhibitors with using dehydroproline that is commercially available (De Zoysa et al., Plant Pathology 62:767-776, 2013).

Toxicity of chemicals used for fire blight control in our studies against three biocontrol agents. Results from our in vitro assays indicated that streptomycin, oxytetracycline, kasugamycin, captan, and mancozeb at 40 ppm were all active against *Streptomyces lydicus* (Actinovate) and *Bacillus amyloliquifaciens* (Double Nickel 55) (Table 2). In contrast, *Aureobasidium pullulans* (Blossom Protect) was not inhibited in growth by the three antibiotics at 40 ppm, but was inhibited by captan and mancozeb. These data indicate that in field applications only Blossom Protect could be safely used in combination with the three antibiotics.

Table 2. Activity of chemicals used for fire blight control against three biocontrol agents

Biocontrol product and agent	Streptomycin	Oxytetracycline	Kasugamycin	Captan	Mancozeb
Actinovate (*Streptomyces lydicus*)	+*	+	+	+	+
Blossom Protect (*Aureobasidium pullulans*)	-	-	-	+	+
Double Nickel 55 (*Bacillus amyloliquifaciens)*	+	+	+	+	+

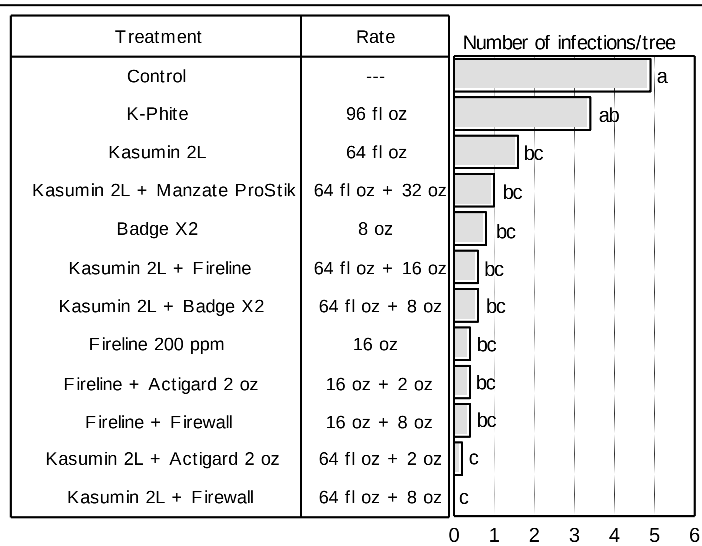
* - Activity was determined using the spiral gradient dilution assay. + = chemical is active against the biocontrol agent, - = chemical is not effective at maximum concentration of 40 ppm tested.

Field studies using protective treatments during the growing season. Fire blight incidence was low at our trial sites in 2013. Thus, in the UC Davis trial, trees were inoculated with the pathogen. In the trial on Bartlett pear in Live Oak, all treatments except K-phite significantly reduced the disease from that of the control where an average of 4.9 fire blight strikes were found per tree (Fig. 1). The remaining treatments which mainly consisted of antibiotics, copper, Actigard, and mixtures between these resulted in statistically similar disease levels. Numerically the lowest amount of disease was observed with the Kasumin-Firewall mixture, followed by Kasumin-Actigard, Fireline-Firewall, and Fireline-Actigard mixtures. Thus, mixtures of Kasumin with other compounds were more effective than Kasumin by itself in this trial. Actigard improved the performance of Kasumin but not of Fireline. Data from a large-scale field trial in a commercial orchard are currently being summarized.

In the trial at UC Davis, all treatments resulted in statistically similar levels of disease (Fig. 2). In contrast to the Live Oak trail, in this trial, K-phite also reduced the disease and Actigard did not improve the effectiveness of Kasumin. The Blossom Protect-Actigard mixture was also very effective.

Thus, although disease pressure was low in 2013, kasugamycin continued to be highly effective in reducing the incidence of fire blight. Once registered, it can be used in resistance management programs with rotations and possibly mixtures. Registration of the product for California is expected for 2014. The effect of the inducer of plant defense mechanisms, Actigard, was variable in our studies. This has been previously shown by others for fire blight, and in our own research for walnut blight caused by *Xanthomonas arboricola* pv. *juglandis*.

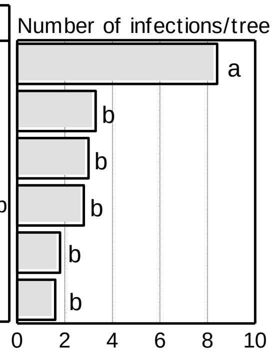
Fig. 1. Evaluation of new bactericides for fireblight management on Bartlett pears in a field trial in Live Oak CA - 2013



Treatments were applied on 3-21 (20% bloom), 3-28 (full bloom), 4-2 (petal fall), and 4-11-2013 (begin rattail) using an airblast sprayer at 100 gal/A. Disease was evaluated on April 17, 2013.

Fig. 2. Evaluation of new bactericides for fireblight management on Comice pears in a field trial at UC Davis, CA - 2013

Treatment	Rate/A/100 gal
Control	-----
Firewall	8 oz
K-Phite	96 fl oz
Actigard + Blossom Protect	2 oz + 1.34 lb + 9.35 lb
Kasumin 2L + Actigard	100 ppm + 2 oz
Kasumin 2L	100 ppm



Treatments were applied on 3-21, 3-28, and 4-3-13 (rattail) using an airblast sprayer at 100 gal/A. On 3-31-13, trees were at 30% bloom. Trees were inoculated with *E. amylovora* using an air-blast sprayer on 3-29-13. Disease was evaluated mid April 2013.