Annual Report - 2014

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. Antibiotic resistance surveys for populations of *Erwinia amylovora* in California pear growing areas were again conducted.
    a. All isolates from seven locations in Lake and Mendocino Co. were found to be sensitive against streptomycin, oxytetracycline, and kasugamycin.
    b. All 130 isolates from 18 locations in Sacramento Co. were sensitive to oxytetracycline and kasugamycin. Only 3 isolates were streptomycin-resistant and these came from a single location.
    c. Thus, similar to 2013, there was a very low level of streptomycin resistance in Sacramento Co. in contrast to some of the previous years. This is presumably related to a reduced fitness of resistant isolates. As we determined previously, streptomycin resistance in California isolates is based on a unique mode of action, different from other pome-fruit growing regions in the United States.
    d. Resistance genes on the current plasmid found in California may have made the pathogen less fit as compared to wild-type, sensitive strains. This may allow the integrated use of streptomycin in mixtures or rotations with oxytetracycline and kasugamycin.
2. Field trials on the management of fire blight were conducted under high disease pressure on Bartlett pear.
    a. Kasugamycin continued to be highly effective in reducing the incidence of fire blight. Kasumin received federal registration on pome fruit and registration for California is expected in 2015 for use in 2015 or 2016.
    b. Among conventional treatments, Kasumin mixed with Fireline, Actigard, or Manzate and a rotation of Kasumin+Captan with Kasumin+Firewall were the most effective.
    c. Among biological treatments, Blossom Protect was most effective. This product has been one of the most consistent biocontrols that we evaluated for fire blight control over the years with general significant reductions in disease from the control.
3. Laboratory assays on the sensitivity of *Aureobasidium pullulans* (Blossom Protect) to copper and sulfur.
    a. Copper (up to 400 ppm MCE tested) did not inhibit the biocontrol organism.
    b. The organism was completely inhibited by 12,000 ppm sulfur (wettable sulfur). Inhibition of growth was noticed at concentrations of $\geq$4000 ppm (no inhibition at 3000 ppm).

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. In California, 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 can be satisfactory when disease severity is low to moderate. On Bartlett pears, copper treatments traditionally have been used only during the dormant and bloom periods because phytotoxicity commonly occurs on fruit as russeting. With the newer formulations of copper, however, reduced rates based on metallic copper equivalent (mce) can be used and thus, extended usage past the bloom period can be an effective rotational treatment or mix-partner without causing russeting. This was also demonstrated in our fire blight trials on Bartlett pear during the past years.

The antibiotics streptomycin and the less effective oxytetracycline (terramycin) have also been used for many years. Because of usage for many years and lack of alternative control materials, resistance developed against streptomycin at many locations in California, mostly in Sacramento Co. 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. Fortunately, these less sensitive populations seem not to be persistent, because in successive samplings over several years they could not be detected at the same locations. Still resistance development in more competitive genotypes of the pathogen is a risk. Surveys on antibiotic resistance monitoring were continued in 2014 in collaboration with farm advisors.

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 or oxytetracycline. 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 and after a long regulatory delay, kasugamycin finally received federal registration on pome fruit in the fall of 2014, and the California registration is expected for later in 2015. Kasugamycin was again effectively used in our field trials in 2014. It was applied by itself or in mixtures or rotations with selected other materials, including other antibiotics or captan. These evaluations are done to identify effective mixture treatments that would reduce the potential for resistance development and for use with other disease management programs such as pear scab treatments. In 2014, we also tested ceragenin (a microbial membrane disrupter), as well as several materials that are either exempt from tolerance or biological treatments. These include K-Phite (a mixture of monopotassium phosphate and dipotassium phosphite) and the biocontrols Actinovate (*Streptomyces lydicus*), Blossom Protect (*Aureobasidum pullulans*), and Serenade Optiva (*Bacillus subtilis*). A buffer containing sugars, whey, and other nutrients was added to some of the biocontrol treatments to potentially enhance growth of the biocontrols agent and make it more effective. Other nutrient additives are under evaluation. Whey is a possible food allergen and this potentially may present a limitation to Blossom Protect buffer usage.

We also 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. A manuscript on this novel mode of resistance is currently being finalized.

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) as well as other treatments for fire blight control in cooperation with UCCE.
   a. 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 Kasumin.
      ii. Plant defense activators (e.g, ProAlexin, Actigard, PM-1) with and without Kasumin.
      iii. Biocontrols (e.g., Blossom Protect) with and without Kasumin.

iv. Large-scale field trials with Kasumin under an RA if necessary.
v. Selected rotation programs

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: Prepare a manuscript for publication based on research done previously by us.
    c. Laboratory in vitro tests to evaluate the activity of biofilm inhibitors such as dehydroproline in combination with copper or antibiotics using spiral gradient dilution assays.

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 2014 from orchards in Sacramento, Lake, and Mendocino 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 156 isolates of *E. amylovora* from 25 orchard locations were obtained in 2014.

***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.).

Field studies using protective treatments during the growing season. In field studies in a commercial cv. Bartlett orchard in Live Oak four applications of selected treatments (see Results) were done using a back-pack airblast sprayer at 100 gal/A. One branch per tree replicate was inoculated with *E. amylovora* (suspension of 2×10^7 cfu/ml) after air-drying of the third application using a backpack sprayer. For evaluation of the natural incidence on April 10, the number infected spurs with developing fruit for a total of 20 to 100 spurs per tree was evaluated. For inoculated blossoms, the number infected blossoms of the total blossoms was evaluated. A small-scale handsprayer trial was also conducted. For this, branches with blossoms were hand-sprayed, allowed to air-dry and were then inoculated with *E. amylovora* by hand-spraying. Disease incidence was determined based on the number of diseased blossoms of the total number of blossoms. All data were analyzed using analysis of variance and LSD mean separation procedures of SAS 9.4.

Evaluation of dehydroproline as a biofilm inhibitor and enhancer of toxicants such as antibiotics and copper to* E. amylovora *in in vitro assays. Strains of *E. amylovora* that are sensitive or resistant to streptomycin were used. Their sensitivity to each of the three antibiotics with or without the addition of the dehydroproline were determined using a filter paper disk assay. Bacterial suspensions were first spread over nutrient agar plates. Four filter paper disks 6 mm in diameter were placed equidistantly on each the agar plates and 10 µl of test solution was added to each disk. Test solutions consisted of dehydroproline 1000 ppm, 10,000 ppm, kasugamycin, oxytetracycline, streptomycin, copper, and combinations of dehydroproline with the latter compounds. Plates were incubated for 24 h and inhibition zones around the filter paper disks were measured.

Laboratory assays on the sensitivity of Aureobasidium pullulans (Blossom Protect) to copper and sulfur. Nutrient agar was amended with copper at selected concentrations up to 400 ppm MCE or with sulfur (wettable sulfur) at 0 ppm (control), 3000 ppm, 4000 ppm, 6000 ppm, and 12,000 ppm. The biocontrol was streaked onto the agar and growth was evaluated after four days of incubation.

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 most isolates of this bacterium. A total of 156 isolates from 25 pear orchard locations were obtained and tested for their sensitivity against streptomycin, oxytetracycline, and kasugamycin. Eighteen of the locations were in Sacramento Co., 6 were in Lake Co., and one was in Mendocino Co. One to 28 isolates per location were obtained.

All isolates from Lake and Mendocino 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 growing area of the state. Among 130 isolates from Sacramento Co., only 3 were streptomycin-resistant and these came from a single location where 7 additional isolates were streptomycin-sensitive. All isolates were sensitive to oxytetracycline and kasugamycin. Thus, similar to 2013, there was a very low level of streptomycin resistance in Sacramento Co. in contrast to some of the previous years where the incidence of resistance was often around 30%, but sometimes up to 70%.

This is of interest because streptomycin resistance has been reported to be stable by others. Possibly, the unique mechanism of streptomycin resistance in current California isolates contributes to reduced fitness of the isolates. If isolates are indeed less fit, the low incidence of resistance can be explained by low disease levels in the past few years that required less intense management programs. With high disease incidence in 2014, the incidence of resistance in sampled populations was still very low. Streptomycin usage has also been lower than in the past because of the perceived widespread resistance to the antibiotic and the high economic incentive to grow an organic crop. This resulted because of the removal of streptomycin as an approved organic treatment by the National Organic Standards Board (i.e., NOSB). The decline in streptomycin resistance is of interest because this indicates that this treatment can be used again in conventional farming operations to obtain effective control and where rotation and mixtures with other modes of action will allow integration of resistance management practices.

Field studies using protective treatments during the growing season. Fire blight incidence was very high in many pome fruit growing areas in California in 2014 and many growers experienced high losses. Natural incidence at our field trial sites ranged from 33% to 45%.

Eleven treatments and one rotation program were compared to the control in an air blast sprayer trial on Bartlett pear. The natural incidence of blight was reduced to low levels by several treatments that contained Kasumin (Fig. 1). These treatments included Kasumin mixed with Fireline, Actigard, or Manzate and a rotation of Kasumin+Captan with Kasumin+Firewall. Additional effective treatments for reducing the natural incidence of disease included ChampIon^{+2}+Mycoshield, Fireline+Firewall, as well as Kasumin, ChampIon^{+2}, and Fireline by themselves. Higher variability among replications of several treatments was found in the inoculation study and no statistical significance was evident although numerical reductions from the control were observed for these treatments. Still, several treatments were very effective and these included Kasumin mixed with Fireline or Manzate, ChampIon^{+2}+Mycoshield, Fireline+Firewall,, Kasumin, and the rotation of Kasumin+Captan with Kasumin+Firewall (Fig. 1). Thus, kasugamycin continued to be highly effective in reducing the incidence of fire blight. Once registered in California (expected in 2015 for use in the 2016 season), it can be used in resistance management programs with rotations and possibly mixtures.

Table 1. In vitro sensitivity of *Erwinia amylovora* populations from pear orchards in Sacramento and Lake Co. CA 2014

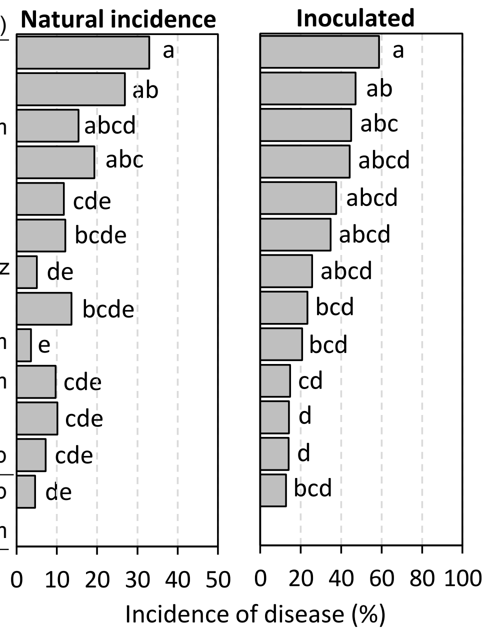
			Streptomycin		Oxytetracycline		Kasugamycin	
Orchard No.	County	No. of isolates	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
1	Sacramento	6	6	0	6	0	6	0
2	Sacramento	12	12	0	12	0	12	0
3	Sacramento	2	2	0	2	0	2	0
4	Sacramento	1	1	0	1	0	1	0
5	Sacramento	10	7	**3**	7	0	7	0
6	Sacramento	12	12	0	12	0	12	0
7	Sacramento	2	2	0	2	0	2	0
8	Sacramento	1	1	0	1	0	1	0
9	Sacramento	28	28	0	28	0	28	0
10	Sacramento	8	8	0	8	0	8	0
11	Sacramento	5	5	0	5	0	5	0
12	Sacramento	7	7	0	7	0	7	0
13	Sacramento	10	10	0	10	0	10	0
14	Sacramento	4	4	0	4	0	4	0
15	Sacramento	5	5	0	5	0	5	0
16	Sacramento	10	10	0	10	0	10	0
17	Sacramento	6	6	0	6	0	6	0
18	Sacramento	1	1	0	1	0	1	0
19	Lake	1	1	0	1	0	1	0
20	Lake	3	3	0	3	0	3	0
21	Lake	5	5	0	5	0	5	0
22	Lake	4	4	0	4	0	4	0
23	Lake	2	2	0	2	0	2	0
24	Lake	4	4	0	4	0	4	0
25	Mendocino	7	7	0	7	0	7	0
	total	156						

In vitro sensitivities were determined using the spiral gradient dilution method.

Biological treatments were compared in an air-blast trial and in a small-scale hand sprayer trial. In the hand sprayer trial, four treatments were compared to the control where 85% of the blossoms became infected after inoculation. Blossom Protect mixed with copper was the most effective treatment, reducing the incidence of disease to 7.4% (Fig. 2). Blossom Protect mixed with wettable sulfur as well as MagnaBon (a low-concentration copper product) showed intermediate efficacy; whereas Actinovate mixed with a nutrient buffer was the least effective. In the air-blast spray trial, Blossom Protect (mixed with nutrient buffer), Actinovate+NuFilm-P, as well as Kasumin reduced the natural incidence of blight significantly from that of the control (Fig. 3). Results for some of the treatments were different when blossoms were inoculated and only Blossom Protect and Serenade Optiva resulted in significant reductions in incidence from the control. Overall, the addition of sulfur to Blossom Protect resulted in numerical (but not statistical) increases in disease as compared to Blossom Protect alone, however no phytotoxicity was observed. Blossom Protect has been one of the most consistent biocontrols that we evaluated for fire blight control over the years with general significant reductions in disease from the control. Possibly, its activity still can be increased, but the BioLink surfactant and penetrant that was evaluated this year did not improve efficacy. Trials on other crops that we conducted have indicated that other nutrients can improve performance of biologicals. We are currently evaluating these nutrients to identify the most effective ones and we plan to test these in 2015 trials.

Fig. 1. Evaluation of new biological treatments for management of fire blight of Bartlett pear in a field trial in Live Oak, CA 2014

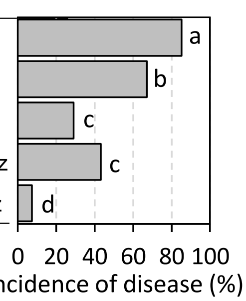
Treatment	Rate (/100 gal)	Natural incidence	Inoculated
Control	---	a	a
Ceragenin + Acetic Acid + NF-P	5.1 + 8 fl oz	ab	ab
Kasumin 2L + Magna Bon	100 + 100 ppm	abcd	abc
Magna Bon	100 ppm	abc	abcd
Fireline	200 ppm	cde	abcd
Champlon	8 oz	bcde	abcd
Kasumin 2L + Actigard	100 ppm + 2 oz	de	abcd
Kasumin 2L	100 ppm	bcde	bcd
Kasumin 2L + Fireline	100 + 150 ppm	e	bcd
Fireline + Firewall	200 + 100 ppm	cde	cd
Champlon + Mycoshield	8 oz + 16 oz	cde	d
Kasumin 2L + Manzate ProStik	100 ppm + 5 lb	cde	d
Rotation: Kasumin 2L + Captan 80WDG	100 ppm + 5 lb	de	bcd
Rotation: Kasumin 2L + Firewall	100 + 100 ppm		



Applications were done on 3-12 (bloom), 3-21 (full bloom), 3-27 (petal fall), and 4-4-14 (rat tail) using an airblast sprayer at 100 gal/A. NF-P = Nufilm P. One branch per tree was inoculated with *E. amylovora* on 3-27-14 after air-drying of the treatments. Disease was evaluated on 4-10-14. For natural incidence, the number infected spurs with developing fruit for a total of 20 to 100 spurs per tree was evaluated. For inoculated blossoms, the number infected blossoms of the total blossoms was evaluated.

Fig. 2. Evaluation of new biological treatments for management of fire blight of Bartlett pear in a small-scale field trial in Live Oak, CA 2014

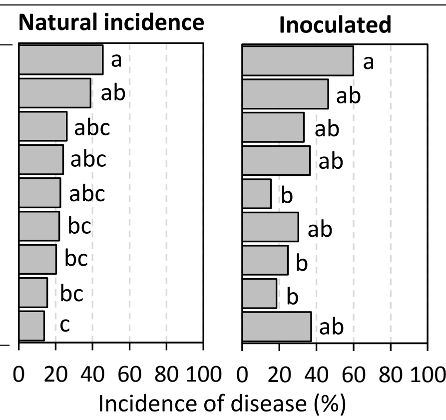
Treatment	Rate (/100 gal)	Incidence of disease
Control	---	a
Actinovate + Buffer	12 + 128 oz	b
Magna Bon	27 fl oz	c
Blossom Protect + Buffer + Wettable sulfur	20 + 128 + 225 oz	c
Blossom Protect + Buffer + Cuprofix	20 + 128 + 12 oz	d



Applications were done to run-off on 3-21-14 to branches with flowers using a hand sprayer. After air-drying branches were inoculated with E. amylovora. Disease was evaluated on 4-4-14. Incidence of disease was based on the number of diseased blossoms per total blossoms.

Fig. 3. Evaluation of new biological treatments for management of fire blight of Bartlett pear in a field trial in Live Oak, CA 2014

Treatment	Rate (/100 gal)
Control	---
K-Phite + WideSpread	96 + 8 fl oz
Actinovate + NF-P	12 oz
Blossom P. + Buffer + WS	1.25 + 8 + 15 lb
Serenade Optiva + NF-P	20 oz + 8 fl oz
Actinovate + NF-P + Buffer	12 oz + 8 fl oz + 36 oz
Blossom P. + Buffer + BioLink	1.25 + 8 lb + 4.5 fl oz
Blossom P. + Buffer	1.25 + 8 lb
Kasumin 2L	100 ppm



Applications were done on 3-12 (bloom), 3-21 (full bloom), 3-27 (petal fall), and 4-4-14 (rat tail) using an airblast sprayer at 100 gal/A. NF-P = Nufilm P, WS = Wettable sulfur, Blossom P = Blossom Protect. One branch per tree was inoculated with *E. amylovora* on 3-27-14 after air-drying of the treatments. Disease was evaluated on 4-10-14. For natural incidence, the number infected spurs with developing fruit for a total of 20 to 100 spurs per tree was evaluated. For inoculated blossoms, the number infected blossoms of the total blossoms was evaluated.

Evaluation of dehydroproline as a biofilm inhibitor and enhancer of toxicants such as antibiotics and copper to* E. amylovora *in in vitro assays. Dehydroproline at 1000 ppm was not inhibitory to strains of *E. amylovora* that are sensitive or resistant to streptomycin, but concentrations of 10,000 ppm resulted in inhibition zones around the treated filter paper disks. Mixtures of dehydroproline (1000 ppm) with streptomycin, oxytetracycline, kasugamycin, or copper did not increase inhibition zones as compared to the bactericides used by themselves. Thus, there was no benefit in using the mixtures. Concentrations of 10,000 ppm of dehydroproline were not further tested because they were considered too high for practical applications and additionally, this chemical is extremely expensive. Another biofilm inhibitor (2-aminoimidazole-triazole) that was tested by other researchers in a publication was not available by any supplier in the US.

Laboratory assays on the sensitivity of Aureobasidium pullulans (Blossom Protect) to copper and sulfur. Copper (up to 400 ppm MCE tested) did not inhibit growth of the biocontrol organism as compared to the control. Sulfur was inhibitory at concentrations of $\geq$4000 ppm and growth was completely inhibited by 12,000 ppm sulfur. Sulfur rates used in the field at 10-20 lb/A/100 gal are equivalent to 12,000 - 24,000 ppm. Thus, field rates of wettable sulfur would be inhibitory to the biocontrol agent and should not be applied in mixtures with Blossom Protect.