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EVALUATION OF NEW BACTERICIDES FOR CONTROL OF FIRE BLIGHT OF PEARS CAUSED BY *ERWINIA AMYLOVORA*

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SUMMARY

1. Antibiotic and copper resistance surveys for populations of *Erwinia amylovora* in California pear growing areas were continued.
   a. Oxytetracycline: All 26 strains from 7 orchards in Sacramento and Lake Co. were sensitive.
   b. Streptomycin: The single strain from Lake Co. was determined to be sensitive. Three strains from Sacramento Co were moderately resistant and five strains were highly resistant. Six of the eight resistant strains came from a single orchard. Thus, populations of *E. amylovora* re-adjust rapidly to selection pressure (i.e., bactericide applications). This indicates that streptomycin can be used strategically and stresses the importance of resistance management with mixtures or rotations without over-using any single active ingredient, and that new alternatives need to be developed.
   c. Copper: Moderate copper resistance was detected in strains of *E. amylovora*. Growth was similar to the control using 20 ppm MCE and was reduced at 30 ppm MCE on nutrient agar. Spontaneous mutants growing at high concentrations of copper were also observed. Management failures with the use of copper under high disease pressure have been attributed to highly favorable environments, low rates of copper registered, moderate copper resistance, and spontaneous mutants with high copper resistance.
2. Field trials on the management of fire blight were conducted under low to moderate disease pressure.
   a. Kasugamycin (Kasumin) continued to be highly effective in reducing the incidence of fire blight. Addition of ε-polylysine numerically improved the performance. California registration of Kasumin was ongoing with compliance of all recent requirements (social insect toxicology), posting for comment in Oct., and registration expected in Dec. in 2017. Kasumin was federally registered in 2014.
   b. Among "biological" treatments, the natural product 1552 and the exempt-from-tolerance antimicrobial ε-polylysine showed efficacy at low disease pressure. The biocontrol Blossom Protect mixed with buffer or molasses was similar in efficacy to Fireline (oxytetracycline) in a trial with moderate disease pressure. The Serenade

Opti-Cueva or -molasses mixtures only resulted a trend for lower disease as compared to the control.

3. Isolates of *Venturia pyrina* from 8 orchard locations were all determined to be highly sensitive to cyprodinil, difenoconazole, dodine, mancozeb, and triflumizole. Therefore, fungicide resistance was not responsible for the high incidence of pear scab in these orchards,

INTRODUCTION

Fire blight, caused by the bacterium *Erwinia amylovora*, is a very destructive disease of pome fruit trees worldwide, especially pears. In California, prolonged rat-tail bloom contributes to a long infection period. Fire blight is one of the most difficult diseases to manage, and very few effective chemicals are available. Integrated programs of sanitation practices and applications with chemical and biological controls are the best approaches. If the disease occurs at low incidence, 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 are based on protective treatments with antibiotics or copper. 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 some newer formulations of copper, however, reduced rates based on metallic copper equivalent (MCE) can be used past the bloom period without causing russeting. Under low disease pressure, copper compounds can provide satisfactory disease control and they can be an effective rotational or mix-partner. In years with high disease pressure such as in 2015, however, commercial copper applications failed to control the disease at many locations. Therefore, in our UCIPM ratings copper is ranked as a +/++ treatment indicating inconsistent performance depending on environmental conditions. In 2015 and 2016, we also reported reduced sensitivity to copper in strains of *E. amylovora* with growth occurring at 20 to 30 ppm MCE on nutrient-rich nutrient agar and 10 to 20 ppm in nutrient-poor CYE agar. These levels indicate moderate copper resistance and can explain the moderate and inconsistent performance of copper. Lack of systemic action and low registered rates are other factors for low efficacy. In 2017, we continued to evaluate copper sensitivity in strains of *E. amylovora* from Lake and Sacramento Co.

Treatments with the antibiotics streptomycin and the less effective oxytetracycline have been employed for many years to manage fire blight. Continued usage for many seasons and lack of alternative control materials caused resistance against streptomycin to develop at high incidence at many locations in California, mostly in Sacramento Co. in 2006 to 2011. With reduced use of streptomycin in the following years, resistance dropped to low levels and was only found in two of 24 orchards surveyed in 2015. In 2016, however, with probably increased streptomycin use, resistance re-emerged to high levels and was detected in 17 of 19 orchards sampled in Sacramento Co. Previously, we also detected isolates of *E. amylovora* with reduced sensitivity to oxytetracycline at several locations. At one of these locations, field treatments with Mycoshield were reported to be ineffective in controlling the disease. Fortunately, these less sensitive populations were not persistent and were not detected in successive samplings at the same locations. Surveys on antibiotic resistance monitoring were continued in 2017 in collaboration with farm advisors.

The incidence of fire blight was variable among California growing regions in the 2017 spring season, and was low to moderate at our field trial locations. In our evaluations of new materials for fire blight control, kasugamycin (Kasumin) again ranked very high in efficacy, either by itself or in selected mixtures. 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, Kasumin received federal registration on pome fruits in 2014, and finally is scheduled for a California registration in Jan. 2018. In 2017, we also tested the copper activity-enhancing compounds ZTD and DAS-1. ZTD is registered in some other countries, and in combination with copper, reduced the incidence of fire blight in our trial to very low levels as compared to copper alone. We also again tested biocontrols (Blossom Protect - *Aureobasidum pullulans*, Serenade Opti - *Bacillus subtilis*) by themselves and in mixtures with growth enhancers, and we evaluated several natural products including Fracture, the antimicrobials ε-polylysine (used commercially as a food preservative in Japan, Korea, and in imported items in the United States) and Nisin, Brandt VegLys, and the numbered compound 1552. This was done because many growers are moving away from antibiotic use, and there is a growing interest in organic pear production. Under low to moderate disease pressure in 2017, some of these 'biological' treatments showed good efficacy.

OBJECTIVES

1. Evaluate and optimize the performance of kasugamycin (Kasumin), other antibiotics such as streptomycin (e.g., Agrimycin-17, Firewall) and oxytetracycline (e.g., Mycoshield, Fireline) and new antimicrobials in field trials.
   a) Kasumin in combination with exempt-from-tolerance antimicrobials (see below), high-sugar compounds, and other bactericides.
   b) Large-scale field trials with Kasumin under an RA (once Kasumin is registered).
   c) Evaluate two new anti-microbials from two US registrants - SBH and ZTD in laboratory and field studies.
2. Evaluate and optimize the performance of selected biocontrols.
   a) Biocontrols (e.g., Blossom Protect, Botector) with and without selected nutrient additives.
3. Evaluate the effectiveness of ε-polylysine, nisin, propionic acid, and lactic acid against *E. amylovora*.
   a) Laboratory assays on different media
   b) Small-scale field studies with the compounds alone or in mixture with other chemicals.

4. Determine the sensitivity of *E. amylovora* populations from pear orchards in California to streptomycin, oxytetracycline, and copper (continuation of antibiotic resistance surveys).
5. In vitro sensitivity of isolates of *Venturia pyrina* from California pear orchards to selected fungicides in 2017.

MATERIALS AND METHODS

Isolation of *E. amylovora* and bacterial culturing. Pear samples with fire blight symptoms were obtained in the spring and early summer of 2017 from 6 orchards in Sacramento and 1 orchard in Lake Co. Infected plant material (fruit, stems, pedicels, twigs) was cut into small sections and incubated in 1 ml of sterile water for 15 to 30 min to allow bacteria to diffuse out of the tissue. Suspensions were streaked onto yeast extract-dextrose-$CaCO_3$ agar (YDC) and single colonies of *E. amylovora* were transferred. A total of 26 strains were obtained and evaluated for their sensitivity to antibiotics and copper.

Laboratory studies on the toxicity of bactericides against *E. amylovora*. Streptomycin and oxytetracycline were evaluated for their in vitro toxicity using the spiral gradient endpoint method. For this, a radial bactericidal concentration gradient was established in nutrient agar in Petri dishes by spirally plating a stock concentration of each antimicrobial using a spiral plater. After radially streaking out suspensions of the test bacteria (10 µl of 108 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 taken visually 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.

Copper sensitivity of strains was determined by streaking bacterial suspensions (70% transmission at 600 nm) on CYE (casitone, yeast extract, glycerol) or nutrient agar amended with 0, 10, 20, or 30 ppm MCE. Growth was recorded after 2 days of incubation at 25°C and was rated as ++ (growth not inhibited, similar to control), + (growth inhibited as compared to the control), or - (no growth).

Field studies using protective treatments during the growing season. In a commercial cv. Bartlett orchard in Live Oak, three applications of selected treatments (see Results) were done using a back-pack air-blast sprayer at 100 gal/A between March 16 and April 4. Natural incidence of disease (i.e., fire blight strikes/tree) was evaluated on April 11 and 25. Data were analyzed using analysis of variance and LSD mean separation procedures of SAS 9.4.

In vitro sensitivity of isolates of* Venturia pyrina *from California pear orchards to selected fungicides in 2017. Leaf and/or fruit samples were obtained from 15 orchard locations. Infected tissues were plated onto potato dextrose agar amended with rifampicin and ampicillin. When sporulating lesions were present, spore suspensions were plated, and fungal colonies were then sub-cultured. In vitro sensitivities for cyprodinil, difenoconazole, dodine, mancozeb, and triflumizole were determined for mycelial growth using the spiral gradient dilution method as previously described. Mycelial growth was measured after 11 days of incubation at 20°C, and EC_{50} values were calculated.

RESULTS AND DISCUSSION

***Survey of antibiotic and copper sensitivity in* E. amylovora *strains from California*.** All 26 strains from seven locations in Sacramento and Lake Co. were determined to be sensitive to oxytetracycline (Table 1); whereas, 8 of 25 strains from Sacramento Co. were resistant to streptomycin. Five of these 8 strains were highly resistant (MIC values >2000 mg/L) and three were moderately resistant (MIC values <30 ppm) (Table 1). Resistance was found in three of the seven orchards sampled, and all six strains from one orchard were either moderately or highly resistant. Results over the years support our recommendation that streptomycin can be used once a year effectively for most growers. In years with high- to moderate disease levels, pathogen populations exposed to multiple applications of streptomycin will be under selection pressure of the antibiotic, and this will allow re-emergence of resistant sub-populations.

All 26 strains from Sacramento and Lake Co. did not grow on CYE (a growth medium with a low copper-binding capacity) amended with 20 ppm MCE (Table 1). They all grew similar to the non-amended control on the nutrient-rich nutrient agar at 20 ppm MCE. One strain still grew well at 30 ppm MCE on nutrient agar, whereas growth of the other strains was reduced at this concentration. Thus, as in 2015 and 2016, we conclude that current *E. amylovora* populations are moderately copper-resistant. Additionally, we again frequently observed the occurrence of spontaneous mutant colonies emerging at higher copper concentrations, especially when using nutrient agar. These mutants were not stable when sub-cultured on copper-free media and reverted back to sensitivity. If these mutants also occur in the field, however, under continued presence of selection pressure (i.e., copper sprays) they may successfully compete and cause disease.

We consider several factors that likely contributed to the failure of copper applications to control fire blight in the past: 1) Highly conducive disease conditions may low allow for the pathogen to overcome the suppressive action of copper; 2) Only low rates of copper are registered for fire blight management (approx. 170 MCE for the 0.5 lb rate of Kocide 3000) and this may allow growth of moderately Cu-resistant strains; 3) There is moderate copper resistance in *E. amylovora;* and 4), Selection of populations (spontaneous mutants) with higher copper resistance after repeated applications may lead to disease in the presence of copper. Furthermore, copper is bacteriostatic and does not kill the pathogen. Thus, use as a pre-bloom/early bloom treatment may have some benefits in suppressing bacterial oozing from cankers. Applying a contact bactericide with low to moderate toxicity will only provide marginal benefits because the pathogen causes a deep internal infection (i.e., cankers) and has a high reproductive capacity. This means that the pathogen will ooze from cankers (unaffected by copper) and disseminate to unprotected tissues if copper is not routinely applied. If several copper applications are done, however, russetting will occur on Bartlett and other pear varieties.

***Field studies using protective treatments during the growing season*.** Fire blight incidence was low to moderate at our field study sites in 2017. In evaluation of conventional bactericides, ZTD-Cu and kasugamycin-streptomycin mixtures significantly reduced the disease to the lowest levels with less than an average of 1 strike per tree as compared to the control where an average of 14.5 blight strikes were present per tree (Fig. 1). ZTD by itself did not significantly reduce the disease from the control, and copper (i.e., Cueva) by itself showed intermediate efficacy. Thus, the copper activity-enhancing compound ZTD was a very promising treatment, however EPA did not accept a biopesticide registration petition from the potential registrant. EPA will only consider registration of ZTD as a conventional pesticide that will require extensive toxicological and environmental fate testing. For the small size of the bactericide market, the potential registrant cannot justify the financial investment. The second enhancer of copper activity that we evaluated, DAS-1, did not improve performance of copper. Furthermore, DAS-2, a pre-mixture of DAS-1 and copper, was also not effective. Currently, we are working with the registrant of these compounds to supply other derivatives of the core molecule SBH.

Addition of the antimicrobial ε-polylysine numerically reduced fire blight as compared to Kasumin by itself, whereas ε-polylysine by itself was less effective (Fig. 1). Very similar results were obtained in 2016. In contrast to 2016, the addition of lactic acid did not improve performance of Kasumin. Other natural products were evaluated as potential alternatives for antibiotics. As in 2016, the experimental 1552 showed good efficacy, significantly reducing the number of strikes per tree to 3. Nisin (Fig. 2), Fracture, and VegLys (Fig. 3) were not effective in statistically reducing the disease. There were numerical reductions in disease after treatment with Serenade+Cueva or Serenade+molasses. Disease, however, was increased as compared to the control after applications with Serenade-NuFilm P. Blossom Protect mixed with the recommended buffer or with molasses reduced the number of fire blight strikes to similar low levels as Fireline in the first evaluation, and showed a statistical trend for reducing fire blight in the second evaluation (Fig. 2).

In vitro sensitivity of isolates of* Venturia pyrina *from California pear orchards to selected fungicides in 2017. Samples were sent to us from orchards where fungicide applications were done, but where scab developed at a high incidence. Thus, fungicide resistance was suspected to be the reason for high disease. Isolates were only obtained from 8 of the 15 locations sampled because non-refrigerated samples were received during periods with very high temperatures. Still, 26 isolates of the pathogen were available for evaluation. All isolates were determined to be sensitive to cyprodinil (EC_{50} range 0.001 – 0.006 ppm), difenoconazole (EC_{50} <0.0015 ppm), dodine (EC_{50} range 0.006 – 0.019 ppm), mancozeb (EC_{50} range 0.14 – 0.56 ppm), and triflumizole (EC_{50} <0.003 ppm) (Table 2). Although no baseline sensitivity data were available for comparison, these EC_{50} values are very low and therefore, all isolates were considered highly sensitive. Possible reasons for high disease incidence in the sampled orchards include not applying liquid lime sulfur dormant treatments to reduce primary inoculum in the spring, poor timing due to microclimate conditions that prevented application, and insufficient number or frequency of in-season applications. In all cases, proper application volumes of carrier are needed to provide sufficient coverage (i.e., minimum of 100 gal/A for tree crops).

Table 1. Sensitivity of *E. amylovora* strains from California pear orchards to streptomycin, oxytetracycline, and copper in 2017.

						Copper sensitivity - growth at:		
No.	Location No.	Isolate code	County	Strepto-mycin	Oxytetra-cycline	20 ppm CYE agar	20 ppm Nutrient agar	30 ppm Nutrient agar
1	1	1-1	Sacramento	S	S	-	++	+
2		1-2		S	S	-	++	+
3		1-3		S	S	-	++	+
4		1-4		S	S	-	++	+
5		1-5		S	S	-	++	+
6	2	2-1	Sacramento	HR	S	-	++	+
7		2-2		HR	S	-	++	+
8		2-3		MR	S	-	++	+
9		2-4		HR	S	-	++	+
10		2-5		HR	S	-	++	+
11		2-6		MR	S	-	++	+
12	3	3-1	Sacramento	HR	S	-	++	+
13	4	4-1	Sacramento	S	S	-	++	+
14	5	5-1	Sacramento	MR	S	-	++	+
15		5-3		S	S	-	++	++
16		5-5		S	S	-	++	+
17		5-6		S	S	-	++	+
18		5-7		S	S	-	++	+
19		5-9		S	S	-	++	+
20	6	6-1	Sacramento	S	S	-	++	+
21		6-2		S	S	-	++	+
22		6-3		S	S	-	++	+
23		6-4		S	S	-	++	+
24		6-5		S	S	-	++	+
25		6-6		S	S	-	++	+
26	7	7-1	Lake	S	S	-	++	+

Sensitivity to streptomycin and oxytetracycline was determined using the spiral gradient endpoint method. S = sensitive, MR = moderately resistant (MIC = <30 ppm), HR = highly resistant (MIC = >2000 ppm). Sensitivity to copper was determined by growth on amended CYE or nutrient agar. ++ = growth similar as in the non-amended control, + = reduction in growth.

Table 2. In vitro sensitivity of isolates of *Venturia pyrina* from California pear orchards to selected fungicides in 2017.

Sample/ Location No.	No. isolates obtained
1	1
2	7
3	7
4	4
5	2
6	2
7	1
8	2
9-15	0
total	26

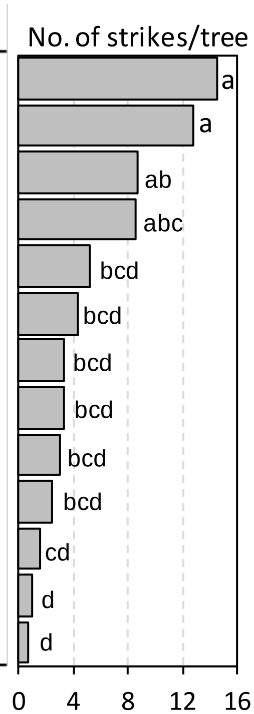
EC_{50} ranges for inhibition of mycelial growth for 26 isolates of *Venturia pyrina* for selcted fungicides

Fungicide	EC_{50} range (ppm)
Cyprodinil	0.001 - 0.006
Difenoconazole	<0.0015
Dodine	0.006 - 0.019
Mancozeb	0.14 - 0.56
Triflumizole	<0.003

In vitro sensitivities were determined using the spiral gradient dilution method. Growth was measured after 11 days of incubation at 20C.

Fig. 1. Evaluation of bactericides for management of fire blight of Bartlett pear in a field trial in Live Oak, CA 2017.

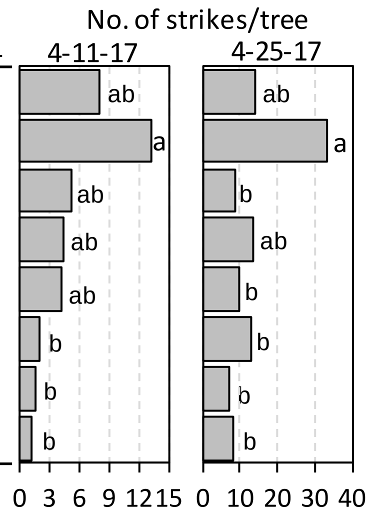
Treatment	Rate (/100 gal)	Applications 3-16	3-29	4-4
Control	---	---	---	---
DAS1 + Cueva	27 + 96 fl oz	@	@	@
ZTD	34 fl oz	@	@	@
DAS2 + Nufilm	27 fl oz	@	@	@
Polylysine	13.5 oz	@	@	@
Cueva	96 fl oz	@	@	@
Kasumin + Lactic acid 1:1	64 fl oz + 15 fl oz	@	@	@
Firewall	8 oz	@	@	@
1552	28 oz	@	@	@
Kasumin	64 fl oz	@	@	@
Kasumin + Polylysine	64 fl oz + 13.5 oz	@	@	@
ZTD + Cueva	34 + 96 fl oz	@	@	@
Kasumin + Firewall	64 fl oz + 8 oz	@	@	@



Applications were done using an air-blast sprayer at 100 gal/A on 3-16 (5% bloom), 3-29 (full bloom), and 4-4-17 (petal fall). There were four single-tree replications per treatment. Disease was evaluated on April 11, 2017.

Fig. 2. Evaluation of biological treatments for management of fire blight of Bartlett pear in a field trial in Live Oak, CA 2017.

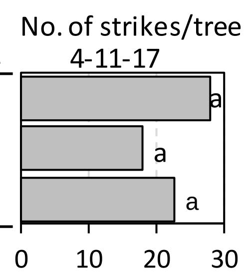
Treatment	Rate (/100 gal)	Applications 3-16	3-30	4-4
Control		---	---	---
Serenade + NuFilm P	16oz + 8 fl oz	@	@	@
Serenade + Cueva	16 oz + 96 fl oz	@	@	@
Serenade + Molasses + NuFilm P	16 oz + 355 + 8 fl oz	@	@	@
Nisin	13.5 oz	@	@	@
Blossom Protect + buffer	20 + 143 oz	@	@	@
Blossom Protect + Molasses	20 oz + 355 fl oz	@	@	@
Fireline	16 oz	@	@	@



Applications were done using an air-blast sprayer at 100 gal/A on 3-16 (5% bloom), 3-30 (full bloom), and 4-4-17 (petal fall). There were four single-tree replications per treatment. Disease was evaluated on April 11 and on April 25, 2017.

Fig. 3. Evaluation of biological treatments for management of fire blight of Bartlett pear in a field trial in Live Oak, CA 2017.

Treatment	Rate (/100 gal)	Applications 3-16	3-29	4-4
Control	'---	---	---	---
Fracture	32 fl oz	@	@	@
Brandt VegLys	6 fl oz	@	@	@



Applications were done using an air-blast sprayer at 100 gal/A on 3-16 (5% bloom), 3-29 (full bloom), and 4-4-17 (petal fall). There were four single-tree replications per treatment. Disease was evaluated on April 11, 2017.