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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 conducted.
   a. Oxytetracycline: All 86 strains from 31 orchards in Sacramento and Lake Co. were sensitive.
   b. Streptomycin: All 18 strains from Lake Co. were determined to be sensitive. However, 48 strains from Sacramento Co were moderately resistant and three strains were highly resistant. Overall, resistance was detected in 17 of 19 orchards. Thus, after several years of low resistance, resistant strains have re-emerged probably with recommended of streptomycin. This was expected but still, the use can be managed with mixtures and rotations. Still, this stresses the need for effective alternative treatments.
   c. The molecular mechanism of moderate resistance to streptomycin was determined to be the same as previously described: resistance genes that are located on plasmid pEU30.
   d. Copper: Reduced copper sensitivity was detected in many strains of *E. amylovora*. At 20 ppm MCE, growth was present using CYE (nutrient-poor) and at 30 ppm MCE, growth was present using nutrient agar (nutrient-rich). These levels indicate moderate copper resistance. Spontaneous mutants growing at high concentrations of copper were also observed frequently again. 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 moderate disease pressure.
    a. Kasugamycin continued to be highly effective in reducing the incidence of fire blight. Addition of lactic acid, ε-polylysine, or molasses numerically improved the performance of kasugamycin. California registration of Kasumin was ongoing in 2016 with deficiencies in state requirements requiring submission of additional data by the registrant in August. A compromised label was proposed by Arysta to reduce the amount of applications from four to two per season. The 18-month state review period concludes in January 2017. The antibiotic is registered by the US EPA since Sept. 2014.
    b. Several types of "biological" treatments were evaluated. The natural product 1552 and the exempt-from-tolerance antimicrobial ε-polylysine showed efficacy at low disease pressure. The biocontrol Blossom Protect mixed with molasses resulted in numerically the lowest disease level in a trial with higher disease pressure. The Serenade Opti-Badge mixture resulted in no improvement of efficacy as compared to Serenade Opti by itself.

INTRODUCTION

Fire blight, caused by the bacterium *Erwinia amylovora,* is a very destructive disease of pome fruit trees worldwide, especially pears. In California, due to prolonged rat-tail bloom, the infection period is long. Fire blight is one of the most difficult diseases to manage, and there are very few effective chemicals available. Integrated programs that combine sanitation and orchard management with chemical and biological controls are the best approaches. If the disease occurs at a 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 schedules with antibiotics, copper, or biocontrols. 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 without causing russeting past the bloom period. 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 we ranked copper as a +/++ treatment indicating inconsistent performance under different environmental conditions. We demonstrated reduced sensitivity to copper in 243 strains of *E. amylovora* with growth occurring at 20 to 30 ppm MCE in nutrient-rich and 10 to 15 ppm in nutrient-poor growth media. 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 known factors contributing to low performance under high disease pressure. In 2016, we also continued to evaluate copper sensitivity in strains of *E. amylovora* from Lake and Sacramento Co. We continued to evaluate copper as a pre-bloom treatment in cooperation with C. Ingels, Farm Advisor, 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. 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 were not persistent and were not detected in successive samplings at the same locations. Surveys on antibiotic resistance monitoring were continued in 2016 in collaboration with farm advisors.

The incidence of fire blight was moderate at many locations in the spring and early summer in 2016. In our evaluations of new materials for fire blight control, the disease was effectively reduced by some treatments. Kasugamycin (Kasumin) again ranked very high in efficacy, either by itself or in selected mixture or rotation treatments. 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. California registration is still in the 18-month review process until Jan. 2017. The registrant has supplied additional data in the summer of 2016 and has proposed a reduced number of applications for their state label. In 2016, we again tested biocontrols (Actinovate - *Streptomyces lydicus*, Blossom Protect - *Aureobasidum pullulans*, Serenade Opti - *Bacillus subtilis*) by themselves and in mixtures with growth enhancers. Additionally, the natural product 1552 that is used commercially as a food preservative in Japan, Korea, and in imported items in the United States was evaluated. This was done because many growers are moving away from antibiotic use, and there is a growing interest in organic pear production. The exempt-from-tolerance, antimicrobial ε-polylysine and lactic acid were also evaluated. Under moderate disease pressure in 2016, these 'biological' treatments were effective in mixtures with antibiotics in reducing fire blight.

OBJECTIVES

1. Evaluate and optimize the performance of kasugamycin (Kasumin) and other antibiotics such as streptomycin (e.g., Agrimycin-17, Firewall) and oxytetracycline (e.g., Mycoshield, Fireline) in field trials.
   a) Kasumin in combination with plant defense activators (e.g., Actigard, PM-1) and other antibiotics.
   b) Large-scale field trials with Kasumin under an RA (once Kasumin is registered).
   c) Facilitate persistence and non-target resistance studies with kasugamycin in the environment in collaboration with Arysta Life Science and G. Sundin (Michigan State University).
   d) New formulations of copper (e.g., Kocide 3000, Badge X2, MagnaBon)
   e) Selected rotation programs.
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, and propionic 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).

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 2016 from 19 orchards in Sacramento and 12 orchards 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 stream 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 86 strains of *E. amylovora* were obtained and evaluated for their sensitivity to antibiotics and copper.

Laboratory studies on the toxicity of bactericides against* E. amylovora*. 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.).

A genetic analysis was performed on selected moderately resistant isolates to determine the mechanism of resistance. PCR amplifications were performed using primers AJ507 (located at the 3' end of *strB*) and pEU30R (located in plasmid pEU30). Amplifications were done at an annealing temperature of 56C, and amplification products were separated in agarose gels. Presence of a band indicated that *strB* is located on plasmid pEU30.

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 25C and was rated as +++ (growth not inhibited, similar to control), ++ (growth inhibited as compared to the control), or + (growth sparse).

Field studies using protective treatments during the growing season. In two field studies in a commercial cv. Bartlett orchard in Live Oak, five applications of selected treatments (see Results) were done using a back-pack air-blast sprayer at 100 gal/A between March 7 and April 20. Natural incidence of disease (i.e., fire blight strikes/tree) was evaluated on random flowers on May 5. Disease incidence was determined based on the number of diseased blossoms or flower clusters of the total number of blossoms or flower clusters. Data were analyzed using analysis of variance and LSD mean separation procedures of SAS 9.4.

RESULTS AND DISCUSSION

***Survey of antibiotic and copper sensitivity among* E. amylovora *strains collected in California*.** All 18 strains from 12 locations in Lake Co. were determined to be sensitive to the antibiotics streptomycin and oxytetracycline (Table 1). All 68 strains from orchards in Sacramento Co. were sensitive to oxytetracycline, but there was a high incidence of resistance to streptomycin (Table 1). Resistance was found in a total of 51 strains in 17 of the 19 orchards sampled. Among resistant strains, 48 were rated as moderately resistant (MR) with MIC values of <30 ppm, and 3 strains that grew at >2000 mg/L streptomycin were rated as highly resistant (HR). After several years with a low to very low incidence of streptomycin resistance, this find was surprising. Streptomycin usage in these orchards is not known to us and spray records from the Sacramento Co. orchards could provide useful information on improving chemical usage. 2015 was a high-disease year, and possibly more applications of streptomycin were done that year and/or in 2016. This would have put the pathogen populations under selection pressure, allowing the resistant sub-population to re-emerge.

A genetic analysis of 24 MR strains from 14 locations was conducted to determine the mechanism of resistance, i.e., if the previously described mechanism in California strains of *E. amylovora* was responsible for resistance. Amplification of a DNA fragment spanning the 3' end of *strB* into pEU30 using primers AJ507 and pEU30R indicated that resistance was conferred by the *str* genes and that these genes are located on plasmid pEU30 (Fig. 1). This is the previously described main mechanism (Förster et al., Phytopathology 105:1302-1310). Therefore, resistance likely did not develop newly. With reduced streptomycin usage, resistant strains were out-competed by wild-type strains over the past few years at these locations, survived at low incidence, and were readily selected for in 2016. In contrast, highly resistant strains only occurred at low incidence. The re-emergence of streptomycin resistance in California orchards stresses the need for new effective rotational fire blight management tools, such as the registration of kasugamycin. Furthermore, continued resistance monitoring in the fire blight populations is important to determine best usage of streptomycin.

Thirteen strains from eight locations in Lake Co. showed reduced growth (as compared to the non-amended control) on CYE (a medium with a low copper-binding capacity) amended with 20 ppm MCE, but there was no growth at 30 ppm MCE (Table 1). The remaining strains did not grow at these copper concentrations. Similarly, 46 of the 68 strains from Sacramento Co. showed growth at 20 ppm MCE, but three strains still grew at 30 ppm MCE. All strains grew well on the nutrient-rich nutrient agar amended with 20 ppm MCE and some showed some growth at 30 ppm MCE. Thus, as in 2015, we conclude that current *E. amylovora* populations are moderately copper-resistant. Additionally, we again frequently observed the occurrence of spontaneous mutants growing 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.

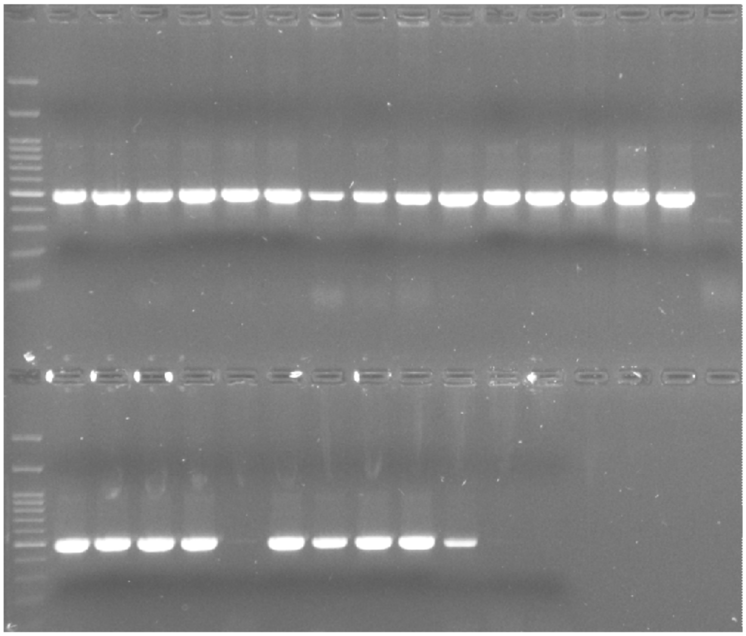
We consider several factors that likely contributed to the failure of copper applications to control fire blight in a cooperative project in 2016 (as well as failures in 2015 trials): 1) Highly conducive disease conditions were present in 2015 at many locations; 2) Low rates of copper are registered for fire blight management (approx. 170 MCE for the 0.5 lb rate of Kocide 3000); 3) There is moderate copper resistance in *E. amylovora;* and 4), Selection of populations (spontaneous mutants) with higher copper resistance after repeated applications. Additionally, copper is bacteriostatic and does not kill the pathogen. Applying a contact will only provide marginal benefits because the pathogen causes a deep internal infection (i.e. cankers) and the bacterium has a high reproductive capacity. This means that the pathogen will ooze out of cankers (unaffected by copper) and disseminate to unprotected tissue if copper is not routinely applied. Numerous copper applications, however, cause russetting in pears.

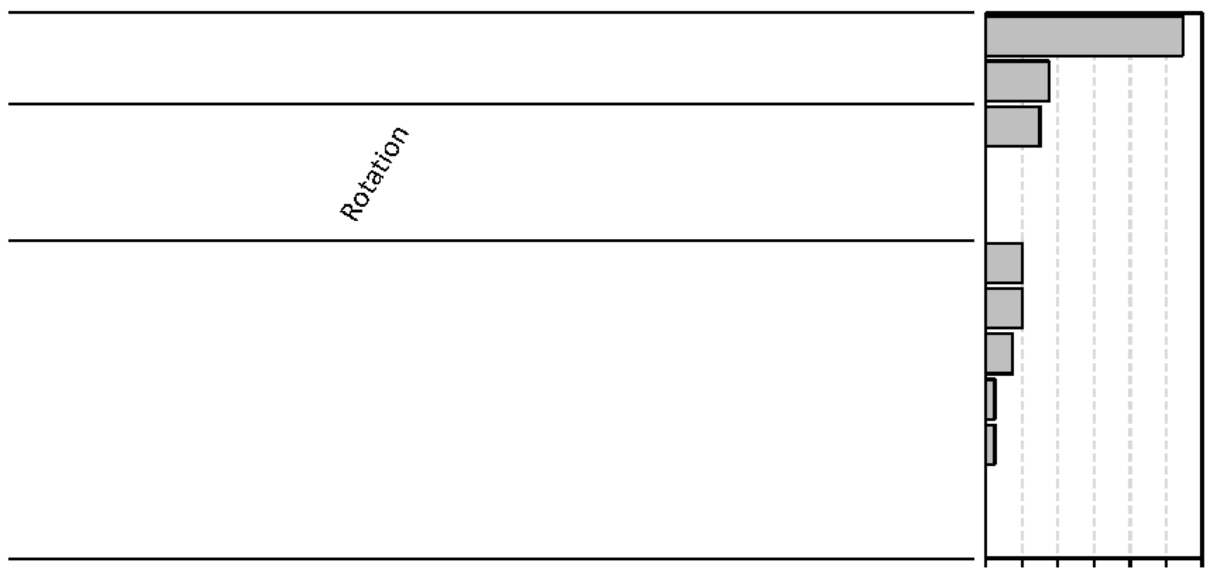
***Field studies using protective treatments during the growing season*.** Fire blight incidence was low to moderate at our field study sites. In evaluation of antibiotics, streptomycin, kasugamycin, and a rotation of antibiotic mixtures all significantly reduced the incidence of disease from the control where an average of 5.5 blight strikes occurred per tree (Fig. 2). There was no statistical difference among treatments. Addition of the antimicrobials lactic acid or ε-polylysine, or of molasses, numerically improved the performance of kasugamycin, but not addition of the acidifying agent LI-700. No disease was detected after five treatments with kasugamycin+ε-polylysine or streptomycin. Thus, treatments that included kasugamycin continued to be highly effective in reducing the incidence of fire blight.

Other treatments were evaluated as potential alternatives for antibiotics, and these included several types of “biologicals”. Among these, the natural product 1552 and the exempt-from-tolerance antimicrobial ε-polylysine were statistically similar effective as the antibiotics in the first trial with low disease pressure (Fig. 2). In the second trial, an average of 20 fire blight strikes was found on untreated control trees. As in the first trial, no statistical differences were found among treatments, but all treatments significantly reduced the disease from the control (Fig. 3). The biocontrol Blossom Protect mixed with molasses resulted in numerically the lowest disease level. Molasses was used because in last year’s in vitro studies it was identified as a promising additive that at selected rates inhibited growth of *E. amylovora*, whereas growth of several biocontrol agents was increased. We speculated that the mode of action of molasses was an increase in water activity. Bacterial growth is generally completely inhibited at a water activity of 0.9. Our measurements of water activity at the application rate of molasses used in field studies, however, indicated that the water activity (e.g., water activity was 0.99) did not significantly change from that of water (i.e., water activity was 1.0). Thus, the cause of the inhibitory effect of molasses is not known. The addition of lactic acid to

Blossom Protect resulted in the same amount of disease as when the recommended buffer was added. The Serenade Opti-Badge mixture resulted in no improvement of efficacy as compared to Serenade Opti by itself (Fig. 3).

County	Orchard No.	No. strains	Streptomycin	Oxytetracycline	Copper sensitivity Growth at 20 ppm MCE	30 ppm MCE
Lake	1	1	S	S	-	
	2	1	S	S	+	
	3	1	S	S	+	
	4	2	S	S	+	
	5	1	S	S	+	
	6	1	S	S	-	
	7	1	S	S	-	
	8	1	S	S	+	
	9	1	S	S	+	
	10	1	S	S	-	
	11	1	S	S	+	
	12	6	S	S	5 x +	
total		18	all S	all S	13	
Sacramento	1	2	S	S	+	
	2	4	3 x MR	S	+	
	3	1	MR	S	+	
	4	3	MR	S	+	
	5	3	MR	S	2 x +	
	6	3	2 x MR, 1 x HR	S	+	
	7	3	MR	S	1 x +	
	8	3	MR	S	-	
	9	3	MR	S	+	
	10	1	S	S		+
	11	5	1 x MR	S	-	
	12	3	MR	S	-	
	13	1	HR	S	-	
	14	4	2 x MR, 1 x HR	S	2 x +	2 x +
	15	1	MR	S	+	
	16	3	MR	S	+	
	17	3	MR	S	+	
	18	4	3 x MR	S	+	
	19	18	11 x MR	S	14 x +	
total		68	48 x MR, 3 x HR	all S	46 x +	3 x +

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