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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. All 243 strains from 63 orchards in five counties (Sacramento (24), Lake (22), Mendocino (14), and Sutter-Yuba (3) Co. were sensitive to oxytetracycline, and a majority was sensitive to streptomycin.
   b. Streptomycin resistance was only detected in two orchards in Sacramento Co. Eight strains were moderately resistant and one was highly resistant. Thus, very low levels of streptomycin resistance has been detected in the last three years and the antibiotic may be effectively used again in rotations with other antibiotics or some biologicals.
   c. Reduced copper sensitivity in strains of *E. amylovora* was detected with growth at 20 to 30 ppm copper in nutrient agar (nutrient-rich) and 10 to 15 ppm in CYE (nutrient-poor) media. These levels indicate moderate copper resistance. Spontaneous mutants that were resistant to copper at high concentrations were also observed frequently, similar to previous reports by others.
   d. Management failures with the use of copper 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 high disease pressure.

a. Kasugamycin continued to be highly effective in reducing the incidence of fire blight. California registration of Kasumin was delayed in 2015 with the state requiring more environmental fate studies. The antibiotic is registered by the US EPA since Sept. 2014.
b. Kasumin mixed with other antibiotics (e.g., AgriMycin 17, Mycoshield), SAR compounds (e.g., Actigard), Manzate, or copper were all highly effective. Kasumin mixed with AgriMycin was the most effective. Copper was ineffective and disease incidence was similar to the untreated control.
c. Among biological treatments, Serenade Opti and Blossom Protect+sugar were the most effective in reducing fire blight after two applications. Blossom Protect was incompatible with Kumulus (a wettable sulfur). Blossom Protect+Magna Bon CS-2500 (copper) and Actinovate + K-Phite were moderately effective. None of the biologicals, however, were effective in evaluations two weeks after the last of three applications.

3. Of the biologicals evaluated in laboratory growth studies:
a. Only *Aureobasidium pullulans* grew well over the optimum temperature range for *E. amylovora* (i.e., 15 to 25C) and it continued to grow well up to 35 C in the 24- and 72-h studies. After 72 h, *Pseudomonas fluorescence* grew best between 10 to 20C and it did not grow at 35C; whereas *Pantoea agglomerans, Bacillus amyloliquefaciens*, and *B. subtilis* had their optimum at 30 to 35C, but grew from 20-35. *Streptomyces lydicus* showed the highest growth at 25 and 30C, but it had a slow growth rate. Although several registrants of biological controls state that growth is not needed for efficacy of their products, in our experience improved performance occurs when conditions favor growth.
b. High amounts of molasses and corn syrup were inhibitory to *E. amylovora*; whereas growth or sporulation of the biologicals continued with high rates of these sources of carbohydrate. Additional testing is planned.

INTRODUCTION

Fire blight, caused by the bacterium *Erwinia amylovora,* is a very destructive disease of pome fruit trees worldwide, especially pears. In California, with continued 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 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 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 as was also demonstrated in our fire blight trials on Bartlett pear during the past years. In 2015, however, commercial copper applications failed to control the disease at many locations. This prompted farm advisors to request copper sensitivity testing in *E. amylovora*, and this was done for many strains sent to us and evaluated in our laboratory. In our UCIPM ratings and in grower meetings, we listed copper as a +/++ indicating inconsistent performance under different environmental conditions. Lack of systemic action and low registered rates are known factors for low efficacy. Reduced sensitivity to copper among strains of the pathogen is another possible explanation for poor fire blight management and thus, this was investigated.

Treatments with the antibiotics streptomycin and the less effective oxytetracycline (terramycin) 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 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 were not persistent and were not detected in successive samplings 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 2015 in collaboration with farm advisors.

The incidence of fire blight was very high at many locations in the spring and early summer in 2015. In our evaluations of new materials for fire blight control, highly effective treatments were identified. Kasugamycin (Kasumin) was 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, but the California registration is still pending due to additional environmental fate data requested by pesticide regulators in the state. Kasugamycin was in 2015 was also applied in mixtures with selected other materials, including other antibiotics, SARs, or mancozeb. 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 2015, we also tested K-Phite (a mixture of monopotassium phosphate and dipotassium phosphite), the biocontrols Actinovate (*Streptomyces lydicus*), Blossom Protect (*Aureobasidum pullulans*), and Serenade Opti (*Bacillus subtilis*) by themselves or in mixtures, and the natural product Companion. This was done because many growers are moving away from antibiotic use, and there is a growing interest in organic pear production. Under high disease pressure in 2015, these 'biological' treatments showed little to no efficacy. To possibly improve the effectiveness of biocontrols, we evaluated several nutrient additives that could be economically used in field applications for their effect on growth of the biocontrol agents as compared to the fire blight pathogen.

We also have been investigating the molecular mechanism of streptomycin resistance in California strains of *E. amylovora*. Several mechanisms have been described for strains 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 strains 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 has been published recently (Phytopathology 105:1302-1310). Interestingly, resistance due to a mutation in the chromosomal *rps*L gene that was found to be the common mechanism in California populations of the pathogen in previous studies by others is rarely found in present populations.

OBJECTIVES

1. Evaluate and optimize the performance of the antibiotic kasugamycin (Kasumin) and other antibiotics such as streptomycin (e.g., Agrimycin-17, 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., Actigard, PM-1) with and without Kasumin.
        iii. Biocontrols (e.g., Blossom Protect) with and without Kasumin and compatibility with organic wettable sulfur, copper, and captan (scab fungicides in pear production).
        iv. Large-scale field trials with Kasumin under an RA.
        v. Selected rotation programs
2. Determine the sensitivity of *E. amylovora* populations from pear orchards in California to streptomycin, oxytetracycline, and kasugamycin (continuation of antibiotic resistance surveys).
    a. Characterization of streptomycin- and oxytetracycline-resistant strains using laboratory and molecular approaches: Finalize a manuscript for publication based on our research.
3. Evaluate the experimental ATD as a novel chemistry derived from a food-grade additive:
    a. Laboratory assays on different media
    b. Field studies with ATD alone or in mixture with antibiotics, copper, or biologicals.

4. Evaluate different additives for improving the efficacy of biologicals including Blossom Protect and Actinovate.
    a. Add different nutrients to spray treatments
    b. Add phosphite compounds to improve activity

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 2015 from 70 orchards in Sacramento, Lake, Mendocino, and Sutter-Yuba Co. Infected plant material (fruit, stems, and pedicels) 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). Single colonies were transferred and the identity of the strains 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). A total of 243 strains of *E. amylovora* from 63 orchard locations were obtained in 2015 (the pathogen could not be recovered from samples from 7 locations) 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.).

Copper sensitivity of strains was determined by streaking bacterial suspensions (70% transmission at 600 nm) on nutrient agar or CYE (casitone, yeast extract, glycerol) agar amended with 0, 10, 15, 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).

***Laboratory studies on growth of selected biocontrols and* E. amylovora.** Studies were conducted to possibly optimize the use of biological control for the management of fire blight. First, the temperature optimum for growth of the biological agents of Blight Ban A506 (*Pseudomonas fluorescens*), Botector and Blossom Protect (both *Aureobasidium pullulans* strains DSM 14940 and 14941), Bloomtime Bio (*Pantoea agglomerans* strain E325), Nacillus (*Bacillus subtilis* and 3 other species 5 strains total), Double Nickel 55 (*B. amyloliquefaciens* strain D747), and Actinovate (*Streptomyces lydicus*) was determined. For this, 20 µl-suspensions of each microorganism (OD600 = 70% transmission) were spread onto 6-cm nutrient agar plates (3 replicated plates for each temperature and organism), and plates were incubated at selected temperatures (12C, 15C, 20C, 25C, 31C, 35C). After 24 h or 72 h, microbial growth was washed off each plate, suspended in 50 ml water, and the OD600 of the suspension was determined. Transmission values were subtracted from a value of 100 to obtain a growth measure. Growth at each temperature was expressed as relative growth as compared to that at the optimum temperature. Because growth of *S. lydicus* is slow and cannot be easily removed from the agar surface, plates were evaluated visually for growth after 2 or 10 days. A rating scale from – (no growth) to ++++ (maximum growth) was used.

The goal of additional studies was to identify nutrient additives that would increase the growth of the biocontrol agents, but not that of *E. amylovora*. A low-nutrient agar (casitone and yeast extract) was used as the base medium and was amended with 0, 1 g, 2 g, 4 g, 8 g, or 16 g/L of sucrose, molasses, corn syrup, or skim milk. Selected mixtures of additives were also tested. Agar plates were inoculated, incubated at 25C for 24 h, and microbial growth was collected and measured as described above. Transmission values were subtracted from a value of 100 to obtain a growth measure. Growth relative to the control for each microorganism was then calculated based on growth on non-amended medium. Cultures of *S. lydicus* were incubated at 30C and evaluated after 1 to 14 days.

Field studies using protective treatments during the growing season. In field studies 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 18 (40% bloom) and March 31. Natural incidence of disease was evaluated on random flowers on 3-31-15 (i.e., incidence of blighted flowers) and on diseased flower clusters on 4-15-15 (i.e., incidence of blighted flower clusters). 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*.** A total of 243 strains from 63 pear orchards (22 in Lake Co., 14 in Mendocino Co., 24 in Sacramento Co., and 3 in Sutter-Yuba Co.) were obtained and tested for their sensitivity against streptomycin, oxytetracycline, and copper. One to 8 strains per location were obtained.

All strains were found to be sensitive against oxytetracycline and the majority was sensitive to streptomycin (Table 1). Streptomycin-resistance was only detected in two orchards in Sacramento Co. In one of the orchards, one out of five strains was resistant, whereas in the other orchard, all eight strains were resistant (8 of them were moderately resistant and one was highly resistant). Thus, similar to the last two seasons (i.e., 2013 and 2014), there was a very low incidence of streptomycin resistance in contrast to some of the previous years where the incidence of resistance was often around 30%, but sometimes up to 70%. It appears that the unique mechanism of streptomycin resistance described by us in recent California isolates contributes to reduced fitness of the isolates. However, streptomycin use (and consequently selection pressure) has also decreased over the past years because of the perceived widespread resistance to the antibiotic. There is also a high economic incentive to grow an organic crop, and because streptomycin is no longer an approved organic treatment by the National Organic Standards Board (i.e., NOSB), usage has decreased. 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 management. With rotation and mixtures of streptomycin with other modes of action (e.g., kasugamycin, oxytetracycline), this will allow integration of resistance management practices. Spray records from the Sacramento Co. orchard with the high level of streptomycin resistance could provide useful information on improving chemical usage. The importance of this highly effective fire blight treatment is evident by the lack of efficacy of alternative treatments in the high-disease season 2015 where streptomycin still provided good disease control (see below). Continued resistance monitoring is important to determine if and how streptomycin can be effectively used again.

Because of widespread failure of copper treatments in managing fire blight in 2015, copper resistance in pathogen populations was considered as an additional possible reason for management failures. In response, we tested most of the *E. amylovora* strains that were sent to us for copper sensitivity. All strains grew well on nutrient agar amended with 20 ppm MCE and some showed some growth at 30 ppm MCE. This growth medium was originally chosen because it is relatively nutrient rich, presumably similar to conditions on the nectaries of pear blossoms. Sensitivity to copper was higher when using other growth media (i.e., CYE) that is thought to have a lower copper-binding capacity. All strains were not inhibited at 10 ppm MCE on this medium, and many showed at least some growth at 15 ppm MCE. Thus, we conclude that current *E. amylovora* populations are moderately copper-resistant. Additionally, we frequently observed the occurrence of spontaneous mutants growing at higher copper concentrations, especially when using nutrient agar. Spontaneous mutants growing at high copper concentrations also were reported previously by others. 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 spring 2015 season: 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. In our studies, *E. amylovora* did not grow on 50 ppm MCE, however, when the bacteria were re-transferred onto copper-free medium, growth resumed.

***Laboratory studies on growth of selected biocontrols and* E. amylovora.** The temperature optimum for growth was determined for seven biocontrol agents and was compared to *E. amylovora*. After 24 h, none of the organisms grew well at ≤15C and only *Aureobasidium pullulans* grew well at 20C (Fig. 1). *Pseudomonas fluorescence* and *E. amylovora* had their optimum at 25C and did not grow at 35C, whereas *Pantoea agglomerans, Bacillus amyloliquefaciens*, and *B. subtilis* (mixed with other strains) had their optimum at 30 to 35C. After 72 h of incubation, only *P. fluorescens* showed high growth at 12C, and there was little growth at 35C. Thus, this confirms that this organism, the biological agent in Blight Ban, is adapted to lower temperatures. *B. amyloliquifaciens* had little growth at 15C, but grew well at 20 to 35C. The other biocontrol agents grew over a wide temperature range from 15 to 35C. *E. amylovora* grew well between 15 and 25C, and growth declined rapidly at 30C. For *Streptomyces lydicus* (biological agent in Actinovate) a different assay had to be used. This organism showed the highest growth at 25 and 30C.

Based on these growth data, *A. pullulans* (biological agent in Botector and Blossom Protect) and *P. agglomerans* (biological agent in Bloomtime Bio) are likely the most suitable organisms to be used for fire blight control in the field under average northern California temperatures in the spring bloom period. These organisms were included in additional studies on the effect of nutrient additives with the goal to enhance their growth but not that of *E. amylovora*.

We selected additives that are readily available and that are not expensive. When a basic nutrient-poor medium was amended with sucrose, growth of *E. amylovora* was highly favored as compared to the three biocontrol agents (Fig. 2A). The addition of skim milk resulted in a similar response of the biocontrols and the pathogen; all had a slight increase in growth. Interesting effects were observed for molasses and corn syrup. Growth of *E. amylovora* was increased at lower rates of both additives, but inhibited at higher rates (16 g/L) relative to the biocontrol agents (Fig. 2A). Several mixtures of additives were effective in suppressing growth of *E. amylovora* and increasing growth of the biocontrol agents (Fig. 2B). Still, molasses at 24 g/L or 32 g/L by itself was similarly effective as the mixtures. *S. lydicus* grew similarly on non-amended and amended growth media (data not shown). Sporulation of the biological agent, however, occurred sooner in the presence of sucrose, corn syrup, and molasses at selected rates. Field studies will determine, if addition of molasses to a spray tank will improve the efficacy of biocontrol treatments. Control of diseases with biocontrol agents is very complex, and laboratory data may not translate into good field efficacy.

Field studies using protective treatments during the growing season. Fire blight incidence was very high in most pome fruit growing areas in California in 2015 and many growers experienced high losses. Natural incidence of flower infections at our field trial sites was approximately 40%.

Evaluation of conventional compounds. Conventional, non-organic treatments were evaluated in one field trial. Several single-active ingredient and mixture treatments of two active ingredients showed high efficacy at the first evaluation after the second application (Fig. 3). Numerically the lowest incidence was in the Kasumin-AgriMycin mixture. Kasumin or AgriMycin by themselves were also very effective. Copper (ChampION++) was not effective. At the second evaluation two weeks later (two weeks after the last application) efficacy of all treatments was lower, but disease was still significantly reduced by most treatments from that of the control. The Kasumin-AgriMycin mixture was still the best treatment. Continued application on a weekly schedule likely would have kept the incidence of disease at lower levels. Thus, kasugamycin continued to be highly effective in reducing the incidence of fire blight. Once registered in California, it can be used in resistance management programs with rotations and possibly mixtures.

Evaluation of biological materials. Biological treatments were compared in one field trial. In the first evaluation, Serenade Opti was the most effective treatment, reducing the incidence of fire blight from 45.3% in the control to 25% incidence. Blossom Protect+sugar and MagnaBon CS-2500 had similar reductions. Intermediate treatments were Blossom Protect + MagnaBon CS-2500 and Actinovate+K-Phite; whereas the remaining treatments were not significantly different from the control. The addition of Kumulus (wettable sulfur) or K-Phite numerically reduced the performance of Blossom Protect as compared to Blossom Protect-Buffer mixtures. In laboratory and small field studies in 2014 we showed that the biological organism *Aureobasidium pullulans* in Blossom Protect was inhibited by sulfur, but not copper. Field data in 2015 support these findings. The new biological Companion was ineffective. Furthermore, rotations of the biological Serenade Opti with Companion reduced the performance of Serenade Opti. Four weeks after the first applications, all of the biological treatments were similar in incidence to the untreated control. However, only three applications were made similar to the conventional bactericide trial and thus, the biological treatments needed more frequent applications at the beginning of bloom and continued through the weeks following bloom (petal fall period).

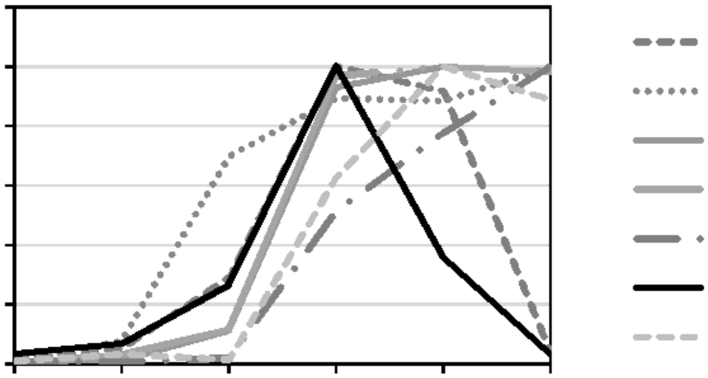
Evaluation of the climatic conditions including precipitation and temperature (high, low, and average) at the trial sites showed that average temperatures were mostly favoring growth of the pathogen (Fig. 5). Rainfall events in late March and mid-April with subsequent warm temperatures provided conducive conditions for fire blight. As stated above, only the biological treatments Blossom Protect, Protector, and Bloomtime Bio have optimal temperature ranges that would allow for growth during these environmental conditions. Although some registrants indicated that growth of the biological agent is not needed for their product (e.g., Serenade Opti) and they are effective; for other products, it seems that growth improves the performance. When temperatures are outside the range of growth, these products may not perform well.

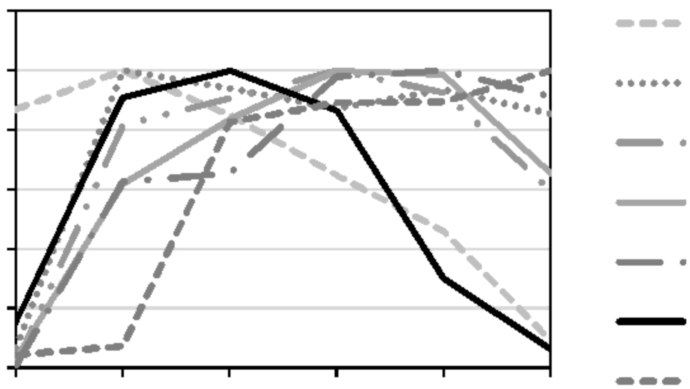
[illegible]

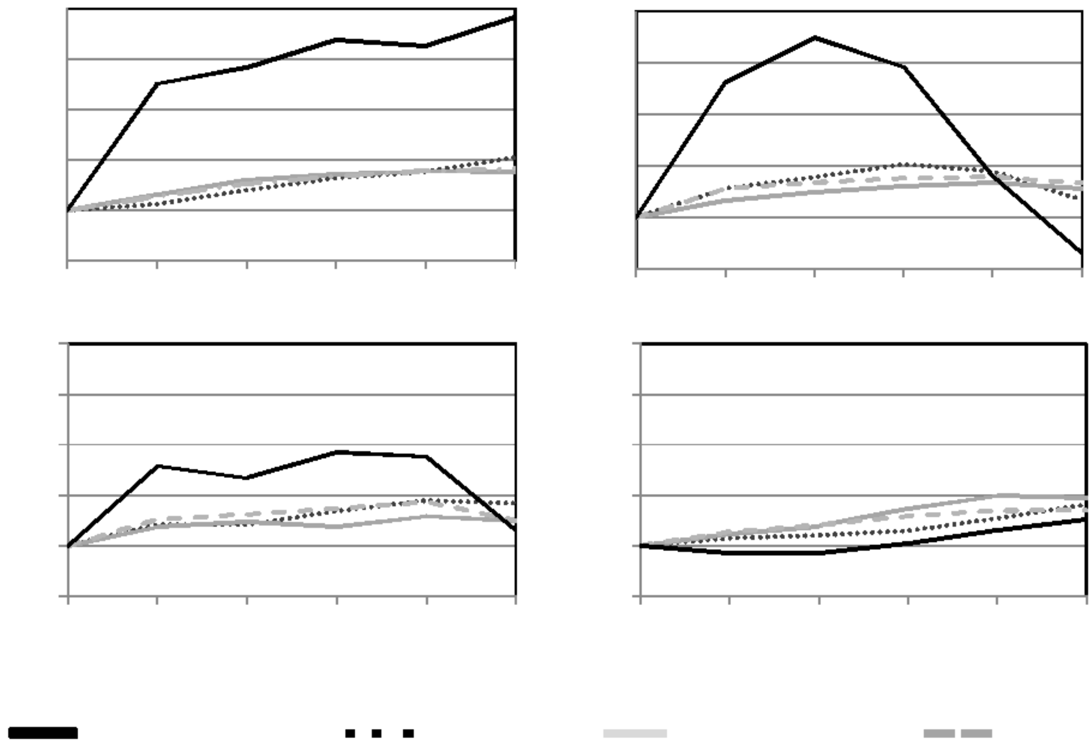
County	No. orchards	No. strains	Sensitivity to		
			Streptomycin*	Oxytetracycline	Copper**
Lake	22	54	S	S	Some strains from 18 locations had at least minimal growth at 15 ppm MCE
Mendocino	14	57	S	S	Some strains from all locations had at least minimal growth at 15 ppm MCE
Sacramento	22	116	S	S	Some strains from all locations had at least minimal growth at 15 ppm MCE
	1	5	1 strain R	S	Some strains had at least minimal growth at 15 ppm MCE
	1	8	all strains R	S	Some strains had at least minimal growth at 15 ppm MCE
Sutter-Yuba	3	3	S	S	All strains had minimal growth at 15 MCE
Total	63	243			

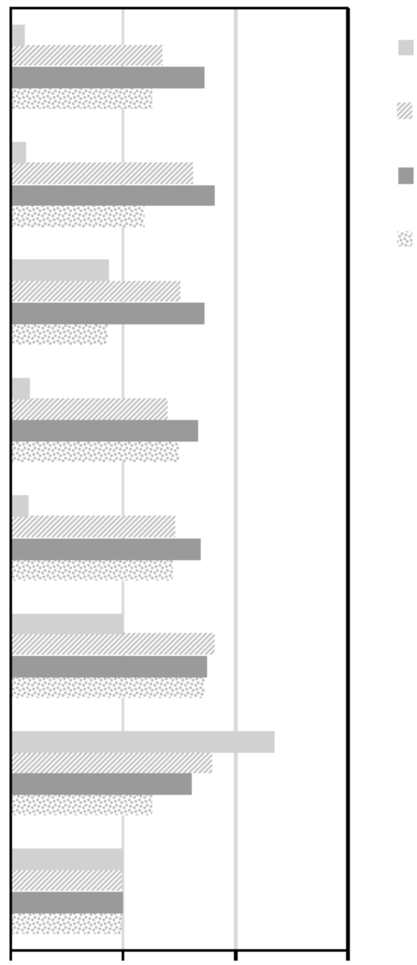
* Sensitivity to streptomycin and oxytetracycline was determined using the spiral gradient dilution method. S = sensitive, R = resistant.

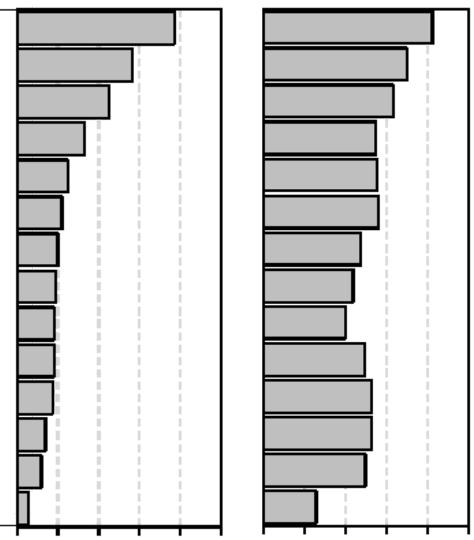
** Sensitivity to copper was determined by growth on amended CYE agar. All strains were not inhibited in growth on nutrient agar amended with 20 ppm MCE and only some inhibition occurred at 30 ppm MCE.

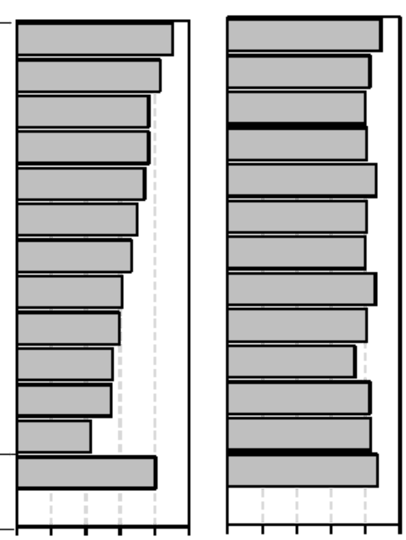


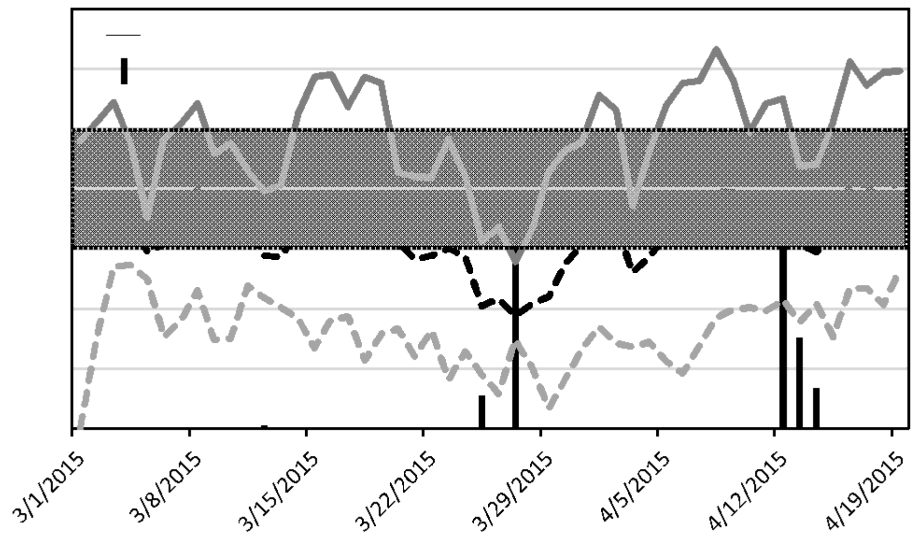








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