Annual Report - 2007

Prepared for the California Pear Board

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

1. In field trials on the management of fire blight, the efficacy of the antibiotics kasugamycin (Kasumin), streptomycin, and oxytetracycline (terramycin, Mycoshield), the biocontrol Bloomtime Bio (*Pantoea agglomerans*), the fungicides Captan and Dithane, as well as copper was evaluated.
    a. In two of the three small-scale trials where branches with blossoms were treated and then inoculated with *E. amylovora*, the three antibiotics performed similarly, whereas in the third trial kasugamycin and streptomycin were more effective than terramycin. The two fungicides and copper also significantly reduced the incidence of disease. Mixtures of antibiotics or of antibiotics with fungicides were similarly effective as individual antibiotics.
    b. In air-blast sprayer field trials, treatments with the biocontrol Bloomtime Bio applied at full bloom and petal fall significantly reduced the disease only when applied at the high labeled rate. Still, the incidence was only reduced by ca. 50% from the untreated control where 5.2% blight incidence was observed.
    c. Two additional air-blast sprayer field trials were done to compare antibiotic, fungicide, mixture, and rotation programs. Very little disease developed in one of the plots. In the other trial, treatments with single-materials (i.e., the three antibiotics and Dithane), mixtures of kasugamycin with streptomycin or Dithane, and a rotation of Kocide, Kasugamycin, and terramycin were similarly effective and reduced disease incidence from 27.3 strikes/tree to between 2.5 (i.e., streptomycin) and 7.3 (i.e. terramycin) strikes/tree. The rotational treatment of Bloomtime Bio, kasugamycin, and terramycin was not as effective.
    d. Phytotoxicity on leaves after three kasugamycin treatments was minor with very slight marginal leaf burn on some leaves.
2. As in 2006, population studies indicated the widespread and high frequency of streptomycin resistance among strains of *E. amylovora* collected from 19 orchards in selected pear-growing districts in California. None of these isolates showed multiple-resistance to kasugamycin.
3. For oxytetracycline, one less-sensitive isolate was detected in our 2006 samplings. Isolates with reduced sensitivity were found again in 2007 at the same location and in addition, in two orchards where terramycin treatments failed to control the disease.
4. Baseline sensitivity data were continued to be developed for kasugamycin using 190 isolates of *E. amylovora* from 19 locations in California.
5. Kasugamycin (Kasumin) registration in the US is still being pursued on pome fruit. Apple field studies were completed in 2007 and submission of a pome fruit registration package to EPA is expected in 2008 by IR-4.

INTRODUCTION

Fire blight, caused by the bacterium *Erwinia amylovora,* is a very destructive disease of pome fruit trees worldwide, especially pears. In addition to cankers, the pathogen overwinters in flower buds, diseased fruit, small twigs, and branches left on the ground after pruning. In the spring, blossoms are infected through natural openings in nectaries and pistils. After destroying the blossoms, the bacteria spread into the peduncles and spurs. During warm, humid weather ooze droplets consisting of new inoculum are exuded from the peduncles. Young fruitlets often become infected, and they also turn black, dry, shrivel, but usually remain attached to the tree. The disease spreads rapidly and the bacteria invade adjacent leaves through stomata, trichomes, hydathodes, but more frequently through wounds caused by hail or wind whipping. Succulent twigs, suckers, sprouts, and shoots are the next tissues infected. Secondary infections may occur throughout the growing season. Inoculum is spread by wind, rain, insects, birds, or by man, e.g. by means of contaminated pruning tools. Primary and secondary infections may spread into the branch. At this time the infection, if walled off, produces a canker or it penetrates further into the branch and then into the trunk. From here the bacteria may move into other branches and finally the trunk. Trunk cankers will eventually girdle the tree and the whole tree will die. The disease can be very severe in some years, causing repeated infections during warm and wet weather.

Control measures. Fire blight is one of the most difficult diseases to manage. Integrated programs that combine sanitation and orchard management with chemical and biological controls are the best known approaches available. 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 disease 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. Copper compounds have been used since the early 1900s, mostly in the form of copper sulfate plus lime (Bordeaux mixture). Control with copper compounds is only satisfactory when disease severity is low to moderate. On Bartlett (summer) pears, copper treatments are widely used only during dormant and bloom periods because phytotoxic effects commonly occur on fruit as russeting. Streptomycin, an antibiotic for fire blight control, came into general commercial use during the late 1950s, followed by the less effective oxytetracycline (Terramycin). Because of the lack of alternative control materials, antibiotics are still being used commercially, although pathogen resistance against the antibiotic streptomycin is widespread. In our 2006 evaluation of isolates from 24 orchards in California 50% of the isolates were found to be resistant to streptomycin and another survey was conducted in 2007. Furthermore, concerns have been expressed by regulatory agencies regarding the use of antibiotics in agriculture that are also used in human medicine.

New, more effective materials for fire blight control with a different mode of action from currently used bactericides have to be developed to combat this destructive disease. These could then be incorporated into a resistance management program. During the past years we evaluated numerous compounds that either were not effective, were inconsistent in their efficacy, or were effective, but were not further developed because of usage concerns (antibiotic classes that are important in human medicine). The antibiotic kasugamycin (Kasumin) showed very promising results in our 2004-2006 field trials with an efficacy equal to terramycin. Members of the kasugamycin antibiotic class are not being used in human and animal medicine. Kasugamycin has a different mode of action from streptomycin or terramycin and there is no cross-resistance known to occur. IR-4 residue studies were done with this antibiotic on pear and apple to allow registration on the pome fruit crop group.

In 2007 we conducted additional field experiments for the evaluation of new potential fire blight control treatments including chemical and biological control treatments. We evaluated the antibiotic kasugamycin alone and in mixtures or rotations with other antibiotics or fungicides. We also continued our studies on the biological control Bloomtime Bio (*Pantoea agglomerans*). In addition, we continued to evaluate the in vitro toxicity of streptomycin, oxytetracycline, and kasugamycin against isolates of *E. amylovora* to establish baseline sensitivities, to detect shifts in sensitivity, and to determine the distribution of streptomycin resistance in pear growing regions of California.

Objectives

1. Evaluate the efficacy of the antibiotic kasugamycin (Kasumin) as compared to streptomycin, oxytetracycline, or terramycin (Mycoshield) in cooperation with UCCE.
    a. Laboratory in vitro tests to evaluate the bactericidal activity with and without adjuvants: Spiral gradient dilution and direct contact assays.

    b. Small-scale hand-sprayer tests using different treatment-inoculation schedules.
    c. Field trials with protective air-blast spray treatments at several locations. Adjuvants, fungicides, product rates, timings, and rotations will be evaluated.
2. Determine the distribution of streptomycin-sensitive and –resistant isolates of *E. amylovora* in pear orchards in California (continuation of 2006 survey)
3. Evaluate the efficacy of Bloomtime Biological FD Biopesticide and of bacteriophage preparations.
    a. Laboratory in vitro tests to evaluate the bactericidal activity with and without adjuvants: Direct contact assays, amended agar assays, and bacterial lysis assays.
    b. Studies with protective spray treatments will be done in field trials. Timings (one vs. two applications at different bloom stages) and selected rates will be evaluated.

MATERIALS AND METHODS

Isolation of E. amylovora and bacterial culturing. Pear samples with fire blight symptoms were collected in the spring and early summer of 2006 and 2007 from orchards in the main pear-growing areas in central and northern California (i.e., Lake, Yuba-Sutter, and Sacramento Co.). Infected plant tissue was macerated in sterile water and aliquots of the suspension were streaked onto nutrient agar. Single bacterial colonies were transferred. Identification of *E. amylovora* was based on cultural appearance on nutrient agar and yeast extract-dextrose-calcium carbonate (YDC) agar. A sub-sample of strains was verified for identity using the Biolog (Hayward, CA) identification system.

***Laboratory studies on the toxicity of bactericides and fungicides against* E. amylovora.** Kasugamycin (Kasumin 2L, Arysta Life Sciences, Cary NC), streptomycin (Sigma, St. Louis, MO), oxytetracycline (Sigma), captan (Captan 80WG, Arysta Life Sciences), and maneb (Maneb 75DF, Cerexagri, Inc., King of Prussia, PA) 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) along the concentration gradient, plates were incubated for 2 days at 25ºC. Minimum inhibitory concentrations (MICs) and endpoint concentrations (EPCs; the concentration where growth is inhibited by 99%) were obtained from radial distances (measured from the center of the plate) of inhibition using the Spiral Gradient Endpoint computer program (Spiral Biotech, Inc.).

***Field studies using protective treatments during the growing season*.** In small-scale field tests on cvs. Hosu, Shinko, and Yoinashi Asian pear at UC Davis, treatments were applied to run-off to open blossoms using a hand sprayer. After 3 days, blossoms were spray-inoculated with *E. amylovora* (10^6 cfu/ml). Disease was evaluated based on the number of diseased blossoms per 40-100 blossoms evaluated per replication.

In field studies in commercial Bartlett orchards in Marysville and Live Oak, the relative efficacy of different treatments was evaluated after air-blast sprayer applications (100 gal/A). Treatments were applied as single-compound, mixture, or rotation programs to develop resistance management practices. All treatment timings are indicated in the Figures of the Results. Disease and potential phytotoxic effects of the treatments were evaluated in mid-April. Data were analyzed using analysis of variance and LSD mean separation procedures of SAS 9.1.

RESULTS AND DISCUSSION

Laboratory studies on the toxicity of bactericides and fungicides against *E. amylovora*. For streptomycin, among 184 strains evaluated in a 2007 survey of 19 California pear orchards, 56 isolates (=30%) were sensitive to this antibiotic with MICs (lowest concentrations of the antibiotic where a reduction of bacterial growth is observed) between 0.24 and 0.53 ppm and endpoint concentrations between 0.47 and 1.32 ppm (Table 1). The remaining isolates were found to be resistant against streptomycin. Thus, 70% of the isolates were resistant to streptomycin as compared to 50% in 2006. MICs for most of the resistant isolates were between 5.3 and 26.8 ppm and endpoint concentrations were between 8.9 and 37.2 ppm. Twelve isolates collected from two orchards, however, had MICs >50 ppm (see below). In comparison of orchards, only one orchard where six isolates were evaluated did not reveal any resistance. The incidence of resistance in two additional orchards (10 isolates evaluated in each location) was 10% and 30%. In the remaining orchards the incidence of resistance was ≥45.5%. Thus, resistance against streptomycin was found to be widespread again in the major California pear growing regions and there was a trend for an increase as compared to our survey in 2006.

For kasugamycin, MICs among isolates collected in 2007 ranged from 4.1 to 25.8 ppm, similar as in 2006. Isolates were found over the entire range of the continuous sensitivity distribution with no distinct sub-groups (Fig. 1A). Concentrations required to inhibit bacterial growth in vitro, however, were much higher for this antibiotic than for streptomycin and oxytetracycline. We used nutrient agar, a medium with moderate nutritional content, in our in vitro toxicity studies. Previously we found that the toxicity of some antimicrobials against bacteria was highly dependent on the culture medium used. Thus, the use of other cultural media in in vitro assays with kasugamycin possibly could result in lower MIC values. Still, our evaluations of in vitro sensitivities for isolates of *E. amylovora* are for relative comparisons to detect shifts in sensitivity, because MIC values derived from in vitro toxicity studies are never absolute and physiological conditions of the host plant cannot be simulated in any agar medium.

For oxytetracycline, we identified one isolate from our 2006 survey with a reduced sensitivity (MIC 1.56 ppm) in one orchard (orchard No. 19, Table 1). Isolates collected in 2007 from this same location displayed a wider range of sensitivities against the antibiotic than isolates from other orchards (except two locations, see below) (Fig. 1 B). Eight of the 38 isolates evaluated had MICs between 0.45 mg/L and 0.69 mg/L as compared to MICs between 0.09 and 0.24 ppm for isolates in orchards where only sensitive isolates were found. This may indicate a shift to reduced sensitivity in this orchard. Furthermore, isolates were collected in two pear orchards (one isolate from orchard No. 17 and 11 isolates from orchard No. 18) where treatments by the grower with terramycin had not controlled the disease. MICs among these twelve isolates ranged from 0.97 to 1.54 ppm. Interestingly, these twelve isolates were also highly resistant to streptomycin with MIC values >50 ppm. Resistance in the fire blight pathogen against oxytetracycline has never been reported previously (McManus et al., 2002). With the identification of isolates in 2006 and 2007 for the first time that are up to ca. 10-times less sensitive to the antibiotic as compared to the majority of isolates collected, our results indicate that currently a shift to reduced sensitivity against oxytetracycline is taken place in California populations of *E. amylovora* with loss of efficacy at some locations. This stresses the critical need for the development and introduction of new protective field treatments before resistance becomes widespread and this antibiotic becomes ineffective as a management tool.

The in vitro toxicity of captan and maneb was evaluated for three isolates of *E. amylovora*. MICs ranged from 2.5 to 6.7 ppm and from 5.75 to 8.31 ppm and endpoint concentrations ranged from 4.1 to 19.2 ppm and from 7.60 to 10.84 ppm for the two fungicides, respectively.

Field studies using protective treatments during the growing season. In small-scale field tests where blossoms of Asian pear were hand-sprayed with different treatments once and inoculated with *E. amylovora* after three days, all treatments significantly reduced the incidence of fire blight. In the first experiment where streptomycin, oxytetracycline, and kasugamycin were compared, the efficacy of the three antibiotics was very similar (Fig. 3). The incidence of blight was 56.6% in the untreated control, and was between 10.1% (kasugamycin) and 15.6% (oxytetracycline) for the treatments. In the second experiment where single-compound treatments with antibiotics and fungicides and mixtures were compared, Kocide

2000 numerically was the least effective treatment on cv. Shinko and the kasugamycin-streptomycin mixture was the most effective (Fig. 4). A similar efficacy was found for all the remaining treatments including Dithane and Captan. On cv. Yoinashi, streptomycin numerically was the most effective and oxytetracycline the least effective treatment (Fig. 4). Again, most of the remaining treatments performed similarly with kasugamycin-captan having the lowest blight incidence among the mixture treatments. These small-scale field studies indicated that kasugamycin in mixtures with oxytetracycline and streptomycin can be very effective and that additionally, Captan and Dithane can be used as effective mixture partners for the registered antibiotics and for kasugamycin.

In a field trial in a commercial orchard where treatments with the biocontrol Bloomtime Bio were applied at full bloom and petal fall using an air-blast sprayer, only the higher rate of the biocontrol provided a significant reduction in the incidence of fire blight as compared to the control (Fig. 5). Incidence of blight was 5.2% in the control and 2.6% in the biocontrol treatment. Thus, in contrast to previous years' trials where Bloomtime Bio was quite effective under low-disease pressure conditions, this year the biocontrol provided only moderate efficacy. Possibly, timing of our applications in 2007 was not optimal.

In the second trial in a commercial orchard, mixture and rotation programs were compared to single-antimicrobial treatments with antibiotics or Dithane. As in some of the small-scale hand-sprayer field tests, oxytetracycline was somewhat less effective than streptomycin or kasugamycin (Fig. 6). Still, there was no significant difference among the three antibiotics. We also confirmed the high efficacy of mixtures of kasugamycin with streptomycin or Dithane. Rotation programs where either Bloomtime Bio or Kocide 2000 treatments were followed by kasugamycin and oxytetracycline also significantly reduced the incidence of disease, however, the program with the initial Bloomtime Bio treatment was less effective. Again, timing of the biocontrol treatment may not have been optimal.

In a trial in another commercial orchard where similar management programs were compared, very little disease developed, and no comparisons among treatments could be made.

In summary, our field trials in 2007 again indicate that kasugamycin is a highly effective treatment against fire blight of pear that can be used in resistance management programs with mixtures and rotations. Kasugamycin has been tested previously by others against fire blight of apple and pear (Aldwinckle and Norelli, 1990; Bonn, 1984). Although efficacy was very good in some of these trials, registration of kasugamycin on these crops was never pursued due to the reported high phytotoxicity (Psallidas and Tsiantos, 2000). In our studies, phytotoxicity was very low after 5 to 6 applications with the 100-mg/L rate and it was negligible when kasugamycin was used in rotation with other compounds. Arysta Life Sciences Corp., the potential registrant, is supporting registration of the material for agricultural use in the United States and we are working closely with this company to proceed with the process. In Sept. 2005 the US-EPA granted an import tolerance for kasugamycin on some agricultural crops and IR-4 residue studies were done on pear in 2006 and on apple in 2007. Registration of kasugamycin for management of fire blight is timely because with the first occurrence of populations of *E. amylovora* with reduced sensitivity to oxytetracycline further selection of the pathogen population and spread of resistance has to be prevented. We also report for the first time on the effective use of the fungicides captan and mancozeb for controlling fire blight. Because captan is registered in the United States on apple and mancozeb on both, apple and pear, both fungicides are available for immediate use and could also be integrated into a resistance management program that should be initiated from the onset of introduction of kasugamycin.