Annual Report - 2008

Prepared for the California Pear Board

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

Project Leaders: Drs. J. E. Adaskaveg and D. Gubler, Department of Plant Pathology, University of California, Riverside CA 92521 and Davis, CA 95616, respectively.

Cooperators: Dr. H. Förster, L. Wade (Arysta Life Science), D. Thompson, D. Felts, C. Ingels (UCCE, Sacramento Co.) and R. Elkins (UCCE, Lake Co.)

Acknowledgements: Special thanks to Naumes Packing, Marysville, CA for their cooperation in this research and for donation of fruit requiring crop destruction.

SUMMARY

1. Several field trials were conducted on the management of fire blight with the antibiotics kasugamycin (Kasumin), streptomycin, and oxytetracycline (terramycin, Mycoshield), the biocontrol Bloomtime Bio (*Pantoea agglomerans*), as well as the fungicides Kocide 3000 (using reduced rates), Syllit, and Dithane. Environmental conditions for fire blight development were not favorable in the spring of 2008 and only a limited amount of data could be obtained.
    a. In a small-scale trial where branches with blossoms were treated with bactericides, simulated rain was applied (mimicking a short rain shower), and blossoms were then inoculated with *E. amylovora*, the three antibiotics demonstrated rain-fastness under these conditions.
    b. In another small-scale trial, kasugamycin and streptomycin showed better post-infection activity (application 24 h after inoculation) than oxytetracycline.
    c. In a field trial, we demonstrated that the new antibiotic kasugamycin was effective in reducing the incidence of fire blight caused by an isolate of the pathogen resistant to streptomycin and oxytetracycline. The latter two antibiotics, in contrast, were ineffective.
    d. There was a trend for higher efficacy of kasugamycin when mixed with the fungicide Syllit. Mixtures of kasugamycin with other materials will need to be continued to be evaluated.
    e. The biocontrol Bloomtime Bio significantly reduced the incidence of disease when applied at 300 g/A during environmental conditions less favorable for disease.
2. Population studies of the pathogen indicated a low incidence of streptomycin resistance in 2008, possibly because of low disease and consequently selection (i.e., streptomycin applications) pressure. Still, all five isolates obtained from one location were highly resistant to streptomycin. No isolates less sensitive to oxytetracycline or kasugamycin were found in 2008.
3. 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 early 2009 by IR-4 with registration expected in 2011.

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 and 2007 evaluations of isolates from orchards in California (24 orchards in 2006, 19 orchards in 2007) 50-70% of the isolates were found to be resistant to streptomycin and another survey was conducted in 2008. 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 2004-2007 field trials with an efficacy equal to terramycin. The antibiotic kasugamycin is not 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 2008 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 initiated trials with a new formulation copper hydroxide, Kocide 3000, that uses less copper (0.5 lb/A*0.3 MCE=0.15 lb MCE/A). We have been successful with this material in reducing rate while maintaining efficacy in walnut in the last three years. Additionally, we are evaluating the bactericidal effects of dodine (Syllit) and mancozeb (e.g., Dithane, Manzate, Penncozeb, etc.) by themselves and in mixtures with kasugamycin. Additive effects of bactericides mixed with EBDC have been reported for other bacterial pathogens. Thus, we are evaluating rotational and mixture programs with copper, fungicides (dodine, mancozeb), and antibiotics to optimize efficacy and prevent resistance developing in pathogen populations by constantly changing the active ingredient during the season.

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 (Kocide 3000, Syllit, and Dithane), product rates, timings, and rotations will be evaluated.
2. Determine the distribution of streptomycin- or terramycin-sensitive and –resistant isolates of *E. amylovora* in pear orchards in California (continuation of 2006-07 surveys)
3. Evaluate the efficacy of Bloomtime Biological FD Biopesticide.
    a. Laboratory in vitro tests to evaluate the bactericidal activity with and without adjuvants: Direct contact assays and amended agar 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*, bacterial culturing, and verification of species identity. Pear samples (blossoms and twigs) with fire blight symptoms were obtained in the spring and early summer of 2008 from orchards in the main pear-growing areas in central and northern California (i.e., Lake, Mendocino, San Joaquin, 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. Initially, identification of *E. amylovora* was based on cultural appearance on nutrient agar and yeast extract-dextrose-calcium carbonate (YDC) agar. All isolates were further verified for their identity using primers specific for the ubiquitous *E. amylovora* plasmid pEA29 as described by Bereswill et al. (Appl. Environ. Microbiol. 58:3522-2536). The presence of a 1-kb DNA fragment after gel electrophoresis confirmed a positive identification.

***Laboratory studies on the toxicity of bactericides against* E. amylovora.** Kasugamycin (Kasumin 2L, Arysta Life Sciences, Cary NC), streptomycin (Sigma, St. Louis, MO), and oxytetracycline (Sigma) were evaluated for their in vitro toxicity using the spiral gradient dilution method. For this, a radial bactericidal concentration gradient was established in nutrient agar media in Petri dishes by spirally plating out a stock concentration of each antimicrobial using a spiral plater (Autoplate 4000; Spiral Biotech, Inc., Norwood MA). After radially streaking out suspensions of the test bacteria (10 µl of 10^8 cfu/ml) along the concentration gradient, plates were incubated for 2 days at 25ºC. Minimum inhibitory concentrations (MICs) 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. Shinko and Yoinashi Asian pear at UC Davis, treatments were applied to run-off to open blossoms using a hand sprayer. Treatments included Kocide 3000 (0.5 lb/A), Syllit (2 pints/A), and Dithane (6.4 /b/A) by themselves, in rotation, or in mixtures with kasugamycin (100 ppm) and compared to oxytetracycline (200 ppm) and streptomycin (100 ppm), After 1 day, 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 studies on the rain-fastness of antibiotic treatments, treatments were applied to run-off to clusters of cv. Yoinashi blossoms using a hand sprayer. After 24 h, simulated rain was applied using an air-blast sprayer and blossoms were then spray-inoculated with a streptomycin-sensitive isolate of *E. amylovora*. Disease incidence was based on the number of infected blossoms per 50-100 blossoms evaluated. The post-infection activity of streptomycin, oxytetracycline, and kasugamycin was also evaluated. For this, blossoms were spray-inoculated with a streptomycin-sensitive isolate of *E. amylovora*. Treatments were applied after

24 h using a hand sprayer. Disease incidence was based on the number of infected blossoms per 50-100 blossoms evaluated.

In field studies in commercial Bartlett orchards in Live Oak, five applications of kasugamycin, streptomycin, Dithane, Syllit (dodine), kasugamycin-Syllit, kasugamycin-Dithane, and the natural product Cerebrocide were done between March 14 and April 10 using an air-blast sprayer at 100 gal/A. In an adjoining orchard, the biocontrol Bloomtime Bio was evaluation in a two-spray program (10% bloom and full bloom). Data were analyzed using analysis of variance and LSD mean separation procedures of SAS 9.1.

A late-season trial (late-May) was performed on cv. Forelle pear to demonstrate that fire blight caused by isolates of *E. amylovora* resistant to streptomycin and oxytetracycline can be managed with the new antibiotic kasugamycin, but not with either streptomycin or oxytetracycline. Because blossoms were no longer available, the study was conducted with succulent shoots. Shoots were first wounded by scraping with a knife and trees were treated using an air-blast sprayer at 100 gal/A. Shoots were then inoculated with *E. amylovora* using a hand sprayer. Disease incidence was based on the number of shoots per total number of shoots evaluated. After evaluation, diseased shoots were cut form the trees and removed from the orchard. Data were analyzed as described above.

RESULTS AND DISCUSSION

Isolation of* E. amylovora *and studies on the toxicity of bactericide. Due to the dry spring in 2008, disease incidence was very low at most locations. Thus, because infected twigs could not be found at many locations, blossom samples were provided by collaborators to isolate for epiphytic *E. amylovora* populations. Many cultures were obtained from these samples and isolate identification was often difficult. Thus, we employed a PCR assay using specific primers for a plasmid that is ubiquitously found in isolates of *E. amylovora*. As shown in Fig. 1, a 1-kb DNA fragment was amplified in some of the isolates. These isolates were considered to be *E. amylovora* and were subsequently tested for their sensitivity against the three antibiotics. For streptomycin, among 109 strains evaluated from 23 California pear orchards (1 to 21 isolates per location), 8 isolates were found to be resistant to this antibiotic with MICs (lowest concentrations of the antibiotic where a reduction of bacterial growth is observed) between 2.455 and >50 ppm (Table 1). The remaining isolates were sensitive against streptomycin. No resistant isolates were collected from blossoms. The five highly resistant isolates (MIC >50 ppm) all originated from one orchard and this comprised the total sampling from this location. The spray history of this orchard needs to be further investigated. Thus, in 2008 only 7.3% of the isolates were resistant to streptomycin as compared to 70% in 2007 and 50% in 2006. This could have two reasons. First, as indicated, many samples of the bacterium came from epiphytic blossom populations. Secondly, because environmental conditions were not highly favorable for fire blight in the spring of 2008, the number of bactericide applications was likely low and the pathogen was not exposed to a high selection pressure. Because in the two previous years of our study the frequency of resistant isolates was much higher, this also indicates (assuming that isolates were obtained from similar locations in the three years) that resistant isolates are not as fit as sensitive isolates, and populations revert back to sensitivity in the absence of selection pressure (i.e., application with streptomycin).

For oxytetracycline, all isolates were found to be sensitive and sensitivity levels were in a similar range as in previous years for sensitive isolates (Fig. 2A). Six isolates of the pathogen from an orchard where we demonstrated a reduced sensitivity against oxytetracycline in 2006 and 2007 were also found to be sensitive. All isolates collected in 2008 were also sensitive against kasugamycin, again with sensitivity levels similar to previous years (Fig. 2B).

In summary, these results on in vitro sensitivity of California populations of *E. amylovora* indicate a low level of streptomycin resistance and no reduced sensitivity against oxytetracycline and kasugamycin in 2008. Thus, populations of the pathogen quickly adapt to changing selection pressure. Because of the high level of streptomycin resistance and first occurrence of reduced sensitivity against oxytetracycline in previous years, there still is a critical need for the development and introduction of new protective field treatments. This we a pursuing as a main focus of our fire blight research.

Field studies using protective treatments during the growing season. Due to environmental conditions not very favorable for fire blight development (very low levels of precipitation) in the spring of

2008, disease incidence in our trials was low. In several trials, however, including one small-scale field test and three trials in commercial orchards, no disease developed and data could not be obtained.

In small-scale field tests where blossoms of Asian pear were hand-sprayed with bactericides, simulated rain was applied after one day, and blossoms were then inoculated with *E. amylovora*, the rain-fastness of streptomycin, oxytetracycline, kasugamycin or mixtures of kasugamycin and Syllit or Dithane was evaluated. Syllit (dodine) was included in our studies because the label of this fungicide indicates some antibacterial activity. The incidence of fire blight was significantly reduced by all treatments (Fig. 3). Incidence was 7.1% in the untreated control and between 0 and 1.7% among the treatments. Thus, these treatments demonstrated a very good persistence under conditions mimicking a short rain shower. Unfortunately, no data for these and additional could be obtained in the neighboring plot where no simulated rain was applied.

In small-scale study on the post-infection activity of the three antibiotics, 3.8% disease incidence was observed in the untreated control (Fig. 4). Mycoshield numerically, but not statistically reduced the incidence, whereas no disease was observed in the kasugamycin and streptomycin treatments. These studies, in addition to the study on rain-fastness above, need to be repeated again under higher disease pressure. In a commercial orchard, all treatments except Syllit were effective in reducing blight from the untreated control (Fig. 5).

In a late-season trial on inoculated shoots of cv. Forelle pear, shoots were found to be highly susceptible and over 70% developed disease symptoms (Fig. 6). None of the treatments applied was highly effective and Mycoshield was not effective at all, possibly due to the high susceptibility of the host. Still, valuable information could be obtained. Thus, whereas disease caused by a streptomycin- and oxytetracycline-resistant isolate of the pathogen could not be reduced by streptomycin or oxytetracycline treatments, there was a significant reduction using kasugamycin. In addition, there was a trend for the kasugamycin-Syllit mixture being more effective than kasugamycin by itself. This same trend (also for the kasugamycin-Dithane mixture) was evident in the small-scale field test discussed above (*see* Fig. 3). Thus, mixture partners with kasugamycin (as also evaluated in 2007) need to be continued to be evaluated in the coming years of field trials.

The only other field trial were data could be obtained in 2008 was a study on the efficacy of the biocontrol agent *Pantoea agglomerans*, i.e., Bloomtime Bio. Two applications of the biocontrol (10% bloom and full bloom) significantly reduced the incidence of blossom blight from 29.9% in the untreated control to 9.8% using the high rate (300 g/A) of the product (Fig. 7). The low rate (150 g/A) numerically, but not significantly reduced the incidence. These results indicate that fire blight can be reduced by this biological treatment when applications are done timely, disease pressure is low, and when no highly favorable environmental conditions for disease development occur during later bloom stages.

In summary, our field trials in 2008 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. 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 expected in 2011 and 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. An emergency registration or Section 18 should be pursued if management practices with terramycin begin to fail in 2009. Mixture partners for kasugamycin and the registered antibiotics need to be continued to be evaluated to maximize the efficacy of treatments and as part of a resistance management program. This should especially be initiated from the onset of introduction of kasugamycin. Thus, identification of integrated fire blight programs with copper, fungicides, and antibiotics is successfully being pursued for the California pome fruit industries.