# Annual Report - 2006

*Prepared for the California Pear Board*

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

1. In two field trials, the efficacy of blossom and foliar air-blast spray treatments with the antibiotics kasugamycin (Kasumin) and terramycin (Mycoshield), as well as the biocontrol Bloomtime Bio (*Pantoea agglomerans*) was evaluated.
    a. The biocontrol Bloomtime Bio was effective and significantly reduced the incidence of fire blight (incidence ca. 1.5-2.0%) from the control (incidence ca. 7%) in a commercial orchard with moderate to low disease incidence. In a second orchard with high incidence of blight, the biocontrol did not significantly reduce blight (incidence ca. 16-17%) from that of the control treatment (incidence ca. 23-24%).
    b. The efficacy of kasugamycin was rate-dependent. Rates of 100 to 125 ppm were equally effective. Different gallonages per acre were also evaluated while maintaining the concentration of kasugamycin (i.e., 100 ppm). The optimum gallonage was 100 gal/A. At 50 and 200 gal/A there was a trend for or significantly higher incidence of disease, respectively.
    c. Significantly more phytotoxicity resulted using higher rates (200 ppm) or higher gallonages of kasugamycin than using lower rates. Some phytotoxicity resulted at all rates but the severity was very low at all rates (slight marginal leaf burn on some leaves).
    d. Rotational treatments of Bloomtime Bio, Kasumin, and Mycoshield were highly effective in both trials similar to when Kasumin or Mycoshield were applied alone.
2. Baseline sensitivity data were developed for kasugamycin using 119 isolates of *E. amylovora* from different locations in California. Minimum inhibitory and endpoint concentrations were determined.
3. Population studies determined the widespread and high frequency of streptomycin resistance in 119 strains of *E. amylovora* in a random collection from 24 orchards in selected pear-growing districts in California. None of these isolates showed multiple-resistance to kasugamycin.
4. IR-4 studies were coordinated for a kasugamycin (Kasumin) registration in the US for pears; whereas apple residue trials were approved for the 2007 season.
5. Studies were initiated to evaluate the potential of a bacteriophage preparation to lyse strains of *E. amylovora* obtained from different locations.


**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. Furthermore, concerns have been expressed by regulatory agencies regarding the use of antibiotics in agriculture that are also used in human medicine. Still, we have been successful in identifying an antibiotic (e.g., kasugamycin) that is effective against fire blight and is used only plant agriculture.

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 have identified a broad-spectrum biocide from Dow Chemicals and the antibiotic Starner. Because of registration costs, however, the manufacturer of the biocide will not proceed with registration. The antibiotic Starner (Valent Biosciences) is not being developed for agricultural use because the class of antibiotics that Starner belongs to is important in human medicine. Other biocides such as acidified hydrogen peroxide had inconsistent performance and are not satisfactory for development. The antibiotic kasugamycin (Kasumin), however, showed promising results in 2004-2006. 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. In 2005, kasugamycin was accepted into the IR-4 program and in 2006 nationwide field residue studies were done on pear. In 2007, apple will also be done nationwide to allow registration of the antibiotic on the pome fruit crop group.

In 2006 we conducted additional field experiments under high and low incidences of fire blight for the evaluation of new potential fire blight control treatments including chemical and biological control treatments. We evaluated the antibiotic kasugamycin alone or in mixtures with other materials. Additional studies that were done included: rates of the antibiotic and different volumes of application (e.g., gal/A) at the same concentration of the antibiotic. We also continued our studies on biological control agents. Bloomtime Bio (*Pantoea agglomerans*) has been reported to be active against *E. amylovora* in vitro and in vivo and its mode of action thought to be based on the production of antibiotics. A commercial preparation of a bacteriophage was also evaluated as a biological control.

**Objectives**

1. Evaluate the efficacy of the antibiotic kasugamycin (Kasumin) as compared to oxytetracycline or terramycin (Mycoshield) in cooperation with UCCE.
    a. Laboratory in vitro tests to evaluate the bactericidal activity with and without other products: Direct contact assays, filter disk assays, amended agar assays.

b. Studies with protective spray treatments will be done in field trials. Combinations, rotations, product rates, and volumes per acre will be evaluated.

2. Evaluate the efficacy of Bloomtime Biological FD Biopesticide as a follow-up to 2005 efficacy studies and other biological control treatments.
   a. Laboratory in vitro tests to evaluate the bactericidal activity with and without adjuvants: Direct contact assays, filter disk assays, 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.
   c. Evaluate a commercial bacteriophage preparation in vitro studies against strains of the pathogen.
3. Develop baseline data for kasugamycin and conduct a survey on the frequency of streptomycin resistance of *E. amylovora* in California pear growing districts (*supplemental mid-season objective*).

## MATERIALS AND METHODS

***Isolation of E. amylovora and bacterial culturing.*** Infected plant tissue was macerated in sterile water and aliquots of the suspension were plated onto nutrient agar. Single bacterial colonies were transferred. Identification of *E. amylovora* was based on cultural appearance on nutrient agar and YDC. A sub-sample of strains was identified using the Biolog (Hayward, CA) identification system.

***Laboratory studies on the toxicity of bactericides or a bacteriophage preparation against* E. amylovora.** Antibiotics (e.g., kasugamycin, streptomycin) were evaluated for their toxicity using the spiral gradient dilution method. For this, a bactericidal concentration gradient was established in agar media in Petri dishes. After inoculation of the media with isolates of the test pathogen and incubation for 2-3 days at 20 C, minimum inhibitory concentrations (MIC) and endpoint concentrations (EPC) or the concentration where growth was inhibited by 99% were obtained using a computer program. MIC and EPC values were plotted for all isolates to obtain a baseline distribution.

In evaluations of the activity of a bacteriophage preparation (supplied by OmniLytics, Inc., Salt Lake City, UT) against different (streptomycin-sensitive or –resistant) isolates of *E. amylovora* obtained from pear orchards in California, bacterial suspensions (50 µl of $2x10^8$ cfu/ml) were spiral-plated onto 10-cm Petri dishes containing nutrient agar. After 24 h, 5-µl drops of phage preparation was placed onto the bacterial lawn. Plates were evaluated for bacterial lysis after 2 days using a rating scale from 0 (= no clearing of bacterial lawn) to 3 (= complete clearing of bacterial lawn). Values were averaged and data were summarized for each isolate of *E. amylovora.*

***Field studies using protective treatments during the growing season.*** In small-scale field tests at UC Davis, treatments of kasugamycin (Kasumin), terramycin (Mycoshield), copper (Kocide 2000), and Bloomtime Biological were applied to run-off to open blossoms using a hand sprayer. After selected time periods, blossoms were spray-inoculated with *E. amylovora* ($10^6$ cfu/ml). In the first experiment blossoms were re-treated and re-inoculated after 6 days. Each replication consisted of one branch on each of four trees. Disease was evaluated based on the number of diseased blossoms per 100 blossoms evaluated per replication or based on the number of diseased flower clusters per 30 clusters evaluated per replication.

In air-blast sprayer field studies (applications at 100 gal/A, unless indicated otherwise) in commercial Bartlett orchards in Marysville and Live Oak, the relative efficacy of different concentrations (100-200 ppm) of kasugamycin was evaluated. The 100-ppm concentration was also evaluated at different gallonages per acre. (75 to 150 gal/A). In some treatments phosphorous acid (ProPhyt) or NuFilm P was added to Kasugamycin. Additionally, the biological control Bloomtime Biological was compared to the chemical treatments at selected rates using one or two application timings. Treatments of Mycoshield, copper, kasugamycin, and Bloomtime biological were also evaluated in 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-May. 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 or a bacteriophage preparation against *E. amylovora*.** For streptomycin, among 117 strains evaluated in a survey of 24 California pear orchards, 59 isolates were sensitive to this antibiotic with minimum inhibitory concentrations between 0.11 and 1.18 ppm and endpoint concentrations between 0.30 and 1.64 ppm (Table 1). The remaining 58 isolates were found to be resistant against streptomycin (minimum inhibitory concentrations >7.5 ppm). Between one and ten isolates could be obtained from each of the remaining 22 orchards. Isolates within orchards were either all sensitive, all resistant, or had mixed populations (Table 1). All 19 isolates evaluated from the Live Oak trial were found to be streptomycin-sensitive. All 11 isolates evaluated from our Marysville trial were also found to be streptomycin-sensitive, although in a survey done in 2001 by the grower in cooperation with another lab, streptomycin-resistance was found at this location (grower pest advisor, *personal communication*). Apparently, the streptomycin-resistant strains were not as fit as the wild-type sensitive strains of the pathogen and dynamic shifts in population structure may occur.

The in vitro toxicity of the antibiotics kasugamycin and streptomycin against *E. amylovora* was evaluated on amended nutrient agar, a medium with moderate nutritional content. Previously we found that the toxicity was dependent on the culture medium used and thus, our assay was standardized. All of the 117 isolates of the pathogen from 24 orchards in California were sensitive to kasugamycin. Minimum inhibitory concentrations (lowest concentrations of kasugamycin where a reduction of bacterial growth is observed) ranged from 4.2 to 17.8 ppm (Fig. 1). Endpoint concentrations (concentrations where growth was inhibited by 99%) ranged from 12.6 to 46.7 ppm.

A bacteriophage solution was prepared by OmniLytics Inc. based on a limited number of *E. amylovora* isolates that we provided. In evaluations of its activity, a range of reactions was observed when drops of the phage solution were added to 1-day old bacterial cultures. Reactions ranged from complete clearing (lysis) of the bacterial lawn to no clearing and a similar range of reactions was found for isolates of the pathogen resistant or sensitive to streptomycin (Fig. 2). Thus, the bacteriophage showed promising *in vitro* activity against *E. amylovora.* The phage, however, could not be evaluated in the field because of the late arrival date of the product (initial contacts by Omnilytics Inc. were not be made before April 2006). For the 2007 season we hope to be able to obtain a preparation that has a wider range of activity against more different strains of the pathogen.

***Field studies using protective treatments during the growing season.*** In small-scale field test where pear blossoms were hand-sprayed and then inoculated with *E. amylovora*, all treatments significantly reduced the incidence of fire blight. In the first experiment where only one treatment and one inoculation were done, 19.8% fire blight incidence were observed on control blossoms. Kocide and the biocontrol Bloomtime Biological were the least effective materials (9.3-10.5% incidence), whereas Kasumin, Kasumin-ProPhyt, and Mycoshield were more effective (1.8-2.5% incidence) (Fig. 3). There was no difference in efficacy between the latter three treatments. In the second experiment with two treatments and two inoculations, all treatments performed similar, reducing fire blight incidence from 41.1% in the control to between 10.7 and 16.3% in the treatments.

Field trials were conducted in commercial Bartlett orchards in Marysville and Live Oak using bactericides and biologicals as protective spray treatments. In an orchard in Live Oak with a high incidence of blight, Bloomtime Bio did not significantly reduce fire blight (incidence ca. 16-17%) from that of the control treatment (incidence 23.4%) (Fig. 4). In the orchard in Marysville with moderate to low disease incidence, however, the biocontrol was effective and significantly reduced the incidence of fire blight from 6.8% in the control to 1.3-2%, similar to Kasumin and Mycoshield (Fig. 5). The numerically best treatments in this latter orchard were the 100- and 150-ppm Kasumin treatments with 0.3 and 0.4% disease, respectively. The copper-Kasumin-Mycoshield rotation was the least effective treatment in this trial (2.7% incidence) and it was less effective than the Bloomtime Biological-Kasumin-Mycoshield rotation with an incidence of blight of 0.9%.

In the Live Oak orchard trial, the efficacy of kasugamycin was rate-dependent. Rates of 100 to 125 ppm were equally effective (Fig. 6). Different gallonages per acre were also evaluated while maintaining the concentration of kasugamycin (i.e., 100 ppm). The optimum gallonage was 100 gal/A. At 50 and 200 gal/A

there was a trend for or significantly higher incidence of disease, respectively. Overall, numerically the best treatments were the Kasumin 100-ppm (100 gal/A) treatment and the rotation Bloomtime Biological-Kasumin-Mycoshield, both with 2.7% incidence of blight. Interestingly, the biological control used in rotation with Kasumin and MycoShield was highly effective in this trial (Fig. 6) but not effective when used by itself under high disease pressure (Fig. 4) without any additional bactericide (or biological) applications.

In both trials, phytotoxicity on pear leaves (slight marginal leaf burn on leaves) was only observed after treatments with Kasumin. The incidence was highest at the highest rate applied (i.e., 150 ppm; Marysville trial) or at the highest gallonage applied (i.e., 100 ppm at 200 gal/A; Live Oak trial) (Figs. 5,6). Still, the severity of phytotoxicity was very low and may have even remained unnoticed, unless leaves were closely examined. Nevertheless, some minor marginal leaf injury was noted.

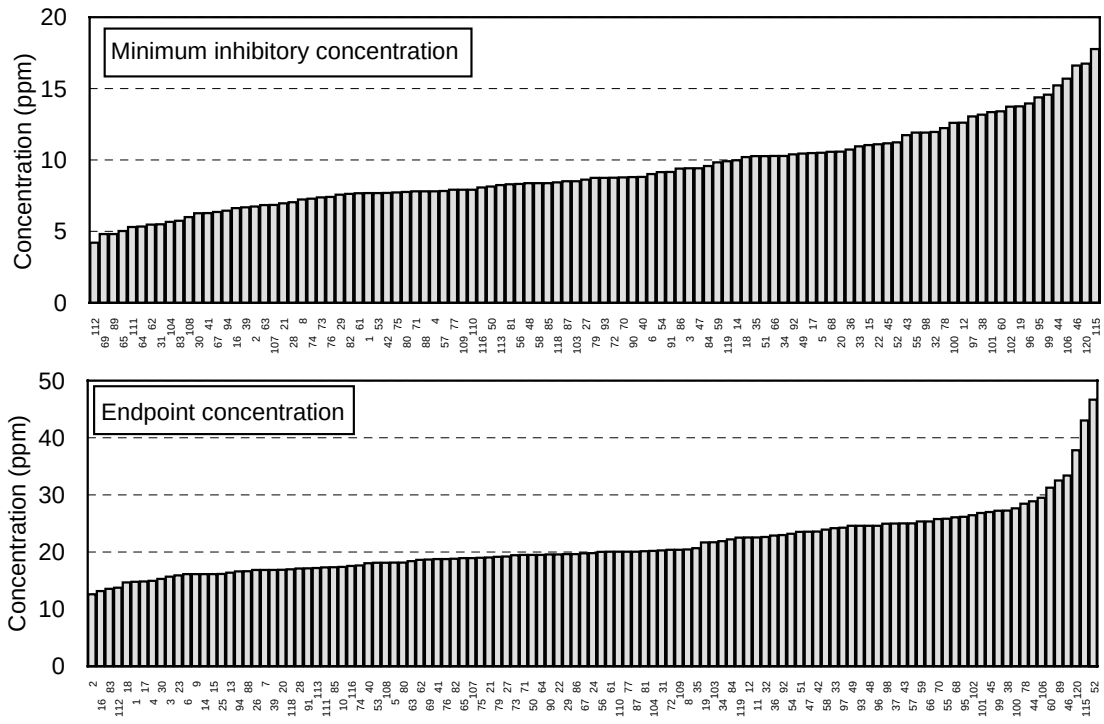
Thus, in several trials in 2006, the antibiotic kasugamycin was found to be highly effective in reducing the incidence of fire blight similar to or lower than that obtained after treatments with Mycoshield. In addition, rotational programs were identified that resulted in a similar high efficacy. Arysta Life Sciences Corp., the potential registrant, is supporting registration of the material for agricultural use in the United States. Furthermore, in Sept. 2005 the US-EPA granted an import tolerance for kasugamycin on some agricultural crops and the IR-4 program accepted our proposal for establishing a domestic residue tolerance. In 2006 nationwide field residue studies were done on pear. In 2007, trials on apple will also be done nationwide to allow registration of the antibiotic on the whole pome fruit crop group. Additional registration requests have been made and are ongoing on other crops (e.g., vegetable crops – pepper, tomato, etc.).

Table 1. Distribtuion of streptomycin-sensitive and -resistant isolates of *Erwinia amylovora* among 24 California pear orchards in 2006 survey.

| Orchard | Total No. of Isolates | Strep.-S | Strep.-R |
|---|---|---|---|
| 1 | 19 | 19 | 0 |
| 2 | 11 | 11 | 0 |
| 3 | 10 | 1 | 9 |
| 4 | 6 | 4 | 2 |
| 5 | 7 | 0 | 7 |
| 6 | 8 | 0 | 8 |
| 7 | 3 | 3 | 0 |
| 8 | 6 | 3 | 3 |
| 9 | 6 | 1 | 5 |
| 10 | 2 | 0 | 2 |
| 11 | 1 | 0 | 1 |
| 12 | 3 | 0 | 3 |
| 13 | 1 | 0 | 1 |
| 14 | 2 | 1 | 1 |
| 15 | 8 | 0 | 8 |
| 16 | 6 | 6 | 0 |
| 17 | 4 | 4 | 0 |
| 18 | 2 | 2 | 0 |
| 19 | 1 | 1 | 0 |
| 20 | 1 | 0 | 1 |
| 21 | 2 | 0 | 2 |
| 22 | 2 | 0 | 2 |
| 23 | 4 | 2 | 2 |
| 24 | 2 | 1 | 1 |
| Total | 117 | 59 | 58 |

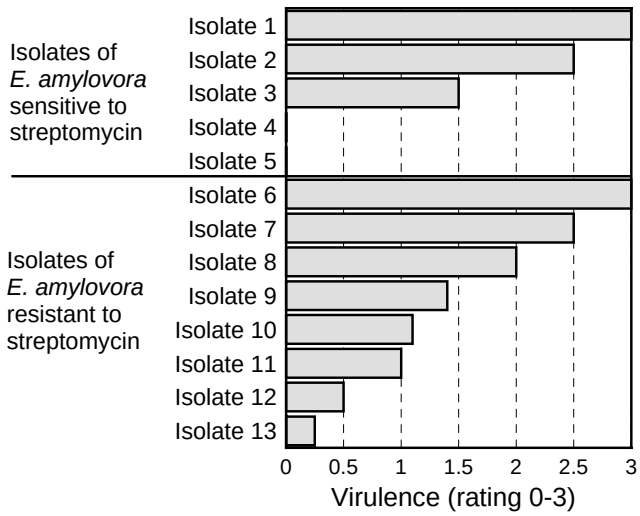
* - Streptomycin sensitivity among the isolates was determined on nutrient agar using the SGD method. Sensitive isolates had minimum inhibitory concentrations between 0.11 and 1.18 ppm and endpoint concentrations between 0.30 and 1.64 ppm; whereas, resistant isolates had minimum inhibitory concentrations >7.5 ppm.

Fig. 1. *In vitro* sensitivty of isolates of *Erwinia amylovora* to kasugamycin



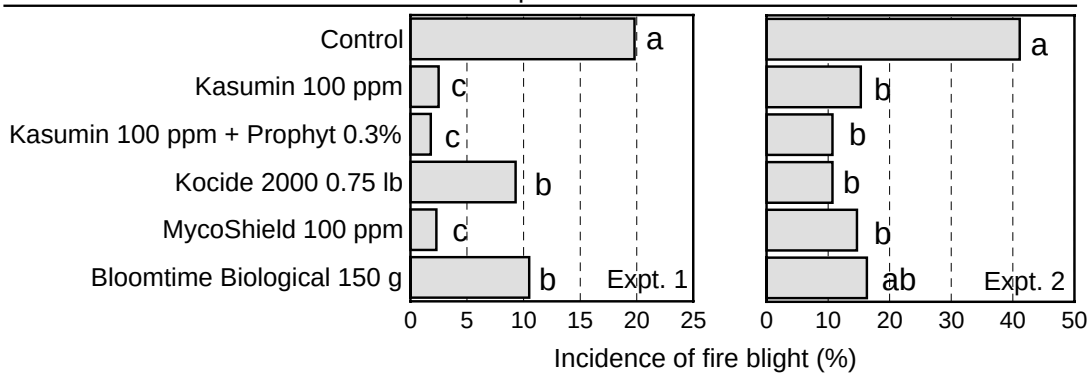

Inhibitory concentrations were determined on nutrient agar using the SGD method. The minimum inhibitory concentration is the lowest concentration of kasugamycin where a reduction of bacterial growth is observed. Endpoint concentration is the concentration where growth was inhibited by 99%.

Fig. 2. Evaluation of the efficacy of a bacteriophage preparation in lysing cells of isolates of *Erwinia amylovora* in laboratory experiments



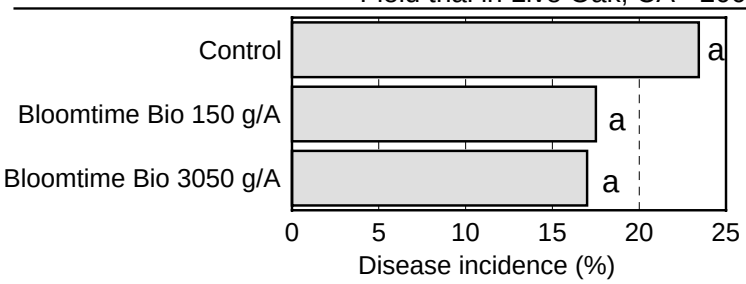

Suspensions of *E. amylovora* (50 ul of $2x10^8$ cfu/ml) were spiral-plated onto 10-cm Petri dishes containing nutrient agar. After 24 h, 5-ul drops of phage preparation were placed onto the bacterial lawn. Plates were evaluated for bacterial lysis after 2 days using a rating scale from 0 (= no clearing of bacterial lawn) to 3 (= complete clearing of bacterial lawn).

Fig. 3. Evaluation of new bactericides and a biocontrol for fireblight management on Shinko Asian pear
Small-scale field experiment in Solano Co. 2006




Treatments were applied to run-off to open blossoms using a hand sprayer on 4-40-06 (expt. 1) or 4-26-06 (expt. 2). Each replication consisted of one branch on each of four trees. After 5 h, blossoms were spray-inoculated with *Erwinia amylovora* ($10^6$ cfu/ml). In the first experiment blossoms were re-treated and re-inoculated on 4-26-06. Disease was evaluated on 5-3-06 (expt. 1) or 5-11-06 (expt. 2). Disease incidence was based on the number of diseased blossoms per 100 blossoms evaluated per replication (expt. 1) or on the number of diseased flower clusters per 30 clusters evaluated per replication (expt. 2).

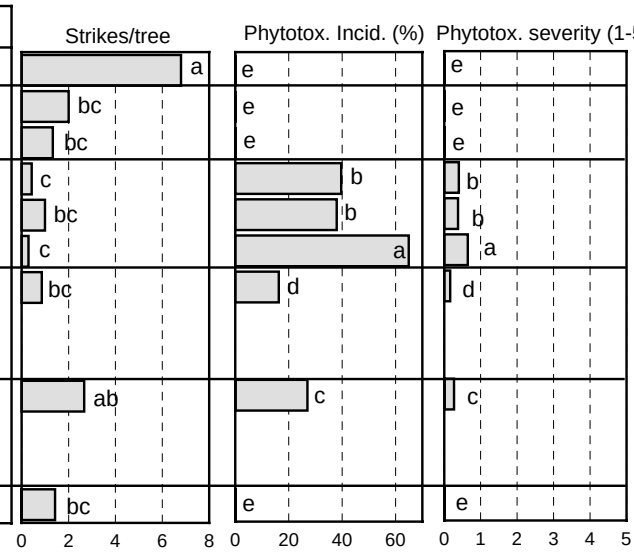
Fig. 4. Evaluation of a biocontrol for fireblight management on Bartlett pears
Field trial in Live Oak, CA - 2006




Treatments were applied on 4-6-06 (both rates) and 4-13-06 (low rate only) using an air-blast sprayer at 100 gal/A. Disease was evaluated on 5-16-06. Disease incidence was based on the number of infected fruiting spurs per 100 spurs evaluated for each of the 6 single-tree replications. No phytotoxicity was observed.

Fig. 5. Evaluation of new bactericides and a biocontrol for fireblight management on Bartlett pears
Field trial in Marysville, CA - 2006

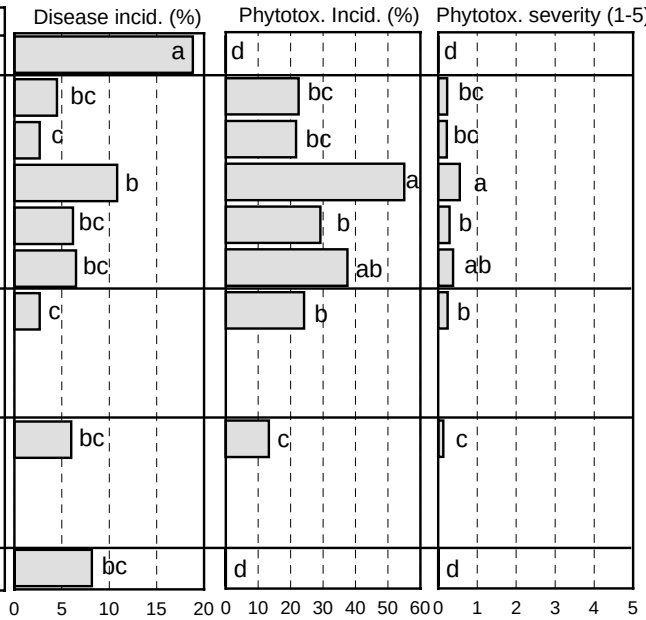
| No. | Treatment | Rate | Strikes/tree | Phytotox. Incid. (%) | Phytotox. severity (1-5) |
|---|---|---|---|---|---|
| 1 | Control | --- | a | e | e |
| 2 | Bloomtime Bio | 150 g | bc | e | e |
| 3 | Bloomtime Bio | 300 g | bc | e | e |
| 4 | Kasumin | 100 ppm | c | b | b |
| 5 | Kasumin | 125 ppm | bc | b | b |
| 6 | Kasumin | 150 ppm | c | a | a |
| 7 | Bloomtime Bio | 150 g | bc | d | d |
| | Kasumin 2L | 100 ppm | | | |
| | Mycoshield | 100 ppm | | | |
| 8 | Copper | 0.75 lb | ab | c | c |
| | Kasumin 2L | 100 ppm | | | |
| | Mycoshield | 100 ppm | | | |
| 9 | MycoShield | 100 ppm | bc | e | e |




Treatments were applied on: 4-6, 4-13, 4-20, 4-26, 5-3, and 5-10-06 using an air-blast sprayer at 100 gal/A. Disease was evaluated on 5-16-06. Disease severity was based on the number of fireblight strikes for each of the 6-7 single-tree replications. Phytotoxicity incidence was based on the number of leaves with marginal necrosis per 50 leaves evaluated for each replication and the severity was based on a rating scale from 0 to 5.

Fig. 6. Evaluation of new bactericides and a biocontrol for fireblight management on Bartlett pears
Field trial in Live Oak, CA - 2006

| No. | Treatment | Rate | Disease incid. (%) | Phytotox. Incid. (%) | Phytotox. severity (1-5) |
|---|---|---|---|---|---|
| 1 | Check | --- | a | d | d |
| 2 | Kasumin 50 gal/A | 100 ppm | bc | bc | bc |
| 3 | Kasumin 100 gal/A | 100 ppm | c | bc | bc |
| 4 | Kasumin 200 gal/A | 100 ppm | b | a | a |
| 5 | Kasumin - Prophyt | 100 ppm -0.3% | bc | b | b |
| | Kasumin - Nufilm P | 100 ppm 6 fl oz | bc | ab | ab |
| 7 | Bloomtime Bio | 150 g | c | b | b |
| | Kasumin 2L | 100 ppm | | | |
| | Mycoshield | 100 ppm | | | |
| 8 | Copper | 0.75 lb | bc | c | c |
| | Kasumin 2L | 100 ppm | | | |
| | Mycoshield | 100 ppm | | | |
| 9 | MycoShield | 100 ppm | bc | d | d |




Treatments were applied on: 4-6, 4-13, 4-20, 4-26, and 5-3-06 using an air-blast sprayer at 100 gal/A (except where indicated otherwise). Disease was evaluated on 5-16-06. Disease incidence was based on the number of infected fruiting spurs per 100 spurs evaluated for each of the 6 single-tree replications. Phytotoxicity incidence was based on the number of fruiting spurs with leaves with marginal injury per 20 spurs evaluated for each replication and the severity was based on a rating scale from 0 to 5.