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EVALUATION OF NEW BACTERICIDES FOR CONTROL OF FIRE BLIGHT OF PEARS CAUSED BY *ERWINIA AMYLOVORA*

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ABSTRACT

1. Antibiotic resistance surveys for populations of *Erwinia amylovora* in California pear orchards were continued.
    a. Kasugamycin: All 117 strains from 14 orchard locations in Sacramento and 18 locations in Lake Co. were sensitive.
    b. Streptomycin: Plasmid-based moderate- and chromosomal-based high-resistance was detected at eight locations in Sacramento Co., and the incidence of resistance including that of high-resistance was sometimes high. Moderate-resistance to streptomycin was also found at three locations in Lake Co. Thus, populations of *E. amylovora* re-adjust rapidly to selection pressure (i.e., bactericide applications). Streptomycin should be used strategically, and these findings stress the importance of resistance management with mixtures or rotations and the development of new alternatives.
    c. Oxytetracycline: Strains with high resistance levels (>40 ppm) were detected at three locations in Sacramento Co. At two of the locations, resistance was already found in 2018. These resistant strains were also highly resistant to streptomycin. In the location with the highest incidence of oxytetracycline resistance, nine applications of the antibiotic were applied between 2017 and 2018. Oxytetracycline resistance in *E. amylovora* has never been reported previously at this level, and this finding is a serious concern.
2. In direct-exposure laboratory studies, the addition of EDTA increased the toxicity of the FDA-approved food preservatives ε-poly-L-lysine and nisin to *E. amylovora.*
3. Field trials on the management of fire blight were conducted under low disease pressure.
    a. The food preservatives ε-poly-L-lysine and nisin in selected mixtures were very effective, but evaluation at higher disease pressure is warranted.
    b. The biocontrol treatment Blossom Protect, the copper product Cueva, and a Serenade ASO-Cueva mixture reduced blight to low levels.
    c. Kasumin continued to perform very well. Its restricted use (maximum of two applications per season) and recommended mixture with another bactericide will help to minimize the risk for resistance development. The mixture of Kasumin with FireWall resulted in the lowest disease incidence.

INTRODUCTION

Fire blight, caused by the bacterium *Erwinia amylovora*, is the most destructive disease of pome fruit trees worldwide, especially pears. In California, prolonged rat-tail bloom contributes to a long infection period. Fire blight is very difficult to manage, and few effective treatments are available. Integrated programs with sanitation practices and applications with chemical and biological controls are the best approaches. If the disease occurs at low incidence, it often can be eliminated by pruning. Thus, aggressive and regular scheduled pruning of diseased tissue is essential for keeping inoculum levels low.

Current chemical control programs for fire blight are based on protective treatments with antibiotics or copper. 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 some newer formulations of copper, however, reduced rates based on metallic copper equivalent (MCE) can be used past the bloom period without causing russeting. Under low disease pressure, copper compounds can provide satisfactory disease control and they can be an effective rotational or mix-partner. In years with high disease pressure, however, copper applications generally fail to control the disease at satisfactory levels. Therefore, in our UCIPM ratings, copper is ranked as a +/++ treatment indicating inconsistent performance depending on environmental conditions. In 2015 - 2018, we reported reduced sensitivity to copper in strains of *E. amylovora* with growth occurring at 20 to 30 ppm MCE on nutrient agar and 10 to 20 ppm on the low copper-binding CYE agar. These levels indicate moderate copper resistance and can also explain the moderate and inconsistent performance of copper. Lack of systemic action and low registered rates are other factors for low efficacy.

Treatments with the antibiotics streptomycin and oxytetracycline have been employed for many years to manage fire blight. Continued usage for many seasons and lack of alternative control materials caused resistance against streptomycin to develop at high incidence at many locations in California, mostly in Sacramento Co., starting in 2006. Since then, the incidence of streptomycin resistance has been fluctuating widely among years from very low to very high levels, and this has been attributed to disease pressure and the intensity of streptomycin use. Strains of *E. amylovora* with reduced sensitivity to oxytetracycline were found several years ago at several locations. In 2018, however, we detected strains highly resistant to this antibiotic for the first time at two locations in Sacramento Co. Surveys on antibiotic resistance monitoring were continued in 2019 in collaboration with farm advisors and orchard managers. Resistance monitoring was also done for kasugamycin (Kasumin) for numerous years, but no resistance has been found. After many years of field evaluations and regulatory battles, this third antibiotic became available for use in the 2018 season in California. Concerns have been expressed by regulatory agencies regarding the use of antibiotics in agriculture, but kasugamycin is not used in human and animal medicine and has a different mode of action from streptomycin or oxytetracycline (no cross-resistance).

Our evaluations in 2019 focused on new natural products that potentially could qualify for organic production for which is a growing interest. We continued to evaluate ε-poly-L-lysine and nisin. ε-poly-L-lysine is used commercially as a food preservative in Japan, Korea and in imported items in the United States. It has demonstrated antimicrobial activity against yeasts, fungi, Gram-positive, and Gram-negative bacteria. Nisin (Niprosin) is used as a preservative in some processed foods. ε-poly-L-lysine and nisin showed promising results in our fire blight trials previously, but our attempts in 2018 to improve their efficacy by encapsulation in alginate were not successful. Thus, other mixture partners were used in 2019, including the UV-protectant zinc oxide.

In 2019, we also evaluated three new experimental antimicrobials (NSA, NS1, NS2), and we continued to evaluate Cueva, Serenade ASO (a liquid formulation), Blossom Protect (with a newly formulated buffer), as well as the three registered antibiotics with selected additives including the foliar fertilizer zinc nitrate (Brandt).

OBJECTIVES

1. Continue antibiotic resistance surveys in *E. amylovora* populations from pear orchards in California for streptomycin, oxytetracycline, and kasugamycin.
2. Evaluate natural products, exempt from tolerance food additives, and biocontrols in laboratory and field studies.
    i. ε-poly-L-lysine, nisin, lactic acid in combination with EDTA, dextran, or sodium diacetate.
    ii. Biologicals including Blossom Protect, Serenade ASO, and others as they become available.
3. Evaluate and optimize the performance of kasugamycin (Kasumin), streptomycin (e.g., Agrimycin-17, Firewall), oxytetracycline (e.g., Mycoshield, Fireline), and new antimicrobials in field trials.
    a) Kasumin in combination with exempt-from-tolerance antimicrobials
    b) Type III secretion system inhibitors (if new ones become available)
    c) Oxytetracycline formulations in combination with selected UV-protecting adjuvants or stabilizers to increase persistence of residues.

MATERIALS AND METHODS

Isolation of *E. amylovora* and bacterial culturing. Samples with fire blight symptoms were obtained in the spring of 2019 from 14 pear orchards in Sacramento Co. and 38 orchards in Lake Co. Infected plant material (fruit, stems, pedicels, twigs) was cut into small sections and incubated in 1 ml of sterile water for 15 to 30 min to allow bacteria to diffuse out of the tissue. Suspensions were streaked onto yeast extract-dextrose-$CaCO_3$ agar (YDC) and single colonies of *E. amylovora* were transferred. A total of 117 strains were obtained (between 1 and 13 strains per location) and evaluated for their sensitivity to antibiotics.

Laboratory studies on the toxicity of bactericides against *E. amylovora*. Streptomycin, oxytetracycline, and kasugamycin were evaluated for their in vitro toxicity using the spiral gradient endpoint method. For this, a radial bactericidal concentration gradient was established in nutrient agar in Petri dishes by spirally plating a stock concentration of each antimicrobial using a spiral plater. 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 taken visually for 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.

The toxicity of ε-poly-L-lysine and nisin by themselves and in mixtures with ethylenediaminetetraacetic acid (EDTA) against *E. amylovora* was evaluated in direct contact assays. For this, suspensions of a standard strain were incubated in final concentrations of 500 ppm of these antimicrobials without or with the addition of selected concentrations of EDTA, dextran, calcium carbonate, or sodium diacetate. Water was used in control treatments. Mixtures were incubated for 30 min, diluted 1:1000 with sterile water, and aliquots were then plated onto nutrient agar. After 2 days, bacterial colonies were enumerated, and percent reduction as compared to the control was calculated. The toxicity of calcium propionate was evaluated using the same assay.

Small-scale field studies on the evaluation of new potential biocontrol agents. Suspensions of the biocontrol agents BC250 and T3-07 were prepared from 1-day-old nutrient agar cultures at a concentration of 4 x 10^7 cfu/ml. On April 18, 2019, flowers were treated (except Oxidate) using a hand sprayer to run-off. Flowers were inoculated with *E. amylovora* (2 x 10^7 cfu/ml) after 4 h by hand-spraying, and Oxidate was applied after another hour. Disease was evaluated after 7 days based on the number of blackened flowers.

Field studies using protective treatments during the growing season. In a trial in a commercial cv. Bartlett orchard in Live Oak, treatments (see Fig. 3) were done using an airblast sprayer at 100 gal/A on 4-4 (5% bloom), 4-12 (full bloom), 4-18 (petal fall), and 4-26-19. NuFilmP was used at 5 fl oz in the first application. There were four single-tree replications per treatment. Natural incidence of disease (i.e., infected spurs of 100 spurs evaluated for each tree) was evaluated on 5-1-19. Data were analyzed using analysis of variance and LSD mean separation procedures of SAS 9.4.

RESULTS AND DISCUSSION

***Survey of antibiotic sensitivity in* E. amylovora *strains from pear in California in 2019*.** In 2019, 65 strains were obtained from 14 orchard locations in Sacramento Co. and

52 strains from 38 locations in Lake Co. All 117 strains were found to be sensitive to **kasugamycin** (Table 1).

In **Sacramento Co.**, resistance to **streptomycin** was detected at eight of the locations with an incidence of 33.3% to 100%. At all eight locations, moderately resistant strains (MIC <20 ppm) with plasmid-based resistance were present, but 6 locations also had strains with high-resistance (MIC >100 ppm) that most likely was chromosomal-based. Strains with high-resistance that once used to be common had declined in recent years; but in in 2018, a high incidence of high-resistance was detected in several locations. One of these locations was re-sampled in 2019, and 41.7% of the strains recovered were highly resistant to streptomycin. In another orchard with a low incidence of high-resistance in 2018, one strain evaluated in 2019 also was highly resistant. In some of the orchards with low- and high-resistance, a rotation of copper – streptomycin + oxytetracycline + mancozeb – Actigard – Kasumin was applied in the 2019 spring season. Thus, resistant strains persisted under this relatively low selection pressure. In three orchards where the same rotation was done in 2019, however, no streptomycin resistance was detected. No resistance was also detected at two locations with organic programs. Over the years, there has been no clear correlation between streptomycin usage in a specific year and the incidence and level of streptomycin resistance present in the pathogen population in the spring season of the respective years. The previous seasons' applications also may need to be considered because they will affect the composition of the overwintering pathogen population. Thus, spray schedules from multiple years will need to be examined. Our current recommendation is to use streptomycin only once a year to reduce selection pressure on the pathogen. Because this was followed in the orchards with high levels of resistance in 2019, it would also be interesting to know if fire blight was successfully managed with the copper – streptomycin + oxytetracycline + mancozeb – Actigard – Kasumin rotation.

High-resistance (>40 ppm) to **oxytetracycline** in *E. amylovora* was detected for the first time at two locations in 2018, and all resistant strains were also highly resistant to streptomycin. These two orchards were re-sampled in 2019, and oxytetracycline resistance was again detected. At the first location, 6 out of 7 strains were resistant in 2018, whereas in 2019, 4 out of 12 strains were resistant. At the other location, 1 of 8 or 12 strains was resistant in 2018 and 2019, respectively. The resistant strains' identity was verified as *E. amylovora* by specific PCR primers. Thus, these resistant strains persisted. Additionally, 1 of 8 strains was resistant to oxytetracycline in a third orchard in 2019. As in 2018, strains resistant to oxytetracycline were also highly resistant to streptomycin. At the location with the highest incidence of oxytetracycline resistance, nine applications of the antibiotic were applied between 2017 and 2018. High dependency on one antibiotic in a two-year period may be responsible for the selection of the resistance detected.

Oxytetracycline resistance in *E. amylovora* has never been reported previously at this high level, and this finding is of serious concern. Considering the wide fluctuations in streptomycin resistance in California pear orchards and the previously described non-persistent population of the pathogen with reduced sensitivity to oxytetracycline, it is currently not known if these new resistant strains are competitively fit and will persist in the absence of selection pressure (i.e., applications with oxytetracycline and streptomycin). We plan to characterize these strains genetically to determine if oxytetracycline resistance genes are similar to those that were previously described from other bacteria (non-plant pathogens). It will also be interesting to determine if there is a molecular association between high-streptomycin and high-oxytetracycline resistance.

In **Lake Co.**, most of the 52 strains evaluated were sensitive to streptomycin and oxytetracycline (Table 1). **Moderate resistance to streptomycin** (MIC <20 ppm), however, was found at 3 locations where streptomycin-oxytetracycline mixtures were applied for fire blight management. This is of interest because except for one strain collected numerous years ago, only sensitive strains have been recovered in our surveys in this California pear growing area.

Laboratory studies on the toxicity of bactericides against *E. amylovora*. In direct contact studies, 30-min exposures of *E. amylovora* to 500 ppm ε-poly-L-lysine or nisin resulted in an approximately 50% inhibition of colony formation (Fig. 1). EDTA was not inhibitory at 50 or 100 ppm, but significantly reduced colony formation at 500 ppm. Mixtures of 500 ppm ε-poly-L-lysine with 500 ppm or 100 ppm EDTA inhibited colony formation by 100 or 79%, respectively. Mixtures of 500 ppm nisin with 500 ppm or 100 ppm EDTA both inhibited colony formation by 100%. These results supported testing these mixtures in field efficacy studies, especially for nisin where lower EDTA concentrations were effective. The addition of dextran, calcium carbonate, or sodium diacetate did not improve the toxicity of ε-poly-L-lysine or nisin.

Small-scale field studies on the evaluation of new potential biocontrol agents. The study was conducted on Comice pear and Fuji apple flowers at UC Davis. When treatments were applied 4 h before inoculation with *E. amylovora*, there was no reduction in the incidence of blighted flowers of both pome fruits by any of the biocontrol treatments (Fig. 2). The addition of an Oxidate treatment did not improve efficacy, and Oxidate by itself was also not effective. The control treatment of streptomycin, however, significantly reduced the incidence of disease by approximately 50% on pear and by nearly 80% on apple.

To be effective, the biocontrols may have needed a longer time period to establish before inoculation, especially when their mode of action is site exclusion. This needs to be considered when re-evaluating these biocontrols. Still, under natural field conditions, infection can occur anytime during bloom and because bloom progresses very quickly on a single flower, there is little time for a biocontrol agent to establish on a flower.

***Field studies using protective treatments during the growing season*.** The incidence of fire blight was low at our field location in the 2019 spring season. Rainfall was high, but low temperatures persisted during the bloom period. On the non-treated control, 7.4% of the flowers were blighted at evaluation time. All treatments evaluated significantly reduced the incidence of blight and most of them were highly effective (Fig. 3).

The food preservatives **ε-poly-L-lysine** or **nisin** were only evaluated in selected mixtures because previously, their efficacy was only moderate when used by themselves. ε-poly-L-lysine mixed with zinc nitrate or Dart (a mixture of capric and caprylic acids) reduced the incidence to ≤1.3%. Nisin mixed with EDTA and zinc oxide (a UV-protectant) was numerically one of the most effective treatments with 0.5% incidence, but when only mixed with EDTA was moderately effective (4.3% incidence). Thus, evaluation at higher disease pressure is warranted for these food preservatives. ε-poly-L-lysine was also very effective in combination with Kasumin (0.8% incidence), but did not improve **Kasumin** efficacy (0.5 - 0.8% incidence). The lowest incidence was observed for the **Kasumin-FireWall** mixture (0.3%) that previously also performed very well under high-disease conditions. Two **Mycoshield** formulations and **FireLine** in mixtures with zinc oxide, zinc nitrate (a foliar fertilizer that showed moderate efficacy by itself in 2018), and/or the non-ionic surfactant LI700 also performed well (0.5 – 1.3% incidence). In addition, the copper product **Cueva**, a **Cueva-Serenade** mixture, and the biocontrol **Blossom Protect** (in combination with a new buffer formulation) also were very effective with 1%, 0.5%, and 0.8% incidence, respectively. Treatments with the **new experimental bactericides** NSA, NS1, and NS2 resulted in 2.5%, 2%, and 1.5% blight incidence, respectively.

In summary, ε-poly-L-lysine and nisin are the most promising bactericides identified in recent years. In collaboration with an agrochemical company, we are trying to develop agricultural formulations for these compounds to improve their field performance. The recently registered Kasumin continues to perform very well. Its restricted use (maximum of two applications per season) and recommended use in mixture with another bactericide will help to minimize the risk for resistance development. Blossom Protect is another fire blight treatment that has performed consistently well over numerous years of our studies. Still, we will continue to evaluate new experimental bactericides and biocontrols in future studies to potentially offer additional effective options for fire blight management and to provide rotation alternatives for effective anti-resistance programs.

Table 1. Sensitivity of *E. amylovora* strains from pear orchards in Sacramento and Lake Co. to streptomycin, oxytetracycline, and kasugamycin in 2019

Sacramento Co.

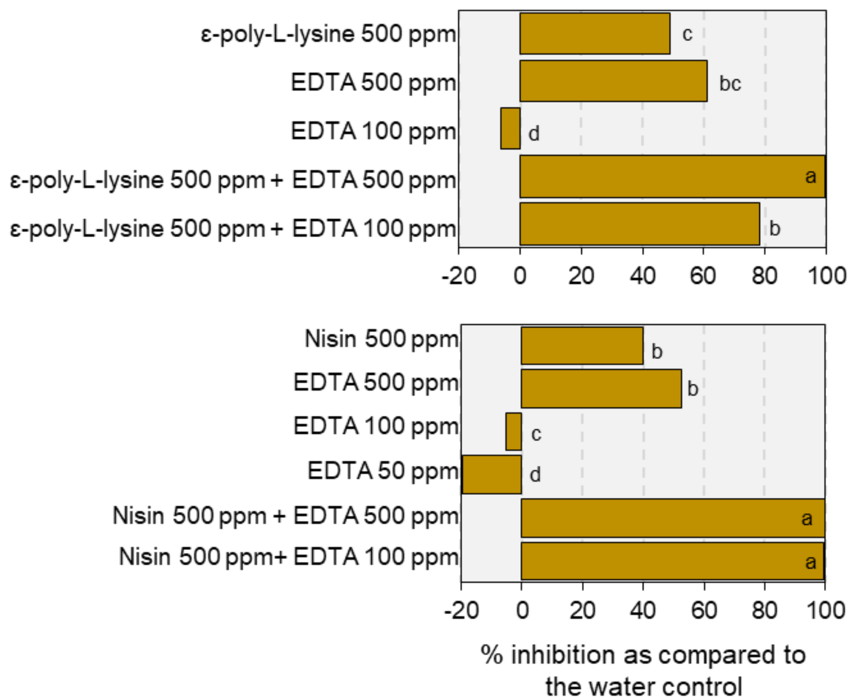
Orchard No.	Streptomycin	Oxytetracycline	Kasugamycin
1	MR	S	S
	MR	S	S
	MR	S	S
2	S	S	S
	S	S	S
3	HR	S	S
	MR	S	S
	MR	S	S
4	MR	S	S
	MR	S	S
	HR	HR	S
	HR	S	S
	MR	S	S
	MR	S	S
	MR	S	S
	HR	S	S
5	MR	S	S
	HR	S	S
	MR	S	S
6	HR	S	S
	MR	S	S
	HR	S	S
	HR	S	S
	MR	S	S
	MR	S	S
7	S	S	S
	S	S	S
	S	S	S
8	S	S	S
	S	S	S
	S	S	S
	S	S	S
9	S	S	S
	S	S	S
	S	S	S
	S	S	S
	S	S	S
	S	S	S
	MR	S	S
	MR	S	S
	HR	HR	S
	S	S	S
	S	S	S
	MR	S	S
10	HR	HR	S
	MR	S	S
	HR	HR	S
	MR	S	S
	HR	S	S
	MR	S	S
	HR	HR	S
	MR	S	S
	MR	S	S
	S	S	S
	MR	S	S
	HR	HR	S
11	MR	S	S
12	S	S	S
	S	S	S
13	S	S	S
14	S	S	S
	S	S	S
	S	S	S
	S	S	S
	S	S	S

Lake Co.

Orchard No.	Streptomycin	Oxytetracycline	Kasugamycin
1	S	S	S
	S	S	S
	S	S	S
2	S	S	S
3	S	S	S
4	S	S	S
5	S	S	S
	S	S	S
6	S	S	S
	S	S	S
7	S	S	S
8	S	S	S
9	S	S	S
10	S	S	S
11	S	S	S
12	S	S	S
	S	S	S
13	S	S	S
14	S	S	S
15	S	S	S
16	S	S	S
17	S	S	S
	S	S	S
	S	S	S
	S	S	S
	S	S	S
18	S	S	S
19	S	S	S
	S	S	S
20	S	S	S
	S	S	S
21	S	S	S
22	S	S	S
23	MR	S	S
24	S	S	S
25	S	S	S
26	S	S	S
27	S	S	S
28	S	S	S
29	S	S	S
30	S	S	S
31	S	S	S
32	S	S	S
33	S	S	S
34	S	S	S
35	S	S	S
	S	S	S
36	MR	S	S
37	MR	S	S
	MR	S	S
38	S	S	S
	S	S	S

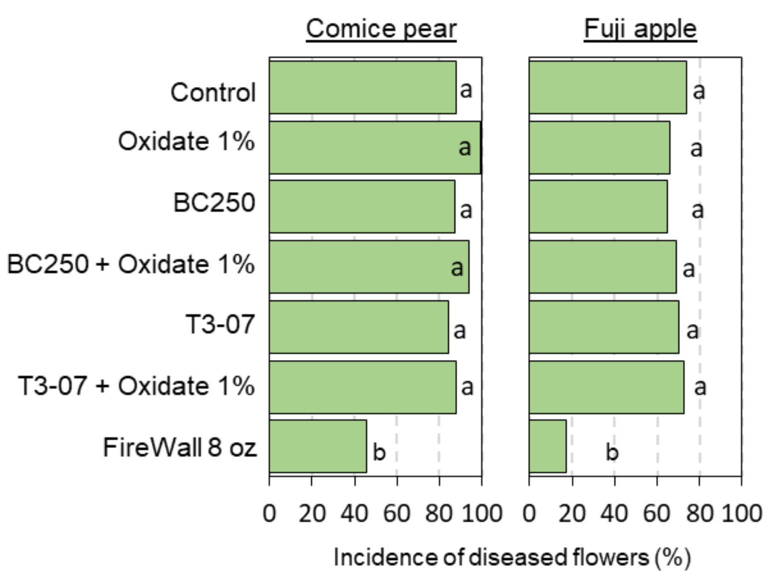
Sensitivity to streptomycin, oxytetracycline, and kasugamycin was determined using the spiral gradient endpoint method. S = sensitive, MR = moderately resistant (MIC = <20 ppm), HR = highly resistant (MIC = >40 ppm).

Fig. 1. In vitro toxicity of epsilon-poly-L-lysine and nisin against *E. amylovora* in direct exposure studies



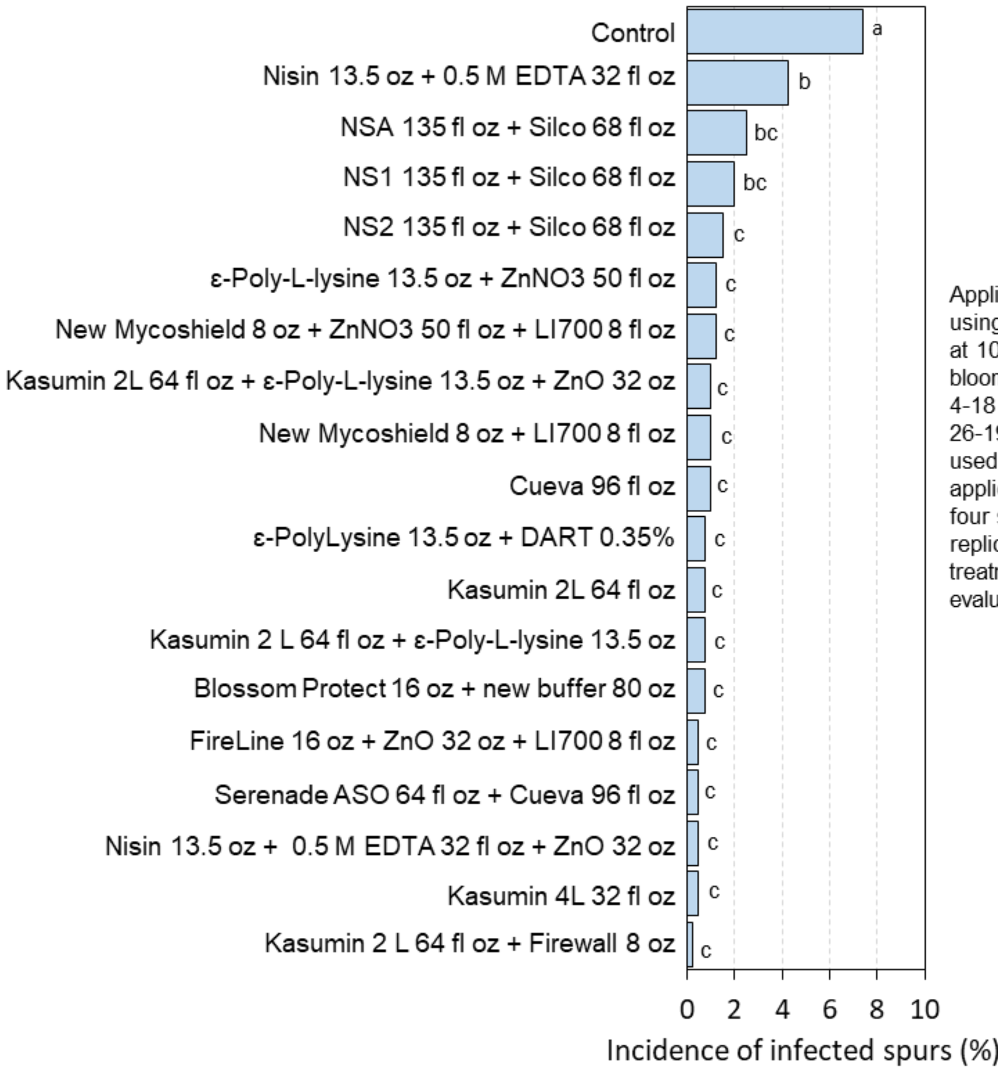
Bacteria were exposed to the test substances for 30 min, diluted 1:100 with water, and then plated onto agar media. Bacterial colonies were quantified after 2 days.

Fig. 2. Evaluation of new potential biocontrol agents for control of fire blight of Comice pear and Fuji apple in a small-scale field trial at UC Davis 2019



Suspensions of the biocontrol agents BC250 and T3-07 were prepared from 1-day-old nutrient agar cultures at a concentration of 4 x 10^7 cfu/ml. On April 18, 2019, flowers were treated (except Oxidate) using a hand sprayer to run-off. Flowers were inoculated with *E. amylovora* (2 x 10^7 cfu/ml) after 4 h, and Oxidate was applied after another hour. Disease was evaluated after 7 days based on the number of blackened flowers.

Fig. 3. Evaluation of biologicals in comparison to an antibiotic for management of fire blight of Bartlett pear in a field trial in Live Oak, CA 2019



Applications were done using an airblast sprayer at 100 gal/A on 4-4 (5% bloom), 4-12 (full bloom), 4-18 (petal fall), and 4-26-19. NuFilmP was used at 5 fl oz in the first application. There were four single-tree replications per treatment. Disease was evaluated on 5-1-19.