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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**
   a. **Surveys** for populations of *Erwinia amylovora* in California pear orchards were continued, but only 10 strains were obtained from 6 locations in Yuba Co. and 3 locations in Lake Co. All 10 strains were sensitive to streptomycin, oxytetracycline, and kasugamycin. No strains were available from locations where high oxytetracycline resistance was detected in recent years.
   b. In **inoculation studies**, treatments with oxytetracycline were significantly less effective, when flowers were inoculated with a resistant as compared to a sensitive strain.
2. **Field trials on the management of fire blight** were conducted under moderate (at a commercial site on Bartlett pear and at UC Davis on Comice pear) or very low (at UC Davis on Asian pears or apple-pear) disease pressure. The latter trial at UC Davis was part of an IR-4 efficacy study.
   a. Among **new bactericides** evaluated, the FDA GRAS bactericide TDA-NC-1 significantly reduced the incidence of disease in a trial where trees were inoculated, but not the natural incidence in another trial. In three studies, a rotation of the biocontrols Blossom Protect and Serenade showed moderate efficacy in reducing the incidence of blight after inoculation or occurring naturally. The new natural antibiotic ningnanmycin (Ninja) showed low or no efficacy at the registrant's low suggested rate, but the essential oil ET-91 was very effective in an inoculation study. The biocontrol Reju-Agro A and aluminum sulfate (Alum) were effective in one, but not in another inoculation study. Overall, none of the new experimental bactericides was as effective as registered antibiotics. New bactericides were also evaluated in studies for their in vitro activity against *E. amylovora*.
   b. Kasumin continued to perform very well, and its efficacy was slightly increased with the addition of Syllit or FireWall. Restrictions for use are now a maximum of four applications per season (no more than two consecutive applications) and recommended use in mixture with another bactericide will help to minimize the risk for resistance development. New formulations of FireLine (in mixture with Dart) and Mycoshield (NUP-17010) provided the highest efficacy in the natural incidence trial.

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 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 copper causes phytotoxicity as fruit russeting. Some newer formulations of copper, however, require reduced rates based on metallic copper equivalent (MCE) and can be used after bloom 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 fire blight at satisfactory levels. Therefore, in our UCIPM ratings, copper is ranked as a +/++ treatment indicating inconsistent performance depending on environmental conditions. In addition, we reported reduced copper sensitivity in strains of *E. amylovora* from pear that can also explain the moderate and inconsistent performance of copper. Lack of systemic action and low registered rates are other factors contributing to low efficacy.

Treatments with the antibiotics streptomycin (STR) and oxytetracycline (OXY) have been employed for many years to manage fire blight. Continued use for many seasons and lack of alternative control materials resulted in resistance to develop against STR at high incidence at many locations in California, mostly in Sacramento Co. Strains with moderate plasmid-based resistance and strains with chromosome-based high resistance have been identified. The incidence of STR 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 STR use. Strains of *E. amylovora* with reduced sensitivity to OXY were found several times during our surveys. Starting in 2018, however, we detected strains with high resistance to this antibiotic for the first time at several locations in Sacramento Co. These strains were found to be similarly virulent and competitive as wildtype strains. We continued to characterize OXY-resistant strains in 2021, and research on the molecular resistance mechanism is ongoing.

Surveys on antibiotic resistance monitoring were continued in 2021 in collaboration with farm advisors and PCAs, but only ten samples were obtained. Resistance to kasugamycin (Kasumin) has not been found in our surveys. This third antibiotic became available for use in 2018 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 STR or OXY (no cross-resistance). In 2020, after 7 years of environmental resistance monitoring with no detected shifts in sensitivity among non-target bacteria, the EPA has suspended this requirement for the kasugamycin registration.

With the current emphasis on identifying alternative bactericides, in 2021, we continued our evaluations of exempt-from-tolerance and potential organic compounds. Some of this research was part of IR-4 efficacy studies. Thus, we continued to evaluate the food preservatives ε-poly-L-lysine and nisin as non-formulated technical compounds. Other products evaluated in 2021 field trials include the biocontrols Blossom Protect, Serenade, Double Nickel, and RejuAgro A, the essential oil product ET-91, the FDA GRAS bactericide TDA-NC1, the natural antibiotic ningnanmycin (Ninja), the experimental QAM, the chlorine dioxide treatment Aqua-Clear 7.5, as well as aluminum sulfate. Some treatments were done in combination with additives such as Dart, ManniPlex Zn, LI700, Regulaid, or NuFilm 17. The essential oil product BacStop was only evaluated in in vitro studies.

OBJECTIVES

1. Continue resistance surveys for streptomycin, oxytetracycline, and kasugamycin in *E. amylovora* populations from pear orchards in California.
    a) Characterize strains of *E. amylovora* provided by farm advisors, PCAs, and growers for resistance
    b) Characterize antibiotic resistance on a molecular basis.
2. Evaluate and optimize the performance of kasugamycin (Kasumin), streptomycin (e.g., Agrimycin-17, FireWall), oxytetracycline (e.g., Mycoshield, FireLine), and new copper formulations in field trials.
    a) Kasumin in combination with exempt-from-tolerance antimicrobials (see below).
    b) Oxytetracycline – new formulations of Mycoshield and FireLine and trials using oxytetracycline with selected adjuvants or stabilizers to increase persistence of residues and overall efficacy.
3. Evaluate new natural products, GRAS food additives, and biocontrols in laboratory and field studies.
    a) ε-poly-L-lysine, nisin, lactic acid in combination with capric/caprylic acids (commercial product Dart). Treatments identified as effective in the laboratory will be included in field studies
    b) Continue evaluating the new FDA GRAS bactericide TDA-NC-1 and new essential plant oils
    c) Registered biologicals including Blossom Protect and Serenade ASO, will be compared to other experimental compounds.

MATERIALS AND METHODS

Isolation of *E. amylovora* and bacterial culturing. Samples with fire blight symptoms were obtained in the spring of 2021 from 6 pear orchards in Yuba Co. and 3 orchards in Lake Co. Infected plant material (fruit, peduncles, 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, and single colonies of *E. amylovora* were transferred. A total of 10 strains were obtained and evaluated for their sensitivity to antibiotics.

Laboratory studies on the toxicity of bactericides against *E. amylovora*. STR, OXY, 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.

Several of the new bactericides were also evaluated for their in vitro toxicity against *E. amylovora*. Different methods were used depending on the formulation of the compound and its toxicity. Amended agar studies where the pathogen was in continuous contact with the test substance were used for ningnanmycin and Dart (i.e., the spiral gradient endpoint assay) and for ET-91 and QAM (i.e., the agar dilution method). Direct exposure studies where the pathogen was exposed to the test substance for a limited time (30 or 60 min) and was then plated onto non-amended agar were done using BacStop, TDA-NC-1, nisin, and ε-poly-L-lysine.

Evaluation of OXY efficacy against fire blight caused by STR-OXY double-resistant strains of *E. amylovora*. In two experiments, twigs with pear flowers were placed in beakers with water on a light bench in the laboratory. Flowers were spray-treated and inoculated after 4-5 h with strains of *E. amylovora* either sensitive to OXA and STR or highly resistant to both antibiotics (10^7 cfu/ml). Disease was evaluated after 7 days.

Molecular analysis of high STR-OXY resistant strains of *E. amylovora*. High molecular-weight genomic DNA was submitted to long- and short-read whole-genome sequencing. Genomes were assembled and annotated, and sequences were compared with known plasmids.

Field studies on the evaluation of new bactericide treatments. In a trial in a commercial cv. Bartlett orchard in Live Oak, treatments (see Fig. 2) were done using an air-blast sprayer at 100 gal/A on 3-23 (white tip, 5% bloom), 3-31 (80% bloom), 4-7 (petal fall), and 4-15-21. There were four single-tree replications per treatment. The natural incidence of fire blight was evaluated on 4-21-21.

New bactericide alternatives were evaluated on Comice pear and Shinko apple pear at UC Davis. On Comice pear, treatments (see Fig. 3) were applied using an air-blast sprayer on 4-1 (full bloom), 4-6, and 4-14-21. Trees were inoculated with *E. amylovora* (5 x 10^6 cfu/ml) after the third application. Disease was evaluated on 4-20-21. Blight incidence was based on the number of flower/fruit clusters with disease of the total number of clusters evaluated.

The trial on Shinko apple pears was part of IR-4 efficacy studies. Treatments (see Fig. 4) were applied using an air-blast sprayer. Application days were 3-17 (5% bloom), 3-23 (50% bloom), 3-24 (80% bloom), and 3-31 and 4-6-21 (petal fall), however, timings differed among treatments depending on the registrants' protocols. Additionally, treatments were carefully mixed based on the provided protocols. Trees were inoculated with *E. amylovora* (10^6 cfu/ml) on 3-24, and disease was evaluated on 4-29-21 by counting the number of fire blight strikes per tree. All 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 2021. Ten fire blight samples were made available in 2021 for our annual resistance monitoring in *E. amylovora.* All strains were determined to be sensitive to STR, OXY, and kasugamycin. Unfortunately, the low number of samples obtained this year is not representative, and this may have been due unfavorable environmental conditions in orchards. Low rainfall and drought during the spring of 2021 contributed to low disease incidence. Furthermore, locations in Sacramento Co. where we found strains with high resistance to both OXY and STR in 2018 to 2020 were not re-sampled, and no information on the persistence of these strains could be obtained. Because 2021 was generally a low-disease year for fire blight, reduced selection pressures (i.e., applications with OXY and STR) may have resulted in the decline of these isolates. Thus, sampling in 2022 will be important to learn more about antibiotic resistance in *E. amylovora*.

Table 1. Sensitivity of *E. amylovora* strains from pear orchards in California to streptomycin, oxytetracycline, and kasugamycin in 2021

Orchard or block (No. isolates)	County	Minimum inhibitory concentration (ppm)		
		Streptomycin	Oxytetracycline	Kasugamycin
1 (1)	Yuba	0.412	0.274	14.58
2 (1)	Yuba	0.387	0.242	13.75
3 (1)	Yuba	0.412	0.258	11.55
4 (1)	Yuba	0.353	0.310	12.24
5 (1)	Yuba	0.425	0.310	7.68
6 (1)	Yuba	0.399	0.228	15.45
7 (1)	Lake	0.563	0.291	20.67
8 (1)	Lake	0.467	0.242	15.90
9 (2)	Lake	0.375	0.208	18.40

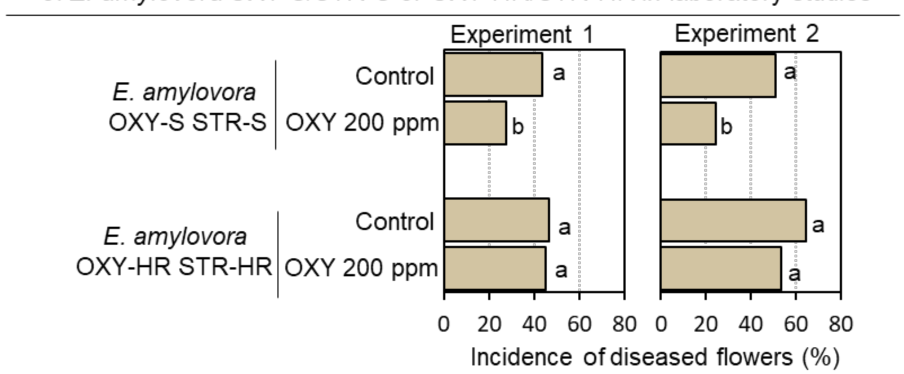
Sensitivity to streptomycin, oxytetracycline, and kasugamycin was determined using the spiral gradient endpoint method. All isolates were considered sensitive to the three antibiotics.

We are currently characterizing resistance mechanisms in the high OXY-STR-resistant strains. PCR amplifications with published primers for STR resistance indicate that a different mechanism is present in these strains. Additionally, a DNA fragment with 98% homology to a *tetA* or *tetB* gene from *Pantoea agglomerans* and other members of the family Enterobacteriacae was amplified in our resistant isolates indicating that OXY resistance in *E. amylovora* is due to the presence of a previously described *tet* gene. Long-read and short-read whole-genome sequencing demonstrated the acquisition of both the *tet, strA,* and *strB* genes into the novel conjugative IncX plasmid pX11-7.

Our previous detections of STR and OXY resistance and the need to protect the efficacy of kasugamycin indicates that the importance of resistance management cannot be overstressed. Antibiotics should only be used in mixtures or rotations, the number of applications of each per season should be limited to two, and new alternatives (e.g., early season copper, biologicals) should be used and developed.

Evaluation of OXY efficacy against fire blight caused by STR-OXY double-resistant strains of *E. amylovora*. In both experiments conducted, the incidence of diseased flowers was significantly reduced in comparison to the control after treatment with OXY and inoculation with a strain of *E. amylovora* sensitive to both antibiotics (Fig. 1). When a double-resistant strain was used for inoculation, however, disease incidence was not reduced. This indicates that disease in the field caused by these resistant strains will not be controlled by OXY applications, and likely also not by a mixture of OXY and STR. Therefore, field resistance may have developed at these locations if the majority of *E. amylovora* strains in the population is resistant.

Fig. 1. Efficacy of oxytetracycline treatments against fire blight caused strains of *E. amylovora* OXY-S/STR-S or OXY-HR/STR-HR in laboratory studies

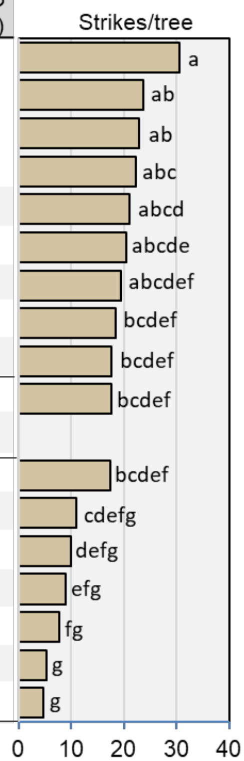


Twigs with pear flowers were placed in beakers with water on a light bench in the laboratory. Flowers were spray-treated and inoculated after 4-5 h with strains of *E. amylovora* either sensitive to oxytetracycline and streptomycin (OXY-S STR-S) or highly resistant to both antibiotics (OXY-HR STR-HR) (10^7 cfu/ml). Disease was evaluated after 7 days.

Field studies on the evaluation of new bactericide treatments. In a trial in a commercial Bartlett orchard, an average of 30.6 fire blight strikes were observed on untreated control trees. In the two UC Davis inoculation studies, 43.8% of flower/fruitlet clusters developed disease on Comice pear, but only an average of 4 clusters with disease per tree were present on Shinko apple pear. Apple pears are highly susceptible to fire blight, however, low temperatures during bloom (which was earlier than for Comice pear) and at the time of inoculation apparently prevented a high level of disease from developing.

Fig. 2. Efficacy of bactericides for management of fire blight of Bartlett pears, Live Oak, 2021

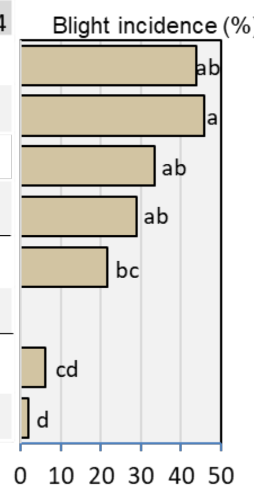
	Treatment	Rate/A	3-23 WT (5%)	3-31 FB (80%)	4-7 (PF)	4-15 (PF)
	Control	---	---	---	---	---
	Nisin + Dart	13 + 24	@	@	@	@
	Cueva + Double Nickel	64 + 128	@	@	@	@
	TDA -NC-1 + NuFilm 17	20 + 8 fl oz	@	@	@	@
	Nisin + ManniPlex	13 + 32	@	@	@	@
	EPL + Dart	13 + 24	@	@	@	@
	EPL + ManniPlex	13 + 32	@	@	@	@
	Mycoshield + LI-700	16 + 32	@	@	@	@
	Ninja (SP2700) + Dart	16 + 24	@	@	@	@
Rotation	Blossom Protect + Buffer	20 + 80	@	@	---	---
	Serenade Opti	96 oz	---	---	@	@
	QAM + Dart	38 oz + 24 fl oz	---	@	@	@
	Kasumin 2L	64	@	@	@	@
	NUP-17010 + LI-700	8 + 32	@	@	@	@
	Kasumin 2L + Syllit	64 + 48	@	@	@	@
	Kasumin 2L + FireWall	64 + 16	@	@	@	@
	NUP-17010	8	@	@	@	@
	New FireLine + Dart	8 + 24	@	@	@	@



Treatments were applied using an air-blast sprayer.
Disease was evaluated on 4-21-21.

Fig. 3. Efficacy of new bactericides for management of fire blight of Comice pear in a field study at UC Davis 2021

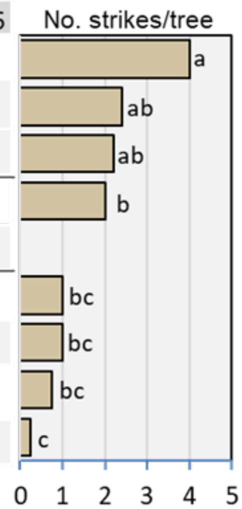
	Treatment	Rate/100 gal	4-1	4-6	4-14
	Control	---	---	---	
	RejuAgro A + Regulaid	20 gal + 16 fl oz	@	@	---*
	Aqua-Clear 7.5 (chlorine dioxide)	40 ppm	@	@	@
	SP2700 (Ninja) + LI700	16 oz + 8 fl oz	@	@	@
Rotation	Blossom Protect + Buffer	20 oz + 80 oz	@	@	---
	Serenade ASO	96 fl oz	---	---	@
	Alum + Regulaid	128 oz + 16 fl oz	@	@	@
	Firewall 17 + LI700	16 oz + 8 fl oz	@	@	@



Treatments were applied using an air-blast sprayer starting at full bloom. Trees were inoculated with *E. amylovora* (5 x 10^6 cfu/ml) after the third application. Disease was evaluated on 4-20-21. Blight incidence is based on the number of flower/fruit clusters with disease of the total number of clusters evaluated.

Fig. 4. Efficacy of new bactericides for management of fire blight of Shinko apple pears in a field study at UC Davis 2021 as part of an IR-4 efficacy study

	Treatment	Rate/100 gal	3-17	3-23	3-24	3-31	4-6
	Control	---	---			---	
	SP2700 (Ninja) + LI700	16 oz + 16 fl oz	@	@**	---	@	@
	Alum + Regulaid	128 oz + 16 fl oz	---	@**	@***	@	---
Rotation	Blossom Protect + Buffer	20 oz + 80 oz	@	@	---		---
Rotation	Serenade ASO	96 fl oz	---	---	---	@	---
	TDA-NC-1 + NuFilm 17	20 oz + 8 fl oz	@	@	---	@	@
	RejuAgro A	20 gal	---	@**	@***	---	---
	ET-91 + LI700	320 + 8 fl oz	---	@**	@***	@	---
	FireWall 17	200 ppm - 16 oz	@	@	---	@	---



Treatments were applied using an air-blast sprayer on 3-17 (5% bloom), 3-23 (50% bloom), 3-24 (80% bloom), and 3-31 and 4-6-21 (petal fall). A non-ionic surfactant was added to the last application of FireWall. Trees were inoculated with *E. amylovora* (10^6 cfu/ml) on 3-24. Disease was evaluated on 4-29-21.

In contrast to previous years, the **<u>food preservatives</u>** ε-poly-L-lysine and nisin that were applied in the Bartlett orchard did not significantly reduce the amount of disease as compared to the control (Fig. 2). ManniPlex Zn or Dart were used as additives, but they did not help to increase efficacy. Because these exempt from tolerance compounds previously showed good field and in vitro activity and because of their favorable safety characteristics, we are moving forward in designing agricultural formulations in collaboration with a chemical company that is interested in being their registrant.

Among other **new bactericides** evaluated, the **<u>FDA GRAS bactericide</u>** TDA-NC1 significantly reduced the disease in the inoculated apple pear (Fig. 4), but not the natural incidence in the Bartlett pear orchard (Fig. 2). This compound completely inhibited in vitro growth after *E. amylovora* was exposed at 500 ppm for 60 min (Table 2). In three field studies, a rotation of the **<u>biocontrols</u>** Blossom Protect and Serenade showed moderate efficacy in reducing blight after inoculation or occurring naturally (Figs. 2,3,4). This supports their use in organic pear production. The biocontrol RejuAgro A (a *Pseudomonas* sp.) was very effective in the apple pear study (Fig. 4) with low disease pressure, but not in the Comice pear study (Fig. 3) where more disease developed in the untreated control. A mixture of the biocontrol Double Nickel with Cueva also did not reduce the amount of disease as compared to the control (study on Bartlett pear, Fig. 2).

The **<u>essential oil product</u>** ET-91 was very effective in the inoculation study on apple pear with low disease pressure (Fig. 4) but was not toxic at 5000 ppm in in vitro studies (Table 2). **Aluminum sulfate** (Alum) was highly effective in the Comice pear (Fig. 3), but not in the apple pear trial (Fig. 4). The new **natural antibiotic ningnanmycin** (Ninja) significantly (Fig. 2) or only numerically (Figs. 3,4) reduced the amount of disease. A minimum inhibitory

Table 2. Summary of in vitro toxicity studies with *E. amylovora* using new antimicrobial compounds

Compound	Type of compound	Inhibition of growth	Assay used
BacStop	Essential oils	No inhibition at 1000 ppm	Direct exposure 30 min
Capric/caprylic acids (Dart)	Natural acids	MIC ca. 1200 ppm	Spiral gradient endpoint assay
ET-91	Essential oils	No inhibition at 5000 ppm	Agar dilution method
Ningnanmycin (Ninja)	Natural antibiotic of *Streptomyces noursei* var. *xichangensis*	MIC = 24.8 ppm	Spiral gradient endpoint assay
QAM	Agave extract	some inhibition at 500 ppm, no growth at 1000 ppm	Agar dilution method
TDA-NC-1	Riboflavin	Complete inhibition at 500 ppm	Direct exposure 60 min
Nisin	Food additive	50% inhibition at 500 ppm	Direct exposure 30 min
Nisin + 100 ppm EDTA	Food additive	Complete inhibition at 500 ppm	Direct exposure 30 min
ε-poly-L-lysine	Food additive	40% inhibition at 500 ppm	Direct exposure 30 min
ε-poly-L-lysine + 500 ppm EDTA	Food additive	Complete inhibition at 500 ppm	Direct exposure 30 min

Sensitivities were determined using amended agar methods (spiral gradient endpoint or agar dilution methods) where the pathogen is exposed to the pathogen for the whole length of the experiment or direct exposure studies where the pathogen is exposed to the bactericide for a limited time period and is then transferred to non-amended growth media.

concentration (MIC) of 24.8 ppm was determined in amended agar studies for this compound (Table 2), which is in the range of kasugamycin. The **new experimental** plant extract QAM reduced the amount of disease on Bartlett pear by approximately half (Fig. 2), although a high MIC of 1000 ppm was determined in direct exposure studies (Table 2). The **chlorine dioxide** product Aqua-Clear 7.5 was not effective in the Comice pear study (Fig. 3). It was also difficult to apply, because the proper amount of chlorine dioxide had to be obtained, and chlorine dioxide fumes are highly toxic.

Kasumin continued to perform very well, and its efficacy was slightly (but not significantly) increased with the addition of Syllit or FireWall (Fig. 2). 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. New formulations of FireLine (in mixture with Dart) and Mycoshield (NUP-17010) provided the highest efficacy in the natural incidence trial on Bartlett pear, but there was no significant difference to the Kasumin treatments (Fig. 2).

In summary, none of the new experimental bactericides was as effective as the registered antibiotics. We will continue to evaluate ε-poly-L-lysine and nisin. New formulations will be made available to us by a collaborating agrochemical company in early 2022. We will perform small-scale inoculation studies with ornamental pears, and the most effective formulations will be used in field studies on European pears. We will continue to evaluate new experimental bactericides and biocontrols in future studies to potentially identify additional effective options for fire blight management and to provide rotation alternatives for effective anti-resistance programs.

Based on our in vitro studies this year (Table 2), pre-screening of new biological treatments for their activity against *E. amylovora* does not always correlate well with field efficacy. For example, ET-91 showed efficacy in a field study, but was not toxic in vitro at 5000 ppm; in contrast, ningnanmycin was relatively toxic in vitro with an MIC of 24.8 ppm, but efficacy in the field was relatively low. In addition to direct toxicity, other modes of action may be involved for these treatments, however, for some compounds improved formulations may improve efficacy. In the absence of year-round availability of pear flowers for which small-scale laboratory tests could be developed, in vitro assays are the only option to obtain toxicity information of new bactericides.