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Project Title: Epidemiology and management of olive knot caused by *Pseudomonas savastanoi* pv. *savastanoi*

Keywords: Bactericides, copper enhancing compounds, antimicrobial natural products, biological controls

JUSTIFICATION/ BACKGROUND

Olive knot caused by the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (*Psv*) is a serious disease of olives (*Olea europaea*) worldwide (8). The pathogen enters through wounds causing outgrowths (knots, tumors, galls) on branches and infrequently on leaves and fruit. Olive knot is one of the most economically important diseases of olives as infection may lead to tree defoliation, dieback, and reduced tree vigor, which ultimately lowers fruit yield and quality (6). *Psv* can survive epiphytically on olives but the main sources of inoculum are bacteria living within knots (7). Large quantities of bacterial ooze can be exuded upon wetting knots. This exudate is disseminated by rain, wind, insects, birds, as well as human activity. The opportunistic pathogen takes advantage of wounds caused by natural leaf abscission (4), frost, and hail, as well as cultural practices such as pruning and harvesting. These latter practices also lead to direct mechanical damage of the knots, exposing and spreading inoculum to healthy tissue. After entering its woody host, the pathogen actively induces knot formation through the production of indoleacetic acid (IAA) and cytokinins (2). In California, infections occur mostly during the rainy season (late fall, winter, and spring) but knots do not develop until new growth starts in the spring. Infections can occur at low temperatures (-5° C) and thus, wetness is the main limiting factor for the disease. None of the currently grown olive cultivars is resistant to the pathogen (5).

Management of olive knot is difficult, and growers rely on applications of copper-based bactericides as the only effective foliar treatment. Manual application of cresol- and xylenol-based compounds (Gallex) to knots can eliminate the knot pathogen but is unfeasible on a commercial scale. Copper has been extensively used in olive production for many years for the control of diseases such as peacock spot and olive knot. Reliance on a single active ingredient has led to our detection of copper resistance in *Psv* strains from a commercial olive orchard. The incidence of copper resistance is currently very low, accounting for only 2% of the total strains collected in different olive growing regions of California. When resistant strains were inoculated to Arbequina and Manzanillo olive wounds, application of copper provided reduced or no control as compared to inoculation with a sensitive strain. Copper-resistant strains caused less disease on leaf scars as compared to Cu-sensitive strains, but still resulted in a high incidence of disease over a range of inoculum concentrations. Therefore, there is a potential risk of copper resistance spreading with continued and sole use of copper. This necessitates the development of new bactericides or copper-activity-enhancing materials to overcome resistance. The latter strategy has proven to be effective for walnut blight management where copper resistance in *Xanthomonas arboricola* pv. *juglandis* is common and copper-mancozeb mixtures have provided exceptional control. Mancozeb can no longer be registered on new crops but other copper-enhancing alternatives can be evaluated. Salicylidene benzoylhydrazone (SBH) was recently discovered to display synergism when combined with copper against *Alternaria solani* causing early blight of tomatoes. We performed preliminary tests with a derivative of this molecule with promising results with several genera of phytopathogenic bacteria including *Psv*. Low concentrations of metallic copper combined with SBH were highly inhibitory in vitro against a copper-resistant *Psv* strain while copper or SBH by themselves at the same concentrations were not effective. Field trials in 2017 on managing olive knot, SBH-copper, however, did not improve performance of copper. Other derivatives of SBH will be supplied by Dow AgroSciences, and these will be tested in 2018.

Other potential bactericides have also been made available to us by agrochemical registrants in 2017. These include a nanoparticle zinc product called Zinkicide and experimental inhibitors of type III secretion systems in plant pathogenic bacteria. The latter compounds are novel in their mode of action. They act on the mechanism that delivers bacterial proteins into the host cells that are necessary for *Pseudomonas* species to cause disease. Currently, we are testing Zinkicide and three experimental type III secretion system inhibitors.

We have been instrumental in the development of the new agricultural antibiotic kasugamycin (commercial name Kasumin) for several bacterial diseases of agronomic crops in the United States. Kasugamycin has high activity against *Erwinia* (1) and *Pseudomonas* species and moderate activity against *Xanthomonas* species and other plant pathogenic bacteria. We found it to be the most promising new treatment for preventing olive knot in our extensive field studies, including in a commercial application to inoculated branches. Kasugamycin is currently federally registered and in 2018 received California registration on pome fruit, cherry, and walnut crops, whereas registration on olives, peaches, and almonds is pending for late 2019. Kasumin – olive is still in the IR-4 program with the final report and submission to the EPA pending in fourth quarter 2018. Kasugamycin would greatly complement current copper sprays and could be used in rotation or mixtures with copper. Oxytetracycline was also submitted to IR-4 and is in the field trial phase of the IR-4 program for establishing tolerances. We will conduct additional studies with oxytetracycline to potentially improve its efficacy by using registrant-recommended adjuvants. New antibiotic registrations, however, find little acceptance with regulatory agencies, and we are currently in discussion with EPA to develop a science-based approach on the use of antibiotics in plant agriculture.

In addition to developing conventional chemical compounds, research on alternative materials such as biopesticides and food additives may provide new modes of action for managing olive knot. Biopesticides such as Serenade contain the gram-positive bacterium *Bacillus subtilis* (strain QST 713) that produces various compounds that are antagonistic against a broad range of fungal and bacterial organisms. In our efficacy trials, Serenade and Serenade-copper mixtures, however, were not effective at recommended rates.

Table 1. Chemicals used for evaluation as potential bactericides against olive knot

Treatments

No.	***Treatment***	***Tradename/experimental name***	***Chemical class/use***
1	Lactic acid	Lactic acid	Organic acid
2	Citric acid	Citric acid	Organic acid
3	Zinc	Zinkicide	Zinc nanoparticle
4	Zinc thiadiazole	ZTD	Zinc thiadiazole
5	Oxytetracycline	Fireline	Antibiotic (tetracycline)
6	Kasugamycin	Kasumin	Antibiotic (aminoglycoside)
7	Copper hydroxide	ChampION++	Copper
8	TS-28 10% SC 5 mM	TS-28	Type III secretion system inhibitor
9	TS-108 20% SC 5 mM	TS-108	Type III secretion system inhibitor
10	TS-153 10% SC 5 mM	TS-153	Type III secretion system inhibitor
11	Nisin	Niprosin	Food preservative
12	Epsilon-poly-L-lysine	e-Polylysine	Food preservative
13	Nisin Alginate	Nisin Alginate	Food preservative
14	Epsilon-poly-L-lysine Alginate	e-poly-L-lysine Alginate	Food preservative
15	DAS1	DAS1	Derivative of salicylidene benzoylhydrazone

Adjuvants

No.	***Treatment***	***Tradename***	***Chemical/use***
A	Adjuvant	Tactic	Surfactant/sticking agent
B	Adjuvant	Nu-Film P	Spreader/sticker agent
C	Adjuvant	Regulaid	Nonionic surfactant
D	UV stabilizer	---	Zinc oxide

Several food additives that are considered ‘generally recognized as safe’ (GRAS) have antimicrobial properties. They are often naturally produced molecules of gram-positive *Streptomyces* species. Although these compounds are typically applied to food products as preservatives, they may have potential for controlling plant diseases when applied as a foliar treatment. Integration of these alternative materials with conventional treatments may improve disease control, reduce the risk of resistance development, and provide olive growers with more resources for managing olive knot. In 2017, we evaluated nisin, Ɛ-poly-L-lysine, and lactic acid and all showed similar efficacy to copper in reducing olive knot on leaf scars, but not on lateral wounds. This information is still valuable because rotational programs could be developed with different modes of actions for different phases of the disease, i.e., leaf scars or lateral wounds occurring during leaf drop or harvest and pruning, respectively. These materials are registerable for conventional and possibly organic treatments.

RESEARCH OBJECTIVES

1) **Develop new bactericides and potential enhancers of copper activity against *Psv***
   a) In-vitro sensitivity of *Psv* to Zinkicide, Type III secretion system inhibitors, and copper mixtures with new SBH derivatives (using selected ratios).
   b) Efficacy of new bactericides in comparison with kasugamycin for the management of olive knot caused by copper-sensitive and -resistant strains of *Psv* in field studies.
      i) Zinkicide
      ii) Potential enhancers of copper activity - new SBH derivatives.
      iii) Type III secretion system inhibitors
      iv) Oxytetracycline formulations in combination with adjuvants recommended by the registrant.
2) **Evaluate several food additives and a sanitizer for the control of olive knot**
   a) Determine the efficacy of the GRAS food additives nisin, Ɛ-poly-L-lysine, and the GRAS sanitizers lactic and citric acid in field studies for the management of olive knot.
3) **Continue to support the registration of the antibiotics kasugamycin and oxytetracycline -** UV blockers and stabilizers and EPA policy**.**

PLANS AND PROCEDURES

1) Develop new bactericides and potential enhancers of copper activity against *Psv*.

1a. In vitro sensitivity of two *Psv* strains against selected food additives and ZTD. The toxicity of nisin, Ɛ-poly-L-lysine, and a zinc thiadiazole (ZTD) to a copper-sensitive and a -resistant strain of *Psv* was evaluated in a direct exposure assay. Bacterial suspensions were mixed with solutions of each of the toxicant as selected concentrations and incubated for 60 s. For the control, water was used instead of the toxicant. Aliquots were then diluted 1:100 with sterile distilled water, and plated onto King's medium B. The number of colonies on each plate were enumerated after 2 days of growth.

1b. Evaluation of bactericides and experimental treatments in field studies. Zinkicide, copper-SBH mixtures, Type III secretion system inhibitors, oxytetracycline, and other treatments (Table 1) were tested in the field on Arbequina and Manzanillo olives at UC Davis or in a commercial planting. Lateral wounds on 1-2-year-old twigs were made using a scalpel and removing the bark to expose cambial tissue. Leaf scars were made by pulling leaves off the same twigs. In addition, wounds from natural leaf drop were used in some studies. Treatments were sprayed onto wounds before inoculation with a suspension of copper-sensitive or -resistant *Psv* strains. Oxytetracycline was used in combination with recommended adjuvants because it is especially vulnerable to UV-degradation. Treatments were compared to Kasumin and copper. The efficacy of treatments was assessed as the percent incidence of knots forming on treated, inoculated wounds as compared to wounds that were treated with water and inoculated (i.e., controls).

2) Evaluate several food additives for the control of olive knot. Field tests were conducted on Arbequina and Manzanillo olives to evaluate the efficacy of nisin, Ɛ-poly-L-lysine, and lactic acid against *Psv*. The same wounding, treatment application, inoculation, and evaluation procedures were used as described above.

3) Continue to support the registration of the antibiotics kasugamycin and oxytetracycline. An inter-commodity and industry group continued to work with the Minor Crop Farmer Alliance to recommend an EPA policy change towards the use of antibiotics in plant agriculture. Specifically, a suggested new internal EPA Guidance Document (GD) for use of antibiotics in plant agriculture was developed based on science and submitted to EPA. Historically, EPA GD 152 for registration of antibiotics in animal husbandry is used for all requests in agriculture. Additionally, we will continue to work with a USDA working group to address CODEX initiatives for establishing policies on all antibiotic use in agriculture including animal and plant uses.

RESULTS AND DISCUSSION

1a. In vitro sensitivity of two *Psv* strains against selected food additives and ZTD.
Direct contact assays demonstrated a high in vitro toxicity of Ɛ-poly-L-lysine against copper-sensitive and -resistant strains of *Psv* (Fig. 1). Incubation in 10 ppm Ɛ-poly-L-lysine for 60 sec completely inhibited growth of both strains. In contrast, a concentration of 1000 ppm of nisin was required to completely inhibit growth of the sensitive strain, and growth of the resistant strain was reduced by 2.5 log. ZTD was not effective in these studies. Thus, Ɛ-poly-L-lysine and possibly nisin were identified as the most promising treatments for managing olive knot. These compounds are antimicrobials for processed food use and have not been formulated for agricultural use.

1b and 2). Evaluation of bactericides, food additives, and other experimental treatments in field studies.
In contrast to the high toxicity of **Ɛ-poly-L-lysine** in in vitro studies, this compound showed only low to no activity in field studies on the management of olive knot (Fig. 2A, B). An attempt was made to make the compound more persistent, and we prepared an alginate formulation that was described in the literature to provide environmental stability. Additionally, we added zinc oxide to this formulation to increase UV stability. Still, this formulation was also not effective (Figs. 3, 4). Thus, in our future studies we will explore other methods to make Ɛ-poly-L-lysine more effective in the field. The other food preservative, **Nisin**, did not show efficacy in reducing knot development (Fig. 2). The nisin-alginate-zinc oxide formulation sometimes improved efficacy (Fig. 4), but not at other times (Figs. 3, 4). The third food preservative, **lactic acid**, was tested only in one trial, and a moderate reduction in knot development was observed (Fig. 2A, B). Thus, this compound deserves further evaluation, possibly including it in mixtures. **Citric acid**, however, was not effective (Fig. 2A, B).

Three **type III secretion system inhibitors** were evaluated in four studies using copper-sensitive and/or -resistant strains of the pathogen (Figs. 3, 4, 5, 6). TS-108 and TS-153 were moderately effective in one study on Manzanillo olive (Fig 6A), TS-108 and TS-153 were moderately effective in another study (Fig. 5), but in other cases, they were not effective. These inhibitors were provided to us by other researchers, and possibly other inhibitor formulations could be more effective.

The **zinc nanoparticle product Zinkicide** was evaluated in two studies and showed good to very good efficacy on lateral wounds (Figs. 5, 6). This product, however, has currently no registrant that is willing to cover the expenses for a registration. Furthermore, nanoparticle products, may be difficult to obtain EPA approval due to the number of toxicity and environmental testing required. **Zinc thiadiazole** was not effective in reducing olive knot development (Figs. 3, 4, 5, 6) and also did not improve copper activity (Figs. 3, 4) in preventing or reducing olive knot.

The **antibiotic oxytetracycline (FireLine)** was evaluated by itself or in combination with the recommended adjuvant Tactic. Results were variable with no efficacy when used by itself (Fig. 2A, B) or with Tactic (Figs. 2B, 3) or resulting in good efficacy using Tactic on lateral wounds (Figs. 2A, 4). Thus, this antibiotic still has potential considering its high in vitro toxicity. The other antibiotic, **kasugamycin (Kasumin)**, was very effective (Figs. 2A, B, 6A) or highly effective (Fig. 5) on lateral wounds and sometimes also showed good efficacy on artificial leaf scar wounds (Fig. 5). Statistically, Kasumin was mostly similar in efficacy to copper. Adding adjuvants like Tactic (Figs. 2A, B) or Nufilm (Figs. 3, 4) did not improve efficacy of Kasumin. Copper (ChampION) was highly effective (Figs. 2A, 3, 4, 5, 6) on lateral and leaf scar wounds when used by itself, but efficacy was reduced when inoculations were done with a copper-resistant strain of *Psv* (Fig. 2B) The addition of **DAS-1, a potential enhancer of copper activity**, did not improve the performance of copper (Figs. 3, 4). **Copper-Kasumin mixtures** were similarly effective as either compound by itself or more effective than either copper alone (Figs. 3, 4). This mixture will provide an excellent strategy for the most effective olive knot management and to prevent or delay the spread of resistance against each component.

One study was done treating inoculated natural leaf scars with ChampION, Kasumin, or a ChampION-Kasumin mixture. In contrast to the studies discussed above with artificial leaf scar injuries where leaves were detached by pulling off, the three treatments were highly effective in protecting natural leaf scars from infection by a copper-resistant strain, and Kasumin completely prevented knot development (Fig. 7). Although these natural leaf scar assays are very laborious to perform (every leaf scar has to be inoculated and treated separately in 20 to 25 replications), these assays need to be repeated and also be used for some of the other treatments under evaluation (e.g., Ɛ-poly-L-lysine, nisin, oxytetracycline).

3) Continue to support the registration of the antibiotics kasugamycin and oxytetracycline.
Kasugamycin was first federally registered on pome fruits, and in 2018, it received California registration on pome fruit, cherry, and walnut crops. Registration on olives, peaches, and almonds is pending for late 2019. The petition for Kasumin on olive was still in the IR-4 program this summer and the final report and submission to the EPA are pending in fourth quarter of 2018. This was due to complications with extractions of kasugamycin from large amounts of oil in some olive samples that caused delays. Working with IR-4 and Arysta, we had additional procedures for alternative extraction procedures sent to the GLP residue lab to resolve the difficulties.

I also attended the IR-4 meeting and met with EPA representatives. I re-emphasized the need for a separate guidance document to GD152 that is specific for registration of bactericides for plant agriculture. Currently, GD152 is for all agriculture, but more importantly, was developed for registration of antibiotics for animal agriculture by USDA and FDA. With the extensive change-over in personnel at EPA, these meetings are critical in moving forward on these issues.

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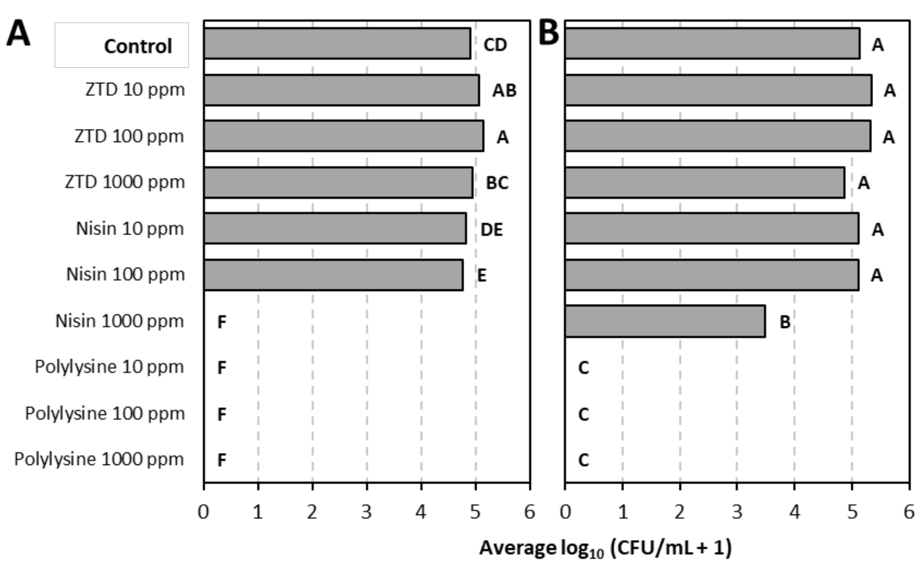

Fig. 1. In-vitro toxicity of selected antimicrobials against *Pseudomonas savastanoi* pv. *savastanoi* strain **(A)** O1-26 (copper-sensitive) or **(B)** O1-113 (copper-resistant), in a direct exposure assay. Bacterial suspensions were exposed to the antimicrobials for 60 s and then plated onto agar media.

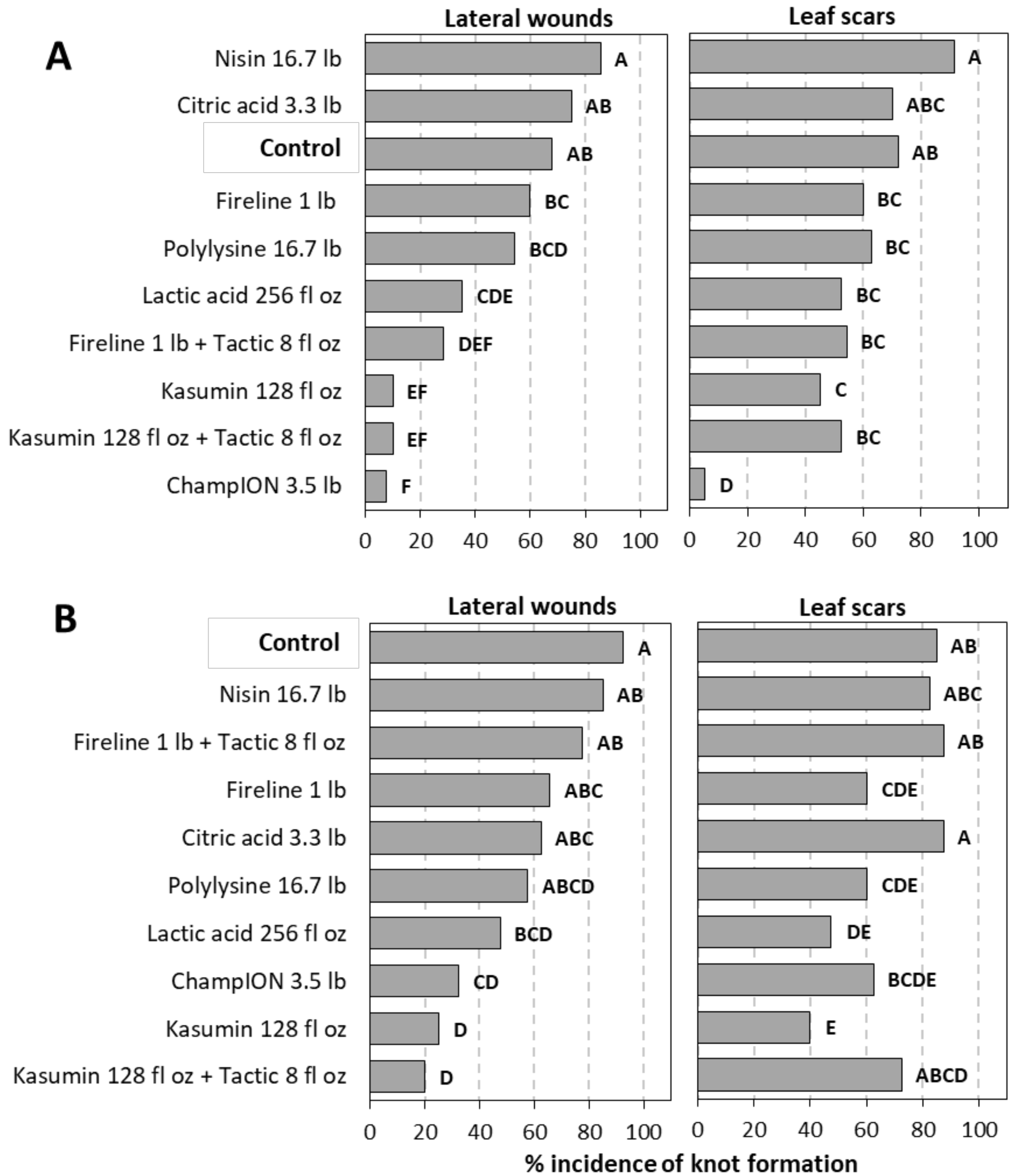

Fig. 2. Field trials on the management of olive knot initiated fall on 'Manzanillo' olives at UC Davis. Treatments were spray-applied to wounds until runoff and allowed to dry. Treatment rates are calculated per acre. Wounds were then spray-inoculated with a **(A)** copper-sensitive, or **(B)** copper-resistant *Pseudomonas savastanoi* pv. *savastanoi* strain.

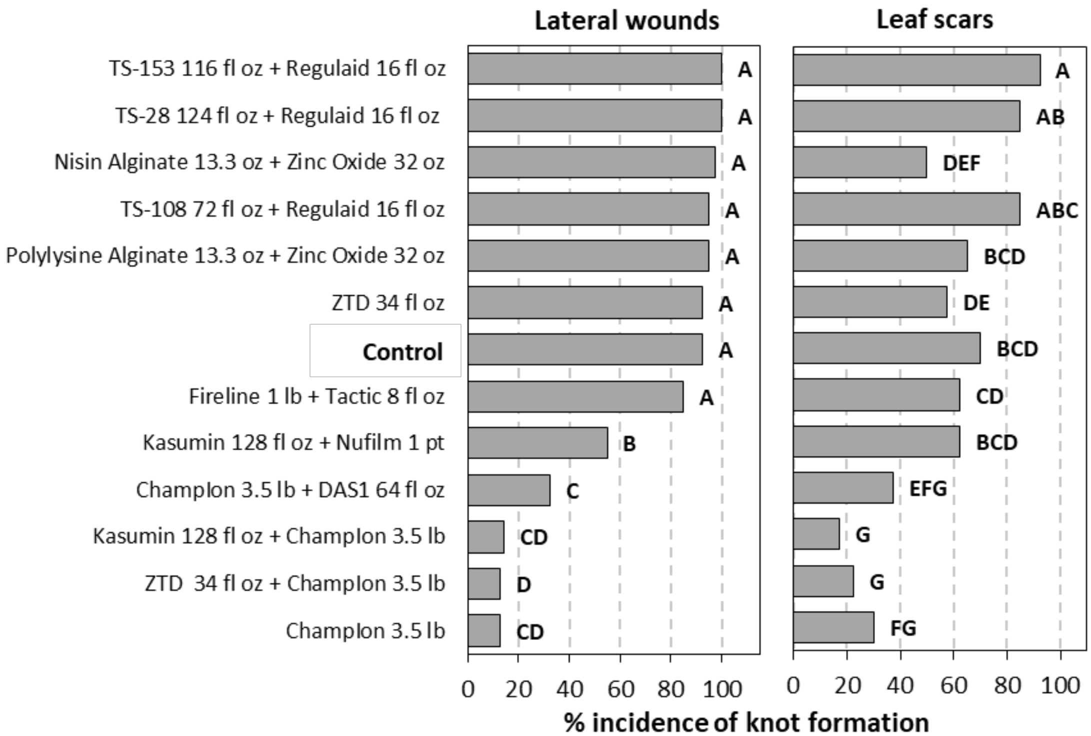

Fig. 3. Field trials on the management of olive knot of 'Manzanillo' olives initiated spring 2018 at UC Davis. Treatments were spray-applied to wounds until runoff and allowed to dry. Treatment rates are calculated per acre. Wounds were then inoculated with a copper-sensitive *Pseudomonas savastanoi* pv. *savastanoi* strain (O1-26).

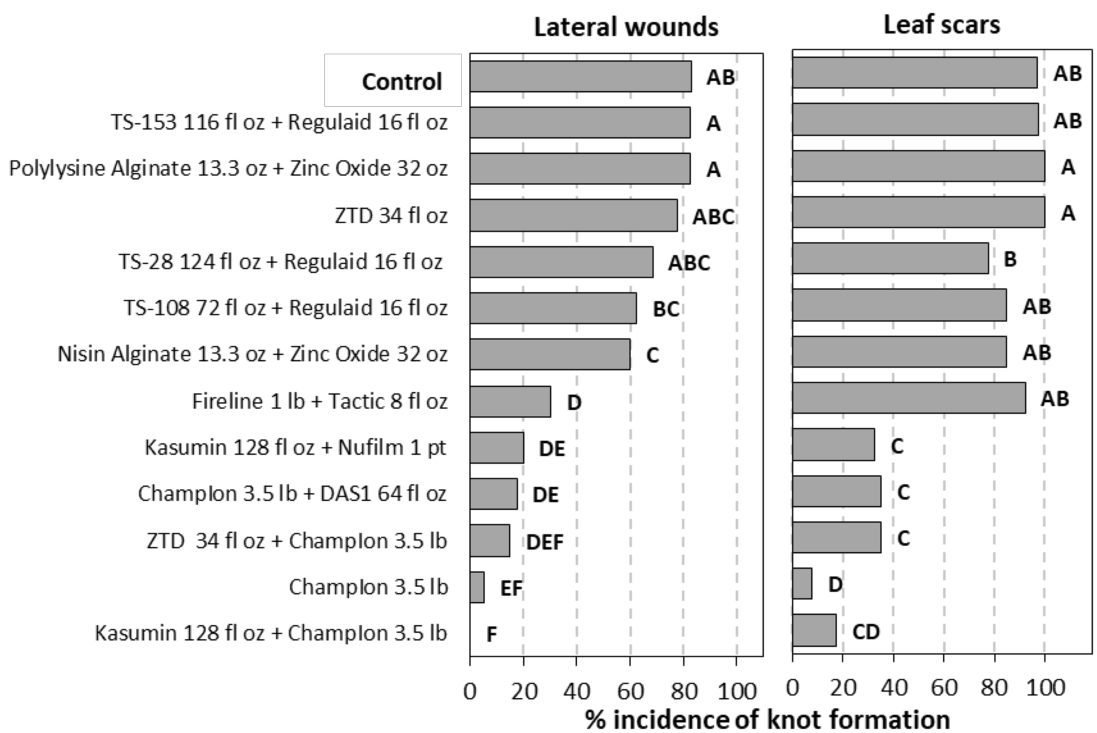

Fig. 4. Field trials on the management of olive knot initiated spring 2018 in a commercial 'Arbequina' orchard. Treatments were spray-applied to wounds until runoff and allowed to dry. Treatment rates are calculated per acre. Wounds were then inoculated with a copper-sensitive *Pseudomonas savastanoi* pv. *savastanoi* strain (O1-26).

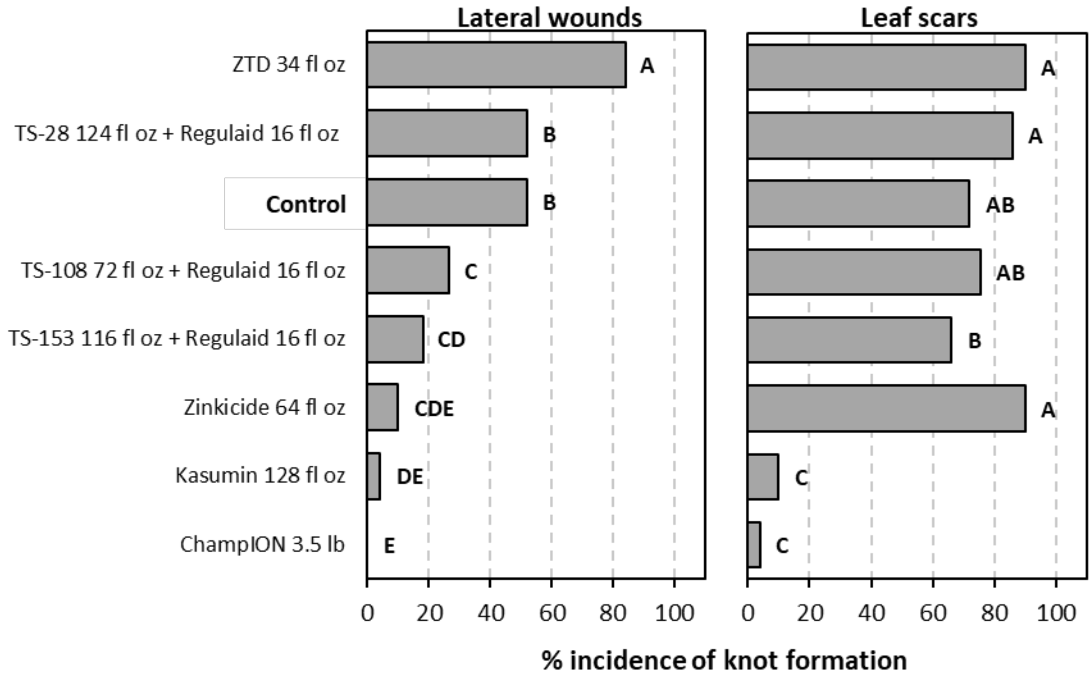

Fig. 5. Field trials on the management of olive knot initiated fall 2017 on 'Arbequina' olives at UC Davis. Treatments were spray-applied to wounds until runoff and allowed to dry. Treatment rates are calculated per acre. Wounds were then spray-inoculated with a copper-sensitive (O1-23) of *Pseudomonas savastanoi* pv. *savastanoi* strain.

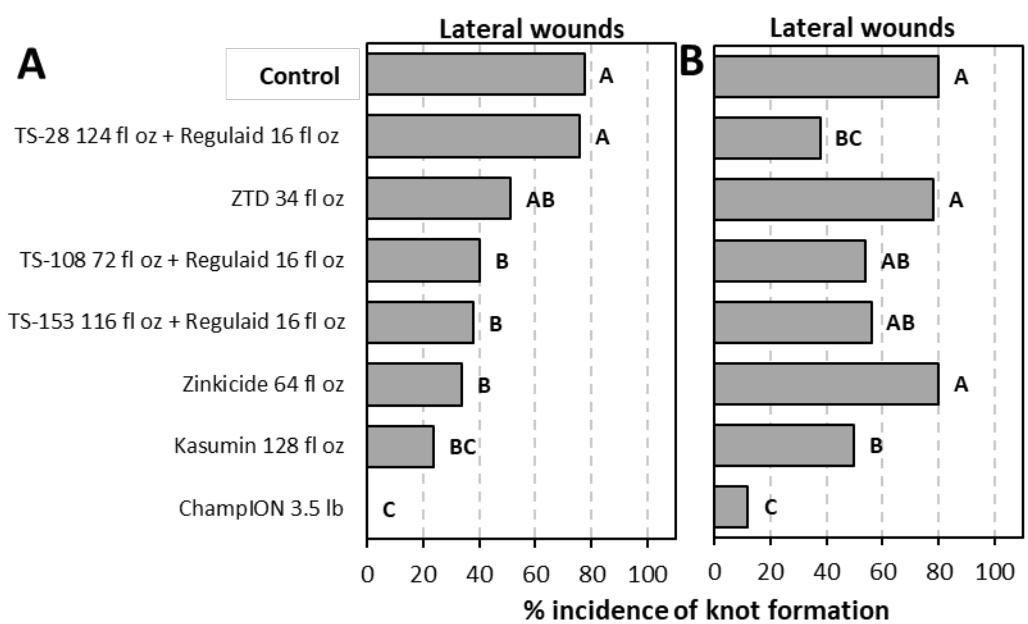

Fig. 6. Field trials on the management of olive knot of 'Manzanillo' olives initiated fall 2018 at UC Davis. Treatments were spray-applied to wounds until runoff and allowed to dry. Wounds were then inoculated with a **(A)** copper-sensitive (O1-23), or **(B)** copper-resistant *Pseudomonas savastanoi* pv. *savastanoi* strain (O1-113).

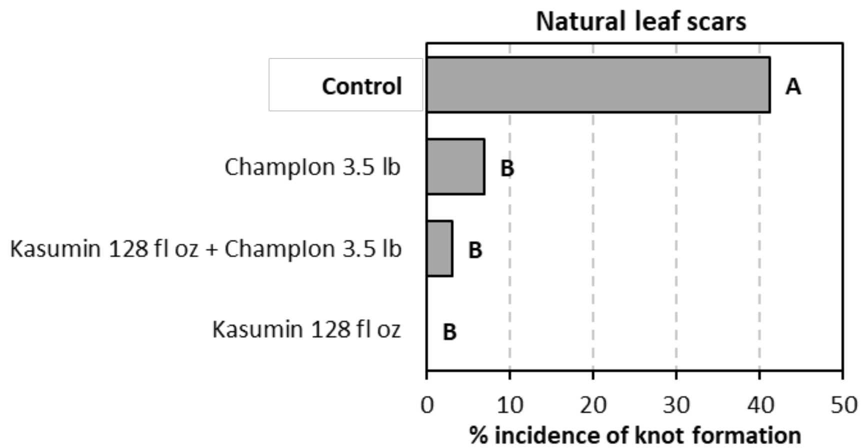

Fig. 7. Field trial on the management of olive knot initiated May 2018 on 'Arbequina' olive at UC Davis. Natural leaf scars were made by removing yellow leaves that readily abscised when gently pulled. Treatments were spray-applied until runoff, allowed to dry, and spray-inoculated with a copper-resistant *Pseudomonas savastanoi* pv. *savastanoi* strain (O1-113). Leaf scars were evaluated for knot development after approximately 3 months.