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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

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JUSTIFICATION/ BACKGROUND

Olive knot caused by the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (*Psv*) is one of the most economically important diseases of olives worldwide (8). The pathogen enters through wounds causing outgrowths (knots, tumors, galls) on branches and sometimes on leaves and fruit. Infection may lead to tree defoliation, dieback, and reduced tree vigor, ultimately lowering 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 that 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 induces knot formation by 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. Manual application of cresol- and xylenol-based compounds (Gallex) to knots can eliminate the knot pathogen but is unfeasible on a commercial scale. Growers rely on applications of copper-based bactericides as the only effective foliar treatment. Copper has been extensively used in olive production for many years for the control of peacock spot and olive knot. Reliance on a single active ingredient has led to our detection of copper resistance in *Psv* strains in a commercial olive orchard. The incidence of resistance is currently 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, copper provided reduced or no control as compared to inoculation with a sensitive strain. Copper-resistant strains showed reduced virulence, but still resulted in a high incidence of disease over a range of inoculum concentrations. Therefore, there is a risk of copper resistance spread with continued and sole use of copper. This necessitates the development of new bactericides.

Numerous potential bactericides, including biological products, have been evaluated by us during this project. The most consistently highly effective compound in our extensive field studies has been the new agricultural antibiotic kasugamycin (Kasumin). Kasugamycin is currently federally registered and in 2018 received California registration on pome fruit, cherry, and walnut crops. Registration on peaches and almonds is pending for late 2019 or early 2020. The olive registration was delayed due to problems with IR-4 residue studies, but these will now be completed in 2019, and registration is expected for 2020/21. Oxytetracycline is also pursued for registration through the IR-4 program. Additional studies with oxytetracycline were conducted in 2019 to potentially improve its efficacy by using selected UV-protecting adjuvants. Antibiotics are currently the most effective alternative to copper. However, because new antibiotic registrations find little acceptance with regulatory agencies, we are in discussion with EPA to develop a science-based approach on the use of antibiotics in plant agriculture.

In an attempt to identify non-antibiotic, non-copper alternatives, the evaluation of antimicrobial food preservatives that are considered ‘generally recognized as safe’ (GRAS) was another focus in our 2019 field studies. We started evaluating nisin, ε-poly-L-lysine, and lactic acid in 2017 and demonstrated efficacy that, however, was not consistent among trials. In 2019, we set up studies to improve their efficacy by using them in mixtures with other bactericides or with additives that potentially could increase their activity and persistence. Treatments evaluated in 2019 are shown in Table 1. New potential enhancers of copper activity and type III secretion system inhibitors that are listed in our proposal were not made available to us.

Table 1. Chemicals used for evaluation as potential bactericides against olive knot in 2019

No.	*Treatment*	*Trade name*	*Chemical class/use*
1	Capric/caprilic acids	Dart	Antimicrobial, adjuvant
2	Copper hydroxide	ChampION++	Fungicide, bactericide
3	Ethylenediaminetetraacetic acid	---	Chelating agent
4	Extract of *Reynoutria sachalinensis*	Serenade	Natural product, antimicrobial
5	ε-poly-L-lysine	---	Food preservative
6	Kasugamycin	Kasumin	Antibiotic (aminoglycoside)
7	Lactic acid	---	Organic acid, antimicrobial
8	Nisin	Niprosin	Food preservative
9	Oxytetracycline	Fireline, Mycoshield	Antibiotic (tetracycline)
10	Zinc nitrate	Brandt Zinc nitrate	Antibacterial
11	Zinc oxide	Brandt Zinc oxide	UV stabilizer

RESEARCH OBJECTIVES

1) **Evaluate new bactericides, potential enhancers of copper activity, food additives, GRAS sanitizers, and other experimentals against *Psv***
   a) Laboratory in-vitro sensitivity studies: copper mixtures with new SBH derivatives as they become available; nisin, ε-poly-L-lysine, and the GRAS sanitizers lactic and citric acid alone or combination and in mixtures with selected adjuvants (see below).
   b) Field efficacy studies with new bactericides in comparison with kasugamycin for the management of olive knot caused by copper-sensitive and -resistant strains of *Psv*.
      i) Potential enhancers of copper activity - new SBH derivatives.
      ii) Type III secretion system inhibitors (as they become available)
      iii) Oxytetracycline formulations in combination with selected UV-protecting adjuvants.
      iv) Nisin and ε-poly-L-lysine alone, in combination with each other, or in mixtures with antimicrobial acids (e.g., lactic, citric, and other acids), chelators (e.g., EDTA), sodium diacetate, buffers (to neutralize acidic carbohydrates), as well as emulsifiers (e.g., dextran) and proprietary fatty acids.
2) **Continue to support the registration of the antibiotics kasugamycin and oxytetracycline**
   a) Administrative support to EPA and other regulatory agencies about registration concerns for kasugamycin and other bactericides
   b) Optimizing the efficacy of oxytetracycline under field conditions (UV blockers and stabilizers) as it goes through the registration process at IR-4.

PLANS AND PROCEDURES

1) Evaluate new bactericides against *Psv.*

1a. In vitro sensitivity of *Psv* against nisin and ε-poly-L-lysine. The toxicity against *Psv* was evaluated in a direct exposure assay. Bacterial suspensions were mixed with solutions of each toxicant at selected concentrations without or with the addition of EDTA and incubated for 30 min. 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 was enumerated after 2 days of growth.

1b. Evaluation of bactericides and experimental treatments in greenhouse and field studies. Treatments listed in Table 1 were tested by themselves and in selected mixtures in greenhouse studies at UC Riverside on Arbequina olives and in the field on Arbequina and Manzanillo olives at UC Davis. 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 one study. Treatments were sprayed onto wounds using a hand sprayer to run-off. After air-drying, wounds were inoculated with a suspension of copper-sensitive or -resistant *Psv* strains (2 x 10^7 cfu/ml) using a hand sprayer. Treatments were compared to Kasumin, copper, and Kasumin-copper mixtures. The efficacy of treatments was assessed after two to three months for greenhouse studies or after five to nine months for field studies and was expressed as incidence of knots forming on treated, inoculated wounds as compared to wounds that were treated with water and inoculated (i.e., controls).

RESULTS AND DISCUSSION

1a. In vitro sensitivity of *Psv* against nisin and ε-poly-L-lysine.

In direct contact assays, 30-min exposures of *Psv* to 500 ppm nisin or 100 ppm ε-poly-L-lysine were not inhibitory. When 500 ppm EDTA was added to these solutions, however, growth was completely inhibited. This demonstrated the potential use of EDTA in enhancing activity, and therefore, EDTA was added to selected treatments with nisin and ε-poly-L-lysine in the field studies.

1b. Evaluation of bactericides and experimental treatments in greenhouse and field studies.

In greenhouse studies, after inoculation with copper-sensitive or –resistant strains of *Psv*, mixtures of **nisin and ε-poly-L-lysine** were not effective on lateral and artificial leaf scar wounds, but when lactic acid was added, the triple-mix was one of the most effective treatments, sometimes surpassing the efficacy of copper (Figs. 1, 2). This treatment, however, was not effective in field studies (Fig. 4), likely because lower concentrations of the food preservatives (1,000 vs. 10,000 ppm) were used. Thus, the high-rate combination treatment will be evaluated again in the field in 2020. Ɛ-poly-L-lysine by itself showed good efficacy in some studies (Figs. 3, 5), but not in others (Fig. 5). In other treatments aimed to improve the efficacy of nisin and ε-poly-L-lysine, the addition, of EDTA (Figs. 1, 5), zinc oxide (Fig. 4), zinc oxide and EDTA (Fig. 4), or Dart (Figs. 3, 4) was not effective. In a study where natural leaf scars were treated and inoculated, however, nisin-EDTA-zinc oxide and ε-poly-L-lysine-Dart were effective (Fig. 6). These results indicate that the two food additives have potential to become treatments for olive knot management, but more studies are needed. We are currently working with a chemist of an agrochemical company that may help us to make the compounds more effective and persistent in the field. Because these treatments potentially could be approved for organic production, their continued evaluation is important.

Kasumin used by itself showed consistent good efficacy in protecting lateral and artificial leaf scar wounds (Figs. 1, 3, 4, 5), but efficacy was reduced when using a copper-resistant strain for inoculation (Figs. 2, 3). The addition of zinc oxide did not improve efficacy (Figs. 1, 2, 4, 5), but a mixture of Kasumin with ε-poly-L-lysine and Dart was highly effective in a greenhouse study (Fig. 3). The **Kasumin-copper mixture** was highly effective in studies with inoculations with a copper-sensitive strain (Figs. 1, 4), whereas copper was not effective when a resistant strain was used (Fig. 2). 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.

Oxytetracycline (Mycoshield, FireLine) was highly effective in greenhouse studies with artificial leaf scar wounds (Figs. 1B, 2B), but efficacy was not improved in trials with artificial lateral or leaf scar wounds when adding zinc oxide (Figs. 1, 4) or Dart (Fig. 4). The Mycoshield-Dart mixture, however, was highly effective in preventing knot development on natural leaf scars (Fig. 6). Thus, this antibiotic has potential considering its high in vitro toxicity, and we need to continue testing other mixture partners. The new ASO formulation of **Serenade** was not effective in two studies where it was included (Fig. 5).

3) Continue to support the registration of the antibiotics kasugamycin and oxytetracycline.

Kasugamycin received California registration on pome fruit, cherry, and walnut crops in 2018. Registration on peaches and almonds is pending for late 2019 or early 2020. The olive registration was delayed due to complications with extractions of kasugamycin from large amounts of oil in some olive samples but these will now be completed in 2019, and registration is expected for 2020/21. Oxytetracycline is pursued for registration through the IR-4 program.

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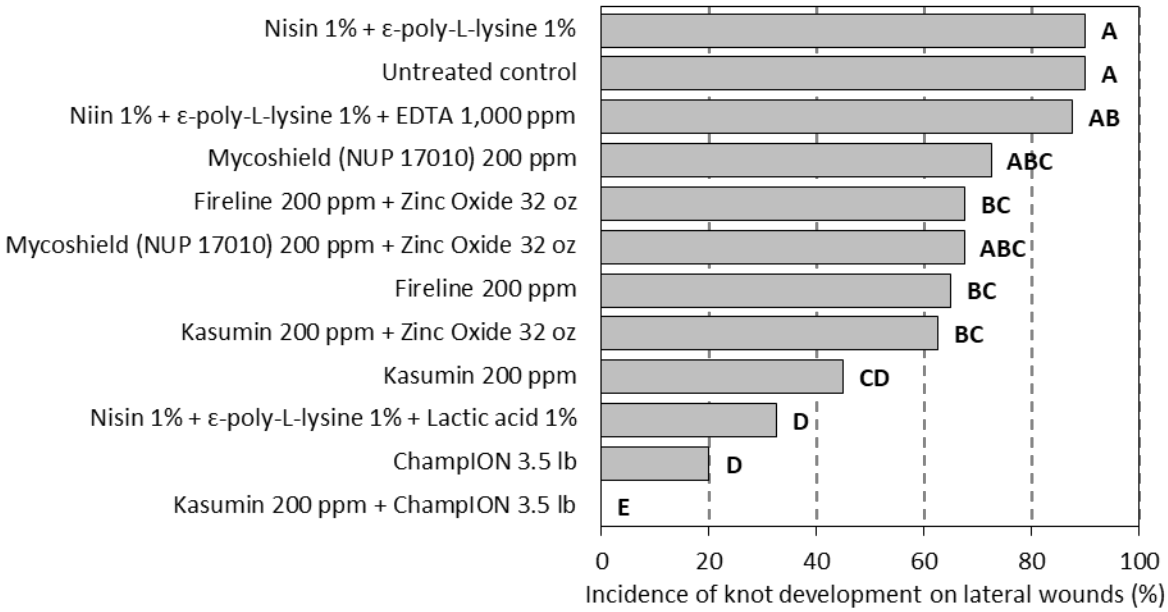
A. Copper-sensitive *Psv* - lateral wounds



B. Copper-sensitive *Psv* - leaf scar wounds

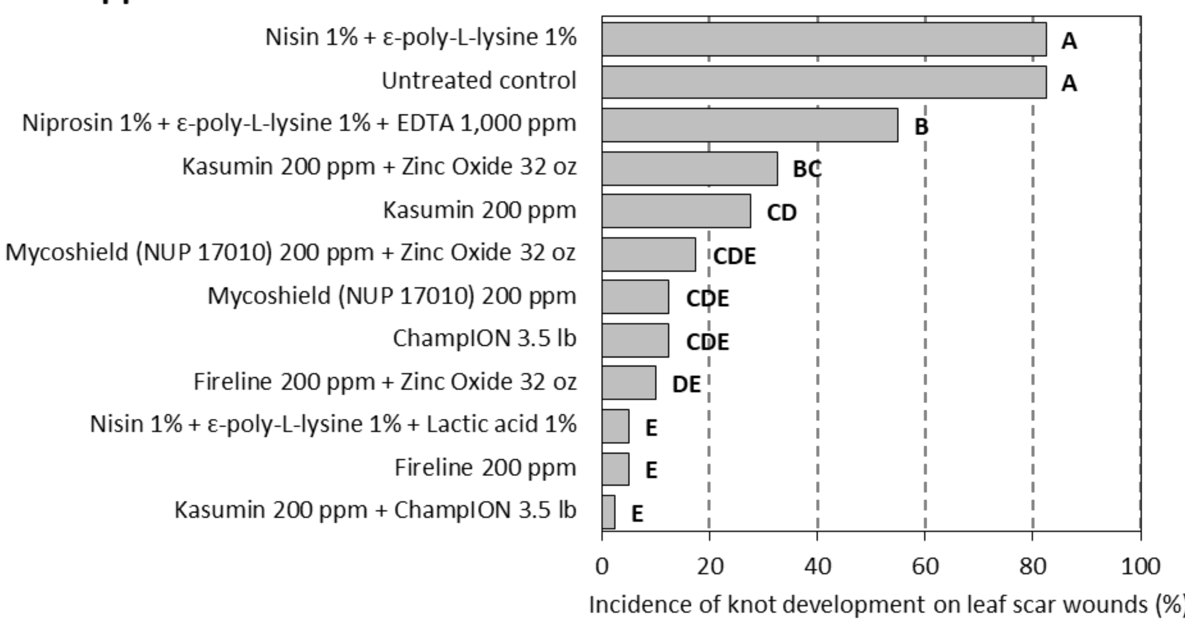
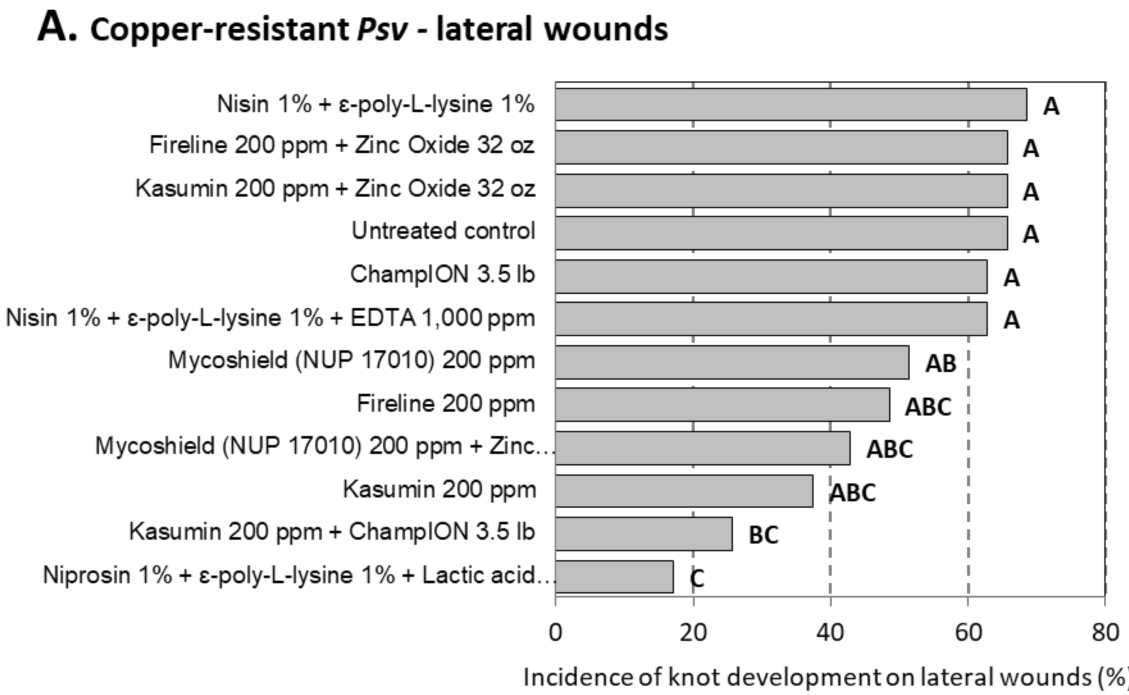


Fig. 1. In greenhouse trials with 'Arbequina' olive, lateral wounds were made by removing a small section of the cambium, and leaf scars were made by pulling off leaves in May 2019. Treatments were applied to wounds until runoff, allowed to dry, and inoculated with a copper-sensitive strain of *Psv*. Disease was evaluated in August 2019. Percent incidence of disease for each treatment followed by the same letter for each wound type is not significantly different based on analysis with GLM and LSD tests using SAS 9.4.



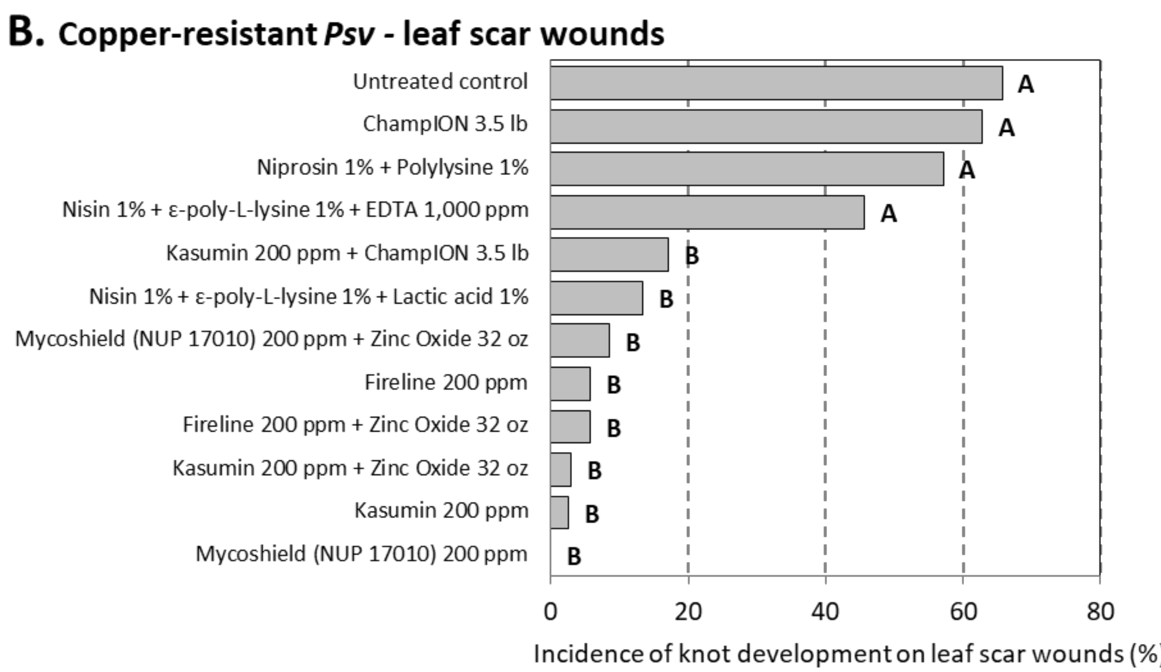


Fig. 2. In greenhouse trials with 'Arbequina' olive, lateral wounds were made by removing a small section of the cambium, and leaf scars were made by pulling off leaves in May 2019. Treatments were applied to wounds until runoff, allowed to dry, and inoculated with a copper-resistant strain of *Psv*. Disease was evaluated in August 2019. Percent incidence of disease for each treatment followed by the same letter for each wound type is not significantly different based on analysis with GLM and LSD tests using SAS 9.4.

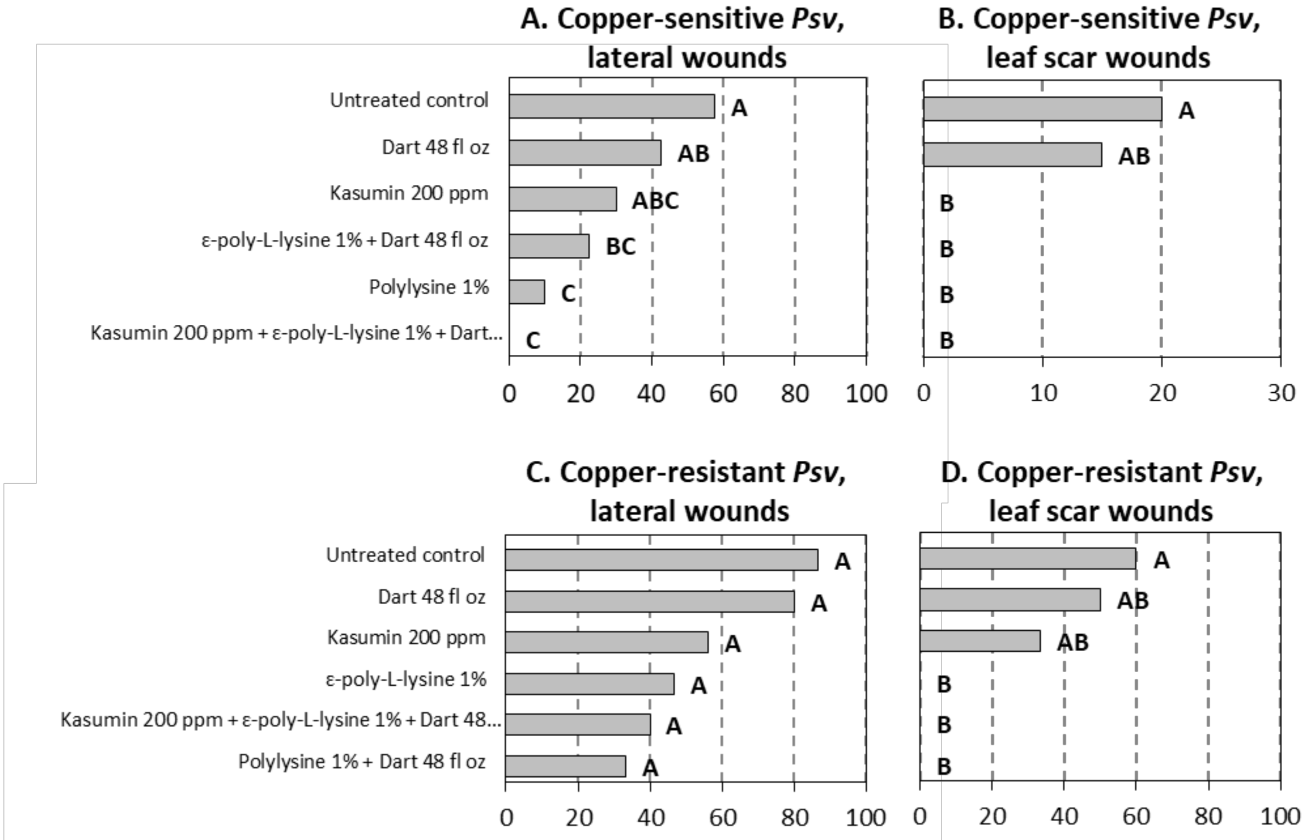


Fig. 3. In greenhouse trials with 'Arbequina' olive, lateral wounds were made by removing a small section of the cambium, and leaf scars were made by pulling off leaves on 8-21-19. Treatments were applied to wounds until runoff, allowed to dry, and inoculated with a copper-sensitive or -resistant strain of *Psv*. Disease was evaluated on 10-24-19. Percent incidence of disease for each treatment followed by the same letter for each graph is not significantly different based on analysis with GLM and LSD tests using SAS 9.4.

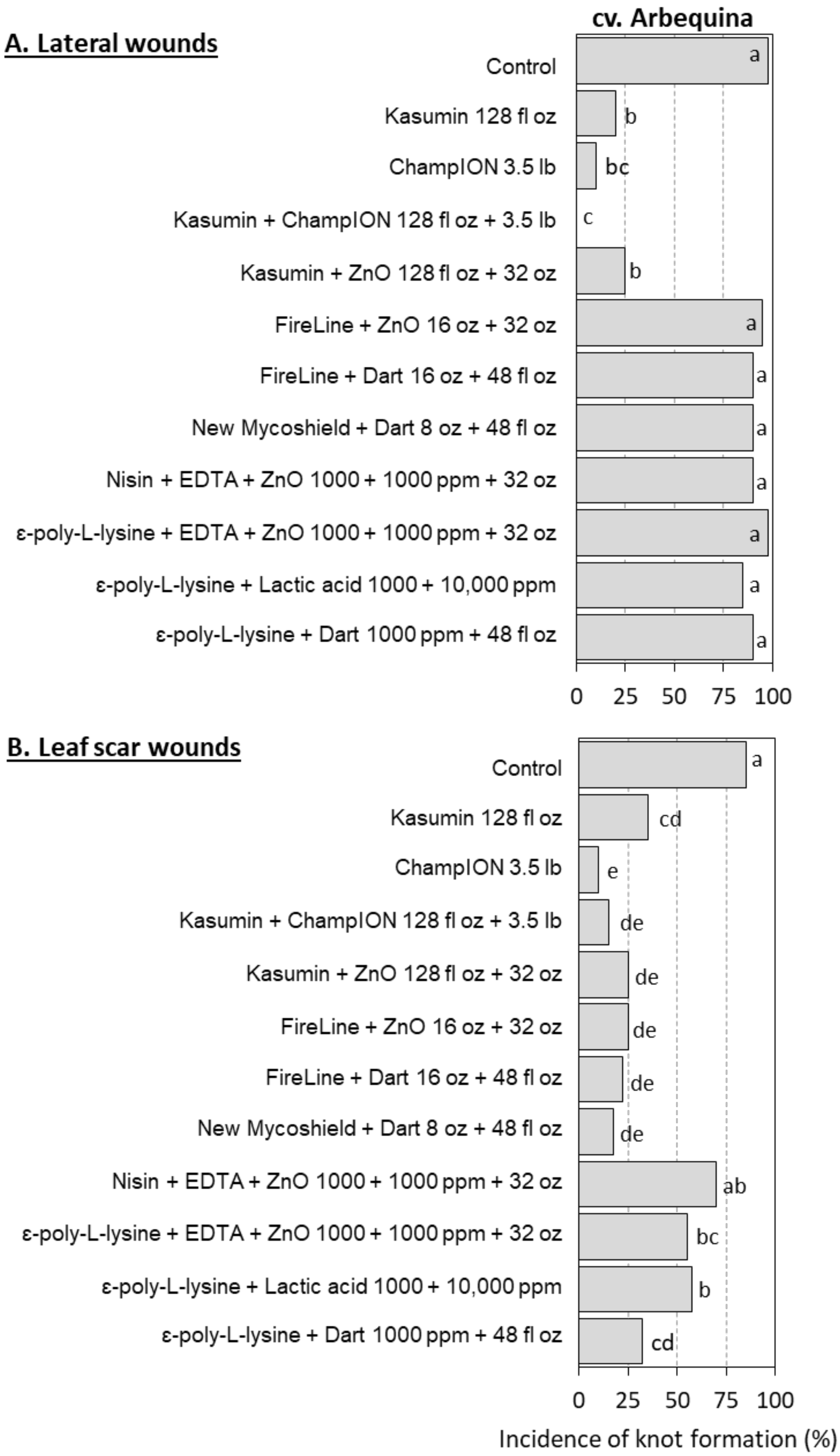


Fig. 4. In May 2019, olive twigs at UC Davis were wounded by lateral wounds, or leaves were removed. Wounds were treated by hand-spraying until runoff. After air-drying, wounds were inoculated with a copper-sensitive strain of Psv ($2x10^7$ cfu/ml). Twigs were evaluated for knot development in October 2019.

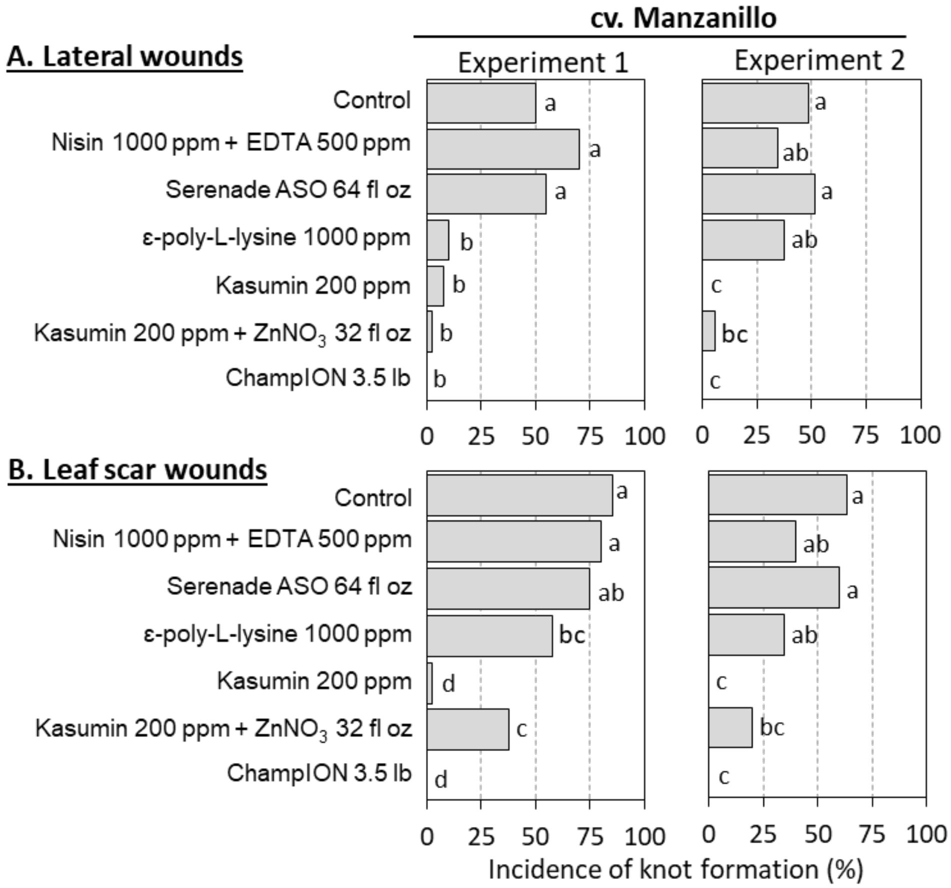


Fig. 5. In Jan. 2019, olive twigs at UC Davis were wounded by lateral wounds, or leaves were removed. Wounds were treated by hand-spraying until runoff. After air-drying, wounds were inoculated with a copper-sensitive strain of Psv ($2x10^7$ cfu/ml). Twigs were evaluated for knot development in October 2019.

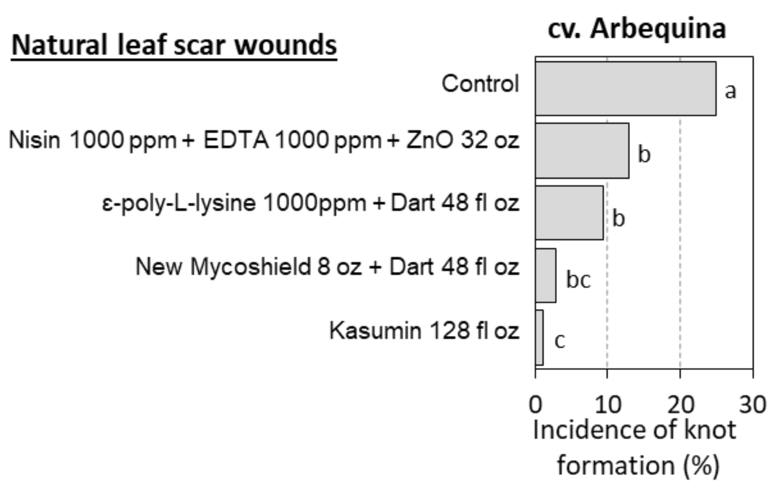


Fig. 6. In May 2019, in an orchard at UC Davis, leaf scars on olive twigs where yellowing leaves abscised by gently touching were marked, treated to run-off with a hand sprayer, and were inoculated after air-drying with a copper-sensitive strain of Psv ($2x10^7$ cfu/ml). Twigs were evaluated for knot development in October 2019.