UNIVERSITY OF CALIFORNIA
RIVERSIDE

Epidemiology and Management of Olive Knot Caused by *Pseudomonas savastanoi* pv. *savastanoi* in California Olive Production

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Plant Pathology

by

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December 2017

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ACKNOWLEDGEMENTS

This research was made possible with the cooperation and financial support of the California Olive Committee and the California Olive Oil Council. The chemistries evaluated in this research were generously provided by Arysta LifeScience, NuFarm Americas Inc., Decco US Post-Harvest, Mason Chemical Company, Pace Chemicals LTD., and DuPont Crop Protection.

I would like to recognize all my collaborators who collected olive knot samples including Dr. Elizabeth Fichtner, Dr. Danielle Lightle, and William Krueger of the University of California Cooperative Extension. I also thank Dr. John Preece for access to olive material at the National Clonal Germplasm Repository; Dave Thompson and Layne Wade for their advice and assistance with field trials; John Post for graciously providing olive orchard space for conducting experimental trials and olive knot samples; and Agromillora California for providing olive seedlings.

Additional funding was provided to me through several awards I was honored to receive including the Chancellor's Distinguished Fellowship Award (2012), the Joseph M. Ogawa Research and Teaching Endowment Award (2015), and the Leo J. Klotz Memorial Endowment Fund (2015 and 2016).

I thank my research committee members Dr. Michael Stanghellini and Dr. Caroline Roper for their advice and guidance during my program.

A very personal and special thank you to Dr. Helga Förster who has been a friend and mentor to me throughout my research. Her expertise, advice, and guidance in the field of plant pathology was invaluable in my education and training.

Finally, I owe a great deal of gratitude to Dr. James E. Adaskaveg who I will always know and address as my Professor. His love, dedication, and enthusiasm for plant pathology is infectious and kept me motivated throughout the program. Research in his lab is always intense and filled with insightful discussion. Professor always provided the necessary equipment and materials as well as a comfortable work environment to ensure high-quality research was accomplished. His experience and knowledge in the subject matter was instrumental in my development as a plant pathologist.

The text of this dissertation, in part, is a reprint of the material as it appears in Plant Disease (Nguyen, K. A., Förster, H., and Adaskaveg, J. E. 2017. Quaternary ammonium compounds as new sanitizers for reducing the spread of the olive knot pathogen on orchard equipment. Plant Dis. 101:1188-1193). The co-author (J.E. Adaskaveg) listed in that publication directed and supervised the research which forms the basis for this dissertation. H. Förster provided technical support and critical review of the manuscript.

DEDICATION

This dissertation is dedicated to all those who have helped, guided, and supported me to reach this point. To my everything, my wife, Ngoc, whose unconditional love and understanding continuously motivates me to always do my best. To my mother, Kim, and father, Anthony, who have sacrificed so much in order to provide their children with the opportunities they never had.

ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Plant Pathology
University of California, Riverside, December 2017
Dr. James E. Adaskaveg, Chairperson

Pseudomonas savastanoi pv. *savastanoi (Psv)* is a wound pathogen causing olive knot disease in California olive production. Sanitation of field equipment with sodium hypochlorite to reduce pathogen dissemination is a strategy to manage the disease but is highly corrosive. In laboratory assays, quaternary ammonium compounds (QACs) were highly toxic to *Psv* at low concentrations (≤5 µg/ml) and brief exposure times (≤60 s). In field trials, pruning equipment contaminated with *Psv* was effectively disinfested with a QAC, greatly reducing disease incidence on cutting wounds. This QAC was registered for use against olive knot on orchard equipment in 2015.

Copper-based bactericides are used as wound protectants against olive knot. Applications made within 24 h of wounding significantly reduced disease. Copper-treated wounds inoculated after 7 days reduced disease incidence by >50 % demonstrating good persistence. Untreated wounds inoculated after ≥10 days resulted in <20% disease, indicating wound healing. ‘Arbequina’ olives were less susceptible to *Psv* than ‘Manzanillo’, but disease was still high on both cultivars.

In-vitro evaluation of 147 California *Psv* strains detected three copper-resistant strains that grew at >150 µg/ml metallic copper in growth media. Copper-treated olive wounds inoculated with a copper-resistant strain resulted in reduced control compared to a -sensitive strain. Copper-sensitive strains were more virulent, but this depended on the type of wound inoculated and inoculum concentration.

Baseline sensitivities were established for kasugamycin and oxytetracycline as copper alternatives. Minimum inhibitory concentrations for 147 strains ranged from 1.86 to 11.52 µg/ml and 0.13 to 0.40 µg/ml for kasugamycin and oxytetracycline, respectively. In field studies, kasugamycin (200 µg/ml) performed equally to copper (1,260 µg/ml metallic copper) in reducing disease on lateral wounds of olives inoculated with a copper-sensitive strain and was better than copper using a -resistant strain. Oxytetracycline (200 µg/ml) was not as effective.

The genetic variability among 152 California strains based on rep-PCR with primers BOX, ERIC, and REP was high within regions, although overall genetic variability among strains was limited (≥82% similarity). Phenetic analyses identified several genotypes, but most strains belonged to one of two groups. The three copper-resistant strains had distinct fingerprints from the other strains.

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CHAPTER I. GENERAL INTRODUCTION

The genus *Pseudomonas* encompasses a diverse group of gram-negative bacteria affecting both animals and plants. Phytopathogenic *Pseudomonas* species are responsible for many diseases of agriculturally important crops. Symptoms are diverse and include blossom, twig, leaf, and kernel blights, cankers, diebacks, leaf spots, and soft rots (Braun-Kiewnick and Sands 2001). Certain pseudomonads have the unique ability of inducing gall or tumor formation on their host. *Pseudomonas savastanoi* pv. *savastanoi* (*Psv,* Gardan et al. 1992) is an important member of this group and is the causal agent of olive knot, a disease found throughout most olive (*Olea europaea* L.) growing regions of the world including California (Young 2004). *Psv* can survive on olives as an epiphyte (Ercolani 1978) and is an opportunistic wound-pathogen. Wounds caused by natural leaf scars, frost and hail damage, and cultural practices such as pruning and harvesting contribute significantly to the spread of the pathogen. *Psv* produces and releases several phytohormones including indole-acetic acid (Smidt and Kosuge 1978) and cytokinins (Surico et al. 1985). These hormones stimulate hyperplasia and hypertrophy of the surrounding host tissue, causing outgrowths (knots, tumors, galls) mainly on olive stems, branches, and trunks, but leaves and fruit can also be affected (Wilson 1935). In addition to phytohormone production, *Psv* relies on a type III secretion system that has a vital role as a virulence factor (Pérez-Martínez et al. 2010; Sisto et al. 2004). Olive knots are the primary inoculum source, containing a substantial concentration of *Psv* cells and can be long-lived. Spread of the pathogen is promoted during periods of rain when it is exuded

from knots as bacterial ooze (Horne et al. 1912). The bacterium is disseminated by splashing water, wind, insects, birds, and human activity (pruning and harvesting). Olive knot is one of the most economically important diseases of olives. Infections often lead to tree defoliation, branch dieback, and reduced tree vigor that ultimately lowers yield, fruit size, and oil quality (Schroth et al. 1963; Schroth et al. 1973).

The disease has been reported as early as the late nineteenth century in California, and was most likely introduced with the import of olive propagation material from Europe (Bioletti 1898). California accounts for more than 95 percent of olive production in the United States. As of 2012, there were approximately 44,000 bearing acres of olives in California, with table olives (canned olives) accounting for 56% of the total production. Mainly two table olive cultivars are grown in California, Manzanillo and Sevillano. The remaining 44% of olives in cultivation consist of a wide assortment of varieties, most of which are used for oil production and include the cultivars Arbequina, Arbosana, and Koroneiki (Vossen 2007). The total production value of olives in California for 2012 was $130 million. It is projected that 3,500 acres of olives for oil production will be added each year until 2020. Varieties of olives currently cultivated vary in their susceptibility to olive knot, but none are resistant to the disease (Penyalver et al. 2006).

The first objective of this dissertation was to examine the genetic variability of *Psv* strains in major olive growing regions of California. The genetic diversity among *Psv* strains in the state has yet to be described and would provide valuable insights about the population. This could include determining if strains are specific to certain olive cultivars

or growing regions, as well as the genetic relatedness of strains expressing differential sensitivity to copper. Repetitive element sequence-based polymerase chain reaction (Rep-PCR; Versalovic 1994) is one technique that has been successfully used for fingerprinting various bacterial populations. Rep-PCR was also utilized to determine the genetic structure of *Psv* populations in Italy and Japan (Scortichini et al. 2004; Tsuji et. al. 2017). Genetic studies on *Psv* strains found in Europe suggest that populations are highly polymorphic and tend to be more genetically similar when isolated from the same geographical region (Krid et al. 2009; Quesada et al. 2008; Sisto et al. 2007). Scortichini et al. (2004) concluded that strains in Italy were identical within orchard locations and that no specific relationship existed between *Psv* genotypes and certain olive cultivars.

The epidemiology of *Psv* has been well studied and reviewed by Ramos et al. (2012). Still, many aspects of the disease cycle need further evaluation in relation to California olive production. This includes understanding the extent of bacteria being exuded from knots and minimal threshold concentrations of bacteria necessary for inducing disease. The duration of wound susceptibility to infection was examined for two commercially important olive cultivars in California, and virulence of *Psv* strains expressing differences in copper sensitivity was compared.

Preventing the introduction and dissemination of *Psv* is the most promising strategy for olive knot control. This, however, can be challenging as the bacterium has the ability to survive epiphytically making detection difficult when nursery plants remain asymptomatic (Ercolani 1978; Penyalver et al. 2006) and knots only occur later on in the field. California is experiencing changes in olive cultivation methods in order to reduce

cost and improve yield for the growing market for olive products (Vossen 2007). Traditionally, olives were grown with an average of 70 trees per acre for oil production. Growers are now shifting towards more intensive methods with high density (HD) and super high density (HSD) production systems where as many as 900 trees are planted per acre (Vossen 2007). Trees in these plantings are trained into hedgerows on a trellis system and are mechanically pruned and harvested. This creates injury sites for *Psv* infection (Tous et al. 2007). Furthermore, equipment coming into contact with diseased trees are likely to be contaminated with *Psv* and can subsequently spread the pathogen to healthy neighboring trees without proper sanitation. Development of sanitation materials and methods is important for reducing the spread of the pathogen due to these mechanized changes. Thus, this was another objective of this research. Potential sanitizers were evaluated including those already registered on agricultural equipment and machinery in other crop systems. These include the quaternary ammonium compounds (QACs) or quats that have been implemented in reducing the spread of citrus canker caused by *Xanthomonas axonopodis* pv. *citri* in Florida (Schubert and Sun 2003). The main goal of this research was to find a non-corrosive, fast-performing sanitizer that is safe for use on food harvesting equipment and that could be readily registered on olives. QACs are cationic surfactants that are odorless, colorless, temperature-stable, non-corrosive to equipment, nonirritating to skin, and able to penetrate food contact surfaces more readily than other sanitizers (Walker and LaGrange 1991). These attributes make QACs ideal candidates for use on olive processing equipment and they were evaluated in this research.

Few chemical treatments currently exist for effective management of olive knot. Copper remains the standard compound of choice as a preventative treatment (Quesada et al. 2010b; Teviotdale and Krueger 2004). Xylenol- and cresol-based materials such as Gallex as mechanically applied wound treatments (Schroth and Hildebrand 1968) are also effective but application is not feasible on a commercial scale (Sibbett and Ferguson 2005). The heavy reliance and exclusive use of copper bactericides increases the risk of resistance development. This has occurred in *Pseudomonas* species affecting other crops (Andersen et al. 1991; Cooksey 1990). A fundamental objective of this research was to collect *Psv* strains from major olive producing areas of California and to determine their sensitivity to copper. Copper has been studied and used for the control of olive knot in California since the early 1900s (Horne et al. 1912; Wilson 1935). It is applied as a foliar spray treatment after harvest during the fall and again in spring to protect leaf scar wounds after natural leaf drop (Teviotdale and Krueger 2004). Applications are carefully timed before the occurrence of rain, the major dissemination route of the bacterium in order to protect susceptible tissue (Horne et al. 1912). With extensive and exclusive use of copper, increased copper tolerance was suspected in California *Psv* populations. Copper resistance that has never been reported from other olive growing locations was confirmed in this study. Another major goal of this dissertation was to identify copper alternatives and improved application strategies for copper. We have studied and are developing the antibiotic kasugamycin for control of other gram-negative bacteria such as *Erwinia*, *Xanthomonas*, and *Pseudomonas* species. Kasugamycin is an aminoglycoside antibiotic that is not currently used in human or animal medicine. The antibiotic exhibits

very low mammalian toxicity (Copping and Duke 2007) and is quickly degraded in the environment (Lu et al. 2012). It is very active against various phytopathogenic bacteria making it an excellent candidate for rotations and mixtures with copper bactericides. Kasugamycin received federal registration based on our previous work for the control of fire blight on pome fruit caused by *Erwinia amylovora* (Adaskaveg et al. 2011). We are currently seeking its registration for additional diseases such as walnut blight caused by *Xanthomonas arboricola* pv. *juglandis* and olive knot. In addition to kasugamycin, the efficacy of oxytetracycline, a tetracycline antibiotic, was explored for the control of olive knot. Oxytetracycline is registered for use against fire blight on apple and pears and for bacterial spot of certain stone fruits caused by *Xanthomonas arboricola* pv. *pruni*. It is used as an injection treatment against phytoplasmas that cause diseases of palm and elm trees.

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CHAPTER II. GENETIC DIVERSITY OF THE OLIVE KNOT PATHOGEN IN CALIFORNIA AND CHARACTERIZATION OF EPIDEMIOLOGICAL FACTORS FOR DISEASE DEVELOPMENT

ABSTRACT

Olive knot, caused by *Pseudomonas savastanoi* pv. *savastanoi*, is a limiting disease in the production of table and oil olives in California. The genetic variability among 152 strains from major production areas of California was determined using BOX, ERIC, and REP primers in rep-PCR. Overall genetic variability among strains was low and strains shared at least 82% similarity. Phenetic analyses identified several genotypes, but most strains belonged to one of two major groups. Three copper-resistant strains had two fingerprints that were distinct from any of the sensitive strains indicating that they may have been introduced from other production areas or hosts. In inoculations, a copper-sensitive strain caused significantly more disease (55% knot formation) than a resistant strain (42.5% knot formation) on leaf scars but not on lateral wounds. Inoculum was produced at high levels (>10^8 CFU/g knot tissue) within 10 min from hydrated olive knots, and concentrations were 2- to 3-log higher than the minimum needed to induce knot formation. 'Arbequina' olive was significantly more susceptible to with a higher incidence of infection of leaf scar and lateral wounds (59.7% to 80.6% incidence) than 'Manzanillo' (47.4% to 68.2% incidence). In wound-healing studies, both types of

wounds were less susceptible to infection ≥10 days after injury indicating a critical period for infection and application of bactericides during favorable environments.

INTRODUCTION

Olive knot, caused by the phytobacterium *Pseudomonas savastanoi* pv. *savastanoi,* has been present in California for over a century. It was likely introduced into the state on olive (*Olea europaea* L.) plants imported from the Mediterranean during the late 1800s when commercial plantings were established (Bioletti 1898). *P. savastanoi* pv. *savastanoi* is a pathogen infecting wounds that are either created mechanically or by natural causes such as leaf drop or cold injury. Wetness from rain or irrigation contacting olive knots, the primary inoculum source, promotes the release and movement of the bacterium to susceptible wounds (Horne et al. 1912). Infections lead to the development of new knots or galls mostly on twigs, trunks, and branches resulting in reduced tree vigor and yield (Schroth et al. 1973; Wilson 1935). Over the last ten years, olive oil production has been increasing in California with the implementation of novel cultural techniques such as high-density planting systems where mechanical pruning and harvesting methods are used (Tous et al. 2010; Vossen 2007). These new methods allow growers to reduce labor costs and remain profitable. However, they also increase the risk of spreading olive knot because pruning and harvesting equipment cause a greater number of susceptible injuries to olives than traditional techniques. Furthermore,

machinery become contaminated with the bacterium from contact with diseased trees and can effectively disseminate the pathogen to healthy trees (Tous et al. 2010).

Olive knot occurs widespread in oil and table olive production areas in California, especially those with higher rainfall, but no information is available on the genetic diversity in *P. savastanoi* pv. *savastanoi* populations among orchards and growing areas. Repetitive element sequence-based PCR (rep-PCR), random amplification of polymorphic DNA, multilocus sequence typing, and amplified fragment length polymorphisms have been used to fingerprint bacterial strains and study the population structure of the pathogen in other areas (Krid et al. 2009; Moretti et al. 2017; Scortichini et al. 2004; Sisto et al. 2007; Tsuji et al. 2017). For example, random amplified polymorphic DNA analyses identified three main clusters among strains from Tunisia that correlated with locations within the country, and only one of the clusters also comprised strains from other European countries and from the United States (Krid et al. 2009). In another study, rep-PCR analyses of strains from several Mediterranean countries detected numerous haplotypes, and clustering of strains was based on their geographic origin (Moretti et al. 2016). Similarly, strains of *P. savastanoi* pv. *savastanoi* from various hosts in several European countries grouped together based on their geographic origin using AFLP banding patterns (Sisto et al. 2007). Using rep-PCR, a high degree of polymorphism with 20 haplotypes was detected among 360 strains from olive in Italy, but no distinct genetic clusters were identified (Scortichini 2004), and a geographic grouping was not attempted. Knowledge about the population structure of a pathogen may provide insight into its spread and possible adaptation to host cultivars or

certain ecological niches with specific microclimates in a geographical region. Such information may also offer clues on the genetic relatedness and origin of copper-resistant strains of *P. savastanoi* pv. *savastanoi* in California that were recently identified (Nguyen et al. 2018).

Important management strategies for olive knot are the use of clean nursery stock, prevention of injuries to trees, pruning and removal of infected plant material, sanitation of field equipment, and protective applications with copper bactericides or one of the few copper alternatives currently available (Nguyen et al. 2017, 2018). To provide the most effective disease control, chemical strategies should be properly timed in respect to environmental conditions, injury and infection events, presence of sufficient inoculum, as well as time of host susceptibility. The epidemiology of olive knot has been well studied (Ramos et al. 2012) but little is known in relation to current California olive production practices that utilize only a few cultivars, mostly of Spanish origin (Vossen 2007). This includes understanding the extent of bacterial inoculum being exuded from knots, inoculum concentrations necessary to induce disease, and the period of wound susceptibility to infection.

The objectives of this study were to i) determine the genetic diversity of *P. savastanoi* pv. *savastanoi* in major olive production areas of California and elucidate the possible origin of copper-resistant strains; ii) quantify natural inoculum concentrations exuding from hydrated olive knots and determine inoculum concentrations necessary to induce disease on major olive cultivars grown in California; iii) compare the virulence of

copper-sensitive and -resistant strains of *P. savastanoi* pv. *savastanoi*, and iv) determine the duration of susceptibility of lateral twig and leaf scar wounds to infection.

MATERIALS AND METHODS

Culturing of *P. savastanoi* pv. *savastanoi* strains and of olive plants in the field. One hundred forty-seven strains of *P. savastanoi* pv. *savastanoi* that were previously isolated from olive knots collected in commercial oil and table olive orchards in major production areas of California (Butte, Colusa, Glenn, Tehama, Tulare, and Yuba Co.) (Nguyen et al. 2018) and an additional 5 strains were isolated from knots collected at the USDA National Clonal Germplasm Repository for olives at the University of California, Wolfskill Experimental Orchards, Yolo Co. (Table 1). A total of 152 strains were molecularly identified with primers targeting the IAA gene (Penyalver et al. 2000) and were maintained in 15% glycerol at -80°C. Strains were grown on King's medium B (KMB, King 1954)) for 48 h at 22 to 25°C before use. Field trials were conducted using 'Arbequina' and 'Manzanillo' olive trees (Agromillora California, Gridley, CA). Trees were planted at the University of California Davis and Riverside field stations similar to high-density commercial olive orchards using a trellis system (Vossen 2007). Tree spacing within a row was approximately 2 m, and the distance between rows was approximately 5 m. Trees were irrigated and fertilized using standard commercial practices.

Genetic fingerprinting of *P. savastanoi* pv. *savastanoi* strains. Rep-PCR was utilized to determine the genetic variability among 152 *P. savastanoi* pv. *savastanoi* strains using BOX, ERIC, and REP primers (Rademaker et al. 1997). PCR reactions contained the following in a total volume of 25 µl: 2.5 µl 10x reaction buffer (Apex; Genesee Scientific, San Diego, CA), 1 u Taq-DNA polymerase (Apex; Genesee Scientific), 500 nM primers BOXA1R, ERIC1R and ERIC2, or REPIR1 and REP2I, 0.25 µl bovine serum albumin (Sigma Aldrich, St. Louis, MO; 10 µg/ml), and 100 µM of each dNTP (Genesee Scientific). One µl of an aqueous bacterial suspension (70% transmittance at OD_{600}) was used as template per reaction, and amplifications were done in a PTC-200 (MJ Research, Inc., Waltham, MA) thermal cycler. Amplification conditions were as described previously (Rademaker et al. 1997), but the extension step was reduced to 2 min, and 35 cycles were performed. Amplification products were separated in 1.5% agarose (containing 1 µg/ml ethidium bromide) in 0.5X Tris-borate-EDTA buffer. A 100-bp ladder (Bioland Scientific, Paramount, CA) was used as DNA size marker. Amplifications were repeated at least once for all strains and primers, and only bands that were consistently amplified were scored.

Inoculum production from wetted olive knots and inoculum concentrations of copper-sensitive and -resistant strains to induce disease on two olive cultivars. Approximately two-year old 'Arbequina' olive twigs were inoculated in the field with *P. savastanoi* pv. *savastanoi* strain O26 as described below. After 6 months, the amount of inoculum released from knots was determined following selected time periods of soaking in water. For this, knots with living green tissue were excised by cutting twigs at opposite

sides of knots and weighed. Knots were surface-sterilized in a 1:10 dilution of household bleach, rinsed three times with sterile distilled water, and each knot was placed into a 50-ml plastic tube containing 5 ml of sterile water. Tubes were kept at 25°C on a G24 incubator shaker (New Brunswick Scientific Co., INC., Edison, NJ) at 100 rpm, and 10-µl samples were taken almost immediately (within 10 min) and after 4, 8, and 24 h. Samples were diluted 1:10,000 with sterile water before plating onto 10-cm KMB media plates with a spiral plater (Autoplate 4000, Spiral Biotech, Inc., Norwood, MA). Colonies that emitted a blue fluorescence under UV light (365 nm) were enumerated after 3 days of growth at 25°C, and colony forming units (CFU) were calculated per gram of knot weight. A sub-sample of 20 colonies was verified as *P. savastanoi* pv. *savastanoi* using specific primers in PCR reactions (Penyalver et al. 2000). In each of the two studies, 10 replicated knots were evaluated for each wetness duration. Concentrations of bacteria recovered were expressed as $\log_{10}$ CFU/g knot tissue.

To determine the effect of inoculum concentration on knot formation and to compare the virulence of copper-sensitive and -resistant strains, lateral and leaf scar wounds were made to 2 years old 'Arbequina' branches 5 mm in diameter. Lateral wounds were made by removing a 5 mm x 10 mm piece of bark with a sterile scalpel so that cambial tissue was exposed. Leaf scar wounds were created by removing leaves by hand. Strains O26 and O113 were identified as copper-sensitive (no growth at >20 µg/ml metallic copper) and -resistant (growth at ≥50 µg/ml metallic copper), respectively, based on culturing on copper-amended casitone-yeast-extract-glycerol agar (Zevenhuizen 1979) as described previously (Nguyen et al. 2017). Aqueous inoculum suspensions

(approximately 10^8 CFU/ml as determined by transmittance at OD_{600}) of the two strains were serially diluted to obtain a range of concentrations from 10^5 to 10^8 CFU/ml. Inoculum was applied to injuries using a spray bottle until runoff. Two repeated studies were done (June 2014 and April 2015) at UC Riverside, and disease was evaluated after 4 to 8 months.

To compare susceptibility of two major commercial olive cultivars, lateral and leaf scar wounds on 'Arbequina' and 'Manzanillo' olive twigs were inoculated with serial dilutions of bacterial suspensions from 10^5 to 10^8 CFU/ml as described above using strain O26. Two repeated studies were done (September 2014 and October 2014) at UC Davis, and disease was evaluated after approximately 10 months.

In each of the two studies, each twig represented an experimental replicate with 5 lateral and 5 leaf scar wounds. For the strain virulence study, 6 replicates were done for each inoculum concentration for each strain (O26 and O113) and wound type on 'Arbequina' olives. For the olive cultivar susceptibility study, 8 replicates were done for each inoculum concentration for each cultivar ('Arbequina' and 'Manzanillo') using strain O26 and trials were arranged in a randomized complete block design (RBCD).

Duration of susceptibility of lateral twig and leaf scar wounds to infection by *P. savastanoi* pv. *savastanoi*. 'Manzanillo' olive twigs were injured to create lateral and leaf scar wounds as described above. Wounds were inoculated immediately or after 10, 20, or 30 days. Wounds were spray-inoculated with *P. savastanoi* pv. *savastanoi* strain O26 (10^7 CFU/ml) until runoff. For each wound age, 5 twig replicates were made, and

each set of timings was on a separate tree. The experiment was done twice on different trees in October of 2013 at UC Davis and evaluations were made after 8 months.

Data analysis. Fingerprint patterns of rep-PCR were scored based on the presence (score = 1) or absence (score = 0) of a band for each strain for the three primer sets used and were entered into a combined binary data matrix. Cluster analysis was done using the NTSYSpc software (ver.2.21q; Rohlf 2009). First, similarities in pair-wise comparisons of fingerprint patterns were generated using Dice's coefficient (SIMQUAL). Clustering was performed using the unweighted pair-group method with arithmetic means (UPGMA) in SAHN. A dendrogram was generated (TREE). To determine how well the dendrogram represented the original similarity matrix, a matrix of cophenetic values was created (COPH) and compared for goodness of fit with the original matrix (MXCOMP, set to 5 permutations).

For additional statistical analyses, data for inoculum production were $\log_{10}$-transformed, whereas percent disease incidence data for the inoculation studies were arcsin-transformed. Variances for repeated studies were evaluated using Bartlett's test of homogeneity. Because all data sets were homogeneous, data for repeated studies were combined. Mean values and standard errors for inoculum production and percent disease incidence were calculated for each inoculum concentration, strain (i.e., copper-sensitive, -resistant), and cultivar (i.e., 'Arbequina', 'Manzanillo') evaluated. A two-way analysis of variance (ANOVA) was done for the variables bacterial strain or cultivar, concentration of inoculum, and the interaction of these variables. A one-way ANOVA was used to test the effects of inoculation time after wounding on the incidence of disease for both lateral

and leaf scar wounds. Homogeneity of variances, ANOVA, and least significant difference (LSD) mean separation procedures were done using a statistical software package (SAS ver. 9.4; SAS Institute, Cary, NC).

RESULTS

Genetic fingerprinting of *P. savastanoi* pv. *savastanoi* strains. The REP primer set showed the majority of polymorphisms among the 152 strains evaluated and was the most discriminatory among the rep-PCR primers utilized. Overall genetic variability among strains, however, was low and most belonged to one of two groups (A and B) based on the presence of a distinct and reproducible DNA fragment approximately 700 bp in length (Fig. 2.1). Two of the three copper-resistant strains (O113 and O145) shared identical fingerprints that were distinct from the third strain (O146), and the latter three strains displayed unique bands not observed in any of the other strains. Several additional strains had unique fingerprints. Using the ERIC primer set, banding patterns were identical for all strains (data not shown). For the BOX primer, most strains were identical except for four strains (i.e., O33, O91, O93, O103) that had a band that was absent in the other strains, and another strain (O121) that amplified a unique band not shared with any of the remaining strains (Fig. 2.2).

The phenetic analysis of 15 polymorphic bands identified in rep-PCR resulted in similarity and cophenetic matrices with a cophenetic correlation value of 0.954. In the UPGMA dendrogram that was generated, several clusters of *P. savastanoi* pv. *savastanoi*

strains group closely together, and no distinct lineages are evident (Fig. 2.3). The majority of strains belong to two groups, A and B. Group A contains strains from all five olive cultivars sampled in Butte, Glenn, Tehama, and Yuba Co.; whereas in group B, strains from four cultivars in Glenn, Tehama, Tulare, Yolo, and Yuba Co. are found (Table 2.1). Overall, Glenn Co. had the highest diversity in *P. savastanoi* pv. *savastanoi* strains with six fingerprint patterns, followed by Tehama county with five patterns, and Yuba Co. with four patterns. In some of the orchards in these counties, more than one genotype was detected (e.g., Glenn-12, Tehama-2, Yuba-1). Strains from Butte, Tulare, and Yolo Co. each had a single fingerprint pattern, but only one orchard was sampled for each of these counties (Table 2.1). A strain from 'Sevillano' olive in Tehama Co. was the most distinct with the lowest similarity coefficient (i.e., 0.8) in comparison with the most dissimilar strains. Two of the three copper-resistant strains from Glenn Co. were identical and they grouped closely with the third strain. These copper-resistant strains did not cluster with any of the other strains, including those from the same orchard (i.e., locations 10 and 12).

Inoculum production from wetted olive knots and inoculum concentrations of copper-sensitive and -resistant strains to induce disease on two olive cultivars. Excised knots that were soaked in water exuded large quantities of bacteria almost immediately (within 10 min) and the bacterial concentration in the water suspension increased from 10^8 CFU/g to $\geq 10^9$ CFU/g knot tissue over a 24-h period.

The amount of inoculum to induce knot development was subsequently evaluated. The overall ANOVA models were highly significant ($P < 0.0001$) for both leaf scars and

lateral wounds. For copper-sensitive and -resistant strains and for both types of wounds, incidence of knot formation increased significantly ($P < 0.0001$) for inoculum concentrations from 10^5 to 10^8 CFU/ml (Fig. 2.4A,B). Using the highest concentration, >80% of both types of wounds developed knots. For leaf scars, there were significant differences in knot formation between the two strains ($P = 0.002$), and there was no interaction ($P = 0.158$) between strains and inoculum concentrations (Fig. 2.4A). Overall, the copper-sensitive strain was more virulent and caused significantly more disease (55% knot formation) than the resistant strain (42.5% knot formation). For lateral wounds, however, there was no significant difference in knot formation between the two strains ($P = 0.195$) with the copper-sensitive and -resistant strains causing 69.2% and 62.5% knot formation, respectively. There was also no interaction ($P = 0.565$) between strains and inoculum concentrations (Fig. 2.4B).

Similar results on the effect of inoculum concentration were obtained when susceptibility of leaf scar and lateral wounds was compared on 'Arbequina' and 'Manzanillo' olive (Fig. 2.5A,B). The overall ANOVA models were highly significant ($P < 0.0001$) for both leaf scars and lateral wounds. Highly significant differences were observed between cultivars and inoculum concentrations for leaf scars ($P = 0.0048$ and $P < 0.0001$, respectively) and for lateral wounds ($P = 0.0112$ and $P < 0.0001$, respectively). There was no interaction between cultivars and inoculum concentration for leaf scars ($P = 0.872$) and lateral wounds ($P = 0.592$). For leaf scars on 'Arbequina' and 'Manzanillo', incidence of knot formation increased from 15.0% and 8.8% to 85% and 70%, respectively (Fig. 2.5A); whereas on lateral wounds incidence increased from 48.8% and

33.8% to 86.3 and 83.8% (Fig. 2.5B) with inoculum concentrations increasing from 10^5 to 10^8 CFU/ml. Overall, 'Arbequina' had a significantly higher incidence of knot formation (59.7% on leaf scars, 80.6% on lateral wounds) as compared to 'Manzanillo' (47.4% on leaf scars, 68.2% on lateral wounds).

Duration of susceptibility of lateral twig and leaf scar wounds to infection by *P. savastanoi* pv. *savastanoi*. In field studies using 'Manzanillo' olives, inoculation period after wounding had a significant effect ($P < 0.0001$) on incidence of knot formation of inoculated lateral and leaf scar wounds (Fig. 2.6). For the 0-day inoculations, incidence of knot formation on both types of wounds was $\geq$80%; whereas after 10 days, the incidence was reduced to $\leq$20%. No significant difference in disease was observed among 10-, 20-, or 30-day-old leaf scar ($P = 0.3544$) and lateral ($P = 0.2352$) wounds.

DISCUSSION

Populations of *P. savastanoi* pv. *savastanoi* from olive in major California growing areas were found to be genetically diverse based on rep-PCR, but overall genetic variability was limited with strains sharing a minimum of 82% similarity. These results are consistent with reports from growing areas in Mediterranean countries (Krid et al. 2009; Moretti et al. 2017; Scortichini et al. 2004; Sisto et al. 2007) and from nurseries and ornamental trees in Japan (Tsuiji et al. 2017) where variability among pathogen strains was evaluated using a range of molecular methods. Still, in the latter studies,

diversity was found mostly among geographic regions and not within locations. Thus, strains from the same orchards in Italy were all identical (Scortichini et al. 2004). Those from plantings in Tunisia were either identical or only two genotypes were identified that were generally not present, however, at other locations (Krid et al. 2009). In contrast in our study, either one of the two major genotypes that we identified was found in orchards in four or five counties in California, respectively, that are separated by distances of up to 300 km. Additionally, there was no association of genotypes with a specific olive cultivar, and this was also observed in Mediterranean growing regions (Scortichini et al. 2004).

This difference in *P. savastanoi* pv. *savastanoi* genotype distribution between California and Mediterranean countries may be indicative of different ways of nursery stock distribution. Thus, whereas trees in Mediterranean countries are often propagated and then planted locally, nursery stock in California is provided by a limited number of propagation facilities that ship plants to growers throughout the state. Epiphytic populations of *P. savastanoi* pv. *savastanoi* on olive plants can therefore be disseminated over long distances (Ercolani 1978).

Interestingly in our study, the three copper-resistant strains of *P. savastanoi* pv. *savastanoi* from two orchards in Glenn Co. did not group closely with any of the California genotypes of copper-sensitive strains that we identified including strains from the same locations. Copper resistance may have been introduced through replants from contaminated nursery stock or from other orchards or growing areas in the state that were not sampled. Because the pathogen is not specific to olive (Sisto et al. 2007), it also could

have been introduced from ornamental hosts such as oleander. Furthermore, because the copper-resistant strains exhibited two different, but related, banding patterns, they may have been introduced independently. Additional sampling of olive knots in California, as well as a worldwide comparison of strains may provide insight into the origin of copper-resistant strains of *P. savastanoi* pv. *savastanoi.*

After soaking olive knots in water, inoculum was produced readily and at concentrations 2- to 3-log higher than the minimum amount needed to induce knot formation in inoculations. These results further support that protective bactericide treatments should be applied before favorable environments for disease occur such as rainfall after mechanical harvesting, leaf drop, hail, or freeze events or immediately afterwards as indicated previously by Nguyen et al. (2018).

'Arbequina' and 'Manzanillo' represent the most widely planted oil and table olive cultivars, respectively, in California. In our studies, disease developed on lateral twig and leaf scar wounds inoculated with bacterial concentrations that were found to be naturally exuding from knots. Our findings are similar to those of Penyalver et al. (2005) who identified 'Arbequina' as highly susceptible to olive knot. 'Manzanillo' was also highly susceptible in our inoculation studies, but significantly less susceptible than 'Arbequina'. A high incidence of disease was obtained in inoculations of both types of wounds. Leaf removal in our assay, however, created an artificial wound, and natural leaf scars likely will be less susceptible to the inoculum concentrations evaluated because a protective abscission zone develops during annual leaf drop of olives (Hewitt 1938). In contrast, the lateral wounds we created were similar to those occurring during mechanical

harvest and pruning. Thus, disease incidence resulting from these inoculations will likely reflect disease occurrence under commercial field conditions.

A copper-sensitive strain was found to be more virulent and induced significantly more disease than the resistant strain, but only on leaf scars. Apparently, copper-resistance has some impact on fitness. In contrast, copper resistance in *P. fluorescens* was found to increase competitive fitness in soils even when copper content was low (Yang et al. 1993). The resistant strain of *P. savastanoi* pv. *savastanoi* was still able to cause a high level of disease on both types of wounds in our study, and probably will be able to compete under field conditions. Furthermore, because copper is widely used in the management of olive knot and is the only highly effective bactericide currently available, copper-resistance is under positive selection pressure during times of the year when copper residues are present on trees (e.g., fall, winter, spring). In fact, these resistant strains were detected after copper treatment failures in orchards were reported in the last several years. Copper resistance may be a recent introduction into California. Based on the fitness of resistant strains to cause disease and because the only effective treatment currently available for managing olive knot is copper, there is a high risk of spread of copper resistance in California olive plantings if alternative management strategies are not developed.

In our wound-healing studies, lateral and leaf scar wounds of 'Manzanillo' olives were less susceptible to infection ≥10 days after injury. Using 'Mission' olives, Hewitt (1938) demonstrated that natural leaf scars were less susceptible to infection 7 days after leaf drop. Based on this and our studies, wound healing is important in relation to timing

of orchard horticultural practices and disease management. The longer the period after pruning or mechanical harvest with no rainfall or wetness from irrigation, the less likely a successful infection will be established by the pathogen because of wound healing. Under California conditions, pruning should be done during low-rainfall periods such as mid- to late spring after leaf drop or early summer, but not after harvest in the fall or in the winter when rains are likely to occur. Secondly, bactericide treatments should be applied during the most susceptible period of injuries, i.e., as soon as possible after they occur.

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Table 2.1. Origin of *P. savastanoi* pv. *savastanoi* strains from California used in this study and their fingerprint patterns based on rep-PCR[1]

Olive cultivar	County (orchard no.)	Fingerprint pattern(s)/no. of strains[2]	Total no. of strains
Koroneiki	Glenn-1	A/13	13
Manzanillo	Glenn-2	A/6, B/1	7
Arbequina	Yuba-1	A/3, B/2	5
Arbosana	Yuba-2	A/4, C/1	5
Manzanillo	Glenn-3	A/3, B/2, D/1	6
Manzanillo	Glenn-4	A/6	6
Manzanillo	Glenn-5	B/10	10
Arbequina	Butte-1	A/8	8
Unknown	Tulare-1	B/6	6
Manzanillo	Tehama-1	A/3, B/2	5
Manzanillo	Glenn-6	A/2	2
Sevillano	Tehama-2	A/1, E/1	2
Arbequina	Glenn-7	A/4, B/3	7
Manzanillo	Glenn-8	A/4	4
Manzanillo	Glenn-9	A/1, B/3, F/2	6
Manzanillo	Tehama-3	A/3, B/2	5
Arbequina	Tehama-4	A/3, B/1, D/1	5
Sevillano	Tehama-5	A/2, B/2, G/1	5
Manzanillo	Glenn-10	B/4, H/1	5
Arbosana	Yuba-3	A/4	4
Arbequina	Yuba-4	A/2	2
Arbequina	Yuba-5	A/4, I/1	5
Arbequina	Yuba-6	A/4	4
Manzanillo	Glenn-11	A/5, B/6	11
Sevillano	Glenn-12	A/1, B/6, H/1, J/1	9
Manzanillo	Yolo-1	B/3	3
Sevillano	Yolo-1	B/1	1
Arbosana	Yolo-1	B/1	1
	Total	A/86, B/55, C/1, D/2, E/1, F/2, G/1, H/2, I/1, J/1	152

[1] - Fingerprint patterns are based on rep-PCR using BOX, ERIC, and REP primers.
[2] - The A and B designations correspond to the two major groups identified in Fig. 2.3, whereas the remaining letters C through J represent the remaining genotypes (not shown in Fig. 2.3).

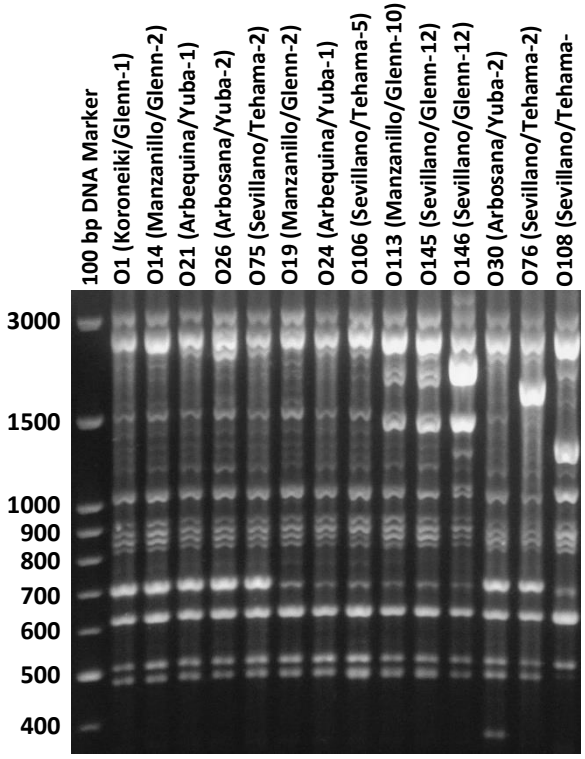

Fig. 2.1. Agarose gel electrophoresis of PCR amplification products of 14 strains of *P. savastanoi* pv. *savastanoi* using primers REP1RI and REP2-I.

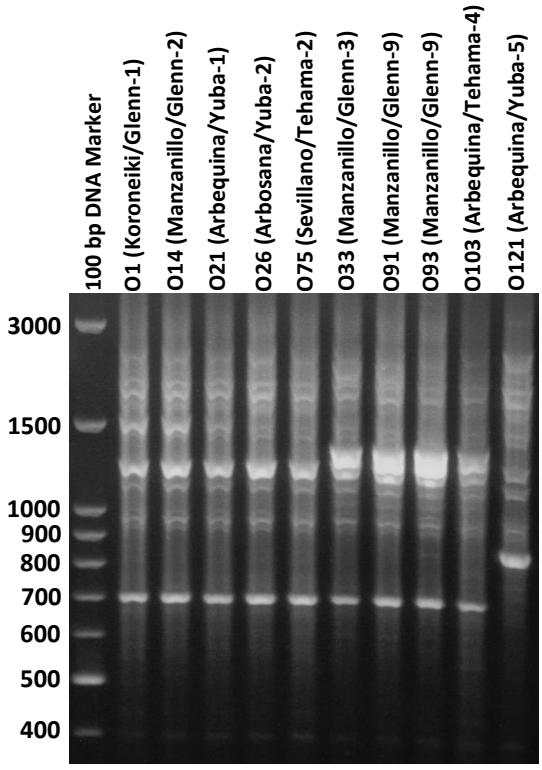

Fig. 2.2. Agarose gel electrophoresis of PCR amplification products of 10 strains of *P. savastanoi* pv. *savastanoi* using primer BOXA1R.

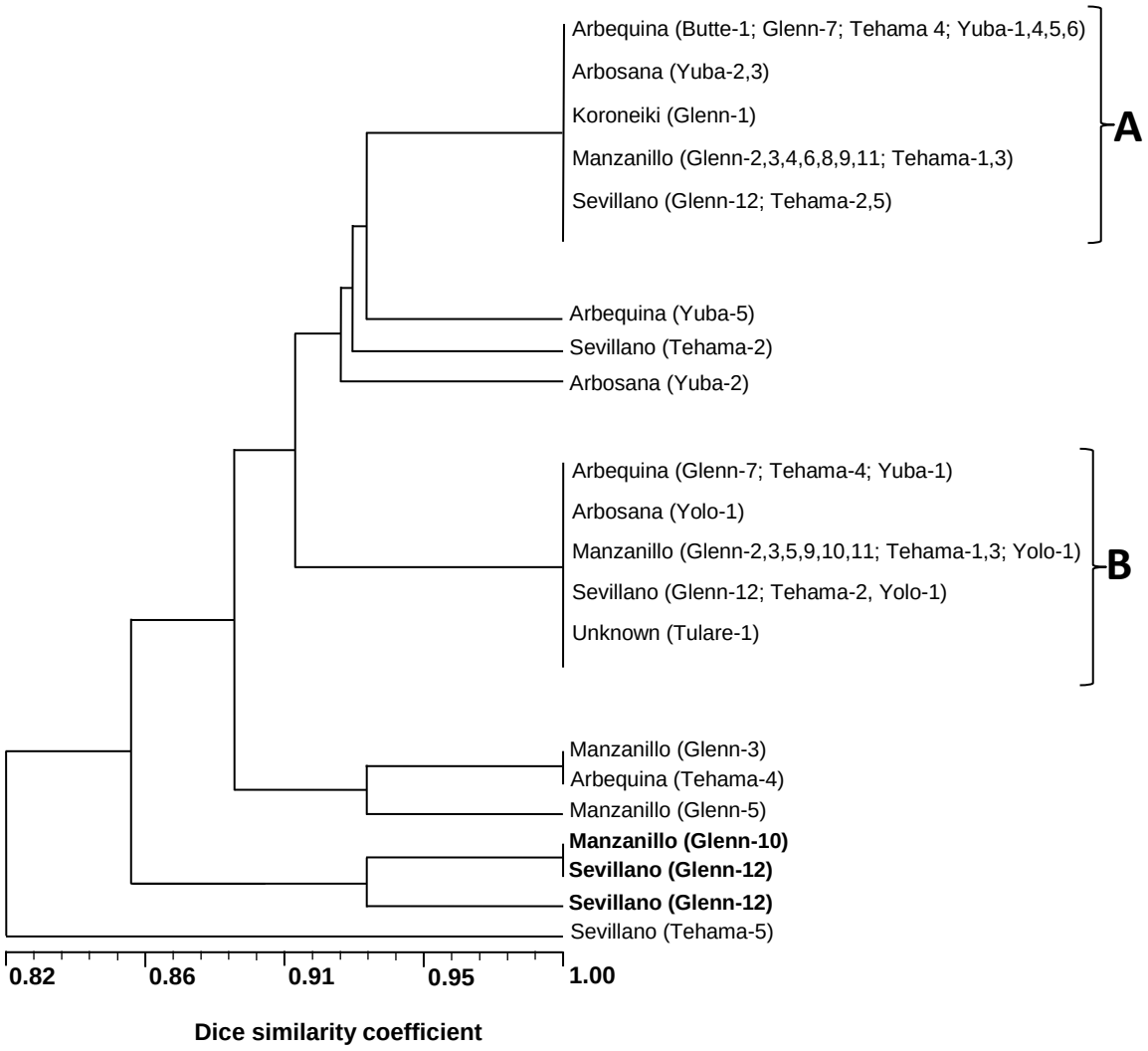

Fig. 2.3. UPGMA clustering of 152 *Pseudomonas savastanoi* pv. *savastanoi* strains from olives in California. Similarity is based on the combined electrophoresis banding patterns of REP, BOX, and ERIC primer sets using Dice's coefficient and UPGMA analysis. A and B indicate the 2 major groups of banding patterns identified. Olive cultivars and locations in bold indicate copper-resistant strains.

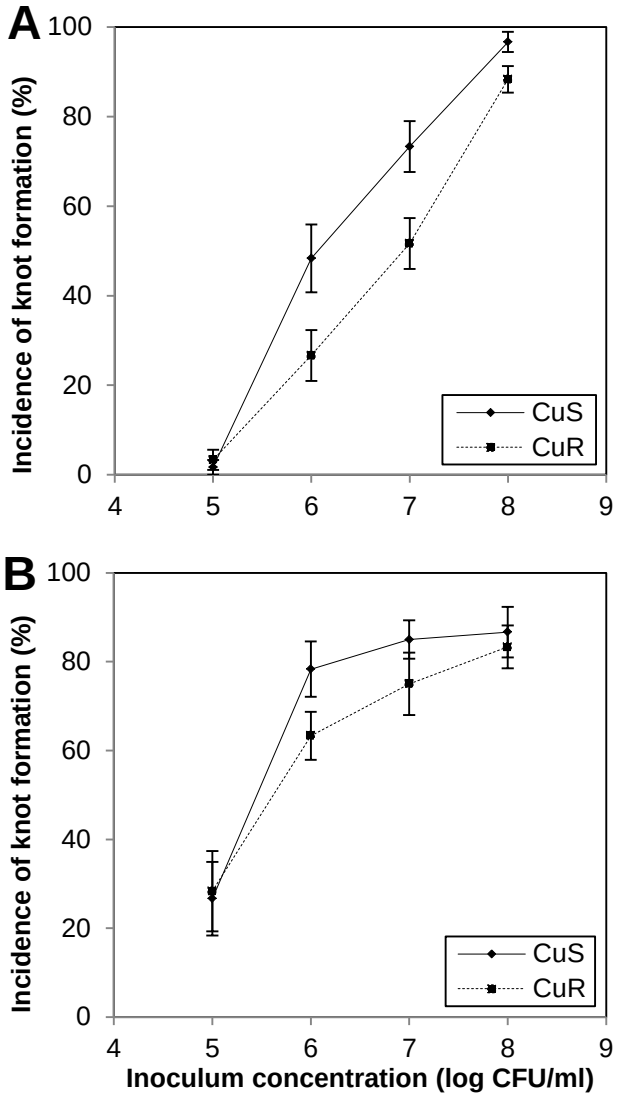

Fig. 2.4. Effect of inoculum concentration of *P. savastanoi* pv. *savastanoi* strains O26 (copper-sensitive, CuS) and O113 (copper-resistant, CuR) on incidence of knot formation on **A**, leaf scars and **B**, lateral wounds of 'Arbequina' olive. Data points are the means of two experiments. Bars indicate the standard error.

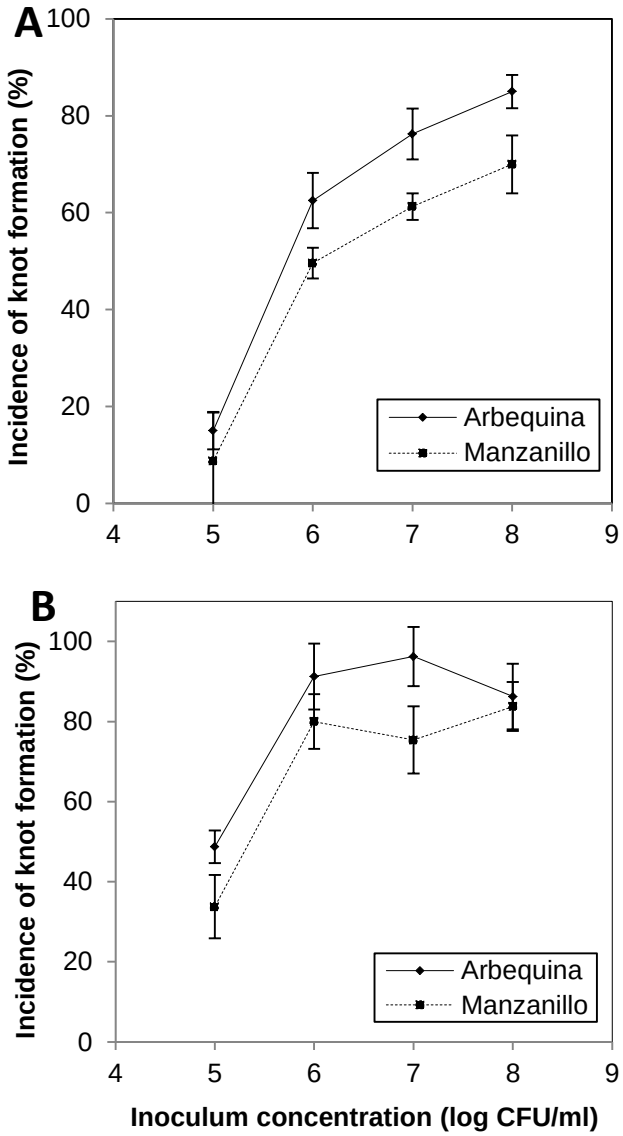

Fig. 2.5. Effect of inoculum concentration of *P. savastanoi* pv. *savastanoi* strain O26 (copper-sensitive, CuS) on incidence of knot formation on **A**, leaf scars and **B**, lateral wounds of 'Arbequina' and 'Manzanillo' olive. Data points are the means of two experiments. Bars indicate the standard error.

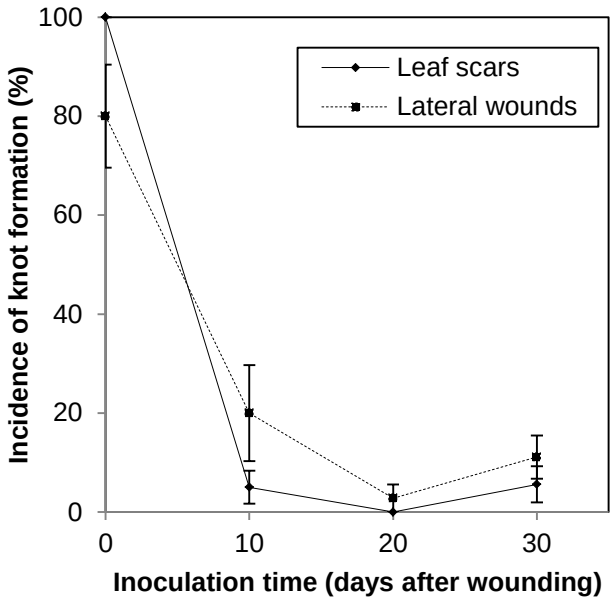

Fig. 2.6. Effect of wound healing duration of leaf scar and lateral wounds of 'Manzanillo' olive on incidence of knot formation after inoculation with *P. savastanoi* pv. *savastanoi* strain O26 (copper-sensitive). Data points are the means of two experiments. Bars indicate the standard error.

CHAPTER III. QUATERNARY AMMONIUM COMPOUNDS AS NEW SANITIZERS FOR REDUCING THE SPREAD OF THE OLIVE KNOT PATHOGEN ON ORCHARD EQUIPMENT

ABSTRACT

Olive knot, caused by the wound pathogen *Pseudomonas savastanoi* pv. *savastanoi*, is a serious bacterial disease that can be disseminated by orchard equipment. Greenhouse studies confirmed that cutting tools contaminated during contact with olive knots are able to disseminate the pathogen to healthy olive tissue. Quaternary ammonium compounds (QACs) were assessed as sanitizing agents for contaminated equipment as a disease management strategy. In laboratory in vitro tests, QACs exhibited high toxicity against the bacterium over a broad pH range from 6 to 9 using short exposure periods (15 to 60 s) and low concentrations (5 µg/ml). QACs applied to contaminated hard surfaces in the presence of an organic load reduced bacterial recovery by $\geq$3.56 $\log_{10}$ CFU/ml. In field trials, sanitation of hedging equipment that was contaminated with the pathogen (2×10^7 CFU/ml) and that was used to prune olives, was successful and sometimes completely prevented new infections from occurring. Application of additional foliar spray treatments of copper or copper-kasugamycin mixtures after hedging significantly improved disease control. In laboratory and field studies, sodium hypochlorite was significantly less effective than QAC compounds in the presence of organic matter. A non-phenolic QAC formulation, however, was ineffective as a preventative treatment when applied prior to inoculation of olive wounds, whereas a copper hydroxide

application was highly effective. Based on data from this research, a QAC formulation was registered for field use as a sanitizer for olive equipment in California in 2015.

INTRODUCTION

Olive knot caused by the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (Gardan et al. 1992) is a disease that has afflicted olive trees (*Olea europaea* L.) since ancient times (Iacobellis 2001) and was likely introduced to California during the early 19th century (Bioletti 1898). The disease is of economic importance because infections result in a decline in tree health, reduced yield, and potentially decreased olive oil quality (Schroth et al. 1963, 1973; Young 2004). Orchards with severe disease are often removed entirely. *P. savastanoi* pv. *savastanoi* is a wound-infecting pathogen that incites the development of knots or galls after invading plant injuries (Wilson 1935; Ramos et al. 2012). Knots serve as the primary inoculum source, releasing copious amounts of bacteria when wetted. Rainfall promotes the dissemination of the bacterium (Horne et al. 1912), but new olive production practices provide an additional dissemination mechanism in the absence of rain. These new methods utilize high-density plantings of olives in hedgerows that are mechanically pruned and harvested (Vossen 2007; Ferguson et al. 2010). Field equipment coming in contact with infected tissues (knots) often become contaminated with *P. savastanoi* pv. *savastanoi* (Wilson 1935). The equipment can subsequently spread the pathogen to healthy trees as it continues to process the hedgerow while also creating injuries necessary for infection (Tous et al. 2010). The

disease can spread rapidly in an orchard from a single infected tree using these types of cultivation practices. Over time, build-up of inoculum will contribute to a decline in orchard health and productivity (Quesada et al. 2010a; Schroth et al. 1973).

Growing consumer demands for olive-based food products with their benefits on human health have led to an increase in production. With the rising cost of labor, growers are relying more on mechanized practices to provide an adequate supply of olives while staying profitable (Vossen 2007; Tous et al. 2010). Eradication of olive knot is nearly impossible once the disease becomes established in an orchard. Management strategies include removal of infected branches by pruning and the use of foliar sprays with copper-based compounds after pruning and harvesting (Teviotdale and Krueger 2004; Quesada et al. 2010b). Copper is a protective treatment that is most effective when applied to plants before infection by the pathogen. Other chemical control options include the manual application of cresol- and xylenol-based compounds (e.g., Gallex) to knots, but this is not feasible on a commercial scale (Schroth and Hildebrand, 1968; Sibbett and Ferguson 2005). The lack of olive cultivars that show resistance to the disease contributes to the problem (Penyalver et al. 2006). Therefore, new management strategies are necessary.

One way to reduce the spread of olive knot could be to routinely sanitize field equipment during usage, and this was evaluated in our research. Among sanitizers that are currently available for commercial use, sodium hypochlorite can be highly effective, but is corrosive to equipment. Quaternary ammonium compounds (QACs) were developed in the 1930s and have been widely used as sanitizers to disinfect food and non-food contact surfaces (Rahn and Van Eseltine 1947). They have been successfully

employed in domestic, agricultural, and industrial applications, as well as in healthcare. QACs are cationic surfactants that are highly toxic against fungi, bacteria, and viruses. The mode of action of QACs against bacteria involves the disruption of the cell membrane through the interaction of the positively charged QAC with the phospholipid bilayer leading to cell leakage and death (Gilbert and Moore 2005). QACs have many beneficial properties that include being colorless, odorless, non-corrosive to metal surfaces, and they are temperature-stable. These compounds are effective in hard water and in the presence of organic matter, and have low mammalian toxicity (Walker and LaGrange 1991). QACs are currently registered as field equipment sanitizers in Florida to prevent the spread of the citrus canker pathogen *Xanthomonas citri* ssp. *citri* (Schubert and Sun 2003) and were shown to be effective in decontaminating some citrus plant surfaces (Bock et al. 2011). These attributes make QACs excellent candidates for use as disinfectants of field equipment to control olive knot. Therefore, the objectives of this study were to evaluate: 1) The toxicity of QACs to *P. savastanoi* pv. *savastanoi* in vitro as compared to other sanitizers including sodium hypochlorite; 2) The effectiveness of QACs and sodium hypochlorite in sanitizing selected equipment surfaces in lab and field trials; and 3) The performance of a QAC formulation as a field treatment for disease prevention.

MATERIALS AND METHODS

Culturing of *P. savastanoi* pv. *savastanoi* and of olive plants in the greenhouse. *P. savastanoi* pv. *savastanoi* strain O1-26, originally isolated from an olive knot in California and positively identified by PCR using primers IAALF and IAALR (Penyalver et al. 2000), was used in all studies. Bacterial stocks were stored in 15% glycerol at -80°C and cultured at 25°C on King's medium B agar (KMB; King et al. 1954) for 2 days before each study. 'Arbequina' and 'Manzanillo' olives (Agromillora California, Gridley, CA) were grown in sterilized soil in 3.78-liter plastic pots under greenhouse conditions at 15 to 30°C. In field trials, the same olive cultivars were grown in a high-density planting system (Vossen 2007) to replicate commercial production at the University of California, Riverside and Davis field stations.

Spread of *P. savastanoi* pv. *savastanoi* from contaminated equipment to healthy olive tissues in greenhouse pruning studies. In order to determine if tools (e.g., scalpels) that were contaminated from cutting diseased tissue (i.e., olive knots) could transmit *P. savastanoi* pv. *savastanoi* to healthy tissue, twigs of 2- to 3-year-old 'Arbequina' olives were inoculated one year in advance to produce the required knots. Knot development was initiated by removing a small section of bark (approximately 2 to 3 mm x 5 to 8 mm) with a scalpel, exposing a portion of cambial tissue. *P. savastanoi* pv. *savastanoi* inoculum was made by suspending 2-day-old cultures in sterile water and adjusted turbidimetrically (OD_{600nm} = 70% light transmittance) to obtain 2 $x10^8$ colony forming units (CFU) per ml. This bacterial suspension was diluted with water (1:20) to a

final inoculum concentration of 10^7 CFU/ml and sprayed onto wounded olive twigs using a hand-held sprayer. For inoculation, a sterile scalpel was contaminated by making a cut through an olive knot (5 to 10 mm in diameter) with a single pass. Subsequently, the scalpel was used to make a small V-shaped notched cut into twigs (1 to 2 years of age) of healthy 3- to 4-year-old 'Arbequina' or 'Manzanillo' olive plants, removing a 5-mm long piece of bark to a depth where cambial tissue was exposed. Between each contamination-inoculation sequence, the blade was sterilized with 70% ethanol. This process was repeated 10 times on each twig, and one twig of each of three plants was used (a total of 30 contamination-inoculation events). In similar tests, scalpel blades were either moistened with water before cutting into a knot followed by wounding a healthy twig; or knots were moistened with water, cut with a dry blade, and then the blade was used to wound a healthy twig. Positive controls were made by dipping a scalpel into a *P. savastanoi* pv. *savastanoi* suspension (10^8 CFU/ml) before wounding a healthy twig, and negative controls were made by wounding healthy olive twigs with a sterile scalpel. Plants were evaluated for knot development after three months. These studies were performed twice.

Direct toxicity of selected sanitizing agents against *P. savastanoi* pv. *savastanoi*. Sanitizers evaluated included QAC-1 containing a mixture of 5% n-alkyl dimethyl benzyl ammonium chlorides and 5% N-alkyl dimethyl ethylbenzyl ammonium chlorides (Deccosan 315; Decco US Post-Harvest, Inc., Monrovia, CA), QAC-2 containing a mixture of 6.51% alkyl dimethylbenzyl ammonium chloride, 3.255% didecyl dimethyl ammonium chloride, 3.255% octyl decyl dimethyl ammonium chloride,

and 8.68% dioctyl dimethyl ammonium chloride (Deccosan 321; Decco US Post-Harvest), polyhexamethylene biguanide hydrochloride (PHMB; Vantocil IB; Arch Chemicals, Inc., Atlanta, GA), and chlorhexidine acetate (Nolvasan; Fort Dodge Animal Health, Fort Dodge, IA). These were compared to sodium hypochlorite (household bleach containing 5.25% sodium hypochlorite). Solutions of sanitizing agents (10 µg total active ingredients/ml) were mixed 1:1 with a *P. savastanoi* pv. *savastanoi* suspension (2×10^8 CFU/ml) to obtain a final concentration of the sanitizing agents of 5 µg/ml in a total volume of 500 µl. Mixtures were gently vortexed and incubated for 60 s. Immediately following exposure, a 1:1000 dilution was made with sterile distilled water to stop the biocidal activity of the sanitizing agents, and the dilution was plated onto 10-cm KMB agar plates using a spiral plater (Autoplate 4000, Spiral Biotech, Inc., Norwood, MA). To confirm that diluted sanitizer solutions no longer had activity against *P. savastanoi* pv. *savastanoi*, bacterial suspensions were incubated in sanitizer solutions at final concentrations of 5 ng/ml for 30 min before plating. Plates were evaluated for bacterial growth after 3 to 4 days at 25°C, and colonies were enumerated. Sanitizer efficacy was determined as the $\log_{10}$ reduction in CFU/ml of the sanitizer treatment as compared to the water control.

In another test, bacterial survival was determined after selected exposure durations to QAC-2. For this, mixtures of QAC-2 and *P. savastanoi* pv. *savastanoi* were prepared as described above with a final QAC-2 concentration of 5 µg/ml. Mixtures were lightly vortexed, incubated for 15, 30, 45, or 60 s, and plated as described previously. The toxicity of QAC-2 was also tested at selected pH conditions. A citric acid buffer was

prepared containing 1.3 g anhydrous citric acid, 1.9 g glycine, and 1.9 g monobasic potassium phosphate in 50 ml of distilled water (Lucas 1955). A mixture of 1.9 ml sterilized buffer and 18.9 ml of sterile distilled water was adjusted to pH 5, 6, 7, 8, or 9 ± 0.1 with 1 N NaOH. Suspensions of *P. savastanoi* pv. *savastanoi* and solutions of QAC-2 (25 µg/ml) were prepared in each of the buffer solutions and mixed using components of the same pH. For the controls, *P. savastanoi* pv. *savastanoi* suspensions were mixed with the respective buffer solutions without QAC. After 60 s of incubation, suspensions were diluted 1:1000 with sterile distilled water, and viable *P. savastanoi* pv. *savastanoi* cells were enumerated by plating as described above. For each treatment in these studies, there was a minimum of 3 replicates, and the experiments were done twice except for the exposure duration study that was performed three times. Sanitizer efficacy was determined as the $\log_{10}$ reduction in CFU/ml by the sanitizer treatment as compared to the untreated control.

Efficacy of two QAC formulations and sodium hypochlorite in disinfecting hard surfaces contaminated with *P. savastanoi* pv. *savastanoi* in macerated olive tissue. Young, succulent, greenhouse-grown 'Arbequina' olive twigs (50 g) were macerated in 250 ml distilled water using a Waring blender for 60 s. The macerate was strained through a 0.34-mm mesh screen, and 50 g of solids were collected in a glass beaker. A bacterial suspension (25 ml) containing 2×10^8 CFU/ml was added to the solids, and mixed thoroughly. Sterilized segments of polyvinyl chloride (PVC) piping (1.2 cm in diameter, 1 cm long) were added to the macerated tissue-bacteria mixture and stirred with a metal spatula until the PVC segments were well coated with macerated

tissue. They were then placed onto a metal screen and sprayed with an aqueous solution of QAC-1, QAC-2 (both at 2,000 µg/ml), sodium hypochlorite (100 µg/ml), or water (control) using an atomizer (DeVilbiss 15-RD, Sunrise Medical, Inc., Somerset, PA) until runoff. After 90 s, each PVC segment was placed into a 50-ml plastic tube containing 5 ml of sterile distilled water and vortexed for 10 s. The suspension was then spiral-plated onto KMB as described for the direct toxicity assays.

Performance of QAC-2 as a sanitizer for pruning equipment contaminated with *P. savastanoi* pv. *savastanoi* in field studies. In springtime and fall studies, the hedging teeth of a handheld gas-powered hedger (Model HL 40, Stihl, Inc., Virginia Beach, VA) were thoroughly sprayed with a suspension of *P. savastanoi* pv. *savastanoi* (1×10^7 CFU/ml) and then with QAC-2 (2,000 µg/ml) or sodium hypochlorite (50 µg/ml) to runoff using a hand-held sprayer. After 90 s, 3- to 4-year-old 'Arbequina' or 'Manzanillo' olive trees were pruned to create lateral cuts on larger limbs and terminal stub cuts of smaller branches. Pruning of olive branches with a non-inoculated hedger was used as a negative control and pruning with a contaminated non-sanitized hedger as a positive control. In some cases, trees wounded with a QAC-2-sanitized hedger received additional applications of foliar spray treatments 1 to 2 h after hedging using an airblast sprayer (Model 420, Stihl). These treatments included copper hydroxide (Kocide 3000; Du Pont de Nemours and Co., Wilmington, DE) at 4,200 µg/ml (1,260 µg/ml metallic copper equivalent) or copper hydroxide 4,200 µg/ml mixed with kasugamycin 100 µg/ml (Kasumin 2L; Arysta LifeScience, Cary, NC). The trials were done using a randomized

complete block design (RCBD) with four single-tree replications per treatment. Trials were performed twice for each olive cultivar.

Efficacy of QAC-3 as a pre-infection, protective treatment for olive injuries in field studies. One- to two-year old twigs (5 to 15 mm in diameter) of 3- to 4-year old 'Manzanillo' or 'Arbequina' trees were wounded with two types of injuries: lateral wounds to replicate mechanical damage sustained from field equipment, and leaf scar wounds to simulate wounds formed after natural leaf drop. Lateral wounds were produced using a scalpel to cut parallel to the length of a twig so that cambial tissue 10 to 20 mm long and several mm wide was removed. For leaf scars, olive leaves were pulled off by hand. Each twig represented one replicate with 5 lateral wounds near the base of the twig (one wound between each leaf internode) followed by 5 leaf scar wounds at consecutive internodes. QAC-3 (7.5% didecyl dimethyl ammonium chloride; KleenGrow; Pace 49, Inc., Delta, B.C., Canada) was evaluated that is labeled for ornamental crops as a pesticide to be applied directly to plant tissue or as an equipment sanitizer. The sanitizer solution was prepared at the maximum labeled rate by mixing 11.2 ml of QAC-3 in 3.78 liters of water (222 µg active ingredient/ml), sprayed with a handheld sprayer on freshly made olive twig wounds until runoff, and allowed to dry. *P. savastanoi* pv. *savastanoi* (1×10^8 CFU/ml) was sprayed onto wounds to run-off. Positive controls included twigs that were wounded, inoculated, and treated with water. A treatment of copper hydroxide (4,200 µg/ml) was included as a negative control. Six to eight replicates were used per trial, and the study was done three times using a RCBD.

Statistical analysis of data. Data were analyzed using SAS software (version 9.4, SAS Institute, Inc., Cary, NC). For direct contact and hard surface disinfectant assays, recovery or reduction of bacteria was expressed as $\log_{10}$ CFU/ml and was analyzed using an analysis of variance (ANOVA). Treatment means were separated using Fisher's least significant difference (LSD) test. For direct contact assays, the loss of biocidal activity of each sanitizer after a 1:1000 dilution (done to neutralize the sanitizer before plating) as compared to the water control was verified using ANOVA and LSD tests. For field and greenhouse tests, percent incidence of disease and percent reduction in disease incidence values were arcsine transformed before performing general linear model (GLM), and LSD analyses.

RESULTS

Spread of *P. savastanoi* pv. *savastanoi* from contaminated equipment to healthy olive tissues in greenhouse pruning studies. Transmission of *P. savastanoi* pv. *savastanoi* and knot formation occurred when a scalpel was used to make an initial cut into an olive knot followed by a cut into a healthy olive twig (Fig. 3.1A, 3.1C, 3.1D). Overall, disease incidence was lower on 'Arbequina' as compared to 'Manzanillo' olives, but knots developed under all conditions evaluated except for the negative control (Fig. 1B). Water applied to a knot or scalpel before making the initial contamination cut significantly ($P < 0.0001$) increased the incidence of knot formation on previously healthy twigs of 'Arbequina' and 'Manzanillo' olives (Table 3.1).

Direct toxicity of selected sanitizing agents against *P. savastanoi* pv. *savastanoi*. There was no significant difference ($P = 0.4333$) in the number of colonies recovered when *P. savastanoi* pv. *savastanoi* was incubated in water or in solutions of QAC-1, QAC-2, chlorhexidine, or PHMB that were diluted 1:1000 with sterile water (i.e., a final concentration of 5 ng sanitizer/ml). Thus, this dilution of the solutions after incubation in the assay was adequate to neutralize their activity before viability of *P. savastanoi* pv. *savastanoi* was assessed. For sodium hypochlorite, however, the 1:1000 dilution did not completely quench the biocidal activity. This dilution was still effective and significantly ($P < 0.0001$) lowered the bacterial populations from the control in contrast to the other diluted sanitizers.

The mean number of bacteria recovered for untreated controls was 1.25×10^5 CFU/ml (i.e., 5.1 $\log_{10}$). QAC-1, QAC-2, chlorhexidine, and PHMB were all highly effective in inactivating *P. savastanoi* pv. *savastanoi* using concentrations of 5 µg/ml and 60-s exposures. There was no significant difference ($P = 0.6018$) in activity among the sanitizers, and the number of colonies was significantly ($P < 0.0001$) reduced from the water control by 3.2, 3.3, 3.6, and 3.8 $\log_{10}$ for QAC-1, QAC-2, chlorhexidine, and PHMB, respectively. Sodium hypochlorite at 5 µg/ml completely inhibited bacterial growth after 60 s of exposure and was significantly more effective than that of the other four sanitizer treatments ($P < 0.0001$).

A range of treatment exposure times was evaluated for QAC-2 at 5 µg/ml, and there were no significant differences ($P = 0.2811$) for 15-, 30-, 45-, or 60-s durations (Fig. 3.2). Numerically, the 15-s exposure reduced the number of *P. savastanoi* pv.

savastanoi colonies by 2.3 $\log_{10}$ as compared to a 3.3-$\log_{10}$ reduction after a 60-s exposure. In evaluating the effect of pH on QAC-2 efficacy, QAC-2 in a solution of pH 5 was significantly ($P = 0.0018$) less effective with a mean reduction in colonies of 0.5 $\log_{10}$ as compared to pH values of 6, 7, 8, or 9. These latter solutions resulted in mean log reductions of $\geq$3.5 $\log_{10}$, and there were no significant differences (Fig. 3.3). A 1:1000 dilution of a 25-µg/ml QAC-2 solution with water was adequate for quenching its activity. There was no significant difference ($P = 0.2372$) in the number of bacterial colonies recovered between diluted QAC-2 (25 ng/ml) and water treatments.

Efficacy of two QAC formulations and sodium hypochlorite in disinfecting hard surfaces contaminated with *P. savastanoi* pv. *savastanoi* in macerated olive tissue. QAC-1 and QAC-2 solutions at 2,000 µg/ml were highly and significantly ($P < 0.0001$) effective in disinfecting *P. savastanoi* pv. *savastanoi*-contaminated PVC piping in the presence of an organic load. After sanitizing contaminated PVC pipes with QAC-2 or QAC-1, 1.22 or 1.42 $\log_{10}$ bacteria cells were recovered, respectively, with no significant difference between the two sanitizing formulations. In contrast, sodium hypochlorite was not effective in the presence of organic matter, with no significant difference in the number of bacterial colonies recovered as compared to the control (Fig. 3.4).

Performance of QAC-2 as a sanitizer for pruning equipment contaminated with *P. savastanoi* pv. *savastanoi* in field studies. Sanitation of a *P. savastanoi* pv. *savastanoi*-contaminated hedger with sodium hypochlorite (50 µg/ml) or QAC-2 effectively and significantly ($P \leq 0.0004$) reduced the incidence of disease on

‘Arbequina’ and ‘Manzanillo’ olives in springtime and fall studies as compared to not sanitizing the hedger before pruning a healthy olive tree (Fig. 3.5A, 3.5B and 3.5C). QAC-2 was significantly ($P \leq 0.0373$) more effective than sodium hypochlorite in reducing knot formation on pruned trees in two of the four studies. Overall, reduction of knot formation from the control was 47.6% to 79.1% for sodium hypochlorite and 54.8% to 100% for QAC-2. When the hedger QAC-2 sanitation treatment was followed by a foliar application with copper or copper-kasugamycin, knot formation was sometimes further reduced as compared to the hedger sanitation alone. Additionally, copper and copper-kasugamycin applications were equally effective (Table 3.2). In these experiments, no disease was observed on pruning wounds created with a hedger that was not contaminated with the pathogen.

Efficacy of QAC-3 as a pre-infection, protective treatment for olive injuries in field studies. Applications of copper hydroxide as a pre-infection protective treatment to lateral and leaf scar wounds significantly reduced the incidence of knot formation on ‘Arbequina’ ($P < 0.0001$) and ‘Manzanillo’ ($P \leq 0.0152$) olives as compared to the controls (Table 3.3). The incidence of knot formation in the controls was 70.0% to 92.5% on leaf scar wounds and 76.3% to 97.5% on lateral wounds, respectively for the two cultivars. Copper hydroxide treatments reduced the incidence to 3.8% to 51% and 10.3% to 65%, respectively. In contrast, treatments with QAC-3 were not effective and there was no significant difference in knot formation from the controls.

DISCUSSION

In this study we demonstrated for the first time that QACs are effective sanitizing agents for *P. savastanoi* pv. *savastanoi*-contaminated field equipment and that sanitation can be a viable management strategy to reduce pathogen dissemination in mechanized olive production. With a limited number of effective chemical treatments available and lack of olive knot-resistant cultivars, disease prevention remains an important aspect in successful olive cultivation (Penyalver et al. 2006). 'Arbequina' and 'Manzanillo' olives were used in our studies because they represent major oil and table cultivars planted in California, respectively. Based on our research, QAC-2 (i.e., Maquat 615-HD or Deccosan 321) was recently approved in California for field use on olive equipment at rates up to 2,000 µg/ml as a Special Local Need (Section 24c) registration.

Several sanitizing agents showed high in vitro toxicity to *P. savastanoi* pv. *savastanoi,* but QACs were chosen for additional laboratory and field studies because agricultural formulations currently exist for other crops such as citrus (Schubert and Sun 2003) that potentially could be registered for use on olives. In direct exposure laboratory assays, sodium hypochlorite was also highly effective. The sanitizers were compared at equivalent active ingredient concentrations (i.e., 5 mg/liter), but the dilution method for quenching in the assay that was adequate for four of the five sanitizers, did not completely eliminate the biocidal activity of sodium hypochlorite. This could have resulted in a slightly over-estimated activity of sodium hypochlorite because the test suspension was plated approximately 30 min after the dilution. Although sodium

hypochlorite is very effective and of low cost, there are several disadvantages with this sanitizer. Sodium hypochlorite is highly corrosive and would cause unacceptable damage to very expensive olive harvesting and pruning equipment. Furthermore, its activity is compromised in the presence of organic material and alkaline environments, and is generally applied at low rates (i.e., 50 to 100 µg/ml) when non-labeled bleach formulations are employed. Currently, there are no other labels of sodium hypochlorite that allow high rates on field equipment. An additional benefit of QACs is that treated surfaces only need to be air-dried in contrast to many other sanitizers that require rinsing.

Previous research by others indicated that *P. savastanoi* pv. *savastanoi* can be disseminated by pruning tools that were contaminated by bacterial suspensions (Wilson, 1935). Our research further confirmed this mechanism of disease spread to healthy trees using naturally produced inoculum from olive knots. Additionally, we demonstrated that the pathogen can be spread by tools in the absence of externally applied wetness. Still, wetness applied to knots or cutting tools resulted in a significant increase in knot development stressing the importance of pruning olives only during dry periods.

In our studies, QAC-2 remained effective over a pH range from 6 to 9, a valuable property when considering that the pH of water sources used for preparing tank mixes in the field can differ by location, and ground water in the main agricultural areas of California is commonly alkaline.

In hard surface, laboratory disinfectant assays, two QAC formulations were challenged against *P. savastanoi* pv. *savastanoi* under conditions that simulated the environment encountered during harvest. Both formulations reduced the number of viable

bacteria recovered from the contaminated PVC piping to low levels although higher concentrations of the sanitizers and longer exposures had to be used in this assay as compared to direct exposure assays. Sodium hypochlorite at 100 µg/ml was not efficacious in the presence of high amounts of organic material. PVC piping was utilized as a hard surface in this assay because automated harvesters commonly use this material as 'beater bars' to remove fruit from trees. These surfaces need to be quickly and effectively disinfected to accomplish orchard practices in a timely manner, and this can be achieved by QAC sanitation.

To evaluate QAC efficacy against olive knot under field conditions, a gas-powered hedger was used to prune 'Arbequina' and 'Manzanillo' olive trees. QAC-2 rates and exposure durations were based on in vitro results and the registrant's recommendations. Sodium hypochlorite was included for comparison at a typical usage rate of 50 µg/ml. The sanitizers displayed good activity even when the hedger was contaminated with a high inoculum concentration (2×10^7 CFU/ml). Sanitation with sodium hypochlorite was effective in this experiment with a minimum amount of contamination with organic material based on the sequence of contamination, treating, and hedging. QAC-2 treatments, however, performed significantly better in most studies (Table 2). Cutting wounds made with a QAC-2-sanitized hedger that were treated with copper hydroxide or copper hydroxide-kasugamycin mixtures one to two hours afterwards further improved control. These results indicate that applications of a sanitation treatment in combination with a foliar spray treatment can help prevent a majority of infections from occurring.

This study also examined the possibility of using a QAC (e.g., QAC-3) as a protective treatment on olive wounds. The non-phenolic QAC formulation was chosen because it is labeled for use on ornamental crops as a pesticide and disinfectant. The QAC-3 treatment proved to be ineffective against *P. savastanoi* pv. *savastanoi* when used to treat two types of wounds on two olive cultivars before inoculation. Field rates were used at the maximum label, and higher rates were not tested due to concerns of phytotoxicity. In contrast, a standard treatment of copper hydroxide was effective to highly effective in these studies.

In summary, the newly registered QAC-2 was a highly effective sanitizer of hard surfaces and orchard equipment in the presence of organic matter in laboratory and field studies. Sanitation practices are an important aspect of olive knot disease management (Quesada et al. 2010b; Teviotdale and Krueger 2004). Growers currently treat olives with a foliar application of copper after harvest and pruning as a standard practice. Integration of copper sprays with an equipment sanitation treatment will further improve disease prevention.

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Table 3.1. Transmission of *Pseudomonas savastanoi* pv. *savastanoi* by contaminated cutting tools to healthy 'Manzanillo' or 'Arbequina' olive plants in greenhouse studies

Contamination-inoculation method[a]	**Incidence of knot formation on twig wounds (%)**[b]	
	'Manzanillo' olive	**'Arbequina' olive**
Scalpel contaminated with bacterial suspension	96.7 a	100 a
Scalpel single pass cut through wetted knot	55.0 b	23.3 b
Wetted scalpel single pass cut through non-wetted knot	58.3 b	28.3 b
Scalpel single pass cut through non-wetted knot	20.0 c	5 bc
Sterile scalpel	0.0 d	0 c

[a] For each method, a sterile scalpel was treated as indicated and subsequently used to make a notched cut into a healthy olive twig. Contamination with *P. savastanoi* pv. *savastanoi* was done using a suspension of 10^8 CFU/ml.

[b] Incidence of disease was recorded after two months and is based on the number of knots that formed of the total number of wounds made with each contamination-inoculation method. Data were analyzed using general linear model (GLM) and Fisher's LSD tests ($P \leq 0.05$). Mean values followed by same letters are not significantly different for each cultivar.

Table 3.2. Efficacy of sodium hypochlorite and a quaternary ammonia formulation (QAC-2) for sanitizing field equipment contaminated with *Pseudomonas savastanoi* pv. *savastanoi* before pruning 'Manzanillo' or 'Arbequina' olives in field studies

Sanitization treatment[a]		Foliar treatment[b]		Reduction in knot incidence (%)[c]			
				Manzanillo		Arbequina	
Sanitizer	Rate (µg/ml)	Bactericide	Rate (µg/ml)	Spring 2015	Fall 2015	Spring 2015	Spring 2016
NaOCl	50	None	---	79.1 b	47.6 b	71.4 b	62.3 b
QAC-2	2,000	None	---	100 a	54.8 b	87.3 ab	90.6 a
QAC-2	2,000	Copper hydroxide (CH)	4,200	100 a	90.5 a	95.2 a	96.2 a
QAC-2	2,000	CH + kasugamycin	4,200 + 100	100 a	85.7 a	93.7 a	96.2 a

[a]A gas-powered hedger was contaminated with *P. savastanoi* pv. *savastanoi* (2×10^7 CFU/ml), sanitization treatments were applied to runoff, and after 90 s, the hedger was used to prune healthy olive twigs and branches. NaOCl = sodium hypochlorite.
[b] Additional foliar sprays were applied using a back-pack sprayer at 933 liters/ha.
[c] Evaluations were done after 7 to 9 months, and the number of knots that developed after sanitation on pruning wounds was enumerated and compared to that developing without sanitation. Values followed by the same letter are not significantly different based on general linear model (GLM) and Fisher's LSD tests ($P \leq 0.05$).

Table 3.3. Efficacy of a non-phenolic quaternary ammonium formulation and copper hydroxide in reducing knot development after field inoculation of leaf scar and lateral twig wounds of two olive cultivars

Cultivar	Treatment[a]	Rate (µg/ml)	Incidence of knot formation (%)[b]			
			2014		2015	
			Leaf scars	Lateral wounds	Leaf scars	Lateral wounds
Arbequina	Water control	---	92.5 a	97.5 a	85.0 a	95.0 a
	QAC-3	222	90.0 a	97.5 a	97.5 a	100.0 a
	Copper hydroxide	4,200	3.8 b	10.3 b	30.0 b	52.5 b
Manzanillo	Water control	---	71.3 a	76.3 a	77.8 ab	96.0 a
	QAC-3	222	71.3 a	79.1 a	83.0 a	100.0 a
	Copper hydroxide	4,200	8.0 b	13.3 b	51.0 b	65.0 b

[a] Spray treatments with QAC-3 (didecyl dimethyl ammonium chloride) were applied to leaf scar and lateral twig wounds in October 2014 or April 2015 using a hand-held sprayer. After air-drying, wounds were spray-inoculated with *Pseudomonas savastanoi* pv. *savastanoi* (2×10^8 CFU/ml).

[b] Knot formation was evaluated after 7 to 9 months, and incidence was based on the number of wounds with knots of the total number of wounds inoculated. Incidence values followed by the same letter for each cultivar in each column are not significantly different based on general linear model (GLM) and Fisher's LSD tests ($P \leq 0.05$).

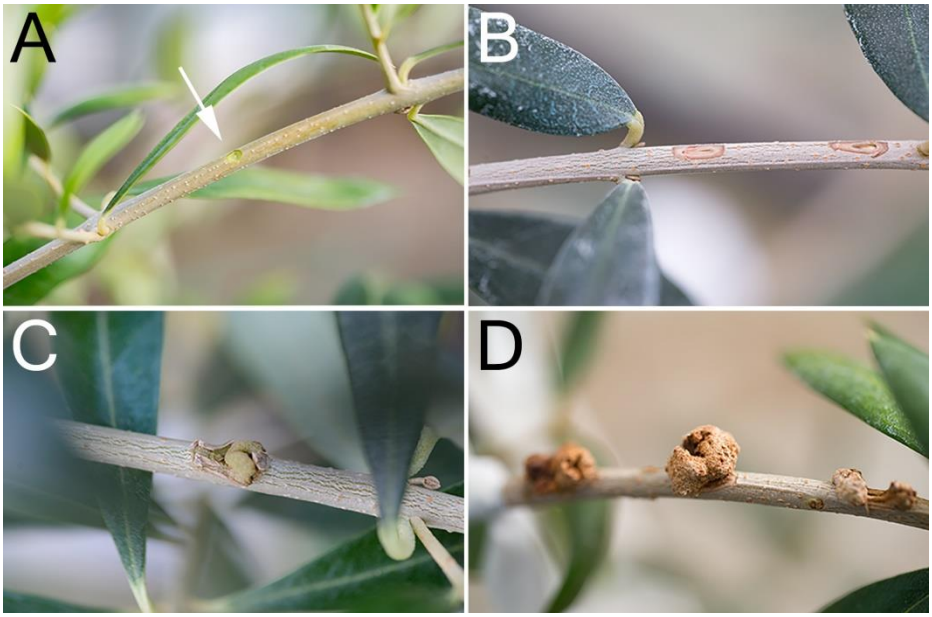

Figure 3.1. **‘**Arbequina’ olive twig injury and subsequent knot development in a greenhouse study. **A,** notched wound made with a scalpel on an olive twig (arrow); **B**, Non-inoculated, healed wound after two months; **C,** Knot that developed on an inoculated wound; and **D**, Knot that developed six months after inoculation.

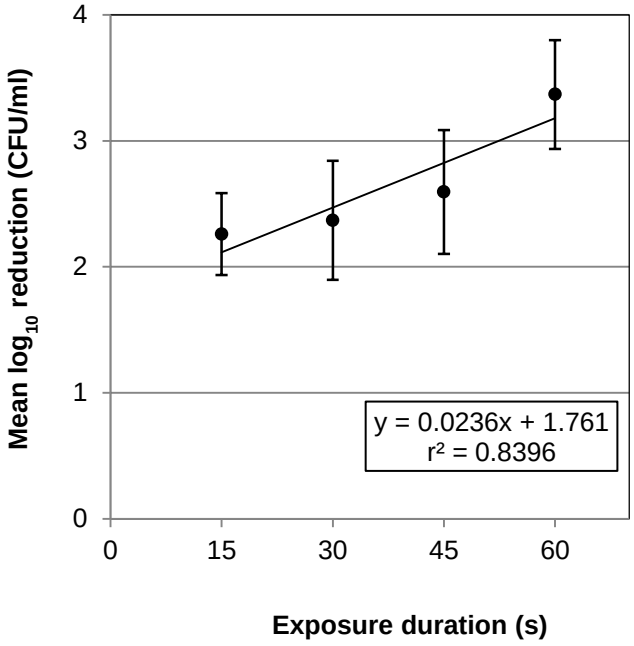

Figure 3.2. Effect of exposure duration on toxicity of QAC-2 (a mixture of alkyl dimethylbenzyl, didecyl dimethyl, octyl decyl dimethyl, and dioctyl dimethyl ammonium chlorides) against *Pseudomonas savastanoi* pv. *savastanoi* in direct contact suspension assays. The mean number of bacterial colonies in the water controls was 5.27 $\log_{10}$.

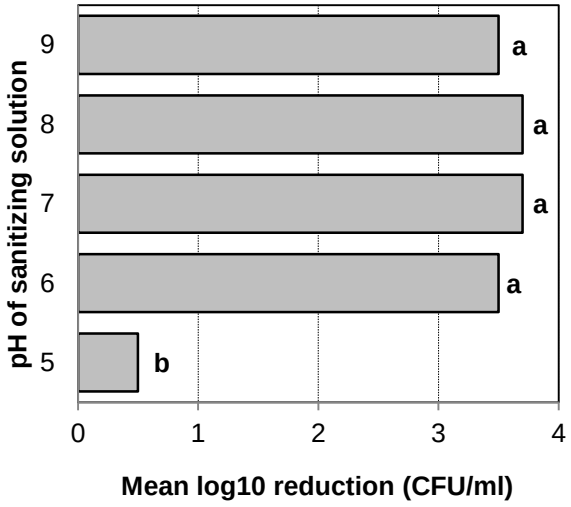

Figure 3.3. Effect of pH on toxicity of QAC-2 (a mixture of alkyl dimethylbenzyl, didecyl dimethyl, octyl decyl dimethyl, and dioctyl dimethyl ammonium chlorides) against *Pseudomonas savastanoi* pv. *savastanoi* in direct contact suspension assays. Bacterial recovery was determined after exposure to 25 µg/ml QAC-2 for 60 s and expressed as mean $\log_{10}$ reduction. Horizontal bars with the same letters are not significantly different based on ANOVA and Fisher's LSD tests ($P \leq 0.05$). Mean bacteria recovered from water controls were 4.65, 5.07, 5.21, 5.17, and 4.93 $\log_{10}$ CFU/ml, for pH 5, 6, 7, 8, and 9, respectively.

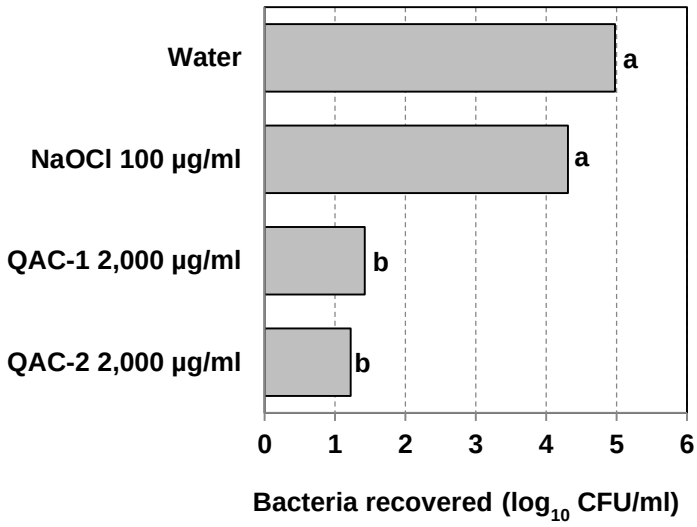

Figure 3.4. Efficacy of QAC-1 (a mixture of n-alkyl dimethyl benzyl and n-alkyl dimethyl ethylbenzyl ammonium chlorides), QAC-2 (a mixture of alkyl dimethylbenzyl, didecyl dimethyl, octyl decyl dimethyl, and dioctyl dimethyl ammonium chlorides), and sodium hypochlorite in disinfecting polyvinyl chloride piping contaminated with *Pseudomonas savastanoi* pv. *savastanoi* in the presence of an organic load (macerated olive tissue). Horizontal bars with the same letters are not significantly different based on ANOVA and Fisher's LSD tests ($P \leq 0.05$). NaOCl= sodium hypochlorite.

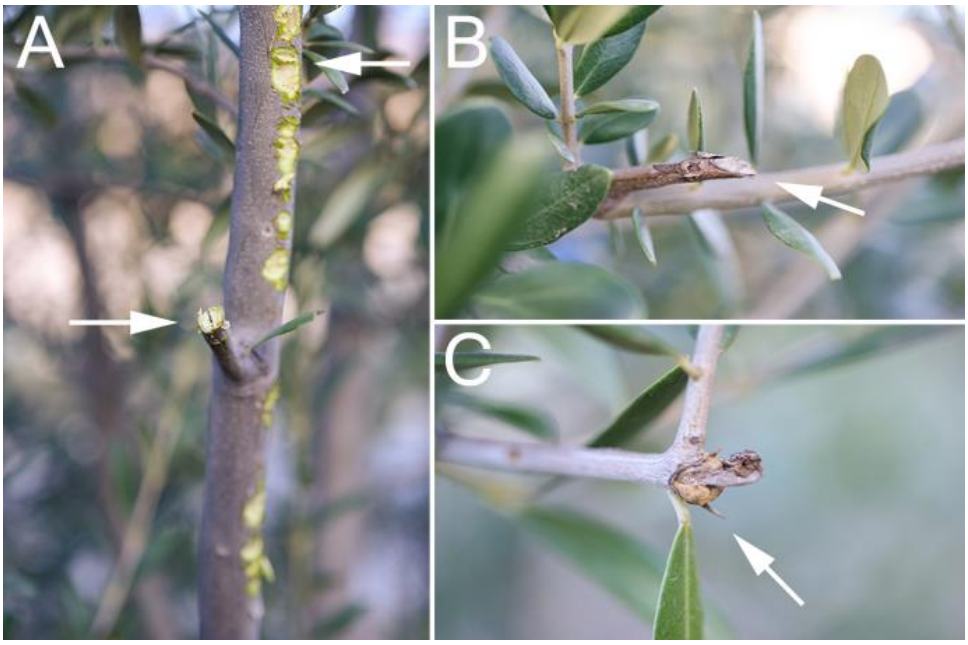

Figure 3.5. Efficacy of a quaternary ammonium compound for decontamination of pruning equipment in a field trial. **A,** Hedger pruning wounds (arrows) on an 'Arbequina' olive branch; **B,** Terminal cut made with a hedger that was effectively sanitized and that healed without knot development (arrow); and **C**, A terminal cut made using a contaminated hedger that resulted in knot development (arrow).

CHAPTER IV. EFFICACY OF COPPER AND NEW BACTERICIDES FOR MANAGING OLIVE KNOT IN CALIFORNIA

ABSTRACT

Baseline sensitivities were established for kasugamycin and oxytetracycline for 147 strains of *Pseudomonas savastanoi* pv. *savastanoi* collected from olive knots throughout California. Minimum inhibitory concentrations (MICs) for ≥95% growth inhibition ranged from 1.86 to 11.52 µg/ml and 0.13 to 0.40 µg/ml for kasugamycin and oxytetracycline, respectively. In copper sensitivity evaluations, 95.3% of the strains collected grew at concentrations of <20 µg/ml metallic copper equivalent (MCE), 2.7% grew between 20 to 30 µg/ml MCE (moderately resistant), and 2% grew at 150 µg/ml MCE (highly resistant). Copper resistance was never reported previously in the olive knot pathogen, and pathogenicity studies confirmed a high virulence of the copper-resistant strains. In comparative field studies, kasugamycin at 200 µg/ml performed equally to the standard copper hydroxide treatment (1,260 µg/ml MCE) for reducing knot development on lateral wounds of 'Arbequina' and 'Manzanillo' olives inoculated with a copper-sensitive strain and was better than copper using a highly copper-resistant strain. Oxytetracycline at 200 µg/ml was not as effective as copper or kasugamycin, but significantly reduced the disease as compared with the untreated control. Field studies on application timings of copper, kasugamycin, and copper-kasugamycin mixtures to inoculated wounds indicated that treatments within 24 h of inoculation resulted in greater

disease control than applications at later times. In greenhouse trials, copper or copper-kasugamycin applied to wounds 7 days before inoculation persisted and reduced knot incidence by >50%. Our findings indicate that kasugamycin is an effective bactericide for controlling olive knot and that the time of bactericide application after inoculation is critical in managing the disease.

INTRODUCTION

Pseudomonas savastanoi pv. *savastanoi* (Gardan et al. 1992) is the causal agent of olive knot, an economically important disease of olives (*Olea europaea* L.) worldwide (Young 2004). The bacterium can survive on its host as an epiphyte (Ercolani 1978) and is an opportunistic pathogen that relies on wounds as entry points to incite disease (Wilson 1935). Wounds may be naturally created by environmental factors such as severe weather that cause injuries to trees (e.g., frost damage, hail, windstorms). Additionally, spring leaf drop and foliar diseases such as peacock spot create leaf scar wounds that are highly susceptible to infection by the pathogen. Changes in California olive production practices that are adapting the Spanish high- and super-high-density (HD and SHD) planting systems with automated hedging and mechanical harvesting (Tous et al. 2010; Vossen 2007) have increased the risk of disease spread by providing more wounds to the pathogen. After invading susceptible tissue, the bacterium produces and releases indole-acetic acid (Comai and Kosuge 1980, 1982) and cytokinins (Surico et al. 1985). These phytohormones stimulate hyperplasia and hypertrophy of the surrounding olive tissue,

leading to the development of outgrowths known as knots or galls (Surico et al. 1985) that can be long-lived and harbor high concentrations of bacteria. The majority of knots form on twigs, trunks, and branches, but leaves, petioles, peduncles, racemes, and fruit occasionally also become infected (Wilson 1935). In addition to knots, the disease may cause tree defoliation, branch dieback, and reduced tree vigor which ultimately lowers yield, and it has also been implicated in reducing oil quality (Schroth et al. 1963, 1973). Knots serve as the primary inoculum source, and high concentrations of *P. savastanoi* pv. *savastanoi* are often recovered from the surfaces of wetted knots (Horne et al. 1912; Wilson 1935). The bacterium is disseminated by splashing water, wind, insects, birds, and human activity. Infections can be initiated year-round when conditions are favorable and are not limited by temperatures as low as -5° C (K. A. Nguyen *unpublished*), although knots will only begin to develop when trees are actively growing during the spring and summer seasons.

The most effective strategy for managing olive knot is prevention by using clean nursery stock and growing olives in areas less conducive for disease development such as locations with minimal rainfall and freezing events (Wilson 1935). Once olive knot becomes established, eradication is often not feasible, and the disease must be controlled to minimize further spread. Disease management may include pruning and removal of infected plant material from the orchard under dry conditions. Field equipment (i.e., pruners and harvesters) should be properly sanitized to reduce the risk of transmitting the pathogen from diseased to healthy plants through contaminated surfaces (Nguyen et al. 2017).

Another important strategy for disease control is the use of properly timed applications of chemical protectants. Painting knots with cresol- and xylenol-based compounds (e.g., Gallex) can be an effective practice. This, however, is not feasible on a commercial scale because individual knots have to be treated which requires excessive labor. Furthermore, the compounds are toxic to leaves and cannot be applied as foliar sprays (Schroth and Hildebrand 1968; Sibbett and Ferguson 2005). Copper is the best foliar treatment currently available for the control of olive knot. It is typically applied twice a year, once in the fall after harvest and again in the spring following natural leaf drop (Teviotdale and Krueger 2004). On other crops, the excessive use and dependence on copper as the only means for controlling phytopathogenic bacteria have led to the development of copper resistance in bacterial pathogens and in a reduction of its effectiveness (Adaskaveg and Hine 1986; Andersen et al. 1991; Marco and Stall 1983). The sole and repeated use of various copper formulations for the control of olive knot in California since the early 1900s (Horne et al. 1912; Wilson 1935) possibly has resulted in the selection of resistance in the pathogen population. Teviotdale and Krueger (1998) evaluated a California *P. savastanoi* pv. *savastanoi* population for copper resistance but only found copper-resistant strains that were not pathogenic on olive. Some California olive growers have reported reduced efficacy of copper, and resistance in the pathogen populations has been considered a possible cause. Other factors such as inappropriate timing of application in respect to wounding and infection events, however, are alternative explanations.

With limited treatment options available for controlling olive knot, we evaluated kasugamycin and oxytetracycline as possible new treatments for the management of the disease. Integration of new modes of action to currently available treatments would reduce the risk of resistance development in the pathogen population and help to prolong the effective use life of these bactericides. Kasugamycin is produced by the gram-positive bacterium *Streptomyces kasugaensis,* and was discovered in the 1960s (Umezawa et al. 1965). Kasugamycin is an aminoglycoside that inhibits bacterial protein synthesis by interfering with the formation of the 30S initiation complex during translation (Okuyama et al. 1971; Tanaka et al. 1966). Kasugamycin is active against many important genera of plant pathogenic bacteria including *Erwinia*, *Xanthomonas*, and *Pseudomonas* (Adaskaveg, *unpublished*). We previously demonstrated the effectiveness of kasugamycin against fire blight of pome fruits caused by *Erwinia amylovora*, and the bactericide was subsequently registered for use on these crops (Adaskaveg et al. 2011). Unlike oxytetracycline, kasugamycin is not used in human and animal medicine (Adaskaveg et al. 2011; McGhee and Sundin 2011) and is not considered to be toxic to mammals or persistent in the environment (Copping and Duke 2007; Lu et al. 2012). The tetracycline compound oxytetracycline is registered as a foliar treatment against bacterial diseases on certain stone and pome fruits and as an injection treatment for lethal yellows of palm and elm phloem necrosis that are both caused by phytoplasmas.

The objectives of this study were to collect strains of *P. savastanoi* pv. *savastanoi* from California olive growing regions, characterize their sensitivity to copper, and establish baseline sensitivities to kasugamycin and oxytetracycline. The efficacy of

kasugamycin, oxytetracycline, and copper hydroxide was evaluated in comparative field trials. Application timing and persistence studies were conducted in greenhouse or field studies to assess pre- and post-infection activity of the treatments and to determine optimum use strategies for managing olive knot.

MATERIALS AND METHODS

Isolation of *P. savastanoi* pv. *savastanoi* strains from diseased plant samples and culturing of the pathogen. Olive twigs and branches with knots were collected between 2011 and 2015 from commercial oil and table olive orchards in major production areas of California (Butte, Colusa, Glenn, Tehama, Tulare, and Yuba Co.) (Table 1). Internal knot tissue was excised with a sterile scalpel and placed into microcentrifuge tubes, 600 µl of sterile deionized water was added, and tubes were vigorously shaken for 5 min on a vortex shaker. The suspension was streaked onto PVF-1 agar (Surico and Lavermicocca 1989). Plates were incubated for 3 days at 25°C before visualization under long wavelength (365 nm) ultraviolet light (Benchtop 2 UV transilluminator; UVP, LLC, Upland, CA). Fluorescent single colonies were transferred onto King's Medium B (KMB; King et al. 1954). Molecular identification was done by polymerase chain reaction (PCR) with species-specific primers IAALF and IAALR (Penyalver et al. 2000) targeting the *iaaL* gene. Isolates positively identified as *P. savastanoi* pv. *savastanoi* were stored in 15% glycerol at -80°C.

laboratory evaluation of copper sensitivity of *P. savastanoi* pv. *savastanoi*

strains. Evaluation of copper sensitivity was performed using an agar dilution-plate assay. Molten casitone-yeast extract-glycerol agar (Zevenhuizen et al. 1979) was amended with cupric sulfate (Mallinckrodt, St. Louis, MO) to final concentrations of 10, 20, 30, or 50 µg/ml metallic copper equivalents (MCE). Two-day-old cultures of *P. savastanoi* pv. *savastanoi* strains grown on KMB agar at 25°C were used to prepare aqueous bacterial suspensions that were adjusted to 70% transmittance (OD_{600}) equaling approximately 2 x 10^8 CFU/ml using a spectrophotometer (DU 730, Beckman Coulter, Inc., Indianapolis, IN). Bacterial suspensions were then transferred to copper-amended media in 15-cm petri dishes by placing a 10-µl droplet at the edge of the plate and streaking towards the center using a sterile plastic pestle. Eight strains were tested per plate with two radially opposing replications per strain (16 streaks/plate). Plates were incubated at 25°C for 2 days and were then visually assessed for growth in comparison to the controls on non-amended media. Strains that only grew in the presence of 10 µg/ml MCE were considered copper-sensitive; strains that grew between 20 and 30 µg/ml MCE were considered moderately sensitive; and strains that had confluent growth at 50 µg/ml MCE similar to the control plates were considered copper-resistant. Strains that grew at 50 µg/ml MCE were further challenged on media amended with 150 µg/ml MCE. This assay was done twice for all 147 *P. savastanoi* pv. *savastanoi* strains that were obtained.

In vitro baseline sensitivities of *P. savastanoi* pv. *savastanoi* strains to kasugamycin and oxytetracycline. To evaluate the toxicity of kasugamycin (Kasumin 2L; Arysta LifeScience, Cary, NC) and oxytetracycline (oxytetracycline hydrochloride; Sigma Aldrich) against *P. savastanoi* pv. *savastanoi*, the spiral gradient endpoint (SGE)

method was utilized (Paton et al. 1990). Aqueous stock suspensions of kasugamycin at 10,000 µg/ml or oxytetracycline at 500 µg/ml were spiraled onto 15-cm Petri dishes containing 50 ml of nutrient agar (NA; Difco Laboratories, Franklin Lakes, NJ) using a spiral plater (Autoplate 4000, Spiral Biotech, Inc., Norwood, MA). Bacterial suspensions were prepared, and 10-µl droplets were streaked radially along the concentration gradient of each bactericide as described above. After 2 days at 25°C, inhibition of growth was determined visually and measured at two points: the lowest inhibitory concentration (LIC; point where a reduction of growth is first observed), and the minimum inhibitory concentration (MIC; point where growth is inhibited by ≥95%). The concentrations at these inhibitory points were determined using the Spiral Gradient Endpoint software (Spiral Biotech) by measuring the distance between the point of inhibition to the center of the plate (radial distance) and entering this measurement into the software. Each isolate was evaluated with two replications per test, and the experiment was performed twice.

Pre-infection activity of bactericide treatments against olive knot in field trials. A field trial was performed in an experimental orchard at the University of California Davis (UC Davis) and in a commercial orchard in Yuba County on ‘Arbequina’ olive trees (Agromillora California, Gridley, CA) in April 2015. For ‘Manzanillo’ olives (Agromillora California), a study was performed in October of 2014 and in April of 2015 at UC Davis. Trees were planted using a high-density design (Vossen 2007). Wounds were made to 1- to 2-year-old olive twigs approximately 5 to 10 mm in diameter. Two types of wounds were made, lateral wounds and leaf scar wounds, both simulating mechanical damage that could be incurred from field equipment. For

lateral wounds, a small section of the bark (10 to 20 mm long x 5 mm wide) was removed using a scalpel exposing the cambial tissue. For leaf scar injuries, leaves were pulled off by hand (Fig. 1A). Each twig represented one experimental replicate with 5 internodal lateral wounds and 5 leaf scar injuries towards the twig tip, and there were 8 replications per treatment. Treatments were prepared as aqueous suspensions and applied to wounded twigs until run-off using a hand-held sprayer. Bactericides included kasugamycin (Kasumin 2L) at 200 µg/ml, oxytetracycline at 200 µg/ml (Fireline; AgroSource, Inc., Mountainside, NJ), copper hydroxide (Kocide 3000, Du Pont de Nemours and Co., Wilmington, DE; or ChampION^{++}, NuFarm Americas, Inc., Alsip, IL) at 1,260 µg/ml or 2,520 µg/ml MCE, and a mixture of copper hydroxide (1,260 µg/ml MCE) and kasugamycin (200 µg/ml). Treatments were allowed to air-dry for 1 to 3 h, and twigs were spray-inoculated to runoff with *P. savastanoi* pv. *savastanoi* strains O1-26 (copper-sensitive) or O1-113 (copper-resistant) using an aqueous suspension at 2 x 10^7 CFU/ml. Olive trees were evaluated after knots had sufficiently formed on untreated-inoculated wounds (typically 6 months). Treatment efficacy was based on the incidence of disease on lateral wounds and leaf scars as compared to nontreated-inoculated wounds.

Post-infection activity of bactericide treatments against olive knot in field trials. Studies were performed on ‘Arbequina’ olives, and treatments had 5 replications per treatment for lateral and leaf scar wounds on 1- to 2-year-old twigs. Wounds were spray-inoculated with copper-sensitive *P. savastanoi* pv. *savastanoi* strain O1-26 (2 x 10^7 CFU/ml) as described above and treated after selected times with 1,260 µg/ml MCE (ChampION^{++}), 200 µg/ml kasugamycin (Kasumin 2L), or a mixture of 2,520 µg/ml

MCE with 200 µg/ml kasugamycin. Untreated, inoculated wounds were used as positive controls. In studies conducted in the spring of 2016 and 2017 at UC Riverside and UC Davis, respectively, treatments were done 1, 4, 8, or 24 h after inoculation. In trials done in fall/winter of 2016/17 at both locations, treatments were applied 2, 24, 48, or 96 h after wound inoculation. Evaluation of disease incidence was performed as described above.

Persistence of protective bactericide treatments against *P. savastanoi* pv. *savastanoi*. In greenhouse studies, 'Arbequina' olive twigs were wounded and treated with the same bactericides as described above. Treated wounds were spray-inoculated with copper-sensitive strain O1-26 (2 x 10^7 CFU/ml) using a hand-held sprayer after 72 or 168 h (7 days). Three plants each with two replications (5 lateral wounds) of each treatment (a total of 6 replications) were inoculated after 72 h, or 168 h. Percent incidence of disease was recorded as the number of knots that developed in lateral wounds. These experiments were repeated once.

Statistical methods and data analysis. For in vitro sensitivity studies, MIC values for kasugamycin and oxytetracycline were plotted in frequency histograms. For this, values were $\log_{10}$-transformed and used to calculate bin widths based on Scott's rule (Scott 1979) with the equation,

$$h = 3.49\hat{\sigma}n^{-\frac{1}{3}}$$

where h is the bin width, $\hat{\sigma}$ is the sample standard deviation, and n is the sample size.

A pairwise comparison between oxytetracycline and kasugamycin sensitivity was performed to determine if multiple resistance was present among strains evaluated. Values of kasugamycin were regressed against those of oxytetracycline using ANOVA

and regression procedures (SAS version 9.4; SAS Institute Inc., Cary, NC) for all 147 olive knot strains.

All treatments in field and greenhouse studies were arranged in a randomized complete block design. Disease incidence values were arcsine-transformed before statistical analyses using general linear model (GLM) and least significant difference (LSD) mean separation procedures (SAS). Data from repeated studies were combined after Bartlett's test of homogeneity of variance was confirmed.

RESULTS

Laboratory evaluation of copper sensitivity of *P. savastanoi* pv. *savastanoi* strains. Of the 147 isolates tested, 140 strains (95.3%) grew at concentrations of ≤20 µg/ml MCE, 4 strains (2.7%) grew between 20 to 30 µg/ml MCE (moderately resistant), and 3 strains (2%) grew at 50 µg/ml and 150 µg/ml MCE (highly resistant). The four moderately-resistant strains came from three locations in Glenn and Tehama Co., and the three resistant strains originated from two locations in Glenn Co. A summary of the copper sensitivity assays is presented in Table 1.

In vitro baseline sensitivities of *P. savastanoi* pv. *savastanoi* strains to kasugamycin and oxytetracycline. For kasugamycin, mean LIC and MIC values were 2.91 and 4.87 µg/ml, respectively; whereas those for oxytetracycline were 0.16 µg/ml and 0.24 µg/ml, respectively (Table 2). Scott's distributions of MIC values were unimodal for both bactericides (Fig. 2A,B). No outliers were present beyond 2.8x of the mean for

kasugamycin or 1.7x of the mean for oxytetracycline. The model of the regression of pairwise MIC values for the two bactericides was highly significant ($P<0.0001$), however, the R^2 value was 0.3411 (Fig. 3).

Pre-infection activity of bactericide treatments against olive knot in field trials. Disease developed at high incidence (67.8 to 97.1%) on untreated lateral and leaf scar wounds of 'Arbequina' and 'Manzanillo' olive trees (Table 3). Approximately 6 months after inoculation, wounds developed typical knots similar in appearance to those developing naturally (Fig. 1B, C). Oxytetracycline significantly reduced the incidence of disease caused by a copper-sensitive strain on lateral wounds of 'Arbequina', but not of 'Manzanillo' olive. This bactericide also was not effective in reducing the incidence of knots caused by the copper-resistant strain on both olive cultivars (Table 3). Kasugamycin significantly reduced the incidence of disease caused by copper-sensitive and -resistant strains on both types of wounds of 'Arbequina' olive. On 'Manzanillo', this treatment significantly reduced the incidence of disease on lateral wounds, but not on leaf scars for sensitive and -resistant strains.

The two copper rates were mostly similar in efficacy and significantly reduced knot formation to low incidence on lateral wounds and leaf scars inoculated with the copper-sensitive strain of the pathogen. Using the copper-resistant strain, the high rate of copper was efficacious in most inoculations except for lateral wounds of 'Manzanillo' (Table 3). The mixture of copper and kasugamycin was highly effective in reducing knots on lateral wounds and leaf scars of 'Arbequina' inoculated with the copper-sensitive strain. It also significantly reduced the disease for the copper-resistant strain except for

the leaf scar inoculations of ‘Manzanillo’. This treatment, however, generally did not result in improved efficacy as compared to either rate of copper alone.

Post-infection activity of bactericide treatments against olive knot in field trials. Spring-time treatments with copper, kasugamycin, or the mixture to both types of wounds within 24 h after inoculation all significantly reduced the incidence of disease from that of the untreated control (Table 4). On lateral wounds, disease was mostly reduced to low levels except for kasugamycin at the 24-h timing, and no disease developed in the mixture treatment applied 1, 4, or 24 h after inoculation. On leaf scar wounds, none of the treatments resulted in zero levels of disease. In general, the 24-h timing had the highest level of disease for each bactericide treatment (Table 4).

The efficacy of fall/winter applications of bactericides at selected times within 24 h after inoculation to leaf scars wounds of ‘Arbequina’ olive (Table 5) was generally lower than those applied in the spring experiments (Table 4), and the 48- or 96-h treatments were often statistically similar to the 24-h treatments. Most timings of copper or the mixture treatments to lateral wounds in Experiment 1 that was conducted in the fall resulted in significantly less knot formation than in the untreated control. For kasugamycin, however, only the 2- and 96-h application timings had significantly less disease than the untreated control. The mixture treatment in this experiment had some of the lowest incidence values on lateral wounds for all timings, but there was no significant difference as compared to most of the copper treatments. In contrast, in Experiment 2 that was done in the winter, all treatments significantly reduced knot formation to low or zero-levels as compared to the untreated control even when applied 96 h after inoculation (Table 5).

In both fall/winter experiments, none of the treatment timings was highly effective in preventing knot formation on leaf scar wounds (Table 5). The 2-h timings were mostly more effective than the later timings in Experiment 1. Additionally, kasugamycin and the copper-kasugamycin mixture were significantly more effective than copper by itself in the winter treatments of Experiment 2.

Persistence of bactericide treatments on wounds for preventing infection by *P. savastanoi* pv. *savastanoi*. In greenhouse studies, copper and copper-kasugamycin applied 3 or 7 days before inoculation reduced disease incidence by ≥50% (Table 6). Kasugamycin treatments did not significantly reduce knot formation at both inoculation timings.

DISCUSSION

Our survey of *P. savastanoi* pv. *savastanoi* strains from California showed differential degrees of sensitivity to copper, but most strains were considered sensitive. Copper resistance is considered a qualitative trait in the ability of *Pseudomonas* spp. to grow on media amended with approximately 50 µg/ml MCE (Andersen et al. 1991; Cazorla et al. 2002), and in previous studies, resistance was not found in evaluations of over 550 strains of the olive knot pathogen in Spain (Quesada et al. 2010). Copper-resistant strains of *P. savastanoi* pv. *savastanoi*, however, were previously reported from California, but these strains were non-pathogenic when inoculated to olives (Teviotdale and Krueger 1998) and were not well characterized using more stringent identification

methods. In our studies, initial isolations from olive knots often resulted in recovery of fluorescent Pseudomonads that were copper-resistant, but *iaaL* was not successfully amplified using the IAAL primers (Penyalver et al. 2000) and these strains were non-pathogenic to olive. Our *P. savastanoi* pv. *savastanoi* strains, including the three highly copper-resistant (growth at 150 µg/ml MCE) ones that we recovered from commercial 'Sevillano' and 'Manzanillo' olive groves in northern California were identified based on their growth on a semi-selective medium, amplification of the *iaaL* gene, and on fulfilling Koch's postulates. Therefore, to our knowledge, this is the first report of copper-resistant, pathogenic strains of *P. savastanoi* pv. *savastanoi* from olive in California and elsewhere.

Development of copper resistance was likely a result of repeated exposure of the pathogen to copper sprays that have been used in California for over a century to control fungal and bacterial diseases of olives (Horne et al. 1912; Wilson 1935). In our field studies, copper treatments at registered rates were highly effective in reducing knot development on two olive cultivars when inoculated with a copper-sensitive strain of the pathogen. Higher registered rates were often more effective in reducing disease incidence. When copper-treated olive wounds were inoculated with a copper-resistant strain, however, disease control was significantly reduced even when using the maximum labeled copper rate, especially on 'Manzanillo' olives. Although the copper-resistant strains only represented 2% of the total isolates collected, continual usage of copper alone will likely increase the incidence of such strains, making the development of new bactericidal treatments and copper-enhancing-materials critical for resistance management and disease control.

Kasugamycin was identified as an effective alternative treatment, and it was the best treatment on lateral wounds inoculated with a copper-resistant strain. Interestingly, kasugamycin was less effective on leaf scars in some experiments, suggesting that the type of wound treated can have a major effect on disease control. Furthermore, as for the copper treatments, disease reduction by kasugamycin was often lower on 'Manzanillo' than on 'Arbequina' olive suggesting that olive cultivar may affect the performance of the bactericide treatment. Mixtures of copper and kasugamycin mostly did not improve disease control compared to using copper by itself, however, this mixture treatment with two ingredients active against the pathogen will help to reduce resistance development against either compound.

In vitro tests demonstrated that oxytetracycline and kasugamycin were toxic to *P. savastanoi* pv. *savastanoi*, however, oxytetracycline was more toxic based on lower MIC values. Distributions of sensitivity values were unimodal, and no strains resistant to either bactericide were identified. No cross-resistance was observed when the inhibitory values of oxytetracycline were compared to those of kasugamycin for the 147 strains because the coefficient of correlation was very low (R^2= 0.3411). This was an expected result because the two bactericides do not share the same modes of action (Copping and Duke 2007). Still, oxytetracycline did not perform consistently in field trials and in most cases showed a low efficacy when compared to copper or kasugamycin treatments.

Oxytetracycline is considered to be sensitive to ultraviolet radiation found naturally in sunlight, and this was suggested to be a contributing factor to the rapid degradation of the bactericide (Christiano et al. 2010). Unfortunately, the same

researchers showed that the addition of several UV protectants to oxytetracycline treatments was not adequate for maintaining effective concentrations against *Xanthomonas arboricola* pv. *pruni* on peach leaves. Still, oxytetracycline has potential for treating olive knot if the negative effects of sunlight can be controlled.

In addition to the direct biocidal activity of treatments, application timing is critical in determining disease control. Our field studies indicated that copper, kasugamycin, and mixtures of copper and kasugamycin applied 24 h or more after inoculation were sometimes less effective than treatments applied earlier. The efficacy of post-infection treatments within this time period varied widely among studies conducted during different times of the year, however, in several trials, earlier applications resulted in significantly lower disease. Treatments in the spring-time were generally more effective than those applied in fall or winter. Copper is known as a contact bactericide with no curative properties. Applications at or shortly after inoculation of wounds during favorable environmental conditions such as rainfall are critical to inhibit bacterial colonization before infections are established. Therefore, it is essential that olives are treated as soon as possible after harvest and at other times when large numbers of new injuries occur (e.g., hail storms, natural leaf drop) and rainfall events are likely under California conditions.

Previous research revealed that oxytetracycline was not likely taken up by plant leaves when applied externally and only moved systemically if delivered through injection (Christiano et al. 2010). Kasugamycin is characterized as a systemic compound on certain crops (Copping and Duke 2007; Lu et al. 2012) although we found its

effectiveness reduced when applied to lateral wounds of olives that were inoculated several days in advance, suggesting that it was not readily absorbed by olive tissue or was diluted to sub-lethal concentrations by movement into the wounds that prevented disease control. It was also less effective on leaf scar wounds (treated before or after inoculation) than on lateral wounds.

The persistence of treatments is another important factor when considering disease prevention. In greenhouse studies, the residual activity of copper and copper-kasugamycin on olive injuries lasted for at least a week, and inoculations of treated wounds resulted in fewer knots than those of untreated wounds. Wounds inoculated after 7 days show reduced susceptibility compared with those inoculated earlier (K. A. Nguyen *unpublished*). Thus, wound healing contributes to preventing infections. Hewitt (1938) showed that wound healing responses are initiated on olive leaf scars within days after leaf drop, preventing most pathogen infections from occurring.

This research presents strong evidence that kasugamycin can be similarly effective as copper in reducing olive knot while providing a different mode of action for disease control. Integration of copper and kasugamycin in mixtures or rotations has the potential to reduce the risk of resistance developing to either bactericide, enabling the prolonged effective use of both compounds. Oxytetracycline was moderately effective in selected studies, and disease control was inconsistent. Future work on optimizing its performance will be necessary to support its use for the control of olive knot.

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Table 4.1. Origin and copper sensitivity of *Pseudomonas savastanoi* pv. *savastanoi* strains from olive knots collected from major olive-producing regions in California

No.	Location by county	Olive cultivar	Olive type	Year collected	No. isolates recovered and copper sensitivity		
					Sensitive	Moderate	Resistant
1	Glenn	Koroneiki	Oil	2011	13	0	0
2	Glenn	Manzanillo	Table/Oil	2011	7	0	0
3	Yuba	Arbequina	Oil	2011	5	0	0
4	Yuba	Arbosana	Oil	2011	5	0	0
5	Glenn	Manzanillo	Table/Oil	2011	6	0	0
6	Glenn	Manzanillo	Table/Oil	2011	6	0	0
7	Glenn	Manzanillo	Table/Oil	2011	10	0	0
8	Butte	Arbequina	Oil	2011	8	0	0
9	Tulare	Unknown	Table	2011	6	0	0
10	Tehama	Manzanillo	Table/Oil	2011	5	0	0
11	Glenn	Manzanillo	Table/Oil	2011	2	0	0
12	Tehama	Sevillano	Table	2011	2	0	0
13	Glenn	Arbequina	Oil	2011	7	0	0
14	Glenn	Manzanillo	Table/Oil	2011	4	0	0
15	Glenn	Manzanillo	Table/Oil	2011	6	0	0
16	Tehama	Manzanillo	Table/Oil	2011	5	0	0
17	Tehama	Arbequina	Oil	2011	5	0	0
18	Tehama	Sevillano	Table	2011	4	1	0
19	Glenn	Manzanillo	Table/Oil	2011	4	0	1
20	Yuba	Arbosana	Oil	2012	4	0	0
21	Yuba	Arbequina	Oil	2012	2	0	0
22	Yuba	Arbequina	Oil	2012	5	0	0
23	Yuba	Arbequina	Oil	2012	4	0	0
24	Glenn	Manzanillo	Table/Oil	2015	9	2	0
25	Glenn	Sevillano	Table	2015	6	1	2
			Total no. of isolates		140	4	3

Copper-sensitivity: Sensitive = growth at <20 µg/ml metallic copper equivalent (MCE); moderate sensitivity = growth between 20 to 30 µg/ml MCE; and resistant = growth at 150 µg/ml MCE. Copper sensitivity based on two repeat experiments using a total of 147 strains.

Table 4.2. Inhibitory concentrations of kasugamycin and oxytetracycline for *Pseudomonas savastanoi* pv. *savastanoi* strains from California

	LIC (µg/ml)[a]		MIC (µg/ml)	
Bactericide	**Range**	**Mean**	**Range**	**Mean**
Kasugamycin	1.20 to 5.87	2.91	1.86 to 11.52	4.87
Oxytetracycline	0.09 to 0.23	0.16	0.13 to 0.40	0.24

[a] Inhibitory values of bactericides were obtained using the spiral gradient endpoint method on nutrient agar; LIC: lowest inhibitory concentration where a reduction of growth is first observed; MIC: minimal inhibitory concentration where growth is reduced by ≥95%. Values are the average of two experiments, each with two replications.

Table 4.3. Efficacy of bactericides as pre-infection wound treatments for reducing the incidence of knot formation after inoculation with copper-sensitive or -resistant *Pseudomonas savastanoi* pv. *savastanoi* strains in field trials on two olive cultivars

		Mean incidence of knots on treated-inoculated wounds (%)[a]							
		Arbequina[b]				**Manzanillo**			
		Cu^S		**Cu^R**		**Cu^S**		**Cu^R**	
Treatment	**Rate (µg/ml)**	**LW**	**LS**	**LW**	**LS**	**LW**	**LS**	**LW**	**LS**
Nontreated control	---	85.7 a	81.1 a	80.0 a	67.8 a	84.6 a	77.1 a	97.1 a	76.3 ab
Oxytetracycline	200	58.6 b	57.8 b	57.3 ab	51.8 ab	80 a	62.8 a	94.2 ab	65.4 ab
Kasugamycin	200	12.9 c	53.3 b	20.0 d	38.9 b	15.3 b	72.8 a	42.8 c	85.4 ab
Copper hydroxide[c]	1,260	2.9 d	12.5 c	38.6 bcd	47.5 ab	29.2 b	27.1 b	97.1 a	76.3 ab
Copper hydroxide	2,520	4.3 cd	5.6 c	38.8 bc	26.7 b	12.3 b	4.2 c	91.4 ab	66.0 b
Copper+kasuga-mycin	1,260 + 200	2.9 d	11.1 c	36.0 cd	42.4 ab	21.5 b	18.5 bc	82.8 b	85.4 a

[a] Data are the average of two experiments after testing for homogeneity of variance using Bartlett's test. Values followed by the same letter within each column are not significantly different based on GLM and LSD tests ($P < 0.05$).
[b] Experiments were conducted in the spring on 'Arbequina' and in the spring and fall seasons on 'Manzanillo' olive. Inoculum (2×10^7 CFU/ml) consisted of a copper-sensitive (Cu^S) or a copper-resistant (Cu^R) strain of *P. savastanoi* pv. *savastanoi*.
[c] For the copper hydroxide treatments at the low rate, Kocide 3000 was used; whereas for the high rate, ChampION^{++} was used. Rates are expressed as metallic copper equivalent (MCE).

Table 4.4. Efficacy of spring-time bactericide treatments applied at selected times within 24 h after inoculation to wounds for reducing the incidence of knot formation of 'Arbequina' olive

			Incidence of knots on inoculated wounds (%)[a]	
Treatment[b]	**Rate (µg/ml)**	**Timing (h)**	**Lateral wounds**	**Leaf scars**
Untreated control	---	---	80.0 a	71.4 a
Copper	1,260	1	2.0 c	18.6 bcd
		4	4.0 c	17.1 bcd
		8	4.0 c	25.7 bc
		24	4.0 c	30.0 b
Kasugamycin	200	1	6.0 c	20 bcd
		4	8.0 c	10 cd
		8	6.0 c	17.1 bcd
		24	27.0 b	27.7 bc
Copper + kasugamycin	1,260 + 200	1	0.0 c	15.7 bcd
		4	0.0 c	10.8 d
		8	4.0 c	10.8 cd
		24	0.0 c	33.3 b

[a] Lateral and leaf scar wounds were inoculated with a copper-sensitive *Pseudomonas savastanoi* pv. *savastanoi* strain and treated after selected timings in March or April of 2017. Data are the average of two experiments based on homogeneity of variance using Bartlett's test. Values followed by the same letter within each column are not significantly different based on GLM and LSD tests ($P < 0.05$).
[b] Copper hydroxide (ChampION^{++}) was used for the copper treatments. For treatments containing copper, rates are expressed as metallic copper equivalent (MCE).

Table 4.5. Efficacy of fall and winter bactericide treatments applied at selected times within 96 h after inoculation to wounds for reducing the incidence of knot formation of 'Arbequina' olive

Treatment	Rate (µg/ml)	Timing (h)	Incidence of knots on inoculated wounds (%)[a]			
			Experiment 1		Experiment 2	
			Lateral wounds	Leaf scars	Lateral wounds	Leaf scars
Untreated control	---	---	93.3 a	90 a	94.2 a	96 a
Copper	1,260	2	28.5 def	60.7 cd	0 c	86 a
		24	46.8 b-f	87.5 ab	0 c	88 a
		48	50 b-e	85.6 ab	0 c	82 a
		96	35.7 c-f	89.2 a	0 c	90 a
Kasugamycin	200	2	31.4 def	37.1 d	0 c	26 bc
		24	78.5 ab	75 abc	2.8 c	30 bc
		48	67.8 abc	85.7 ab	14.2 b	40 bc
		96	57.1 bcd	75 abc	2.8 c	42 b
Copper + kasugamycin	1,260 + 200	2	15.6 f	34.3 d	0 c	18 c
		24	17.8 ef	67.8 bc	0 c	32 bc
		48	39.2 c-f	75 abc	0 c	34 bc
		96	43.8 c-f	78.1 abc	0 c	28 bc

[a] Lateral and leaf scar wounds were inoculated with a copper-sensitive *Pseudomonas savastanoi* pv. *savastanoi* strain and treated after selected timings in September 2016 (Experiment 1) or January 2017 (Experiment 2). Values followed by the same letter within each column are not significantly different based on GLM and LSD tests ($P < 0.05$).

[b] Copper hydroxide (ChampION^{++}) was used for the copper treatments. For treatments containing copper, rates are expressed as metallic copper equivalent (MCE).

Table 4.6**.** Persistence of bactericides applied to lateral twig wounds of 'Arbequina' olive to prevent knot formation by *Pseudomonas savastanoi* pv. *savastanoi* in greenhouse studies

		Incidence of knot formation (%)[a]	
		Inoculation timing after treatment	
Treatment[b]	**Rate (µg/ml)**	**3 days**	**7 days**
Untreated control	---	80 a	81.8 a
Copper	1,260	40 bc	34.5 b
Kasugamycin	200	74.4 ab	90 a
Copper + kasugamycin	1,260 + 200	36.7 c	37.3 b

[a] Lateral wounds were treated and inoculated with a copper-sensitive *Pseudomonas savastanoi* pv. *savastanoi* strain after 3 or 7 days. Data are the average of two experiments after testing for homogeneity of variance using Bartlett's test. Values followed by the same letter within each column are not significantly different based on GLM and LSD tests ($P < 0.05$).
[b] Copper hydroxide (Kocide 3000) was used for the copper treatments. For treatments containing copper, rates are expressed as metallic copper equivalent (MCE).

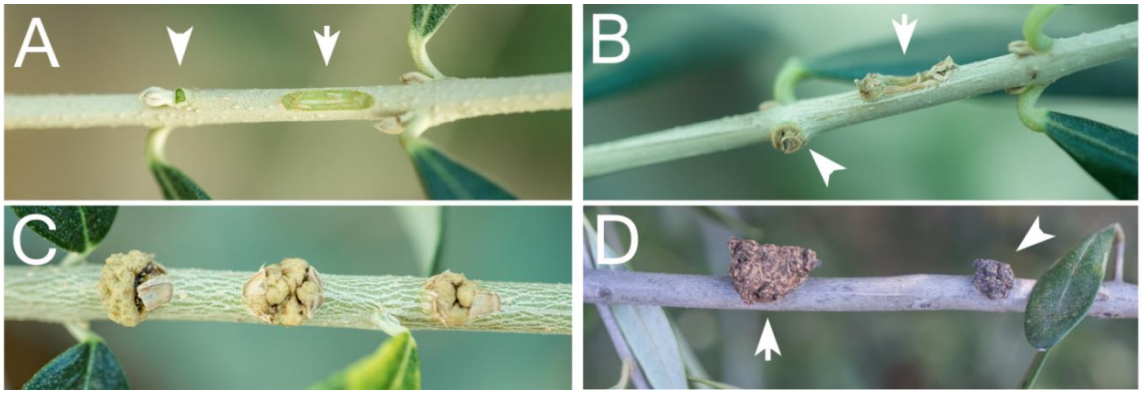

Figure 4.1. Knot development on inoculated olive wounds and after natural infections. **A**, Leaf scar (arrowhead) and lateral (arrow) wounds made on an 'Arbequina' olive twig; **B**, Developing olive knot on leaf scar (arrowhead) and lateral (arrow) wounds; **C**, Knots on lateral wounds 6 months after inoculation; and **D**, Knot forming on naturally occurring leaf scar (arrowhead) and lateral wound (arrow).

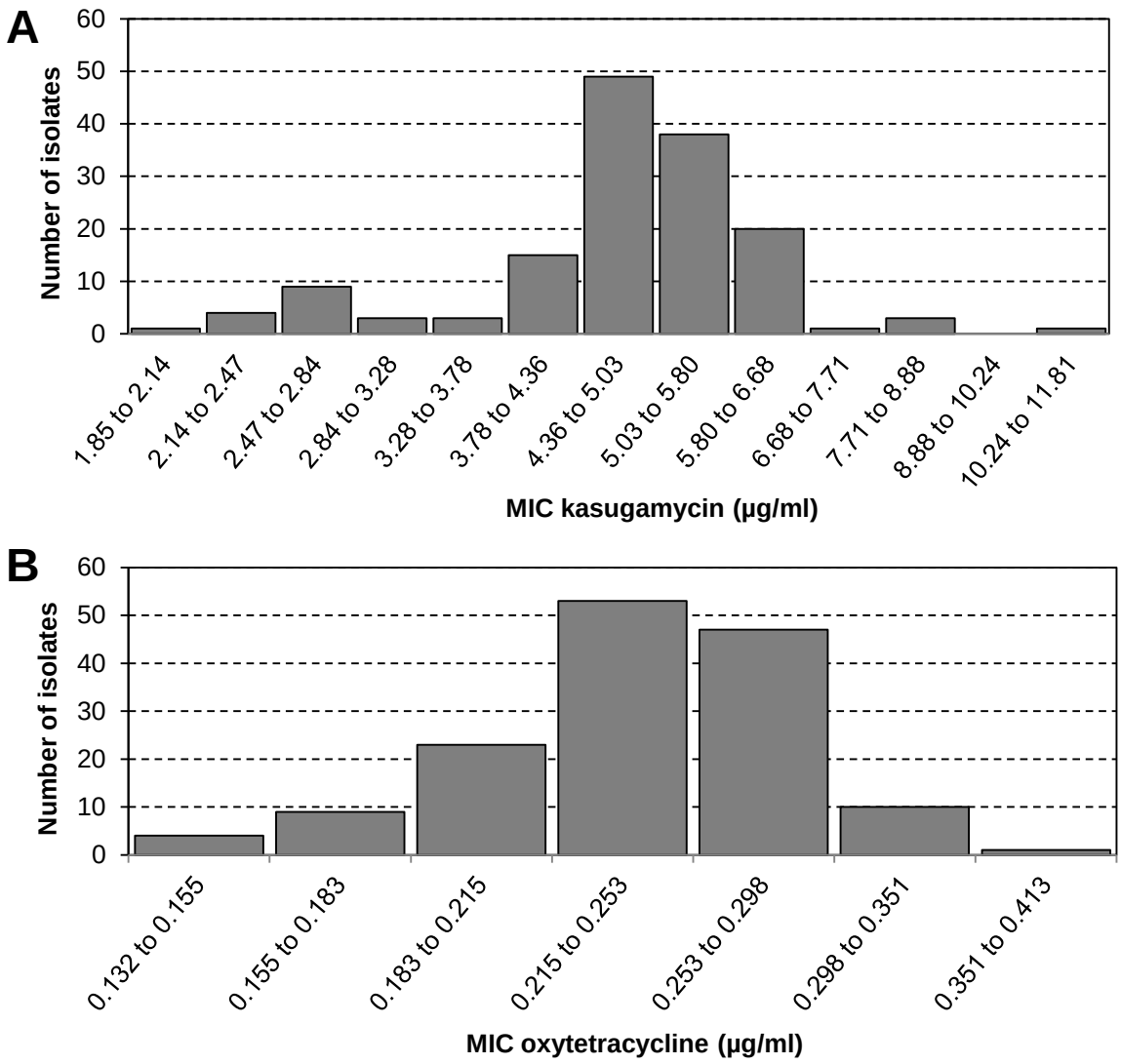

Figure 4.2. Scott's distributions of minimum inhibitory concentration (MIC) values (concentrations that inhibit growth by ≥95%) of 147 *Pseudomonas savastanoi* pv. *savastanoi* strains from California for **A,** kasugamycin, and **B**, oxytetracycline. Bar height indicates the number of strains within each bin, and bin width was calculated using Scott's method (Scott 1979).

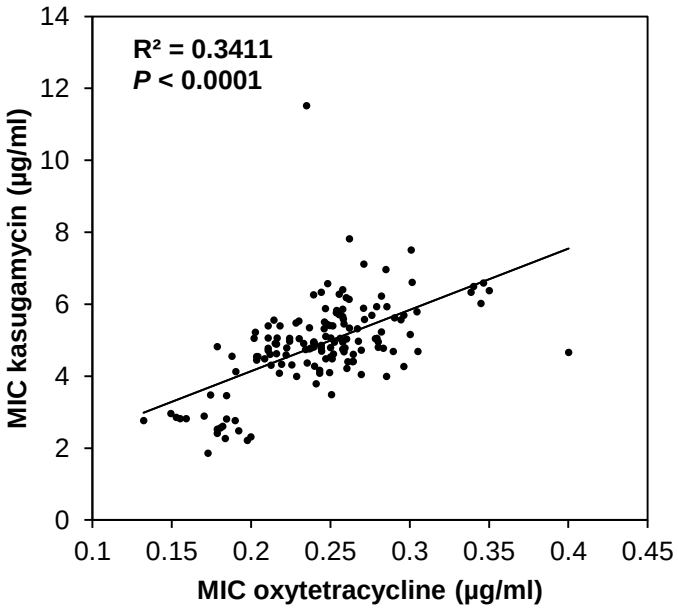

Figure 4.3. Scatter plot of minimal inhibitory concentration (MIC, concentration that inhibits growth by ≥95%) values of kasugamycin plotted against those of oxytetracycline for 147 *Pseudomonas savastanoi* pv. *savastanoi* strains.

CHAPTER V. GENERAL CONCLUSIONS

Changes in olive cultivation methods and increases in production acreages in California will likely result in an increase in the incidence of olive knot and a higher risk of spreading the pathogen among growing areas. Management of olive knot is difficult, and only a limited number of treatments are currently available for its control. Therefore, studies on the epidemiology of olive knot were conducted to better understand the disease for more effective management, and new sanitation and bactericide treatments were evaluated. We determined that a non-corrosive QAC was a safe and effective sanitizing agent for *P. savastanoi* pv. *savastanoi*-contaminated field equipment to reduce the spread of the bacterium. The detection of copper resistance in the pathogen population prompted the evaluation of new antimicrobials. Kasugamycin was identified as an effective foliar protective treatment that complements the use of copper-based bactericides in current olive knot management strategies and will help to reduce the spread of copper resistance. The major research objectives reported in the dissertation were:

i. Characterize the genetic diversity of *P. savastanoi* pv. *savastanoi* populations in major olive producing regions of California and determine if certain genotypes are associated with geographic regions or specific olive cultivars and if the origin of copper resistance can be elucidated .
ii. Compare the virulence of copper-sensitive and -resistant strains of *P. savastanoi* pv. *savastanoi* and the susceptibility of two major olive cultivars grown in

California in inoculation studies using different types of wounds and inoculum concentrations.

iii. Assess if QACs can be effective sanitizing agents against the olive knot pathogen in laboratory and field studies.

iv. Establish in-vitro sensitivities of *P. savastanoi* pv. *savastanoi* strains to copper, kasugamycin, and oxytetracycline.

v. Determine the best application strategies for copper, kasugamycin, and oxytetracycline for preventing olive knot in greenhouse and field trials.

This research provides new information on the epidemiology of olive knot in California and on new management tools. The following results were obtained and conclusions were made:

i. The genetic diversity of 152 *P. savastanoi* pv. *savastanoi* strains in California was determined using rep-PCR. Overall, the genetic variability of the population was low with most strains belonging to one of two major groups. The copper-resistant strains had similar fingerprint patterns and were not closely related to any of the –sensitive strains. Genetically heterogeneous populations were found in Glenn, Yuba, and Tehama counties. Fifteen of the 26 orchards sampled contained a genetically mixed population, and no association of a specific haplotype with a specific olive cultivar was established.

ii. High concentrations of *P. savastanoi* pv. *savastanoi* were exuded from olive knots 10 min after rehydration, and concentrations were sufficient to cause

disease on wounds. Inoculum concentration had a significant effect on disease incidence, and more disease developed at higher concentrations. This effect was observed in all inoculation studies with different olive cultivars, *P. savastanoi* pv. *savastanoi* strains, and types of olive wounds. A copper-sensitive strain was significantly more virulent than a copper-resistant strain based on the incidence of disease on 'Arbequina' leaf scar wounds, but not on lateral wounds. Still, both strains caused a high incidence of disease, especially at higher concentrations ($\geq 10^6$ CFU/ml). 'Manzanillo' olives were significantly less susceptible to infection than 'Arbequina' olives for leaf scar and lateral wounds, but both cultivars were considered highly susceptible.

iii. In laboratory tests, two QAC formulations were highly toxic to *P. savastanoi* pv. *savastanoi* over a broad pH range (6 to 9) using short exposure periods (15 to 60 s) and low concentrations (5 µg/ml). When a hard surface that was contaminated with bacteria in the presence of an organic load was treated with the same formulations at 2,000 µg/ml, bacterial recovery was reduced by ≥ 3.6 $\log_{10}$ CFU/ml after 90 s of exposure. These QACs were significantly more effective than sodium hypochlorite at 100 µg/ml. In field trials, hedging equipment contaminated with a heavy load of bacteria and used to prune olives was successfully sanitized using a QAC, and in some trials, new infections were completely prevented. QACs were not effective as a protectant treatment for preventing knot development on inoculated olive wounds. Based on these results, a QAC formulation was registered for use on olive field equipment in 2015.

iv. Baseline sensitivities were established for kasugamycin and oxytetracycline for 147 strains of *Pseudomonas savastanoi* pv. *savastanoi* collected from olive knot samples throughout California. Minimum inhibitory concentrations (MICs) for ≥95% growth inhibition ranged from 1.86 to 11.52 µg/ml and 0.13 to 0.40 µg/ml for kasugamycin and oxytetracycline, respectively. In copper sensitivity evaluations, 95.3% of the strains collected only grew at concentrations of <20 µg/ml metallic copper equivalent (MCE), 2.7% grew at 20 to 30 µg/ml MCE (moderately resistant), and 2% grew at 150 µg/ml MCE (highly resistant). Copper resistance was never reported previously in the olive knot pathogen, and pathogenicity studies confirmed that copper-resistant strains were highly virulent.

v. In comparative field studies, kasugamycin at 200 µg/ml performed equally to the standard copper hydroxide treatment (1,260 µg/ml MCE) in reducing knot development on lateral wounds of 'Arbequina' and 'Manzanillo' olives inoculated with a copper-sensitive strain and was better than copper using a highly copper-resistant strain. Oxytetracycline at 200 µg/ml was not as effective as copper or kasugamycin, but significantly reduced disease incidence as compared with the untreated control. Field studies on application timings of copper, kasugamycin, and copper-kasugamycin mixtures to inoculated wounds indicated that treatments within 24 h of inoculation resulted in higher disease control than applications at later times. In greenhouse trials, copper or copper-kasugamycin applied to wounds 7 days before inoculation persisted and reduced knot incidence by >50%. Lateral and leaf scars wounds were less susceptible to infection when inoculated

after ≥10 days indicating wound healing. Our findings demonstrate that kasugamycin is an effective bactericide for controlling olive knot and that the time of bactericide application after inoculation is critical in managing the disease.