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Improved Management Strategies for New and Re-Emerging Diseases of Almond and Strawberry in California

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

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by

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DEDICATION

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ABSTRACT OF THE DISSERTATION

Improved Management Strategies for New and Re-Emerging Diseases of Almond and Strawberry in California

by

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Changes in pathogen populations, standard production practices, and regulatory restrictions have rendered current management strategies insufficient in pathosystems of strawberry and almond in California. New, improved and integrated strategies seek to reduce the economic impact of disease, reduce the risk of pathogen spread, and prevent development of pathogen resistance to chemical strategies.

Anthracnose crown rot of strawberry, caused primarily by *Colletotrichum acutatum* in California, is an important disease impacting nursery and fruit production. Pre-plant dip treatment with azoxystrobin failed during a recent disease outbreak, and QoI-resistance was confirmed. In evaluation of alternative treatments, the biofungicide natamycin was identified and was highly effective at reducing disease severity and mortality caused by QoI-sensitive or resistant isolates, and based on this work, was registered federally as a new dip treatment of nursery plants.

Angular leaf spot of strawberry, caused by *Xanthomonas fragariae*, has impacted export of California fruit due to quarantine restrictions. Pre-harvest chemical

management is limited to copper, which is not very efficacious and can be phytotoxic. The bactericide amino thiadiazole was highly effective by itself or in select mixtures, and reduced disease incidence to low levels. Post-harvest fumigation with propylene oxide at select doses significantly reduced bacterial populations in infected leaflet tissues by at least 2.5-log compared with controls, with no phytotoxic effect to leaves or fruit. Together, this systems-management approach could reduce the risk of *X. fragariae* on fruit destined for export.

Bacterial spot, caused by *Xanthomonas arboricola* pv. *pruni*, is herein reported as a new disease of almond in California. Epidemiological studies demonstrated susceptibility of 'Fritz' almond over the spring season following inoculation of fruit and leaves, as well as flowers with subsequent disease development on fruit. Mummified fruit and associated spurs were identified as pathogen overwintering sites. Dormancy application of copper or copper-mancozeb significantly reduced disease in high-rainfall years. In-season bactericide application at petal fall or full bloom and petal fall also significantly reduced disease incidence, but was associated with phytotoxicity in some trials. Dormancy and in-season treatments of copper-mancozeb mixtures integrated with removal of mummified fruit are currently the best strategies for managing the disease.

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GENERAL INTRODUCTION

Today's agricultural commodities must adapt to address new challenges that arise in disease systems due to changes in pathogen populations, standard production practices, and state, federal, and international regulations. Management strategies employed, therefore, need to be efficacious, economical, safe for the consumer, and environmentally responsible. Additionally, with an increasingly global market in the movement of both planting materials and food products, efforts made toward disease reduction also reduce the risk of long-distance pathogen dissemination and avoid market closures when quarantine laws are enacted. Within this dissertation, new practices for the management of two strawberry diseases, (i) anthracnose crown and root rot caused by fungi in the genus *Colletotrichum*, and (ii) angular leaf spot caused by the bacterium *Xanthomonas fragarie*, as well as a herein reported new almond disease in California (i.e., bacterial spot caused by *X. arboricola* pv. *pruni*) are presented. This research sought to address one to several of the challenges listed above recently faced by the respective commodities.

Strawberry and almond are among the most economically important crops grown in the state of California. The strawberry industry represents over 80% of fresh-market fruit production in the United States with an approximate value of over 1.8 billion dollars in 2016, and was the tenth largest valued agricultural export in the state (CDFA 2017; USDA/NASS 2017). The almond industry represents 100% of United States production and over 80% of the world production, with an approximate value of over 5.1 billion dollars in 2016 and was the largest valued agricultural export in the state (CDFA 2017).

In the management of anthracnose crown and root rot of strawberry, caused primarily by *C. acutatum* in California, a disease outbreak occurred in transplant nursery production areas in the 2015-2016 growing season. This was in-part attributed to reported failure of the extensively-applied quinone-outside inhibitor (QoI) azoxystrobin, a single-site mode of action fungicide, following dipping of transplants in a suspension with this compound prior to planting (Forcelini et al. 2016). This nursery-level outbreak resulted in dissemination and planting of asymptomatic transplant stock with latent infections, and following overhead watering as is common for early plant establishment (Daugovish et al. 2009), significant economic losses occurred as plants were stunted or died due to crown infections. The only other compound registered for this use, a pre-mixture of the anilinopyrimidine cyprodinil and the phenylpyrrole fludioxonil, is reported to cause phytotoxicity and stunting of plants under some environmental conditions or following off-label plant exposure durations.

The objectives of Chapter 1 (accepted for publication in Plant Disease, Haack et al. 2018), were therefore to confirm QoI-resistance in California, evaluate alternative compounds for efficacy as a pre-plant fungicide dip treatment, and present baseline sensitivity data for a new fungicide, natamycin, for agricultural use. Specifically, natamycin was investigated for its efficacy in managing anthracnose crown rot and its toxicity against *C. acutatum.* Natamycin is classified as a biopesticide because it is a commercial fermentation product of *Streptomyces natalensis* (Aparicio et al. 2016; EPA 2017). The US Environmental Protection Agency has designated biopesticides as natural occurring compounds, products that are naturally produced or derived from the

culturing/fermentation of a biological organism (i.e., biochemicals), or plant incorporated protectants. The registration of a biopesticide still requires an EPA review but it is less costly compared to that of a synthetically manufactured active ingredient, and is exempt from residue tolerances. Additionally, biopesticides are generally recognized as inherently reduced-risk pesticides, being safer for the environment, handlers, and consumers.

Natamycin has been used as an additive in cheese and other food products since the 1960s (Aparicio et al. 2016; Delves-Broughton 2014) and is designated as 'generally recognized as safe' (i.e. 'GRAS'), by the United States Food and Drug Administration. This biopesticide has a unique mode of action from any other fungicide used in strawberry, directly binding to ergosterol, the primary sterol in fungi, leading to disruption of membrane function and cellular transport (Aparicio et al. 2016; Hamilton-Miller 1974). Natamycin is also used in United States mushroom cultivation and was recently registered for post-harvest fruit disease management (Chen et al. 2016). Development of preharvest foliar uses has not been possible because it is sensitive to breakdown following ultraviolet (UV) light exposure (Koontz et al. 2003).

In California and elsewhere, angular leaf spot of strawberry is not associated with significant crop losses but the pathogen, *Xanthomonas fragariae,* is a quarantine-regulated organism, and is therefore economically important due to trade restrictions (EPPO 1997; Turechek and Peres 2009). Specific trade losses for fresh-market California fruit occurred during exports to Australia in 2010 and 2011, where shipments were rejected following detection of the pathogen, and such exports have since been suspended

(AGDA 2018; AQIS 2011). Angular, water-soaked lesions are the primary disease symptom, and all green plant parts can be infected, including fruit calyxes where the disease was detected on exported strawberry fruit (Kennedy and King 1962; Maas 1998).

Systems-based approaches with multiple independent and additive management practices enacted to reduce the risk of pathogen or pest movement is used as the basis for trade mitigation and regulation (Heather and Hallman 2007). Such approaches in insect quarantine management include both pre-harvest chemical and cultural management, as well as post-harvest fumigation treatment of commodities (Follett and Neven 2006). Therefore, as presented in Chapter 2, pre- and post-harvest management strategies were investigated for managing angular leaf spot of strawberry. For pre-harvest management of angular leaf spot, copper is the only registered option for chemical control, and efficacy is inconsistent and phytotoxicity common (Roberts et al. 1997). Additional bactericides with high efficacy and different modes of action are needed. Thiazole- and thiadiazole-derived compounds have demonstrated antibacterial activity, thought to interfere with cell peroxidation and inhibit lipid synthesis or transport in the cell membrane (FRAC 2018; Hu et al. 2014). A commercial formulation with zinc is used in China against other Xanthomonad diseases (Chen et al. 2014; Zhang et al. 2013). Technical-grade amino thiadiazole was therefore investigated alone and in mixture with copper for pre-harvest preventative management of angular leaf spot.

Methyl bromide is used for post-harvest management of quarantine insect pests of strawberry (Walse et al. 2012) but is herein confirmed to have no effect against *X. fragariae.* Thus, an alternative compound, i.e. propylene oxide, was evaluated because of

its long-term use in California nut industries as a post-harvest antimicrobial fumigant for pasteurization against foodborne pathogen contamination (EPA 2006), and because of its demonstrated activity against quarantine mites and insects of strawberry, indicating potential for co-registration. Phytotoxicity is often a limiting factor for many postharvest chemical fumigations, and effects of rate, duration, and temperatures on both efficacy and phytotoxicity are considered, while maintaining relevance to current commercial practices used at post-harvest fumigation facilities.

In strawberry, methyl bromide has been ubiquitous in its use as a pre-plant soil fumigant for both nursery transplants as well as fruit production fields. Following the phase-out of methyl bromide in 2005, strawberry growers have received critical use exemption, allowing limited and highly regulated continued use, but such exemption is reviewed annually and it is unclear whether use will be allowed in the future (Federal Register 2015). Methyl bromide is highly effective against *C. acutatum* populations that can survive in the soil for up to 9 months and against *X. fragariae* populations on leaf litter and debris that may persist for over 1 year (Eastburn and Gubler 1990; Gubler et al. 2005; Maas 1998). In California, due to high land prices and decreasing availability, strawberry are planted annually and often with minimal crop rotation to lesser-value crops. Therefore, continued phase-out of this compound at the fruit-production level and especially at the nursery-transplant production level could result in increased pathogen persistence and dissemination, and corresponding increased disease levels, further highlighting the need for diverse management strategies and tools in strawberry.

Bacterial spot of almond, caused by *Xanthomonas arboricola* pv. *pruni,* was first diagnosed in the San Joaquin Valley almond growing region in 2013 and is herein reported as a new disease of almond in California. In almond, a high-input system in regards to water and fertilization management has emerged in the past decade, driven by high almond prices and low labor costs due to horticultural mechanization. Additionally, movement from low density (70 trees/acre) intensely pruned orchards to high density (110 to 140 trees/acre) minimally pruned orchards to increase yield per acre has also increased humidity in the orchard microclimate, favoring disease development (Hendricks 1996; Arquero and Jarvis-Shean 2017). The most profitable and popular almond cultivar 'Nonpareil' requires the cross-pollination with a different cultivar for proper nut set (Socias i Company et al. 2017). Cultivar 'Fritz', itself having a high yield and nut quality as well as a late harvest, has grown in popularity, with multiple orchards with Nonpareil/Fritz coming into full production maturity in the past decade (ABC 2017; Asai et al. 1996). 'Fritz' almond, however, is highly susceptible to bacterial spot (Palacio-Bielsa et al. 2017). The disease, characterized by light to dark-colored amber gumming that exudes from the fruit hull, was likely present for some time but misdiagnosed as being caused by other gumming-agents, including insect feeding damage and almond anthracnose. On peach and other *Prunus* hosts in other growing regions of the United States, the disease is common and the epidemiology is well understood under high rainfall and humidity (Ritchie 1995; Ritchie et al. 2008). These environmental conditions are quite different than those commonly present in California. Historically, copper applications are considered only moderately efficacious against *X.*

arboricola pv. *pruni* and phytotoxicity limits use (Brannen et al. 2007; Ritchie 1999). Copper is also used as a management tool against walnut blight in California caused by *X. arboricola* pv. *juglandis,* though it must often be mixed with the ethylene bis-dithiocarbamate fungicide mancozeb to overcome copper-resistant strains (Nguyen et al. 2016). Because mancozeb is registered on almonds for several fungal diseases but is not registered on stone fruit, the compound needs to be evaluated for managing bacterial spot of almond caused by a different pathovar of *X. arboricola*. The objectives of this study, therefore, were to confirm the presence and causality of the disease following Koch's postulates, apply modern identification methods of the pathogen, evaluate infection timing and host susceptibility of different phenological stages, determine overwintering sites of the pathogen, and determine the efficacy of dormancy and/or in-season bactericide applications of copper or copper-mancozeb.

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CHAPTER I.

Natamycin, a New Biofungicide for Managing Crown Rot of Strawberry Caused by QoI-Resistant *Colletotrichum acutatum*

ABSTRACT

Anthracnose crown rot of strawberry, caused by *Colletotrichum acutatum*, is an important disease impacting California nursery and fruit production. Pre-plant dip treatments of transplants with fludioxonil-cyprodinil or azoxystrobin are industry standards for managing the disease and have been used extensively. Following reports of reduced efficacy of azoxystrobin in the field, high levels of QoI resistance were detected in California isolates of the pathogen. Resistance was associated with the G143A mutation in *cytb*, similar to a previous report from Florida, and there were no detected fitness penalties in pathogenicity or virulence. Therefore, several alternative fungicides were investigated in laboratory and field studies. Subsequently, the new biofungicide natamycin was identified. Baseline sensitivities of 74 isolates of *C. acutatum* to natamycin were determined to be unimodal with a range from 0.526 to 1.996 μg/ml (mean 0.973 μg/ml). Although this toxicity was considerably lower than that of azoxystrobin (using sensitive isolates), fludioxonil, or cyprodinil, dip treatments of transplants with natamycin (at 500 or 1000 mg/liter) were highly effective. Disease severity and plant mortality in field studies with inoculated transplants were reduced to similar low levels as treatments containing fludioxonil, whereas azoxystrobin failed in inoculations with QoI-resistant isolates of *C. acutatum*. Fruit yield was also significantly increased by natamycin as compared with the inoculated control. Differences in disease

susceptibility were observed among cultivars evaluated, with ‘Monterey’ and ‘Portola’ more susceptible as compared with ‘Fronteras’. Natamycin has a unique mode of action that is different from other fungicides registered on strawberry and, based on this research, was registered in the United States as a pre-plant dip treatment of strawberry transplants for management of anthracnose crown rot.

INTRODUCTION

Anthracnose is a major disease of strawberry (*Fragaria* x *ananassa* Duch.) in California nursery and fruit production (Gunnell and Gubler 1992; Smith 1998), an industry that represented 83% of fresh market fruit production in the United States and is estimated to have a value of over $1.8 billion annually (CDFA Agricultural Statistics 2015-16). All parts of the plant are susceptible to infection, resulting in significant yield loss from any of the different phases of the disease including crown rot, root rot, flower blight, fruit rot (i.e., black spot), petiole lesions, and leaf spots (Daugovish et al. 2009; Howard et al. 1992; Smith 2008). Members of the fungal genus *Colletotrichum* are associated with strawberry anthracnose worldwide, with *C. gloeosporioides* (Penz) Penz. & Sacc. and occasionally *C. fragariae* A. N. Brooks most commonly causing crown rot and root rot, and *C. acutatum* J. H. Simmonds most commonly causing foliar and fruit rot (Freeman and Rodriguez 1995; Howard et al. 1992; Smith and Black 1990). In California, however, *C. acutatum* is the most common causal agent for all above- and below-ground phases of the disease (Daugovish et al. 2009; Koike et al. 2008). More

recently, *C. gloeosporioides* and *C. acutatum* are considered to be species-complexes (Damm et al. 2012; Weir et al. 2012).

Salmon- to orange-colored conidial masses are produced by *C. acutatum* in acervuli on infected tissues at high humidity. They are spread short distances by water splash from overhead sprinkler irrigation or rainfall, and long-distance by infected plant material, soil associated with transplants, or contaminated equipment (Debode et al. 2015; Eastburn and Gubler 1990; Howard et al. 1992). The pathogen can survive in infected plant debris and soil for at least nine months in the absence of a host (Eastburn and Gubler 1990; Freeman et al. 2002). In fields that have been fumigated, disease outbreaks are attributed to planting of infected nursery stock. Transplants that appear healthy may have quiescent infections that are activated once conditions are favorable (Debode et al. 2015; Horn and Carver 1968; Leandro et al. 2001), typically under warm, wet environments (King et al. 1997). Crown infections can cause the entire plant to wilt and die during early establishment of fruit production fields where overhead irrigation is common (Eastburn and Gubler 1990). Significant anthracnose crown rot outbreaks occurred in California nurseries during the 2000-2001 (Daugovish et al. 2009) and 2015-2016 (K. Ivors, *unpublished*) seasons, resulting in high economic losses.

For anthracnose crown rot management, water wash or fungicide treatments of bare-root transplants have been used in the nursery or immediately before planting in production fields (Daugovish et al 2009; Freeman et al. 1997). Running water removes soil from root and crown tissues, potentially removing inoculum, but does not control any infections already present (Koike et al. 2008). Pre-plant fungicide dips have been shown

to decrease anthracnose crown rot, but only two products are currently registered for this use in the United States. The single-site mode of action quinone outside inhibitor (QoI) azoxystrobin has been used extensively as a pre-plant dip and as a foliar treatment for management of anthracnose and other fungal diseases (Bartlett et al. 2002; Koike et al. 2008; Turechek et al. 2006). Resistance, however, has been reported against this fungicide group in *C. acutatum* populations from strawberry in Florida (Forcelini et al. 2016). During the 2015-2016 California nursery crown rot outbreak, growers reported reduced efficacy of azoxystrobin dips, suggesting that resistance may also be present in California (K. Ivors, *unpublished*). A pre-mixture product of the anilinopyrimidine cyprodinil and the phenylpyrrole fludioxonil is also registered, but phytotoxicity and stunting of plants have been reported under some environmental conditions or following off-label plant exposure durations (G. Holmes, *unpublished*). In addition to chemical treatments, heat treatment of transplants may reduce mortality from anthracnose, but this practice is not widely used because it can reduce plant vigor (Freeman at al. 1997). In-season fungicide spray applications are common to prevent foliar and fruit infections (Koike et al. 2008; MacKenzie and Peres 2012), but these are ineffective against crown rot and root necrosis (Daugovish et al. 2009).

Natamycin, also known as pimaricin, is a natural polyene macrolide derived from fermentation of *Streptomyces* species (i.e., *S. natalensis, S. chattanoogensis, S. gilvosporeus,* and *S. lydicus*), and has been used as an anti-fungal additive in the food industry since the 1960s (Aparicio et al. 2016; Lu et al. 2008). Currently, it is registered in the United States as a biopesticide on mushroom beds and recently, as a postharvest

treatment on stone fruit and citrus. Because of its unique mode of action and because resistance has never been reported in filamentous fungi during extensive use in the food industry (Aparicio et al. 2016), this fungicide could be valuable in strawberry production where QoI resistance has been reported in *C. acutatum* and new rotational products are needed for dip treatments.

The purpose of this research was to evaluate new fungicides with registration potential for use as pre-plant dip treatments of bareroot strawberry transplants for the management of anthracnose crown rot. The specific objectives were to: (i) determine if QoI resistance is present in *C. acutatum* populations in California and confirm with molecular tools; (ii) establish in-vitro sensitivity ranges of EC_{50} values for selected new fungicides for QoI-sensitive and –resistant isolates of *C. acutatum*; (iii) evaluate the efficacy of fungicide dip treatments on disease severity, plant mortality, and fruit yield; and (iv) evaluate susceptibility of selected cultivars to disease.

MATERIALS AND METHODS

Fungal isolates and culture methods. Seventy-four isolates of *C. acutatum* were obtained in 2015 and 2016 from commercial strawberry fields in Santa Barbara, San Luis Obispo, Ventura, Santa Cruz, Stanislaus, Tehama, San Joaquin and Merced counties of California (Table 1.1). The transplant material used to establish these fields were originally sourced from five independent nursery companies that have operations throughout the state (Table 1.1). Crowns and petioles of symptomatic plants were sectioned into small pieces (5 mm^2) using a scalpel, surface disinfested in 1% sodium

hypochlorite for 2 min, triple-rinsed with sterile distilled water, and plated onto potato dextrose agar (PDA) acidified with 1 ml 85% lactic acid per liter (APDA). Isolates were obtained from fruit lesions by touching a sterile cotton swab onto the lesion and then streaking the swab onto the surface of APDA plates. Colonies with morphological characteristics of *Colletotrichum* spp. were sub-cultured onto PDA and examined microscopically. Colonies of *C. acutatum* were differentiated from other species of *Colletotrichum* by conidial morphology (Adaskaveg and Hartin 1997; Smith and Black 1990) and a subset of 26 isolates (Table 1.1) was confirmed to belong to the *C. acutatum* species complex using CaInt-1 and CaInt-2 primers targeting the internal transcribed sequence (ITS) region (Adaskaveg and Hartin 1997; Sreenivasaprasad et al. 1996). Cultures were stored in liquid nitrogen, and working stocks were kept as PDA plugs in sterile water at 12°C. For conidial production, a mycelial suspension was prepared from 4- to 7-day-old PDA cultures, spread onto new PDA plates, allowed to air-dry, and 5 to 10, 1.5-cm long autoclaved strawberry petiole pieces were randomly placed onto the agar surface. Orange conidial sporulation was present after 3 to 4 days of incubation at 25°C and was used for inoculations or in-vitro assays within 4 weeks.

Fungicides. Formulated products of natamycin (DelvoCid Instant; DSM, Heerlen, The Netherlands), azoxystrobin (Abound Flowable; Syngenta Crop Protection Inc., Greensboro, NC), chlorothalonil (Bravo Weather Stick; Syngenta Crop Protection Inc.), cyprodinil (Vangard 75WG; Syngenta Crop Protection Inc.), fludioxonil (Scholar 50WP; Syngenta Crop Protection Inc.), and pydiflumetofen (experimental product A19649B; Syngenta Crop Protection Inc.) were used for in-vitro sensitivity studies as indicated in

Fig. 1.1. For treatment of plants, these products were used by themselves or in pre-mixtures of fludioxonil and cyprodinil (Switch WG; Syngenta Crop Protection Inc.) or of pydiflumetofen and fludioxonil (experimental product A20560C; Syngenta Crop Protection Inc.) as indicated in Fig. 1.2. Additionally, the biological control *Streptomyces lydicus* WYEC 108 (minimum of 10^3 cfu/g formulated product; Actinovate AG; Valent U.S.A. LLC, Walnut Creek, CA) was included in field trials. Aqueous solutions were used in all studies.

Evaluation of in vitro sensitivity of fungicides. Fungicide sensitivities for all isolates of *C. acutatum* were determined using the spiral gradient dilution method (Förster et al. 2004). Briefly, an exponential concentration gradient for each fungicide was obtained by depositing appropriate stock concentrations radially onto 15-cm Potato Dextrose Agar (PDA) plates using a spiral plater (Autoplate 4000; Spiral Biotech, Inc.). For azoxystrobin, PDA was amended with 100 µg/ml salicylhydroxamic acid (SHAM). Stock concentrations were 1,000 µg/ml for natamycin, 100 or 5,000 µg/ml for azoxystrobin, 200 µg/ml for chlorothalonil, 50 µg/ml for cyprodinil, 20 µg/ml for fludioxonil, and 10,000 µg/ml for pydiflumetofen. Sterile water was applied to control plates. After 2 to 4 h, 10 µl of each conidial suspension (5 x 10^5 spores/ml) was streaked radially from the outer edge towards the center of the plate using a sterile pestle. Each isolate was replicated on opposite sides of the plate, and four isolates were evaluated on each plate. Plates were incubated in the dark for 20 h at 20°C for evaluation of conidial germination (azoxystrobin only) or for 72 h at 25°C for evaluation of mycelial growth. Effective concentrations to inhibit mycelial growth or conidial germination by 50% (EC_{50}

values) were determined as described previously (Förster et al. 2004). Each isolate was evaluated twice for each fungicide.

Molecular characterization of QoI resistance in *C. acutatum.* A subset of 5 QoI-sensitive and 11 QoI-resistant *C. acutatum* isolates (Table 1.1) were grown for 7 to 10 days on PDA. DNA was extracted from mycelia using the MPBio FastDNA kit (MPBio, Santa Ana, CA). PCR amplification was done using primers C.gramcytb-bf1 and C.gramcytb-br1 targeting the cytochrome b gene (*cytb*) according to Forcelini et al. (2016). Product amplification was confirmed by agarose (1%) gel electrophoresis in 0.5x Tris Borate EDTA buffer. Amplicons were purified using ExoSap-IT (Affymetrix Inc., Santa Clara, CA) and sequenced at the Core Instrumentation Facility of the University of California Riverside Institute for Integrative Genome Biology. Sequences were aligned using the BioEdit software (www.mbio.ncsu.edu/BioEdit/bioedit.html).

Effect of pre-plant dip treatments on disease severity and plant mortality. Two trials were conducted using 'Portola' and 'Fronteras' (trial 1) or 'Monterey' and 'Fronteras' (trial 2) strawberry cultivars. Bareroot transplants were obtained from two nurseries and dipped for 4 min with agitation in aqueous conidial suspensions (10^6 conidia/ml) of a mixture of four each of QoI-sensitive or -resistant isolates of *C. acutatum* (Table 1.1). Plants were stored at 20°C for 8 h to allow conidia to germinate and infect, and then up to 3 days at 5°C. Plants were then dipped in aqueous fungicide solutions for 4 min with agitation. Inoculated control plants were dipped in water or were left untreated. Excess solution was allowed to drain for approximately 2 min, and transplants were stored in plastic bags at 5°C overnight. Planting in the field was done on

March 4, 2016 (trial 1), and October 29, 2016 (trial 2), at the University of California Riverside Agricultural Operations facility. The field area had been fallow for over one year, and had not been planted with strawberry previously. No fumigation treatment was applied prior to planting. Plants were spaced 30 cm apart in double-row beds in a randomized complete block design. Each bed represented a block (replication) of plots, and there were four blocks each for plants inoculated with QoI-sensitive or resistant isolates in each study. Each replication of treatments consisted of a plot of 10 plants of each cultivar. Overhead irrigation was applied three days each week for 30 min each in the morning and afternoon for the first month, and drip irrigation was applied for two days each week for 2 h throughout the study. Fertilizer was applied through drip irrigation weekly or bi-weekly. No foliar fungicide or insecticide applications were made.

Plants were evaluated periodically for disease. Fungal isolations were done from diseased tissues of fifteen randomly selected, symptomatic plants each that were inoculated with QoI-sensitive or –resistant isolates to validate that *C. acutatum* was the causal agent. Isolations from crown, leaf, and fruit tissues were done as described above, except that tissues were plated onto PDA amended with ampicillin (130 µg/ml) and rifampicin (20 µg/ml). Plants in the field were evaluated after 5 (trial 1) or 14 weeks (trial 2) for disease severity on above-ground growth using the following scale: 0 = healthy, vigorous plant; 1 = growth reduced; 2 = growth and vigor reduced, less than 50% the size of healthy plants; 3 = growth and vigor extremely reduced, less than 25% the size of healthy plants, and 4 = plant dead. Severity ratings were averaged for each 10-plant

replication. The incidence of plant mortality was based on the number of dead plants (rating = 4) of the 10 plants of each cultivar in each replication.

Effect of pre-plant dip treatments on fruit yield. Crop yield was determined using 'Fronteras'. In trial 1, a single harvest of all mature red, commercially acceptable fruit for each treatment replication was conducted 10 weeks after planting, and fruit were weighed. In trial 2, flowers and immature fruit were removed 7 and 12 weeks after planting, similar to commercial practices to encourage vegetative growth during the winter period. All mature red, commercially acceptable fruit were harvested 20 and 23 weeks after planting, and fruit weights were combined for the two harvests for each treatment replication.

Statistical analysis. For in-vitro fungicide toxicity experiments, variances were homogeneous for the two experiments according to Bartlett's test, and data were combined for each isolate-fungicide combination. This data set was used to determine mean values and the range of sensitivity. Mean EC_{50} values for mycelial growth or spore germination were plotted for each fungicide in frequency distributions according to Scott's method (Scott 1979).

Data for incidence of mortality were arcsin-transformed, whereas yield data were transformed using $y=\log_{10}(x+1)$ prior to statistical analysis for both trials. All data for disease severity, incidence of mortality, and yield were normally distributed according to Shapiro-Wilk's test. Data were combined from different experiments when variances were homogeneous according to Bartlett's test. Analyses of variance (ANOVA) or generalized linear model (GLM) procedures were performed following a factorial design

with cultivar, inoculum type, and fungicide treatment as the main factors. When interactions between cultivar and inoculum type were significant, data were analyzed separately for each combination with treatment as the single factor. Multiple comparisons for treatment means were conducted using Fisher's Least Significant Difference (LSD) test. All statistical procedures were done using SAS (version 9.4; SAS Institute, Inc., Cary, NC) with a significance value of α=0.05.

RESULTS

In vitro sensitivity of isolates of *C. acutatum* to fungicides. Of the 74 isolates tested, 29 were determined to be QoI-resistant, with EC_{50} values for mycelial growth and conidial germination higher than 40 μg/ml (Table 1.1). The remaining 45 isolates were QoI-sensitive, with mean EC_{50} values for mycelial growth and germination inhibition of 0.034 μg/ml (range 0.015 to 0.064 μg/ml) and 0.129 μg/ml (range 0.060 to 0.267 μg/ml), respectively. Mean EC_{50} values for mycelial growth for all 74 isolates for chlorothalonil, cyprodinil, fludioxonil, and natamycin were 0.267 μg/ml (range 0.115 to 0.549 μg/ml), 0.025 μg/ml (range 0.012 to 0.390 μg/ml), 0.001 μg/ml (range 0.0006 to 0.0014 μg/ml), and 0.973 μg/ml (range 0.526 to 1.996 μg/ml), respectively. Sensitivity data are presented in Table 1.2 for QoI-sensitive and -resistant isolates separately. For azoxystrobin, a greater than 1000 times difference in sensitivity between sensitive and resistant groups was observed. No significant differences in sensitivity to cyprodinil (P=0.3468), fludioxonil (P=0.2556), and natamycin (P=0.1010) were found for QoI-sensitive and -resistant isolates. For chlorothalonil, QoI-sensitive isolates were significantly (P=0.0375)

less sensitive (mean EC_{50} value 0.276 μg/ml) than QoI-resistant isolates (mean EC_{50} value 0.250 μg/ml; Table 1.2). Pydiflumetofen was not inhibitory to mycelial growth at concentrations of up to 80 μg/ml tested.

Frequency distributions of EC_{50} categories for each fungicide are shown in the histograms of Fig. 1.1A-F. Distributions for all fungicides were unimodal except for azoxystrobin where a bimodal distribution was observed.

Molecular characterization of QoI resistance in *C. acutatum.* All 11 QoI-resistant isolates selected for *cytb* sequencing were found to contain a single amino acid substitution from glycine (G) to alanine (A) at codon 143 (G143A mutation), resulting from a base pair substitution from guanine to cytosine. None of the QoI-sensitive isolates sequenced showed this mutation.

Effect of pre-plant dip treatments on disease severity and plant mortality. The overall models for severity and mortality disease measurements, as well as for the independent variables cultivar, type of inoculum (i.e., QoI-sensitive or –resistant isolates of *C. acutatum*), and treatment were highly significant ($P<0.0001$). There were significant ($P<0.0001$) interactions between cultivar and treatment, as well as between type of inoculum and treatment, but not between cultivar and type of inoculum ($P=0.8133$ and $P=0.0564$ for severity and mortality, respectively). Due to these interactions, data were analyzed separately for each cultivar and inoculum type. In trial 1, 'Fronteras' was significantly ($P<0.0001$) less susceptible to anthracnose crown rot (mean severity 1.4, mean mortality 24.9%) than 'Portola' (mean severity 2.3, mean mortality 52.2%). Similarly, in trial 2, 'Fronteras' was significantly ($P<0.0001$) less susceptible

(mean severity 1.6, mean mortality 22.4%) than ‘Monterey’ (mean severity 2.2, mean mortality 39.9%). Inoculation with QoI-resistant isolates resulted in significantly (P<0.0001) higher disease severity (mean 2.1) and incidence of mortality (mean 40.7%) than when using QoI-sensitive isolates (mean severity 1.6, mean mortality 29.0%).

Disease severity and incidence of mortality of untreated, inoculated control plants were high at evaluation time five weeks after springtime planting of ‘Portola’ or 14 weeks after fall planting of ‘Monterey’ strawberry. After inoculation with QoI-sensitive isolates, disease severity was 3.5 and 3.2, and incidence of mortality was 85.0% and 69.1% for untreated ‘Portola’ and ‘Monterey’ controls, respectively (Figs. 1.2,1.3A-D). After inoculation with QoI-resistant isolates, these values were 4.0 and 3.4, and 100% and 74.6% for the two cultivars, respectively. In contrast, average disease severity and mortality for two studies with ‘Fronteras’ planted in the spring or fall were 2.1 and 32.9%, and 2.5 and 47%, respectively, for QoI-sensitive and -resistant inoculum (Figs. 1.2E, F; 1.3E, F). Fungal isolations from diseased tissues of randomly selected symptomatic plants confirmed *C. acutatum* as the causal agent of disease symptoms. Water dip treatments of transplants were generally ineffective in reducing disease and mortality as compared to the untreated control, and only resulted in a significant (P<0.05) reduction in disease severity for ‘Monterey’ inoculated with QoI-resistant isolates of *C. acutatum* (Fig. 1.2D).

Transplants of all three strawberry cultivars that were inoculated with QoI-sensitive or -resistant isolates and dip-treated with natamycin at either rate or with the pydiflumetofen-fludioxonil or fludioxonil-cyprodinil pre-mixtures in all cases showed

significantly (P<0.05) lower disease severity (Fig. 1.2) and plant mortality (Fig. 1.3) as compared to the controls. Furthermore, there were generally no significant (P>0.05) differences among these four treatments, and the new fungicides provided similar control to the registered pre-mixture of fludioxonil and cyprodinil. Among the six cultivar-inoculum combinations, the 0.5 g/liter-rate of natamycin was significantly (P>0.05) less effective than the 1 g/liter-rate only for 'Portola' inoculated with QoI-resistant isolates (Figs. 1.2B, 1.3B). No stunting of plants or other signs of phytotoxicity were observed in any treatment in these trials.

Treatments with azoxystrobin provided a high level of disease control when transplants were inoculated with QoI-sensitive isolates of the pathogen, and efficacy was similar to natamycin or the two pre-mixtures containing fludioxonil (Fig. 1.2A, C, E; Fig. 1.3A, C, E). Azoxystrobin, however, was not effective when plants were inoculated with QoI-resistant isolates, and there was no reduction in crown rot severity and plant mortality as compared with the controls (Fig. 1.2B, D, F; Fig. 1.3B, D, F).

Among the remaining treatments, chlorothalonil showed inconsistent performance. The fungicide significantly reduced disease severity as compared with the untreated control in five of the six cultivar-inoculum combinations (Fig. 1.2) and plant mortality in four of the six combinations (Fig. 1.3). Efficacy of chlorothalonil was often statistically lower than that of natamycin and the two pre-mixture treatments containing fludioxonil. Pydiflumetofen was among the least effective treatments, and disease severity was significantly reduced from the untreated control only on 'Monterey' using

QoI-sensitive inoculum (Fig. 1.2C). The biocontrol *S. lydicus* was ineffective in all studies.

Effect of pre-plant dip treatments on fruit yield. Because harvest frequency and timing were different for the two trials conducted in different growing seasons with 'Fronteras', data were analyzed separately for each study and inoculum type. Yield data generally reflected performance of the treatments in reducing disease severity and plant mortality (Table 1.3). Water dip treatments did not result in increased yield as compared with the untreated control except in the first trial with QoI-sensitive inoculum. In both studies, natamycin treatments had statistically similar yields as the industry standard fludioxonil-cyprodinil or the experimental pydiflumetofen-fludioxonil pre-mixture. Yield of azoxystrobin-treated transplants was statistically higher ($P<0.05$) than the untreated control and statistically similar to natamycin and fludioxonil-containing treatments in inoculations with QoI-sensitive isolates (Table 1.3). Yield for azoxystrobin, however, was similar to the untreated control in both studies after inoculation with QoI-resistant isolates.

Fruit yields of the remaining treatments (i.e., water control, *S. lydicus*, pydiflumetofen, chlorothalonil) were not significantly different from the untreated control in trial 1. In trial 2, results were similar, but chlorothalonil and *S. lydicus* significantly ($P<0.05$) increased yield from the untreated control for transplants inoculated with QoI-sensitive isolates. Still, natamycin at both rates, fludioxonil-containing treatments, and azoxystrobin (in inoculations with QoI-sensitive isolates) had the highest numerical yields among all treatments.

DISCUSSION

Pre-plant dip treatments of strawberry transplants for the management of anthracnose crown rot have relied on the use of fludioxonil-cyprodinil and azoxystrobin since the early 2000s. With extensive use of these fungicides for pre- and post-plant disease management, there is a high risk for selection for resistance. Thus, new pre-plant fungicide treatments are needed that have different modes of action from registered products. In this study, we report the distribution and genetic basis for QoI resistance in California populations of *C. acutatum,* and we identify a new highly effective pre-plant treatment, the biofungicide natamycin, that has a unique mode of action from all other fungicides registered on strawberry worldwide.

QoI-resistance was first described in *C. acutatum* from strawberry in Florida in 2013 (Forcelini et al. 2016). G143A and F129L mutations in *cytb* were identified conferring high and moderate resistance levels, respectively. In our study on California isolates, only the G143A mutation was detected although only 11 resistant isolates were sequenced of the total of 29 isolates that we identified. Because all our resistant isolates had EC_{50} values for azoxystrobin of >40 µg/ml and thus, were highly resistant, other resistance genotypes may not be present in California at this time. Diseased plants that yielded resistant isolates originated from two of five California nursery sources and were collected in three of eight counties sampled. Therefore, resistance currently may still be limited in distribution, but could spread quickly with frequent movement of plant material. Population genetic analyses of isolates may provide information if resistance developed independently in the two nurseries and in Florida. Resistance can be managed

by using treatments with different modes of action. The availability of alternative fungicides is important because resistance conferred by the G143A mutation characteristically cannot be mitigated using higher, off-label application rates of QoI compounds in the field (Bartlett et al. 2002; Fernández-Ortuño et al. 2012; Kim et al. 2003; Lesniak et al. 2011). Additionally, this mutation was shown to be stable in *C. acutatum* populations and did not result in a fitness penalty in laboratory assays (Forcelini et al. 2017). In our study, QoI-resistant and -sensitive isolates were similarly pathogenic, but disease severity and mortality after inoculation with resistant isolates were higher than for QoI-sensitive isolates. Several studies on other fungal pathogens have indicated that resistant isolates harboring the G143A mutation did not have reduced fitness as compared to wild-type sensitive isolates (Corio-Costet et al. 2011; Karaoglanidis et al. 2011; Rallos et al. 2014). Fitness of resistant isolates of *Magnaporthe oryzae*, however, was reduced (Ma and Uddin 2009).

In vitro toxicities of chlorothalonil, cyprodinil, fludioxonil, and natamycin, all exhibited unimodal distributions of sensitivities. Although cyprodinil and fludioxonil have been used for many years in California strawberry production, no resistant isolates were detected in our samplings, and results reflect current sensitivity levels in *C. acutatum* populations in the sampling locations. A high in-vitro activity of the fludioxonil-cyprodinil pre-mixture was previously determined for *C. truncatum* (Torres-Calzada et al. 2015), and values were in a similar range as in our evaluation of the individual active ingredients. Because natamycin has never been used in commercial

strawberry production, EC_{50} values determined can be considered a baseline sensitivity that can be used as a reference in future resistance monitoring.

Although in vitro toxicity of natamycin *to C. acutatum* was considerably lower than that of azoxystrobin (using sensitive isolates), fludioxonil, and cyprodinil, dip treatments of strawberry transplants with natamycin were highly effective and similar to those with azoxystrobin (when disease was caused by QoI-sensitive isolates) or fludioxonil-cyprodinil. Chlorothalonil was inconsistent in reducing anthracnose crown rot and plant mortality in our studies. This confirms previous studies on strawberry anthracnose where chlorothalonil was not very effective (Daugovish et al. 2009). Fruit yields increased as disease severity and plant mortality decreased after treatment with fungicides that were effective against anthracnose crown rot. Yields overall were much lower in the first trial that was initiated in the spring because high temperatures that sporadically occur at the inland California field site in the spring caused plants to decline. The second trial that was initiated in fall/winter and was harvested in the spring allowed time for greater plant growth. Additionally, flowers during early growth were removed to encourage vegetative growth, and two consecutive harvests were combined in the second study. For both spring- and fall-planted trials, the lack of foliar fungicide sprays that are standard in commercial field settings also likely contributed to lower overall yield values.

Treatments were evaluated as dips of transplants because planting material is a common source of introducing *C. acutatum* into a field and currently, dip treatments of transplants are a common practice in California in the establishment of new strawberry fields. As shown in our results, plant mortality was reduced significantly by natamycin

and fludioxonil-cyprodinil when transplants were inoculated with QoI-sensitive or -resistant isolates. Under highly favorable disease conditions, additional foliar treatments will have to be applied to protect plants from petiole and leaf lesions, as well as fruit rot throughout the season, and these can be integrated with those for gray mold management caused by *Botrytis* spp. The experimental fungicide pydiflumetofen was not effective against *C. acutatum* in our in vitro and field studies. This fungicide, however, is very active against *B. cinerea* and has shown high efficacy in reducing powdery mildew of strawberry (J. Adaskaveg, *unpublished*). The pydiflumetofen-fludioxonil pre-mixture was among the most effective of our pre-plant dip treatments for crown rot of strawberry, but this was most likely due to the fludioxonil component. Therefore, this pre-mixture has potential as a broad-spectrum post-plant field fungicide for strawberry.

Natamycin is classified as a biofungicide because it is a commercial fermentation product of *S. natalensis* (Delves-Broughton 2014). The dip application of transplants developed in this study led to its registration (as the product Zivion M, DSM, Heerlen, The Netherlands) on strawberry, representing the first use of natamycin in field agriculture. Natamycin previously was registered for management of dry bubble disease of mushroom caused by *Verticillium fungicola* var. *fungicola*, and as a postharvest treatment for managing citrus and stone fruit decays caused by *Monilinia, Penicillium, Geotrichum,* and *Botrytis* species (Chen et al. 2016). Its unique mode of action is the direct binding to ergosterol, the primary sterol in fungi, leading to disruption of membrane function and cellular transport (Aparicio et al. 2016; Hamilton-Miller 1974). It does not inhibit ergosterol production as do sterol biosynthesis- or demethylation-

inhibiting fungicides. Thus, in 2017, it was assigned its own Fungicide Resistance Action Committee Code, No. 48 (FRAC 2017). Natamycin is designated as a biopesticide by the United States Environmental Protection Agency and therefore, it is exempt from pesticide tolerances in the United States, and is "generally recognized as safe" (GRAS) by the US Food and Drug Administration. The registration of natamycin is furthermore important for the strawberry industry because after many years of use in the food industry, resistance in filamentous fungi has not been reported (Delves-Broughton 2014).

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Table 1.1. Isolates of *Colletotrichum acutatum* used in this study

No.	Isolate code	Date of collection	Origin			QoI sensitivity[b]
			Tissue type	County in CA	Nursery company[a]	
1	Ca117[ce]	5/25/2016	petiole	Merced Co.	D	S
2	Ca103	4/26/2016	petiole	San Joaquin	B	R
3	Ca104	4/26/2016	petiole	San Joaquin	B	R
4	Ca105	4/26/2016	petiole	San Joaquin	B	R
5	Ca128	8/5/2016	petiole	San Joaquin	B	R
6	Ca3[cde]	9/18/2015	crown	San Luis Obispo	A	S
7	Ca11a[cde]	9/18/2015	crown	San Luis Obispo	A	S
8	Ca12a	9/18/2015	crown	San Luis Obispo	A	S
9	Ca67[c]	4/7/2016	crown	San Luis Obispo	A	S
10	Ca75	4/10/2016	crown	San Luis Obispo	A	S
11	Ca76[c]	4/10/2016	crown	San Luis Obispo	A	S
12	Ca1	9/9/2015	crown	Santa Barbara	A	S
13	Ca5	9/22/2015	crown	Santa Barbara	A	S
14	Ca6[d]	9/22/2015	crown	Santa Barbara	A	S
15	Ca7[c]	9/22/2015	crown	Santa Barbara	A	S
16	Ca9[c]	9/22/2015	crown	Santa Barbara	A	S
17	Ca10	9/9/2015	crown	Santa Barbara	A	S
18	Ca130	8/7/2016	crown	Santa Barbara	A	S
19	Ca134[c]	9/6/2016	petiole	Santa Barbara	B	S
20	Ca131[d]	8/22/2016	crown	Santa Barbara	E	R
21	Ca132[d]	8/22/2016	crown	Santa Barbara	E	R
22	Ca137[de]	8/22/2016	crown	Santa Barbara	E	R
23	Ca21[ce]	10/30/2015	crown	Santa Cruz	D	S
24	Ca24[c]	10/30/2015	crown	Santa Cruz	D	S
25	Ca25	10/30/2015	crown	Santa Cruz	D	S
26	Ca26[c]	10/30/2015	crown	Santa Cruz	D	S
27	Ca48	10/30/2015	crown	Santa Cruz	D	S
28	Ca59	1/7/2016	petiole	Stanislaus	A	S
29	Ca81	4/18/2016	petiole	Stanislaus	A	S
30	Ca92	4/18/2016	petiole	Stanislaus	A	S
31	Ca94	4/18/2016	petiole	Stanislaus	A	S
32	Ca126[c]	7/8/2016	petiole	Stanislaus	A	S
33	Ca127	7/8/2016	petiole	Stanislaus	A	S
34	Ca129[c]	8/5/2016	petiole	Stanislaus	A	S
35	Ca83[de]	4/17/2016	petiole	Tehama	C	S
36	Ca84	4/17/2016	petiole	Tehama	C	S
37	Ca85	4/17/2016	petiole	Tehama	C	S
38	Ca88	4/17/2016	petiole	Tehama	C	S
39	Ca89	4/17/2016	petiole	Tehama	C	S
40	Ca90[c]	4/17/2016	petiole	Tehama	C	S
41	Ca91	4/17/2016	petiole	Tehama	C	S
42	Ca12b[cde]	10/26/2015	crown	Ventura	B	R
43	Ca13[cde]	10/26/2015	crown	Ventura	B	R
44	Ca14[cde]	10/26/2015	crown	Ventura	B	R
45	Ca17[d]	10/6/2015	crown	Ventura	B	R
46	Ca28	10/30/2015	crown	Ventura	B	R
47	Ca29	10/30/2015	crown	Ventura	B	R
48	Ca30[c]	10/30/2015	crown	Ventura	B	R
49	Ca31	10/30/2015	crown	Ventura	B	R
50	Ca32	10/30/2015	crown	Ventura	B	R
51	Ca33	10/30/2015	crown	Ventura	B	R
52	Ca49[cde]	10/6/2015	crown	Ventura	B	R
53	Ca51	12/23/2015	crown	Ventura	B	R
54	Ca52[d]	12/23/2015	crown	Ventura	B	R
55	Ca55[c]	1/29/2016	fruit	Ventura	B	R
56	Ca60	3/9/2016	crown	Ventura	B	R
57	Ca61	3/9/2016	crown	Ventura	B	R
58	Ca62[d]	3/9/2016	crown	Ventura	B	R
59	Ca65[c]	4/5/2016	crown	Ventura	B	R
60	Ca120[c]	6/29/2016	crown	Ventura	B	R
61	Ca121	6/29/2016	crown	Ventura	B	R
62	Ca123[d]	6/29/2016	crown	Ventura	B	R
63	Ca124	6/29/2016	crown	Ventura	B	R
64	Ca15[cde]	10/6/2015	crown	Ventura	B	S
65	Ca16	10/4/2015	crown	Ventura	B	S
66	Ca18	10/4/2015	crown	Ventura	B	S
67	Ca19	10/4/2015	crown	Ventura	B	S
68	Ca53	12/23/2015	crown	Ventura	B	S
69	Ca57[c]	1/29/2016	fruit	Ventura	B	S
70	Ca69	4/5/2016	crown	Ventura	B	S
71	Ca70	4/5/2016	crown	Ventura	B	S
72	Ca82	6/2/2016	petiole	Ventura	B	S
73	Ca122	6/29/2016	crown	Ventura	B	S
74	Ca125	6/29/2016	crown	Ventura	B	S

[a] Nursery companies in CA are coded A to E and describe the origin of plants in each field.

[b] Sensitivity to azoxystrobin was determined using the spiral gradient dilution method, S = sensitive, R = resistant (threshold $EC_{50} \geq 1$ µg/ml).

[c] Isolate used in a mixture of 4 QoI-sensitive or -resistant isolates for inoculation of transplants.

[d] Isolate used for molecular characterization of QoI resistance.

[e] Isolate molecularly confirmed as *C. acutatum* using CaInt-1 and CaInt-2 primers (Adaskaveg and Hartin 1997).

Table 1.2. Effective concentrations of fungicides to inhibit mycelial growth or conidial germination of QoI-sensitive and -resistant isolates of *Colletotrichum acutatum* by 50% (EC_{50}).

		EC_{50} (µg/ml)			
		QoI-sensitive, n=45		QoI-resistant, n=29	
Fungicide	**Stage**	**Mean**	**Range**	**Mean**	**Range**
Azoxystrobin	mycelium	0.034	0.015 - 0.064	>40[a]	NA[b]
Azoxystrobin	conidia	0.129	0.060 - 0.267	>40	NA
Chlorothalonil	mycelium	0.276	0.155 - 0.549	0.250	0.115 - 0.400
Cyprodinil	mycelium	0.026	0.019 - 0.360	0.025	0.012 - 0.390
Fludioxonil	mycelium	0.0009	0.0006 -0.0013	0.0010	0.0006 - 0.0014
Natamycin	mycelium	0.996	0.526 - 1.778	0.902	0.565 - 1.996
Pydiflumetofen	mycelium	>80[a]	NA	>80	NA

[a] Inhibition was not observed using the highest concentration tested (i.e., 40 or 80 µg/ml) in the spiral gradient dilution method.
[b] NA= not applicable

Table 1.3. Effect of fungicide dip treatments of 'Fronteras' strawberry transplants on fruit yield.

		Mean fruit weight/replication[ab] (g)							
	Rate[c]	Trial 1				Trial 2			
Treatment	(g/liter)	QoI-sensitive		QoI-resistant		QoI-sensitive		QoI-resistant	
Untreated control	--	23.9	f	81.2	bc	834.6	d	640.9	c
Water control	--	125.0	cde	101.8	ab	992.8	cd	813.0	c
Streptomyces lydicus WYEC 108	1.8	57.4	ef	29.0	c	1220.0	bc	924.4	bc
Pydiflumetofen	0.08	63.3	def	47.8	bc	988.6	cd	747.1	c
Chlorothalonil	1.35	102.4	cdef	116.7	ab	1294.0	bc	1136.9	abc
Natamycin	0.5	161.8	bc	261.7	a	1826.8	ab	1979.7	a
Natamycin	1.0	161.1	bc	145.2	ab	1738.4	ab	1670.6	ab
Azoxystrobin	0.16	378.1	a	16.4	c	1946.5	ab	682.6	c
Fludioxonil/cyprodinil	0.15 / 0.22	190.2	bc	254.9	a	1998.8	ab	1651.5	ab
Pydiflumetofen/fludioxonil	0.08 / 0.14	243.1	b	217.7	a	2325.2	a	1921.3	a

[a] For each treatment, 10 plants were used for each of four replications for each inoculum mixture (QoI-sensitive or -resistant isolates of *Colletotrichum acutatum*). Fruit weight for trial 1 was determined in a single harvest approximately 10 weeks after planting and for trial 2 in two combined harvests approximately 20 and 23 weeks after planting.

[b] Data were transformed using the equation $y=\log_{10}(x+1)$ and evaluated using analysis of variance and least significant difference (LSD) mean separation procedures ($P < 0.05$). Mean values followed by the same letter within each column are not significantly different.

[c] Rates are based on active ingredient, except for *S. lydicus* where the rate is based on formulated product.

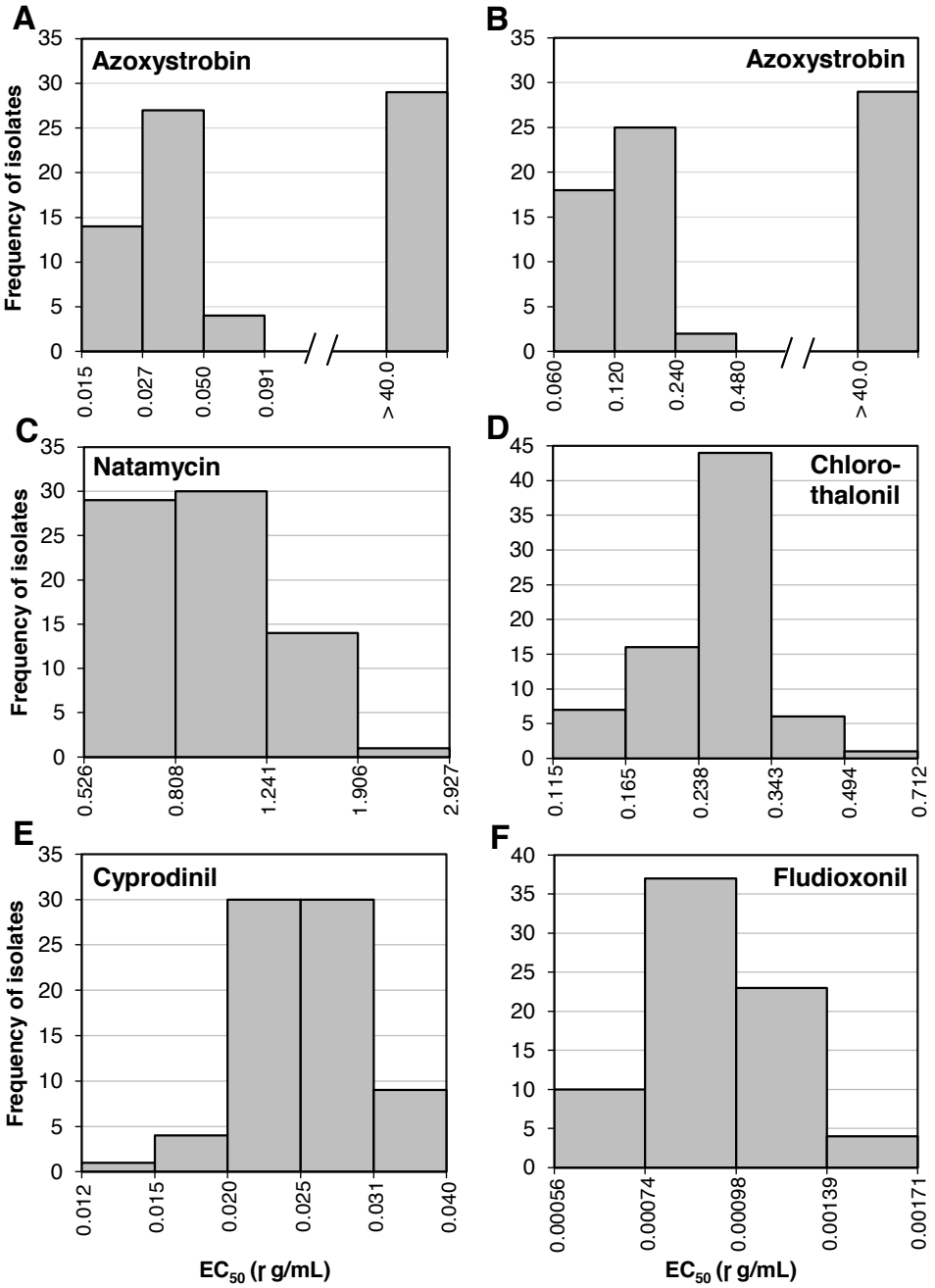

Fig. 1.1. Frequency histograms of effective concentrations to inhibit **A, C-F,** mycelial growth or **B,** spore germination of 74 isolates of *Colletotrichum acutatum* by 50% (EC_{50} values) as determined by the spiral gradient dilution method. Bar height represents the number of isolates within each bin, and bin widths were calculated using Scott's method (Scott 1979). For determination of bin width for azoxystrobin evaluations, only sensitive isolate values were considered.

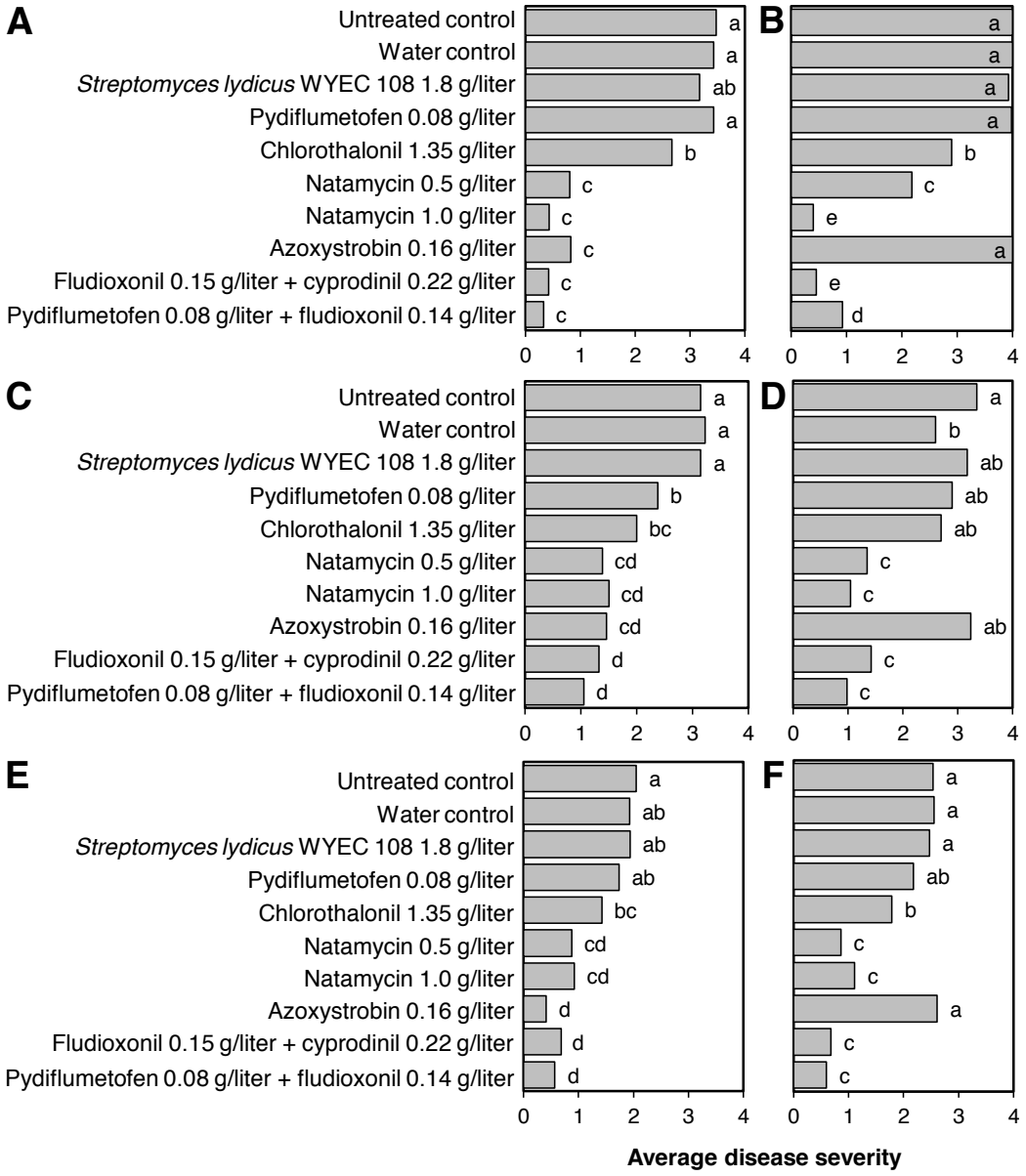

Fig. 1.2. Effect of fungicide dip treatments of **A, B,** 'Portola', **C, D,** 'Monterey', or **E, F,** 'Fronteras' strawberry transplants inoculated with a mixture of four isolates each of **A, C, E,** QoI-sensitive or **B, D, F,** -resistant isolates of *Colletotrichum acutatum* on disease severity. Plants were evaluated after five or fourteen weeks for above ground vegetative growth based on a rating scale of 0=healthy, vigorous plant, 1=growth reduced, 2=growth and vigor reduced, 3= growth and vigor extremely reduced, 4= dead. Average disease severity was determined based on 10 plants for each of four replications in each experiment. Bars followed by the same letter within a graph are not significantly different based on least significant difference mean separation (P>0.05) procedures. Rates are based on active ingredient, except for *S. lydicus* where the rate is based on formulated product.

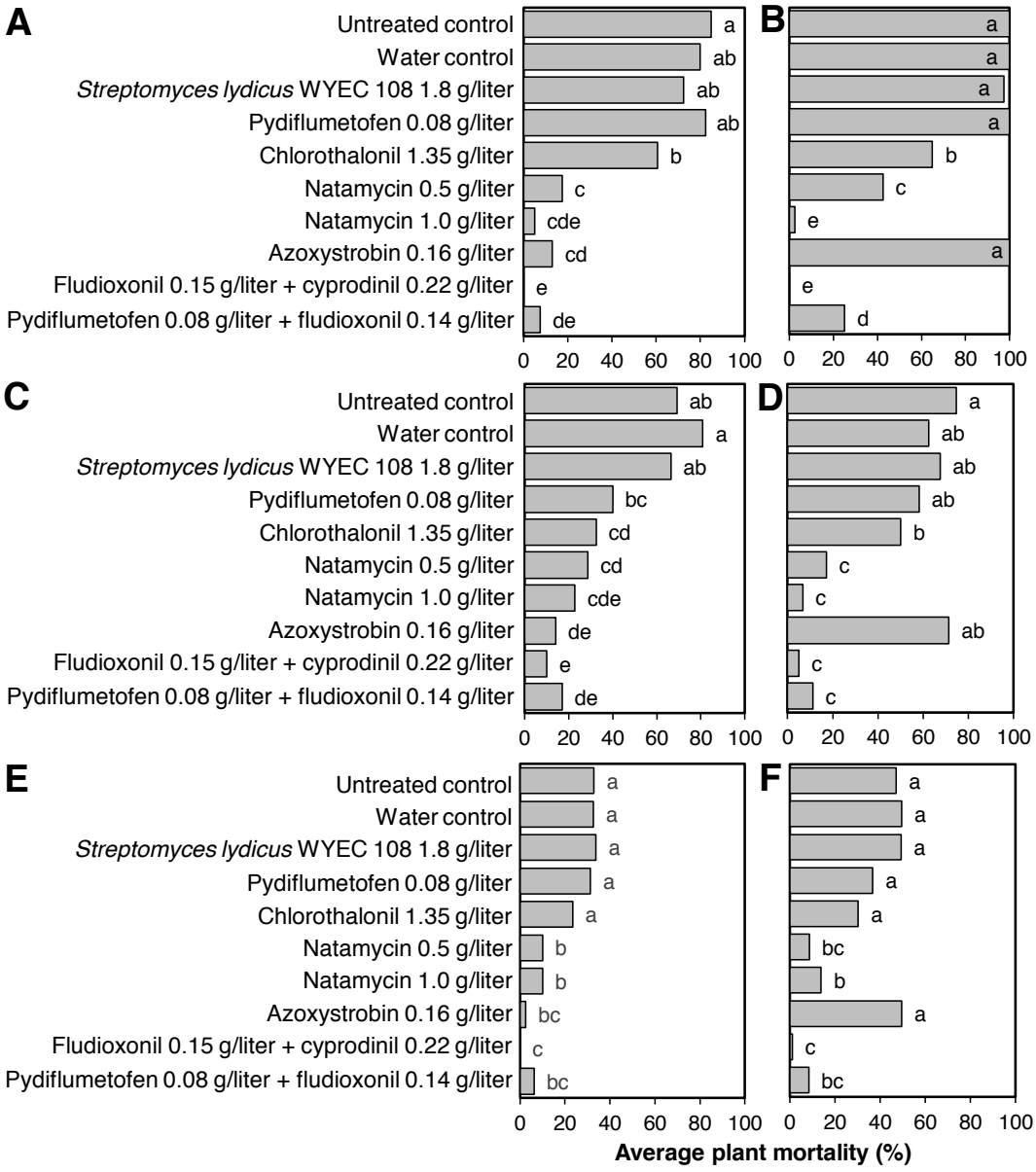

Fig. 1.3. Effect of fungicide dip treatments of **A, B,** 'Portola', **C, D,** 'Monterey', or **E, F,** 'Fronteras' strawberry transplants inoculated with a mixture of four isolates each of **A, C, E,** QoI-sensitive or **B, D, F,** -resistant isolates of *Colletotrichum acutatum* on plant mortality. Plants were evaluated after five or fourteen weeks. Average plant mortality is based on the number of dead plants out of 10 plants for each of four replications in each experiment. Bars followed by the same letter within a graph are not significantly different based on least significant difference mean separation (P>0.05) procedures. Rates are based on active ingredient, except for *S. lydicus* where the rate is based on formulated product.

CHAPTER II.

Management of *Xanthomonas fragariae* with pre- and postharvest treatments to overcome trade barriers for California strawberries

ABSTRACT

Xanthomonas fragariae, the causal agent of angular leaf spot (ALS) of strawberry, is a quarantine pathogen in some export markets, causing trade restrictions and economic loss to the California fresh-market strawberry industry. Pre-harvest chemical management options are limited to copper, and there are no post-harvest treatments available that reduce populations of the pathogen if ALS is detected at an export destination. Here, we report high pre-harvest efficacy for the experimental bactericide amino thiadiazole, alone and in mixtures with low rates of copper or the antibiotic kasugamycin, with average disease incidence reduction up to 92.8% compared with the control. Although effective against quarantine insect pests of strawberry, postharvest methyl bromide fumigation was ineffective against *X. fragariae* in diseased plant tissue at a standard commercial rate. Postharvest propylene oxide (PPO) fumigation, used for decades by the California nut industries for insect and microbial disinfestation, significantly reduced *X. fragariae* populations in infected leaflet tissues by at least 2.5-log compared with controls at a dose of ≥142 mg/liter for 2 h at 15 to 20°C. Fumigated leaflets showed little to no phytotoxicity at effective rates, and fumigated fruit were not significantly affected in appearance or susceptibility to postharvest gray mold or Rhizopus rot following storage at 2°C for 3 d and at 15°C for an additional 5 d. Together, these new treatments offer potential strategies for establishing a systems approach with

preharvest treatments significantly reducing the risk of ALS on plants and, in response to quarantine detections, a postharvest fumigation treatment that reduces viable pathogen populations in existing lesions.

INTRODUCTION

Angular leaf spot (ALS), caused by *Xanthomonas fragariae* Kennedy and King, is the most economically important bacterial disease of strawberry (Kennedy and King 1962a; Maas 1998). Characteristic symptoms include angular, water-soaked lesions 1 to 4 mm in diameter that are translucent when viewed using transmitted light and dark green when viewed using reflected light (Kennedy and King 1962a). Lesions may later coalesce, causing reddish spots on the upper-side of leaves that eventually become necrotic (Gubler et al. 2005). All green plant tissues are susceptible to infection, including leaves, stems, crowns, sepals, and calyxes (Maas 1998; Gubler et al. 1999). Calyx infections will occasionally extend into the pedicel, causing blight of the associated flower tissues (Gubler et al. 1999). The bacterium can move systemically within the plant's vascular system and may cause collapse in extreme infections (Bestfleisch et al. 2015; Hildebrand et al. 1967; Milholland et al. 1996).

X. fragariae is not free-living in soil but is able to survive between growing seasons on infected plant debris (Kennedy and King 1962b; Roberts et al. 1996). Pathogen populations can be reduced to low levels by pre-plant fumigation with methyl bromide/chloropicrin mixtures (Gubler et al. 2005). Disease outbreaks still may occur in fumigated fields due to planting of infected nursery stock. Therefore, the main pre-plant

strategy of ALS management is the use of disease-free planting material (Gubler et al. 2005; Roberts et al. 1997; Zimmerman et al. 2004). Transplants that appear healthy, however, may contain latent, asymptomatic infections that become activated once conditions are favorable (Mahuku and Goodwin 1997; Turechek and Peres 2009). Lesions exude bacterial ooze that is disseminated short distances by splashing water (Kennedy and King 1962b). In the low-rainfall California climate, this within-field pathogen spread is exacerbated by overhead irrigation that is applied throughout the growing season in nursery production and in early plant establishment for fruit production (Daugovish et al. 2009). Although ALS occurs at a wide range of temperatures, development is generally favored by cool, moist days with cold nights near freezing (Maas 1998).

Chemical controls applied during the growing season are considered only marginally effective against *X. fragariae* (Gubler et al. 2005). Thus, if the pathogen is introduced in the field, in-season management relies heavily on avoiding favorable, wet micro-climate environments by using drip or sub-surface irrigation. Copper-containing products are registered and are used especially in nursery transplant production, but additional modes of action with high efficacy are needed to develop bactericide rotation programs and resistance management strategies. Thiazole- and thiadiazole-derived compounds have potential applications as antimicrobials in agriculture (Frija et al. 2016; Hu et al. 2014). In preliminary laboratory assays, technical-grade amino thiadiazole (ATD) was inhibitory against *X. arboricola* pvs. *pruni* and *juglandis*, causal agents of bacterial spot of almond and walnut blight, respectively (J. E. Adaskaveg, *unpublished*).

The antibiotic kasugamycin was recently registered for use against *Erwinia amylovora*, causal agent of fire blight of apple and pear, federally (2014) and in California (2018), and against walnut blight and bacterial blast and canker (*Pseudomonas syringae* pv. *syringae*) of sweet cherry in 2018 federally and in California. These compounds show potential for evaluation and registration, offering additional tools for pre-harvest management of ALS in California.

Yield losses due to ALS can be high, especially in the southeastern United States where wet environmental conditions are favorable for disease development. In California, Florida, and other states, however, the disease is primarily of regulatory concern because *X. fragariae* is designated a quarantine pathogen of concern in some countries (EPPO 1997; Turechek and Peres 2009). Detection of viable *X. fragariae* in calyxes of fruit destined for high-value export markets has caused quarantine restrictions, trade losses, and market closures. Specifically, Australia was a market for California strawberry fruit in 2010 and 2011 with an export volume of 150,000 to 200,000 boxes annually with an estimated current value of approximately $10 million (California Strawberry Commission, *personal communication*). Australian government officials repeatedly detected viable *X. fragariae* in ALS lesions on calyxes of fresh-market fruit in 2010 during routine import inspections and again in 2011 during a pre-clearance program (AGDA 2018; AQIS 2011). When quarantine insect pests are detected, infested shipments can be fumigated at the ports, re-inspected, and allowed to be imported once disinfested. Because there is no post-harvest curative treatment to eradicate *X. fragariae* and because of the high cost of re-routing the highly perishable crop to alternative lower-

value markets, shipments to Australia were considered high-risk and were suspended in 2012 by the California strawberry industry.

In post-harvest fumigation against insect pests for quarantine purposes, methyl bromide is still permitted on a number of fresh-market crops, including strawberries (Federal Register 2003). Several other fumigation compounds are also registered or under evaluation (Heather and Hallman 2007b; USDA 2010). Propylene oxide (PPO) was identified as a fumigant of interest for evaluation against ALS due to its registration for more than 40 years and its use in the California nut industries for insect and microbial control (EPA 2006). PPO is an alkylating agent that reacts with cellular macromolecules including DNA, resulting in cell death (Sweeney et al. 2009).

Trade regulation and mitigation are intricately dependent on pre- and post-harvest management strategies in a systems-approach to reduce the risk of pest or disease occurrence on the crop. Therefore, the purpose of this research was to evaluate new management options for ALS with an overall goal of reducing the presence of *X. fragariae* on calyxes of fresh-market strawberry fruit. The specific pre-harvest management objective was to evaluate the efficacy of low-metallic copper equivalent (MCE) formulations, ATD, and kasugamycin using selected rates and mixtures against ALS on strawberry plants. Post-harvest management objectives were to (i) evaluate the efficacy of PPO fumigation in reducing viable *X. fragariae* cells in infected leaf tissue, (ii) evaluate phytotoxic effects of the fumigation on strawberry fruit tissues, and (iii) investigate effects of the fumigation on fruit susceptibility to the important post-harvest fungal pathogens *Rhizopus stolonifer* and *Botrytis cinerea*.

MATERIALS AND METHODS

Bacterial strain and culturing. *X. fragariae* strain 1298 (*Xf*1298, original designation *Fa*P21) was obtained from ALS-symptomatic 'Portola' strawberry plants from a Northern California nursery in December 2011 by Dr. John Leveau at the University of California, Davis using described methods (Henry et al. 2016). For all experimentation, *Xf*1298 was re-grown from long-term storage at -80°C in 20% glycerol and plated onto Wilbrink's medium with nitrate (WBN) agar plates (Koike 1965; EPPO 2006). After 3 to 4 d growth at 25°C, colonies were sub-cultured and allowed to grow for an additional 3 to 4 d. A bacterial suspension in sterile deionized water was adjusted to approximately 1 x 10^7 bacterial cells/mL (70% OD_{600} transmission) and used immediately for inoculations. *Xf*1298 sensitivity to copper was evaluated using WBN agar plates amended with copper sulfate to final concentrations of 0 (control), 10, 20, and 30 mg MCE/liter. Ten µl of bacterial suspension was streaked radially in duplicates onto three replicated plates using a sterile pestle. Growth was evaluated after 3 days of incubation at 25°C and compared with the control.

Plant inoculation. Bare-root 'Splendor' (Plant Sciences Inc., Watsonville, CA) strawberry plugs were stored at 2°C for up to 4 months prior to planting in 10-cm-diameter pots and maintained in a greenhouse at 22° to 27°C at the University of California, Riverside. Plants were fertilized using 14-14-14 slow-release fertilizer (Osmocote Classic; Everris North America, Inc., Dublin, OH) that was applied to the soil surface following planting, drip-irrigated daily, and treated with foliar insecticides/miticides approximately every 2 to 3 weeks. Leaf undersides of plants with at

least three mature leaves were inoculated with the bacterial suspension using an atomizer (DeVilbiss 15-RD; Sunrise Medical, Inc., Somerset, PA). Leaves were sprayed until a film of small droplets was visible, just before run-off. Plants were then moved into a bench-top chamber (3.3 m x 2 m x 1 m) with clear plastic tarping. Inside the chamber, six upward-facing misters (30 ml/min/mister) mounted approximately 60 cm above the pots were operated twice daily for 1 min to provide leaf wetness and high relative humidity (RH) (Hildebrand et al. 2005). After 5 days, plants were moved to greenhouse benches. ALS infection was confirmed for each inoculation by re-isolation and pathogen identification techniques described below. Infected tissues were used for fumigation tests within 6 weeks of inoculation.

Bactericides. Copper hydroxide (Kocide 3000; DuPont, Wilmington, DE) or a pre-mixture of copper hydroxide and copper oxychloride (Badge X2; Gowan Co., Yuma, AZ), kasugamycin (Kasumin 2L; Arysta LifeScience, Cary, NC), and technical-grade adenine thiadiazole (5-amino-1,3,4-thiadiazole-2-thiol; ATD; Sigma Aldrich, St. Louis, MO) were used by themselves or in selected mixtures at rates indicated in Fig. 2.1. ATD (0.5 g) was first dissolved in a mixture of 1.35 ml dimethyl sulfoxide and 5.4 ml 95% ethanol before diluting with water. Formulated compounds were mixed directly in water and used immediately.

Efficacy of pre-harvest bactericide applications to manage ALS in greenhouse studies. In each of the three repeated trials, bactericides were applied to all above-ground plant surfaces at rates indicated in Fig. 2.1 using a hand-held spray bottle until run off. Controls were sprayed with water. After 12 h of drying, plants were

inoculated as described above. Plants were arranged in a randomized-complete-block design with 5 to 6 blocks of single-plant replications. After 14 to 20 days, each treated leaf was evaluated for the presence of ALS. Disease incidence was based on the number of leaves with at least one lesion of the total number of leaves treated per replication. The three trials were inoculated on Oct. 10, 2014, July 24, 2015, and Aug. 14, 2015, and evaluated on Oct. 30, 2014, Aug. 7, 2015, and Sept. 1, 2015, respectively.

Methyl bromide fumigation of plant material with ALS symptoms. Infected leaflets were grouped so that each treatment unit contained leaflets with approximately similar disease severity. The first fumigation trial was performed on July 16, 2014, at the USDA-Parlier, CA, facility per the exploratory fumigation methods of Walse et al. (2016). Twelve leaflets were treated with 35 mg/liter methyl bromide for 3 h at 12.7°C or 15.6°C. Control leaflets were placed in a chamber at 15.6 °C but did not receive any fumigant, placed on the counter-top at the same temperature, or were kept at 2.0 ± 0.5°C. The second study was conducted among commercial strawberry fruit that were destined for export at a fumigation facility in Watsonville, CA on July 22, 2014. For this, six leaflets each were placed among the fruit load at five locations in the chamber (2.3 m x 2.3 m x 6 m), resulting in a total of 30 treated leaflets. The fruit and leaflets were commercially treated with methyl bromide (Cardinal Professional Products, Hollister, CA) for the control of quarantine mite insects, at an applied rate of approximately 1362 g/chamber (43.9 mg/liter), equaling an approximate target dose of 35 mg/liter, accounting for sorption (Walse et al. 2013, 2016). After 3 h of fumigation at 18 to 26°C and a 2-h venting period, leaflets were collected, and bacterial isolations were conducted as

described below. Control leaflets (n=12) were not exposed to the fumigant but were set on a counter outside of the fumigation chamber for the duration of treatment period.

Propylene oxide fumigation of plant material with ALS symptoms. Infected leaflets were sorted visually according to disease severity as described above and acclimated to the appropriate fumigation temperature for 4 to 12 h prior to treatment. Fumigant concentrations are indicated in Figs. 2.2 and 2.3. In laboratory tests at the University of California, Riverside, using static-chambers, leaflets were placed inside 1-liter glass jars containing a 25-ml glass vial. Appropriate volumes of PPO fumigant were loaded into the inner vial, and glass jars were closed immediately with a rubber-sealed lid and incubated at 15°C or 20°C. Control jars received no fumigant. After 2 h, jars were opened and vented for 30 min in a fume hood. There were three replicate jars per factorial treatment unit, each with 3 or 4 strawberry leaflets. The experiment was done three times (trials 1, 2, and 3, respectively).

In laboratory trials at USDA-Parlier, leaflets were placed inside 28-liter, modified vacuum chambers (Labconco Corp., Kansas City, MO) with multiple ports for evacuation, air-sampling, and fumigant introduction as described previously (USDA 2010; Walse et al. 2016). The chambers were located inside a walk-in environmental incubator maintained at 15°C (USDA 2010). Nine to twelve leaflets were placed inside perforated stainless-steel baskets. Doses required to achieve target PPO fumigation concentrations were calculated for each experiment, and calibration studies were conducted prior to fumigation. A vacuum was applied to the chamber to obtain rapid volatilization of the fumigant that was introduced via a syringe. The vacuum was

removed with air after approximately 3 min following complete vaporization of the fumigant. For each treatment, air samples were taken from the head-space of the chamber at regular intervals during the 2-h fumigation period to confirm the gaseous fumigant concentration in the chamber using gas chromatography (Walse et al. 2013). After fumigation, lids were opened, and chambers were aerated. Control chambers were injected with water equivalent to the largest volume of PPO used. For each trial, a single chamber was used for each PPO rate. A total of 4 experiments were conducted (trials 1 to 4).

Rating of leaflet phytotoxicity and re-isolation and enumeration of viable bacteria after fumigation. After fumigation, leaves were either processed immediately or stored at 2°C for up to 48 h. Leaflets were visually rated for PPO phytotoxicity using a scale based on leaf surface darkening/purpling of non-diseased areas. The scale was: 0 = healthy, no visible darkening; 1 = leaflet slightly darkened, but still appearing healthy; 2 = obvious darkening, but leaflet still mostly green; 3 = leaflet mostly purple and/or brown (necrosis) on the upper leaf surface (Fig. 2.4).

Bacterial isolations were done for each replicate leaflet to enumerate viable bacterial populations present in tissues. For this, leaflets were surface-sterilized for 1 min with 1% sodium hypochlorite and rinsed twice with sterile, deionized water. From each leaflet, a 14-mm-diameter disc was removed from the area of highest disease severity, cut into pieces, and placed into a 1.7-ml microcentrifuge tube containing 500 µl sterile deionized water and a 5-mm-diameter stainless-steel ball. Tubes were vortexed for 10 min to macerate tissues. Suspensions were diluted serially to a final dilution of 1:500 and

plated radially in an exponential deposition onto 10-cm WBN medium plates using a spiral plater (Autoplate 4000; Spiral Biotech, Inc.). *X. fragariae* colonies were enumerated after 5 to 6 days at 25°C using templates and enumeration tables (Spiral Biotech, Inc.). Colonies were verified as *X. fragariae* using morphological and growth rate characteristics, and a subset of colonies were subjected to polymerase chain reaction (PCR) using species-specific primers 245A and 245B (Pooler et al. 1996).

Effect of post-harvest propylene oxide fumigation on sepal and fruit appearance and susceptibility of fruit to fungal decays. Two studies were conducted using fruit from an untreated strawberry field at the University of California, Riverside, or organically-grown fruit from a local market. Fruit were placed horizontally into plastic trays and the upper-sides were inoculated using an atomizer (DeVilbiss) with a spore suspension (5×10^4 spores/ml) of *Botrytis cinerea* (isolate 1356) grown on King's medium B (King et al. 1954). Fruit were incubated at 25°C and >95% RH for 2 h then air-dried for 2 h. Spore germination was confirmed at the time of fumigation by microscopic observation of a potato dextrose agar plate that was inoculated with the same spore suspension. Fruit were loaded into jars with 9 to 12 fruit per jar, the fumigant was applied, and jars were vented as described above for UCR-conducted leaflet fumigation trials. Fruit were stored at 2°C for 3 days. Fruit were visually rated for PPO phytotoxicity using a scale based on darkening/purpling of veins of fruit sepals. The scale was: 0 = healthy, no visible damage; 1 = <50% of the calyx with purple veining, still appearing healthy; 2 = ≥50% of the calyx with purple veining; 3 = purple veining and necrosis on <50% of the calyx; and 4 = ≥50% of the calyx brown and necrotic. Fruit receptacles were

also rated for any discoloration or necrosis using a plus/minus rating. Fruit were then placed into plastic trays and incubated for 5 days at 15°C, >95% RH. The incidence of gray mold and naturally developing *Rhizopus* rot was assessed based on the number of diseased berries with characteristic mycelial growth of the total number of berries in each jar replication. Fungal isolations were done on subsamples of infected fruit to confirm the pathogens.

Statistical analysis of data. All statistical analyses were performed using SAS (ver. 9.4, SAS Institute, Cary, NC) with a significance value of α=0.05. Data were combined from different trials when variances were homogeneous according to Levene's test and interactions were not significant. Data were subjected to analysis of variance (ANOVA) or generalized linear model (GLM) procedures followed by comparisons of treatment means using Fisher's Least Significant Difference (LSD) test. For greenhouse efficacy trials, leaf incidence data were arcsine transformed prior to GLM analysis on combined data sets with treatment as the single factor. For methyl bromide fumigation trials, GLM procedures were used for overall model analysis of bacterial population data with treatment and temperature factors included for trial 1, and treatment and replicate (spatial location) factors included for trial 2.

For leaflet fumigation trials with PPO in static chambers, GLM procedures were used for overall model analyses of bacterial population and phytotoxicity data with treatment, replicate, and treatment*replicate interaction as factors for trial 1, and trial, temperature, treatment, replicate, and all interactions as factors for trials 2 and 3. When individual factors were not significant in the overall model, they were combined.

Population data were averaged by replicate, and regression procedures were performed following transformation using $y=\log_{10}(count/ml + 1)$. GLM procedures were performed on combined phytotoxicity data followed by multiple comparisons for treatment means using Fisher's LSD test. For leaflet fumigation trials with PPO in active infiltration chambers, bacterial population and leaf phytotoxicity data were initially analyzed with trial, treatment, and trial*treatment interaction as factors using GLM for overall or combined model analyses with treatment as the single factor. Presented population values are the average for each treatment combination that were transformed using $y=\log_{10}(average\ count/ml + 1)$.

For fruit fumigation studies, fungal disease incidence was arcsin-transformed prior to analysis, whereas phytotoxicity ratings were not transformed. Trials were initially analyzed with trial, treatment, temperature, replicate, and all interactions as factors using ANOVA for overall or combined model analyses with treatment as the single factor.

RESULTS

***X. fragariae* strain sensitivity to copper and infection of strawberry leaves.** Strain 1298 was found to be copper-sensitive with growth similar to the control at 10 mg MCE/liter, reduced growth at 20 mg MCE/liter, and no visible growth at 30 mg MCE/liter. In producing infected leaves for fumigation tests, angular, water-soaked lesions (0.5 to 2.5 mm in diameter) characteristic of ALS were present approximately 10 to 14 d after inoculation in the greenhouse. *X. fragariae* infection was confirmed by re-isolation where colonies with typical morphology on WBN medium were visualized as

described previously (EPPO 2006). Colonies were initially off-white, becoming light yellow, convex, shiny, and mucoid after 4 to 6 days at 25°C. PCR amplification with *X. fragariae*-specific primers 245A and 245B resulted in a single band of the expected 300-bp length.

Efficacy of bactericide applications to manage ALS in greenhouse studies. The overall model for disease incidence was highly significant ($P<0.0001$) for treatment ($P<0.0001$) and trial ($P<0.0001$), but not for their interaction ($P=0.1209$). Average incidence was significantly higher in trial 3 (37.2%) than in trials 1 (23.6%) and 2 (25.3%).

Homogeneity of variance of disease incidence was upheld among all three trials, and because there were no significant interactions between treatment and trial, data were combined by treatment. There was a significant ($P<0.0001$) difference among treatments, and multiple comparisons were conducted (Fig. 2.3). Kasugamycin, copper, and copper/kasugamycin mixture treatments resulted in a statistically similar disease incidence (average 60.2%, 46.7%, and 46.9%, respectively) as the control (average 56.3%), whereas all treatments containing ATD significantly reduced disease incidence. ATD at 500 mg/liter by itself or in mixture with copper resulted in the lowest numerical incidence, both with an average of 4.1%. These were statistically similar to the kasugamycin/500 mg ATD/liter (average 10.1%) and copper/250 mg ATD/liter (average 11.9%) mixture treatments. When the ATD rate in mixture with copper was reduced to 50 mg/liter, disease incidence significantly increased (average 22.9%) as compared with higher rates (Fig. 2.3).

Effect of methyl bromide fumigation of plant material with ALS symptoms on survival of *X. fragariae*. The overall model for bacterial population recovery data was not significant in small-scale chamber (trial 1, P=0.2779) and commercial (trial 2, P=0.6286) experiments. There was no statistical difference between *X. fragariae* populations recovered from control (average 1.7 x 10^5 cfu/ml in trial 1 and 2.2 x10^5 cfu/ml for trial 2) and methyl bromide-treated (average 2.2 x 10^5 cfu/ml in trial 1 and 1.9 x 10^5 cfu/ml for trial 2) leaflets. No phytotoxicity was observed for the methyl bromide treatments at commercial rates, but some dehydration of leaf tissues occurred in the commercially-conducted experiment.

Effect of PPO fumigation of plant material with ALS symptoms on survival of *X. fragariae*. In treatments using static-chambers with passive fumigant infiltration, treatment concentrations in trial 1 differed from those in trials 2 and 3; therefore, data are presented separately (Fig. 2.2A,B). The overall model for trial 1 was significant (P=0.0035) with treatment significantly (P<0.0001) contributing to the model, but not replication (P=0.1965) or treatment*replication interaction (P=0.5496). Therefore, data were averaged for each replicate and combined by treatment. The regression of PPO concentration on viable bacterial population size was highly significant (P<0.0001) and had an R-square value of 0.9627 (Fig. 2.2A).

The overall model for trials 2 and 3 was highly significant (P<0.0001), with treatment significantly (P<0.0001) contributing to the model. Data were averaged for all replicates of a treatment because no significant (P=0.9329) difference was observed. Fumigation temperature (average populations at 15°C and 20°C were 3.8 x 10^4 cfu/ml

and 5.4 x 10^4 cfu/ml, respectively) did not significantly contribute to the model (P=0.0636); thus, data were combined. Similarly, 'trial' did not significantly (P= 0.8834) contribute to the model, and because homogeneity of variance between the two trials was upheld (P= 0.4904), data from different trials were combined by treatment. The regression of PPO concentration on viable bacterial population size was highly significant (P<0.0001) and had an R-square value of 0.8315 (Fig. 2.2B).

In treatments using active chambers with vacuum infiltration of the fumigant, trials 1 and 2 differed in a single treatment rate compared with trials 3 and 4; thus, analyses were conducted separately for the two sets of studies. The overall models for both sets of trials were highly significant (P<0.0001) with treatment significantly (P<0.0001) contributing to the models. Trial (P=0.7915 and P= 0.9873 for the first and second sets of studies, respectively) and trial* treatment interaction (P= 0.9630 and P=1.0000, respectively) factors were not significant, and because homogeneity of variance was upheld for each set of trials (P=0.7948 and P=0.7336 for the first and second set, respectively), data were combined. In both sets of studies, bacterial populations were high in untreated control plants with averages of 5.2 and 5.3 $\log_{10}$(cfu/ml +1) (Fig. 2.3A). In the first two studies, PPO at 40 mg/liter with an average population size of 5.1 $\log_{10}$(cfu/ml +1) was not significantly different from the control. PPO rates ranging from 213 to 427 mg/liter resulted in a significant, 2.8 to >5-log reduction in viable bacteria (i.e., 211 to 0 cfu/ml, respectively) compared with the control. In the second set of studies, all five PPO rates evaluated, ranging from 142 to 427 mg/liter, resulted in a significant, 4- to >5-log reduction of viable bacteria (i.e., 12.9

to 0 cfu/ml, respectively) compared with the control (Fig. 2.3A). Many re-isolations resulted in no *X. fragariae* recovery and thus, viable populations were reduced to below the threshold of detection for this method used.

Effect of PPO fumigation on leaflet appearance (phytotoxicity). In trial 1 using static infiltration, the overall model for phytotoxicity was not significant (P=0.5743), with replicate (P=0.9449) and treatment*replicate interaction (P=0.9991) not contributing to the overall model. Treatment was significant (P=0.0462), and replicate values were therefore combined by treatment. In the one-way analysis, treatment was significant (P=0.0142) (Fig. 2.2C), and multiple comparisons between treatment means showed that PPO at 427 mg/liter (average rating 0.4) resulted in significantly (P<0.05) higher phytotoxicity compared with the control, as well as the 40- and 213-mg/liter rates of PPO where no phytotoxicity was observed on any of the leaflets. In trials 2 and 3 using static infiltration, the overall model for phytotoxicity ratings was not significant (P=0.1313), but treatment significantly (P=0.0003) contributed to the model. Fumigation temperature did not significantly (P=0.1770) contribute to the model and thus, data were combined. Similarly, data were combined for all replicates because no significant (P=0.9499) difference was observed. Lastly, 'trial' did not significantly (P= 0.6512) contribute to the model, and because homogeneity of variance between the two trials was upheld (P= 0.6639), data from different trials were combined by treatment. In the one-way analysis, treatment was significant (P=0.0002), and multiple comparisons between treatment means indicated that PPO at 427 mg/liter resulted in significantly (P<0.05) higher phytotoxicity (average rating 0.5) compared

with the untreated control (average rating 0.1) and the 213-mg/liter rate of PPO (average rating 0.2). Visually, phytotoxicity was present as a slight darkening of the still green leaves in some leaves at the highest PPO rate evaluated in these trials.

The overall models for leaflet phytotoxicity in the vacuum-infiltration trials were highly significant ($P<0.0001$) with treatment significantly ($P<0.0001$) contributing to the models. Trial ($P=0.7397$ and $P= 0.8513$ for the first and second sets of studies, respectively) and trial* treatment interaction ($P= 0.7847$ and $P=0.5388$, respectively) factors were not significant, and because homogeneity of variance was upheld for each pair of trials ($P=0.5566$ and $P=0.1115$ for the first and second sets of studies, respectively), data were combined by treatment. Overall models with treatment as the single factor were highly significant ($P<0.0001$) for both sets of studies. Control leaflets had a healthy green appearance (average rating of 0.21 and 0.14 for the two sets, respectively), and were statistically similar in appearance to leaflets exposed to PPO at 40, 142, or 213 mg/liter with average severity ratings of $\leq$0.43 (Figs. 2.3B,2.4). Average phytotoxicity ratings for leaflets treated with 284, 356, or 427 mg/liter PPO were significantly higher, and phytotoxicity increased with increasing rates. In the first set of studies, leaflet ratings for PPO at 284 mg/liter (average rating 1.25) and 356 mg/liter (average rating 1.63) were statistically similar and evident as darkened leaf tissue. These ratings were significantly ($P<0.05$) lower than ratings for PPO treatments at 427 mg/liter (average rating 2.88) where nearly all leaflets were very dark and purplish in color (Figs. 2.3B,2.4). Similar phytotoxicity was observed in the second set of studies, but ratings using the three PPO high rates of 284 (average rating 1.36), 356 (average rating 1.80),

and 427 (average rating 2.80) mg/liter were all significantly (P<0.05) different from each other (Figs. 2.3B,2.4).

Effect of postharvest PPO fumigation on sepal and fruit appearance and fruit susceptibility to fungal decays. The overall model for phytotoxicity ratings on sepals in trials 1 and 2 was not significant (P=0.1122). Average phytotoxicity ratings of the control and the 213- and 427-mg/liter PPO fumigations were 1.77, 1.70, and 1.63 for all studies and temperatures, respectively. No phytotoxicity was observed on fruit in any of the trials.

The overall models for gray mold and Rhizopus rot incidence were not significant (P=0.7579 and P=0.2555, respectively), and no individual or interaction factor tested resulted in significant (P>0.05) contributions to the model. Therefore, there was no statistical difference in decay incidence for all trials and temperatures between control fruit with an average of 66.0% and 44.5%, fruit treated with 213 mg/liter PPO with an average of 72.7% and 41.5%, and fruit treated with 427 mg/liter PPO with an average of 70.7% and 46.5%, for gray mold and Rhizopus rot, respectively.

DISCUSSION

In this study, we identified new treatments for the pre-harvest management of angular leaf spot and for the postharvest fumigation of infected strawberry tissues to eradicate viable cells of the pathogen *X. fragariae*. These management tools have the potential for further evaluation and registration, demonstrating a systems-approach

(Heather and Hallman 2007a) with the end goal of a reduced risk of exporting a quarantine pathogen in a diseased commodity.

Bacterial diseases can increase rapidly under favorable environments, and field treatments are needed in an integrated disease management program to protect plants from infection and delay the onset of an epidemic. Thus, pre-harvest bactericide treatments were evaluated in this study. Copper formulations are among the few registered products for use against ALS in the United States. These, however, are only moderately efficacious (Merteley 2010; Roberts et al. 1997). In the present study where a rate of 175 mg/liter MCE was used, disease incidence was not significantly reduced as compared with the control although in vitro, *X. fragariae* strain *Xf*1298 was sensitive to copper with no growth at 30 ppm MCE. In contrast to previous reports (Merteley 2010; Roberts et al. 1997), no copper phytotoxicity was observed in our studies, likely because only one application was made to plants and the lowest labeled rate was used. The antibiotic kasugamycin, when applied alone at 100 mg/liter or in mixtures with copper, was also not effective in reducing ALS. In contrast, in studies on bacterial spot of tomato (caused by *X. perforans* and other species), kasugamycin was effective (Vallad et al. 2010). For walnut blight caused by *X. arboricola* pv. *juglandis,* efficacy of kasugamycin-copper mixtures was higher than for kasugamycin by itself and was similar to the industry standard copper-mancozeb (Nguyen et al. 2016).

Treatments containing ATD consistently were highly effective in reducing ALS incidence. ATD when applied alone at 500 mg/liter significantly reduced the disease and no additional benefits in performance were observed when ATD was used in mixtures

with kasugamycin or copper. This compound therefore has excellent potential as a new treatment for managing ALS. Additionally, zinc thiazole has demonstrated activity against *Xanthomonas* spp., including *X. oryzae* pv. *oryzae*, causal agent of bacterial leaf blight of rice (Chen et al. 2014; Zhang et al. 2013), and a commercial formulation is registered for agricultural use in China (Zhejiang Xinnong Chemical Co. Ltd, Hangzhou, Zhejiang, China). Evaluation of this formulated zinc thiazole product may support registration in the United States if a registrant is identified. The specific mode of action is not known for ATD (5-amino-1,3,4-thiadiazole-2-thiol) or the commercial zinc thiazole (zinc-bis (2-amino-5-mercapto-l, 3, 4 thiadiazole); Chen et al. 2014; Zhang et al. 2013). However, an isomer of ATD, 1,2,4-thiadiazole, is proposed to interfere with cell peroxidation and inhibit lipid synthesis or transport in the cell membrane, while other thiadiazole derivatives are believed to be involved in host plant defense induction (FRAC 2018; Hu et al. 2014). Registration of ATD or similar products should be pursued for nursery use to reduce the initial introduction of *X. fragariae* into fruit-bearing fields, as well as for fruit production to reduce the disease on harvested fruit that may be exported. Additional evaluations of thiazole derivatives against *X. fragariae* and other bacterial plant pathogens may provide a new non-antibiotic, non-copper-based treatment for managing bacterial diseases including ALS.

Methyl bromide had no effect in reducing viable *X. fragariae* populations in infected leaf material when tested at commercial fumigation rates. This reflects industry experience where commercial quarantine treatments with methyl bromide for insect pests in strawberry destined for export to restrictive markets (APHIS 2016; Walse et al. 2012)

still result in ALS detections (AQIS 2011). In our studies, we identified PPO as a potential new commercial fumigant for strawberries that can dramatically reduce viable populations of *X. fragariae* in diseased plant tissues while causing little to no phytotoxicity at effective rates. Diseased leaves were used because they could be consistently produced in large enough amounts for replicated experiments. The primary export concern for the fresh-market California strawberry industry, however, are infected fruit calyces. Calyxes, likely due to their smaller size, had a low disease incidence following our inoculations. Leaves are acceptable surrogates because they are anatomically similar to calyxes. Additionally, in our re-isolations of *X. fragariae* from symptomatic calyx tissues, bacterial and fungal contaminants often inhibited growth of *X. fragariae* in vitro, as observed and described by Henry et al. (2016), resulting in additional complications in obtaining reliable data.

Temperatures and fumigation duration in our studies were selected based on current commercial fumigation practices for insect control in the export strawberry fruit industry. PPO was tested using static- and vacuum-infiltration systems with slightly different results for efficacy and phytotoxicity at corresponding rates, temperatures, and durations. In general, static-infiltration of PPO at lower rates (e.g., 213 mg/liter) resulted in lower efficacy in reducing viable bacterial populations and lower phytotoxicity to leaflets compared with vacuum-infiltration, whereas higher rates in the static system provided similar 3- to 5-log reductions in viable bacteria with less phytotoxicity. Vacuum infiltration replaces air in the chamber and leaf mesophyll with PPO, resulting in increased exposure of the bacterial pathogen and inner leaf tissues to PPO.

Commercially, both static and vacuum systems are used for post-harvest fumigation of different commodities (APHIS 2016) although for strawberries, methyl bromide is usually applied using a static system with fans to aid in the dispersal of the fumigant. At the same temperatures and treatment duration, the PPO rates to reduce viable *X. fragariae* were higher than rates that provide control of mites and insects of quarantine concern that infest strawberry and other types of fruit (S. Walse, *unpublished*). This work provides evidence to support continued efforts to register PPO as a fumigant for multiple pests as well as ALS of strawberry.

Fruit phytotoxicity and fungal decay studies approximated the temperature regime and timelines of fumigation treatments for exported fruit, with 1 to 3 days cold storage (2°C) after treatment during transportation and moderate temperatures during the market and consumer shelf-life. No significant phytotoxicity to fruit and calyxes was observed and only minor phytotoxicity was observed on leaves in static-infiltration systems. PPO fumigation did not significantly reduce decay or change fruit susceptibility to the major postharvest decays evaluated. Effective post-harvest fumigation control of *B. cinerea* and *R. stolonifer* was demonstrated in strawberry using acetaldehyde, but this has not been commercialized (Prasad and Stadelbacher 1974).

To our knowledge, this is the first demonstration that fumigation of a fresh-market commodity can result in a several log-scale reduction in populations of a plant pathogenic bacterium that may potentially be used in response to a quarantine detection. The majority of fumigation research has focused on reducing quarantine insect pests (Follett and Neven 2006; Heather and Hallman 2007b), whereas other studies involved

pathogens causing foodborne illnesses (Danyluk et al 2005; Kasler and Yousef 2017), postharvest fungal decays (Gabler et al. 2010; Sholberg 1998), or the improvement of postharvest shelf life and fruit quality (Mahajan et al. 2014; Wills et al. 2000). Plant diseases of quarantine concern are more commonly mitigated by chemical, physical (e.g., hot water or steam), or irradiation treatments, or a combination of these (APHIS 2016; Eckert and Ogawa 1988; Mahajan et al. 2014). Strawberries, however, cannot be treated with any aqueous-based postharvest treatment because wetting of fruit reduces fruit quality and shortens shelf-life (Eckert and Ogawa 1988).

Additional studies on the evaluation of PPO fumigation on a range of strawberry cultivars from different growing seasons and regions is warranted. Other factors that will need to be considered include effects of increased fruit load on efficacy, PPO sorption into the organic load, and chemical residues (Jimenez et al 2015; Walse et al. 2013, 2016). PPO is registered for nut crops with residue limits of 300 mg/kg in the United States (EPA 2012) and 100 mg/kg in Australia for almonds (APVMA 2018). PPO readily volatilizes at ambient temperatures, breaks down relatively quickly into propylene glycol and water, and is not known to persist in the environment or pose major environmental risks (EPA 2006). Furthermore, because infected transplants are the primary mechanism of introduction of *X. fragariae* into production fields, fumigation of transplants with PPO could also be evaluated.

In international trade, a science-based decision-making process is the foundation for quarantine regulations. For insects, required pest reductions are based on ‘Probit 9’ where 99.9968% mortality (3 survivors per 100,000 treated) is achieved (Follet and

Neven 2006), whereas in food safety, an approximately 3- to 5- log reduction in bacterial populations in a food product is considered 'pasteurized' (Breidt et al. 2013; FDA 2001). In the present study, a 50-µl aliquot of diseased leaf tissue extract was plated onto selective medium resulting in a detection threshold of 1 cfu/50 µl or 20 cfu/ml following a 1:500 dilution of the original leaf macerate. This corresponds to approximately 3.3 x 10^6 cfu/cm^2 diseased leaf tissue used in our study. Plating of additional replicates, larger volumes, or using lower dilutions would increase the sensitivity of detection. PPO fumigation resulted in a multi-log, sometimes 5-log, reduction of viable *X. fragariae* populations compared with controls and thus, is similar to reductions targeted in food-safety.

Currently, PCR techniques are the standard for *X. fragariae* detection in quarantine inspection, but these cannot differentiate between live and dead microbial cells. Confirmatory bacterial isolation that enables a better estimate of the live population is too slow for a perishable fruit such as strawberries and for quarantine purposes because *X. fragariae* takes 4 to 7 days to develop medium-sized colonies (EPPO 2006; Henry et al. 2016). DNA-intercalating dyes can reduce PCR signals from dead cells (Fittipaldi et al. 2012). These are currently being evaluated for *X. fragariae* detection in strawberry tissues (Wang and Turechek 2015) and are used for live detection or evaluation of pasteurization/sterilization efficacies for other regulated bacterial plant (Temple et al. 2013) and foodborne (Fang et al. 2018) pathogens. If ALS was detected during quarantine inspections, standardized PPO fumigation protocols to obtain a multiple-log

reduction in the pathogen population and confirmatory PCR detection methods for living bacteria could be part of an international trade agreement.

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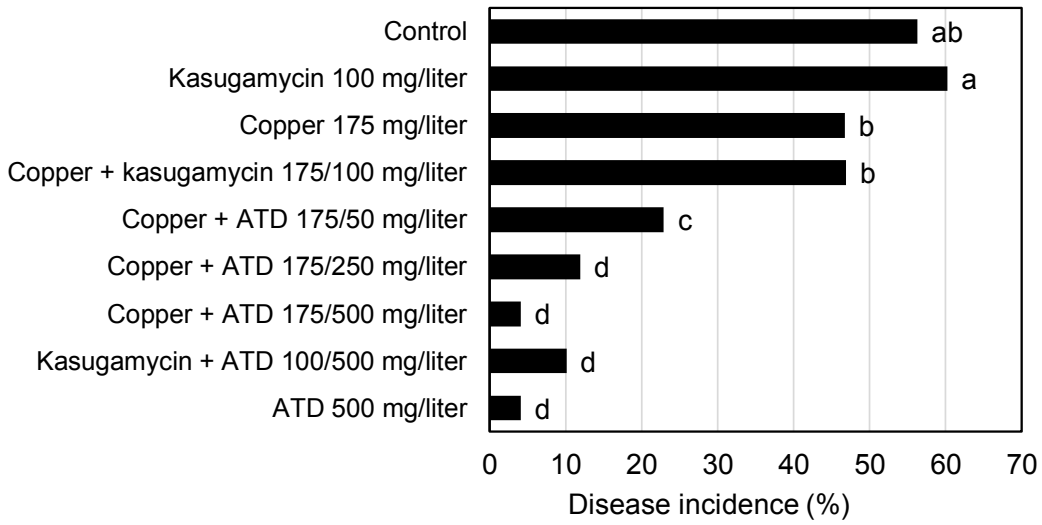

Fig. 2.1. Efficacy of protective foliar bactericide treatments on incidence of angular leaf spot (ALS) of strawberry in greenhouse studies. Plants were treated, air-dried, inoculated with a bacterial suspension, and rated after 14 to 20 days. Incidence of disease is based on the number of leaves with ALS lesions of the total number of leaves. Values are the mean of three experiments. Bars followed by the same letter are not significantly different based on least significant difference mean separation ($P > 0.05$). Copper rates are based on metallic copper equivalent. In the first experiment, copper hydroxide was used, and in the second and third experiments, a mixture of copper hydroxide and copper oxychloride was used.

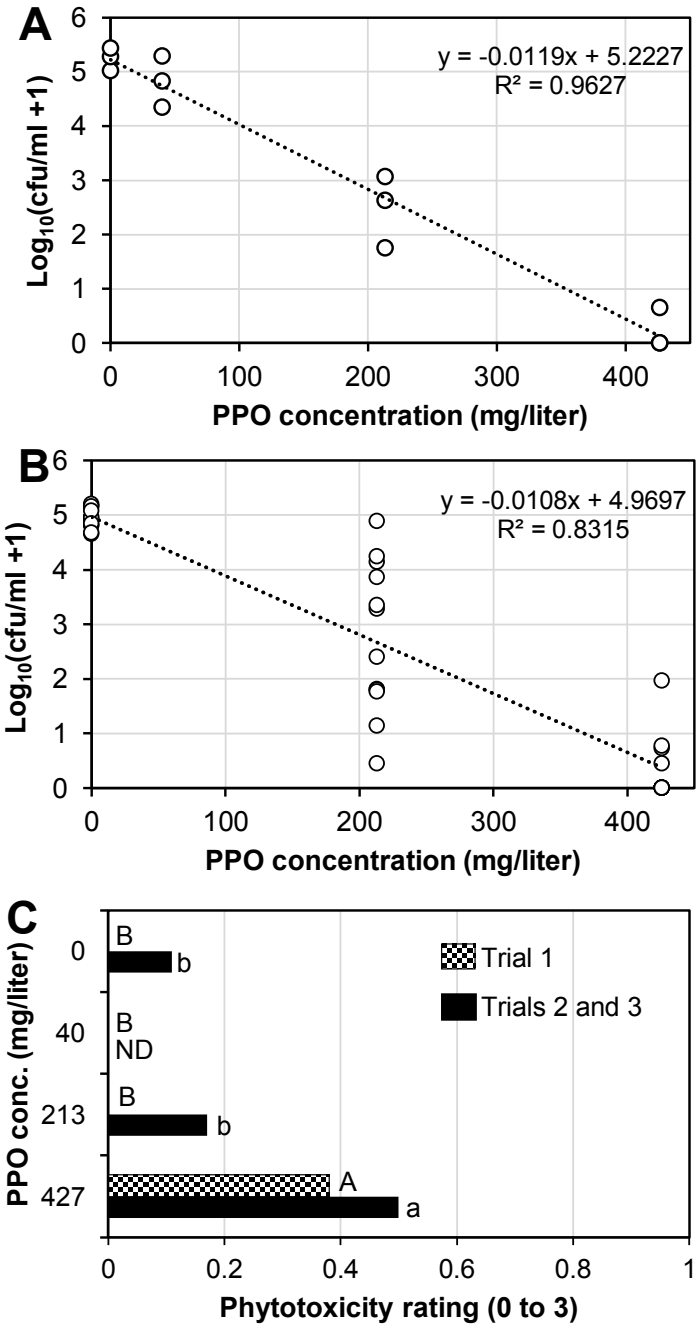

Fig. 2.2. Regressions of propylene oxide (PPO) concentration applied to diseased strawberry leaflets in a static fumigation chamber on *Xanthomonas fragariae* populations recovered in **A)** trial 1, **B)** trials 2 and 3. Each open circle represents the average bacterial population recovered from leaflets in a single chamber replicate. **C)** Phytotoxicity on leaflets after PPO fumigation; bars followed by the same letter in the same case (upper case for trial 1, lower case for trials 2 and 3) are not significantly different based on least significant difference mean separation. ND = not done.

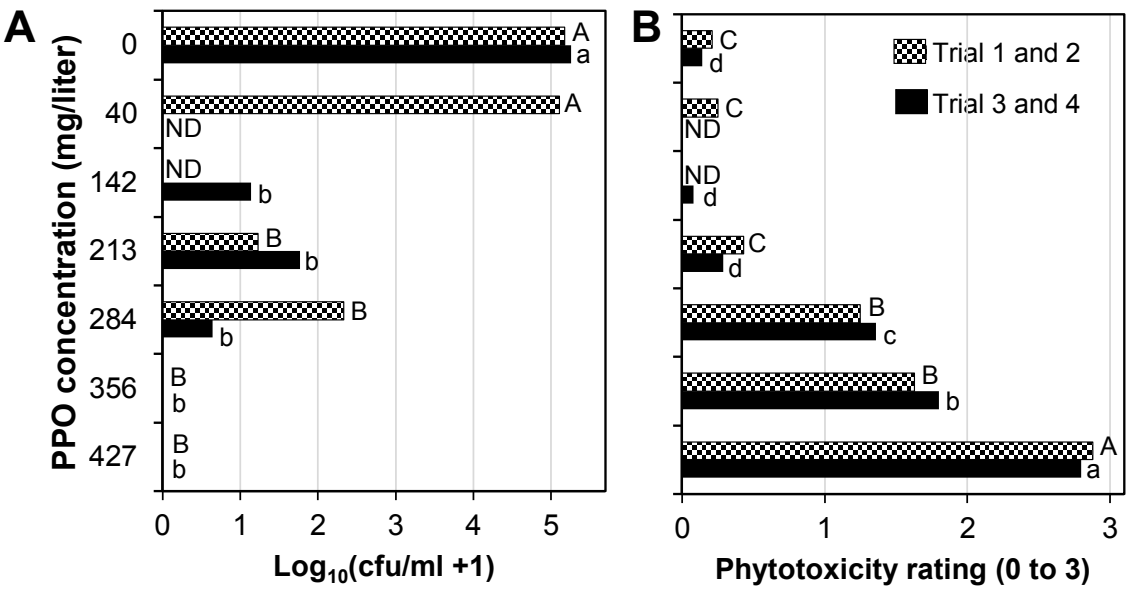

Fig. 2.3. Effect of propylene oxide (PPO) fumigation concentrations applied to diseased strawberry leaflets in a vacuum-fumigation chamber on **A)** *Xanthomonas fragariae* populations recovered from treated leaflets and **B)** leaflet phytotoxicity. Bars followed by the same letter in the same case (upper case for trials 1 and 2, lower case for trials 3 and 4) are not significantly different based on least significant difference mean separation. ND = not done.

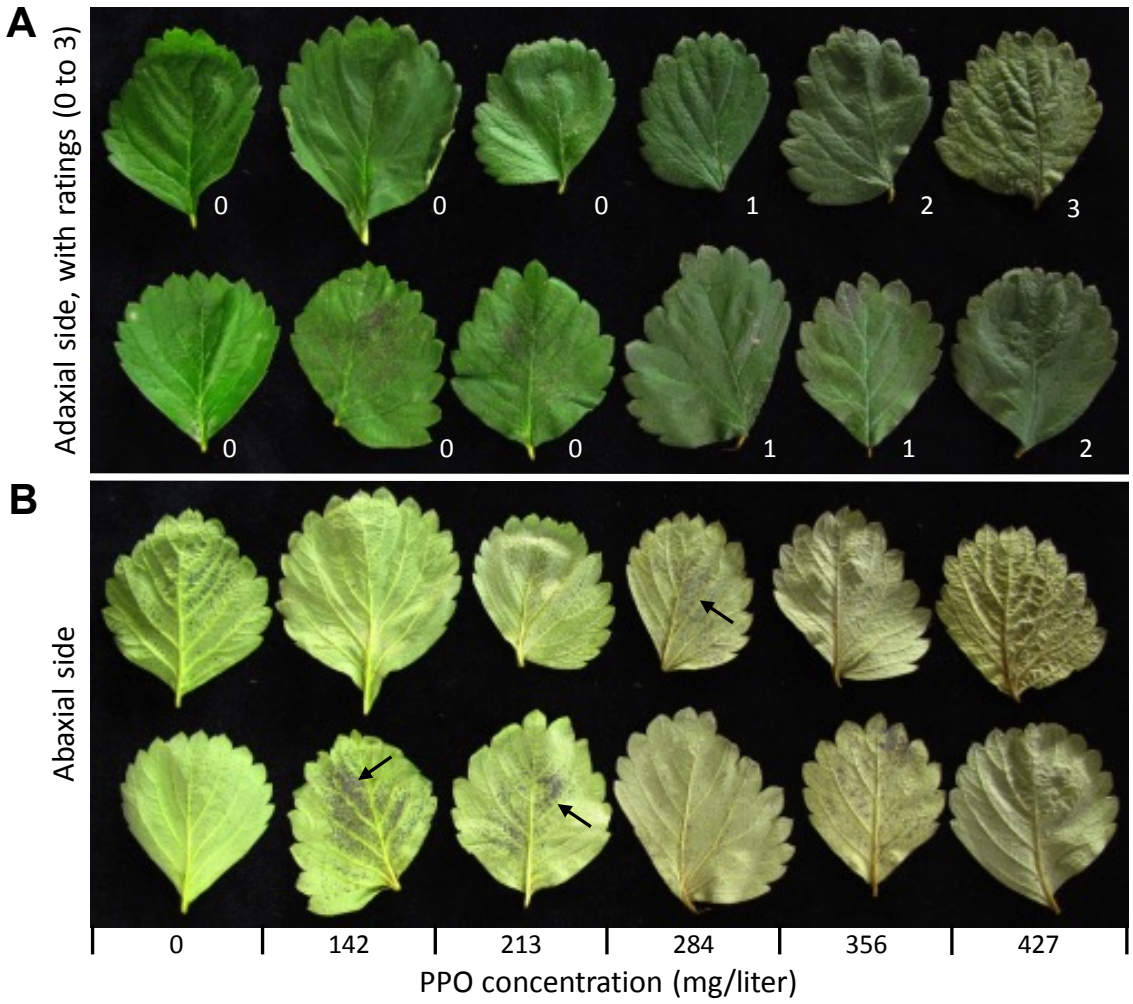

Fig. 2.4. Examples of phytotoxicity of **A)** adaxial or **B)** abaxial sides of leaflets that were treated with propylene oxide in a vacuum-infiltration chamber. Leaflets were rated for phytotoxicity on non-diseased tissues of the adaxial sides using a scale from 0 to 3. The rating as indicated in **A,** on the lower right-hand corner of each leaflet ranged from: 0 = healthy, no visible darkening; 1 = leaflet slightly darkened, but still appearing healthy; 2 = obvious darkening, but leaflet still mostly green; to 3 = leaflet very dark with purple and/or brown tones. Examples of severe disease symptoms are indicated by arrows on the abaxial side.

CHAPTER III.

Host Susceptibility, Pathogenicity, and Overwintering Sites of *Xanthomonas arboricola* pv. *pruni* and Management of Bacterial Spot of Almond, a New Disease in California

ABSTRACT

Bacterial spot caused by *Xanthomonas arboricola* pv. *pruni* was first detected on almond in California in 2013 and is reported herein as a new disease in California based on fulfilling Koch's postulates and identification of the pathogen using species-specific PCR primers. Infected mummified fruit from the previous growing season and their peduncles were identified as primary overwintering sites of the bacterium on the tree. Twig cankers were not observed, and the pathogen was not recovered from dormant buds. Isolation from flowers and emerging leaves was only successful when they were collected within 20 cm of an infected, mummified fruit on the tree. Inoculation of flowers, immature fruit, as well as immature and mature leaves resulted in disease development, indicating a long period of host susceptibility in the spring. Disease incidence was highest in fruit inoculations. In split-plot trials over three years, dormancy applications in December or January with copper or copper-mancozeb significantly reduced the disease compared to untreated controls in seasons with high rainfall but had no effect in seasons with low rainfall. In-season applications of copper-mancozeb at petal fall or at full bloom and petal fall were also effective in reducing the disease. Copper phytotoxicity was observed after repeated applications of copper bactericides, especially in low-rainfall seasons. Dormancy and in-season treatments of copper-mancozeb

mixtures integrated with removal of mummified fruit are currently the best management strategies for bacterial spot of almond in California

INTRODUCTION

Bacterial spot of almond (*Prunus dulcis* (Mill.) D. A. Webb) and other *Prunus* species has been reported in most regions of the world where these crops are grown (EPPO 2015; Ritchie 1995). The pathogen, *Xanthomonas arboricola* pv. *pruni* (syn. *X. campestris* pv. *pruni* Smith; Smith 1903; Vauterin et al. 1995) was first reported in Michigan on Japanese plum in 1903 (Smith 1903). Among commercially grown *Prunus* species, bacterial spot has the highest economic impact on Japanese plum, peach, and nectarine (Stefani 2010). Although *X. arboricola* pv. *pruni* is listed as a quarantine organism (list A2; locally present but with limited distribution) by the European and Mediterranean Plant Protection Organization (EPPO 1997), it is not identified as such by other regional quarantine regulatory agencies (Lamichhane 2014).

The disease was reported on almond in New Zealand in the 1970s (Young 1977), in Australia, India, and Pakistan in the 1990s (Akhatar et al. 1995; EPPO 1997; Jindal et al. 1990), and in Spain in 2010 (Palacio-Bielsa et al. 2010). In Australia, exasperated by common spring rains, the disease is of major concern especially on the susceptible cultivars ‘Fritz’ and ‘NePlus Ultra’. These cultivars are now rarely planted, and many existing orchards have been removed due to excessive crop losses (Palacio-Bielsa et al. 2017).

Fruit symptoms on almond are somewhat distinct from those observed in other *Prunus* hosts (Roselló et al. 2012). Infections start as small, watery sunken blemishes on the hull (i.e., mesocarp) that darken, enlarge, and exude light to dark amber gumming that accumulates as a gum drop or continues to profusely exude gum to form a long tendril on the hull surface. Upon removal of the hull epidermis at the gumming sites, diagnostic brown corky lesions can be observed extending inward. These infections sometimes reach the shell and kernel, causing darkening of the endosperm and therefore down-grading the quality of the crop. Multiple infections can occur on a single fruit, and infected fruit may drop prematurely or remain on the tree (i.e., 'stick tights') following harvest. Infected fruit on the tree develop into mummified fruit ('mummies') that may remain on the tree throughout dormancy and into the next growing season. Leaf spots are concentrated in areas of the leaf that remain wet for longer periods of time, including the leaf tip, margins, and along the mid-rib. Lesions begin as angular, water-soaked areas surrounded by chlorotic tissue, that later may coalesce, become necrotic, and may abscise creating 'shot-holes' in the leaves. Leaf symptoms can appear similar to copper phytotoxicity or shot hole caused by the fungal pathogen, *Wilsonomyces carpophilus*.

Disease symptoms were first observed in California during the spring 2013 season at high incidence on fruit in some orchard locations in Colusa, San Joaquin, Stanislaus, Merced, and Madera counties (Holtz et al. 2013). It was especially prevalent on 'Fritz' cultivar, but also observed on 'Nonpareil', 'Butte', 'Carmel', and 'Price'. Following consistent isolation of *Xanthomonas arboricola* pv. *pruni* as identified by morphological (EPPO 2005) and molecular (Pagani 2004) techniques, the disease was diagnosed as

bacterial spot of almond (Holtz et al. 2013), and Koch's postulates are reported herein. Because fruit symptoms appear similar to leaf-footed bug (*Leptoglossus* spp.) damage or anthracnose (*Colletotrichum acutatum* species complex), it is possible that bacterial spot was present for some time in these regions in California but was misdiagnosed. Since then, the disease has additionally been observed on cultivars 'Aldrich', 'NePlus Ultra', 'Winters' and 'Wood Colony', and confirmed in additional counties in California (e.g., Butte, Kern; Adaskaveg *unpublished*).

On almond, copper and copper-based mixtures are reported to have marginal efficacy against the disease (Palacio-Bielsa et al. 2017), but there have not been any comprehensive studies evaluating application efficacy or timing. On peach in the eastern United States, three to five applications of copper products or the antibiotic oxytetracycline are done prior to shuck split. This program is efficacious under low to moderate disease pressure based on environmental conditions (Ritchie 1999; Ritchie et al. 2008). Later application of copper-based compounds increases the risk of copper phytotoxicity on *Prunus* spp., with increased risk following repeated applications (Brannen et al. 2007; Ritchie et al. 2008). Copper ions function in many ways to cause bacterial cell death, including disruption of the integrity of cell membranes, induction of reactive oxygen species, DNA damage, and respiratory inhibition (FRAC 2018; Fones and Preston 2013; Grass et al. 2011), and are contact-dependent for efficacy.

In the United States, the coordination zinc and manganese ethylene bis-dithiocarbamate fungicide mancozeb is also used alone or in mixture with copper and other bactericides for the management of bacterial diseases, including those caused by

Xanthomonas spp. (Buchner et al. 2010; Guillino et al. 2010). It is also classified as having a multi-site mode of action, with active break-down products thought to interfere with enzymes in the cell cytoplasm and mitochondria (FRAC 2018; Guillino et al. 2010). Mancozeb is not often associated with crop injury. Copper products and mancozeb are currently registered for use on almond in California and labels can potentially be amended to include usage for managing bacterial spot caused by *X. arboricola* pv. *pruni.*

Although much is known about the epidemiology and management of bacterial spot on other *Prunus* hosts worldwide, information on the disease on almond in California is limited. Therefore, studies were conducted over several growing seasons to better understand the epidemiology of bacterial spot in California almond orchards and to investigate management options. Specific objectives were to: (i) demonstrate Koch's postulates and provide definitive identification of the pathogen using molecular methods; (ii) identify overwintering sites of the bacterium on almond trees; (iii) determine timing of infection and compare susceptibility of flowers, fruit, and leaves; and (iv) determine if dormancy bactericide applications targeting overwintering populations of the pathogen reduce disease in the following spring season and determine relative efficacy of in-season bactericide applications for the most efficacious disease management strategy.

MATERIALS AND METHODS

Collection of potential overwintering inoculum sources of *X. arboricola* pv. *pruni*, and bacterial isolation and identification. Collections were made between 2014 and 2018 from two 15- to 20-year-old commercial almond orchards in San Joaquin Co.,

CA, inter-planted with ‘Fritz’ and ‘Nonpareil’ cultivars. Symptomatic tissues collected included mummified fruit (dehydrated fruit from the previous season) with raised lesions and amber-colored gumming and peduncles (i.e., ‘spurs’ once fruit are removed) associated with these mummies, also with amber-colored gumming that was visible once mummies were removed. Mummies were collected from the tree or from the orchard floor if sufficient numbers could not be found in the tree. Asymptomatic tissues collected included dormant buds, flowers, emerging leaves, and fruit mummies without raised lesions or gumming. Flowers and emerging leaves were collected within 20 cm or more than 2 m from a symptomatic mummified fruit, or from a tree without mummified fruit (see Table 3.1). Dormant buds were collected from trees without symptomatic mummies. Sampling of tissues was done between December and July. Mummies and associated spurs were surface-disinfested in 1% sodium hypochlorite for 1 min and triple-rinsed with sterile distilled water. Mummies were soaked in sterile deionized water for 15 to 30 min to soften tissues, the outer hull layer was removed using a sterile razor blade, and tissues of raised brown corky lesions of symptomatic mummies or tissues of asymptomatic mummies were excised. Spurs were split longitudinally, and pieces approximately 2 mm x 5 mm in size were excised. Asymptomatic buds, emerging leaves, and flowers were removed from twigs and cut into pieces (approximately 2 mm x 5 mm). Samples were added to 1.7-ml microcentrifuge tubes containing 500 µl sterile deionized water. Tubes were vortexed for 10 min to aid in the suspension of any bacteria present. Suspensions were plated onto yeast extract-dextrose-calcium carbonate media (YDC; Schaad 1988) or Tween medium with 0.3 g/liter boric acid (McGuire et al. 1986), both

amended with 100 mg/liter cycloheximide, 33 mg/liter cephalexin, 3.4 mg/liter 5-fluorouracil, and 0.34 mg/liter tobramycin after autoclaving (YDCM and TWM, respectively). Plates were evaluated for colonies of *X. arboricola* pv. *pruni* after 3 to 5 days for YDCM and after 5 to 7 days for TWM at 25°C. Candidate colonies were sub-cultured onto YDC and verified as *X. arboricola* pv. *pruni* using morphological characteristics, and a subset of isolates were subjected to PCR using species-specific primers Y17CoF and Y17CoR (Pagani 2004).

Koch's postulates and inoculation of almond tissues with *X. arboricola* pv. *pruni* in the field. Inoculations were conducted in 2016 and 2017 on approximately 10- to 11-year-old 'Fritz' almond trees in San Joaquin Co., CA, on flowers at full bloom (16 Feb. 2016 and 22 Feb. 2017) and on immature fruit (16 Apr. 2016 and 15 Apr. 2017) near the end of hull expansion (Kester et al. 1996). *X. arboricola* pv. *pruni* strains *Xap*942 and *Xap*1789 obtained from naturally-infected symptomatic 'Nonpareil' and 'Fritz' fruit in Stanislaus and San Joaquin county in 2013 and 2015, respectively, were cultured from long-term storage at -80°C in 20% glycerol on YDC medium for 24 h at 25°C. Following sub-culturing and additional growth for 24 to 48 h, bacterial suspensions were made using single strains or a mixture of the two strains in approximately equal proportions in sterile deionized water and adjusted to 1 x 10^8 (80% OD_{600} transmission) bacterial cells/ml. Suspensions were used immediately for inoculations at selected phenological stages as indicated in Table 3.2A,B. For each replicate, a single branch 30 to 70 cm long with approximately 12 to 100 flowers or 3 to 40 fruit was selected. Treatments were applied to run-off using a hand-held spray bottle. Control treatments were sprayed with

water. Branches were covered with white plastic bags that were misted with water inside for 18 to 20 h to provide a high-humidity microenvironment. For each treatment, 8 to 12 replications were done using 13 trees. Evaluations were conducted 4 to 8 weeks after inoculation. For this, the number of fruit with at least one sunken corky lesion characteristic of bacterial spot was determined out of total number of fruit per replicate. Disease incidence on leaves was evaluated based on the number of leaves with at least one lesion on terminal 30-cm ends of each replicate branch with approximately 200 leaves. Bacterial isolations were conducted from representative samples of symptomatic diseased tissues as described above to verify the presence of the pathogen.

Bactericides. Copper hydroxide (Kocide 3000, DuPont, Wilmington, DE; ChampION^{++} Nufarm Americas, Inc., Alsip, IL) or a mixture of copper hydroxide and mancozeb (Manzate 45DF or Max; United Phosphorus, Inc., King of Prussia, PA), were used for dormant and in-season applications at rates indicated in Tables 3.3 – 3.6. For in-season timings with multiple copper applications, copper rates were reduced in some trials with sequential applications to mitigate phytotoxic effects (Brannen et al. 2007).

Dormancy and timing of in-season bactericide treatments. Trials were conducted in a commercial, 15- to 20-year-old 'Fritz' almond orchard on 'Nemaguard' peach rootstock in San Joaquin Co., CA, with sprinkler-irrigation. Dormancy and in-season bactericide treatments were evaluated in 2014/15, 2015/16, and 2016/17 using a split-plot design with dormancy treatments as the main plots, and in-season applications as the sub-plots. There were four single-tree replications for each treatment combination. Dormant applications with copper or copper-mancozeb were done in December (e.g., 18

Dec. 2014, 4 Dec. 2015, and 14 Dec. 2016), and delayed dormant treatments were done at bud-swell in January (e.g., 27 Jan. 2015, 28 Jan. 2016, and 25 Jan. 2017).

In 2015 studies, dormant and delayed-dormant applications were replicated in separate rows within the same orchard, and in-season applications were conducted for each. In the split-plot with dormant treatments, in-season timings evaluated included one-application (11 Mar.), two-application (4, 21 Apr.), and four-application (25 Feb., 6 Mar., 17 Mar., 6 Apr.) spray programs with copper-mancozeb. In the split-plot with delayed dormant treatments, efficacy of copper and copper-mancozeb was compared in a four-application program (25 Feb., 6 Mar., 17 Mar., 6 Apr.).

In 2016 and 2017, dormant and delayed-dormant applications were applied within the same row, and in-season bactericide application timings were done as a split-plot using a copper-mancozeb mixture at full bloom (FB; 16 Feb. 2016, and 22 Feb. 2017), petal fall (PF; 7 Mar. 2016, and 15 Mar. 2017) or both FB and PF. All applications were made using an air-blast backpack sprayer (Model SR420; Stihl Inc., Virginia Beach, VA) at 935 liter/ha. Trials were evaluated on 1 July 2015, 17 June 2015, 15 June 2016, and 6 June 2017. Disease incidence on fruit was based on the number of symptomatic fruit per 100 fruit evaluated per tree. Copper phytotoxicity of leaves was evaluated for all treatments in 2015 and 2016, and in 2017, for the FB+PF in-season program as well as for the untreated control. For this, the terminal 30-cm ends of four branches per tree were evaluated using a rating scale as follows: 0 = healthy; 1 = <5% spotted leaves; 2 = 5 to 50% leaves with noticeable spotting; 3 = >50% spotted leaves; and 4 = severe spotting, tattered appearance, and leaf-drop. Fruit symptoms were verified to be caused by *X.*

arboricola pv. *pruni* by bacterial isolation from a sub-sample (n=10 to 12) of fruit and confirmed using PCR methods as described above. Absence of *X. arboricola* pv. *pruni* or of fungal infections was confirmed by isolation from leaf spots attributed to copper phytotoxicity. Following surface disinfestation as described above, small (4 to 20-mm^2) leaf pieces from the margin of lesions were suspended in sterile deionized water as above, vortexed, and the suspension was plated onto YDC for bacterial isolations, or leaf tissue was placed onto potato dextrose agar amended with 130 mg/liter ampicillin and 20 mg/liter rifampicin for fungal isolations.

Environmental monitoring. For each winter-spring season where field studies were conducted, data for average daily temperatures and precipitation were obtained for Stations 70 (Manteca, CA) and 249 (Ripon, CA) from the California Irrigation Management Information System (CIMIS, https://cimis.water.ca.gov/WSNReportCriteria.aspx) and were summarized graphically.

Statistical analysis. Isolation data were summarized by mean and range of percent recovery of the pathogen among sampling years for each tissue type and location within a tree in relation to a symptomatic mummified fruit.

Inoculation data for 2016 and 2017 were analyzed separately. Fruit and leaf incidence were arcsine transformed prior to analysis. When the two *X. arboricola* pv. *pruni* strains were used separately, a generalized linear model (GLM) analysis and Levene's test for homogeneity of variance were conducted. Data were combined prior to further analysis when homogeneity of variance was upheld. GLM was used for overall model analysis following a factorial design with phenological stage, treatment, and

stage*treatment interaction as factors. When the overall models were found to be significant, one-way classification of data was used for comparing treatments and phenological stages for each year following GLM and Fisher's LSD pairwise mean separation procedures.

The four bactericide trials were analyzed separately. Disease incidence data were arcsine-transformed prior to analysis, and phytotoxicity severity ratings were not transformed. Analysis of variance (ANOVA) or GLM procedures were conducted for the overall models according to a split-plot design as described above. When the overall models were found to be significant, one-way classification of data was used for comparing in-season treatments separately for each dormant treatment, and dormant treatments separately for each in-season program following ANOVA or GLM and Fisher's LSD mean separation procedures. Fisher's LSD was also conducted each for main- and sub-plot factor means using combined data.

All statistical procedures were done using SAS (version 9.4; SAS Institute, Inc., Cary, NC). A significance value of $\alpha=0.1$ was used for GLM or ANOVA overall and one-way classification models, and a significance value of $\alpha=0.05$ was used for multiple comparison procedures.

RESULTS

Identification of overwintering structures. *X. arboricola* pv. *pruni* was recovered from 40% of the symptomatic mummies collected and from 51.4% of their associated spurs. The pathogen was isolated from 19.4% of asymptomatic flowers and

3.4% of emerging leaves when these tissues were collected adjacent to a symptomatic mummy in the tree canopy. *X. arboricola* pv. *pruni* was not isolated from asymptomatic mummies, dormant bud samples, or from flowers that were collected either more than 2 m from a symptomatic mummy or from a tree where mummified fruit were not present.

On YDCM, *X. arboricola* pv. *pruni*-candidate colonies were yellow, convex, mucoid, and shiny (EPPO 2005). On TWM, they were light yellow, convex, mucoid, shiny, and surrounded by a white halo of minute crystals in the agar as a result of Tween lipolysis by the bacterium (McGuire et al. 1986). On both media, yellow coloration darkened over time. PCR amplification with *X. arboricola* pv. *pruni*-specific primers Y17CoF and Y17CoR resulted in a single band of the expected 943-bp size.

Mummified fruit, especially those collected from the orchard floor, resulted in high levels of bacterial contamination even on semi-selective media. For these samples, *X. arboricola* pv. *pruni* was more easily recovered from symptomatic mummies on TWM medium compared to YDCM (*data not shown*). On YDCM, different bacteria with yellow pigmentation were isolated from symptomatic and asymptomatic mummified fruit, but with various shades of yellow (e.g., neon-yellow, orange-yellow), consistency, and morphological characteristics distinct from *X. arboricola* pv. *pruni*. These bacterial contaminants did not result in positive PCR identification using *X. arboricola* pv. *pruni*-specific primers.

Effect of in-season inoculations on disease incidence. In 2016 inoculations of flowers, disease incidence on fruit was not significantly (P=0.3278) different for strains *Xap*942 (35.0%) and *Xap*1789 (28.8%). In inoculations of emerging leaves, disease

incidence was also not significantly (P= 0.2732) different for the two strains (6.5% and 4.3%, respectively). Because homogeneity of variance was upheld (P=0.1452 and 0.7951 for fruit and leaf evaluations, respectively), data were combined.

All inoculations resulted in disease development with fruit and leaf symptoms similar to those described previously in almond and observed in natural infections in California (Table 3.2). *X. arboricola* pv. *pruni* was consistently re-isolated from inoculated fruit and leaves, and PCR amplification with *X. arboricola* pv. *pruni*-specific primers Y17CoF and Y17CoR resulted in a single band of the expected 943-bp size, fulfilling Koch's postulates.

The overall models for 2016 and 2017 inoculation studies were significant for disease incidence of fruit (P= <0.0001), with treatment (i.e., inoculation) (P<0.0001), phenological stage (P=0.0024), and interaction (P=0.0035) factors significantly contributing to the model in 2016, but only the treatment (P<0.0001) factor contributed significantly to the model in 2017. Thus, phenological stage and interaction factors were not significant (P= 0.7716 and 0.4841, respectively). Following one-way data classification, inoculations resulted in significantly higher disease levels compared to the water control for all four year*stage fruit disease evaluation groups (P<0.0001 to 0.0006).

In 2016, inoculations of flowers resulted in a significantly (P<0.0001) lower disease incidence compared to inoculation conducted at the immature fruit stage, whereas in 2017, inoculations at both stages were statistically similar (P= 0.7461) (Table 3.2). Some water controls showed low levels of fruit disease incidence in 2016 (mean 1.5%

and 2.4% for flower and immature fruit stages, respectively) and moderate levels of disease in 2017 (mean 21.2% and 21.8% for flower and immature fruit stages, respectively). These were statistically similar among inoculation stages for each year (P=0.8897 and 0.5293 for 2016 and 2017, respectively).

For incidence of spotted leaves, the overall models for 2016 and 2017 were significant (P <0.0001), with treatment (P <0.0001) significantly contributing to the model, but not phenological stage or the interaction (P=0.7696 and 0.7169 for 2016; P=0.7556 and 0.9068 for 2017, respectively). No significant differences were observed in diseased leaf count for similar treatments inoculated at emerging or mature leaf stages for either year (P=0.4112 to 0.9589). All inoculations with *X. arboricola* pv. *pruni* resulted in significantly (P=<0.0001 to 0.0091) higher disease compared with the respective water controls.

Effect of dormancy and timing of in-season bactericide treatments on disease incidence. Fruit lesions with amber gumming were the predominant disease symptom observed in each year. *X. arboricola* pv. *pruni* was consistently isolated from these lesions, verifying the presence of bacterial spot. None of the leaf spot isolations resulted in the recovery of the pathogen, indicating that copper phytotoxicity was the cause.

Following a relatively wet December and dry spring season in 2014-2015 (total rainfall 1 Dec. to 30 Apr. was 207.5 mm, with 148.1 mm in Dec.; Fig. 3.1A), disease was first observed in late May and the incidence was overall low at evaluation time. An average of 10.3% and 4.5% of fruit on control trees were diseased in trials 1 and 2, respectively (Tables 3.3, 3.4). Still, the overall models for disease incidence were

significant (P=0.0009 and 0.0787 for trials 1 and 2, respectively), and because the interaction between dormancy and in-season treatments was not significant (P=0.5928 and 0.8674, respectively), data were combined and interpreted across in-season and dormancy treatments for each trial for evaluation of overall factor effects (Tables 3.3, 3.4). Neither dormant nor delayed-dormant applications significantly reduced the disease compared with the untreated control in trials 1 (P=0.4214) and 2 (P=0.1236). In-season applications with copper-mancozeb (Table 3.3) or with copper and copper-mancozeb (Table 3.4) significantly (P=0.0029 and 0.0007, respectively) reduced the disease compared with the untreated control.

In the 2015-16 and 2016-17 trials, following relatively wet winter and spring seasons with total rainfall from 1 Dec. 2015 to 30 Apr. 2016 of 311.0 mm (Fig. 3.1B) and from 1 Dec. 2016 to 30 Apr. 2017 of 338.3 mm (Fig. 3.1C), the disease was first observed in late April. Disease incidence on untreated trees was 9.0% and 41.3% in the two trials, respectively. Overall models (P<0.0001 and 0.0027 for the two studies, respectively), dormancy (P=0.0700 and P=0.0045) and in-season (P=0.0072 and P<0.0001) factors, as well as the interaction between dormancy and in-season bactericide timings in both trials were significant (P=0.0020 and P=0.0124).

Following one-way classification of data according to in-season bactericide timing, (Tables 3.5, 3.6), dormancy applications significantly (P=0.0088 for 2015-16 and P=0.0231 for 2016-17) reduced disease incidence when no in-season timings were applied and when copper/mancozeb was applied at FB in 2015-16 (timing 2; P=0.0299), but not in 2016-17 (P=0.1842). For these, dormancy timings were statistically similar

(P>0.05). Dormancy treatments were not significant when followed by a single bactericide treatment at PF (timing 3; P=0.1155 for 2015-16 and P=0.4526 for 2016-17) or by two treatments at FB and PF (timing 4; P=0.7362 for 2015-16 and P=0.4873 for 2016-17).

Following one-way classification of data according to dormancy timing (Tables 3.5, 3.6), in-season bactericide applications significantly reduced the disease when no dormancy treatment was applied in 2015-16 (P=0.0697) and 2016-17 (P=0.0001) and following the dormant bactericide application in 2015-16 (P=0.0388). When no dormancy treatment was applied in 2015-16, timing 4 (FB + PF) significantly (P<0.05) reduced the disease compared with the control (timing 1) but was not statistically different (P>0.05) from in-season timings 2 and 3. In 2016-17, all three in-season timings significantly (P<0.05) reduced the disease compared with the control without a dormancy treatment, with timings 3 (PF only) and 4 (FB and PF) resulting in a statistically lower disease incidence compared with timing 2 (FB only). Following dormant application of copper/mancozeb, in-season timings 2 (FB) and 3 (PF) resulted in statistically (P<0.05) lower disease compared with the control (timing 1) in 2015-16 but were not statistically significant (P=0.3695) in 2016-17. In-season treatments following delayed-dormant timing of copper/mancozeb application were not significant in the 2015-16 and 2016-17 studies (P=0.8638 and 0.1623, respectively).

Effect of dormancy and in-season bactericide treatments and timings on copper phytotoxicity on leaves. No phytotoxicity was observed on trees receiving no in-season bactericide applications. The overall models for phytotoxicity were significant

in all trials (P<0.0001, P=0.0016, P<0.0001, and P=0.0418 for 2014-15 trial 1, 2014-15 trial 2, 2015-16, and 2016-17, respectively), and because there was no interaction between dormancy and in-season treatments (P=0.3313, 0.8599, 0.1909, and 0.4402, respectively), data were combined and interpreted across levels by trial for evaluation of overall factor effects (Tables 3.3- 3.5). Dormancy treatments were only found to significantly contribute to the model in trial 1 in 2014-15 (P= 0.0066), where a dormant application of copper – mancozeb had significantly lower phytotoxicity averaged across in-season applications compared to the control and copper alone. In-season bactericide application programs had a significant effect in all trials (P<0.0001, P<0.0001, P<0.0001, and P=0.0003 for 2014-15 trial 1, 2014-15 trial 2, 2015-16, and 2016-17, respectively), and all in-season bactericide application timings and treatments resulted in significantly (P>0.05) higher phytotoxicity compared with the respective controls. In trial 1 in 2014-15, phytotoxicity was highest in the 4-application program (timing 4, rating 2.6), followed by the single-application (timing 2, rating 1.2) and two-application (timing 3, rating 0.8) spray programs (Table 3.3). In trial 2 in 2014-15, no differences (P>0.05) were observed between copper and copper – mancozeb mixtures in a 4-application program (Table 3.4). In 2015-16, timings 3 (PF, rating 2.6) and 4 (FB + PF, rating 2.9) had the highest phytotoxicity and were significantly higher than for timing 2 (FB, rating 0.9; Table 3.5). In 2016-17, phytotoxicity was low, and the two-spray FB + PF program resulted in a mean rating of 0.8.

DISCUSSION

Bacterial spot is reported herein as a new disease of almond in California. Koch's postulates were fulfilled, and the pathogen *X. arboricola* pv. *pruni* was identified using specific PCR primers. The disease has been previously reported from almond and stone fruits in other parts of the world (Palacio-Bielsa et al. 2017; Ritchie 1995) and the pathogen was first detected and confirmed on almond in California in 2013 (Holtz et al. 2013). Symptomatic mummified fruit and their peduncles/spurs were identified as the primary overwintering site for *X. arboricola* pv. *pruni* in California orchards. Mummified fruit are also implicated as overwintering sites of bacterial spot of almond in Spain and Australia (Palacio-Bielsa et al. 2017; Roselló et al. 2012). Removal of mummified fruit during dormancy, a cultural practice that isalso important for the management of navel orangeworm (*Amyelois transitella*; Zalom et al. 1984),can therefore reduce primary inoculum of *X. arboricola* pv. *pruni* in the following spring. To our knowledge, this is also the first report of the presence of *X. arboricola* pv. *pruni* in spurs associated with infected mummified fruit. This is important because the peduncle of an almond fruit remains attached to the tree, forming a woody spur. Therefore, even when mummified fruit are removed, the infected spur remains a potential source of primary inoculum in the orchard canopy. In other growing regions of almond, twig lesions/cankers and dormant buds are reported as overwintering sites of *X. arboricola* pv. *pruni* (Palacio-Bielsa et al. 2017; Roselló et al. 2012), and these, in addition to leaf scar infections, are the primary overwintering locations in other hosts such as peach (Battilani et al. 1999; Ritchie et al. 2008; Zaccardelli et al. 1995). We did not observe such twig lesions, and the bacterium

was not recovered from buds. It remains possible that these overwintering sites exist in California orchards, but they probably have a minor role. Recovery of the bacterium from asymptomatic flowers and emerging leaves was dependent on their location within 20 cm of a symptomatic mummified fruit, suggesting that the bacterium may have spread from the infected mummy, likely by rain-splash or dripping dew.

Recovery of *X. arboricola* pv. *pruni* from symptomatic mummified fruit was low for some collections possibly due to high levels of contamination even when semi-selective media were used or due to low bacterial levels in the tissues. Population sizes of *X. arboricola* pv. *pruni* in the various tissues were not quantified, but even small amounts of viable bacterial cells may exponentially increase to high concentrations in overwintering structures during favorable temperature and wetness conditions. Additionally, because multiple yellow-pigmented bacteria other than *X. arboricola* pv. *pruni* were isolated from mummified fruit, proper bacterial species identification with species-specific primers is important for diagnostic purposes.

Inoculations of flowers, fruit, as well as immature and mature leaves of 'Fritz' almond resulted in disease development, indicating a long period of host susceptibility to infection during the spring season. Disease incidence was low on inoculated leaves and low to high on fruit following flower and fruit inoculations. These disease levels are similar to those observed naturally on leaves and fruit in California. Disease development on fruit was lower after flower than after immature fruit inoculations in 2016; but in 2017, there was no difference between the two inoculations. Wetness conditions were similar during the incubation period in both years, but temperatures were lower in 2017.

Optimum environmental conditions for bacterial spot development on almond are not known, although the disease is more common in areas with temperate, humid climates (Palacio-Bielsa et al. 2017). Thus, environmental conditions cannot explain the difference in disease development between the years. The high disease levels observed after flower inoculations in 2017, however, may be due to the presence of high natural inoculum levels in the orchard that led to fruit infections. High natural inoculum levels were evident by the relatively high disease incidence (21.2% and 21.8%) on branches with flowers or fruit that served as non-inoculated controls and by high disease levels in our management plots (41.3% in the untreated control; Table 3.6) in 2017. Alternatively, in flower inoculations, *X. arboricola* pv. *pruni* possibly did not infect immediately, but instead remained epiphytic for a period of time (Shepard and Zehr 1994). Thus, disease observed after flower inoculation may have resulted from fruit infection by epiphytic populations. Microscopic visualization of the infection process may lead to a better understanding of the specific infection timing and mechanism in almond, although our studies demonstrate that a mechanical wound is not necessary for infection. Previous studies on other hosts indicate that the bacterium can enter tissues via natural openings such as stomata and lenticels or through wounds such as leaf scars, and the pathogen has the potential to move systemically (Du Plessis 1986; Roselló et al. 2012).

Successful inoculations of immature fruit demonstrate the potential for high disease development under natural conditions when springtime rains occur. Infections later in the season may have less impact on yield and kernel quality because kernels and endocarps are developed, and there is not enough time for the pathogen to grow into these

tissues to cause losses. Still, the disease has been observed to reach epidemic levels in orchards where high-angle impact sprinkler irrigation in May, June, and July wets the lower tree canopy, creating a conducive microclimate (Adaskaveg, *unpublished*). Therefore, use of microsprinkler or drip irrigation that do not wet tree canopies should be beneficial to reduce bacterial spot.

Development of bacterial spot on *Prunus* spp. depends greatly on climactic conditions, with warm temperatures and wet conditions favoring disease (Battilani et al. 1999; Palacio-Bielsa et al. 2017; Stefani 2010). In the present study, dry spring conditions in 2015 resulted in relatively low disease in both trials. In 2016, disease was observed at similar levels as in 2015 despite the occurrence of more rainfall. This may be because low disease levels in 2015 resulted in a small overwintering population of the bacterium and correspondingly low primary inoculum populations in the spring of 2016.

Dormancy applications significantly reduced the disease in years with more rainfall (i.e., 2015-16 and 2016-17 trials), even without in-season applications, but not in a dry year (i.e., 2014-15 trials). Copper and copper-mancozeb treatments showed high efficacy against bacterial spot in our trials under California conditions in dormant and delayed dormant applications. In high-rainfall spring seasons (2015-16 and 2016-2017 trials), dormancy as well as in-season full bloom plus petal fall or petal fall applications alone significantly reduced the disease as compared with the untreated control in the overall main effects model of the split plot. In low-rainfall seasons (2014-15), dormancy treatments were not effective, but in-season applications of copper-mancozeb at petal fall or later significantly reduced the disease from the untreated control. This differs from

reports from other geographical regions and on other hosts where chemical treatments are considered only moderately effective under low to moderate disease pressure (Palacio-Bielsa et al. 2017; Ritchie 1999; Ritchie et al. 2008). Still, in our study, an interaction was observed between dormancy and in-season timings in the high-rainfall seasons indicating that the efficacy of specific combinations of these applications were probably dependent on microclimatic conditions and phenological stages during application of the bactericides.

Determination of optimal spray timings is important for most efficient and cost-effective disease management as compared with a calendar-based spray schedule. Optimal timing may also reduce chemical load in the environment by reducing the number of sprays. Lastly, optimal timings have been shown to prevent resistance development in a pathogen population (van den Berg et al. 2013). Because copper and mancozeb are currently registered in the United States for management of diseases of almond, preserving their efficacy for bacterial spot is relevant. Furthermore, our efficacy data will support the addition of bacterial spot to labels of these compounds. Evaluation of population-level sensitivities of *X. arboricola* pv. *pruni* to these compounds and monitoring of any changes in sensitivity is warranted, especially because copper resistance is present in Michigan populations of *X. arboricola* pv. *pruni* (McGrath et al. 2009) as well as in California populations of *X. arboricola* pv. *juglandis* (causal agent of walnut blight; Nguyen et al. 2016).

Copper use is limited by the risk of copper phytotoxicity especially if multiple applications have to be done. This is demonstrated in some of our studies, and differences

in phytotoxicity levels are likely due to annual differences in microclimates. For example, rain can reduce copper residues on plant surfaces, minimizing build-up. Phytotoxicity was generally higher with repeated in-season applications when foliage was present, as observed also in peach (Brannen et al. 2007; Richie et al. 2008). With phytotoxicity as a limiting factor for copper usage and label restrictions in the use of mancozeb that prohibits its use later than five weeks after petal fall, evaluation of other, new bactericide treatments with differing modes of action and that cause no phytotoxicity for the management of bacterial spot of almond in California is warranted.

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Table 3.1. Recovery of *Xanthomonas arboricola* pv. *pruni* from almond tissues collected in the field

Structure/tissue	Years collected	No. samples	Percent recovery	
			Mean	Range among years
Symptomatic mummy	2013 - 2018	201	40.0	10.0 - 67.7
Asymptomatic mummy[a]	2015, 2016	29	0.0	0.0 - 0.0
Spur of a mummy[b]	2017, 2018	44	51.4	42.9 - 60.0
Dormant buds[a]	2016, 2018	66 (132)[c]	0.0	0.0 - 0.0
Flowers[a]	2016, 2018	46 (66)	0.0	0.0 - 0.0
Flowers (within 20 cm of a mummy[b])	2017, 2018	56 (138)	19.4	11.5 - 46.7
Emerging leaves (within 20 cm of a mummy[b])	2016, 2017	71 (175)	3.4	0.0 - 6.7

[a] Asymptomatic tissues collected more than 2 m from a symptomatic mummified fruit or from a tree without mummified fruit.
[b] Mummies showed symptoms of bacterial spot.
[c] Values in parentheses represent the number of individual units of each tissue that were used for composite samples.

Table 3.2. Inoculation of 'Fritz' almond with *Xanthomonas arboricola* pv. *pruni* at selected phenological stages

		Disease incidence (%) on fruit[a] resulting from inoculations of:					
		Flowers (full bloom)[b]			Immature fruit		
Year	Treatment[c]	Range	Mean	LSD[d]	Range	Mean	LSD
2016	Control	0.0 - 5.3	1.5	A b	0.0 - 14.3	2.4	A b
	X. arboricola pv. *pruni*	0.0 - 66.7	31.8	B a	50.0 - 90.9	77.7	A a
2017	Control	0.0 - 71.4	21.2	A b	0.0 - 30.4	21.8	A b
	X. arboricola pv. *pruni*	50.0 - 100.0	74.9	A a	50.0 - 100.0	71.4	A a

		Disease incidence (%) on leaves resulting from inoculations of:					
		Emerging leaves			Mature leaves		
Year	Treatment	Range	Mean	LSD	Range	Mean	LSD
2016	Control	0.0 - 1.0	0.1	Z y	0.0 - 1.5	0.4	Z y
	X. arboricola pv. *pruni*	0.0 - 26.5	5.3	Z z	0.5 - 11.5	5.4	Z z
2017	Control	0.0 - 3.0	0.9	Z y	0.0 - 2.5	0.8	Z y
	X. arboricola pv. *pruni*	1.5 - 16.5	7.6	Z z	3.5 - 10.5	6.6	Z z

[a] Diseased fruit and leaves had at least one lesion. Incidence was based on the total number of fruit per branch or leaves on the terminal 30-cm ends of each branch.
[b] Inoculations were done on 16 Feb 2016 and 22 Feb 2017 for flower/emerging leaves and on and 14 April 2016 and 15 April 2017 for immature fruit/mature leaves.

[c] Controls were sprayed with distilled water. For inoculations, a suspension (1×10^8 cfu/ml) of an approximately equal mixture of *X. arboricola* pv. *pruni* strains *Xap* 942 and *Xap* 1789 was used. In 2016, the two strains were used separately for flower/emerging leaf inoculations, and data were combined.
[d] Mean incidence values were compared using analysis of varianace and least significant difference (LSD) mean separation procedures. Values followed by the same lowercase letter within a column or values followed by the same uppercase letter within a row for each tissue evaluation and year are not significantly different for different inoculation timings.

Table 3.3. Effect of dormant and timing of in-season bactericide treatments on the incidence of bacterial spot on fruit and copper phytotoxicity on leaves of 'Fritz' almond in San Joaquin Co. 2014-2015

	Disease incidence (%)[a]											
	Timing 1		Timing 2		Timing 3		Timing 4		Dormant mean			
	IS[b]: none		IS: 3/11		IS: 4/6, 4/24		IS: 3/11, 4/6, 4/24, 5/13		Treatment		Phytotoxicity[c]	
Dormant treatments (12/18/14)[d]	Mean	LSD[e]	Mean	LSD	Mean	LSD	Mean	LSD	**Incid.**	**LSD**	**Severity**	**LSD**
Control	10.3	A a	2.0	A a	4.3	A a	2.5	A a	**4.8**	**a**	**1.3**	**a**
Copper	4.5	A a	2.3	A a	1.8	A a	1.0	A a	**2.4**	**a**	**1.2**	**a**
Copper - mancozeb	3.3	A a	0.8	A a	2.0	A a	1.0	A a	**1.8**	**a**	**1.0**	**b**
IS timing mean disease	**6.0**	**A**	**1.7**	**B**	**2.7**	**B**	**1.5**	**B**				
IS timing mean phytotoxicity	**0.0**	**D**	**1.2**	**B**	**0.8**	**C**	**2.6**	**A**				

[a] Fruit were evaluated for the presence of bacterial spot and leaves for phytotoxicity on 7/1/15.

[b] IS = in-season treatments of copper (ChampION++) - mancozeb (Manzate Max) mixtures were applied to the sub-plots using a backpack sprayer. Rates were 1.2 + 3.6, 0.6 + 2.4, 0.3 + 2.4, and 0.3 + 2.4 g/liter for MCE + mancozeb for 3/11, 4/6, 4/24, and 5/13 applications, respectively.

[c] Copper phytotoxicity of leaves was evaluated on the terminal 30-cm ends of four branches per tree using a rating scale as follows: 0 = healthy; 1 = <5% spotted leaves; 2 = 5 to 50% leaves with noticeable spotting; 3 = >50% spotted leaves; and 4 = severe spotting, tattered appearance, and leaf-drop. Severity values are averaged according to main and sub-plot effects (right-most column and bottom row, respectively).

[d] Dormant treatments were applied to the main plot using a backpack sprayer on 12/18/14. Copper (Kocide 3000) was applied at a rate of 2.15 g/liter metallic copper equivalent (MCE), alone and mixed with mancozeb (Manzate 75DF) at a rate of 3.2 g/liter.

[e] Values followed by the same letter in the same case and weight (nonbold versus bold) are not statistically different based on analysis of variance and Fisher's LSD mean separation (P >0.05) procedures. Statistical comparisons for nonbold data by column are with lower-case letters, and those by row are with upper-case letters. Dormant treatment and phytotoxicity severity averages over all in-season timings are in the right columns in bold. In-season timing and phytotoxicity severity averages over all dormant treatments are in the bottom rows in bold.

Table 3.4. Effect of delayed dormant and in-season bactericide treatments on the incidence of bacterial spot on fruit and copper phytotoxicity on leaves of 'Fritz' almond in San Joaquin Co. 2015

	Disease incidence (%)[a]									
	IS[b]: none		IS: copper		IS: copper + manzate		**Delayed dormant mean**			
							Treatment		**Phytotoxicity[c]**	
Delayed dormant treatments (1/27/15)[d]	Mean	LSD[e]	Mean	LSD	Mean	LSD	**Incid.**	**LSD**	**Severity**	**LSD**
Control	4.5	A a	1.3	B a	0.8	B a	**2.2**	**a**	**0.6**	**a**
Copper	2.0	A a	1.0	A a	0.5	A a	**1.2**	**a**	**0.7**	**a**
Copper + mancozeb	2.3	A a	0.8	AB a	0.0	B a	**1.0**	**a**	**0.8**	**a**
IS treatment mean disease	**2.9**	**A**	**1.0**	**B**	**0.4**	**B**				
IS treatment mean phytotoxicity	**0.0**	**B**	**0.9**	**A**	**1.1**	**A**				

[a] Fruit were evaluated for the presence of bacterial spot and leaves for phytotoxicity on 7/1/15.

[b] IS = in-season treatments of copper (ChampION++) or copper - mancozeb (Manzate Max) mixtures were applied to the sub-plots. Copper rates were 1.2, 0.6, 0.3, and 0.3 g/liter metallic copper equivalent (MCE), and mancozeb rates were 3.6, 2.4, 2.4, and 2.4 mg/liter for 3/11, 4/6, 4/24, and 5/13 applications, respectively.

[c] Severity of copper phytotoxicity on leaves was evaluated on the terminal 30-cm ends of four branches per tree using a rating scale with 0 = healthy; 1 = <5% spotted leaves; 2 = 5 to 50% leaves with noticeable spotting; 3 = >50% spotted leaves; and 4 = severe spotting, tattered appearance, and leaf-drop. Severity values were averaged based on main and sub-plot effects (right-most column and bottom row, respectively).

[d] Delayed dormant treatments were applied to the main plot on 1/27/15. Copper (Kocide 3000) was applied at a rate of 2.15 g/liter MCE, alone and mixed with mancozeb (Manzate 45DF) at a rate of 3.2 g/liter.

[e] Values followed by the same letter in the same case and weight (nonbold versus bold) are not statistically different based on analysis of variance and Fisher's LSD mean separation (P >0.05) procedures. Statistical comparisons for nonbold data by column are with lower case letters, and those by row are with upper case letters. Dormant treatment and phytotoxicity severity averages over all in-season timings are in the right columns in bold. In-season timing and phytotoxicity severity averages over all delayed dormant treatments are in the bottom rows in bold.

Table 3.5. Effect of dormancy and in-season bactericide treatment timings on the incidence of bacterial spot on fruit and copper phytotoxicity on leaves of 'Fritz' almond in San Joaquin Co. 2015-2016

	Disease incidence (%)[a]											
	Timing 1		Timing 2		Timing 3		Timing 4		Dormancy timing mean			
	IS[b]: none		IS: 2/16 (FB)		IS: 3/7 (PF)		IS: 2/16, 3/7 (FB, PF)		Treatment		Phytotoxicity[c]	
Dormancy treatment timing[d]	Mean	LSD[e]	Mean	LSD	Mean	LSD	Mean	LSD	**Incid.**	**LSD**	**Severity**	**LSD**
Control	9.0	A a	7.5	AB a	5.0	AB a	1.0	B a	**5.4**	**a**	**1.5**	**a**
Dormant (12/4/15)	4.3	A b	0.5	B b	0.3	B a	2.7	AB a	**1.9**	**ab**	**1.5**	**a**
Delayed dormant (1/28/16)	0.8	A b	0.8	A b	0.5	A a	1.5	A a	**0.9**	**b**	**1.6**	**a**
IS treatment mean disease	**4.3**	**A**	**2.9**	**AB**	**1.9**	**B**	**1.6**	**B**				
IS timing mean phytotoxicity	**0.0**	**C**	**0.9**	**B**	**2.6**	**A**	**2.9**	**A**				

[a] Fruit were evaluated for the presence of bacterial spot and leaves for phytotoxicity on 6/15/16.

[b] IS = in-season treatments of copper (ChampION++) - mancozeb (Manzate 45DF) were applied to the sub-plots. Copper was applied at a rate of 1.2 g/liter metallic copper equivalent (MCE), mixed with mancozeb at a rate of 3.6 g/liter at full bloom (FB) and/or petal fall (PF).

[c] Copper phytotoxicity of leaves was evaluated on the terminal 30-cm ends of four branches per tree using a rating scale as follows: 0 = healthy; 1 = <5% spotted leaves; 2 = 5 to 50% leaves with noticeable spotting; 3 = >50% spotted leaves; and 4 = severe spotting, tattered appearance, and leaf-drop. Severity values are averaged according to main and sub-plot effects (right-most column and bottom row, respectively).

[d] Dormancy treatments were applied to the main plot. Copper (ChampION++) was applied at a rate of 2.15 g/liter metallic copper equivalent (MCE), alone and mixed with mancozeb (Manzate 45DF) at a rate of 3.2 g/liter.

[e] Values followed by the same letter in the same case and weight (nonbold versus bold) are not statistically different based on analysis of variance and Fisher's LSD mean separation (P >0.05) procedures. Statistical comparisons for nonbold data by column are with lower-case letters, and those by row are with upper-case letters. Dormant treatment and phytotoxicity severity averages over all in-season timings are in the right columns in bold. In-season timing and phytotoxicity severity averages over all dormant treatments are in the bottom rows in bold.

Table 3.6. Effect of dormancy and in-season bactericide treatment timings on the incidence of bacterial spot on 'Fritz' almond fruit in San Joaquin Co. 2016-2017

	Disease incidence (%)[a]									
	Timing 1		Timing 2		Timing 3		Timing 4		**Dormancy timing mean**	
	IS[b]: none		IS: 2/22 (FB)		IS: 3/15 (PF)		IS: 2/22, 3/15 (FB, PF)			
Dormancy treatment timing[c]	Mean	LSD[d]	Mean	LSD	Mean	LSD	Mean	LSD	**Incid.**	**LSD**
Control	41.3	A a	16.3	B a	0.8	C a	3.0	C a	**13.6**	**a**
Dormant (12/14/16)	10	A b	4.3	A a	1.3	A a	3.3	A a	**4.7**	**b**
Delayed dormant (1/25/17)	8.8	A b	9.0	A a	2.5	A a	1.8	A a	**5.5**	**b**
IS treatment mean disease	**18.1**	**A**	**10.4**	**A**	**1.5**	**B**	**2.7**	**B**		

[a] Fruit were evaluated for the presence of bacterial spot on 6/6/17.

[b] IS = in-season treatments of copper (ChampION++) - mancozeb (Manzate 45DF) mixtures were applied to the sub-plots using a backpack sprayer. Rates were 1.2 + 2.4 g/liter for MCE + mancozeb for single applications at full bloom (FB) and petal fall (PF) (timings 2, 3), whereas for FB+PF (timing 4), the copper rate was reduced to 0.6 g/liter MCE for the PF application.

[c] Dormancy treatments were applied to the main plot. Copper (ChampION++) was applied at a rate of 2.15 g/liter metallic copper equivalent (MCE), alone and mixed with mancozeb (Manzate 45DF) at a rate of 3.2 g/liter.

[d] Values followed by the same letter in the same case and weight (nonbold versus bold) are not statistically different based on analysis of variance and Fisher's LSD mean separation (P >0.05) procedures. Statistical comparisons for nonbold data by column are with lower-case letters, and those by row are with upper-case letters. Dormant treatment averages over all in-season timings are in the right columns in bold. In-season timing averages over all dormant treatments are in the bottom rows in bold.

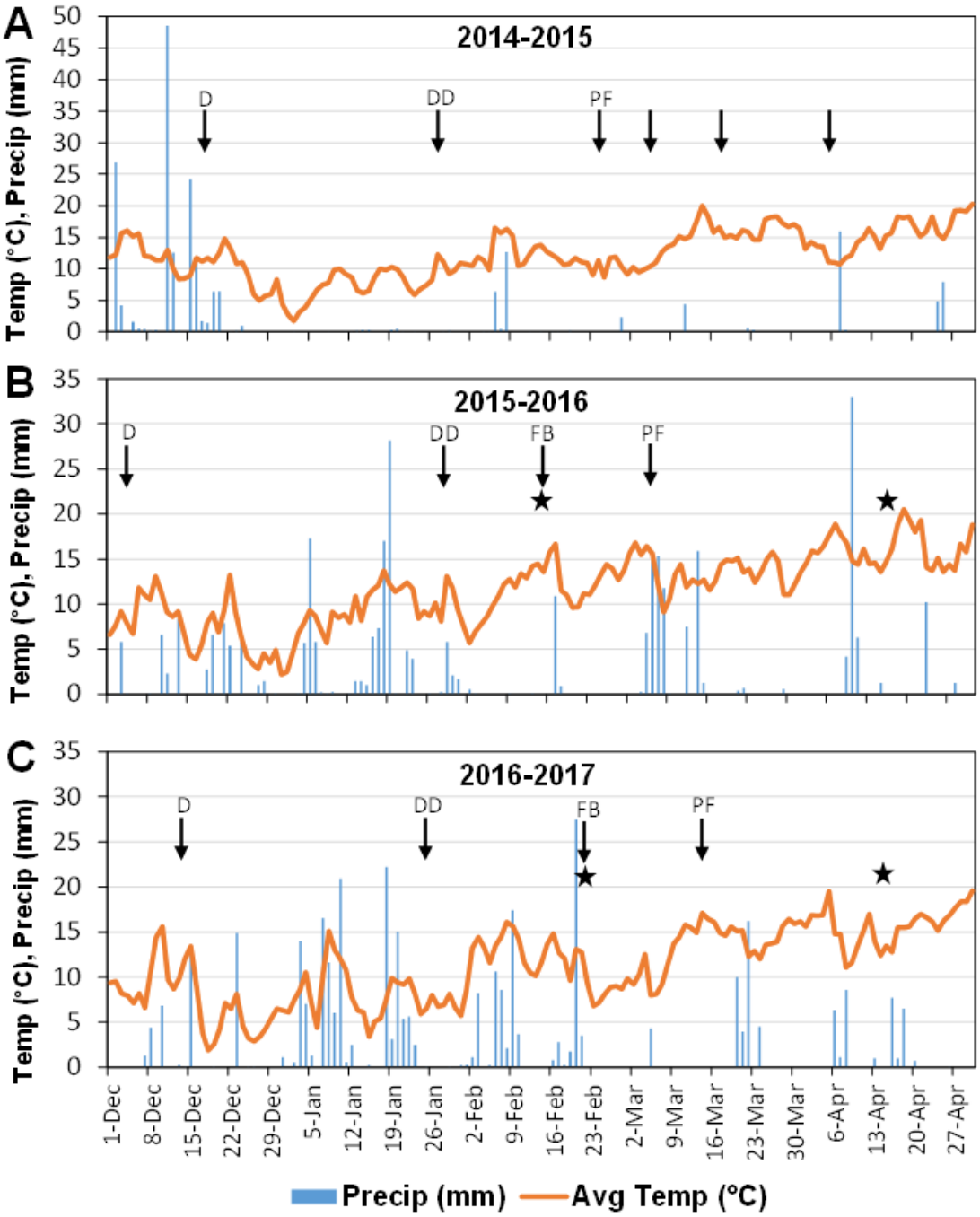

Fig. 3.1. Environmental conditions near field trial location in Ripon, CA during the winter and spring of **(A)** 2014-2015, **(B)** 2015-2016, and **(C)** 2016-2017 seasons. Black arrows indicate bactericide application timings for D=dormant, DD=delayed dormant, FB= full bloom, PF=petal fall, and post-petal fall in-season applications for trials conducted each year. Stars indicate bacterial inoculation dates for flower/emerging leaf and immature fruit/mature leaf inoculation dates in February and April for B and C, respectively.

GENERAL CONCLUSION

Integrated disease management relies on an understanding of the biology of the host and the pathogen, including pathogen survival and dissemination mechanisms, as well as the environmental conditions favorable for disease development. The availability of multiple management tools, including fungicides and bactericides, is essential. These antimicrobials, when used at an appropriate timing, can result in significant reduction of disease incidence and severity. The studies presented in this dissertation identify new disease management tools and techniques in response to evolving challenges in the production of strawberry and almond commodities in California. These new approaches have the potential to reduce the economic impact of the pathogens in current and future crop years, reduce spread of the pathogens to new geographical areas, and/or support the prevention of pathogen resistance development to antimicrobials.

For anthracnose crown rot of strawberry caused by *C. acutatum*, QoI-resistance, associated with the G143A mutation in *cytb,* was confirmed in California populations of the pathogen and there were no detected fitness penalties in pathogenicity or virulence. A bimodal distribution of sensitive (0.027-0.050 mg/liter) and resistant (>40 mg/liter) populations was identified. The detection of QoI-resistance highlights the need for rotational materials with different modes of action. As a result of screening new fungicides, the biofungicide natamycin was identified as a new pre-plant dip treatment for strawberry transplants. Mean EC_{50} values for the baseline sensitivity of mycelial growth of 45 QoI-sensitive and 29 -resistant *C. acutatum* isolates to natamycin were 0.996 and 0.902 mg/liter, respectively, and were unimodal. Although this toxicity was

considerably lower than that of azoxystrobin (using sensitive isolates), fludioxonil, or cyprodinil, dip treatments of transplants with natamycin (at 500 or 1000 mg/liter) were highly effective and similar to industry standards. Dipping transplants inoculated with QoI-sensitive or –resistant *C. acutatum* for 5 minutes in 500 or 1000 mg/liter natamycin reduced plant mortality in the field by 58 to 95% as compared to the control. Fludioxonil/cyprodinil reduced mortality by 86 to 100%. Azoxystrobin was only effective against QoI-sensitive isolates, reducing mortality by 80 to 92%. Fruit yield was also significantly increased by natamycin as compared with the inoculated control. Differences in disease susceptibility were observed among cultivars evaluated, with 'Monterey' and 'Portola' more susceptible as compared with 'Fronteras'. Natamycin has a unique mode of action that is different from other fungicides registered on strawberry and, based on this research, was registered in the United States as a pre-plant dip treatment of strawberry transplants for management of anthracnose crown rot. Additionally, despite decades of use as an antifungal food additive, resistance to natamycin has never been reported in filamentous fungi.

Xanthomonas fragariae, the causal agent of angular leaf spot (ALS) of strawberry, is a quarantine pathogen in some export markets, causing trade restrictions and economic loss to the California fresh-market strawberry industry. Pre-harvest management is difficult and limited to the use of disease-free planting material, applications of copper bactericides that can be phytotoxic, and proper irrigation practices. Here, we report high pre-harvest efficacy for the experimental bactericide amino thiadiazole, alone and in mixtures with low rates of copper or the antibiotic kasugamycin,

with average disease incidence reduction up to 92.8% compared with the control. Currently, there are no post-harvest treatments available that reduce populations of the pathogen if ALS is detected at an export destination. Although effective against quarantine insect pests of strawberry, methyl bromide fumigation was ineffective against *X. fragariae* in diseased plant tissue at a standard commercial rate. In this study, propylene oxide, used for decades by the California nut industries for insect and microbial disinfestation/pasteurization, was identified as a potential commercial fumigant of strawberries. In fumigation chamber trials, 2 h exposure of diseased strawberry leaves to propylene oxide at a dose of ≥142 mg/liter consistently reduced bacterial populations by 2.5 to >5 log units in infected tissues compared with controls. Fumigated leaflets showed little to no phytotoxicity at effective rates, and fumigated fruit were not significantly affected in appearance or susceptibility to postharvest gray mold or Rhizopus rot following storage at 2°C for 3 d and at 15°C for an additional 5 d. Together, these new treatments offer potential strategies for establishing a systems approach with preharvest treatments significantly reducing the risk of ALS on plants and, in response to quarantine detections, a postharvest fumigation treatment that reduces viable pathogen populations in existing lesions. Additionally, this represents the first report of a postharvest, post-infection fumigation-based management treatment against a bacterial phytopathogen.

Bacterial spot caused by *Xanthomonas arboricola* pv. *pruni* was first detected on almond in the San Joaquin Valley in California in 2013 and is reported herein as a new disease in California based on fulfilling Koch's postulates and identification of the

pathogen using species-specific PCR primers. Because fruit symptoms appear similar to leaf-footed bug (*Leptoglossus* spp.) damage or anthracnose (*Colletotrichum acutatum* species complex), it is possible that bacterial spot was present for some time in California but was misdiagnosed. Viable *X. arboricola* pv. *pruni* was consistently recovered from infected mummified fruit from the previous growing season (mean 40%) and their associated peduncles/spurs (mean 51%). These host tissues were therefore identified as primary overwintering sites of the bacterium on the tree. Twig cankers were not observed, and the pathogen was not recovered from dormant buds, both of which are reported as important overwintering mechanisms in other *Prunus* hosts of this pathogen. Isolation from flowers and emerging leaves was only successful when they were collected within 20 cm of an infected, mummified fruit on the tree. Inoculation of flowers, immature fruit, as well as immature and mature leaves resulted in disease development, indicating a long period of host susceptibility in the spring. Disease incidence was highest in fruit inoculations. In split-plot trials over three years, dormancy applications in December or January with copper or copper-mancozeb significantly reduced the disease incidence compared to untreated controls in seasons with high rainfall but had no effect in a single season with low rainfall. In-season applications of copper-mancozeb at petal fall or at full bloom and petal fall were also effective in reducing the disease. Copper phytotoxicity was observed after repeated applications of copper bactericides in some trials. Dormancy and/or in-season treatments of copper-mancozeb mixtures integrated with removal of mummified fruit are currently the best management strategies for bacterial spot of almond in California. Specific numbers of

applications required, then, depends on environmental conditions, especially rainfall. This research represents the first studies conducted to better understand the epidemiology and management of bacterial spot in California almond orchards.