Title
Management of Canker and Dieback Diseases of Citrus and Sycamore in California

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Publication Date
2017

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA
RIVERSIDE

Management of Canker and Dieback Diseases of Citrus and Sycamore in California

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

Doctor of Philosophy

in

Plant Pathology

by

Joey Sal Mayorquin

December 2017

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ACKNOWLEDGEMENTS

Chapter I in its entirety is reprinted with permission as it appears in Mayorquin, J.S., Wang, D.H., Twizeyimana, M. and Eskalen, A. 2016. Identification, Distribution, and Pathogenicity of Diatrypaceae and Botryosphaeriaceae Associated with Citrus Branch Canker in the Southern California Desert. Plant Disease. 100(12), 2402-2413. Financial support for Chapters I and II was provided by the California Citrus Research Board (CRB), support for Chapter III was provided by the California Citrus Research Board (CRB) and Wonderful Citrus, and support for Chapter IV was provided by Orange County (OC) Parks and USDA National Institute of Food and Agriculture Hatch project (CA-R-PPA-5061-H). Citrus plants used in Chapter III were kindly donated by Wonderful Citrus.

I would like to first thank my advisor, Dr. Akif Eskalen, for all of his mentorship and support throughout my PhD studies. I would also like to acknowledge my committee members, Drs. M. Caroline Roper and Michael Stanghellini, for their support and guidance during these research projects and the crafting of this dissertation. I would also like to thank the various scientists who have given suggestions and helpful tips over the course of these research projects including Drs. Deborah Mathews, Philippe Rolshausen, and Greg W. Douhan.

To my lab family, both past and present, this dissertation would not be possible without your support and encouragement throughout the years. To Dr. Mathias “Twizey” Twizeyimana, I am forever grateful for your guidance and support throughout the citrus projects. You have truly had the greatest impact on my life, both professionally and

personally, and I hope to be as successful a scientist as you are, although I may need a little more of your "African magic". To Drs. Virginia McDonald and Tony Adesemoye, thank you for your patience and support in the beginning of my career as I was learning how to be a plant pathologist. To "my boys" Danny, Francis, and Kameron, I could not have made it through all these years without your support and friendship. To my lab sister Shannon "Mother Earth" Lynch, thank you for always being a source of encouragement and positivity no matter how tough things got. To Beth, thank you for all the silly moments like our random discussions and Daiso shopping trips, but most of all thank you for sharing your words of wisdom and the good book with me. To Luis and Cedric for their friendship in my final year. To my undergraduates Lisa Yeung, Jei-Jai Lee, and Ryan Yuan, thank you for your assistance in the lab with various tasks. To our visiting scientists throughout the years, Drs. D. Soner Akgül and Mukaddes Kayim, thank you for your friendship and for teaching me some Turkish which I plan to use when I make my way out there to see you guys! And last, but certainly not least, to Joe my friend and colleague, thank you for always being there when I needed you, whether it was assistance in the field, help with statistics, or just grabbing a beer and shooting the moon.

I would like to thank all of the students in the PLPA graduate program, especially the "Fawcett Crew", Stacey, Kevin, and Daniel for their friendship and support throughout the years. To Jeannette my forever friend, colleague, and "grad student wife", know that I could not have made it this far without your support and encouragement. You are my <3. To my "citrus sister" Morgan, although we did not get close until the "final

hours" of my PhD studies, know that our friendship and lunch room shenanigans have helped me to keep my sanity in these final days.

Finally, I would like to thank my family and friends for being supportive of me throughout this journey. I love you all.

DEDICATION

This dissertation is dedicated to my mother Laurie, for giving me every opportunity she never had and for always being there when I needed her most.

ABSTRACT OF THE DISSERTATION

Management of Canker and Dieback Diseases of Citrus and Sycamore in California

by

Joey Sal Mayorquin

Doctor of Philosophy, Graduate Program in Plant Pathology
University of California, Riverside, December 2017
Dr. Akif Eskalen, Co-Chairperson
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Canker and dieback diseases are commonly caused by fungal pathogens and affect hardwoods which include ornamentals, native tree species, and agricultural commodities. A resurgence of interest and research in these diseases in the past two decades has determined that many of these canker and dieback diseases for a particular host involve multiple species of fungal pathogens both within and between fungal families, suggesting that canker and dieback pathosystems are more complicated than previously thought and thus the management of these diseases may be more complicated given the diversity of fungal pathogens involved. Therefore, we sought to determine the fungal pathogens involved in canker and dieback diseases of citrus and ultimately to develop chemical management strategies for these diseases in citrus in addition to a new, invasive pest-disease complex, Shot hole borer-Fusarium Dieback, affecting California sycamore in California.

The results presented in this dissertation identify a new canker and dieback disease of citrus, Eutypella canker of citrus, in the Desert Region of California caused by three species of *Eutypella*: *E. citricola*, *E. microtheca*, and a *Eutypella* sp. Fungicide field trials determined strobilurin fungicides to be most effective in reducing vascular necrosis caused by *Eutypella* species in addition to vascular necrosis caused by *N. dimidiatum*, the causal agent of Hendersonula, an established canker and dieback disease in the Desert Region. A new twig dieback and gumming disease of citrus, Colletotrichum Dieback, was identified in the San Joaquin Valley Region of California and is caused by *Colletotrichum gloeosporioides* and *C. karstii*. Fungicide field trials determined pyraclostrobin fungicides to be the most effective in reducing the prevalence of Colletotrichum Dieback symptoms. For the management of Shot hole borer-Fusarium Dieback, pesticide field trials determined the insecticide emamectin benzoate and the fungicides metconazole, tebuconazole, and carbendazim plus debacarb to significantly reduce shot hole borer attacks in infested California sycamore.

The results of these works establish the presence of two new canker and dieback disease of citrus in California and provide chemical management strategies for the control of these diseases in addition to the management of Shot hole borer-Fusarium Dieback.

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GENERAL INTRODUCTION

Canker and dieback diseases of hardwoods are a general term used to refer to a multitude of diseases primarily caused by fungal pathogens, and in some cases bacterial pathogens, which share a common set of symptoms, i.e., vascular necrosis (canker) of phloem and xylem tissues along with terminal branch and scaffold limb dieback. Additional symptoms can be associated with these diseases; however, this is dependent upon the biology of individual canker, dieback pathosystems and the response of the host to these diseases (Blanchette and Biggs, 2013). Canker and dieback diseases can be found to affect tree hosts in agriculture, urban forests, and native wildlands. The concept of overlapping symptomatology, despite individual or unique canker and dieback pathosystems, presents challenges to the accurate identification of canker and dieback diseases in hardwood hosts and ultimately to the implementation of successful management strategies for these individual pathosystems as no specific strategies exist for emerging diseases. This is particularly true when the presence of more than one pathosystem for an individual host is unknown or suspected. Evidence from current canker and dieback research shows the complexity of many of these pathosystems, often involving a suite of fungal pathogens from a single fungal family (Adesemoye et al. 2014) to more complex pathosystems involving several fungal pathogen suites from more than one fungal family (Mayorquin et al. 2016b; Freeman et al. 2016). This complicates management strategies for these diseases as several pathogens must be taken into account when developing management strategies focused on controlling the suite of fungal pathogens associated with a particular canker and dieback disease. The scope of work

presented in this dissertation is focused on aspects of chemical management strategies for canker and dieback diseases present in both citrus and California sycamore in the urban environment in California. Specifically, these studies are focused on chemical management strategies for: (i) two emerging diseases of citrus, (ii) one established disease in citrus, and (iii) an emerging pest-disease complex threatening the urban forests of Southern California.

The California citrus industry is currently valued at over $3 billion with California accounting for 47% of total U.S. citrus production, with the majority of citrus fruit destined for fresh market (USDA-NASS 2016). The economic impact that canker and dieback diseases have on citrus production has not been determined, however the complexity of these canker and dieback diseases in terms of their fungal pathogen suites have suggested that the overall economic impact is greatly underappreciated in light of this new evidence (Adesemoye et al. 2014). Canker and dieback disease of citrus has been documented for well over 100 years throughout several countries, including the United States and overviews of these diseases in citrus, particularly as they pertain to California citrus production, have been reviewed by Adesemoye et al. (2014) and Mayorquin et al. (2016b). To summarize, it is now known that several canker and dieback diseases of citrus exist in the state of California; Adesemoye et al. (2014) determined the established disease "Dothiorella gummosis" is caused by 10 species of botryosphaeriaceous fungi. Adesemoye and Eskalen (2011) and Mayorquin et al. (2016b) found that three species of *Eutypella* are associated with an emerging disease of citrus known as Eutypella canker of citrus, which occurs alongside an established canker and

dieback disease of citrus known as Hendersonula (Calavan and Wallace 1954). In addition to these mentioned canker and dieback diseases, Mayorquin et al. (2016a) detected an emerging twig and shoot dieback disease of citrus involving two species of *Colletotrichum*, one of which had not been previously recorded from citrus in California or other citrus growing regions of the United States. These findings of unrecorded canker and dieback diseases of citrus in California and the revision of an established disease to include more fungal pathogens highlight the need for appropriate management strategies not only for established diseases, but for emerging diseases for which no strategies are in place.

One of the main concerns for the California citrus industry is the limited number of pesticides currently registered for use on citrus for various pests and diseases (Anonymous 2003). This is particularly true for the management of canker and dieback diseases of citrus as no chemical management strategies exist for these diseases as they are often considered minor diseases (Whiteside 1980). The limited number of pesticides available to the citrus industry and the lack of chemical management strategies for citrus canker and dieback diseases places the California citrus industry in a precarious situation as not only are chemical strategies not available for documented canker and dieback diseases, but establishment of management strategies for emerging canker and dieback diseases is hindered by the lack of chemical availability and management procedures.

The California urban forest industry is estimated to be worth $3.6 billion and provides a number of benefits to the community including protection from unfavorable environmental conditions such as heat and air pollution, watershed renewal, and

increased property values which in turn drive consumer spending habits. Despite the benefits provided by urban forests, this industry is facing a number of challenges including limited funding at city and county levels, environmental stresses like global warming, and increased care and maintenance required to maintain these plantings in unnatural habitats. Additionally, urban forests are more susceptible to pests and diseases due to the lack of tree species diversity in the urban landscape; these pests and diseases can have significant impacts on urban forests which can reduce tree growth and longevity which in turn effects the benefits provided by the urban forest to the community (Hurt 2012). Invasive Shot hole borer-Fusarium Dieback (ISHB-FD) is an emerging, invasive pest-disease complex of over 300 hardwood tree species in California (Eskalen et al. 2013); this pathosystem is unique as its fungal pathogens are vectored by two related, but distinct ambrosia beetles (Stouthamer et al. 2017) and these fungal pathogens are used as a food source to support the life cycle of the beetle (Freeman et al. 2013; 2016). The canker and dieback caused by the fungal pathogens of SHB is like that of the fungal pathogens causing canker and dieback in citrus. The most significant threat this pest-disease complex poses to California is due to the wide host range of the vector and includes landscape trees, native tree species, and agricultural commodities. Of particular concern is the aggressiveness of SHB-FD on native California sycamore trees which are commonly found in the urban forests of southern California. Management of this complex is difficult as strategies must be tailored to suit individual hosts from a wide range of plant species and locations. Current practices are limited to the removal of heavily-infested trees which is not practical for large-scale land management in urban

forests nor is it ideal for native tree species. Therefore, chemical management practices are needed which aim to control both the vector and fungal pathogens through the use of insecticides and/or fungicides to suppress vector populations and reduce the fungal colonization of the pathogens within their hosts.

The objectives of these studies were to: (i) identify the fungi associated with citrus branch canker and dieback symptoms in the Desert Regions and determine their pathogenicity; (ii) determine the efficacy of commercially-available fungicides for use as pruning protectants against fungal canker pathogens in the Desert Regions; (iii) identify the fungi associated with twig dieback and gumming of citrus in the San Joaquin Valley Region, determine their pathogenicity, and evaluate fungicide efficacy in reducing the prevalence of this disease; and (iv) determine the efficacy of insecticides, fungicides, and combinations thereof in reducing attacks of Shot hole borer on California sycamore in landscape areas.

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CHAPTER I. Identification, Distribution, and Pathogenicity of Diatrypaceae and Botryosphaeriaceae Associated with Citrus Branch Canker in the Southern California Desert

Reprinted with permission from: Mayorquin, J.S., Wang, D.H., Twizeyimana, M. and Eskalen, A. 2016. Identification, Distribution, and Pathogenicity of Diatrypaceae and Botryosphaeriaceae Associated with Citrus Branch Canker in the Southern California Desert. Plant Disease. 100(12), 2402-2413.

ABSTRACT

Several members of the Botryosphaeriaceae and Diatrypaceae are known as canker and dieback pathogens of a number of woody hosts. As desert citrus production in California can occur in proximity to table grape production, it was suspected that fungi associated with grapevine cankers might also be associated with citrus branch canker and dieback decline. To determine the fungi associated with branch canker and dieback disease of citrus in the southern California desert regions, surveys were conducted from 2011 to 2013 in the major citrus growing regions of Riverside, Imperial and San Diego counties. Cankered tissues were collected from branches showing symptoms typical of branch canker and dieback. Various fungal species were recovered from necrotic tissues and species were identified morphologically and by phylogenetic comparison of partial sequences of the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2), β-tubulin gene, and elongation factor 1-α genes with those of other species in GenBank. Four fungi, including *Neoscytalidium dimidiatum*, *Eutypella citricola*, *E. microtheca* and an un-named *Eutypella* sp. were associated with branch canker. *N. dimidiatum* was the most frequently recovered fungus from symptomatic tissues (31%) followed by *E. citricola* (10%), *E. microtheca* (4%) and *Eutypella* sp. (2%). In pathogenicity tests, all fungi caused lesions when inoculated on 'Lisbon' lemon (citrus) branches.

Lesions caused by *Eutypella* sp. were significantly longer than those of the other *Eutypella* spp.; however, they did not differ significantly from those produced by *N. dimidiatum*. The most parsimonious unrooted trees based on the combined data of ITS and partial β-tubulin gene region sequences showed three distinct clades of *Eutypella* spp. (*E. citricola*, *E. microtheca*, and an unidentified *Eutypella* sp.). Similarly, ITS and partial translation elongation factor 1-α gene region sequences differentiated two species of *Neoscytalidium*, *N. dimidiatum* and *N. novaehollandiae*. Identifying the diversity, distribution and occurrence of these fungal pathogens is useful for the management of citrus branch canker and dieback disease in the desert citrus growing regions of California.

INTRODUCTION

Citrus production in California is valued at over $3.4 billion dollars and California is ranked second in overall production value in the United States (Anonymous 2015). Citrus production in California extends from Central to Southern California with orchards located in the desert areas of Coachella Valley (Riverside County), Imperial Valley (Imperial County), and Borrego Springs (San Diego County) primarily focused on lemon and grapefruit varieties. Over half of California's total grapefruit production and nearly one-fifth of the total lemon production occur in the desert regions of California (Anonymous 2009).

Canker and dieback diseases of woody hosts are common and continue to be extensively studied both in agricultural and non-agricultural fields.

Many of these studies have shown the diversity of Botryosphaeriaceae and/or Diatrypaceae occurring on a number of agricultural commodities in California (almond, avocado, cherry, citrus, grapevine) and worldwide (almond, apricot and grapevine); in some cases, host overlap exists between these two families further requiring a better understanding of the overall dieback disease complex throughout the world where this occurs (Adesemoye et al. 2014; Carter 1957; Gramaje et al. 2012; Inderbitzin et al. 2010; McDonald and Eskalen 2011; Munkvold and Marois 1994; Trouillas et al. 2010; Trouillas et al. 2011; Urbez-Torres et al. 2006). The economic impact of these canker diseases on crop productivity has not been enumerated for every crop host, but it is suggested that the effect of these diseases could be financially substantial. In California, cordon and trunk canker diseases of grapevine account for the primary cause of plant mortality (Trouillas et al. 2010) and the economic losses are estimated to be up to 260 million dollars (Siebert 2001) per year. Of these diseases, Eutypa dieback caused by *Eutypa lata* (Diatrypaceae) is considered one of the most important canker diseases of grapevine in California and worldwide (Moller and Kasimatis, 1978) and is also of economic importance in apricot (Carter 1957). Other members of the Diatrypaceae are known to be associated with grapevine trunk diseases (Luque et al. 2012; Trouillas et al. 2010; Trouillas et al. 2011; Urbez-Torres et al. 2009; Urbez-Torres et al. 2012), however their individual impact to the overall economic loss from these diseases is not known. The economic loss resulting from branch canker pathogens of citrus in California has not been determined and it is likely that the significance of these pathogens on citrus both in

California and worldwide is more appreciable than currently understood (Adesemoye et al. 2014).

Branch canker and dieback diseases of citrus have been reported since the early 1900's (Punithalingham 1980). Symptoms of these diseases generally include the presence of dead, sunken bark around dark colored cankers, twig and branch dieback, and gummosis. Susceptibility to these diseases are influenced by wounding and a weakening of trees which can include frost damage, sunburn and other abiotic factors (Calavan and Wallace 1954; Whiteside 1980). Various Botryosphaeriaceae species are known to cause branch canker and dieback in citrus, including *Diplodia natalensis* Pole-Evans, *Dothiorella gregaria* Sacc., and *Hendersonula toruloidea* Nattrass (Calavan and Wallace 1954; Punithalingham 1980; Whiteside 1980). The disease known as Hendersonula branch wilt, or simply Hendersonula, caused by the botryosphaeriaceous fungus *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers (formerly *H. toruloidea* and *N. hyalinum*) has been reported to be affecting desert citrus production in California (Calavan and Wallace 1954) and in Israel (Oren et al. 2001). Symptoms include characteristic black spore masses underneath the bark, branch cankers, wilt and dieback. Recently, ten species in the Botryosphaeriaceae have been identified associated with branch canker and dieback of citrus in California (Adesemoye et al. 2014). In addition, *Eutypella microtheca* and a *Eutypella* sp. were reported for the first time on 'Allen Eureka' lemon and 'Red Blush' grapefruit in Riverside and San Diego counties, respectively in 2011 (Adesemoye and Eskalen, 2011).

A number of *Eutypella* spp. has been previously recorded from citrus species including *Citrus aurantium*, *C. limon*, *C. maxima*, *C. paradisi*, and *Citrus* sp. in Argentina, Brazil, Philippines, Cote d'Ivoire, and Australia (Hanlin 1992; Rappaz 1987; Reinking 1918; Resplandy et al. 1954; Trouillas et al. 2011). *Eutypella citricola* Speg. was reported on dead twigs of a *Citrus* sp. in Florida (Anonymous 1960). However, there is no other record of this genus occurring on citrus in North America other than the study by Adesemoye and Eskalen, 2011. Although it appears that *Eutypella* has been implicated in the dieback of citrus in various regions worldwide, to our knowledge, it has not been formally investigated as a potential pathogen of citrus at least in California (Anonymous 1960; Hanlin 1992; Rappaz 1987; Reinking 1918; Resplandy et al. 1954; Trouillas et al. 2011). Several species of *Eutypella* have been reported from other woody hosts in California including grapevine and (Trouillas et al. 2010) and non-agricultural trees and shrubs like California buckeye, baccharises, white alder and box elder (French 1989, Rappaz 1987; Trouillas et al. 2010) and those associated with grapevine in California were shown to produce lesions when artificially inoculated on grapevine confirming these species as pathogens on grapevine. *Eutypella* comprised over 60% of all fungi isolated from grapevine cankers in the Coachella Valley (Trouillas et al. 2010), a region which is home to table grape and citrus production, both of which can occur in close proximity to each other. Hence, it is likely that citrus in the Coachella Valley are susceptible to infection by *Eutypella* spp.

The objectives of this study were to (i) characterize the symptomatology of citrus branch canker in the southern California desert regions; (ii) identify fungi associated with

citrus branch canker based on morphological traits and molecular phylogenetic analysis; (iii) assess the pathogenicity of recovered fungi; and (iv) determine the distribution of fungi associated with this disease among counties.

MATERIALS AND METHODS

Field survey and isolation. Surveys were conducted in nine commercial citrus orchards within the desert production region of California, including Coachella Valley (4 in Riverside County), Imperial Valley (4 in Imperial County), and Borrego Springs (1 in San Diego County) between summer of 2011 and spring of 2013 and in summer of 2015. Approximately 30 trees were sampled from each orchard and cankered tissues from a single branch were collected from each tree showing symptoms typical of branch canker and dieback including, but not limited to, depressed bark on cankered branches, bark cracking and splitting, gumming and a layer of black sooty spores directly under the bark. When possible, citrus trees growing in close proximity to grapevine were sampled. Surrounding hardwood trees (Tamarix, *Tamarix ramosissima*) in the vicinity of citrus groves were examined for symptoms of branch canker and dieback and samples were taken. In total, 273 samples were collected.

Samples were rinsed with distilled water to remove organic debris, dipped in 95% ethanol, and flamed to burn off excess alcohol. The bark was removed to expose the necrotic and healthy tissues and small pieces from the margin of necrotic lesions were placed onto the surface of potato dextrose agar (Difco Laboratories) plates amended with 0.0% tetracycline hydrochloride (Fisher Scientific) (PDA-tet). Isolation plates were

incubated in the dark at room temperature for 3 to 5 days. Pure cultures from emerging fungi were obtained by transferring hyphal tips from the colony margin to water agar; single hypha were excised with the aid of a stereomicroscope and subsequently transferred to acidified PDA plates. Fungal isolates were stored as mycelial plugs in sterile water (4°C) and 30 % glycerol (-80°C).

Morphological identification. Fungal isolates were identified initially to genus based on gross colony morphology (colony color, colony growth pattern and rate of growth) and those belonging to the Diatrypaceae and Botryosphaeriaceae were further studied using published descriptions (Crous et al. 2006; Phillips et al. 2013; Slippers et al. 2013; Trouillas et al. 2010). To induce conidial production (van Niekerk et al. 2004), isolates were grown on oatmeal agar (OA) (30g oat flakes/liter) and incubated at room temperature under continuous fluorescent light until fruiting structures (pycnidia) developed. In some cases, autoclaved pine needles or citrus wood chips were added to the surface of agar media to enhance fruiting body production. Oozing conidial masses were collected with a sterile inoculating loop and mounted on a microscope slide in a drop of lactoglycerol. The length (arc length for filiform conidia) and width of 30 conidia were measured for each isolate on a compound microscope using SPOT Imaging software (Diagnostic Instruments Inc., MI). The mean, standard deviation, minimum, mode and maximum conidial lengths and widths were calculated for each isolate. To determine the effect of temperature on fungal radial growth, two isolates for each fungal species were grown on PDA and incubated at 8 different temperatures (5, 10, 15, 20, 25, 30, 35 and 40

°C) for 4 days. Colony diameter was measured every day for four days and this experiment was repeated once. Average daily growth was calculated by computing the average growth for fungal isolates at two and three days of growth at the indicated temperatures.

DNA isolation, amplification and phylogenetic analysis. Genomic DNA for each isolate was extracted from pure cultures following the method of Cenis with slight modifications (Cenis 1992). In brief, mycelia were scraped from agar plates and placed into 2.0 ml conical vials containing extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and glass beads. Tubes were bead bashed using a FastPrep-24 machine (MP Biomedicals Inc.) at 4 M/s for 30s. Oligonucleotide primers ITS4 and ITS5 (White et al. 1990) were used to amplify the ITS1-5.8S-ITS2 region of the rDNA, primers Bt2a and Bt2b (White et al. 1990) were used to amplify a partial region of the nuclear beta-tubulin gene, and primers EF1F and EF2R (Jacobs et al. 2004) were used to amplify a portion of the translation elongation factor 1-α gene. Polymerase chain reaction (PCR) was carried out as previously described (Slippers et al. 2004). Reaction mixtures consisted of 1x reaction buffer, 0.2 mM dNTP's, 0.2 µM forward and reverse primers, 2.5 U of standard Taq polymerase (New England Biolabs, MA), and template DNA ranging from 50-100 ng/µl. Cycling conditions were as follows: an initial preheat at 95°C for two minutes, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C (63°C for EF1F/EF2R) for 30 s, and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min. PCR products were verified by gel electrophoresis in

1x TBE buffer and photographed under UV light after staining with SYBR Green (Invitrogen, OR). PCR products were purified using ExoSAP-IT (Affymetrix, CA) and sequenced in both directions using the primers mentioned above at the Institute for Integrative Genome Biology of the University of California, Riverside.

Sequences were edited using Sequencher 4.6 (Gene Codes Corp., MI) and local alignments done using ClustalX (Thompson et al. 1997). Phylogenetic analysis was performed with MEGA6 (Tamura et al. 2013) and sequences in the present study (Table 1.1) were compared to known sequences in GenBank (Table 1.2). Analyses were run separately for ITS, β-tubulin, and EF1-α datasets; and ITS and β-tubulin and ITS and EF1-α datasets were combined and analyzed for the Diatrypaceae and Botryosphaeriaceae, respectively. Maximum parsimony analysis was performed for each dataset using the Subtree-Pruning-Regrafting (SPR) search method with gaps and missing data treated as a complete deletion. Bootstrap values were obtained from 1,000 bootstrap replications and tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasmy index (HI) were recorded.

Pathogenicity tests and effect of temperature on fungal colonization. Pathogenicity tests were conducted on three-year-old 'Lisbon' lemon (*Citrus limon*) grafted on C-35 citrange rootstock maintained in a greenhouse (average temperature range of 21-32°C) from December 2013 to August 2014. To assess pathogenicity, plants were stem wound-inoculated on the scion (approximately 1cm diameter) with a 4 mm diameter cork borer to remove bark; there was one inoculation site per scion shoot inoculated. Then, an agar

plug was taken from the colony margin of a one-week old fungal culture for each isolate (Table 1.1) and placed mycelia side down on the wound. Wounds were covered with petroleum jelly and wrapped with Parafilm. Control shoots were inoculated with sterile agar plugs. Inoculated plants and controls were arranged in a randomized complete block design with four replications and the experiment was repeated once. Shoots were destructively sampled after 8 months to measure internal vascular necrosis (lesions). Necrotic tissue developing outside the area of inoculation were cultured on PDA-tet and fungal isolates obtained from re-isolation were identified based on their morphology (colony shape, color, growth rate) and by sequencing the ITS region as described earlier to fulfill Koch's postulates.

To assess the effect of temperature on fungal colonization, healthy twigs from Lisbon lemon trees were collected and transported back to the laboratory, University of California, Riverside. Twigs were rinsed in distilled water to remove surface debris and then soaked in a solution of 10% household bleach for 10 mins to surface disinfect. Leaves were removed and twigs were cut into approximately 12cm long segments. The ends of these segments were dipped into paraffin wax to prevent desiccation. A 4 mm cork borer was used to make a wound by removing bark and then a fungal plug from a one-week old culture (Table 1.1) was placed in the wound mycelial side down. Control twigs were inoculated with sterile agar plugs. Wounds were covered with petroleum jelly and wrapped with Parafilm. The experiment design was a 2-level factorial design with temperature and isolate as factors. Inoculated twigs and controls were placed in containers containing a moistened paper towel to provide humidity and incubated at 25°C

or 32°C in microbiological incubators for 10 days. Each temperature and isolate combination had 5 replications and the experiment was repeated once. After incubation, segments were destructively sampled by removing the bark and measuring internal vascular necrosis. Necrotic tissue from the margin of lesions was taken and placed onto PDA-tet to confirm fungal presence. Fungal colonies were identified based on gross colony morphology.

Statistical analysis. Internal lesion lengths from the pathogenicity test were analyzed using JMP Pro 11 (SAS Institute, NC). One-way Analysis of Variance (ANOVA) was used to compare lesion length means. All pairs of means were compared using Tukey's honest significant difference (HSD) where α=0.05. Data from the independent trials was checked for homogeneity error of variance; no heterogeneity was detected, thus data from each independent trial were combined. To assess the effect of temperature on fungal colonization, lesions lengths were analyzed by an independent-samples t-test with confidence level α=0.05 using JMP Pro 11. To construct fungal radial growth curves, daily growth was plotted against temperature and fit to a Weibull five-parameter regression curve using the probability density function (Miller et al. 2003) in SigmaPlot (version 11, Systat Software Inc., CA).

RESULTS

Field survey, fungal recovery and distribution. Disease was observed on lemon and grapefruit trees ranging in age from 20- to 40- year old on the following varieties:

'Lisbon' and 'Allen Eureka' lemon (*C. limon*), 'Rio Red', 'Ruby Red' and 'Pink' grapefruit (*C. paradisi*). Disease expression appeared to be more severe in *C. paradisi* cultivars than on *C. limon* cultivars. Tamarisk (*Tamarix* sp.) trees that were commonly observed lining citrus groves in San Diego County also displayed symptoms of branch canker and dieback.

Symptoms of branch canker on citrus in the desert regions were observed primarily on twigs and scaffold branches. On severely declining trees, lesions could be seen extending into the trunk. Diseased branches showed typical symptoms of depressed bark covering cankered areas; necrotic tissues ranging from dark, chocolate brown to nearly black and in some cases a salmon-colored zone at the margin of necrotic tissue were observed. Symptoms also included cracking or peeling of the bark, often associated with gumming. A layer of black fungal spores was observed directly underneath the bark, indicative of infection by *Neoscytalidium dimidiatum*. In most cases there was a dieback of the affected limb. Examination of fallen, decaying branches on orchard floors revealed numerous fruiting bodies resembling those of Diatrypaceous fungi.

Both *Neoscytalidium* and *Eutypella* were detected in all three counties sampled (Fig. 1.1). At least two species of *Eutypella* were detected in each county. The frequency of *Eutypella* species recovered between counties varied, but *E. citricola* was the most frequently recovered (Fig. 1.1).

N. dimidiatum was the most frequently isolated in Riverside and Imperial Counties (Table 1.3). In Riverside County, *N. dimidiatum* and *Eutypella* spp. were recovered from 38 % and 14 % of necrotic tissues collected from branches. In Imperial

County, *N. dimidiatum* and *Eutypella* spp. were recovered from 27 % and 16 % of necrotic tissues collected from branches. *N. dimidiatum* and *Eutypella* spp. were recovered from 10 % and 27 % of necrotic tissues collected from branches in San Diego, respectively. Both *N. dimidiatum* and *Eutypella* spp. were isolated from necrotic tissues on the same branch at almost 3 % of the total branches collected.

In Riverside County, 55 % of *Eutypella* isolates were identified as *E. citricola*, 30% as *E. microtheca* Trouillas, W.M. Pitt & Gubler, and 15 % as a *Eutypella* sp. (Table 1.3). In Imperial County, 56 % of isolates were identified as *E. citricola*, 31 % as *E. microtheca*, and 13 % as a *Eutypella* sp. In San Diego County, 75 % of isolates were identified as *E. citricola* and 25 % as a *Eutypella* sp. *E. microtheca* was never recovered from San Diego County. *Eutypella* spp. were recovered from necrotic tissues collected from branches of tamarisk (Table 1.1).

Morphological identification. Three groups of *Eutypella* were distinguished based on colony and conidial morphology (Table 1.4) and classified according to the system used by Trouillas et al. (2010). The most frequent group found associated with branch cankers in this area consisted of white colonies with moderate aerial mycelia later developing spots of melanized hyphae. Pycnidia of these isolates produced orange colored chirri of filiform conidia with a mean length and width of 14.8±1.6 x 1.1±0.1 μm. Based on descriptions from isolates of grapevine, these isolates were morphologically similar to *Eutypella* sp. group 2 (Trouillas et al. 2010). The second group of *Eutypella* was characterized by colonies with abundant, white aerial mycelia that develop tufts of

mycelium with age. These cultures were unique in the production of a pink pigment in culture on both PDA and OA. Pycnidia of these isolates exuded orange chirri of filiform conidia, which were strongly curved with a mean length and width of 14.1±2.4 x 1.2±0.2 μm. These isolates were morphologically similar to the description of *Eutypella* sp. group 3 (Trouillas et al. 2010). Colonies from the third group of *Eutypella* were white producing moderate aerial mycelia over time that developed a greenish, black center. Pycnidia exuded white conidial masses with filiform conidia with a mean length and width of 20.2±2.0 x 1.3±0.3 μm. These isolates were morphologically similar to *Eutypella* sp. group 1 (Trouillas et al. 2010).

Only one group of *Neoscytalidium* sp. was identified based on colony and conidial morphology. This group consisted of colonies with mycelia initially light in color, developing dark olivaceous to black with age. Disarticulating hyphae were observed, forming 0 to 2 septate spores. Conidia from these isolates were initially hyaline and aseptate, later forming two septate brown spores, with a characteristic darker central cell. These isolates were morphologically similar to *N. dimidiatum* (Phillips et al. 2013). Other fungal genera isolated at low frequencies from branch cankers included *Aspergillus*, *Alternaria, Coniophora, Fusarium, Hypoxylon* and *Spencermartinsia*.

Results from the radial growth experiment revealed that all fungi could grow between 15-35°C (Fig. 1.2). *N. dimidiatum* and *E. microtheca* were capable of growth at 40°C, however *E. microtheca* isolates grew very little (< 3mm) by the end of three days. Optimal temperatures for *N. dimidiatum*, *E. microtheca* and *E. citricola* occur between 30-35°C whereas the optimum temperature range for *Eutypella* sp. is close to 30°C.

Phylogenetic analyses. Phylogenetic analysis of individual loci produced similar tree topology and thus data sets for the ITS and β-tubulin (for the Diatrypaceae) and ITS and EF1-α (for the Botryosphaeriaceae) genes were combined and analyzed. The ITS tree topology for the Diatrypaceae indicated that the *Eutypella* sp. was indistinguishable from *Peroneutypa scoparia* (Schwein.) Carmarán & A.I. Romero, however individual analysis of the β-tubulin dataset revealed the *Eutypella* sp. to be distinct from *P. scoparia* (not shown). The combined dataset for Diatrypaceae consisted of 975 nucleotides with 328 nucleotides being parsimony informative and maximum parsimony analysis returned 10 most parsimonious trees with the following scores: CI=0.73, RI=0.95 and RCI=0.70. The combined data set for Botryosphaeriaceae consisted of 796 nucleotides of which 185 nucleotides were parsimony informative. Analysis of the combined dataset returned 6 most parsimonious trees with the following scores: CI=0.85, RI=0.93 and RCI=0.80.

The *Eutypella* spp. from this study belonged to three distinct clades based on the combined data set (Fig. 1.3). The first clade (96 % bootstrap support) corresponds to *E. citricola* (*Eutypella* species group 1, (Trouillas et al. 2010)). The second *Eutypella* clade (99 % bootstrap support) corresponds to *E. microtheca* Trouillas, W.M. Pitt & Gubler (*Eutypella* species group 2, (Trouillas et al. 2010)). The third clade (99 % bootstrap support) corresponds to an unidentified *Eutypella* sp. (*Eutypella* species group 3, (Trouillas et al. 2010)). This species is most closely related to *P. scoparia,* however isolates from this study form a subclade (69 % bootstrap support) that is distinct from *P. scoparia* (93 % bootstrap support).

For *Neoscytalidium*, one major clade (99 % bootstrap support) was formed from the combined dataset (Fig. 1.4). The first subclade (83 % bootstrap support) corresponds to *N. dimidiatum* which includes isolates obtained from citrus in this study and other vouchered specimens from walnut, mango and human and the second subclade (95 % bootstrap support) corresponds to *N. novaehollandiae* which includes vouchered specimens from acacia, mango, blue grevillea, boab and rattlepod in Australia.

Pathogenicity tests and effect of temperature on fungal colonization. Two to four weeks after fungal inoculation, gum production originating from the point of inoculation was present in shoots inoculated with fungi, but was not present in control shoots. Bark splitting at the site of inoculation was frequently observed on shoots inoculated with *N. dimidiatum*, but less common on shoots inoculated with species of *Eutypella*; No bark splitting was observed at the point of inoculation on control shoots. All fungi tested were capable of causing lesions when inoculated on citrus branches (Fig. 1.5). Lesions from pathogen inoculated shoots were light to dark brown in color and extended acro- and basipetally from the point of inoculation. Lesions were not observed on control branches. No significant differences in lesion lengths were observed between isolates of the same species; however, significant differences in lesion lengths were observed between different fungal species ($P<0.05$). Lesions produced by the *Eutypella* sp. were significantly larger ($P<0.05$) than those of *E. citricola* and *E. microtheca*.; however, they did not differ significantly from those produced by *N. dimidiatum*. Lesions produced by *N. dimidiatum* were significantly larger ($P<0.05$) than those produced by *E. microtheca*

and *E. citricola*. Lesions produced by *E. microtheca* and *E. citricola* did not differ significantly from each other. All isolates were re-isolated from inoculated branches and confirmed morphologically as identical to the isolates used in inoculations using the colony characteristics described earlier. Representative isolates were chosen for further characterization by sequencing of the ITS region as described earlier. Fungal recovery ranged from 75 to 100 % for all isolates.

Significant differences ($P<0.05$) in lesion length were observed among fungal species and between temperatures (Fig. 1.6). For *N. dimidiatum*, a significant difference ($P<0.001$) was observed in lesion lengths between shoots incubated at 25 and 32°C. Average lesion length at 25°C was 23.3mm whereas the average lesion length at 32°C was 94.4mm. For isolates of *E. microtheca*, a significant difference ($P<0.01$ for isolate DC09 and $P<0.05$ for isolate DC37) was observed in lesion lengths when shoots incubated at 32°C were compared to those incubated at 25°C. Larger lesions were produced on segments incubated at 32°C then when incubated at 25°C. For *E. citricola* a significant difference in lesion length ($P<0.05$) was observed in only one isolate (DC91). This isolate showed a significant decrease in lesion length from segments incubated at 32°C in comparison to those segments incubated at 25°C. The other isolate of *E. citricola* showed no significant differences in lesion length at either temperature. For isolates of *Eutypella* sp., no significant difference was observed in lesion lengths at either temperature. Fungal recovery from these segments ranged from 95-100 %.

DISCUSSION

This study is the first comprehensive assessment on the distribution, identification and pathogenicity of fungal species, associated with citrus branch canker in the Southern California desert regions. Using morphological and molecular sequence data (ITS and β-tubulin), three distinct *Eutypella* species, including *E. citricola, E. microtheca* and an un-identified *Eutypella* sp. were identified from citrus branch samples showing cankers and dieback. These species have also been confirmed as pathogens of citrus as they were capable of producing lesions on citrus shoots. The unidentified *Eutypella* sp. is reported for the first time in citrus in California. *Neoscytalidium dimidiatum* has been shown to be the predominate fungus found associated with citrus branch cankers in the Southern California desert regions followed by *E. citricola, E. microtheca*, and *Eutypella* sp.

The distribution of *Eutypella* spp. occurring on citrus has been reported from a number of regions including Argentina, Australia, Brazil, China, Cote d'Ivore, Philippines and the United States (California and Florida) (Adesemoye and Eskalen 2011; Anonymous 1960; Hanlin 1992; Rappaz 1987; Reinking 1918; Reinking 1921; Resplandy et al. 1954; Trouillas et al. 2011). In California, two *Eutypella* spp. were previously reported as associated with branch cankers of citrus in Riverside and San Diego Counties; however, these *Eutypella* spp. were not identified to species level (Adesemoye and Eskalen 2011). Three species of *Eutypella* in three citrus growing counties in California were detected in the current study, including *E. citricola*, *E. microtheca*, and an un-identified *Eutypella* sp. These same *Eutypella* spp. are also associated with grapevine in the Coachella Valley (Trouillas et al. 2010) and *E. citricola*

and *E. microtheca* have also been reported from citrus and grapevine in Australia (Trouillas et al. 2011). *Eutypella* spp. were the most frequently recovered fungi from grapevine cankers in the Coachella Valley (Trouillas et al. 2010), whereas *N. dimidiatum* was the most frequently recovered canker pathogen of citrus in the current study. It is unknown how the occurrence of multiple susceptible hosts in a single area contributes to fungal distribution and prevalence in a particular host, but it is clear that more work is needed to further evaluate these pathosystems to assess the potential risk imposed by plurivorous pathogens in areas with multiple hosts.

Identification of diatrypaceous fungi has largely been based on teleomorph morphology; however, in the absence of a sexual state, anamorph morphology provides very little in species delimitation as these asexual states often exhibit considerable morphological overlap (Glawe and Rogers 1984, Rappaz 1987). Studies using a multi-gene phylogenetic approach have successfully distinguished members of the Diatrypaceae associated with various hosts, particularly in grapevine (Acero et al. 2004; Gramaje et al. 2012; Luque et al. 2012; Trouillas et al. 2011; Trouillas et al. 2010; Urbez-Torres et al. 2012). In this study, all three *Eutypella* spp. could be distinguished based on multi-gene phylogenetic analysis and this was further supported by differences in conidial and colony morphology. Little genetic variation was observed among isolates of *E. citricola* and no variation was seen for isolates of *E. microtheca* and the *Eutypella* sp. More robust methods of genetic analysis (e.g. AFLP, PCR fingerprinting or microsatellite loci) may be necessary to detect intraspecific variation within these populations. To study intraspecific variation of *Diplodia seriata* De Not. occurring on grapevine, Elena et al.

(2015) used the inter-simple sequence repeat (ISSR) technique and determined two main groups of *D. seriata,* however isolates obtained from different hosts and/or different geographic locations did not group into distinct clusters suggesting a shared genetic background irrespective of host or geographic origin. The use of such a technique can provide insight into the population structure and diversity of *Eutypella* spp. occurring on citrus and grapevine in the southern California desert regions and can determine whether certain genotypes are host specific.

Concerning agricultural commodities in this area, it is unclear how transmission of these *Eutypella* spp. occurs between citrus and grapevine which can be found growing in close proximity in the Coachella Valley. Perithecia (sexual fruiting bodies) of *E. citricola* and *E. microtheca* have been reported from both grapevine and citrus (Trouillas et al. 2010; Trouillas et al. 2011). Numerous perithecia were observed on dead, decaying citrus branches in the present study, suggesting that both citrus and grapevine are primary hosts for these pathogens as they are able to complete their life cycles. It is unclear at this time if other woody hosts (i.e. Tamarisk) serve as primary hosts for these pathogens. Various studies have speculated on the factors contributing to fungal pathogen distribution including the effects of temperature, physiological stresses, availability of hosts and host overlap (Adesemoye et al. 2014; Leavitt 1990; Urbez-Torres 2011). It is essential that further studies be conducted to investigate the factors contributing to pathogen distribution and persistence by determining the effects among pathogen distribution and the relative abundance of primary hosts, cultural practices in use, and spore dispersal within and between hosts. This information is invaluable in understanding

the seemingly complex epidemiology of these canker pathogens and will lead to better management strategies particularly where there is host overlap in a region.

Several studies have proven pathogenicity of *Eutypella* spp. on their respective hosts including *E. parasitica* which causes Eutypella canker on *Acer* spp. and *E. vitis* and *Eutypella* sp. on grapevine (Davidson and Lorenz 1938; French 1989; Trouillas 2009; Urbez-Torres et al. 2009). Studies assessing *E. vitis* and *Eutypella* sp. as pathogens on grapevine concluded that these species were capable of infecting host tissues, and recent work in Australia indicated both *E. citricola* and *E. microtheca* were pathogenic on grapevine (Pitt et al. 2013; Trouillas 2009; Urbez-Torres et al. 2009). Two species of *Eutypella* were able to produce lesions on detached citrus shoots (Adesemoye and Eskalen 2011), corroborating results in the present study. The influence of temperature on lesion development suggests that, for *E. microtheca*, temperatures close to the optimal fungal growth temperature lead to larger lesions suggesting enhanced disease development under permissive conditions (i.e. higher temperatures). The *Eutypella* sp. in the present study produced the largest lesions out of all fungi tested in contrast to previous studies where various *Eutypella* spp. were not the most virulent species in pathogenicity assays on grapevine (Pitt et al. 2013; Trouillas 2009; Urbez-Torres et al. 2009). This stresses the importance of this *Eutypella* sp. as a pathogen of citrus and future studies should be aimed at further identifying and characterizing the virulence and host colonization of this species.

N. dimidiatum is a well-known pathogen of desert citrus in California dating back to the 1950's when it was determined to be the cause of branch dieback and canker of

grapefruit in Southern California (Calavan and Wallace 1954). *N. dimidiatum* is frequently observed in (semi-)arid/tropical regions around the globe where it can be found on apple, apricot, citrus, ficus, plum, pitahaya and poplar (Chuang et al. 2012; Nattrass 1933, 1934; Oren et al. 2001; Polizzi et al. 2009; Ray et al. 2010, Sadowsky et al. 2007). It has also been reported from other woody hosts in California, including *Ficus* sp., *Juglans regia*, and *Prunus amygdalus* (Chen et al. 2013; English et al. 1974; Inderbitzin et al. 2010). Regarding the effect of heat-stress on disease development of Scytalidium wilt, Sadowsky et al. (2007) showed that "very hot" (34°C) temperature pre-treatment was most conducive to infection with *Scytalidium lignicola*. The results of lesion development caused by *N. dimidiatum* from the temperature study herein corroborates these results suggesting increased temperatures allow for more aggressive infection by this pathogen. Ten species of Botryosphaeriaceae associated with citrus branch canker were reported in California (Adesemoye et al. 2014); however, only two of these species were found associated with citrus in the desert regions, namely *N. dimidiatum* and *Spencermartinsia viticola* (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous. During a survey of canker disease in California vineyards, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. was most abundant in vineyards in the Coachella Valley (Urbez-Torres et al. 2006) and has been reported previously from citrus (French 1989). At present, it is unclear why other members of the Botryosphaeriaceae previously reported on citrus were not detected during this survey, but it is unlikely that any one factor such as temperature, host availability, geographic location, etc., can be responsible for fungal pathogen distribution; instead a combination of these factors governs pathogen

distribution (Adesemoye et al. 2014; Leavitt 1990; Urbez-Torres 2011). Expanded surveys of more groves may be necessary to determine if other members of the Botryosphaeriaceae are present. *N. dimidiatum* was recently reported as a pathogen of grapevine from the Coachella Valley, citing an estimated 15 % incidence of *N. dimidiatum* of affected grapevines (Rolshausen et al. 2013), further supporting the need for continued research into the epidemiology of this pathogen as an important pathogen of agricultural commodities in the Southern California desert regions.

Branch canker and dieback of citrus is an ongoing problem for citrus growers located in the desert areas of Southern California. It is well known that physiological stresses can predispose trees to infection by canker pathogens and that injuries including sunburn, frost damage and mechanical damage serve as points of entry for these pathogens (Whiteside 1980; Calavan and Wallace 1954). Management of branch canker is primarily achieved through various cultural practices including but not limited to, proper irrigation/fertilization, pruning during dry weather, removal of decaying/dead branches and twigs and prompt disposal of infected material. Although cultural practices are the current management strategies recommended for citrus branch canker, areas with a high incidence of disease may benefit from the use of chemical control. Studies conducted in avocado and grapevine have shown a number of fungicides to be successful in inhibiting members of the Botryosphaeriaceae and Diatrypaceae respectively and the use of these as pruning protectants has been studied (Gramaje et al. 2011; Pitt et al. 2012; Twizeyimana et al. 2013). In vitro studies and field trials are underway to determine the

efficacy of various commercial products for use as pruning protectants on citrus against *N. dimidiatum* and *Eutypella* spp.

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Table 1.1 Representative isolates of *Neoscytalidium dimidiatum* and *Eutypella* spp. recovered from citrus branch cankers from desert citrus in Southern California

Species	Isolate	County	Host	Variety	GenBank number	
					ITS[a]	EF1-α or β-tubulin[b]
Neoscytalidium dimidiatum	UCRDC08[c]	Imperial	*Citrus paradisi*	Rio Red	KF620362	KF620398
N. dimidiatum	UCRDC29	Imperial	*C. paradisi*	Rio Red	KF620363	KF620399
N. dimidiatum	UCRDC59	Riverside	*C. paradisi*	Pink	KF620364	KF620400
N. dimidiatum	UCRDC65	Riverside	*C. paradisi*	Pink	KF620365	KF620401
N. dimidiatum	UCRDC132	Riverside	*C. limon*	Allen Eureka	KF620366	KF620402
N. dimidiatum	UCRDC170	Imperial	*C. paradisi*	Ruby Red	KF620367	KF620403
N. dimidiatum	UCRDC176	Imperial	*C. paradisi*	Rio Red	KF620368	KF620404
N. dimidiatum	UCRDC280	San Diego	*C. limon*	Lisbon	KF620369	KF620405
N. dimidiatum	UCRDC284	San Diego	*C. limon*	Lisbon	KF620370	KF620406
Eutypella citricola	UCRDC06	Imperial	*C. paradisi*	Rio Red	KF620371	KF620407
E. citricola	UCRDC83	Riverside	*C. limon*	Allen Eureka	KF620372	KF620408
E. citricola	UCRDC88	Riverside	*C. limon*	Allen Eureka	KF620373	KF620409
E. citricola	UCRDC90	Riverside	*C. limon*	Allen Eureka	KF620374	KF620410
E. citricola	UCRDC91[c]	Imperial	*C. paradisi*	Rio Red	KF620375	KF620411
E. citricola	UCRDC112	Imperial	*C. paradisi*	Rio Red	KF620376	KF620412
E. citricola	UCRDC113	Imperial	*C. paradisi*	Rio Red	KF620377	KF620413
E. citricola	UCRDC117[c]	Imperial	*C. paradisi*	Rio Red	KF620378	KF620414
E. citricola	UCRDC186	Imperial	*C. paradisi*	Ruby Red	KF620379	KF620415
E. citricola	UCRDC272	San Diego	*C. limon*	Lisbon	KF620380	KF620416
E. citricola	UCRDC291	San Diego	*Tamarix* sp.	...	KF620381	KF620417
E. citricola	UCRDC293	San Diego	*C. limon*	Lisbon	KF620382	KF620418
E. citricola	UCRDC333	Riverside	*C. limon*	Allen Eureka	KF620383	KF620419
Eutypella microtheca	UCRDC09[c]	Imperial	*C. paradisi*	Rio Red	KF620384	KF620420
E. microtheca	UCRDC37[c]	Imperial	*C. paradisi*	Rio Red	KF620385	KF620421
E. microtheca	UCRDC67	Riverside	*C. paradisi*	Pink	KF620386	KF620422
E. microtheca	UCRDC103	Riverside	*C. paradisi*	Pink	KF620387	KF620423
E. microtheca	UCRDC106	Riverside	*C. paradisi*	Pink	KF620388	KF620424
E. microtheca	UCRDC148	Imperial	*C. limon*	Lisbon	KF620389	KF620425
E. microtheca	UCRDC325	Riverside	*C. limon*	Allen Eureka	KF620390	KF620426
Eutypella sp.	UCRDC142	Riverside	*C. limon*	Allen Eureka	KF620391	KF620427
Eutypella sp.	UCRDC143	Riverside	*C. limon*	Allen Eureka	KF620392	KF620428
Eutypella sp.	UCRDC210[c]	Imperial	*C. paradisi*	Ruby Red	KF620393	KF620429
Eutypella sp.	UCRDC211	Imperial	*C. paradisi*	Ruby Red	KF620394	KF620430
Eutypella sp.	UCRDC276[c]	San Diego	*C. limon*	Lisbon	KF620395	KF620431
Eutypella sp.	UCRDC287	San Diego	*Tamarix* sp.	...	KF620396	KF620432
Eutypella sp.	UCRDC335	Riverside	*C. limon*	Allen Eureka	KF620397	KF620433

[a] ITS=internal transcribed spacer region

[b] EF1-α=translation elongation factor 1-α. Accession numbers given for *N. dimidiatum* correspond to the ef1-α gene. Accession numbers given for gene *Eutypella* spp. correspond to the β-tubulin

[c] Isolates used in pathogenicity test and the effect of temperature experiment

Table 1.2. Sequences and their description of the fungal species obtained from GenBank used in the phylogenetic study

Isolate	Species	Host	Origin	GenBank Number	
				ITS[a]	EF1-α or β-tubulin[b]
HVGRF01	*Eutypella citricola*	*Citrus paradisi*	Australia	HQ692589	HQ692521
HVVIT07[c]	*E. citricola*	*Vitis. Vinifera*	Australia	HQ692579	HQ692512
WA06FH	*E. citricola*	*V. vinifera*	Australia	HQ692588	HQ692518
UCD2342Co	*E. citricola*	*V. vinifera*	California	GQ293968	GQ294022
UCD2349Co	*E. citricola*	*V. vinifera*	California	GQ293969	GQ294021
MSUELM13	*Eutypella vitis*	*V. vinifera*	United States	DQ006943	DQ006999
UCD2428TX	*E. vitis*	*V. vinifera*	United States	FJ790851	GU294726
HVFIG02[c]	*Eutypella cryptovalsoidea*	*Ficus carica*	Australia	HQ692573	HQ692524
HVFIG05	*E. cryptovalsoidea*	*F. carica*	Australia	HQ692574	HQ692525
HVGRF02[c]	*Eutypella microtheca*	*C. paradisi*	Australia	HQ692569	HQ692533
UCD2Co	*E. microtheca*	*V. vinifera*	California	GQ293958	GQ294018
UCD3Co	*E. microtheca*	*V. vinifera*	California	GQ293957	GQ294019
YC17	*E. microtheca*	*V. vinifera*	Australia	HQ692562	HQ692537
YC24	*E. microtheca*	*V. vinifera*	Australia	HQ692565	HQ692530
UCD713SJ	*Eutypella* sp.	*V. vinifera*	California	GQ293955	GQ294016
BAFC3321	*Peroneutypa scoparia*	N/A	Argentina	EU702431	EU728698
BAFC3322	*P. scoparia*	N/A	Argentina	EU702432	EU728699
DFMAL100	*P. scoparia*	*Robinia pseudoacacia*	France	GQ293962	GQ294029
UCD2334Co	*Eutypella* sp.	*V. vinifera*	California	GQ293963	GQ294027
UCD2335Co	*Eutypella* sp.	*V. vinifera*	California	GQ293964	GQ294028
EP18	*Eutypa lata*	*V. vinifera*	Australia	HQ692611	HQ692501
RGA01	*E. lata*	*Fraxinus angustifolia*	Australia	HQ692614	HQ692497
ADSC300	*E. lata*	*Schinus molle*	Australia	HQ692610	HQ692493
HVGRF03[c]	*Diatrypella vulgaris*	*C. paradisi*	Australia	HQ692590	HQ692502
HVPT01	*D. vulgaris*	*S. molle*	Australia	HQ692594	HQ692506
RGA02	*Eutypa leptoplaca*	*F. angustifolia*	Australia	HQ692602	HQ692483
TUQU01	*E. leptoplaca*	*Quercus* sp.	Australia	HQ692598	HQ692491
CBS251.49	*Neoscytalidium dimidiatum*	*Juglans regia*	United States	KF531819	KF531797
CBS145.78[c]	*N. dimidiatum*	*Homo sapien*	United Kingdom	KF531816	KF531795
WAC12693	*N. dimidiatum*	*Mangifera indica*	Australia	EF585538	EF585576
WAC12690	*N. dimidiatum*	*M. indica*	Australia	EF585537	EF585577
CBS499.66	*N. dimidiatum*	*M. indica*	Mali	KF531820	EU144063
CBS122610	*Neoscytalidium novaehollandiae*	*Acacia synchronicia*	Australia	EF585536	EF585578
WAC12691	*N. novaehollandiae*	*M. indica*	Australia	EF585543	EF585574
WAC13303	*N. novaehollandiae*	*M. indica*	Australia	GU172398	GU172430
CBS122070	*N. novaehollandiae*	*Grevillea agrifolia*	Australia	EF585539	EF585579
WAC13304	*N. novaehollandiae*	*M. indica*	Australia	GU172399	GU172431
WAC12688	*N. novaehollandiae*	*M. indica*	Australia	EF585542	EF585575
CBS122072	*N. novaehollandiae*	*Adansonia gibbosa*	Australia	EF585535	EF585581
WAC13273	*N. novaehollandiae*	*M. indica*	Australia	GU172397	GU172429
CBS122071[c]	*N. novaehollandiae*	*Crotalaria medicaginea*	Australia	EF585540	EF585580
WAC13275	*N. novaehollandiae*	*M. indica*	Australia	GU172400	GU172432
MFLUCC11-0425[c]	*Cophinforma eucalypti*	*Eucalyptus* sp.	Thailand	JX646800	JX646865
MFLUCC11-0655	*C. eucalypti*	*Eucalyptus* sp.	Thailand	JX646801	JX646866
CMW22674	*Cophinforma atrovirens*	*Pterocarpus angolensis*	South Africa	FJ888473	FJ888456
MFLUCC10-0051	*Botryosphaeria agaves*	*Agave* sp.	Thailand	JX646790	JX464855
MFLUCC11-0125[c]	*B. agaves*	*Agave* sp.	Thailand	JX646791	JX646856
ATCC22927	*Botryosphaeria corticis*	*Vaccinium* sp.	United States	DQ299247	EF614931
CBS119047[c]	*B. corticis*	*V.corymbosum*	United States	DQ299245	EU017539
MFLUCC10-0098[c]	*Botryosphaeria fusispora*	*Entada* sp.	Thailand	JX646789	JX646854
CBS116741	*Botryosphaeria dothidea*	*Olea europaea*	Greece	AY640254	AY640257
CMW8000[c]	*B. dothidea*	*Prunus* sp.	Switzerland	AY236949	AY236898
CBS110302	*B. dothidea*	*Vitis* sp.	Portugal	AY259092	AY573218
CBS162.25	*Macrophomina phaseolina*	*Eucalyptus* sp.	N/A	KF531826	KF531803
CBS22733	*M. phaseolina*	*Zea mays*	N/A	KF531825	KF531804
CBS447.68	*Phyllosticta foliorum*	*Taxus baccata*	Netherlands	FJ824768	FJ824773

[a] ITS= internal transcribed spacer region

[b] EF1-α= translation elongation factor 1-α. Accession numbers given for the Botryosphaeriaceae correspond to the ef1-α gene. Accession numbers given for Eutypella spp. correspond to the β-tubulin gene

[c] Denotes type-specimen

Table 1.3. Percent (%) Recovery of *Neoscytalidium dimidiatum* and *Eutypella* spp. in the southern California desert regions

			Number (%) of branch cankers yielding[a]					
County	Orchards[b]	Branch cankers[c]	*N. dimidiatum* (%)	*Eutypella* spp. (%)	Both[d] (%)	*E. citricola* (%)	*E. microtheca* (%)	*Eutypella* sp. (%)
Riverside	4	144	55 (38)	20 (14)	1 (1)	11 (55)	6 (30)	3 (15)
Imperial	4	99	27 (27)	16 (16)	2 (2)	9 (56)	5 (31)	2 (13)
San Diego	1	30	3 (10)	8 (27)	1 (3)	6 (75)	0 (0)	2 (25)
Total number:	9	273	85 (31)	44 (16)	4 (1)	26 (59)	11 (25)	7 (16)

[a] Branch samples per county yielding indicated fungi

[b] Number of orchards surveyed by county

[c] Number of branch cankers collected by county

[d] Number of branch cankers yielding both *N. dimidiatum* and a *Eutypella* spp.

Table 1.4. Conidial size of isolates from citrus branch canker used in this study and comparison with previous studies

Species	This Study		Previous Studies	Source
	Conidial Size (µm)	Mean ± SD (µm)	Conidial Size (µm)	
Eutypella citricola	(9)-14-19 x (1)-1-2	14.8±1.6 x 1.1±0.1	10.5-16(-20) x 1.5	Trouillas et al. 2010
E. microtheca	(8)-13-23 x (1)-1-2	14.1±2.4 x 1.2±0.2	(14.00-)17.63-18.82(-23.79) x (1.33-)1.45-1.52(-1.64)	Trouillas et al. 2011
Eutypella sp.	(12)-20-27 x (1)-1-2	20.2±2.0 x 1.3±0.3	(17.5-)20-25(-27.5) x 1-1.5	Trouillas et al. 2010

Conidial size, means, and standard deviation were determined from 30 conidia from at least five isolates from each species in the table.

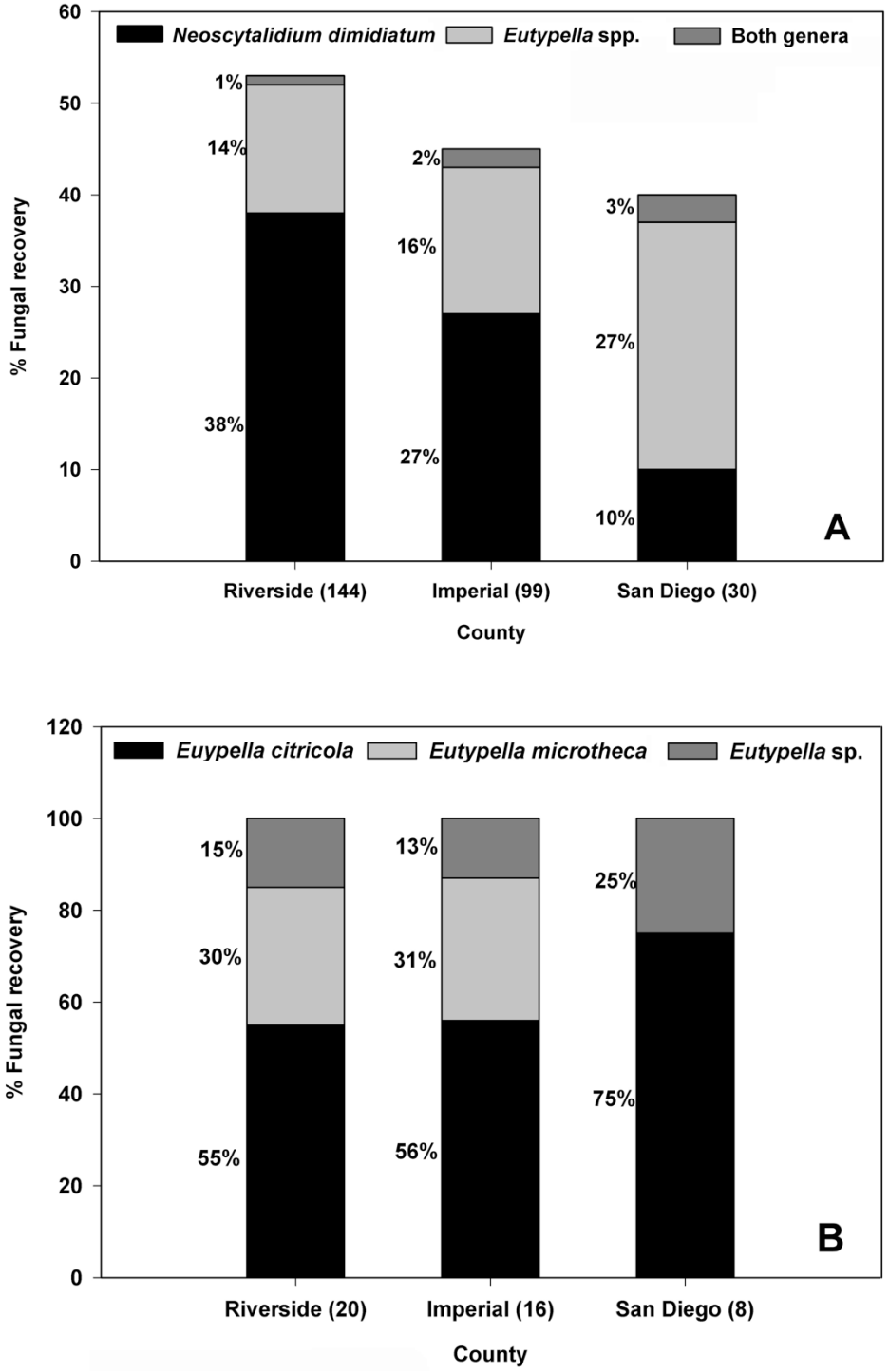

Figure 1.1. Fungal recovery (%) of (a) *Neoscytalidium dimidiatum* and *Eutypella* spp. and (b) *Eutypella* spp. by county. Numbers in parentheses following county names indicate the number of symptomatic branches collected in that county. Percentages were rounded to the nearest whole number.

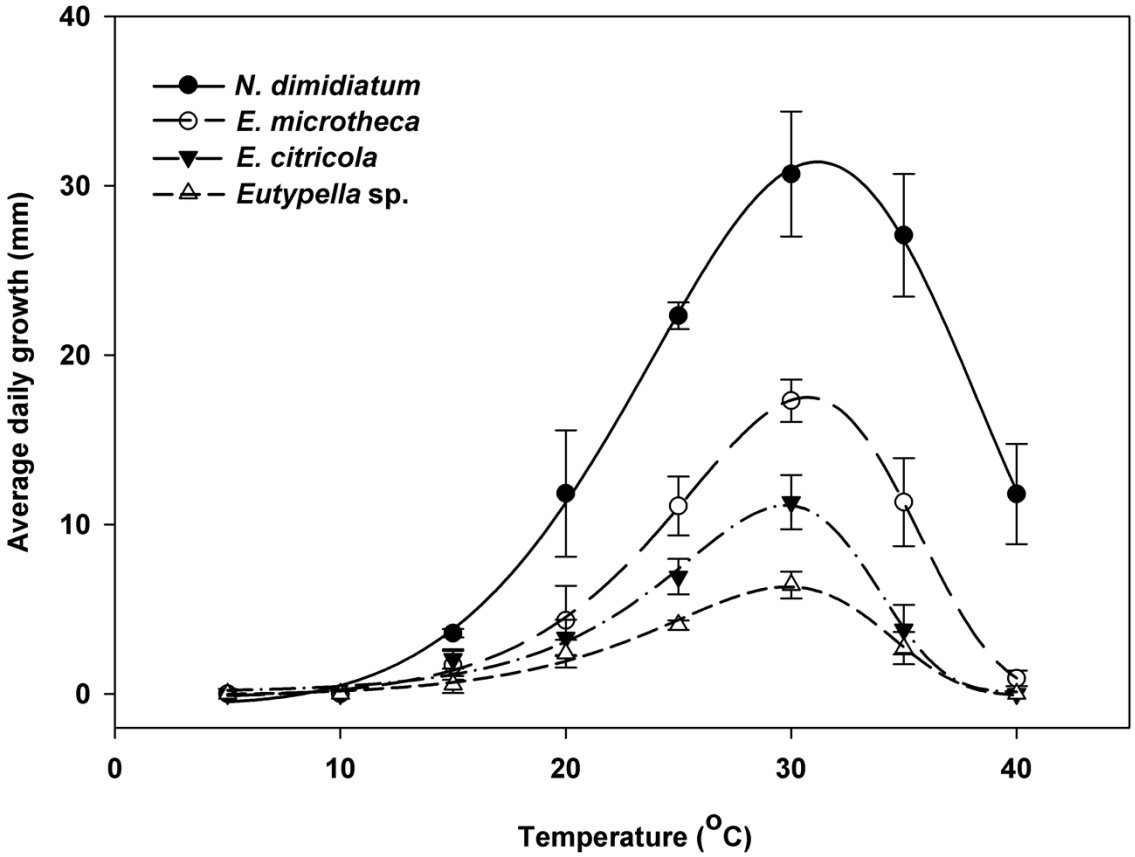

Figure 1.2. Optimum temperature of *Neoscytalidium dimidiatum*, *Eutypella citricola*, *E. microtheca* and *Eutypella* sp. Average daily growth (mm) was derived from measurements at two and three days. Vertical bars represent standard errors of the mean.

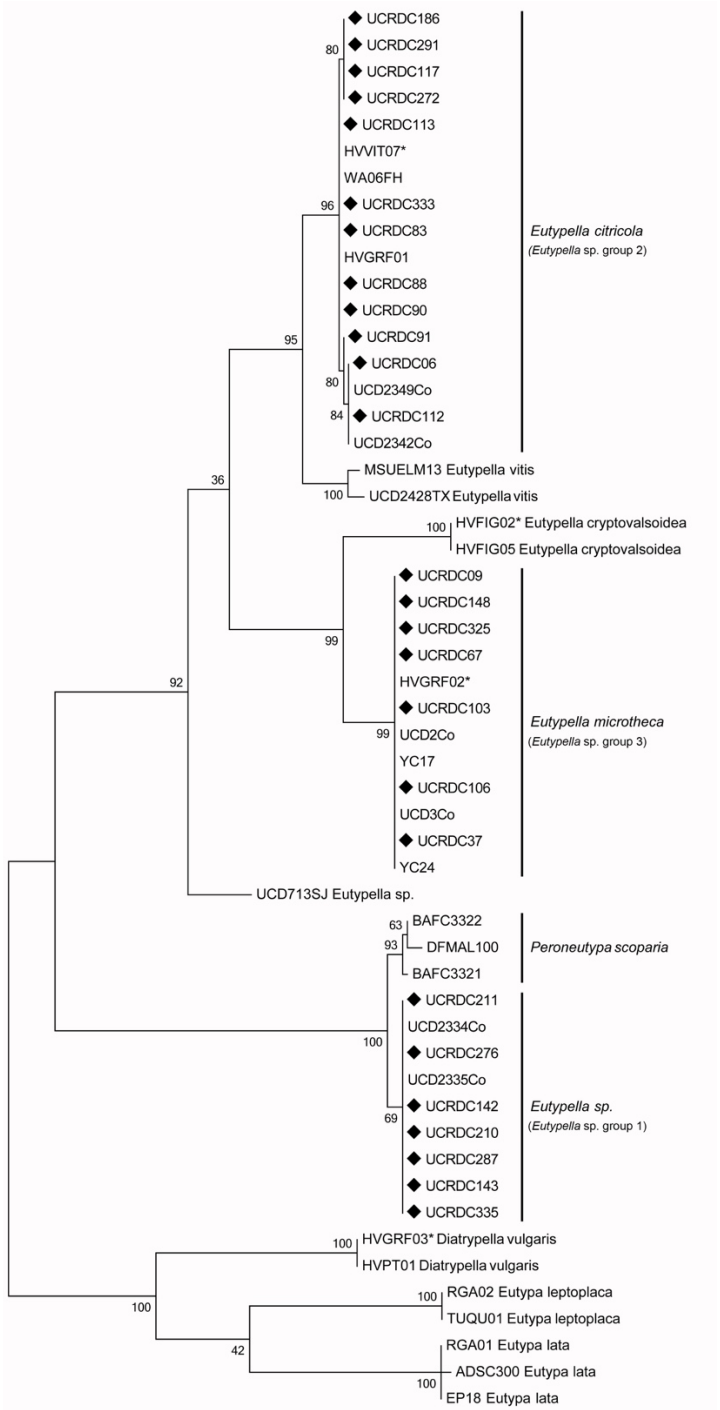

Figure 1.3. One of the most parsimonious unrooted trees based on internal transcribed spacer 1(ITS1), 5.8S ribosomal DNA, ITS2 and partial β-tubulin gene region sequences for isolates of species of the Diatrypaceae inferred from maximum parsimony analysis. Numbers on branches are bootstrap values from 1,000 bootstrap replicates. Diamonds indicate isolates from this study and asterisks denote type-specimens.

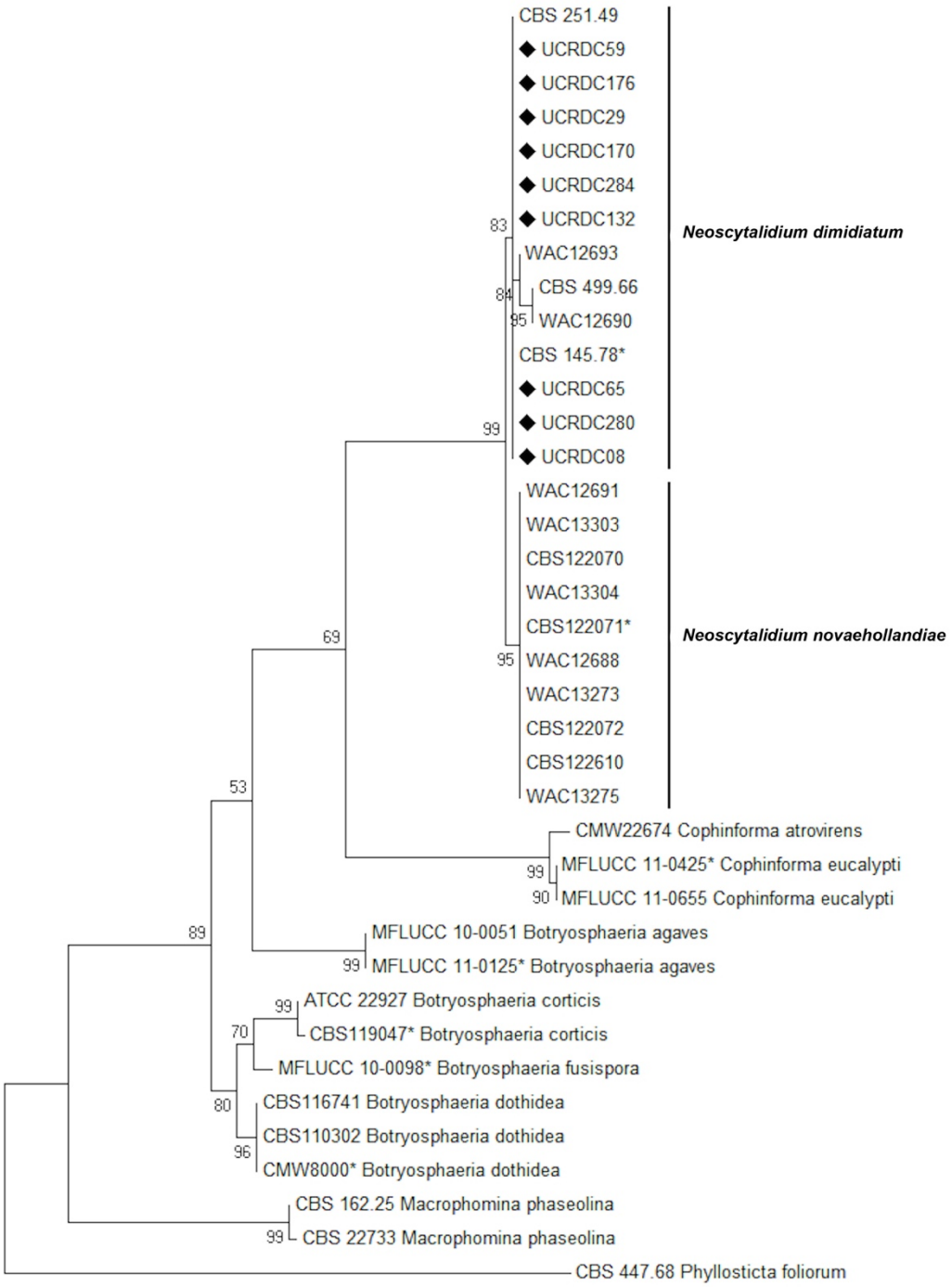

Figure 1.4. One of the most parsimonious unrooted trees based on internal transcribed spacer 1(ITS1), 5.8S ribosomal DNA, ITS2 and partial translation elongation factor 1-α gene region sequences for isolates of species of the Botryosphaeriaceae inferred from maximum parsimony analysis. Numbers on branches are bootstrap values from 1,000 bootstrap replicates. Diamonds indicate isolates from this study and asterisks denote type-specimens. CBS 447.68 (*Phyllosticta foliorum*) was added as an outgroup.

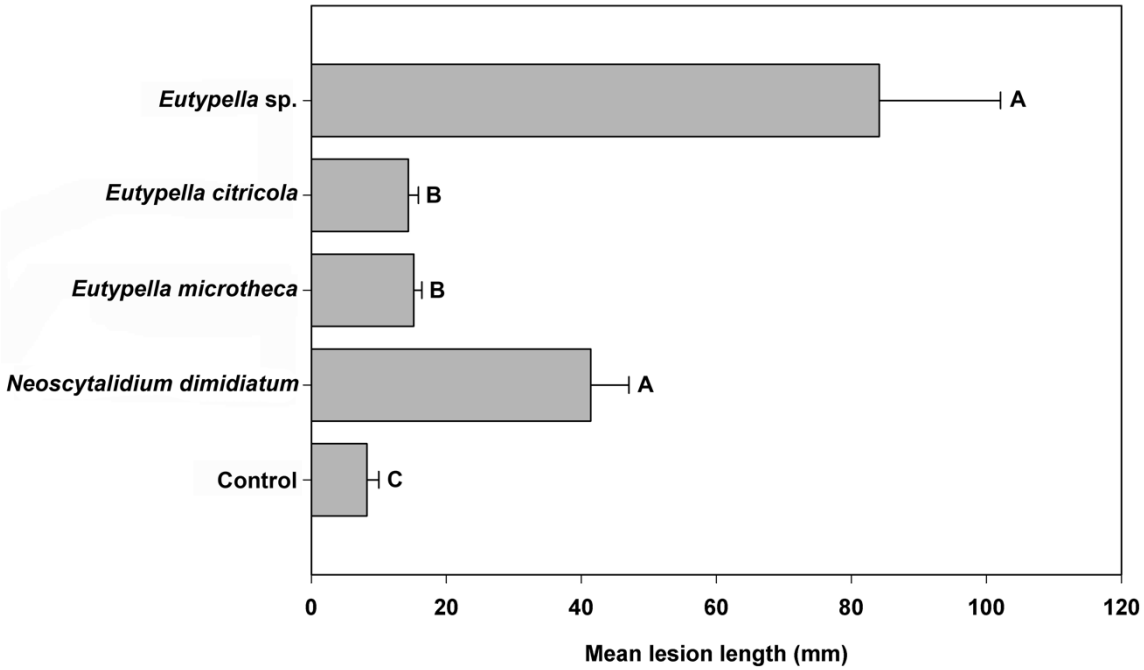

Figure 1.5. Pathogenicity of *N. dimidiatum* and *Eutypella* spp. on Lisbon lemon plants after 8 months. Horizontal lines represent standard error of the mean according to Tukey's honestly significant difference mean separation test at α=0.05. Levels not connected by the same letter are significantly different.

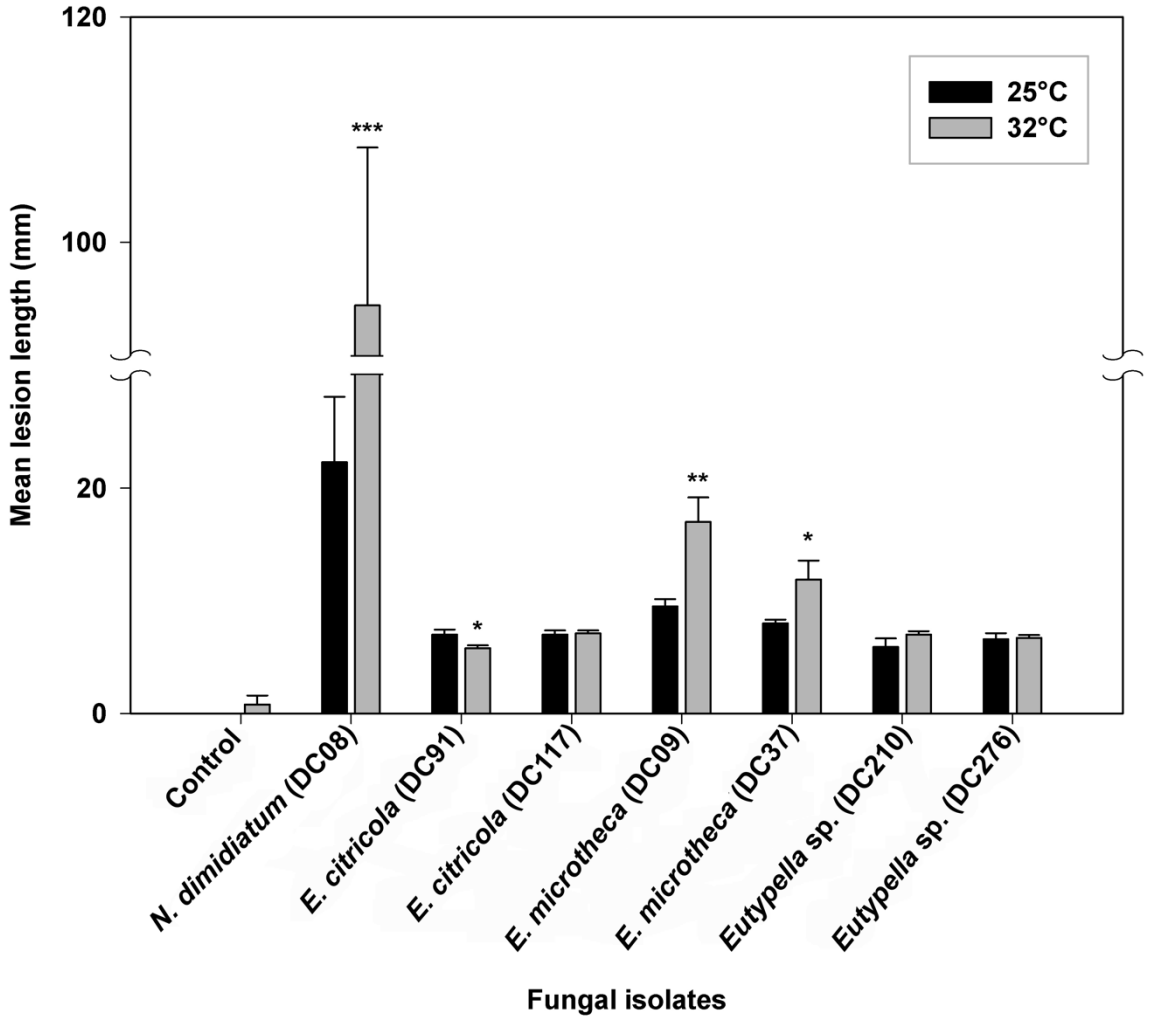

Figure 1.6. Temperature study of *N. dimidiatum* and *Eutypella* spp. on detached shoots of Lisbon lemon incubated at 25°C or 32°C. Vertical lines represent standard error of mean from an independent-samples t-test with confidence level α=0.05. Asterisks indicate statistical significance (* = P<0.05, ** = P<0.01, and *** = P<0.001) between temperature treatments within a fungal isolate.

CHAPTER II. *In Vitro* and Field Evaluation of Fungicides for use as Pruning Protectants Against *Eutypella* spp. and *Neoscytalidium dimidiatum* on Citrus and Spore Trapping Studies

ABSTRACT

Citrus branch canker and dieback diseases are considered minor diseases; however, they remain a concern for citrus growers, especially for growers in desert citrus production regions faced with two canker and dieback diseases: Hendersonula and Eutypella canker of citrus. At present, management of these diseases rely on cultural practices, however the use of a chemical management strategy may be beneficial to desert citrus production as no chemical strategies exist for these diseases. *In vitro* screens of nine fungicides identified azoxystrobin, pyraclostrobin, and trifloxystrobin to have lower EC_{50} values overall compared to other fungicides when tested against *N. dimidiatum*, *E. citricola*, *E. microtheca*, and *Eutypella* sp., whereas metconazole, fenbuconazole, and propiconazole supported higher EC_{50} values on average when compared to other fungicides tested. Protection assays determined fluazinam to limit the colonization of all fungal species on treated citrus branch pieces more effectively than pieces treated with strobilurins. Field trials found applications of pyraclostrobin as a pruning protectant to significantly ($P < 0.001$) reduce the vascular necrosis caused by *N. dimidiatum* and application of trifloxystrobin to significantly reduce ($P = 0.01$) vascular necrosis caused by *E. citricola* when compared to water treated controls; no significant reductions in lesion lengths were observed for *E. microtheca* or *Eutypella* sp. Spore trapping studies revealed spores of *Eutypella* spp. to be present almost year-round with the majority of spores trapped during Fall and Winter months. These results suggest that the use of

strobilurins as pruning protectants against *N. dimidiatum* and *E. citricola* are effective in reducing vascular necrosis caused by these pathogens and may be useful in the management of other *Eutypella* spp. on citrus. Continued studies are necessary to determine the most effective times of fungicide application for the management of these canker and dieback diseases.

INTRODUCTION

California citrus production is primarily consumed as a fresh market commodity and California's citrus production is ranked second in the U.S. with the state contributing 47% of the total U.S. production; the current value of U.S. citrus production is just over $3 billion (USDA-NASS 2016). Citrus production in the state occurs in four major growing areas: San Joaquin Valley Region, Coastal-Intermediate Region, Interior Region, and Desert Region. Citrus varieties produced in each region vary, but the majority of citrus production in the San Joaquin Valley Region is focused on navels, Valencia, and tangerines; lemons and limes are primarily produced in the Coastal-Intermediate Region; citrus production in the Interior Region is vastly smaller than other growing regions and produces lemons, navels, Valencia, and grapefruit; and the Desert Regions primarily produces grapefruit, tangerine, and lemon. Pests of citrus in California, as well as their management strategies, vary by growing region and the California citrus industry relies heavily on close monitoring of its pests to develop appropriate and cost-effective approaches to management through integrated pest management (IPM) programs. Of concern to the industry are the limited number of pesticides approved for use on citrus for

various diseases and pests (Anonymous 2003). The need for continued research into pesticides for the management of citrus diseases is imperative to maintain lucrative citrus production in the state.

Branch canker and dieback diseases of citrus refer to a group of diseases characterized by overlapping symptoms (twig and branch dieback, dark-colored cankers, and gummosis) and are considered to have complex etiologies due to the wide range of fungal pathogens associated with these branch and twig diebacks. Many of these pathogens have been identified as belonging to the Botryosphaeriaceae (Adesemoye et al. 2014; Calavan and Wallace 1954; Graham and Menge 2000). However, these fungi are also found in other crop hosts such as almond (Olmo et al. 2017), avocado (Twizeyimana et al. 2013), and grapevine (Pitt et al. 2012) where they are known to cause various dieback and canker diseases. Other fungi outside of the Botryosphaeriaceae have also been shown to cause branch canker and dieback diseases in citrus including members of the Diatrypaceae (Mayorquin et al. 2016), which are also known to be pathogens of grapevine (Gramaje et al. 2012; Pitt et al. 2012; Rolshausen and Gubler 2005; Sosnowski et al. 2008). The economic losses attributed to all known canker and dieback diseases has not been determined for every host crop, but the losses caused by these diseases in grapevine are estimated to be up to $260 million per year (Siebert 2001). Currently it is unknown what the economic burden of branch canker and dieback diseases are for the citrus industry, but it is likely that the burden is greater than previously thought (Adesemoye et al. 2014).

In the desert citrus production areas of California, two branch canker and dieback diseases have been identified which are of concern to citrus growers; these diseases are Hendersonula, caused by the fungus *N. dimidiatum* (Penz.) Crous & Slippers (Calavan and Wallace 1954), and Eutypella canker of citrus, caused by three species of *Eutypella: E. citricola* Speg., *E. microtheca* Trouillas, W.M. Pitt & Gubler, and a *Eutypella* sp. (Mayorquin et al. 2016). Despite the complexity of these pathosystems involving multiple fungal species from different fungal families, these diseases are considered minor as physiological stress is almost always associated with these dieback diseases and appear to be necessary for canker formation by normally weak pathogens; typically, these diseases are not lethal unless cankers extend into the main branches or trunk. Additionally, these fungi are considered primary colonizers of wounds which can be caused by, but are not limited to mechanical damage, pruning, and environmental conditions such as sunburn and frost injury (Graham and Menge 2000).

Management strategies for citrus branch canker and dieback diseases are limited; the primary means of management relies on cultural practices to avoid stress to trees or to reduce the pathogen population once established in an orchard. General recommended practices include: maintaining tree vigor through appropriate irrigation, pruning of infected twigs and branches below cankered areas during dry periods, prompt disposal of infected tissues, and disinfection of pruning materials (Calavan and Wallace 1954; Graham and Menge 2000). Several vague recommendations have been made concerning the use of fungicides as pruning protectants for these diseases and range from “suitable fungicides” and “fungicidal pruning compound” to more specific compounds such as

Benomyl (Calavan and Wallace 1954; Graham and Menge 2000; Punithalingham 1980), but chemical management strategies are not currently practiced for the management of canker and dieback diseases in citrus (http://ipm.ucanr.edu/). Despite the lack of evidence and perhaps interest in fungicides as pruning protectants for these diseases in citrus, chemical management for dieback diseases in other woody commodities such as almond (Olmo et al. 2017), avocado (Twizeyimana et al. 2013), and grapevine (Pitt et al. 2012) for botryosphaeriaceous fungi and grapevine (Gramaje et al. 2012; Sosnowski et al. 2008) for diatrypaceous fungi have been studied and several candidate fungicides have been identified for use in these pathosystems.

The lack of chemical options available to the citrus industry for the management of citrus branch canker and dieback diseases caused by *N. dimidiatum* and *Eutypella* spp. and the ongoing findings of these diseases throughout the desert citrus production areas prompted this study. The objectives of this study were: (i) to evaluate commercially available fungicides *in vitro* against *N. dimidiatum* and three *Eutypella* spp.; (ii) to determine the protective ability of these fungicides in reducing or preventing fungal colonization of citrus wood pieces; (iii) to determine the ability of candidate fungicides in reducing vascular necrosis caused by canker pathogens; and (iv) to determine spore dispersal of these pathogens to identify 'windows' of fungicide application preceding pathogen dispersal.

MATERIALS AND METHODS

Fungal isolates. Isolates of *N. dimidiatum*, *E. citricola*, *E. microtheca*, and *Eutypella* sp. used in the *in vitro* and field experiments were obtained from branch canker samples collected from symptomatic citrus orchards in Riverside, Imperial, and San Diego counties in California; Fungal identification was confirmed using the morphological and molecular methods described by Mayorquin et al. (2016). Five isolates of *N. dimidiatum* (UCRDC08, UCRDC59, UCRDC132, UCRDC170, and UCRDC280), *E. citricola* (UCRDC91, UCRDC117, UCRDC272, UCRDC293, and UCRDC333), *Eutypella* sp. (UCRDC142, UCRDC210, UCRDC211, UCRDC276, and UCRDC287), and four isolates of *E. microtheca* (UCRDC09, UCRDC67, UCRDC103, UCRDC106) were used for *in vitro* fungicide screens; one isolate each of *N. dimidiatum* (UCRDC08), *E. citricola* (UCRDC91), *E. microtheca* (UCRDC09), and *Eutypella* sp. (UCRDC210) were used for field evaluations. Before use in *in vitro* and field experiments, isolates were grown and maintained on potato dextrose agar amended with 0.01% tetracycline hydrochloride (PDAt) for seven days at 25°C in a microbiological incubator.

***In vitro* fungicide screens.** Nine commercial fungicides from five chemical groups (Table 2.1) were evaluated for their ability to reduce mycelial growth by 50% (EC_{50}) of *N. dimidiatum* and *Eutypella* spp. using the spiral gradient dilution (SGD) method described by Förster et al. (2004) and Twizeyimana et al (2013). To obtain fungal inoculum for each fungal species, plugs of seven-day-old cultures were transferred to PDA plates on which eight cellophane strips were placed and allowed to incubate at 25°C in a microbiological incubator for five days to allow mycelium to completely cover the

cellophane strips. Stock solutions of fungicides were prepared and diluted to various concentrations (10-100 μg/ml) prior to deposition based on pilot screens (*unpublished data*). Plates were spiraled using an AutoPlate4000 (Spiral Biotech, MA) and allowed to incubate for four hours in order for the applied fungicides to diffuse and form a gradient throughout the media; following incubation, mycelium-covered cellophane strips were placed radially on fungicide amended plates. For each fungal isolate, two replicate cellophane strips were placed on a single fungicide amended plate for each fungicide tested and inoculated plates were incubated for three days at 25°C in the dark before being measured to determine EC_{50} values; these experiments were repeated once.

Protection assay. To assess the potential of fungicides in reducing fungal colonization on citrus branch pieces, a modified protocol from Rolshausen and Gubler (2005) was used. Twigs (approx. 2cm diameter) of three-year-old 'Allen Eureka' lemon plants grown in a lath house at the University of California, Riverside were harvested and rinsed in distilled water to remove surface debris before being cut into 4-cm-long segments. Branch segments were placed into beakers of distilled water and sterilized in an autoclave for 20 min. at 121°C followed by an additional 20 min. at 121°C 24 hrs after initial sterilization. French square bottles (250ml) were filled with 15ml of PDA and autoclaved for 15 min. at 121°C before being laid flat on the side to cool. Fungal plugs (5 mm in diameter) of *N. dimidiatum* (UCRDC08), *E. citricola* (UCRDC91), *E. microtheca* (UCRDC09), and *Eutypella* sp. (UCRDC210) were taken from five-day-old cultures and placed into French square bottles and allowed to grow for four days (*Eutypella* spp.) or two days (*N. dimidiatum*) prior to citrus branch introduction. Sterilized citrus branch

segments were blotted dry and soaked in fungicide solutions at labeled rates (Table 2.1) for 15 min. before being blotted dry to remove residual fungicide and placed into French square bottles 1 cm away from the advancing fungal margin; control branch segments were soaked in sterile distilled water. Three branch segments per fungal species and fungicide were used and bottles were arranged in a completely randomized design; this experiment was repeated once. Bottles were incubated for one week (*N. dimidiatum*) or four weeks (*Eutypella* spp.) before branch segments were assessed for external fungal colonization using the following scale (1-5): 1, 0-20% branch segment colonized; 2, 21-40% branch segment colonized; 3, 41-60% branch segment colonized; 4, 61-80% branch segment colonized; and 5, 81-100% branch segment colonized. After rating, bark was removed from branch segments and segments were surface sterilized in 10% household bleach for 10 mins, rinsed twice in sterile distilled water for 2 mins, and blotted dry. Branch segments were cut longitudinally and 10 wood chips (2 x 2 mm) were removed and plated onto PDAt plates for all branch segments. Plates were incubated for 4 days at 25°C before being assessed for fungal recovery calculated as the percentage of pieces from which inoculated fungi were recovered.

Field trial. Based on the registration status of tested fungicides for citrus, four of the nine fungicides (i.e., azoxystrobin, fenbuconazole, pyraclostrobin, and trifloxystrobin) were chosen for field evaluations as potential pruning protectants (Table 2.1). Trials were established in a commercial citrus orchard of 'Allen Eureka' lemons in Riverside county and each plot (two plots) was setup in a randomized complete block design with 10 blocks of four trees. Fungicides were applied at maximum labeled field rates in addition

to a control treatment (distilled water) and untreated control treatment. One tree in each block was assigned to one fungal pathogen and all trees within a block were treated with each fungicide and control treatment on separate branches (6 branches per tree); both plots were treated and inoculated one week apart during September 2013.

Fungicides and pathogen inocula were applied according to the methods of Twizeyimana et al. (2013): (i) woody citrus branches (approx. 3-cm-diameter) were randomly selected, pruned distally, and sprayed with fungicides using handheld sprayers until runoff was achieved (approx. 4 ml); (ii) pruned branches were inoculated with fungal pathogens, distilled water (control), or no treatment (untreated control) 24hrs after fungicide application. Pathogen inoculum was produced as follows: (i) fungal pathogens were initially grown on PDAt for seven days at 25°C before fungal plugs were removed and used to inoculate flasks containing 250 ml of potato dextrose broth (PDB); (ii) inoculated PDB flasks were incubated on a rotary shaker at 25°C and 125 rpm for seven days; (iii) fungal mats were filtered from PDB using four layers of cheesecloth, rinsed twice with sterile distilled water, ground in sterile distilled water using a blender, and filtered through two layers of cheesecloth; and (iv) a drop of TritonX100 was added to mycelial suspensions and mycelial fragments were quantified using a hemocytometer and adjusted to approximately 10^5 fragments/ml.

To determine the extent of vascular colonization, treated citrus branches were removed six months post-application and transported to the lab in a cooler. Branches were rinsed in distilled water to remove surface debris and flame sterilized by spraying

branches with 95% ethanol and passing through a flame before being split longitudinally to examine and measure internal vascular necrosis (lesions); external necrosis was also measured. To determine fungicide efficacy in reducing lesion lengths, percent lesion reduction was calculated for each treated branch in comparison to the water-treated control branch for each tree using the following formula: (control lesion length – treated lesion length) / control lesion length x 100. To determine the presence of the inoculated fungi, five small wood pieces (4 x4 mm) from the margin of lesions (or point of inoculation for control branches) were removed and plated onto PDAt. Plates were incubated at 25°C in the dark for six days before being assessed for fungal identity (morphological observations) and recovery calculated as the percentage of pieces from which inoculated fungi were recovered.

Spore Trapping. Spore trapping was performed as described by Eskalen and Gubler (2001) with slight modifications. Two citrus orchards previously surveyed by Mayorquin et al. (2016) were chosen for trapping studies during August 2013 to July 2014; one orchard was located in Riverside County and consisted primarily of Persian lime (two-year-old orchard) and the second orchard was located in Imperial County and consisted primarily of 'Allen Eureka' lemon (10-year-old orchard). A single spore trap was randomly placed within the canopy on ten trees in each grove and spore traps were replaced every other week. To make a spore suspension from traps, 5ml of sterile distilled water was added to tubes containing spore traps and vortexed for 30 s. Two 100 µl aliquots of the resulting suspension was spread plated onto two PDAt plates and plates were air dried in a laminar flow hood to dry excess moisture from plates. Plates were

incubated at 25°C for five days before fungal colonies were enumerated; colonies resembling *Eutypella* spp. or *N. dimidiatum* were identified morphologically as described by Mayorquin et al. (2016). Individual colonies were assumed to be derived from a single conidium. Colony forming units (CFU) are reported as the combined number of fungal colonies from two plates for each spore trap. Weather data (temperature and precipitation) was taken from local weather stations in each county.

Statistical analysis. Statistical analysis was performed using SPSS v. 24.0 (IBM Corp., NY). Prior to analyzing EC_{50} values from independent *in vitro* trials and lesion lengths from field trials, EC_{50} values and lesion lengths were $\log_{10}$ transformed according to Liang et al. (2015) and data were evaluated for normality and homoscedasticity using the Shapiro-Wilk test of normality and Levene's test respectively. As no heterogeneity was detected, data from independent trials were combined and evaluated by ANOVA using a general linear model (GLM) and post-hoc comparisons were performed using Tukey's honest significant difference (HSD) at $\alpha = 0.05$ for *in vitro* studies and for the field study. For field trial data, Pearson's correlation coefficients were calculated to determine any relationships between internal and external lesions in addition to branch diameter.

RESULTS

***In vitro* fungicide screens.** Significant differences were observed among the nine fungicide active ingredients tested ($P < 0.001$) and between the fungal pathogens tested ($P < 0.001$) (Table 2.2); a significant interaction was also observed between fungal

pathogen and fungicide ($P < 0.001$). Overall, *N. dimidiatum* had the lowest EC_{50} values among all fungi tested followed by *E. microtheca*, *E. citricola* and *Eutypella* sp. EC_{50} values across all fungicides and fungal pathogens ranged from 0.001 to 0.547. Strobilurins supported lower EC_{50} values when tested against *N. dimidiatum*, however those values did not differ significantly from each other. Similarly, triazole fungicides supported low EC_{50} values, but did not differ significantly from each other. Cyprodinil was the least effective fungicide in reducing mycelial growth of *N. dimidiatum* and was significantly higher than any other fungicide tested. Pyraclostrobin supported the lowest EC_{50} values when tested against *E. citricola* and was significantly lower than other strobilurins tested. All triazoles tested had significantly higher EC_{50} values when compared to other fungicides tested against *E. citricola*. For *E. microtheca*, pyraclostrobin produced the lowest EC_{50} values. However, it was not significantly lower than values for trifloxystrobin, but it was significantly lower than EC_{50} values for azoxystrobin. Fludioxonil EC_{50} values were found to be significantly lower than those of other triazoles tested, however it did not differ significantly from any strobilurin tested. Fluazinam supported the lowest EC_{50} values when tested against *Eutypella* sp. and was significantly lower than all other fungicides tested except for fludioxonil. No significant differences in EC_{50} values were found between strobilurins, however metconazole had significantly lower values than any other triazole tested. Cyprodinil was not tested for any species of *Eutypella* as growth of these species were abnormal on un-amended minimal media plates which are used to assess the activity of cyprodinil.

Protection assay. Control branch segments for all fungal species were found to be completely colonized (100%, rating 5) after one week post treatment (Fig. 2.1). Overall, *N. dimidiatum* had lower colonization ratings than *Eutypella* spp. and *E. microtheca* generally had higher colonization ratings for all fungicides tested. For all fungal species tested, only treatment with fluazinam resulted in colonization ratings of 1 (0-20% colonized). For all strobilurins tested, only *N. dimidiatum* and *Eutypella* sp. had colonization ratings of 1 (0-20% colonized); *E. microtheca* had colonization ratings of 3 (41-60% colonized) for pyraclostrobin and azoxystrobin and a rating of 4 (61-80% colonized) for trifloxystrobin, whereas *E. citricola* had a rating of 1 (0-20% colonized) for pyraclostrobin and ratings of 2 (21-40% colonized) for trifloxystrobin and azoxystrobin. Treatment of branch segments with fenbuconazole resulted in colonization ratings of 1 (0-20% colonized) for both *N. dimidiatum* and *Eutypella* sp. followed by ratings of 2 (21-40% colonized) for *E. citricola* and 5 (81-100% colonized) for *E. microtheca*. Cyprodinil treatment resulted in ratings ≥3 (≥41% colonized) for all fungi tested: a rating of 3 (41-60% colonized) for *Eutypella* sp., rating 4 (61-80% colonized) for *N. dimidiatum* and *E. citricola*, and rating 5 (81-100% colonized) for *E. microtheca*. Fungal recovery for *N. dimidiatum*, *E. citricola*, and *E. microtheca* ranged from 0-100% followed by *Eutypella* sp. from 30-100%.

Field trial. Lesions were observed on all fungicide treated branches and water control treated branches; no lesions were observed on untreated control branches. Resulting lesions varied in the intensity of discoloration, but were generally light brown to dark

chocolate brown and progressed away from the pruned surface. External lesions (depressed, dead bark) were not observed on all treated branches. Significant differences were observed among the fungicides tested ($P < 0.001$) and between fungal pathogens tested ($P = 0.004$); a significant interaction was observed between fungicide and pathogen ($P = 0.017$). Pyraclostrobin was the most effective fungicide in reducing lesion lengths of *N. dimidiatum* (Table 2.3); lesion lengths (average lesion length reduction of 53.4%) were significantly smaller than the untreated control ($P < 0.001$), however no significant reductions in lesion length ($P > 0.05$) were observed for azoxystrobin (28.6% reduction), trifloxystrobin (19.9% reduction), or fenbuconazole (12.7% reduction). Trifloxystrobin was the most effective in reducing lesions lengths of *E. citricola* with an average lesion length reduction of 33.0% ($P = 0.01$). Treatments of azoxystrobin (26.0% reduction), pyraclostrobin (22.9% reduction), and fenbuconazole (2.5% reduction) were not found to significantly reduce ($P > 0.05$) lesion lengths caused by *E. citricola*. Fungicide treatment with azoxystrobin (6.0% reduction), fenbuconazole (8.0% reduction), pyraclostrobin (19.7% reduction), and trifloxystrobin (7.9%) did not significantly reduce lesion lengths ($P > 0.05$) caused by *E. microtheca*. No significant reductions in lesion lengths ($P > 0.05$) caused by *Eutypella* sp. were observed for azoxystrobin (14.8% reduction), fenbuconazole (7.5% reduction), or pyraclostrobin (22.8% reduction); treatment with trifloxystrobin (27.6% reduction) was marginally insignificant ($P = 0.06$). No correlation was found between external and internal lesions or branch diameter and internal lesions ($P > 0.05$). Fungal recovery from water control branches was over 70% for the fungi used in this field study; no fungi used in this study

were recovered from untreated control branches. All branches treated with fungicides yielded lower percentages of the inoculated fungi than those of the controls. For *N. dimidiatum* treatments, branches treated with fenbuconazole yielded less of this pathogen (<20%), whereas those branches treated with trifloxystrobin, pyraclostrobin and azoxystrobin had recovery rates between 20-40%. For *Eutypella* spp., branches treated with azoxystrobin, trifloxystrobin and pyraclostrobin had fungal recovery rates between 34-43%. Branches treated with fenbuconazole had an average recovery rate of 68%.

Spore Trapping. Spores of *Eutypella* are present almost all year round in both Riverside (Fig. 2.2.1) and Imperial counties (Fig. 2.2.2). In Riverside Co., the highest amount of spores were trapped during the months of September, January and May and in Imperial Co., the highest amount of spores were trapped during the month of March. Overall more spores were present in Riverside Co. during winter months (December-March), followed by Spring (March-June), Fall (September-December) and finally the lowest amount of spores were present during summer months (June-September). In Imperial Co., the majority of spores were trapped during the Fall and Winter months followed by Spring months and finally the lowest amount of spores were trapped during summer months. No obvious correlation was observed between spores trapped and temperature or precipitation events. Considering data from both locations, spores of *Eutypella* are most abundant during the Fall and Winter months followed by Spring and Summer. No colonies of *N. dimidiatum* were observed on trap plates in any month from either location.

DISCUSSION

To our knowledge, this study is the first comprehensive assessment of various fungicides as potential pruning protectants for potential use in the management of two branch canker and dieback diseases of citrus. *In vitro* fungicide studies show that all tested strobilurins (currently registered for use on citrus in California) were generally more effective in reducing mycelial growth of *N. dimidiatum* and *Eutypella* spp. when compared to other fungicides and when used as a protective treatment, they reduced fungal colonization on citrus wood, particularly external colonization by *N. dimidiatum*. Fluazinam was also found to reduce external colonization (< 20% colonization) by all fungal species tested. However, this fungicide is not currently registered on citrus. Fungicide field data were supported by *in vitro* fungicide data as significant reductions in lesion lengths were observed for branches treated with pyraclostrobin when challenged with *N. dimidiatum* and with trifloxystrobin when challenged with *E. citricola*. No significant reductions in lesions lengths were observed for any fungicide when challenged with *E. microtheca* or *Eutypella* sp. Based on spore trapping studies, spores of *Eutypella* spp. are known to be present almost year-round with the majority of spores captured during Fall and Winter months. This suggests that fungicide applications should be applied just prior to Fall and perhaps again before winter to achieve adequate disease reduction. However, further field studies will be necessary to determine the timing and number of applications for the management of Hendersonula and Eutypella canker of citrus in the desert production areas of California.

In vitro fungicide screens and fungicide field applications showed strobilurins to be most effective in reducing the mycelial growth, colonization, and lesion development of *N. dimidiatum*, particularly pyraclostrobin. Overall, *N. dimidiatum* was most sensitive to all fungicides tested *in vitro* as *N. dimidiatum* had the lowest EC_{50} values overall when compared to *Eutypella* spp. To our knowledge, no fungicide susceptibility screens have been performed for *N. dimidiatum* in citrus prior to this study. However, several other reports on susceptibility of this fungus to fungicides exist in the medical literature (Madrid et al. 2009) and in crop hosts such as pitaya (Ni et al. 2013). Madrid et al. (2009) found the polyene amphotericin to be most effective in inhibiting the growth of *N. dimidiatum*, however several triazoles were also found to inhibit the growth of this fungus. Although triazoles in this study were found to support low EC_{50} values when tested against *N. dimidiatum in vitro*, no significant reduction in lesion length was observed when branches were treated with fenbuconazole. Similar to the results of this study, Ni et al. (2013) found azoxystrobin, pyraclostrobin, and trifloxystrobin to inhibit the mycelial growth of *N. dimidiatum*, however greater inhibition with strobilurins was observed in treatments with a pre-mix product of azoxystrobin and difenoconazole at any discriminatory dose applied in the study; additionally, treatment with pyraclostrobin, trifloxystrobin, and azoxystrobin were found to reduce spore germination in *N. dimidiatum* by 97-100%.

The efficacy of fungicides for the management of canker and dieback diseases caused by members of the Botryosphaeriaceae has been studied in several other crop systems. Studies in almond (Olmo et al. 2017), avocado (Twizeyimana et al. 2013), and

grapevine (Pitt et al. 2012) identified pyraclostrobin as one of the most effective fungicides in reducing fungal growth of several species of Botryosphaeriaceae *in vitro*. However, in field studies, pyraclostrobin was not the most effective fungicide in reducing disease severity when applied as a protectant in almond (Olmo et al. 2017); in avocado, a pre-mix of azoxystrobin and propiconazole was the most effective treatment applied in reducing lesion lengths caused by botryosphaeriaceous fungi, however a reduction in lesion lengths was observed in treatments with pyraclostrobin alone (Twizeyimana et al. 2013). No field trials with pyraclostrobin were conducted by Pitt et al. (2012), however Rolshausen et al. (2010) found pyraclostrobin to be effective in reducing infection of botryosphaeriaceous fungi when applied as a pruning protectant to grapevine. Pyraclostrobin was found to be effective in reducing lesion lengths caused by *N. dimidiatum* in citrus. Although fluazinam was found to be effective in reducing the mycelial growth of *N. dimidiatum in vitro* in this study, it was not tested in the field as this product is not currently registered for use on citrus. The use of strobilurins as pruning protectants against members of the Botryosphaeriaceae is supported by this study and studies from other cropping systems; further work will be necessary to determine the use of pre-mix fungicides containing strobilurins as pruning protectants.

Species of *Eutypella* in this study were generally more susceptible to strobilurins, particularly pyraclostrobin, as these fungicides had lower EC_{50} values compared to other fungicides. Efficacy studies in grapevine revealed pyraclostrobin to be effective in reducing mycelial growth of several diatrypaceous fungi including *E. citricola* (Gramaje et al. 2012) and mycelial growth and ascospore germination of *Eutypa lata* (Sosnowski et

al. 2008). Sosnowski et al. (2008) reported that although pyraclostrobin was capable of reducing colonization of *E. lata* when used as a pruning protectant, it was not the most efficacious product tested and further testing at higher concentrations would be necessary, similar to the study by Rolshausen et al. (2010) indicating pyraclostrobin as the least effective fungicide tested in reducing infection of grapevine pruning wounds by *E. lata*. Although trifloxystrobin significantly reduced lesion lengths caused by *E. citricola* in this study, it did not significantly reduce lesion lengths caused by *E. microtheca* or *Eutypella* sp. Azoxystrobin and pyraclostrobin were also ineffective in significantly reducing lesion lengths of *Eutypella* spp. suggesting that higher rates may be necessary to achieve adequate control, however maximum label rates were applied in this study so it is unlikely that these products would be further investigated for *Eutypella* spp. if higher rates would be necessary. Although Gramaje et al. (2012) reported efficacy of pyraclostrobin in reducing the mycelial growth of *E. citricola*, pyraclostrobin was ineffective in reducing the growth of *E. microtheca* at two concentrations (1 and 10 ppm) tested; additionally, no greenhouse or field studies were conducted to determine the efficacy of these products as pruning protectants. This is contradictory to the results of this study where pyraclostrobin was found to reduce mycelial growth of *E. microtheca in vitro* at the highest concentration (10 ppm) tested in that study. At present, it is unclear why inhibition was not observed for *E. microtheca* in the study by Gramaje et al., however it is possible that isolates tested in that study are insensitive to pyraclostrobin, at least to some degree. In this study, not all isolates responded to fungicides in the same manner in terms of their sensitivities (a range of sensitivities was observed), however

inhibition was observed for all isolates for all products tested (Mayorquin *pers. obser.*). It is imperative that fungicide baseline studies be conducted to study pathogen sensitivity to various compounds, especially with plurivorous pathogens of agricultural commodities such as *Eutypella*. This information will aid in the development of successful chemical management strategies for these pathogens, particularly in areas where there is host overlap.

In this study, fluazinam was effective in reducing mycelial growth of both *N. dimidiatum* and *Eutypella* spp. and was found to reduce fungal colonization (< 20% colonization) of citrus wood pieces by all fungal species tested. *In vitro* susceptibility screens of 10 species of botryosphaeriaceous fungi collected from citrus (Adesemoye et al. 2014) indicated that fluazinam was one of the most effective fungicides in reducing mycelial growth compared to other fungicides (J.S. Mayorquin *unpublished*). Fluazinam is a QiI-fungicide targeting cellular respiration as an uncoupler of oxidative phosphorylation and has been studied as a potential pruning protectant for both botryosphaeriaceaous fungi (Pitt et al. 2012) and diatrypaceous fungi (Gramaje et al. 2012; Sosnowski et al. 2008). Gramaje et al. (2012) and Pitt et al. (2012) found fluazinam to be one of the most effective fungicides in reducing mycelial growth of various canker pathogens at rates ≤ 1.0 mg/L. Pitt et al. (2012) found fluazinam to be one of the most effective treatments in reducing infection by *Diplodia* spp., however Sosnowski et al. (2008) found that although fluazinam could reduce the colonization of *E. lata* in treated grapevine, further studies using higher concentrations would be necessary. Currently, fluazinam is not registered for use on citrus, but it does have registration for other crops

in California. Future studies evaluating the efficacy of fungicides for the management of canker pathogens should include fluazinam as a potential candidate fungicide given the growing evidence of its inhibitory effects, often at low rates.

Spores of *Eutypella* spp. from this study were trapped almost year-round with the majority of spores trapped during the Fall and Winter months; no spores of *N. dimidiatum* were trapped as no colonies resembling this pathogen were observed on agar media after plating spore trap washes. Spore dispersal studies of botryosphaeriaceous fungi in California grapevine (Úrbez-Torres et al. 2010) and avocado (Eskalen at al. 2013) revealed the majority of spores to be trapped during the months of December through February; furthermore, a strong relationship was observed between spore capture and precipitation events in vineyards (Úrbez-Torres et al. 2010) and higher frequencies of spores were captured during or soon after precipitation events in avocado groves (Eskalen et al. 2013). Although there are fewer precipitation events in the Desert Region than compared to other citrus growing regions such as the Coastal-Intermediate Region, it was discovered that spores of *N. dimidiatum* were not trapped at any point during the course of this study; however, the lack of *N. dimidiatum* spore capture may be due to the toruloid form of this fungus being more predominate than the pycnidial form in citrus (Calavan and Wallace 1954). This raises questions regarding the mechanism of dispersal of *N. dimidiatum* in citrus orchards and may suggest that the toruloid form of this fungus, and thus arthrospores, are the primary propagules of this pathogen as its pycnidial form is rarely encountered and no sexual state is known to exist. Spore dispersal studies of the diatrypaceous fungus *E. lata* in apricot (Ramos et al. 1975) and grapevine (Petzoldt et al.

1982) in California show most spores are trapped during the Fall, coinciding with precipitation events. The presence of *Eutypella* spp. almost year-round, despite the lack of abundant precipitation events in the Desert Region, may be due to several factors. Irrigation regimens in citrus orchards in the Desert Region are often extended in response to the desert climate and this increase in irrigation duration may contribute to continued spore dispersal especially in groves where proper grove hygiene is not adhered to as numerous perithecia of *Eutypella* spp. can be found on dead branches littering orchard floors (Mayorquin et al. 2016). Although no correlative studies between irrigation practices and spore dispersal were conducted in this study, a positive relationship was observed between irrigation and spore dispersal of botryosphaeriaceous fungi in vineyards in Riverside Co. (Úrbez-Torres et al. 2010), suggesting that irrigation practices can be as influential as precipitation on spore dispersal. Further studies in this region will be necessary to establish which factor(s) contribute to spore dispersal of *Eutypella* spp. as well as other canker pathogens to develop more relevant and effective management strategies which account for varying cultural practices.

Wounds are known to serve as infection courts for various canker and dieback pathogens (Graham and Menge 2000), however the time of infectivity for these wounds (i.e. pruning wounds) is not known for Hendersonula or Eutypella canker of citrus. Studies in California vineyards on the susceptibility of pruning wounds to *E. lata* revealed that wounds are most susceptible to infection after pruning, but the time of infectivity can continue for up to seven weeks depending on environmental conditions (Munkvold and Marois 1995). In studies on avocado, no significant differences were

observed when branches were inoculated with pathogens 1 day or 1 week after fungicide application (Twizeyimana et al. 2013). As suggested by Sosnowski et al. (2008) and Twizeyimana et al. (2013), long-term field trials might be necessary to evaluate the length of time fungicides can provide protection to pruning wounds. The present study did not evaluate the length of time fungicides can provide adequate protection to pruning wounds from several fungal pathogens, however future studies investigating this parameter would be beneficial to establishing a chemical management strategy for citrus branch canker and dieback. Moreover, unlike grapevine, citrus is not necessarily pruned yearly although hedging of rows is fairly common and this hedging creates wounds on young shoots as well as mechanical damage to woody branches. Therefore, studies looking at broadcast sprays directly after hedging may be more appropriate than targeted fungicide applications on woody branches.

This study demonstrates the efficacy of several fungicides in reducing the growth of *N. dimidiatum* and *Eutypella* spp. *in vitro* and identified strobilurins as the most effective fungicides in reducing the vascular colonization of *N. dimidiatum* and *E. citricola* on citrus when used as pruning protectants. For the chemical management of Hendersonula, pyraclostrobin products are considered strong candidates and will need to be further evaluated for their use as pruning/wounding protectants against *N. dimidiatum*. Further studies are also warranted to identify fungicides which could be used for the management of Eutypella canker of citrus, with emphasis on trifloxystrobin. Studies on the use of fluazinam as a potential wounding protectant for several other canker pathogens in citrus are warranted due to its effectiveness at low rates *in vitro*. The

identification of candidate fungicides for use in the management of citrus branch canker and dieback is a significant step towards an integrated management strategy for these diseases in citrus.

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Table 2.1. Fungicides evaluated in vitro and in the field for citrus dieback caused by *Eutypella* spp. and *N. dimidiatum*

Active ingredient	Chemical group[a]	Trade name	Manufacturer	Field rate
Azoxystrobin	QoI	Abound Flowable	Syngenta Crop Protection	15.5 fl oz/A[bc]
Cyprodinil	AP	Vangard	Syngenta Crop Protection	10 oz/A[b]
Fenbuconazole	DMI	Enable 2F	Dow AgroSciences	8.0 fl oz/A[bc]
Fluazinam	QiI	Omega 500F	Syngenta Crop Protection	13.8 fl oz/A[b]
Pyraclostrobin	QoI	Headline	BASF	15.0 fl oz/A[bc]
Trifloxystrobin	QoI	Gem	Bayer CropScience	3.8 fl oz/A[bc]
Fludioxonil	PP	Graduate	Syngenta Crop Protection	…
Metconazole	DMI	Tourney	Valent U.S.A.	…
Propiconazole	DMI	Tilt	Syngenta Crop Protection	…

[a] AP: Anilino-pyrimidines; DMI: DeMethylation Inhibitors; QiI: Quinone inside Inhibitors QoI: Quinone outside Inhibitors; PP: PhenylPyrroles

[b] Rates applied for protection assay

[c] Rates applied for field trials

Table 2.2. In vitro sensitivity of *N. dimidiatum* and *Eutypella* spp. to select fungicides for potential management of citrus dieback

		EC_{50} for mycelial growth inhibition (µg/ml)[a]			
Fungicide	**Concentrations applied (µg/ml)**	***N. hyalinum***	***E. citricola***	***E. microtheca***	***Eutypella* sp.**
Pyraclostrobin	10	0.001 (0.000) a	0.003 (0.001) a	0.001 (0.001) a	0.048 (0.014) bc
Trifloxystrobin	10	0.002 (0.001) ab	0.007 (0.004) b	0.003 (0.002) ab	0.049 (0.019) bc
Azoxystrobin	10	0.002 (0.001) ab	0.009 (0.005) b	0.006 (0.004) b	0.058 (0.018) c
Fluazinam	10	0.002 (0.001) bc	0.009 (0.007) b	0.004 (0.003) ab	0.029 (0.013) a
Metconazole	100	0.004 (0.003) bcd	0.145 (0.090) c	0.082 (0.070) c	0.053 (0.026) bc
Fludioxonil	10	0.007 (0.002) cd	0.008 (0.004) b	0.005 (0.004) ab	0.033 (0.012) ab
Fenbuconazole	100	0.008 (0.010) cde	0.140 (0.131) c	0.205 (0.206) c	0.158 (0.117) d
Propiconazole	100	0.008 (0.008) cde	0.133 (0.103) c	0.112 (0.125) c	0.213 (0.130) d
Cyprodinil	100	0.091 (0.074) f	NT[b]	NT	NT
HSD (α=0.05)[c]		**0.103**	**0.094**	**0.182**	**0.054**

[a] Means and standard deviations (in parentheses) are for all isolates of each species. Means are ordered based on values for *N. dimidiatum*. Levels not connected by the same letter are significantly different

[b] NT= not tested

[c] Tukey's Honest Significant Difference (HSD)

Table 2.3. Efficacy of select fungicides in reducing lesion lengths caused by *N. dimidiatum* and *Eutypella* spp. in citrus

	Mean percent (%) lesion reduction ± SE							
Treatment	***N. hyalinum***	***P***	***E. citricola***	***P***	***E. microtheca***	***P***	***Eutypella* sp.**	***P***
Azoxystrobin	28.6 ± 6.0	0.35	26.0 ± 3.9	0.07	6.0 ± 2.7	0.99	14.8 ± 5.2	0.41
Fenbuconazole	12.7 ± 5.3	0.97	2.5 ± 4.4	0.99	8.0 ± 3.3	0.99	7.5 ± 5.0	0.76
Pyraclostrobin	53.4 ± 5.4	<0.001	22.9 ± 4.5	0.15	19.7 ± 5.5	0.66	22.8 ± 5.0	0.30
Trifloxystrobin	19.9 ± 4.2	0.64	33.0 ± 3.4	0.01	7.9 ± 4.8	0.92	27.6 ± 4.1	0.06

Mean precent reductions and SE were calculated from fungicide treated branches compared to un-treated (control) branches. *P*-values were determined using Tukey's honest significant difference (HSD) at α=0.05

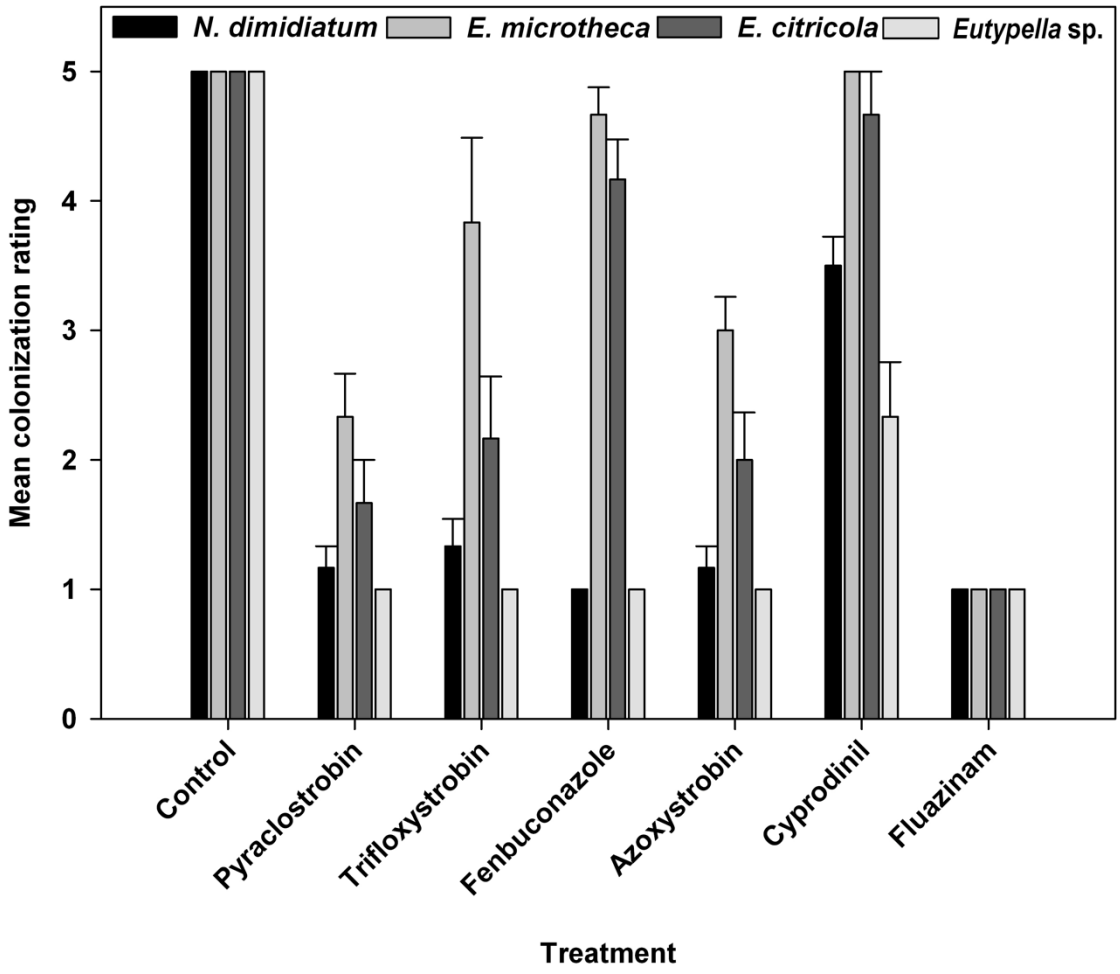

Figure 2.1. Protection of citrus branch segments with several fungicides challenged with *Eutypella* spp. and *N. dimidiatum*. Vertical bars represent mean colonization ratings. Colonization ratings (1 to 5) are as follows: 1, 0-20% branch segment colonized; 2, 21-40% branch segment colonized; 3, 41-60% branch segment colonized; 4, 61-80% branch segment colonized; and 5, 81-100% branch segment colonized.

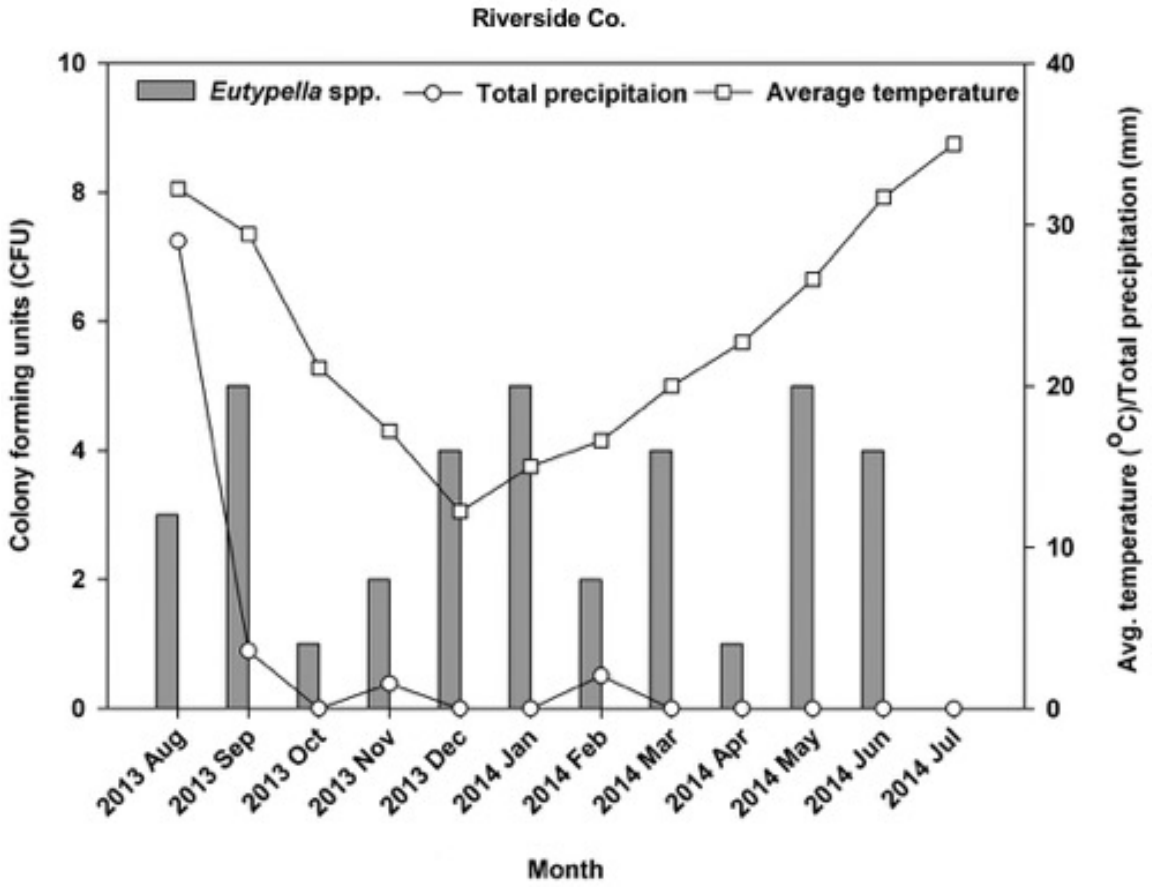

Figure 2.2.1. Monthly spore trap counts with temperature (°C) and precipitation (mm) for Riverside county. Vertical bars represent total number of colony forming units counted from all traps for indicated month.

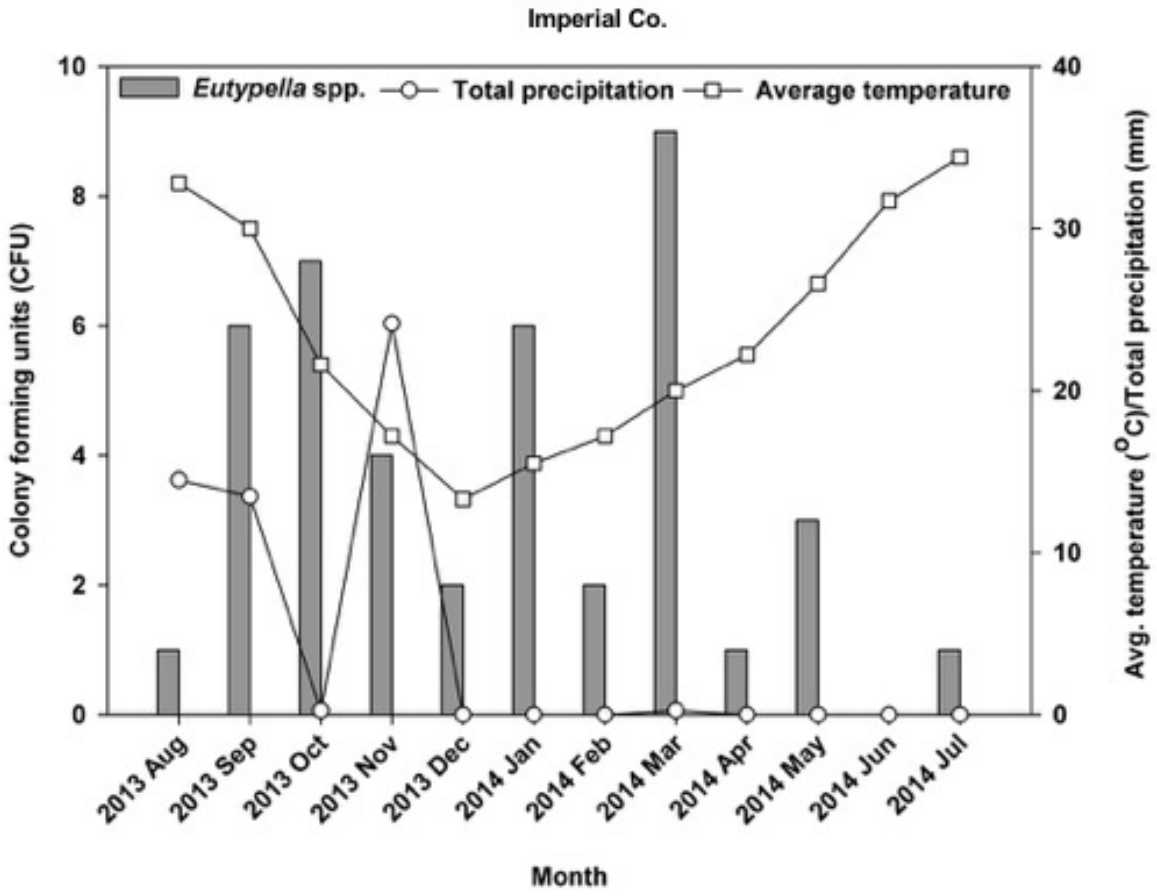

Figure 2.2.2. Monthly spore trap counts with temperature (°C) and precipitation (mm) for Imperial county. Vertical bars represent total number of colony forming units counted from all traps for indicated month.

CHAPTER III. Identification, Pathogenicity, Spore Trapping, and Chemical Management of *Colletotrichum karstii* Associated with Citrus Shoot Dieback in California

ABSTRACT

Colletotrichum species are well-known pathogens of citrus that are associated with leaf and fruit anthracnose diseases, however their role in twig and shoot dieback diseases of citrus has recently become more prominent. Recent isolations from orchards in the Central Valley have revealed *C. gloeosporioides* (3-22%.) and *C. karstii* (3-31%) to be associated with 6-57% of samples showing signs of shoot dieback collected between four counties. Initial pathogenicity tests using clementine (cv. 4B) indicated that both *C. karstii* and *C. gloeosporioides* are capable of producing lesions on citrus stems. Expanded pathogenicity tests revealed *C. karstii* to be the most aggressive fungal species tested as it produced the longest lesions after 15 months. Spore trapping studies indicated that spores of *Colletotrichum* spp. are present during the spring and summer months and dispersal occurs during or soon after rain events with spore counts during trapped months ranging from 1 spore/month in Madera Co., 1-32 spores/month in Kern Co., and 1-152 spores/month in Tulare Co. Fungicide trials determined that pyraclostrobin-containing products were most effective in reducing the prevalence of symptoms associated with this disease in a one-season field trial. These findings confirm *C. karstii* as a new pathogen of citrus in California and highlight the need for further investigation into this pathosystem to determine the extent of this disease throughout all major citrus growing regions in California and the impact these fungi will have on citrus production.

INTRODUCTION

From 2015 to 2016, the United States (U.S.) citrus industry was valued at 3.34 billion dollars with California's citrus production accounting for 47 percent of the overall U.S. production. Much of California's bearing acreage is devoted to orange production, however other citrus varieties of tangerine, mandarin, lemon and grapefruit are grown throughout the state (USDA-NASS, 2016). Since the California citrus industry contributes to over one-third of the total U.S. citrus production, the identification and management of new diseases threatening the state's citrus production is crucial.

Colletotrichum constitutes a large genus of ascomycete fungi which are known for having broad ecological roles ranging from endophytes to plant pathogens. *Colletotrichum* includes some important pathogenic fungi which occur on numerous plant hosts including native and agricultural plant species occurring in tropical and subtropical regions (Cannon et al. 2010; Dean et al. 2012; Hyde et al. 2009b). *Colletotrichum* is well-known for causing various anthracnose diseases, with general anthracnose symptoms including necrotic lesions, often sunken, on various plant parts including stems, leaves, flowers and fruit (Agrios, 2005). Although *Colletotrichum* is primarily described as causing anthracnose diseases, other diseases such as rots caused by *Colletotrichum* spp. have been documented (Cannon et al., 2012; Lenne 2002). The taxonomy of *Colletotrichum* spans well over a century and has gone through many revisions since this time, especially with the advances in DNA technologies, and there are currently over 100 accepted species of *Colletotrichum*. A thorough review on the history of *Colletotrichum* classification can be found in Cannon et al. (2012). Phylogenetic studies focused on three

well established *Colletotrichum* species in the literature (*C. gloeosporioides*, *C. acutatum*, and *C. boninense*) revealed these species belong to species complexes and collectively 71 phylogenetic species were identified from these three species complexes (Damm et al. 2012[a]; Damm et al. 2012[b]; Weir et al. 2012) highlighting the importance of species identification from hosts, especially from new and emerging diseases.

Historically, two species of *Colletotrichum* have been associated with causing anthracnose diseases of citrus (Timmer et al. 2000), *C. gloeosporioides* (Penz.) Penz. & Sacc. and *C. acutatum* J.H. Simmonds. These anthracnose diseases, post-harvest anthracnose, Postbloom Fruit Drop (PFD), and Key lime anthracnose (KLA) are of great importance as postharvest diseases (Damm et al. 2012; Timmer et al. 2000). Additionally, twig dieback (known as withertip) is known to occur in citrus (Klotz 1961) and shoot blight can occur in advanced stages of KLA (Peres et al. 2008). Recent evidence is suggesting that species of *Colletotrichum* previously unknown from citrus are associated with and potentially causing several new diseases of citrus globally, particularly from the *C. boninense* species complex. *Colletotrichum karstii* You L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai (*C. boninense* species complex) has been increasingly reported from anthracnose symptoms of citrus globally and is often found to occur with other *Colletotrichum* spp., particularly *C. gloeosporioides* which is found to be the predominate *Colletotrichum* species associated with citrus (Huang et al. 2013; Peng et al. 2012; Ramos et al. 2016). Despite the predominance of *C. gloeosporioides* associated with citrus from these studies, *C. karstii* has been increasingly reported from anthracnose diseases of other commodities including avocado, mango, and persimmon (Ismail et al.

2015; Lima et al. 2013; Silva-Rojas and Vila-Quezada, 2011; Wang et al. 2016) and is considered the most common and geographically diverse species of its complex (Damm et al. 2012). Although *C. karstii* has been reported from citrus in China, Italy, and Portugal (Aiello et al. 2014; Huang et al. 2013; Peng et al. 2012; Ramos et al. 2016), in the United States *C. karstii* has only been reported from a *Leucospermum* sp. (Crous et al. 2013) and from *Phalaenopsis* spp. (Jadrane et al. 2012). In the rest of North America, *C. karstii* has been reported only from two additional hosts in Mexico, *Annona cherimola* (Damm et al. 2012) and a *Musa* sp. (Velazquez-del Valle et al. 2016).

In California, disease symptoms associated with *Colletotrichum* spp. in various *Citrus* spp. have appeared to be increasing in the San Joaquin Valley citrus growing regions compared to historical observations and based on isolations from diseased plant tissues yielding typical *Colletotrichum* colonies (Eskalen *per obs*.). Symptoms that have been observed include leaf chlorosis, twig dieback, crown thinning, wood necrosis in branches and in some cases death of young plants. However, it is not clear which species are associated with these diseases as *C. gloeosporioides sensu stricto* has been the only species associated with anthracnose diseases of citrus in California. Therefore, the objectives of this study were to: (i) identify which *Colletotrichum* species are associated with canker and dieback of *Citrus* spp. in California; (ii) assess the pathogenicity of *Colletotrichum* spp. on citrus; and (iii) to determine when and under what environmental conditions spores of *Colletotrichum* spp. are released in citrus orchards in California; and (iv) to evaluate fungicides for their efficacy in reducing symptom prevalence in clementine.

MATERIALS AND METHODS

Field survey and fungal isolations. Field surveys were conducted in 10 commercial citrus orchards throughout Madera County (1 orchard), Tulare County (4 orchards), and Kern County (5 orchards) beginning in the spring of 2014 to fall 2015 for Madera, Tulare, and Kern counties and spring 2017 for two orchards in both Tulare and Kern Co. The dominant citrus varieties in citrus orchards from Kern and Tulare counties were 'Clemenules' clementine, 'Fukumoto' and 'Washington' navel, Valencia orange and 'Fisher' navels for Madera County, with an average orchard age of 11 years. Citrus orchards were sampled once during the spring and re-sampled during the fall of that same year for orchards in Madera, Tulare, and Kern Co. Approximately 16 trees were sampled from each orchard during each sampling period, with twigs/shoots collected from trees showing signs of blighted twigs/shoots and cankered shoots. Twig and shoot samples were collected and transported on ice to the laboratory the same day of collection and stored at 5°C until processed for isolations.

Prior to isolation, twig/shoot samples were rinsed with distilled water to remove surface debris and sterilized by spraying samples with 95% ethanol and briefly flaming. To isolate from the margin of necrotic tissues, bark was removed with a flame sterilized paring knife and small pieces of tissue containing both necrotic and healthy wood were plated onto potato dextrose agar (PDA) (Difco Laboratories) amended with 0.01% tetracycline hydrochloride (Fisher Scientific). Plates were incubated at room temperature (~ 23-25°C) in a cabinet in the dark for approximately 5-7 days. Single spore cultures were obtained from colonies with developed acervuli by using a sterile inoculation loop

to scoop chirri from acervuli and streak onto water agar plates. After 24 hours with the aid of a stereomicroscope, isolated germinating conidia were picked off with a sterile inoculating needle and transferred to PDA plates. For long term storage of cultures, mycelial plugs from actively growing colonies were placed in tubes of sterile water and stored at 4°C.

Morphological characterization. Fungi were initially identified to the genus level based on gross colony morphology (colony color, colony growth pattern, growth rate and conidia shape) and those resembling *Colletotrichum* spp. were further studied using published descriptions (Damm et al. 2012). To examine spore morphology, isolates were grown on synthetic nutrient-poor agar medium (SNA), to which filter paper had been added, Oatmeal Agar (OA) and/or PDA to induce conidial production and incubated under continuous fluorescent light for approximately two weeks at an average temperature between 23-25 °C as described by Damm et al. (2012). Conidial masses were scooped up with a toothpick and mounted in a drop of lactoglycerol on a microscope slide. The length and width of approximately 30 conidia per isolate were measured using SPOT Imaging software (Diagnostic Instruments Inc., MI). Appressoria were also measured from cultures using a modified slide culture technique (Harris 1986) with plugs of SNA as the culture medium. Slide cultures were incubated under the conditions described above and mounted in lactoglycerol for microscopic examination. The length and width of at least 15 appressoria were measured for each isolate examined. The mean, standard deviation, minimum, mode and maximum conidial lengths and widths, in

addition to the mean and standard deviation of appressoria lengths and widths, were calculated for each isolate using Microsoft Excel (Microsoft Corporation, WA).

DNA isolation, amplification and phylogenetic analysis. Genomic DNA for each isolate was extracted using a modified method of Cenis (Cenis 1992). Mycelia scraped from agar plates was placed into 2.0 ml conical vials containing extraction buffer and glass beads and were macerated using a FastPrep-24 machine (MP Biomedicals Inc.) at 4 M/s for 30s. Oligonucleotide primers ITS4 and ITS5 (White et al. 1990) were used to amplify the ITS1-5.8S-ITS2 regions of the rDNA, primers T1 and Bt2b (White et al. 1990) were used to amplify a partial region of the nuclear beta-tubulin gene (TUB2), and primers GDF1 and GDR1 (Jacobs et al. 2004) were used to amplify a 200 bp intron of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). Polymerase chain reaction (PCR) was carried out essentially as described by Damm et al. (2012). Reaction mixtures consisted of 1x reaction buffer, 0.2 mM dNTP's, 0.2 μM forward and reverse primers, 2.5 U of standard Taq polymerase (New England Biolabs, MA), and template DNA ranging from 50-100 ng/μl. For ITS, cycling conditions were as follows: an initial preheat at 95°C for two minutes, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min. For TUB2 and GAPDH, cycling conditions were as described by Damm et al. (2012). PCR products were purified using ExoSAP-IT (Affymetrix, CA) following the manufacturer's instructions and sequenced at the Institute for Integrative Genome

Biology of the University of California, Riverside using both forward and reverse primers for each locus.

Sequences were edited using Sequencher 4.6 software (Gene Codes Corp., MI) and local alignments done using ClustalX (Thompson et al. 1997). Phylogenetic analysis was performed with MEGA6 (Tamura et al. 2013) and sequences in the present study (Table 3.1) were compared to known sequences in GenBank (Table 3.2). Separate analyses were run for each gene region dataset in addition to a combined dataset of all three genes after visually checking individual gene tree topology for congruence among the three genes. Maximum parsimony analysis was performed for each dataset using the Subtree-Pruning-Regrafting (SPR) search method with gaps and missing data treated as a complete deletion. Bootstrap values were obtained from 1,000 bootstrap replications and tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasmy index (HI) were recorded. Additionally, Neighbor-joining (NJ) and Maximum likelihood (ML) analyses were performed for the combined dataset using the maximum composite likelihood model with uniform rates (NJ) and the Kimura 2-parameter model with gamma distribution and Nearest-Neighbor-Interchange heuristic method (ML); for both ML and NJ analyses, gaps and missing data were treated as complete deletions and bootstrap values were obtained from 1,000 bootstrap replications.

Pathogenicity tests. Both preliminary and long-term pathogenicity tests were conducted using two-year-old clementine '4B' grafted to Carrizo and maintained in a greenhouse at the University of California, Riverside. For preliminary pathogenicity tests, four trees

were stem wound inoculated six inches above the soil line using a 5mm cork borer to remove bark and expose the cambium beneath. Mycelial plugs were taken from the edge of a one-week-old colony (Table 3.1) and were placed into the wounds; un-infested plugs were used for control inoculations. Wounds were covered with petroleum jelly and wrapped with Parafilm. Plants were maintained in the greenhouse for ten days before being destructively sampled to measure internal vascular necrosis (lesions). Small pieces of tissue from the margin of lesions were plated onto PDA amended with 0.01% tetracycline hydrochloride. Emerging fungal colonies were recorded and identified by sequencing the ITS region as mentioned previously. This experiment was conducted twice. For long term pathogenicity tests, a sterile scalpel was used to remove bark and expose the cambium beneath six inches from the soil line from four plants. A 20 µl drop of a conidial suspension in sterile water ($1x10^6$ spores/ml) was pipetted onto the wound and a sterile 18-gauge needle was inserted through the droplet and into the xylem to facilitate suspension uptake; control plants were inoculated with sterile water. Resulting wounds were covered with petroleum jelly and wrapped with Parafilm. Inoculated plants were maintained in the greenhouse for approximately 15 months (Dec 2015- Mar 2017) before being destructively sampled to measure internal vascular necrosis. Small pieces of tissue from the margin of lesions were plated onto PDA amended with 0.01% tetracycline hydrochloride. Emerging fungal colonies were recorded and identified by sequencing the ITS region.

Spore trapping. Spore trapping was done using glass microscope slide traps as described by Eskalen and Gubler (2001). Traps were placed in five citrus orchards previously surveyed beginning in April 2014 and concluding in November 2014; ten traps were deployed in each orchard for a total of fifty traps. One spore trap was randomly placed on a branch of a tree and traps were collected every two weeks and shipped to the laboratory for further processing. Once received, slide traps were rinsed with sterile de-ionized water and two 100 μl aliquots of this wash were spread plated onto PDA amended with 0.01% tetracycline hydrochloride and incubated in a dark cabinet for five days in order to enumerate fungal species. Fungal colonies resembling *Colletotrichum* were identified by gross colony morphology as described previously, counted and sub-cultured. A single colony was assumed to be derived from a single spore. Weather data (mean temperature, total monthly rainfall and mean relative humidity) were collected from the nearest weather stations from each orchard to determine possible correlation between the release of fungal spores and weather conditions.

***In vitro* fungicide screen and field trial.** Six formulated fungicides were screened using the Spiral Gradient Dilution (SGD) method according to Förster et al. (2004); Three QoI-fungicides (azoxystrobin, pyraclostrobin, and trifloxystrobin), one DMI-fungicide (fenbuconazole), one SDHI-fungicide (fluopyram), and one QiI-fungicide (fluazinam) were chosen for screening. Fungicides were screened against five isolates of each *Colletotrichum* spp. associated with CD: *C. gloeosporioides* and *C. karstii*. Fungicides were applied at various stock concentrations (Table 3.4) with a spiral plater (Spiral

Biotech Inc.) using an exponential deposition mode; control plates were treated with sterile water in the manner above. Fungicide treated plates were incubated for three hours to allow a fungicide gradient to form before a 10µl drop of fungal spore suspensions were radially streaked across plates. Spore suspensions were made by flooding one-week-old cultures of *C. gloeosporioides* and *C. karstii* with 0.01% Triton X100 and loosening spores with a glass rod; resulting spore suspensions were filtered through one layer of sterile Kimwipes® to remove hyphae and adjusted to a final concentration of 10^5 spores/mL with a hemocytometer. Each fungal isolate was replicated twice on a single plate and this experiment was repeated once. Plates were incubated at 25°C for 3 days and EC_{50} values were determined as described by Förster et al. (2004). A one-season field study was established in one block of a commercial clementine orchard (cv. 4B) consisting of ~2800 citrus trees in Tulare Co. Prior to pesticide application, trees were surveyed for symptoms of CD in May 2016 and the prevalence of twig dieback and gumming typical of CD were recorded; treatments were randomly assigned to rows based on CD prevalence and treatments were blocked using a randomized complete block design with each block (half of the citrus orchard block) consisting of all six treatments (including untreated control) with 4 half row replications for each product. Five fungicides (three QoI-fungicides, one biopesticide, and one premix QoI/SDHI-fungicide) were applied at labeled rates (Table 4) with an air-blast sprayer in June 2016 with each product being applied to eight half-rows. Evaluations for CD prevalence were conducted in July, September, and October following fungicide application by recording the presence/absence of twig dieback in combination with gumming for every other tree in a

row (approx. 40 trees per row evaluated) in addition to the presence of other dieback (abiotic) and cardinal direction (east- and west- facing) to determine if CD symptom prevalence was more common on one side of a tree.

Statistical analysis. Lesion lengths for pathogenicity tests were analyzed using JMP Pro 12 (SAS Institute). Levene's test was used to determine the homogeneity of variance between independent trials; no heterogeneity was detected and data from independent trials was combined. Lesion length data was $\log_{10}$ transformed to normalize data, however data from the initial pathogenicity test could not be normalized after transformation and thus lesion lengths were analyzed by a Kruskal-Wallis test with confidence level $\alpha = 0.05$. Mean lesion lengths of fungal inoculated plants compared to control plants were evaluated using the Steel with Control method with confidence level $\alpha = 0.05$. For the long-term pathogenicity test, mean lesion lengths were compared using a one-way analysis of variance. Mean lesion lengths of fungal inoculated plants compared to control plants were evaluated using Dunnett's method with confidence level $\alpha = 0.05$. Data for *in vitro* fungicide screens were tested by ANOVA under a general linear model (GLM) and mean EC_{50} values of all fungicides were compared using Tukey's honest significant difference (HSD) at confidence level $\alpha = 0.05$. Data for the field trial was analyzed using a generalized linear model and comparisons were made between treated groups to control groups using Dunnett's method with confidence level $\alpha = 0.05$. Figures were made using SigmaPlot (version 11; Systat Software Inc.).

RESULTS

Field survey. Twig and shoot dieback symptoms were observed in all 10 orchards surveyed. Main symptoms included twig/shoot dieback, crown thinning and in some cases branch dieback and wood cankers. The single orchard surveyed from Madera County had a low incidence of shoot dieback compared to the other 9 orchards surveyed in this study. Disease was observed on all rootstock/scion combinations sampled in this study: 'Fisher' navel on trifoliate, '4B' clementine on Carrizo, 'Clemenules' clementine on Carrizo, 'Fukumoto' navel on carrizo, and 'Washington' navel on troyer, and Valencia on trifoliate. From all 10 orchards, 274 symptomatic samples were collected; Out of the 274 samples collected during this survey, 88 samples yielded species of *Colletotrichum*. Approximately 6% of samples collected from Madera County yielded *Colletotrichum* spp.; approximately 57% of samples collected from Tulare County yielded *Colletotrichum* spp.; and approximately 18% of the samples collected from Kern County yielded *Colletotrichum* spp. *Colletotrichum karstii* was recovered from approximately 3% of samples collected from Madera County, approximately 12% of samples collected from Kern County, and 31% from samples collected from Tulare County. *Colletotrichum gloeosporioides* was recovered from approximately 3% of samples collected from Madera County, approximately 10% of samples collected from Kern County, and approximately 22% of samples collected from Tulare County. Other fungi isolated from symptomatic tissues were identified using gross colony characteristics and sequencing the ITS regions of representative isolates as described previously and included *Alternaria* spp., *Penicillium* spp., *Fusarium* spp., *Quambalaria* spp., Botryosphaeriaceae spp. and

Diatrypaceae spp. Botryosphaeriaceae spp. were recovered from 27 out of the 274 samples collected and Diatrypaceae spp. were recovered from eight out of the 274 samples collected. Both Botryosphaeriaceae and Diatrypaceae spp. were never co-isolated with species of *Colletotrichum*.

Morphological characterization. Isolates of *Colletotrichum* from this study could be divided into two distinct groups based on colony morphology. The first group consisted of colonies which were white to cream colored, on reverse a pale orange color was present and various conidiomata (orange colored) developed over time throughout the colony. Conidia from these isolates were hyaline, straight and cylindrical with rounded ends. These isolates closely matched published descriptions of *C. karstii* (Damm et al. 2014) (Table 3.3). The second group consisted of colonies which were cream to gray colored, with dark gray aerial mycelium developing over time, on reverse colonies were cream to gray. Conidiomata (orange colored) were abundant throughout the colony. Conidia from these isolates were hyaline, straight and cylindrical with rounded ends. These isolates resembled descriptions of *C. gloeosporioides* (Damm et al. 2014).

Phylogenetic analysis. Individual analysis of the ITS and GAPDH datasets yielded similar topologies (data not shown). Isolates from this study formed one major clade, which includes vouchered specimens of *C. karstii*, with 98% bootstrap support (ITS) and 89% bootstrap support (GAPDH), however vouchered specimens of *C. phyllanthi* and *C. annellatum* also grouped in this clade. Individual analysis of the TUB2 dataset showed

isolates from this study clustering in one major clade with 89% bootstrap support. Vouchered specimens of *C. phyllanthi* and *C. annellatum* did not cluster in this group as they did in the ITS and GAPDH phylogenetic trees. Due to the overall similar tree topologies produced by the individual datasets, all three genes were combined into one dataset for analysis. The combined data set consisted of 1078 nt, with 737 conserved sites, 324 variable sites and 275 parsimony-informative sites. Maximum parsimony analysis generated 10 parsimony trees. Tree scores were as follows: CI=0.75, RI=0.96 and RCI=0.72. The combined data set (Fig. 3.1) revealed that isolates from this study grouped into two well supported clades. The first major clade (96% bootstrap support) consisted of isolates from this study and other vouchered specimens of *C. karstii* which included reference isolate CBS106.91 of *C. karstii*. *Colletotrichum phyllanthi* and *C. annellatum* were the most closely related species to *C. karstii*. The second major clade (99% bootstrap support) consisted of the remaining isolates from this study and the type specimen of *C. gloeosporioides* (CBS112999). Maximum likelihood and neighbor-joining analysis of the combined dataset produced trees (data not shown) with similar topology to the MP analysis; two major clades were formed with the first clade consisting of isolates from this study and other vouchered specimens of *C. karstii* with bootstrap values of 88% (ML and NJ) and the second clade consisting of isolates from this study and the type specimen of *C. gloeosporioides* with bootstrap values of 99% (ML) and 94% (NJ). Little genetic variation was observed within isolates of the same species based on these phylogenetic analyses.

Pathogenicity tests. Ten days after inoculation, no external lesions, indicated by depressed, necrotic bark surrounding the inoculation site were present on shoots, however fruiting bodies resembling those of *Colletotrichum* were present surrounding the inoculation site on more than half of the plants inoculated with *C. karstii* and only one plant from those inoculated with *C. gloeosporioides*. There was a significant difference ($\chi^2 = 17.627$; $P < 0.001$) in lesion lengths produced between the various fungal treatments (Fig. 3.2); no internal lesions were observed in control shoots; however, a wound response was observed. Lesions lengths produced by both *Colletotrichum* spp. were brown to chocolate brown in color and extended from both ends of the inoculation point. A significant difference in lesion lengths was observed for both *C. karstii* ($P = 0.003$) and *C. gloeosporioides* ($P = 0.015$). Fungal recovery of *Colletotrichum* from inoculated shoots ranged between 25-100%; no *Colletotrichum* spp. were recovered from control shoots. Fifteen months after inoculation, plants inoculated with either *Colletotrichum* spp. showed no dieback symptoms nor were any external lesions observed surrounding the site of inoculation. Unlike the initial pathogenicity test, no fruiting bodies were observed at the point of inoculation. Internal vascular necrosis was observed in plants inoculated with *Colletotrichum* spp. (Fig. 3.3) which ranged in color from brown to chocolate brown and extended from both ends of the inoculation point; no lesions were observed in control plants; however, a wound response was observed. A significant difference [$F(2,10) = 18.625$, $P < 0.001$] was observed between fungal treatments and there was a significant difference in lesion lengths for lesions produced by *C. karstii* ($P < 0.001$) and *C.*

gloeosporioides (P = 0.035). Fungal recovery from inoculated shoots ranged between 25-100% and no *Colletotrichum* spp. were recovered from control plants.

Spore trapping. The majority of *Colletotrichum* spp. spores were trapped during the Spring (Mar-Jun) and Summer (Jun-Sep) months for at least two months. In Madera County (Fig. 3.4.1), spores (total spores counted) of *Colletotrichum* spp. were trapped in July and September (1 spore each month); in Kern County (Fig. 3.4.2), spores of *Colletotrichum* were trapped in May (1 spore), June (2 spores) and September (32 spores); in Tulare County (Fig. 3.4.3), spores of *Colletotrichum* were trapped in March (152 spores), April (25 spores), May (44 spores), June (1 spore), September (11 spores) and October (1 spore). The majority of fungal spores were trapped during or after precipitation events; In Kern County, the majority of spores were trapped closely following a precipitation event in September and May and in Tulare County the majority of spores were trapped during precipitation events between March and May and September through October. Across all locations, the majority of spores were trapped when average temperatures were between 15-25° C.

***In vitro* fungicide screen and field trial.** *In vitro* fungicide experiments revealed that all products tested *in vitro*, except for fluopyram, could reduce fungal growth of *Colletotrichum* spp. at the concentrations tested (Table 3.4). EC_{50} values for spore germination inhibition and mycelial inhibition for the strobilurins and fenbuconazole did not differ significantly ($P > 0.05$) and thus only EC_{50} values for mycelial inhibition are presented; spore germination inhibition was not recorded for fluazinam or fluopyram.

There was a significant difference ($P < 0.05$) between EC_{50} values of fungal species in response to chemical treatment. Pyraclostrobin produced the lowest EC_{50} values overall followed by fluazinam, trifloxystrobin, azoxystrobin, and fenbuconazole. Strobilurins had lower EC_{50} values (approximately <100-fold lower) than fenbuconazole values, however the three strobilurins did not differ significantly from each other ($P > 0.05$) nor from fluazinam. For the field study, symptoms of CD were observed in approximately 10% of trees evaluated for the presence of twig dieback accompanied by gumming prior to pesticide application; dieback caused by shading was also present, however it was more common than dieback associated with CD or other canker pathogens (data not shown). No significant difference ($P > 0.05$) was observed between CD symptom prevalence for east- or west-facing sides of trees, therefore direction was not considered in further analyses. After pesticide application, products containing pyraclostrobin as an active ingredient (Headline® and Pristine®) were capable of reducing CD symptoms for one month (Fig. 3.5) when tested in the field. Pristine® treatment showed a significant reduction ($P = 0.004$) in CD symptoms during the month of July when compared to untreated (control) trees; however, this was the only month in which Pristine® treated trees showed a reduction in CD symptom. Headline® treated trees showed a significant reduction ($P < 0.05$) in CD symptoms for September when compared to untreated control trees. All other treatments showed higher disease prevalence when compared to untreated controls for the months of July and September; CD symptom prevalence for all pesticide treatments were found to be higher than the untreated control during the month of October (data not shown).

DISCUSSION

This is the first study to report the finding of *Colletotrichum karstii* as a pathogen of citrus in the state of California within the San Joaquin Valley and warrants further studies of anthracnose diseases occurring in the other three main citrus growing regions (Coastal-Intermediate, Interior, and Desert) within the state. Both morphological and molecular identification as well as pathogenicity tests on mandarin confirm that *Colletotrichum karstii* is a putative new pathogen of citrus in CA. This study also confirms the presence of *C. gloeosporioides* within the state and that both species are associated with various anthracnose symptoms. Moreover, both species of *Colletotrichum* reported herein can be found co-infecting symptomatic tissues. A fungicide field trial determined fungicides containing pyraclostrobin to be the most effective in reducing symptom prevalence compared to non-treated trees for one month post application.

The association of *C. karstii* with citrus twig and shoot dieback in California represents a significant finding as a new pathogen of citrus in the state and to our knowledge a new pathogen of citrus in the United States. In citrus, *Colletotrichum* is synonymous with anthracnose diseases and is attributed to *C. gloeosporioides* and *C. acutatum* which are considered important foliar and fruit pathogens (Timmer et al. 2001). Although symptoms of anthracnose caused by *C. gloeosporioides* in citrus include twig dieback, leaf drop and post-harvest decay, a progression to shoot dieback and association with wood cankers has not been observed (Timmer et al. 2001; J.E. Adaskaveg, personal communication). However, shoot blight is a symptom of Key lime anthracnose caused by *C. acutatum*, but this disease affects only Key lime (Peres et al. 2008). Shoot dieback

caused by species of *Colletotrichum* is not a new phenomenon however; several studies have shown the ability of *Colletotrichum* to cause shoot dieback in olive, red stinkwood, poplars, camellia, coprosma, almond, apple and persimmon (Moral et al. 2009; Mwanza et al. 1999; Marks et al. 1965; Sun et al. 2014; Forbes and Pearson, 1987; Adaskaveg and Förster, 2000; Roberts 1915 and Zhang 2008). Recently, Ramos et al. (2016) determined *C. karstii*, in addition to *C. gloeosporioides*, to be preferentially associated with a twig dieback of lemon in Portugal and found both species capable of causing dieback and branch necrosis of sweet orange and mandarin. Although results from pathogenicity tests in this study did not reproduce the dieback observed in commercial citrus orchards, the ability of both *Colletotrichum* spp. to cause internal vascular necrosis of mandarin supports the findings of Ramos et al. and confirms the status of *C. karstii* as an emerging pathogen of citrus. At present, it is unclear if *C. karstii* will play major roles in leaf anthracnose in California citrus since it has been isolated from leaf lesions and confirmed to infect leaves of several citrus varieties (Peng et al. 2012; Ramos et al. 2016) or pre/post-harvest fruit decay as *C. karstii* has been reported as causing preharvest anthracnose on sweet orange (Aiello et al. 2014). The climate of California and the cultural practices implemented in the state may not be conducive to leaf and fruit infection which may explain the lack of leaf and fruit symptoms encountered during field surveys in this study.

During this study, *C. karstii* was isolated from citrus wood canker samples which resembled wood canker symptoms typical of those caused by *Botryosphaeria* and its allied members (Adesemoye et al. 2013). Isolations from the wood canker samples

collected during this study yielded only *C. karstii and C. gloeosporioides* suggesting these species may act as canker pathogens or as secondary invaders, or possibly saprobes, colonizing tissues previously killed by branch canker pathogens like *Botryosphaeria spp*. The latter seems unlikely as no *Botryosphaeria* spp. or other canker pathogens typically associated with citrus were recovered from those samples. However, it is unclear at this time if *C. karstii* is the primary pathogen responsible for the branch canker observed in this study. In hosts such as apple and persimmon, anthracnose symptoms can include both shoot dieback and wood cankers (Roberts 1915 and Zhang 2008). In these cases, the dieback observed in twig and shoots can also be seen to progress into woody branches if the infection is severe. Pathogenicity results from this study confirm *C. karstii* as a pathogen of citrus and suggest that this fungus may in fact be responsible for causing typical branch canker symptoms (gumming, depressed bark with the presence of fruiting bodies, and vascular necrosis) observed in lath house inoculated plants. Nevertheless, long term field pathogenicity studies will be necessary to determine the severity of vascular cankers caused by these fungi.

Colletotrichum karstii has a worldwide distribution with known occurrences throughout Africa, Asia, North and South America, Europe, and Oceania on a variety of plant species including agricultural commodities such as apple (Velho et al. 2014), avocado (Velazquez-del Valle et al. 2016), citrus (not in CA), mango (Lima et al. 2013), olive (Schena et al. 2014) and pistachio (Lichtemberg et al. 2017) and is considered the most common and diverse 'species' in the *C. boninense* complex (Damm et al. 2012). However, with respect to *C. karstii* associated with citrus, this 'species has only been

reported from China, Italy and Portugal (Aiello et al. 2014; Huang et al. 2013; Peng et al. 2012; Ramos et al. 2016). *Colletotrichum karstii* is a plurivorous pathogen and within citrus specifically, it does not appear to be host specific since it was found on several varieties of citrus during this study in addition to other studies (Huang et al. 2013; Peng et al. 2012; Ramos et al. 2016). Damn et al. (2012) reported high sequence variability within strains of *C. karstii* which were sequenced from various hosts throughout the world, however in this study, strains collected from various citrus species and locations throughout California showed very little genetic variability. It is unknown at this time if this lack of genetic variability could be attributed to a clonal population of *C. karstii* on citrus in California, however sequencing individual loci may not provide enough genetic resolution to answer this question, therefore more robust methods such as AFLP or RAD-sequencing will be necessary to further characterize the population of *C. karstii* infecting citrus in California.

Although little is known regarding the epidemiology of this pathogen on citrus, several factors are likely important for the dissemination and progression of this disease. Relative humidity and precipitation in citrus orchards in California play an important role in the epidemiology of *Colletotrichum* infection whereby conidia dispersed by rain and humidity are conducive to pathogen spread (Jeffries et al. 1990). Our spore trap study showed that spore trapping of *Colletotrichum* species occurred most frequently during the months with the highest precipitation (Fig. 4), however *Colletotrichum* spp. were not always correlated with rainfall. Similar results were found with other spore traps studies in California (Eskalen and Gubler 2001; Eskalen et al. 2013; Urbez-Torres et al. 2010).

The rainy season during our spore trap surveys confirmed that spores of *Colletotrichum* spread as airborne inoculum primarily during the winter and spring months in California and coincide with or soon after precipitation events and also by irrigation sprinkler. However, most spores were trapped during months when precipitation was short, but intense. Several other studies involving spore trapping also suggest that large number of spores of *Colletotrichum* spp. are produced and released during prolonged precipitation events (Hunter and Buddenhagen 1972) or during short, intense precipitation events (Dodd et al. 1989).

Wounding is also known to predispose plants to infection by *Colletotrichum* (Muimba-Kankolongo and Bergstrom, 1992; Shaw 1995) and an increase in susceptibility to both infection and lesion formation of *Protea* to *Colletotrichum* spp. was observed when plants were wounded (Lubbe et al. 2006). Lubbe et al. also showed that the phenological state of the host is an important factor for the pathogenicity of *Colletotrichum* since inoculations of shoots made during winter with *Colletotrichum* spp. did not develop lesions (2006). During this study, symptoms were observed during the late spring and summer months, with no new symptoms being observed into fall, winter and early spring. This may suggest that young, tender flush is necessary for initial pathogen colonization as late spring through the summer months would coincide with citrus flushing. Further studies will be necessary to elucidate the aspects which are conducive to pathogen infection and persistence in this pathosystem.

Currently no strategies exist for the management of this emerging disease in citrus, however the *in vitro* fungicide screen and field trial in this study determined that

pyraclostrobin was most effective in reducing fungal growth *in vitro* and when used in the field alone or in combination with another fungicide, were effective in reducing symptom prevalence of Colletotrichum Dieback. Mondal et al. (2005) determined that ED_{50} (effective dose) values for *C. acutatum* on citrus were lower for pyraclostrobin than for azoxystrobin; additionally, fenbuconazole was found to be more active against *C. acutatum* than azoxystrobin, but less active than pyraclostrobin. These results support the findings of this study where pyraclostrobin was found to be most effective *in vitro*, however no field studies were conducted by Mondal et al. nor were these fungicides screened against *Colletotrichum* species present in this study. When pyraclostrobin and pyraclostrobin + boscalid were applied as preharvest treatments for postharvest decay of citrus caused by *C. gloeosporioides*, Salvatore and Ritenour (2007) found these treatments to significantly reduce anthracnose incidence in grapefruit; It is unclear if foliar anthracnose was reduced in the study by Salvatore and Ritenour, however these results support the findings of this study in that pyraclostrobin containing products were more effective in reducing symptom prevalence of CD. Although it is unclear if CD is or will become an important fruit decay disease in California, it may be possible that the fungicides used in controlling foliar symptoms of CD may be of use in the management of fruit decay if caused by the species of *Colletotrichum* collected from this study. Although the use of fungicides for the management of CD is continuing, adherence to cultural practices recommended for the management of canker and dieback pathogens should be followed. These practices include maintaining trees in good condition through appropriate irrigation regimens and proper fertilization, removal of infested twigs and

branches during dry periods followed by immediate disposal of infested material, and sanitizing pruning equipment. These methods will likely become part of an integrated pest management strategy for CD in California in addition to chemical management strategies as further chemical studies are ongoing.

This study serves as a baseline for future research regarding the role of *Colletotrichum* spp., specifically *C. karstii*, as a potentially important pathogen of citrus. Research focused on the biology of this pathogen as well as the epidemiology will further our understanding of this pathosystem as it may relate to citrus anthracnose and will provide information that is essential in the management of this new disease. Additionally, the identification of pyraclostrobin as a potential fungicide for use in the chemical management of Colletotrichum Dieback is promising for the California citrus industry and further trials should be considered in further exploring the use of pyraclostrobin-containing products for the decrease of disease prevalence in commercial citrus.

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Table 3.1. Representative isolates of *Colletotrichum* spp. collected from citrus shoot cankers in California

Species	Isolate[b]	County	Host	Cultivar	GenBank accession[a]		
					ITS	GAPDH	TUB2
Colletotrichum karstii	UCR1761	Tulare	*Citrus sinensis*	Cara Cara	KY076528	KY304050	KY086311
C. karstii	UCR1763	Tulare	*C. sinensis*	Cara Cara	KY076529	KY304051	KY086312
C. karstii	UCR2263	Madera	*C. sinensis*	Fisher	KY076530	KY304052	KY086313
C. karstii	UCR2264	Madera	*C. sinensis*	Fisher	KY076531	...	...
C. karstii	UCR2275	Madera	*C. sinensis*	Fisher	KY076532	...	...
C. karstii	UCR2456	Tulare	Spore trap	...	KY076533	...	...
C. karstii	UCR2457	Tulare	Spore trap	...	KY076534	...	...
C. karstii	UCR2473	Tulare	Spore trap	...	KY076535	...	...
C. karstii	UCR2475	Tulare	Spore trap	...	KY076536	...	...
C. karstii	UCR2510	Tulare	Spore trap	...	KY076537	...	...
C. karstii	UCR2545	Tulare	Spore trap	...	KY076538	...	...
C. karstii	UCR2553	Tulare	*C. reticulata*	Clemenules	KY076539	...	...
C. karstii	UCR2556	Tulare	*C. reticulata*	Clemenules	KY076540	...	...
C. karstii	UCR2563	Tulare	*C. reticulata*	Clemenules	KY076541	KY304053	KY086314
C. karstii	UCR2571	Tulare	*C. reticulata*	4B	KY076542	KY304054	KY086315
C. karstii	UCR2572	Tulare	*C. reticulata*	4B	KY076543	...	...
C. karstii	UCR2576	Tulare	*C. reticulata*	4B	KY076544	KY304055	KY086316
C. karstii	UCR3552	Kern	*C. reticulata*	Clemenules	KY076545	KY304056	...
C. karstii	UCR3553[c]	Kern	*C. reticulata*	Clemenules	KY076546	KY304057	KY086317
C. karstii	UCR3554	Kern	*C. reticulata*	Clemenules	KY076547	KY304058	KY086318
C. karstii	UCR3561	Kern	*C. reticulata*	Clemenules	KY076548	KY304059	KY086319
C. gloeosporioides	UCR2544	Tulare	Spore trap	...	KY076563	...	...
C. gloeosporioides	UCR2552[c]	Tulare	*C. reticulata*	Clemenules	KY076564	KY304073	KY086333
C. gloeosporioides	UCR2564	Tulare	*C. reticulata*	Clemenules	KY076565	...	...
C. gloeosporioides	UCR2569	Tulare	*C. reticulata*	Clemenules	KY076566	KY304074	KY086334
C. gloeosporioides	UCR2575	Tulare	*C. reticulata*	4B	KY076567	KY304075	KY086335
C. gloeosporioides	UCR3551	Kern	*C. reticulata*	Clemenules	KY076568	KY304076	KY086336

[a] ITS = internal transcribed spacer; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; TUB2 = beta-tubulin

[b] Isolate codes correspond to University of California, Riverside (UCR)

[c] Isolates used in pathogenicity tests

Table 3.2. Sequences and descriptions of fungal species obtained from GenBank for use in the phylogenetic study

Species	Isolate	Country	Host	GenBank accession[a]		
				ITS	GAPDH	TUB2
Colletotrichum boninense	CBS112115	Australia	*Leucospermum* sp.	JQ005160	JQ005247	JQ005594
C. boninense	CBS123755[b]	Japan	*Crinum asiaticum* var. *sinicum*	JQ005153	JQ005240	JQ005588
Colletotrichum sp.	CBS123921	Japan	*Dendrobium kingianum*	JQ005163	JQ005250	JQ005597
C. cymbidiicola	CBS123757	Japan	*Cymbidium* sp.	JQ005168	JQ005255	JQ005602
C. cymbidiicola	CBS128543	New Zealand	*Cymbidium* sp.	JQ005167	JQ005254	JQ005601
C.oncidii	CBS129828[b]	Germany	*Oncidium* sp.	JQ005169	JQ005256	JQ005603
C.oncidii	CBS130242	Germany	*Oncidium* sp.	JQ005170	JQ005257	JQ005604
C. toruosum	CBS102667	New Zealand	*Passiflora edulis*	JQ005165	JQ005252	JQ005599
C. toruosum	CBS128544[b]	New Zealand	*Solanum melongena*	JQ005164	JQ005251	JQ005598
C. brassicicola	CBS101059[b]	New Zealand	*Brassica oleracea* var. *gemmifera*	JQ005172	JQ005259	JQ005606
C. beeveri	CBS128527[b]	New Zealand	*Brachyglottis repanda*	JQ005171	JQ005258	JQ005605
C. colombiense	CBS129817	Colombia	*P. edulis*	JQ005173	JQ005260	JQ005607
C. colombiense	CBS129818[b]	Colombia	*P. edulis*	JQ005174	JQ005261	JQ005608
C. petchii	CBS118193	China	*Dracaena sanderana*	JQ005227	JQ005314	JQ005661
C. petchii	CBS118774	China	*D. sanderana*	JQ005225	JQ005312	JQ005659
C. annellatum	CBS129826[b]	Colombia	*Hevea brasiliensis*	JQ005222	JQ005309	JQ005656
C. phyllanthi	CBS175.67[b]	India	*Phyllanthus acidus*	JQ005221	JQ005308	JQ005655
C. karstii	CBS106.91	Brazil	*Carica papaya*	JQ005220	JQ005307	JQ005654
C. karstii	CBS110779	South Africa	*Eucalyptus grandis*	JQ005198	JQ005285	JQ005632
C. brasiliense	CBS128501[b]	Brazil	*P. edulis*	JQ005235	JQ005322	JQ005669
C. brasiliense	CBS128528	Brazil	*P. edulis*	JQ005234	JQ005321	JQ005668
C. parsonsiae	CBS128525[b]	New Zealand	*Parsonsia capsularis*	JQ005233	JQ005320	JQ005667
C. hippeastri	CBS125377[b]	China	*Hippeastrum vittatum*	JQ005230	JQ005317	JQ005664
C. hippeastri	CBS125376	China	*H. vittatum*	JQ005231	JQ005318	JQ005665
C. dacrycarpi	CBS130241[b]	New Zealand	*Dacrycarpus dacrydioides*	JQ005236	JQ005323	JQ005670
C. constrictum	CBS128503	New Zealand	*S. betaceum*	JQ005237	JQ005324	JQ005671
C. constrictum	CBS128504[b]	New Zealand	*Citrus limon*	JQ005238	JQ005325	JQ005672
C. gloeosporioides	CBS112999[b]	Italy	*C. sinensis*	JX010152	JX010056	JX010445

[a] ITS = internal transcribed spacer; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; TUB2 = beta-tubulin

[b] Denotes type-specimen

Table 3.3. Conidial dimensions of representative *Colletotrichum* spp. from citrus shoot cankers in this study and comparison with pervious studies

		Conidial size (µm mean ± SD)[a]			Appressoria size (µm mean ± SD)[b]	Previous Studies (µm)		
Species	**Isolate**	**PDA[c]**	**SNA**	**OA**	**SNA**	**Conidia**	**Appresorria**	**Source**
Colletotrichum karstii	UCR3550	15.3±1.1 x 5.0±0.3	16.1±0.8 x 5.2±0.2	14.8±0.8 x 5.2±0.2	…			
C. karstii	UCR3552	14.3±0.8 x 5.2±0.4	14.0±1.3 x 5.6±0.5	12.4±0.8 x 5.8±0.4	8.0±1.6 x 6.1±1.1			
C. karstii	UCR3553	13.6±0.8 x 5.9±0.3	13.6±1.2 x 5.6±0.5	14.0±0.6 x 5.8±0.4	9.2±1.7 x 5.5±0.6	14.5-17.0 x 5.0-6.5	8.9±2.9 x 5.4±1.5	Damm et al. 2012
C. karstii	UCR1717	13.5±1.1 x 7.5±0.3	…	…	…			
C. karstii	UCR1763	13.5±1.1 x 7.5±0.3	…	…	…			
C. gloeosporioides	UCR2410	13.5±1.1 x 7.5±0.3	16.3±0.6 x 5.2±0.4	15.6±0.7 x 5.4±0.3	11.9±2.4 x 6.5±1.0			
C. gloeosporioides	UCR2411	13.5±1.1 x 7.5±0.3	16.2±1.2 x 5.2±0.5	15.3±0.7 x 5.4±0.4	10.5±1.9 x 7.0±1.3			
C. gloeosporioides	UCR2412	13.5±1.1 x 7.5±0.3	15.8±0.9 x 5.2±0.4	15.1±0.3 x 5.4±0.3	11.0±2.2 x 6.7±1.3			
C. gloeosporioides	UCR2552	13.5±1.1 x 7.5±0.3	15.5±1.4 x 5.2±0.4	14.4±1.2 x 5.1±0.5	11.7±1.9 x 7.3±1.6	10-16 x 5-7	5-8 (diameter)	Timmer et al. 2000
C. gloeosporioides	UCR2564	13.5±1.1 x 7.5±0.3	15.3±1.3 x 4.6±0.5	14.3±1.0 x 4.7±0.3	11.0±2.3 x 6.8±1.5			
C. gloeosporioides	UCR2569	13.5±1.1 x 7.5±0.3	15.4±0.8 x 4.7±0.4	15.2±1.9 x 5.1±0.4	11.6±2.8 x 7.2±1.3			
C. gloeosporioides	UCR3551	13.5±1.1 x 7.5±0.3	15.5±0.7 x 4.6±0.3	14.3±0.9 x 4.9±0.4	…			

[a] Conidial means and standard deviation (SD) were determined from 30 conidia for each isolate

[b] Appressoria means and standard deviation (SD) were determined from 15 appressoria for each isolate

[c] PDA = Potato dextrose agar; SNA = Synthetic nutrient-poor agar; OA = Oatmeal agar

Table 3.4. EC_{50} values of fungicides tested *in vitro* and field rates for C*olletotrichum* spp. associated with Colletotrichum Dieback

				C. karstii	***C. gloeosporioides***	
A.I.	**Trade name**	**Manufacturer**	***In vitro* concentration (*μ*g/ml)**	**EC_{50} (*μ*g/ml)**	**EC_{50} (*μ*g/ml)**	**Field rate**
pyraclostrobin	Insignia	BASF	10	0.002±0.001 a	0.002±0.001 a	8 oz/A
fluazinam	Omega 500F	Syngenta	10	0.003±0.001 a	0.006±0.002 a	…
trifloxystrobin	Gem 500SC	Bayer	10	0.008±0.004 a	0.008±0.004 a	3.8 oz/A
azoxystrobin	Abound Flowable	Syngenta	10	0.039±0.019 a	0.055±0.021 a	12 oz/A
fenbuconazole	Enable 2F	Dow Agro	500	0.518±0.201 b	0.622±0.252 b	…
fluopyram	Luna Privelage	Bayer	5000	NI	NI	…
boscalid+pyraclostrobin	Pristine	BASF	…	…	…	16 oz/A
B. subtilis	Serenade ASO	Bayer	…	…	…	2 qt/A

Levels not connected by the same letter are significantly different using Tukey's honest significant difference (HSD) ($\alpha = 0.05$).

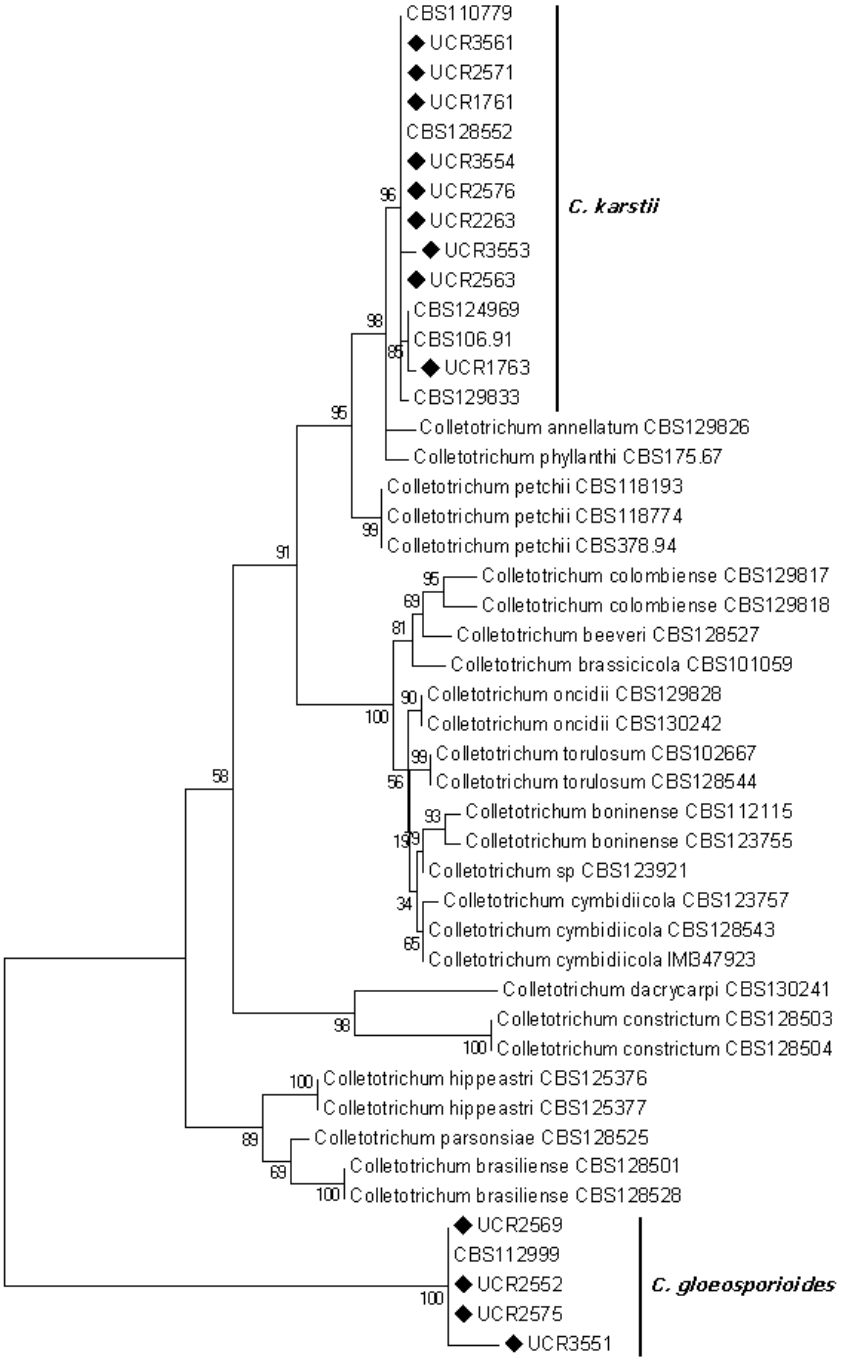

Figure 3.1. One of the most parsimonious unrooted trees based on internal transcribed spacer 1, 5.8S ribosomal DNA, ITS2 (ITS); 200 bp intron of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH); and partial β-tubulin gene region (TUB2) sequences for isolates of species of the *C. boninense* species complex inferred from maximum parsimony analysis. Numbers on branches are bootstrap values from 1,000 bootstrap replicates. Diamonds indicate *Colletotrichum* spp. isolates from this study.

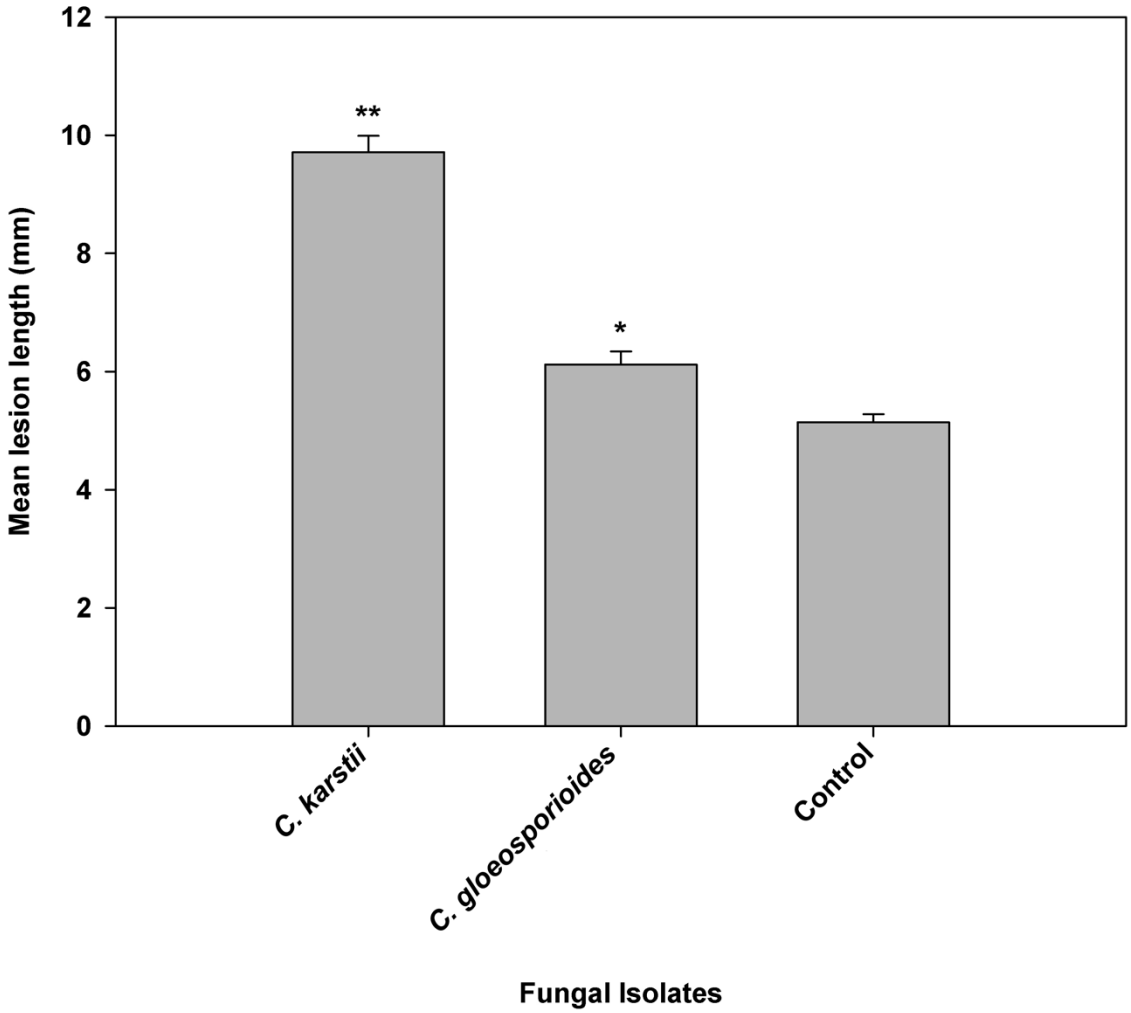

Figure 3.2. Pathogenicity of *Colletotrichum* spp. on '4B' clementine after 10 days. Vertical lines represent standard error of the mean and multiple comparisons were performed using Steel with Control method (α= 0.05). Asterisks (*) denote the following significance: '*' $P < 0.05$ and '**' $P < 0.01$.

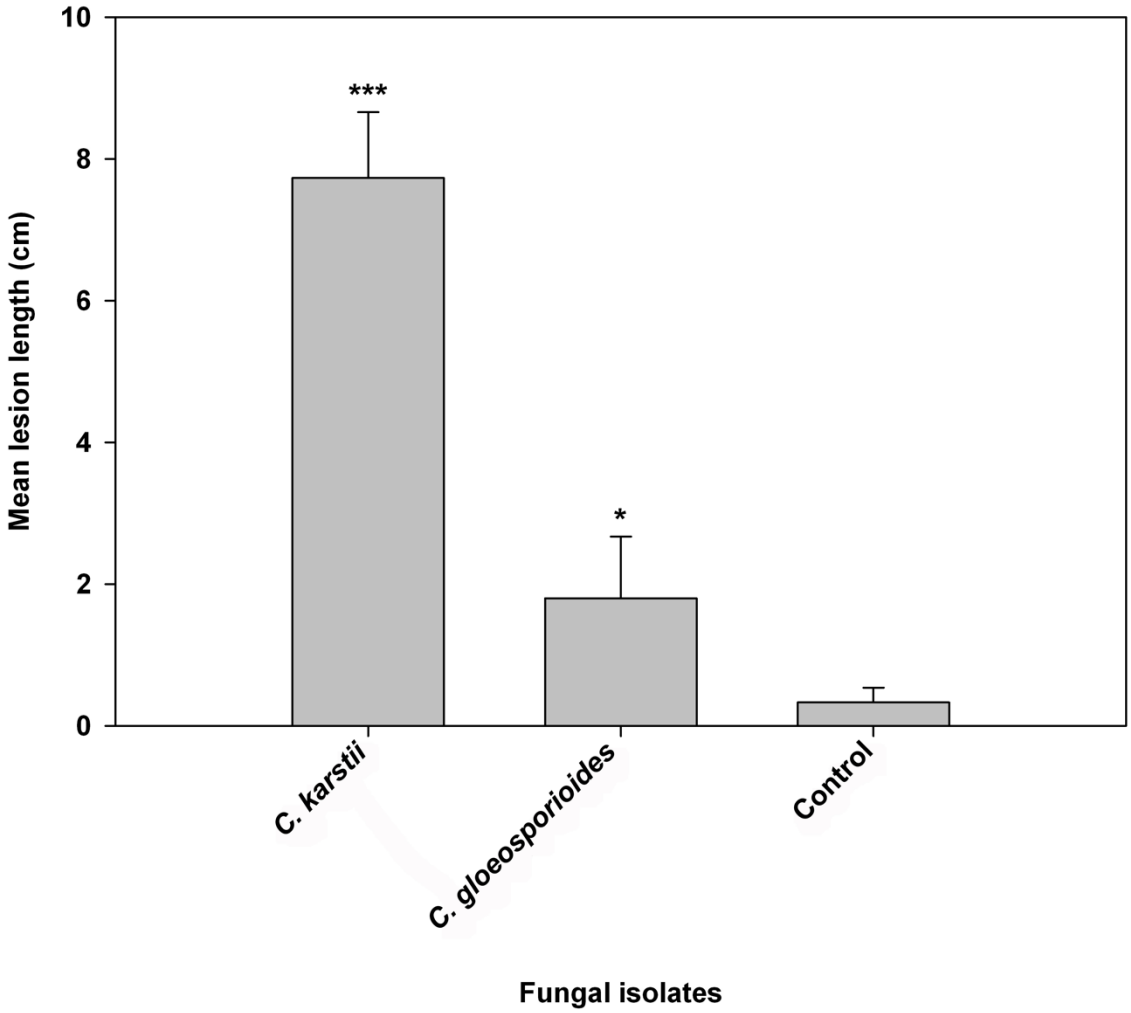

Figure 3.3. Pathogenicity of *Colletotrichum* spp. on '4B' clementine after 15 months. Vertical lines represent standard error of the mean and multiple comparisons were performed using Dunnett's test ($\alpha = 0.05$). Asterisks (*) denote the following significance: '*' $P < 0.05$ and '***' $P < 0.001$.

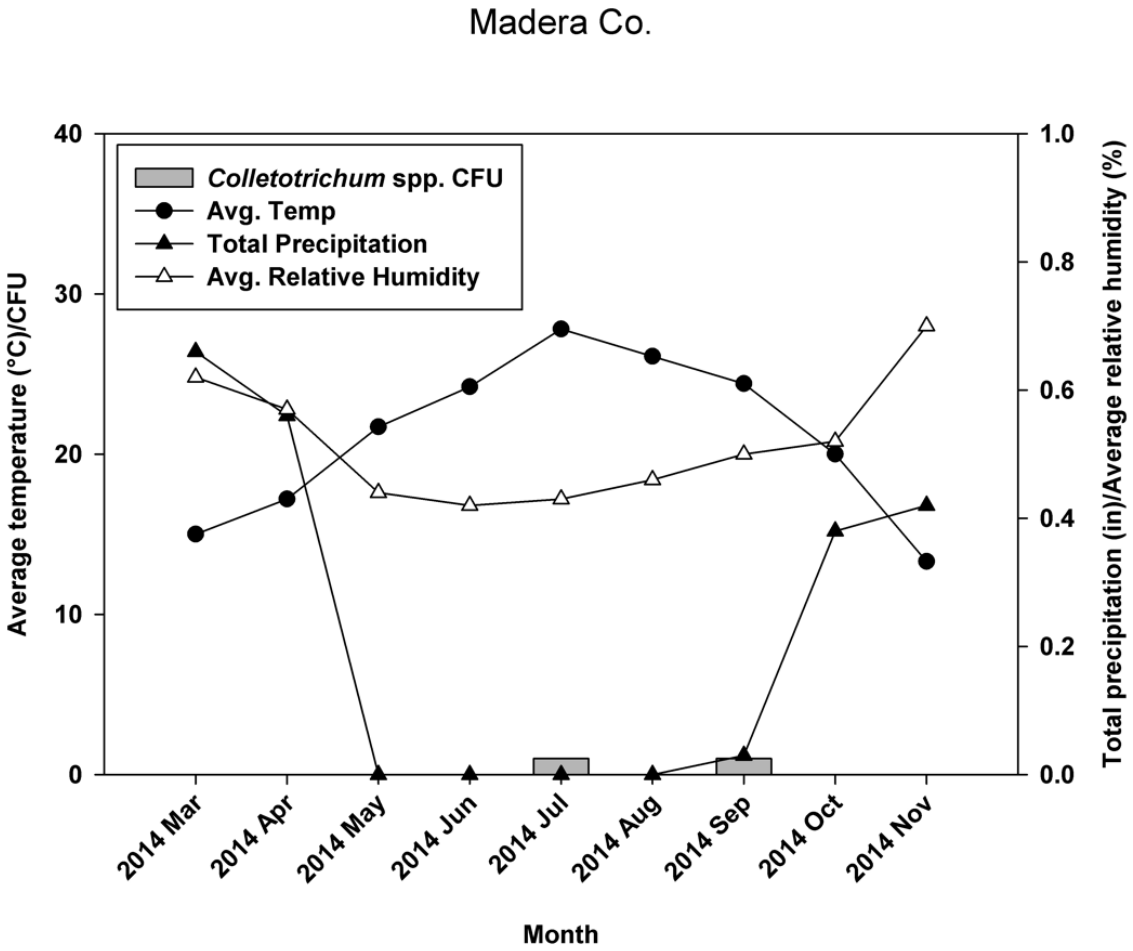

Figure 3.4.1. Monthly spore trap counts with temperature (°C), precipitation (mm), and relative humidity (%) for Madera county. Vertical bars represent total colony forming units (CFU) counted from each citrus orchard by month. Lines represent average monthly temperature (°C) and relative humidity (%) and total monthly precipitation (mm).

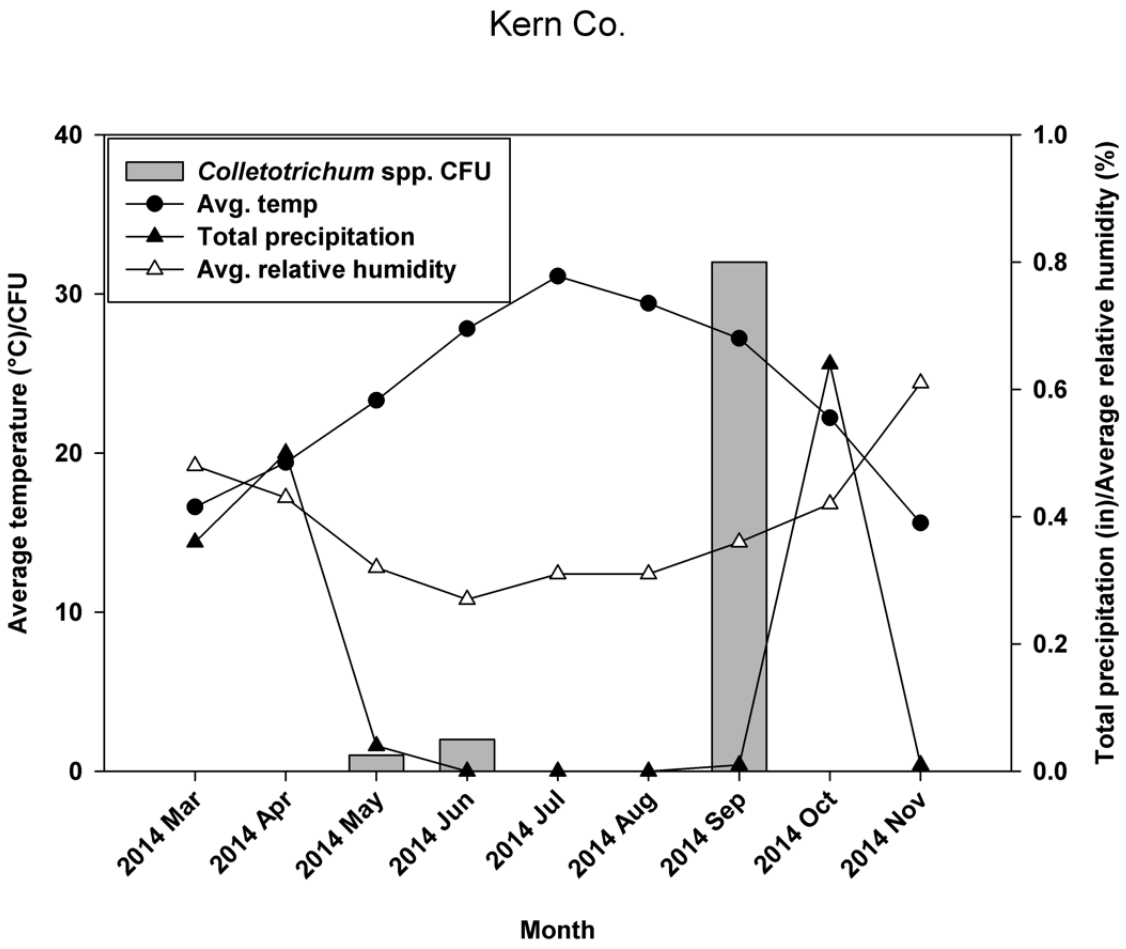

Figure 3.4.2. Monthly spore trap counts with temperature (°C), precipitation (mm), and relative humidity (%) for Kern county. Vertical bars represent total colony forming units (CFU) counted from each citrus orchard by month. Lines represent average monthly temperature (°C) and relative humidity (%) and total monthly precipitation (mm).

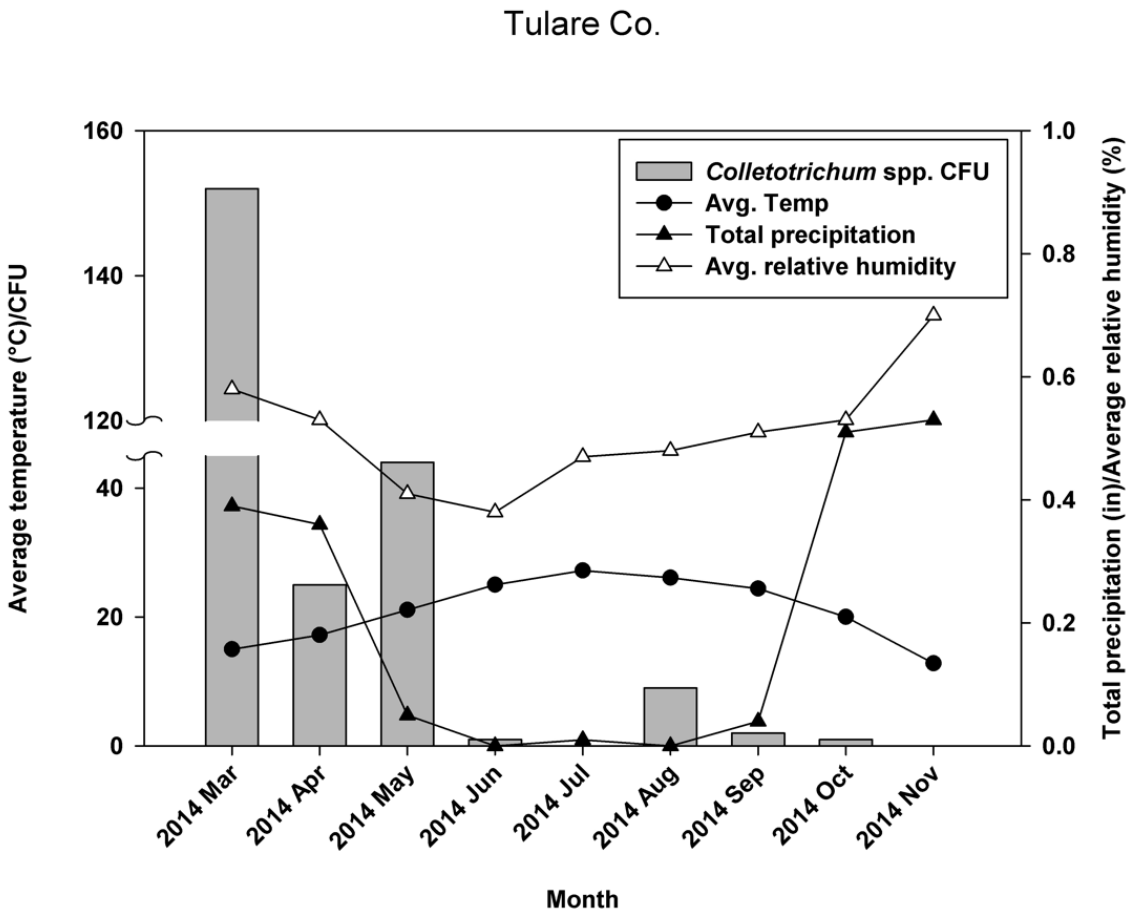

Figure 3.4.3. Monthly spore trap counts with temperature (°C), precipitation (mm), and relative humidity (%) for Tulare county. Vertical bars represent total colony forming units (CFU) counted from each citrus orchard by month. Lines represent average monthly temperature (°C) and relative humidity (%) and total monthly precipitation (mm).

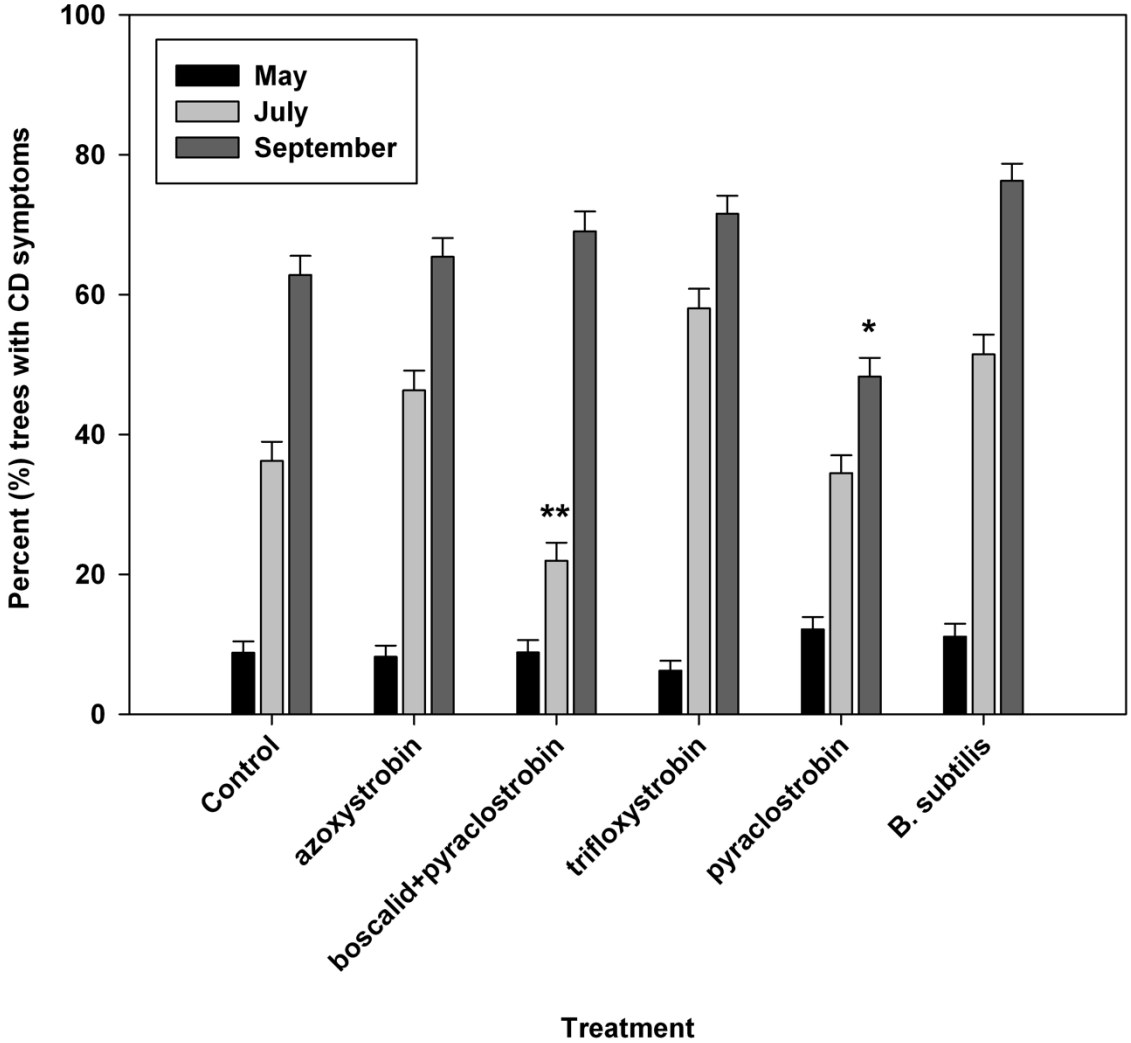

Figure 3.5. Prevalence of Colletotrichum Dieback (CD) symptoms in citrus trees by month after fungicide application. Vertical bars represent the mean percent of trees displaying symptoms of CD. Vertical lines represent the standard error of mean and multiple comparisons were performed using Dunnett's test ($\alpha = 0.05$). Asterisks (*) denote the following significance: '*' $P < 0.05$ and '**' $P < 0.01$.

CHAPTER IV. Chemical Management of Invasive shot hole borer and Fusarium Dieback in California sycamore (*Platanus racemosa*) in Southern California

ABSTRACT

Fusarium Dieback (FD) is a new vascular disease of hardwood trees in southern California and is part of a highly invasive pest-disease complex threatening the avocado industry, urban forests and native wildlands in California. This disease is vectored by two recently introduced invasive ambrosia beetles (*Euwallacea* spp. nr. *fornicatus*) known as Polyphagous Shot Hole Borer (PSHB) and Kuroshio Shot Hole Borer (KSHB) and collectively form the pest-disease complex known as Shot Hole Borer-Fusarium Dieback (SHB-FD) complex. Mitigation of this pest-disease complex currently relies on tree removal; however, this practice is expensive and impractical given the wide host range and rapid advancement of the beetles throughout hardwoods in southern California. This study reports on the assessment of various pesticides for use in the management of SHB-FD. *In vitro* screening of 13 fungicides revealed pyraclostrobin, trifloxystrobin, and azoxystrobin to generally have lower EC_{50} values across all fungal symbionts of PSHB and KSHB; metconazole was found to have lower EC_{50} values for *Fusarium* spp. and *Paracremonium pembeum*. Triadimefon and fluxapyroxad were not capable of inhibiting any fungal symbiont at the concentrations tested. A one-year field study identified two insecticides, emamectin benzoate alone and in combination with propiconazole, and bifenthrin to significantly reduce SHB attacks in addition to two injected fungicides (metconazole and a combination of carbendazim and debacarb) and one spray fungicide

(tebuconazole). Bioassays designed to assess fungicide retention one year post application revealed six out of the seven fungicides to exhibit some level of inhibition *in vitro* with all thiabendazole treated trees sampled exhibiting inhibition. This study has identified several pesticides which can be implemented as part of an IPM strategy for not only California sycamore, but for other landscape trees currently affected by SHB-FD in an effort to reduce SHB infestation in low- to moderately- infested trees.

INTRODUCTION

Shot Hole Borer-Fusarium Dieback (SHB-FD) is a pest-disease complex vectored by ambrosia beetles (*Euwallacea* spp. nr. *fornicatus*), which attack numerous hardwood tree species, and is rapidly spreading throughout California. First identified in Los Angeles in 2012 (Eskalen et al. 2013), SHB-FD has since spread into the surrounding counties of Orange, Riverside, San Bernardino, San Diego, Ventura, and Santa Barbara; More recently, SHB have been collected from beetle traps in the coastal county of San Luis Obispo (Eskalen et al., unpublished data). The destructive nature of SHB-FD is a result of the combined effects of its vectors Polyphagous Shot Hole Borer (PSHB) and Kuroshio Shot Hole Borer (KSHB) [*Euwallacea* spp. nr. *fornicatus* Coleoptera: Scolytinae] with their ambrosial fungal symbionts *Fusarium euwallaceae* S. Freeman, Z. Mendel, T. Aoki & O'Donnell (Freeman et al. 2013) and *Fusarium kuroshium* F. Na, J. D. Carrillo & A. Eskalen (Na et al. 2017) respectively in addition to other fungal associates of *Graphium* spp. and *Paracremonium pembeum* (PSHB only). As the beetle burrows into host trees to construct galleries, it simultaneously inoculates host tissue with

its ambrosial symbionts which invade the host vasculature resulting in the disruption of water and nutrient transport leading to dieback and eventually, tree death in susceptible hosts (Eskalen et al. 2013; Mendel et al. 2012).

First reported in California in 2003 from insect traps, PSHB was collected from several hosts in the Los Angeles area, however no record of host disease symptoms was reported from this collection (Rabaglia et al. 2006). Beginning in 2012, several backyard avocado trees and ornamentals in the Los Angeles (LA) area exhibited symptoms of an unknown dieback disease associated with the presence of wood-boring beetles; These beetles were later identified as the same beetles initially detected in 2003 and subsequently identified as PSHB (Eskalen et al. 2012; 2013); furthermore, these beetles were also determined to be identical to beetles found causing the same disease in Israeli avocado groves (Mendel et al. 2012). The beetle was initially restricted to the LA and Orange county (OC) areas, but continued to spread throughout southern California when in 2013, it was detected in San Diego county. Although initially believed to be an extension of the initial LA/OC infestation, it was later confirmed that the beetles in San Diego county, although morphologically indistinguishable from PSHB, were in fact a separate, but closely related species of PSHB later named KSHB (Stouthamer et al. 2017). These findings confirmed the occurrence of two related, but separate invasive ambrosia beetle introductions in California and are a cause of great concern over the existence of two highly destructive pathosystems.

Fusarium Dieback is characterized by several traits: signs of beetle boring activity which include small entry holes approximately 0.85mm in diameter and the presence of

frass, while symptoms include: wet spots, gumming, frass, or powdery exudate at entry holes and dieback symptoms of affected trees. These signs and symptoms vary by host, but a combination of the symptoms above may show in a host or no obvious symptoms may be seen initially (Eskalen et al. 2013). The host range of SHB has been increasing overtime since its discovery; in 2012, 207 out of 335 hosts examined had signs and symptoms consistent with FD. From these 207 hosts, which belonged to 58 plant families, 11 were species native to California, 13 were agricultural commodities currently in production in California, and 19 were determined to be reproductive hosts, in which the beetle could complete its full life cycle, and includes both native plant species and agricultural commodities (Eskalen et al. 2013). As of 2017, the number of hosts has increased to 342 tree species encompassing 63 plant families and the number of reproductive hosts has more than doubled to include 55 tree species with new additions being added regularly (Eskalen 2017: https://eskalenlab.ucr.edu/pshb). The economic and ecological burden of SHB-FD on trees planted throughout California, agricultural production, and native wildland communities will be substantial. In the absence of effective control measures for this pest disease complex, it has been suggested that the economic costs related to SHB-FD damage would exceed $36 billion (McPherson, n.d.) for the removal of dying trees.

The management of ambrosia beetles is particularly challenging since these beetles spend most of their life within their hosts, emerging only to colonize nearby hosts which drastically limits the exposure of these beetles to contact insecticides. Furthermore, the xylomycetophagous ambrosia beetles do not consume the wood of host trees as bark

beetles do (Batra 1966; Beaver 1989; Francke-Grosmann 1967) which could be problematic if using injectable insecticides as it is unclear how much contact exposure occurs between beetle and insecticide. Cooperband et al. (2016) observed that PSHB adults develop within 22 days at 24°C and produce an average of 32 viable female offspring and it is thought that adult beetles and larvae are present throughout the year suggesting that beetle reproduction takes place year-round (Eatough-Jones and Paine 2015). The high and constant reproductive output of PSHB suggests that multiple spray applications of contact insecticides would likely need to be made throughout the year as no clear window of beetle emergence can be accurately predicted in a given area. Thus, the use of injectable systemic insecticides would be more appropriate for management of this vector to circumvent these issues. Several studies have shown the efficacy of various insecticides in the management of various bark and ambrosia beetles in avocado (Peña et al. 2011), oak (Svira et al. 2004), and elms (Pajares and Lanier 1989). However, the association of these beetles with symbiotic and auxiliary fungi presents another challenge as the symbionts of both PSHB and KSHB are known to be pathogenic on their hosts (Lynch et al. 2016; Mendel et al. 2012; Na et al. 2017). This highlights the need for two levels of management; one to directly manage vector populations and a second level which aims to manage the fungal populations established by SHB once a host has been invaded.

The symbiosis between SHB and their associated fungi is essential to the survival and persistence of these ambrosia beetles as they feed exclusively on the symbiotic mutualists. In addition to *F. euwallaceae,* the primary symbiont of PSHB, which is

known to be essential for the beetle to complete its full life cycle and is likely required for gallery establishment in new hosts (Freeman et al. 2013; Freeman et al. 2016), PSHB is also associated with two other fungi: *Graphium euwallaceae* M. Twizeyimana, S.C. Lynch & A. Eskalen and *Paracremonium pembeum* S.C. Lynch & A. Eskalen (Lynch et al. 2016). *Graphium euwallaceae* likely serves as the primary food source for immature beetle stages, but may also play a prominent role during initial gallery formation. *P. pembeum* is not required as a source of food for any stage of beetle development nor is it currently considered a symbiont, but it could play a role as a fungal antagonist towards contaminating fungi within beetle galleries (Freeman et al. 2016, Lynch et. al. 2016). Similarly, KSHB is known to associate with two symbiotic fungi: *Fusarium kuroshium* and *Graphium kuroshium* F. Na, J. D. Carrillo & A. Eskalen, both of which presumably serve the same function in KSHB as *F. euwallaceae* and *G. euwallaceae* serve in PSHB. This complex, but required association between beetle and fungi highlights an intrinsic vulnerability of this system whereby suppression of fungal symbionts and associates could have severe impacts on beetle establishment and development. Exploiting this obligatory relationship between SHB and their associated fungi could be useful in the management of SHB-FD as fungicides could be used to restrict the growth of fungal populations within tree hosts which in turn would impact the endurance of SHB.

No management strategies outside of cultural practices are in place for the management of SHB-FD; currently tree removal is recommended for heavily infested trees followed by either chipping and/or solarization of infested material (Eatough-Jones and Paine 2015) to reduce beetle populations in localized areas in addition to eliminating

physical hazards imposed by declining, heavily infested trees. Although these practices are useful in the absence of other management strategies and will continue to be used as part of an integrated pest management (IPM) program, the removal of infested trees is not only laborious and costly (estimated between $650-1000 per tree), but it is not practical for large scale land management. The objectives of this study were to: (i) identify fungicides which inhibit the growth of SHB symbionts *in vitro*; (ii) assess pesticide efficacy *in planta* in reducing PSHB populations; and (iii) recommend an immediate chemical management strategy for SHB-FD in California which could be used as part of an IPM program for SHB-FD on landscape trees in California.

MATERIALS AND METHODS

Fungal isolates. Twelve isolates of *Fusarium euwallaceae*, nine isolates of *Fusarium kuroshium*, five isolates of both *Graphium euwallaceae* and *Graphium kuroshium*, and four isolates of *Paracremonium pembeum* were used in the *in vitro* fungicide screening experiment. These isolates were recovered from samples collected between 2012-2015 from avocado (*Persea americana*), box elder (*Acer negundo*), castor bean (*Ricinus communis*), California sycamore (*Platanus racemosa*), weeping acacia (*Acacia floribunda*), and coast live oak (*Quercus agrifolia*) showing symptoms of FD in Los Angeles, Orange, and San Diego counties in California (Table 1). Fungal isolation from plant material was carried out by briefly flaming plant samples and removing the outer surface with a sterile paring knife to reveal internal vascular necrosis. Wood pieces were excised from the leading margin of necrotic tissue and placed onto Potato Dextrose Agar

amended with 0.01% tetracycline hydrochloride (PDA-t). Cultures were incubated at 25°C for 3 to 5 days and pure cultures were obtained by scraping emerging fungal colonies with a sterile inoculation loop and streak plating onto water agar and incubating plates at 25°C for 24 hours. After 24 hours, single germinating conidia were transferred from water agar plates with the aid of a stereomicroscope to fresh PDA plates to establish pure cultures. To confirm fungal identity, morphological and molecular methods were followed as described by Freeman et al. (2013), Lynch et al. (2016), and Na et al. (2017).

***In vitro* fungicide screening.** Eleven fungicides belonging to different chemical families (Table 2) were tested *in vitro* to determine the effective concentration that reduces 50% of mycelial growth (EC_{50} values) of the 35 isolates using the spiral gradient dilution (SGD) method (Förster et al., 2004). Briefly, PDA (50 ml) was poured into each 15-cm-diameter petri dish at least 24 h before each fungicide suspension was applied. A 62.5 µl fungicide suspension from each of the stock suspensions was applied with a spiral plater (SGE; Spiral Biotech, Inc.) using the exponential deposition mode. The plates were incubated for approximately 3 h to allow the fungicides to diffuse into the agar medium and form a concentration gradient along the radius of the plate. Droplets (10 µl) of conidial suspensions (5×10^5ml) made from each fungal isolate were spread across the radial lines in predetermined plate positions using a sterile plastic pestle. There were control treatments consisting of PDA plates without fungicides added, to which the appropriate conidial suspension of each isolate was applied with a sterile plastic pestle. There were two replications per isolate and plates were incubated at 25°C for between 3

to 5 days depending on fungal species and at the end of this period EC_{50} values were determined as described by Förster et al. (2004).

Pesticide field trial. Field trials were established at three county regional parks throughout Orange County California: Yorba Regional Park (33°52'14.4" N, 117°45'45.2" W); Carbon Canyon Regional Park (33°55'19.9" N, 117°50'13.2" W); and Ted Craig Regional Park (33°54'10.2" N, 117°53'04.7" W). Sites were chosen based on two criteria: availability of SHB hosts and level of infestation. The most common host trees available at all three sites included: *Platanus racemosa*, *Platanus* x *acerifolia*, *Alnus rhombifolia*, *Liquidambar styraciflua*, *Quercus agrifolia*, *Salix laevigata*, and *Populus fremontii*, with *P. racemosa* being the most abundant host tree available (Arbor Access Tree Inventory, West Coast Arborists). To evaluate level of infestation, trees were classified by number of beetle entry holes on trunks and dieback presence according to the following criteria: low infestation (<30 entry holes and no dieback); moderate infestation (≥30 entry holes and no dieback); and heavy infestation (≥30 entry holes and dieback present). Trees from all three sites ranged from 18 to 71 cm diameter at breast height (DBH) and levels of infestation were as follows: Yorba Regional Park (low infestation), Ted Craig Regional Park (moderate infestation), and Carbon Canyon Regional Park (high infestation). Based on these criteria, 80 trees from each park were randomly selected for inclusion into the pesticide trial using a randomized complete block design with each park functioning as a block. On 25 July 2015, the DBH of each tree was recorded and the number of beetle entry holes counted from an approximately 1

m length section on the bole beginning approximately 1 m from the soil line. Each of the 80 eighty trees were randomly assigned to one of 10 pesticide treatments for a total of 8 trees per treatment. Assigned trees were sorted by DBH and initial entry holes to check for similar DBH and entry holes across all treatments using ANOVA; no significant differences ($P > 0.05$) in initial entry holes were detected between treatments. On 11-12 August 2015, trees were treated with the pesticides and rates in Table 2. Trees treated with thiabendazole, emamectin benzoate, propiconazole, and emamectin benzoate + propiconazole were injected using the Arborjet QUIK-jet Air injector (Arborjet, Woburn, MA); trees treated with carbendazim + debacarb and tebuconazole were injected using Chemjet tree injectors (Queensland Plastics, Australia); and trees treated with *Bacillus subtilis* (strain QST713), metconazole, and bifenthrin were mixed with a bark penetrant (Pentra-Bark®) at a rate of 2.9 ml/cm DBH and applied with a spray rig until run-off. Trees were evaluated every month (approximately 28 days), for 12 months, following pesticide application by counting the number of beetle entry holes on the bole as described above. Loose bark from trees was removed with a plastic putty knife. Oil-based paint pens (Diagraph MSP) were used to count entry holes by dotting to the right of beetle entry holes and a unique color pen was used for every month.

Fungicide retention bioassay. Bioassays were conducted according to the method of Mayfield et al. (2008) to determine pesticide retention in field injected trees. At 12 months post-injection, tree cores were taken from all treated trees at Yorba Regional Park for all treatments except bifenthrin and emamectin benzoate treatments as these

pesticides are insecticides. Briefly, wood cores (approximately 1 cm x 8 cm, each) were removed from pesticide treated and untreated trees using an increment borer in four cardinal directions at approximately 1.2 m above the soil line. Wood cores were surface disinfested with 95% ethanol and flamed to burn off excess ethanol. Bark was removed and remaining xylem cores were placed onto PDA-t seeded with spores (1×10^6 ml) of *F. euwallaceae* (UCR4082) which were applied using an atomizer. Plates were incubated for six days and scored (ratings 0-3) using a modified rating-scale from Stennes and French (1987) as follows: 0, fungal growth over entire plug; 1, fungal growth on part of the plug; 2, no fungal growth on plug; 3, no fungal growth on plug + presence of zone of inhibition (ZOI) in agar medium. Scores ≥1 are considered to show inhibition. The number of trees showing inhibition was calculated from the presence of at least one core with a score ≥1 and percent inhibition was calculated as the percent of plugs per tree with scores ≥1.

Statistical analyses. All statistical analyses were performed using R (Version 3.2.3). EC_{50} values were transformed using logarithmic transformation ($\log_{10}$) prior to analysis to normalize data as suggested by Liang et al. (2015) and evaluated using the Shapiro-Wilk test of normality. Homoscedasticity for all independent, *in vitro* runs for each fungal species tested was evaluated using Levene's test; no heterogeneity was detected and thus data from individual runs were combined. EC_{50} values were tested by ANOVA under a linear regression model and mean EC_{50} values of all fungicides were compared using Tukey's honest significant difference (HSD) at $\alpha = 0.05$. Count data from the fungicide

applications *in planta* were transformed to attacks of SHB/m^2 based on the total area of the individual trees that were counted for the duration of the experiment. The transformed data was not normally distributed and zero inflated when analyzed using the "lattice" package (Sarkar 2008) in R; therefore, regression analysis was performed using a generalized linear model with a negative binomial link function and performed using the "MASS" package (Venables and Ripley, 2002) in R. The model was blocked by location. Post-hoc analysis was done by first constructing a contrast matrix in order to compare all treatments to the control then by using the "multcomp" package (Holthorn et al. 2008) in R with link function glht for comparison of all fungicide treatments to the control based on the matrix. Bioassay inhibition data were tested using a generalized linear model with binomial distribution and logit link function after coding individual tree core inhibition scores as either 0 (inhibition rating 0) or 1 (inhibition rating ≥1) for each treatment. Independent contrasts were performed between control treatment and each pesticide treatment, except for *B. subtilis* treatment, to determine significance at $\alpha = 0.05$.

RESULTS

***In vitro* fungicide screening.** There were significant differences ($P < 0.001$) among 13 fungicides used in the *in vitro* fungicide screening experiment; however, no significant differences were recorded among the 12 *Fusarium euwallaceae* isolates ($P = 0.791$) (Table 3). Metconazole had the lowest EC_{50} value (0.031); however, its EC_{50} did not significantly differ ($P > 0.05$) to that of pyraclostrobin. Pyraclostrobin had significantly

lower EC_{50} values than other strobilurins tested, azoxystrobin and trifloxystrobin. Pyrimethanil had highest EC_{50} while two other fungicides fluxapyroxad and trifloxystrobin had 0% (or no) inhibition when tested against 12 *F. euwallaceae* isolates. Like *F. euwallaceae*, there were significant differences ($P < 0.001$) among the 13 fungicides tested *in vitro* against *Fusarium kuroshium* (Table 3); however, no significant differences were detected between the nine isolates of *Fusarium kuroshium* ($P = 0.299$). Pyraclostrobin had the lowest EC_{50} value (0.005) and differed significantly ($P < 0.05$) from the mean value for metconazole (0.0190). Fluopyram had the highest EC_{50} value while pyrimethanil, fluxapyroxad, and triadimefon showed no inhibition of *Fusarium* spp. at the concentrations tested. Pyrimethanil was observed to inhibit growth of *F. euwallaceae*, but not for *Fusarium kuroshium*.

There were significant differences ($P < 0.001$) among 12 fungicides (*G. euwallaceae*) and 11 fungicides (*Graphium kuroshium*) used in the *in vitro* fungicide screening experiment; however, no significant differences were recorded within species for the five *Graphium euwallaceae* isolates ($P = 0.862$) or *Graphium kuroshium* isolates ($P = 0.341$) (Table 3). Pyraclostrobin had the lowest EC_{50} values for both *G. euwallaceae* (0.007) and *Graphium kuroshium* (0.004); however, its EC_{50} did significantly differ ($P < 0.05$) to those of the other strobilurins tested (pyraclostrobin and azoxystrobin). Triflumizole had highest EC_{50} while three other fungicides myclobutanil, fluxapyroxad, and triadimefon had no inhibition when tested against 10 *Graphium* spp. isolates. Furthermore, no inhibition with fluopyram was observed when tested against *G. euwallaceae*.

There were significant differences ($P < 0.001$) among 13 fungicides used in the *in vitro* fungicide screening experiment against *Paracremonium pembeum*; however, no significant differences were recorded among the four *P. pembeum* isolates ($P = 0.217$) (Table 3). Pyraclostrobin had the lowest EC_{50} (0.023); however, its EC_{50} did not significantly differ ($P > 0.05$) from trifloxystrobin. Thiabendazole had the highest EC_{50} while three other fungicides pyrimethanil, fluxapyroxad, and triadimefon had no inhibition when tested against four *P. pembeum* isolates.

Pesticide field trial. During this study, two trees (one treated with bifenthrin and one treated with metconazole) were removed in April 2016 at Yorba Regional Park and three trees (one each from treatments of emamectin benzoate, metconazole, and bifenthrin) were removed in February 2016 from Carbon Canyon Regional Park due to hazard concerns; data collected from these five trees prior to death/removal were excluded from data analysis. Analysis of mean attacks/m^2 of untreated control trees plotted by month by site revealed overall increases in the number of attacks/m^2 (Fig. 1). Yorba Regional Park (low infestation) attacks/m^2 steadily increased throughout the twelve-month study. Craig Regional Park (moderate infestation) attacks/m^2 increased throughout the initial count period to the third month (Nov), with a slower increase in attacks/m^2 between the fourth month throughout the ninth month (May), and increases in attacks/m^2 continued again from the tenth month to the 12th month (August). Carbon Canyon Regional Park (heavy infestation) attacks/m^2 increased throughout the initial count period to the third month (Nov), followed by a plateau of overall attacks/m^2 between the forth through the tenth

month (June), and an increase in attacks/m^2 observed during the 11th month (July). A reduction in attacks/m^2 at Carbon Canyon Regional Park was observed during the 12th month (August) due to bark sloughing off resulting in lower attacks/m^2 recorded at this site. Analysis of attacks/m^2 by quarter for all sites (Q1: Sep-Nov; Q2: Dec-Feb; Q3: Mar-May; and Q4: Jun-Aug) revealed Q3 to have significantly more increases in attacks/m^2 than all other quarters.

Significant differences in attacks were observed when comparing parks, therefore each park was analyzed individually. Trees at Yorba Regional Park revealed no treatments to have significantly fewer attacks/m^2 than untreated control trees (Fig. 2). Trees at Craig Regional Park treated with tebuconazole and bifenthrin had significantly fewer ($P < 0.001$) attacks/m^2 than untreated controls (Fig. 2). Trees at Carbon Canyon Regional Park treated with tebuconazole and metconazole had significantly fewer ($P < 0.001$) attacks/m^2 than untreated controls followed by carbendazim + debacarb ($P < 0.01$), emamectin benzoate, and emamectin benzoate + propiconazole ($P < 0.05$) (Fig. 2); However, emamectin benzoate and emamectin benzoate + propiconazole did not differ significantly ($P = 0.059$) from each other.

Fungicide retention bioassay. All treatments except for *Bacillus subtilis* treated trees and untreated (control) trees exhibited some level of inhibition (Table 4). Out of the eight trees treated for each pesticide, thiabendazole treated trees had the highest levels of inhibition with all eight trees showing some level of inhibition followed by five trees for propiconazole, propiconazole + emamectin benzoate, carbendazim + debacarb, and

tebuconazole, and one tree for metconazole. Mean percentages of tree cores showing some level of inhibition per tree ranged from 25-50 % with the highest mean observed in trees treated with propiconazole (50%) followed by thiabendazole (47%), propiconazole + emamectin benzoate (45%), and carbendazim + debacarb, tebuconazole, and metconazole all with means of 25%. Tree cores taken from thiabendazole treated trees showed ZOIs on agar plates (score of 3) with a mean percent of samples per tree ranging from 25-50%; however, no tree cores taken from other pesticide treated trees produced ZOIs on agar plates. A significant difference ($P < 0.001$) was observed among the eight treatments tested. Independent contrast between untreated and pesticide treated trees showed a significant difference in inhibition for thiabendazole ($P < 0.001$), propiconazole ($P = 0.0001$), propiconazole + emamectin benzoate ($P = 0.0004$), carbendazim + debacarb ($P = 0.0127$), and tebuconazole ($P = 0.0127$). Inhibition observed in metconazole treated trees was not significantly different ($P > 0.05$) from untreated control trees nor was inhibition significantly different ($P > 0.05$) between propiconazole and propiconazole + emamectin benzoate treated trees.

DISCUSSION

Shot hole borer-Fusarium Dieback is a destructive pest-disease complex causing significant damage to hardwood trees in southern California and until recently, no management strategies outside of cultural practices (tree removal) existed. This study provides evidence for the efficacy of several pesticides, including fungicides, insecticides, and a combination thereof, in reducing SHB attacks in California sycamore.

Overall, three triazole fungicides: tebuconazole, metconazole, and propiconazole (in combination with an insecticide) were found to significantly reduce the number of beetle attacks on California sycamore treated trees in addition to trees treated with a mixture of carbendazim and debacarb in combination and one insecticide (emamectin benzoate). Although only one field application was done for each of the pesticides tested, bioassays of fungicide treated trees 12 months post application revealed six of the seven treatments to exhibit some level of inhibition (retention) *in vitro*.

The management of wood boring ambrosia beetles is difficult in the traditional context of insect chemical management since the use of contact insecticides can be negated by the lifestyle of these beetles as they spend most of their life within their host tree and relatively little time outside of the host limiting the chance of exposure to sprayed insecticides and also because they are not phloeophagous such as bark beetles (Beaver 1989). Due to the "cryptic" nature of these beetles, management strategies are primarily focused on an integrated approach which can include the use of injectable, systemic pesticides in addition to sanitation and silviculture techniques which include removing infested branches and immediate treatment of infested material either through burning, burying, or chipping to reduce beetle populations in the area (USDA New Pest Response Guidelines: Exotic Wood-Boring and Bark Beetles 2011). However, the rapid expansion of the beetle population and wide host range of these beetles throughout southern California limits the reliability of sanitation and silviculture techniques alone in managing this pest disease complex and therefore necessitates the need for chemical control strategies in landscape systems. This, combined with the fact that ambrosia

beetles are dependent on their symbiotic fungi for survival, suggests that the use of fungicides may be better suited to the control of ambrosia beetles as any reduction in fungal vigor may potentially negatively affect the survival of ambrosia beetles.

In this study, both insecticides tested (emamectin benzoate and bifenthrin) were found to significantly reduce beetle attacks in infested trees; however, bifenthrin treated trees had significantly reduced beetle attacks in moderately-infested trees and not in heavily-infested trees. Similarly, emamectin benzoate when applied alone or in addition to combination injections with propiconazole had reduced beetle attacks in heavily-infested trees and not in moderately infested trees. The reduction of attacks with emamectin benzoate treatment alone are contrary to Fettig et al. (2013) whereby emamectin benzoate applications alone were not sufficient in reducing attacks of *Dendroctonus ponderosae* on *Pinus contorta* if applications were not made a year before protection was needed, however injections of emamectin benzoate in combination with propiconazole were found to significantly reduce *D. ponderosae* attacks when injected the same year, but before beetle flight. Out of several injected systemic insecticides, only injection with emamectin benzoate into avocado provided partial control of redbay ambrosia beetle (*Xyleborus glabratus*) attacks (Ploetz et al. 2017). The findings of this study suggest that emamectin benzoate alone may be sufficient in reducing attacks from SHB as combination treatments with propiconazole did not result in significantly lower SHB attacks when compared together. Bifenthrin is a pyrethroid insecticide which is both a contact and stomach poison, but is non-systemic. The use of pyrenthroids as a management option for the control of bark and ambrosia beetles has been studied in

several systems and found to be successful in the management of bark and ambrosia beetles in oak (Svira et al. 2004), elms (Pajares and Lanier 1989), and apple (Ciglar and Baric 2000), however not all pyrenthroids have been found to be consistently effective, especially for bifenthrin. Mizell and Riddle (2004) reported that bifenthrin-treated bolts consistently reduced the number of Asian ambrosia beetle attacks compared to untreated control bolts. Bifenthrin was also found to suppress *D. brevicomis* and *Ips confuses* in conifers, but multi-year applications were suggested depending on the pest system (Fettig et al. 2006). Peña et al. (2011), studying the chemical control of *X. glabratus*, found that avocado trees treated with bifenthrin and zeta-cypermethrin had more *X. glabratus* attacks than any other treated tree and Carrillo et al. (2013) found bifenthrin to only be effective in suppressing *X. glabratus* for four days post application under field conditions. Eatough-Jones et al. (2017) found bifenthrin treated trees to not significantly reduce the number of PSHB attacks in California sycamore despite previous studies suggesting bifenthrin treated cut logs were more effective in preventing PSHB attacks than several of the other insecticides tested (Eatough-Jones and Paine, 2017). Additionally, emamectin benzoate treated trees were found to have reduced numbers of PSHB attacks during the second month following insecticide application; however, this was the only time point after application which showed a reduction in PSHB attacks for emamectin benzoate treatment (Eatough-Jones et al. 2017).

In this study, treatment of California sycamore with carbendazim in combination with debacarb were found to significantly reduce SHB attacks in heavily infested trees. Carbendazim is a benzimidazole and breakdown product of benomyl and has been

extensively studied as an injectable in the treatment of Dutch Elm Disease (Gregory and Jones 1973) and other diseases such as oak wilt (Jones et al. 1973). The use of carbendazim in these studies found that injection as a prophylactic was more efficacious in reducing or even preventing symptom development than when used as a therapeutic treatment. Although a systemic fungicide, carbendazim has not been observed to move into new wood growth, thus necessitating the need for annual treatments (Nishijima and Smalley 1978; Stennes and French 1987). Debacarb, like carbendazim, is a benzimidazole which has been studied in the management of various diseases including Diplodia Tip Blight of pine (Hartman et al. 2009) and in combination with carbendazim for Dutch Elm Disease (Lanier 1987). Hartman et al. (2009) reported that debacarb treatment led to the slowest increase in disease incidence, but was not effective in preventing tip blight. Similarly, Lanier (1987) observed that debacarb did not prevent infection and was also not effective as a therapeutic, but prophylactic injections appeared to effect symptom progression. Results from the bioassay in this study suggest that the combination product of carbendazim and debacarb is retained after one year of treatment as inhibition was observed *in vitro* in five out the eight trees sampled, however it is not possible to determine which compound or if both compounds were still present at high enough concentrations in the wood to inhibit fungal growth nor is it known whether carbendazim and/or debacarb is responsible for the fungal inhibition observed as neither product was tested singly *in vitro* on fungal growth.

Both metconazole and tebuconazole were found to significantly reduce SHB attacks in treated sycamores in this study and metconazole was found to have lower EC_{50} values

across all fungi tested *in vitro*. Tebuconazole has been used in the management of *D. ponderosae* on pine and was found to reduce mortality and blue stain of pine treated with a combination of tebuconazole and abamectin to a degree less than that of abamectin treated trees alone (Fettig et al. 2013). To our knowledge, metconazole has not been used in the management of bark or ambrosia beetles until recently. Eatough-Jones et al. (2017) did not find metconazole treatment alone to be effective in reducing PSHB attacks in California sycamore although reductions were observed in combination treatments of metconazole, bifenthrin, and emamectin benzoate when compared to untreated control trees, however the lack of efficacy of single metconazole applications may be due to the short duration of the study (six months) and adequate time had not passed for the effects of this compound to be seen. Although metconazole is known to be xylem mobile, this treatment was not injected during this study, but applied as a spray mixed with a bark penetrant. No treatment of metconazole without penetrant was applied in this study, however it is possible that the bark penetrant facilitates the movement of metconazole into the host vasculature for a period of time. Results from the bioassay showed little retention (one out of seven trees) of metconazole after 12 months post application, suggesting that spray treatments of metconazole although efficacious in a season, likely need to be applied yearly. The triazole, propiconazole, by itself was not found to significantly reduce beetle attacks, but was found to reduce beetle attacks when combined with the insecticide emamectin benzoate. This contrasts with the *in vitro* fungicide results where propiconazole was found to inhibit fungal growth of all symbionts tested. Propiconazole has been studied for the treatment of Laurel Wilt disease in Florida and

was found to be effective *in vitro* at a concentration of 0.1 ppm in completely inhibiting *Raffaelea lauricola* and trees treated with injections of propiconazole did not develop crown wilt symptoms for at least 30 weeks post injection (Mayfield et al. 2008). Ploetz et al. (2011) reported similar results with several triazoles *in vitro* and *in planta* against *R. lauricola* and determined that several triazoles, including propiconazole, had the most activity against *R. lauricola*. It is unclear why single propiconazole treatments were not effective during this study given that propiconazole is systemic and showed activity in both *in vitro* screens and in trees receiving combination treatments of propiconazole and emamectin benzoate.

Thiabendazole has been studied for the management of Dutch Elm Disease (Stennes and French 1987) and Laurel Wilt (Mayfield et al. 2008; Ploetz et al. 2011). Preventative treatments with thiabendazole prior to inoculation with *Ophiostoma novo-ulmi* provided protection to trees for two seasons and thiabendazole was found to persist in new wood growth for three growing seasons (Stennes 1981; Stennes and French 1987). Thiabendazole was found to inhibit *R. lauricola* growth *in vitro* at 10ppm (Mayfield et al. 2008) and greenhouse studies indicated that thiabendazole could reduce symptom severity in *R. lauricola* inoculated plants, but to a lesser extent than that of propiconazole treated plants. The results from this study indicate that thiabendazole was not effective in reducing attacks of SHB on California sycamore even though thiabendazole was effective *in vitro* against the various fungi and that all thiabendazole treated trees sampled exhibited inhibition in bioassays. It has been suggested that products with longer retention times, but reduced fungitoxicity may be more important for the management of

these diseases (Ploetz et al. 2011; Stennes 2000). Although not effective in significantly reducing beetle attacks, the use of thiabendazole due to its retention should be further investigated for FD especially in non-reproductive hosts where beetle colonization is not a concern, but the potential for fungal deposition is.

The use of insecticides and/or fungicides is promising for the management of SHB-FD in southern California especially when used as part of an IPM strategy for this pest-disease complex. Despite the efficacy of several pesticides shown in this study, several considerations must be given to the recommendations/conclusions to be drawn from the results of this trial. Further chemical studies using other hosts of SHB-FD and including longer trial times (> 1 year) will be necessary to determine the efficacy of these pesticides for use in particular hosts as well as to determine the length of time a product can provide adequate protection for. Fungicide retention bioassays in avocado treated with several compounds used in this study show no inhibition against fungal symbionts tested despite these trials being active for one year (Carrillo et al. *unpublished*). This lack of inhibition may suggest a difference in host vascular structure which is affecting the uptake and distribution of various pesticides. Other factors such as tree health, soil moisture, and temperature can all affect the uptake and distribution of a product (Fettig et al. 2013, 2014). Additionally, the degree of SHB-FD infection of a host is likely to affect the uptake and distribution of a product as heavily infested trees have more vasculature compromised by both beetle boring and fungal colonization thereby reducing the efficacy of a product. As noted by Eatough-Jones et al. (2017) the limited pesticide efficacy from their study may be due to both the advance level of infestation of the trees in addition to

the length of time (6 months) the trees were monitored for. Future long-term studies involving multiple SHB-FD hosts will be necessary to further evaluate product efficacy as well as determine specific management protocols for individual hosts.

RECOMMENDATIONS

Based on the result of this study, the following recommendations are given for the chemical management of SHB-FD: (i) careful monitoring of trees of interest including surrounding trees is recommended at all times; (ii) pesticides should only be applied to trees with low to moderate levels of infestation; applications should not be made on non-infested trees as no preventative treatments have been reported nor is it likely that applications to heavily- infested trees will "rescue" these trees from SHB-FD; (iii) after pesticide application, trees should be monitored regularly and any routine maintenance (i.e. pruning) should be performed as necessary and (iv) heavily infested trees serve as "reservoirs" of shot hole borer and contribute to local beetle populations and may also pose a physical hazard and should therefore be removed and infested wood sanitized appropriately. For up-to-date information regarding the management of SHB-FD, please visit http://eskalenlab.ucr.edu/pshb.html.

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Table 4.1. Fungal isolates used in the *in vitro* fungicide screening experiment

Species	Isolate	Host	Host scientific name	Location	County[a]
Fusarium euwallaceae	UCR4082	Avocado	*Persea americana*	La Habra	LA
F. euwallaceae	UCR4109	Avocado	*P. americana*	San Marino	LA
F. euwallaceae	UCR4147	Avocado	*P. americana*	San Marino	LA
F. euwallaceae	UCR4152	Avocado	*P. americana*	Hacienda Heights	LA
F. euwallaceae	UCR4128	Box elder	*Acer negundo*	Hacienda Heights	LA
F. euwallaceae	UCR4175	Box elder	*A. negundo*	San Marino	LA
F. euwallaceae	UCR3200	Box elder	*A. negundo*	San Marino	LA
F. euwallaceae	UCR4336	Box elder	*A. negundo*	San Marino	LA
F. euwallaceae	UCR4060	Castor bean	*Ricinus communis*	Orange Co. River	Orange
F. euwallaceae	UCR4086	Castor bean	*R. communis*	Azusa	LA
F. euwallaceae	UCR4100	Castor bean	*R. communis*	Orange Co. River	Orange
F. euwallaceae	UCR4149	Castor bean	*R. communis*	La Habra	LA
F. kuroshium	UCR3645	Avocado	*P. americana*	Fallbrook	SD
F. kuroshium	UCR3654	Avocado	*P. americana*	Bonsall	SD
F. kuroshium	UCR3661	Avocado	*P. americana*	Escondido	SD
F. kuroshium	UCR3062	Avocado	*P. americana*	Escondido	SD
F. kuroshium	UCR3641	California sycamore	*Platanus racemosa*	El Cajon	SD
F. kuroshium	UCR3643	California sycamore	*P. racemosa*	El Cajon	SD
F. kuroshium	UCR3644	California sycamore	*P. racemosa*	El Cajon	SD
F. kuroshium	UCR3616	California sycamore	*P. racemosa*	San Diego	SD
F. kuroshium	UCR3615	Castor bean	*R. communis*	San Diego	SD
Graphium euwallaceae	UCR2974	Castor bean	*R. communis*	San Marino	LA
G. euwallaceae	UCR2975	Box elder	*A. negundo*	San Marino	LA
G. euwallaceae	UCR2977	Weeping acacia	*Acacia floribunda*	San Marino	LA
G. euwallaceae	UCR2979	Coast live oak	*Quercus agrifolia*	San Marino	LA
G. euwallaceae	UCR2980	Avocado	*P. americana*	San Marino	LA
G. kuroshium	UCR4593	Avocado	*P. americana*	Fallbrook	SD
G. kuroshium	UCR4606	Avocado	*P. americana*	Bonsall	SD
G. kuroshium	UCR4609	Avocado	*P. americana*	Bonsall	SD
G. kuroshium	UCR4616	Avocado	*P. americana*	Escondido	SD
G. kuroshium	UCR4618	Avocado	*P. americana*	Escondido	SD
Paracremonium pembeum	UCR2982	Box elder	*A. negundo*	San Marino	LA
P. pembeum	UCR2991	California sycamore	*P. racemosa*	San Marino	LA
P. pembeum	UCR2983	Avocado	*P. americana*	San Marino	LA
P. pembeum	UCR2994	Castor bean	*R. communis*	San Marino	LA

[a]LA: Los Angeles County; SD: San Diego County

Table 4.2. Fungicides tested in the *in vitro* screening and field trial for their ability to manage Fusarium Dieback

Active ingredient	Trade name	Chemical Family	Manufacturer	Field rate applied[a]	Application method[b]
Bacillus subtilis	Cease	Microbial	BioWorks	1% (v/v) solution	Trunk spray
Bifenthrin	Onyx	Pyrethroid	FMC	240 g/L	Trunk spray
Carbendazim+debacarb	Fungisol	Benzimidazole	Mauget	2.4 ml/cm DBH	Injection
Emamectin benzoate	Tree-äge	Avermectin	ArborJet	2.9 ml/cm DBH	Injection
Metconazole	Quash	Triazole	Valent USA	18.1 g/cm DBH	Trunk spray
Propiconazole	Propizol	Triazole	ArborJet	3.9 ml/cm DBH	Injection
Tebuconazole	Tebuject 16	Triazole	Mauget	2.4 ml/cm DBH	Injection
Thiabendazole	Arbotect 20-S	Benzimidazole	Syngenta	2.4 ml/cm DBH	Injection
Azoxystrobin	Abound	Strobilurin	Syngenta	...	...
Fluopyram	Luna Privelage	Benzamide-pyridine	Bayer	...	...
Fluxapyroxad	Xemium	Carboxamide	BASF	...	...
Myclobutanil	Rally	Triazole	DowAgroSciences	...	...
Pyraclostrobin	Cabrio	Strobilurin	BASF	...	...
Pyrimethanil	Scala	Anilopyrimidine	Bayer	...	...
Triadimefon	Bayleton Flo	Triazole	Bayer	...	...
Trifloxystrobin	Flint	Strobilurin	Bayer	...	...
Triflumizole	Procure	Imidazole	Chemtura	...	...

[a] DBH= diameter at breast height; applications of B. subtilis, bifenthrin, and metconazole were mixed with 2% (v/v) penetrant (Pentra-Bark, Quest Products Corp.)

[b] Trunk sprays were applied with a trunk-mounted sprayer until run off

Table 4.3. Mean and standard deviation of EC_{50} values for fungal associates of SHB in in vitro fungicide experiment

Active ingredient	*Fusarium euwallaceae*	*Fusarium kuroshium*	*Graphium euwallaceae*	*Graphium kuroshium*	*Paracremonium pembeum*
	EC_{50} (µg/ml)[ab]	EC_{50} (µg/ml)	EC_{50} (µg/ml)	EC_{50} (µg/ml)	EC_{50} (µg/ml)
Metconazole	(25) 0.031±0.019 a	(100) 0.019±0.007 b	(1000) 1.392±1.089 d	(1000) 0.943±0.136 e	(100) 0.102±0.054 b
Pyraclostrobin	(100) 0.037±0.019 a	(100) 0.005±0.002 a	(10) 0.007±0.006 a	(10) 0.004±0.001 a	(100) 0.023±0.009 a
Tebuconazole	(100) 0.055±0.025 b	(100) 0.240±0.103 d	(5000) 4.859±1.515 e	(5000) 4.424±1.386 f	(500) 1.031±0.567 c
Trifloxystrobin	(100) 0.058±0.023 b	(100) 0.072±0.031 c	(100) 0.081±0.065 b	(100) 0.042±0.016 b	(100) 0.030±0.013 a
Thiabendazole	(500) 0.189±0.039 c	(1000) 1.400±0.195 f	(1000) 4.444±0.695 e	(1000) 0.616±0.143 d	(5000) 14.178±1.813 f
Azoxystrobin	(1000) 0.697±0.420 e	(1000) 1.171±0.489 f	(100) 0.113±0.056 b	(100) 0.047±0.022 b	(500) 0.967±0.615 c
Fluopyram	(1000) 1.779±0.595 f	(5000) 2.814±1.092 g	(5000) NI	NT	(500) 0.812±0.217 c
Myclobutanil	(1000) 2.171±1.695 f	(1000) 1.291±0.341 f	(5000) NI	(5000) NI	(5000) 4.467±1.400 e
Propiconazole	(1000) 0.423±0.262 d	(1000) 0.483±0.206 e	(1000) 0.234±0.084 c	(1000) 0.242±0.035 c	(5000) 4.573±2.507 e
Triflumizole	(1000) 0.647±0.574 de	(1000) 0.381±0.060 e	(5000) 4.891±3.848 e	(5000) 5.157±1.703 f	(1000) 1.857±0.801 d
Fluxapyroxad	(5000) NI[c]	(5000) NI	(5000) NI	(5000) NI	(5000) NI
Pyrimethanil	(5000) 4.768±2.959 g	(5000) NI	NT[d]	NT	(5000) NI
Triadimefon	(5000) NI	(5000) NI	(5000) NI	(5000) NI	(5000) NI
HSD[e] (α= 0.05)	0.052	0.029	0.064	0.014	0.041

[a] In brackets, concentrations applied (µg/ml)

[b] Numbers are mean and standard deviation of EC50 values. Levels connected by the same letter are not significantly different.

[c] No inhibition

[d] Not tested

[e] Tukey's honest significant difference (HSD)

Table 4.4. Fungicide retetntion bioassay from treated California sycamore trees

Treatment	No. of trees	No. of trees with samples exhibiting inhibition	% of samples per tree exhibiting inhibition			% of samples per tree exhibiting ZOI[a] in agar	
			Mean	Range	P-value[b]	Mean	Range
Thiabendazole	8	8	47	25-75	< 0.0001	16	25-50
Propiconazole	8	5	50	25-100	0.0001	0	0
Propiconazole + emamectin benzoate	8	5	45	25-75	0.0004	0	0
Carbendazim + Debacarb	8	5	25	25	0.0127	0	0
Tebuconazole	8	5	25	25	0.0127	0	0
Metconazole	7	1	25	25	> 0.05	0	0
B. subtilis	8	0	...	...	...	...	...
Untreated	8	0	...	...	...	...	...

[a] Zone of inhibition

[b] p-values for treatments are in comparison to untreated controls using independent contrast

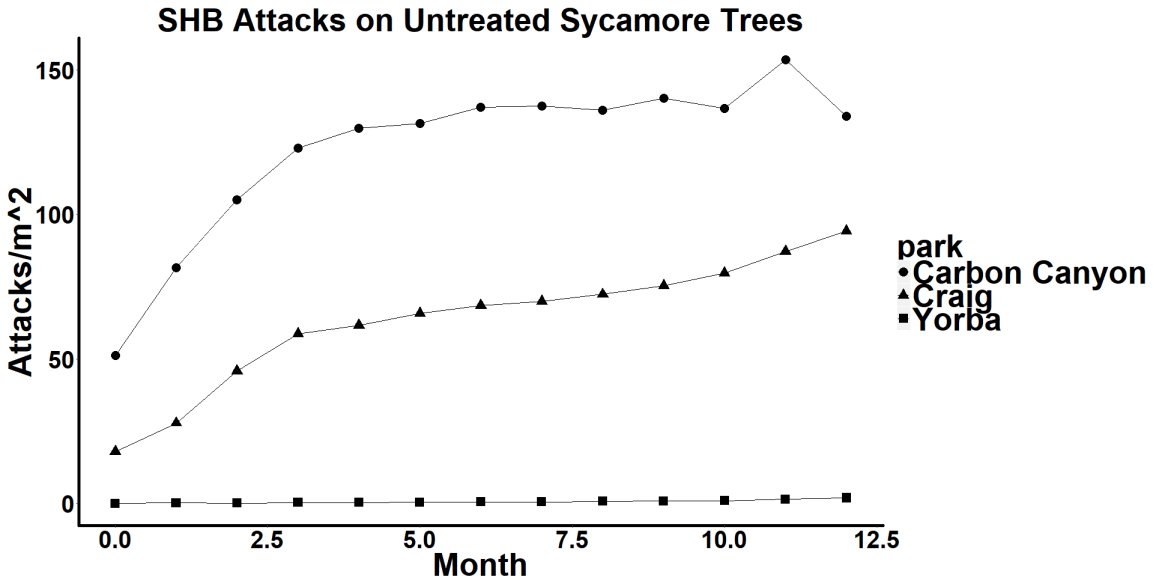

Figure 4.1. Mean SHB attacks/m^2 of untreated control trees over twelve months at three sites with various infestation rates (Yorba: low; Craig: moderate; and Carbon Canyon: heavy).

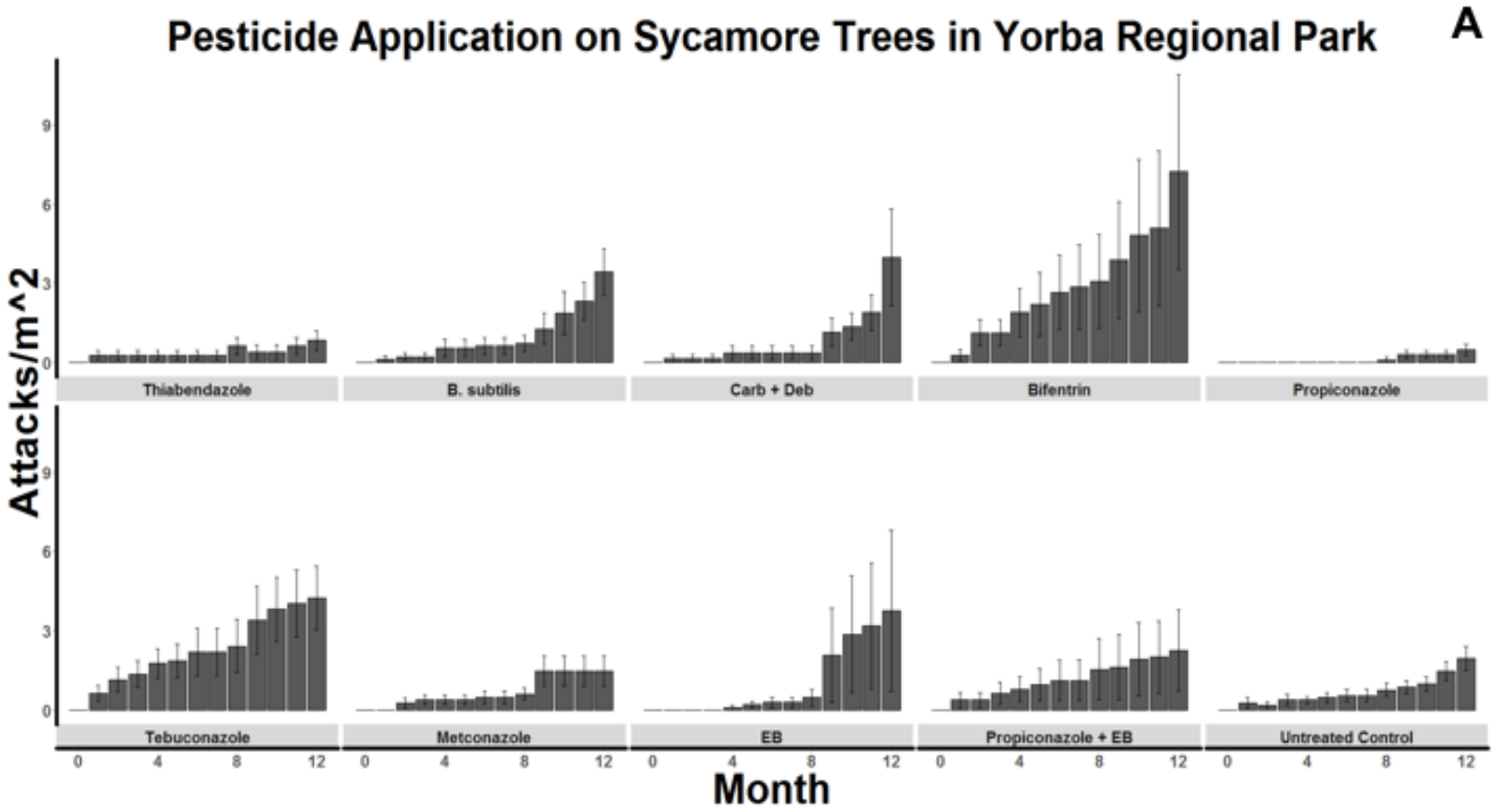

Figure 4.2.1. Mean SHB attacks/m^2 for pesticide treated trees at Yorba Regional Park. Vertical bars represent standard error of means. P-values are derived from comparisons between individual treatments and untreated controls at α=0.05.

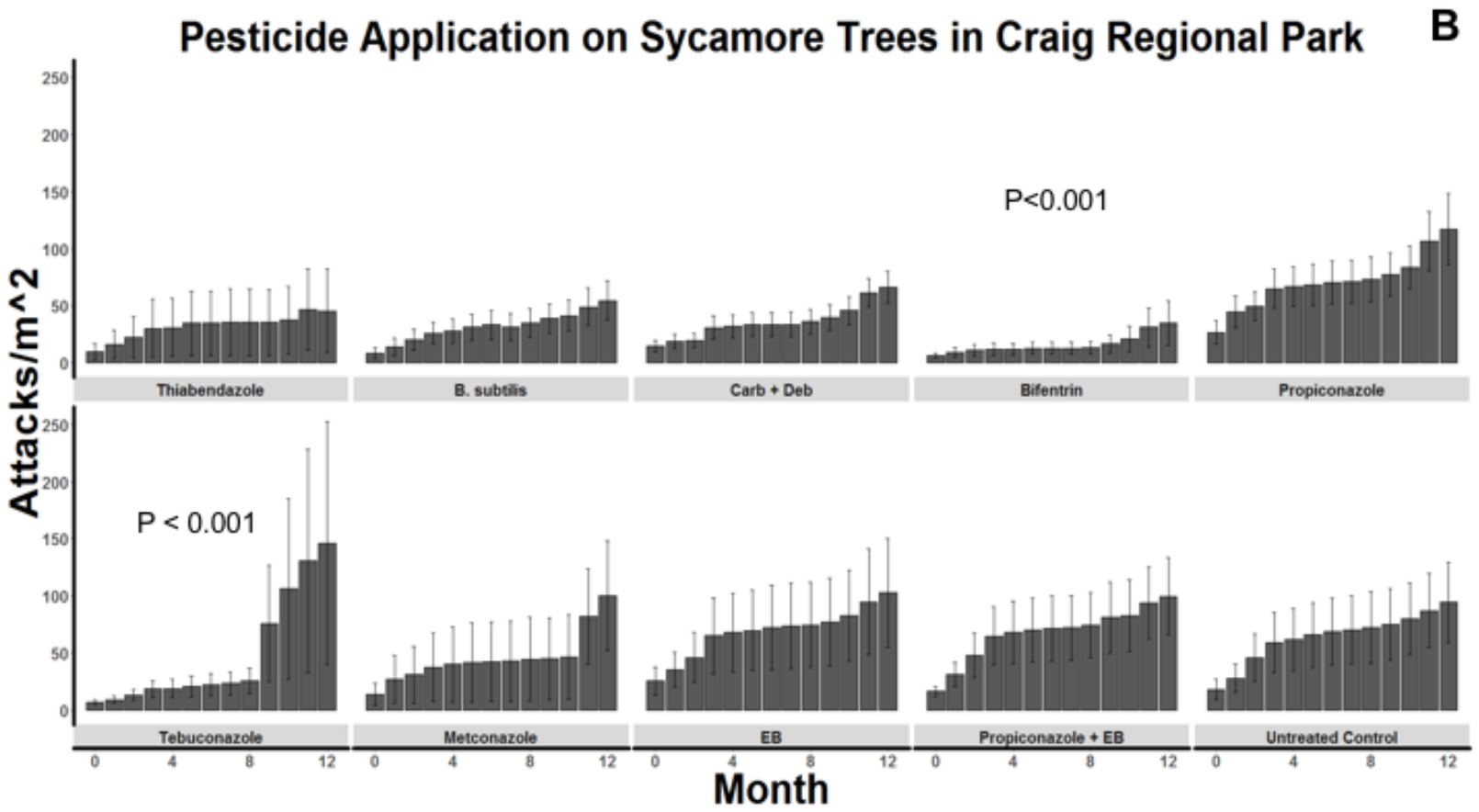

Figure 4.2.2. Mean SHB attacks/m^2 for pesticide treated trees at Craig Regional Park. Vertical bars represent standard error of means. P-values are derived from comparisons between individual treatments and untreated controls at α=0.05.

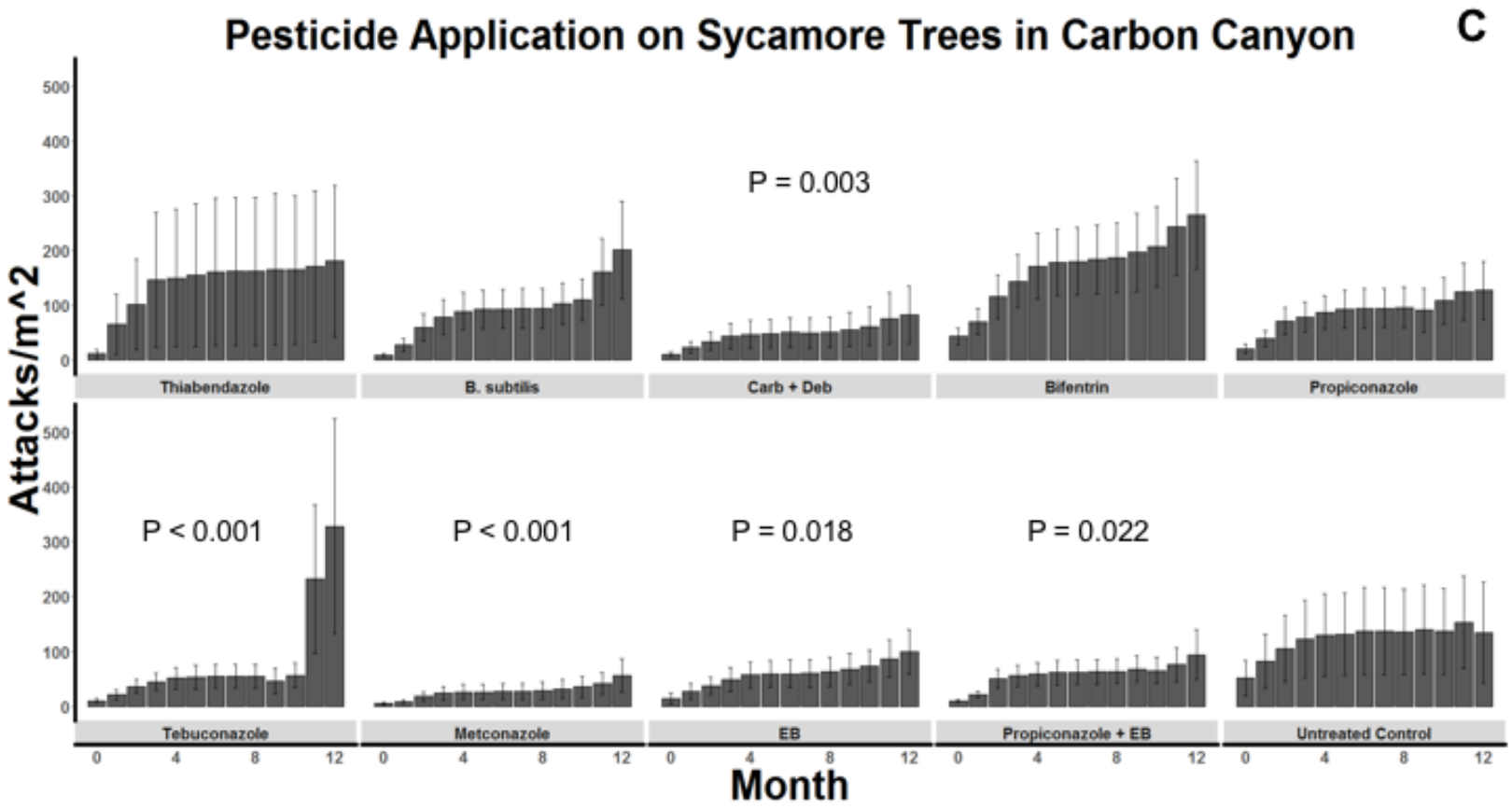

Figure 4.2.3. Mean SHB attacks/m^2 for pesticide treated trees at Carbon Canyon Regional Park. Vertical bars represent standard error of means. P-values are derived from comparisons between individual treatments and untreated controls at α=0.05.

GENERAL CONCLUSION

The studies presented within this dissertation highlight the diversity of canker and dieback diseases present in citrus and California sycamore in California and provide strategies for the management of these diseases. This work identified two new diseases of citrus in California: Eutypella canker of citrus which was found to be predominate in the Desert Region of California, but can also be found in the San Joaquin Valley Region and Colletotrichum Dieback which has only been found to occur in the San Joaquin Valley Region. Hendersonula, an ongoing canker and dieback disease of citrus since the early 1950's, was revisited and determined to be more predominate than Eutypella canker of citrus in the Desert Region of California. Chemical management strategies were studied for both Eutypella canker of citrus and Hendersonula and several candidate fungicides were determined to be efficacious in reducing disease severity of these two pathosystems. Finally, chemical management strategies were investigated for a new dieback disease of sycamore in California known as Shot hole borer-Fusarium Dieback (SHB-FD) and several fungicides and insecticides were determined to be efficacious in reducing SHB attacks in California sycamore.

Hendersonula and Eutypella canker of citrus continue to be pervasive canker and dieback diseases in citrus growing areas of California, particularly in the Desert Region. *Neoscytalidium dimidiatum* was determined to be the most abundant fungal pathogen associated with branch canker and dieback symptoms in the Desert Region followed by *Eutypella citricola*, *E. microtheca*, and *Eutypella* sp. In less than 3% of cases, both *N. dimidiatum* and at least one *Eutypella* species could be co-isolated from the same

cankered branch suggesting that a complex etiology of branch canker and dieback diseases in general may exist whereby distinct pathogens may participate as one "super pathogen complex" within the same plant host. *Eutypella* sp. was determined to be the most aggressive fungal pathogen in pathogenicity tests followed by *N. dimidiatum*, *E. microtheca*, and *E. citricola* suggesting that Eutypella canker of citrus may be more aggressive in terms of disease severity when comparing the virulence of fungal canker pathogens in this area. Additionally, temperature studies revealed that fungal colonization is enhanced for at least *N. dimidiatum* and *E. microtheca* when environmental temperatures are closer to the maximum fungal growth temperatures. This establishes the importance of environment (i.e. temperature) and its influence on fungal aggressiveness/colonization.

Management strategies for Hendersonula and Eutypella canker of citrus are limited to cultural practices which are focused on preventing environmental stress to a host and on the reduction of pathogen inoculum. In the absence of good orchard hygiene however, branch canker and dieback diseases can become problematic and severe decline or even death of trees is possible if these diseases are unmanaged, especially when considering the wound-invading nature of these pathogens. The identification of several fungicides, particularly strobilurins, which were capable of reducing fungal growth at low rates show promise as pruning/wound protectants in citrus. Overall, strobilurins were shown to reduce vascular necrosis caused by *N. dimidiatum* and *Eutypella citricola* when applied as pruning protectants 24 hours prior to pathogen introduction suggesting that application of these fungicides can help to reduce fungal colonization of wounds. This is

an important management tool for the citrus industry as hedging remains a common practice and creates multiple wounding sites allowing for greater chances of infection. Additionally, if high-density plantings are desired, this would increase the amount of training (pruning) needed for young orchards and could lead to significant decline if good grove hygiene is not adhered to which includes maintaining tree vigor through appropriate irrigation, pruning of infected twigs and branches below cankered areas during dry periods, prompt disposal of infested material, and disinfection of pruning materials. Therefore, a chemical strategy which could be used alongside current cultural practices forms the basis for an integrated pest management program for the management of canker and dieback diseases in citrus.

Colletotrichum Dieback is new disease of citrus and little is known regarding neither the epidemiology of this pathosystem nor the impact this disease will have on citrus production in California. *Colletotrichum gloeosporioides* and *C. karstii* are associated with all major citrus varieties surveyed in the San Joaquin Valley; *C. karstii* is also reported here as a new pathogen of citrus not previously recorded in citrus in the United States. It remains unclear at this time is *C. karstii* is a recent introduction to the state of California or if this pathogen was misidentified as *C. gloeosporioides* sensu stricto in other studies of *Colletotrichum* occurring on citrus. Moreover, the determination of *C. karstii* as a more aggressive pathogen than *C. gloeosporioides* raises concerns regarding the economic impact this pathogen will have on citrus production. The need for early management strategies for Colletotrichum Dieback is imperative as

little is known regarding this pathosystem, especially as it is unclear if this pathogen behaves as a "canonical" canker pathogen similar to *Eutypella* or *Neoscytalidium*.

One of the most devastating dieback diseases to affect California hardwoods in recent years is the new invasive pest-disease complex Shot hole borer-Fusarium Dieback (SHB-FD). The wide host range of this pest-disease complex together with the seemingly year-round reproductive cycle of its beetle vector, SHB-FD remains a major concern not only for urban forests, but for wildlands and avocado production in southern California. The need for effective pesticide management for this pest-disease complex is paramount to limiting the spread of both the vector as well as suppressing the fungal colonization of its pathogenic fungal symbionts in the host. The identification of several insecticides and fungicides capable of reducing SHB attacks in California sycamore provides hope for immediate use of a chemical management strategy alongside cultural methods not only for this native landscape tree, but for other hardwoods currently planted in urban landscapes. Invasive pests of this nature pose significant ecological threats as they are highly destructive and disrupt organisms on many levels in the ecosystem especially when current management strategies are not effective or robust enough to limit disease progression and severity. Although it is unlikely that any curative or preventative treatments will be found for SHB-FD, the current findings provide several options for chemical management which can ultimately be tailored to various hosts and host locations so that suitable management strategies are available to a variety of state and private agencies and appropriate for various site locations and conditions.

One of the greatest challenges to pest management is the emergence of new pests and pathogens and the need for effective management strategies which can be implemented. Although canker and dieback diseases can share common features in their symptomatology, epidemiology, and pathogen biology, these diseases can be quite distinct from each other and may function as truly singular pathosystems or they may function under a much broader pathosystem involving several different pathogens known to occur on the same host or within several hosts in a defined area. Understanding the basic biology and epidemiology of these diseases is the most important step in being able to develop effective management strategies which can be enacted in a relatively short period of time. Investigations presented wherein highlight the diversity of canker and dieback diseases of citrus and California sycamore in California and provide respective industries and agencies with chemical management options for the management of these diseases.