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

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Abstract

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Several members of the families Botryosphaeriaceae and Diatrypaceae are known as canker and dieback pathogens of a number of woody hosts. Because 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 hyalinum*, *Eutypella citricola*, *E. microtheca*, and an

unnamed *Eutypella* sp., were associated with branch canker. *N. hyalinum* was the most frequently recovered fungus from symptomatic tissues (31%) followed by *E. citricola* (10%), *E. microtheca* (4%), and the *Eutypella* sp. (2%). In pathogenicity tests, all fungi caused lesions when inoculated on 'Lisbon' lemon (citrus) branches. Lesions caused by the *Eutypella* sp. were significantly longer than those of the other *Eutypella* spp.; however, they did not differ significantly from those produced by *N. hyalinum*. 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. hyalinum* 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.

Citrus production in California is valued at over \$1.9 billion 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 in both agricultural and nonagricultural fields. Many of these studies have shown the diversity of members of the families Botryosphaeriaceae or Diatrypaceae occurring on a number of agricultural commodities in California (almond, avocado, cherry, citrus, and 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, 2011; Úrbez-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 (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 family are known to be associated with grapevine trunk diseases (Luque et al. 2012; Trouillas et al. 2010, 2011; Urbez-Torres et al. 2009, 2012); however, their individual impact on 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 1900s (Punithalingam 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 is 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; Punithalingam 1980; Whiteside 1980). The disease known as Hendersonula branch wilt, or simply Hendersonula, caused by the botryosphaeriaceous fungus *Neoscytalidium hyalinum* (C. K. Campb. & J. L. Mulder) A. J. L. Phillips, M. Groenew. & Crous (formerly *H. toruloidea* and *N. dimidiatum*) has been reported to be affecting desert citrus production in California

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(Calavan and Wallace 1954) and Israel (Oren et al. 2001). Symptoms include characteristic black spore masses underneath the bark, branch cankers, wilt, and dieback. Recently, 10 species in the Botryosphaeriaceae family 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 a *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). *E. citricola* Speg. was reported on dead twigs of a *Citrus* sp. in Florida (Anonymous 1960). However, there is no record of this genus occurring on citrus in North America other than the study by Adesemoye and Eskalen (2011). Although it appears that the genus *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 (Trouillas et al. 2010) and nonagricultural trees and shrubs such as 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* spp. made up 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 (four orchards in Riverside County), Imperial Valley (four in Imperial County), and Borrego Springs (one in San Diego County) between summer 2011 and spring 2013 and in summer 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 (PDA; 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 families 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; oat flakes at 30 g/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.). 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 eight different temperatures (5, 10, 15, 20, 25, 30, 35, and 40°C) for 4 days. Colony diameter was measured every day for 4 days and this experiment was repeated once. Average daily growth was calculated by computing the average growth for fungal isolates at 2 and 3 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, and 0.5% sodium dodecyl sulfate) and glass beads. Tubes were bead bashed using a FastPrep-24 machine (MP Biomedicals Inc.) at 4 M/s for 30 s. Oligonucleotide primers internal transcribed spacer (ITS)4 and ITS5 (White et al. 1990) were used to amplify the ITS1-5.8S-ITS2 region of the ribosomal DNA, primers Bt2a and Bt2b (White et al. 1990) were used to amplify a partial region of the nuclear β -tubulin gene, and primers EF1F and EF2R (Jacobs et al. 2004) were used to amplify a portion of the translation elongation factor (EF) 1- α gene. Polymerase chain reaction (PCR) was carried out as previously described (Slippers et al. 2004). Reaction mixtures consisted of 1 $\times$ reaction buffer, 0.2 mM dNTP, 0.2 μ M forward and reverse primers, 2.5 U of standard *Taq* polymerase (New England Biolabs), and template DNA ranging from 50 to 100 ng/ μ l. Cycling conditions were as follows: an initial preheat at 95°C for 2 min; 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 1 $\times$ Tris-borate-EDTA buffer and photographed under UV light after staining with SYBR Green (Invitrogen). PCR products were purified using ExoSAP-IT (Affymetrix) 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.) and local alignments were done using Clustal X (Thompson et al. 1997). Phylogenetic analysis was performed with MEGA6 (Tamura et al. 2013) and sequences in the present study (Table 1) were compared with known sequences in GenBank (Table 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 families, respectively. Maximum-parsimony analysis was performed for each dataset using the Subtree-Pruning-Regrafting 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 (RCI), and homoplasy index were recorded.

Pathogenicity tests and effect of temperature on fungal colonization. Pathogenicity tests were conducted on 3-year-old 'Lisbon' lemon (*C. limon*) grafted on C-35 citrange rootstock maintained in a greenhouse (average temperature range of 21 to 32°C) from December 2013 to August 2014. To assess pathogenicity, plants were stem wound-inoculated on the scion (approximately 1 cm in 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 1-week-old fungal culture for each isolate (Table 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 tissues developing outside the area of inoculation were cultured on PDA-tet and fungal isolates obtained from reisolation were identified based on their morphology (colony shape, color, and 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 at the 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 min to surface disinfect. Leaves were removed and twigs were cut into segments approximately 12 cm long. 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 1-week-old culture (Table 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 two-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 or 32°C in microbiological incubators for 10 days. Each temperature and isolate combination had five 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). One-way analysis of variance was used to compare lesion length means. All pairs of means were compared using Tukey's honestly significant difference

Table 1. Representative isolates of *Neoscytalidium hyalinum* 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
<i>Neoscytalidium hyalinum</i>	UCRDC08 ^c	Imperial	<i>Citrus paradisi</i>	Rio Red	KF620362	KF620398
<i>N. hyalinum</i>	UCRDC29	Imperial	<i>C. paradisi</i>	Rio Red	KF620363	KF620399
<i>N. hyalinum</i>	UCRDC59	Riverside	<i>C. paradisi</i>	Pink	KF620364	KF620400
<i>N. hyalinum</i>	UCRDC65	Riverside	<i>C. paradisi</i>	Pink	KF620365	KF620401
<i>N. hyalinum</i>	UCRDC132	Riverside	<i>C. limon</i>	Allen Eureka	KF620366	KF620402
<i>N. hyalinum</i>	UCRDC170	Imperial	<i>C. paradisi</i>	Ruby Red	KF620367	KF620403
<i>N. hyalinum</i>	UCRDC176	Imperial	<i>C. paradisi</i>	Rio Red	KF620368	KF620404
<i>N. hyalinum</i>	UCRDC280	San Diego	<i>C. limon</i>	Lisbon	KF620369	KF620405
<i>N. hyalinum</i>	UCRDC284	San Diego	<i>C. limon</i>	Lisbon	KF620370	KF620406
<i>Eutypella citricola</i>	UCRDC06	Imperial	<i>C. paradisi</i>	Rio Red	KF620371	KF620407
<i>E. citricola</i>	UCRDC83	Riverside	<i>C. limon</i>	Allen Eureka	KF620372	KF620408
<i>E. citricola</i>	UCRDC88	Riverside	<i>C. limon</i>	Allen Eureka	KF620373	KF620409
<i>E. citricola</i>	UCRDC90	Riverside	<i>C. limon</i>	Allen Eureka	KF620374	KF620410
<i>E. citricola</i>	UCRDC91 ^c	Imperial	<i>C. paradisi</i>	Rio Red	KF620375	KF620411
<i>E. citricola</i>	UCRDC112	Imperial	<i>C. paradisi</i>	Rio Red	KF620376	KF620412
<i>E. citricola</i>	UCRDC113	Imperial	<i>C. paradisi</i>	Rio Red	KF620377	KF620413
<i>E. citricola</i>	UCRDC117 ^c	Imperial	<i>C. paradisi</i>	Rio Red	KF620378	KF620414
<i>E. citricola</i>	UCRDC186	Imperial	<i>C. paradisi</i>	Ruby Red	KF620379	KF620415
<i>E. citricola</i>	UCRDC272	San Diego	<i>C. limon</i>	Lisbon	KF620380	KF620416
<i>E. citricola</i>	UCRDC291	San Diego	<i>Tamarix</i> sp.	...	KF620381	KF620417
<i>E. citricola</i>	UCRDC293	San Diego	<i>C. limon</i>	Lisbon	KF620382	KF620418
<i>E. citricola</i>	UCRDC333	Riverside	<i>C. limon</i>	Allen Eureka	KF620383	KF620419
<i>Eutypella microtheca</i>	UCRDC09 ^c	Imperial	<i>C. paradisi</i>	Rio Red	KF620384	KF620420
<i>E. microtheca</i>	UCRDC37 ^c	Imperial	<i>C. paradisi</i>	Rio Red	KF620385	KF620421
<i>E. microtheca</i>	UCRDC67	Riverside	<i>C. paradisi</i>	Pink	KF620386	KF620422
<i>E. microtheca</i>	UCRDC103	Riverside	<i>C. paradisi</i>	Pink	KF620387	KF620423
<i>E. microtheca</i>	UCRDC106	Riverside	<i>C. paradisi</i>	Pink	KF620388	KF620424
<i>E. microtheca</i>	UCRDC148	Imperial	<i>C. limon</i>	Lisbon	KF620389	KF620425
<i>E. microtheca</i>	UCRDC325	Riverside	<i>C. limon</i>	Allen Eureka	KF620390	KF620426
<i>Eutypella</i> sp.	UCRDC142	Riverside	<i>C. limon</i>	Allen Eureka	KF620391	KF620427
<i>Eutypella</i> sp.	UCRDC143	Riverside	<i>C. limon</i>	Allen Eureka	KF620392	KF620428
<i>Eutypella</i> sp.	UCRDC210 ^c	Imperial	<i>C. paradisi</i>	Ruby Red	KF620393	KF620429
<i>Eutypella</i> sp.	UCRDC211	Imperial	<i>C. paradisi</i>	Ruby Red	KF620394	KF620430
<i>Eutypella</i> sp.	UCRDC276 ^c	San Diego	<i>C. limon</i>	Lisbon	KF620395	KF620431
<i>Eutypella</i> sp.	UCRDC287	San Diego	<i>Tamarix</i> sp.	...	KF620396	KF620432
<i>Eutypella</i> sp.	UCRDC335	Riverside	<i>C. limon</i>	Allen Eureka	KF620397	KF620433

^a ITS = internal transcribed spacer region.

^b EF1- α = translation elongation factor 1- α . Accession numbers given for *N. hyalinum* correspond to the EF1- α gene and accession numbers given for *Eutypella* spp. correspond to the β -tubulin gene.

^c Isolates used in pathogenicity test and the effect of temperature experiment.

(HSD), where $\alpha = 0.05$. Data from the independent trials was checked for homogeneity error of variance and no heterogeneity was detected; thus, data from each independent trial were combined. To assess the effect of temperature on fungal colonization, lesion

lengths were analyzed by an independent-samples *t* test with confidence level $\alpha = 0.05$ 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

Table 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	<i>Eutypella citricola</i>	<i>Citrus paradisi</i>	Australia	HQ692589	HQ692521
HVVIT07 ^c	<i>E. citricola</i>	<i>Vitis vinifera</i>	Australia	HQ692579	HQ692512
WA06FH	<i>E. citricola</i>	<i>V. vinifera</i>	Australia	HQ692588	HQ692518
UCD2342Co	<i>E. citricola</i>	<i>V. vinifera</i>	California	GQ293968	GQ294022
UCD2349Co	<i>E. citricola</i>	<i>V. vinifera</i>	California	GQ293969	GQ294021
MSUELM13	<i>E. vitis</i>	<i>V. vinifera</i>	United States	DQ006943	DQ006999
UCD2428TX	<i>E. vitis</i>	<i>V. vinifera</i>	United States	FJ790851	GU294726
HVFIG02 ^c	<i>E. cryptovalsoidea</i>	<i>Ficus carica</i>	Australia	HQ692573	HQ692524
HVFIG05	<i>E. cryptovalsoidea</i>	<i>F. carica</i>	Australia	HQ692574	HQ692525
HVGRF02 ^c	<i>E. microtheca</i>	<i>C. paradisi</i>	Australia	HQ692569	HQ692533
UCD2Co	<i>E. microtheca</i>	<i>V. vinifera</i>	California	GQ293958	GQ294018
UCD3Co	<i>E. microtheca</i>	<i>V. vinifera</i>	California	GQ293957	GQ294019
YC17	<i>E. microtheca</i>	<i>V. vinifera</i>	Australia	HQ692562	HQ692537
YC24	<i>E. microtheca</i>	<i>V. vinifera</i>	Australia	HQ692565	HQ692530
UCD713SJ	<i>Eutypella</i> sp.	<i>V. vinifera</i>	California	GQ293955	GQ294016
BAFC3321	<i>Peroneutypa scoparia</i>	N/A ^d	Argentina	EU702431	EU728698
BAFC3322	<i>P. scoparia</i>	N/A	Argentina	EU702432	EU728699
DFMAL100	<i>P. scoparia</i>	<i>Robinia pseudoacacia</i>	France	GQ293962	GQ294029
UCD2334Co	<i>Eutypella</i> sp.	<i>V. vinifera</i>	California	GQ293963	GQ294027
UCD2335Co	<i>Eutypella</i> sp.	<i>V. vinifera</i>	California	GQ293964	GQ294028
EP18	<i>Eutypa lata</i>	<i>V. vinifera</i>	Australia	HQ692611	HQ692501
RGA01	<i>E. lata</i>	<i>Fraxinus angustifolia</i>	Australia	HQ692614	HQ692497
ADSC300	<i>E. lata</i>	<i>Schinus molle</i>	Australia	HQ692610	HQ692493
HVGRF03 ^c	<i>Diatrypella vulgaris</i>	<i>C. paradisi</i>	Australia	HQ692590	HQ692502
HVPT01	<i>D. vulgaris</i>	<i>S. molle</i>	Australia	HQ692594	HQ692506
RGA02	<i>E. leptoplaca</i>	<i>F. angustifolia</i>	Australia	HQ692602	HQ692483
TUQU01	<i>E. leptoplaca</i>	<i>Jugercus</i> sp.	Australia	HQ692598	HQ692491
CBS251.49	<i>Neoscytalidium hyalinum</i>	<i>Juglans regia</i>	United States	KF531819	KF531797
CBS145.78 ^c	<i>N. hyalinum</i>	<i>Homo sapiens</i>	United Kingdom	KF531816	KF531795
WAC12693	<i>N. hyalinum</i>	<i>Mangifera indica</i>	Australia	EF585538	EF585576
WAC12690	<i>N. hyalinum</i>	<i>M. indica</i>	Australia	EF585537	EF585577
CBS499.66	<i>N. hyalinum</i>	<i>M. indica</i>	Mali	KF531820	EU144063
CBS122610	<i>N. novaehollandiae</i>	<i>Acacia synchronicia</i>	Australia	EF585536	EF585578
WAC12691	<i>N. novaehollandiae</i>	<i>M. indica</i>	Australia	EF585543	EF585574
WAC13303	<i>N. novaehollandiae</i>	<i>M. indica</i>	Australia	GU172398	GU172430
CBS122070	<i>N. novaehollandiae</i>	<i>Grevillea agrifolia</i>	Australia	EF585539	EF585579
WAC13304	<i>N. novaehollandiae</i>	<i>M. indica</i>	Australia	GU172399	GU172431
WAC12688	<i>N. novaehollandiae</i>	<i>M. indica</i>	Australia	EF585542	EF585575
CBS122072	<i>N. novaehollandiae</i>	<i>Adansonia gibbosa</i>	Australia	EF585535	EF585581
WAC13273	<i>N. novaehollandiae</i>	<i>M. indica</i>	Australia	GU172397	GU172429
CBS122071 ^c	<i>N. novaehollandiae</i>	<i>Crotalaria medicaginea</i>	Australia	EF585540	EF585580
WAC13275	<i>N. novaehollandiae</i>	<i>M. indica</i>	Australia	GU172400	GU172432
MFLUCC11-0425 ^c	<i>Cophinforma eucalypti</i>	<i>Eucalyptus</i> sp.	Thailand	JX646800	JX646865
MFLUCC11-0655	<i>C. eucalypti</i>	<i>Eucalyptus</i> sp.	Thailand	JX646801	JX646866
CMW22674	<i>C. atrovirens</i>	<i>Pterocarpus angolensis</i>	South Africa	FJ888473	FJ888456
MFLUCC10-0051	<i>Botryosphaeria agaves</i>	<i>Agave</i> sp.	Thailand	JX646790	JX646855
MFLUCC11-0125 ^c	<i>B. agaves</i>	<i>Agave</i> sp.	Thailand	JX646791	JX646856
ATCC22927	<i>B. corticis</i>	<i>Vaccinium</i> sp.	United States	DQ299247	EF614931
CBS119047 ^c	<i>B. corticis</i>	<i>V. corymbosum</i>	United States	DQ299245	EU017539
MFLUCC10-0098 ^c	<i>B. fusispora</i>	<i>Entada</i> sp.	Thailand	JX646789	JX646854
CBS116741	<i>B. dothidea</i>	<i>Olea europaea</i>	Greece	AY640254	AY640257
CMW8000 ^c	<i>B. dothidea</i>	<i>Prunus</i> sp.	Switzerland	AY236949	AY236898
CBS110302	<i>B. dothidea</i>	<i>Vitis</i> sp.	Portugal	AY259092	AY573218
CBS162.25	<i>Macrophomina phaseolina</i>	<i>Eucalyptus</i> sp.	N/A	KF531826	KF531803
CBS447.68	<i>Phyllosticta foliorum</i>	<i>Taxus baccata</i>	Netherlands	FJ824768	FJ824773
CBS22733	<i>M. phaseolina</i>	<i>Zea mays</i>	N/A	KF531825	KF531804

^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 and accession numbers given for *Eutypella* spp. correspond to the β -tubulin gene.

^c Denotes type-specimen.

^d N/A = not available.

density function (Miller et al. 2003) in SigmaPlot (version 11; Systat Software Inc.).

Results

Field survey, fungal recovery, and distribution. Disease was observed on lemon and grapefruit trees ranging in age from 20 to 40 years old on the following varieties: Lisbon and Allen Eureka lemon (*C. limon*) and '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* spp.) 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 *N. hyalinum*. 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 diatrypeaceous fungi.

Both *Neoscytalidium* and *Eutypella* spp. were detected in all three counties sampled (Fig. 1A and B). At least two species of *Eutypella* were detected in each county. The frequency of *Eutypella* spp. recovered between counties varied but *E. citricola* was the most frequently recovered (Fig. 1B).

N. hyalinum was the most frequently isolated in Riverside and Imperial Counties (Table 3). In Riverside County, *N. hyalinum* and *Eutypella* spp. were recovered from 38 and 14%, respectively, of necrotic tissues collected from branches. In Imperial County, *N. hyalinum* and *Eutypella* spp. were recovered from 27 and 16%, respectively, of necrotic tissues collected from branches. *N. hyalinum* and *Eutypella* spp. were recovered from 10 and 27%, respectively, of necrotic tissues collected from branches in San Diego. Both *N. hyalinum* 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 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).

Morphological identification. Three groups of *Eutypella* spp. were distinguished based on colony and conidial morphology (Table 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 by 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 by 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 by 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. hyalinum* (Phillips et al. 2013). Other fungal genera isolated at low frequencies from branch cankers included *Aspergillus*, *Alternaria*, *Coniophora*, *Fusarium*, *Hypoxylon*, and *Spenceriartinsia*.

Results from the radial growth experiment revealed that all fungi could grow between 15 and 35°C (Fig. 2). *N. hyalinum* and *E. microtheca* were capable of growth at 40°C; however, *E. microtheca* isolates grew very little (<3 mm) by the end of 3 days. Optimal temperatures for *N. hyalinum*, *E. microtheca*, and *E. citricola* occur between 30 and 35°C whereas the optimum temperature range for the *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 family Diatrypeaceae) and ITS and EF1- α (for the family Botryosphaeriaceae) genes were combined and analyzed. The ITS tree topology for the family Diatrypeaceae 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 the family Diatrypeaceae 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 the family Botryosphaeriaceae consisted of 796 nucleotides, of which 185 nucleotides were parsimony informative. Analysis of the combined dataset returned six 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. 3). The first clade (96% bootstrap support) corresponds to *E. citricola* (*Eutypella* sp. group 1) (Trouillas et al. 2010). The second *Eutypella* clade (99% bootstrap support) corresponds to *E. microtheca* Trouillas, W. M. Pitt & Gubler (*Eutypella* sp. group 2) (Trouillas et al. 2010). The third clade (99% bootstrap support) corresponds to an unidentified *Eutypella* sp. (*Eutypella* sp. 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* spp., one major clade (99% bootstrap support) was formed from the combined dataset (Fig. 4). The first subclade (83% bootstrap support) corresponds to *N. hyalinum*, 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, boob, and rattlepod in Australia.

Pathogenicity tests and effect of temperature on fungal colonization. At 2 to 4 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. hyalinum* 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. 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. hyalinum*. Lesions produced by *N. hyalinum* 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 reisolated 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. 6).

For *N. hyalinum*, 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 23.3 mm whereas the average lesion length at 32°C was 94.4 mm. 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 with 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 with those segments incubated at 25°C. The other isolate of *E. citricola* showed no significant differences in lesion length at either temperature. For isolates

Fig. 1. Fungal recovery (%) of **A**, *Neoscytalidium hyalinum* 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.

of the *Eutypella* sp., no significant difference was observed in lesion lengths at either temperature. Fungal recovery from these segments ranged from 95 to 100%.

Discussion

This study is the first comprehensive assessment of 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* spp., including *E. citricola*, *E. microtheca*, and an unidentified *Eutypella* sp., were identified from citrus branch samples showing cankers and dieback. These species have also been confirmed as pathogens of citrus because they were capable of producing lesions on citrus shoots. The unidentified *Eutypella* sp. is reported for the first time in citrus in California. *N. hyalinum* 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 the *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'Ivoire, Philippines, and the United States (California and Florida) (Adesemoye and Eskalen 2011; Anonymous 1960; Hanlin 1992; Rappaz 1987; Reinking 1918, 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 unidentified *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. hyalinum* 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

because these asexual states often exhibit considerable morphological overlap (Glawe and Rogers 1984; Rappaz 1987). Studies using a multigene phylogenetic approach have successfully distinguished members of the Diatrypaceae family associated with various hosts, particularly in grapevine (Acero et al. 2004; Gramaje et al. 2012; Luque et al. 2012; Trouillas et al. 2011, 2010; Urbez-Torres et al. 2012). In this study, all three *Eutypella* spp. could be distinguished based on multigene 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., amplified fragment length polymorphism, 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 intersimple-sequence repeat technique and determined two main groups of *D. seriata*; however, isolates obtained from different hosts or different geographic locations did not group into distinct clusters, suggesting a shared genetic background regardless of host or geographic origin. The use of such a technique can provide insight into the population

Fig. 2. Optimum temperature of *Neoscytalidium hyalinum*, *Eutypella citricola*, *E. microtheca*, and a *Eutypella* sp. Average daily growth (in millimeters) was derived from measurements at 2 and 3 days. Vertical bars represent standard errors of the mean.

Table 3. Percent (%) recovery of *Neoscytalidium hyalinum* and *Eutypella* spp. in the southern California desert regions

County	Orchards ^b	Branch cankers ^c	Number (%) of branch cankers yielding ^a					<i>Eutypella</i> sp.
			<i>N. hyalinum</i> (%)	<i>Eutypella</i> spp. (%)	Both ^d	<i>Eutypella citricola</i>	<i>E. microtheca</i>	
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. hyalinum* and a *Eutypella* spp.

Table 4. Conidial size of isolates from citrus branch canker used in this study and comparison with previous studies^a

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

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

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, 2011). Numerous perithecia were observed on dead, decaying citrus branches in the present study, suggesting that

Fig. 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.

both citrus and grapevine are primary hosts for these pathogens because 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 cancer 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 the *Eutypella* sp. on grapevine (Davidson and Lorenz 1938; French 1989; Trouillas 2009; Urbez-Torres et al. 2009). Studies assessing *E. vitis* and the *Eutypella* sp.

as pathogens on grapevine concluded that these species were capable of infecting host tissues, and recent work in Australia indicated that 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. hyalinum is a well-known pathogen of desert citrus in California dating back to the 1950s, when it was determined to be the cause

Fig. 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.

of branch dieback and canker of grapefruit in southern California (Calavan and Wallace 1954). *N. hyalinum* is frequently observed in (semi)arid and 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 a *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 a “very hot” (34°C) temperature pre treatment was most conducive to infection with *Scytalidium lignicola*. The results of lesion development caused by *N. hyalinum* from the temperature study herein corroborates these results, suggesting that 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. hyalinum* and *Spencermartinsia viticola* (A. J. L. Phillips & J. Luque) A. J. L. Phillips, A. Alves & Crous. During a survey of

Fig. 5. Pathogenicity of *Neoscytalidium hyalinum* 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 $\alpha = 0.05$. Levels not connected by the same letter are significantly different.

Fig. 6. Temperature study of *Neoscytalidium hyalinum* and *Eutypella* spp. on detached shoots of Lisbon lemon incubated at 25 or 32°C. Vertical lines represent standard error of mean from an independent-samples *t* test with confidence level $\alpha = 0.05$. Asterisks indicate statistical significance (*, **, and *** = $P < 0.05$, 0.01, and 0.001, respectively) between temperature treatments within a fungal isolate.

canker disease in California vineyards, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. was most abundant in vineyards in the Coachella Valley (Úrbez-Torres et al. 2006) and has been reported previously from citrus (French 1989). At present, it is unclear why other members of the Botryosphaeriaceae family 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 govern pathogen distribution (Adesemoye et al. 2014; Leavitt 1990; Urbez-Torres 2011). Expanded surveys of more groves may be necessary to determine whether other members of the Botryosphaeriaceae family are present. *N. hyalinum* was recently reported as a pathogen of grapevine from the Coachella Valley, citing an estimated 15% incidence of *N. hyalinum* 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 (Calavan and Wallace 1954; Whiteside 1980). Management of branch canker is primarily achieved through various cultural practices, including but not limited to proper irrigation and fertilization, pruning during dry weather, removal of decaying or 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 families, 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. hyalinum* and *Eutypella* spp.

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