Identification, Pathogenicity, and Spore Trapping of *Colletotrichum karstii* Associated with Twig and Shoot Dieback in California

Joey S. Mayorquin,[1] Mohamed T. Nouri,[2] Beth B. Peacock,[1] Florent P. Trouillas,[2] Greg W. Douhan,[3] Craig Kallsen,[4] and Akif Eskalen[5,†]

[1]Department of Microbiology and Plant Pathology, University of California, Riverside, CA 92521; [2]Department of Plant Pathology, University of California, Davis, Kearney Agricultural Research and Extension Center, Parlier, CA 93648; [3]University of California Cooperative Extension, Tulare, CA 93274; [4]University of California Cooperative Extension, Bakersfield, CA 93307; and [5]Department of Plant Pathology, University of California, Davis, CA 95616

Abstract

Colletotrichum Corda, 1831 species are well-documented 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 surveys of orchards in the Central Valley of California have revealed *C. gloeosporioides* and a previously undocumented species, *C. karstii*, to be associated with twig and shoot dieback. Pathogenicity tests using clementine (cv. 4B) indicated that both *C. karstii* and *C. gloeosporioides* are capable of producing lesions following inoculation of citrus stems. Pathogenicity tests also revealed *C. karstii* to be the most aggressive fungal species producing the longest lesions after 15 months. The majority of spores trapped during this study were trapped during or closely following a precipitation event with the majority of spores being trapped from January through May. These findings confirm *C. karstii* as a new pathogen of citrus in California.

The 2017–2018 U.S. citrus crop was valued at $3.28 billion, with California's citrus production accounting for 59% 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 2018). As the California citrus industry is important, the identification and management of new disease threats is crucial.

Colletotrichum constitutes a large genus of ascomycetous fungi that are known for having diverse ecological roles ranging from endophytes to plant pathogens. *Colletotrichum* includes some important fungal pathogens of numerous plant hosts including native and agricultural plant species occurring in tropical and subtropical regions (Cannon et al. 2012; Dean et al. 2012; Hyde et al. 2009). *Colletotrichum* is well known for causing various anthracnose diseases, with general anthracnose symptoms including necrotic lesions on various plant parts including stems, leaves, flowers, and fruits (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 of history and has gone through many revisions, especially since recent advances in DNA technologies. Currently, over 100 species of *Colletotrichum* have been described and a thorough review of the history of *Colletotrichum* classification can be found in Cannon et al. (2012). Recent phylogenetic studies of three well established *Colletotrichum* species (*C. gloeosporioides*, *C. acutatum*, and *C. boninense*) revealed that these species represent species complexes and collectively account for at least 71 phylogenetic species (Damm et al. 2012; Weir et al. 2012). The large species diversity within the *Colletotrichum* genus highlights the importance of DNA phylogenies to identify species accurately.

Historically, two species of *Colletotrichum*, *C. gloeosporioides* (Penz.) Penz. & Sacc. and *C. acutatum* J.H. Simmonds, have been associated with anthracnose diseases of citrus (Timmer et al. 2000). These anthracnose diseases, which include postharvest anthracnose, postbloom fruit drop (PFD), and key lime anthracnose (KLA), are of great economic importance (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 suggests that additional species of *Colletotrichum* previously unknown from citrus are causing diseases of citrus globally, particularly from the *C. boninense* species complex. *C. 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 worldwide and is often found to occur in association with other *Colletotrichum* spp., particularly *C. gloeosporioides*, which generally predominates within citrus hosts (Guarnaccia et al. 2017; Huang et al. 2013; Peng et al. 2012; Ramos et al. 2016). *C. karstii* has been increasingly reported also from anthracnose diseases of other crops including avocado, mango, and persimmon (Ismail et al. 2015; Lima et al. 2013; Silva-Rojas and Ávila-Quezada 2011; Wang et al. 2016) and is considered the most common and widely distributed species of the *C. boninense* species complex (Damm et al. 2012). Although *C. karstii* has been reported from citrus in China, Italy, and Portugal (Aiello et al. 2015; Guarnaccia et al. 2017; Huang et al. 2013; Peng et al. 2012; Ramos et al. 2016), in the United States, *C. karstii* has only been reported from *Leucospermum* (Crous et al. 2013) and *Phalaenopsis* spp. (Jadrane et al. 2012). In North America, *C. karstii* has been reported in Mexico from two additional hosts, *Annona cherimola* (Damm et al. 2012) and *Musa* sp. (Velázquez-del Valle et al. 2016).

Recently, unusual disease symptoms associated with *Colletotrichum* spp. were observed frequently in various *Citrus* orchards in the San Joaquin Valley of California (Eskalen, personal observation). Symptoms included leaf chlorosis, twig and shoot dieback, crown thinning, wood cankers in branches, and in some cases, death of young plants. Isolation from diseased tissues yielded typical *Colletotrichum* species based on colony morphology, but slight differences also suggested that more than one species might be present. To date, *C. gloeosporioides* has been the only species associated with anthracnose diseases of citrus in California. Therefore, the objectives of this

†Corresponding author: A. Eskalen; aeskalen@ucdavis.edu

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study were to: (i) identify *Colletotrichum* species associated with twig and shoot dieback as well as branch canker of *Citrus* spp. in California; (ii) assess the pathogenicity of *Colletotrichum* spp. in twigs of *Citrus* spp.; and (iii) to determine when and under what environmental conditions spores of *Colletotrichum* spp. were dispersed within CA citrus orchards based on spore trapping.

Materials and Methods

Field survey and fungal isolations. Field surveys were conducted in 13 commercial citrus orchards throughout Madera Co. (one orchard), Fresno Co. (three orchards), Tulare Co. (four orchards), and Kern Co. (five orchards) from 2014 to 2016. The main citrus varieties sampled 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. Orchards were sampled during both the spring and fall of that same year for orchards in Madera, Tulare, and Kern counties. Approximately 16 trees were sampled from each orchard during each sampling period. Samples included portions of blighted twigs and shoots, and cankered branches. The samples were transported in an ice chest to the laboratory and stored at 5°C until processed for isolations.

Prior to isolation, twig and shoot samples were rinsed with distilled water to remove surface debris and disinfested by spraying 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 tissue were plated onto potato dextrose agar (PDA) (Difco Laboratories) amended with 0.01% tetracycline hydrochloride (Fisher Scientific). Plates were incubated at room temperature (~23 to 25°C) in the dark for approximately 5 to 7 days. Single-spore cultures were obtained from fungal colonies with developed acervuli by using a sterile inoculation loop to scoop spore masses from acervuli and streak them onto water agar plates. After 24 h, isolated germinating conidia were picked off with a sterile inoculating needle and transferred to new PDA plates with the aid of a stereomicroscope. Each culture derived

Table 1. Representative isolates of *Colletotrichum* spp. collected from citrus shoot cankers in California and fungal species obtained from GenBank

					GenBank accession[c]		
Species[a]	**Isolate[b]**	**Location**	**Host**	**Cultivar**	**ITS**	**GAPDH**	**TUB2**
Colletotrichum karstii	KARE523	Fresno	*Citrus reticulata*	...	KY076519	KY304041	KY086302
C. karstii	KARE524	Fresno	*C. reticulata*	...	KY076520	KY304042	KY086303
C. karstii	KARE525	Fresno	*C. reticulata*	...	KY076521	KY304043	KY086304
C. karstii	KARE526	Fresno	*C. reticulata*	...	KY076522	KY304044	KY086305
C. karstii	KARE530	Fresno	*C. reticulata*	...	KY076523	KY304045	KY086306
C. karstii	KARE531	Fresno	*C. reticulata*	...	KY076524	KY304046	KY086307
C. karstii	KARE534	Fresno	*C. reticulata*	...	KY076525	KY304047	KY086308
C. karstii	KARE536	Fresno	*C. reticulata*	...	KY076526	KY304048	KY086309
C. karstii	KARE545	Fresno	*C. reticulata*	...	KY076527	KY304049	KY086310
C. karstii	UCR1717	Tulare	*C. sinensis*	Cara Cara	MK101014	...	...
C. karstii	UCR1761	Tulare	*C. sinensis*	Cara Cara	KY076528	KY304050	KY086311
C. karstii	UCR1763[d]	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	UCR3550	Kern	*C. reticulata*	...	MK101015	...	...
C. karstii	UCR3552[d]	Kern	*C. reticulata*	Clemenules	KY076545	KY304056	...
C. karstii	UCR3553[de]	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	KARE31	Solano	*C. sinensis*	...	KY076549	KY304060	KY086320
C. gloeosporioides	KARE36	Solano	*C. sinensis*	...	KY076550	...	...
C. gloeosporioides	KARE37	Solano	*C. sinensis*	...	KY076551	KY304061	KY086321
C. gloeosporioides	KARE527	Fresno	*C. reticulata*	...	KY076552	KY304062	KY086322
C. gloeosporioides	KARE528	Fresno	*C. reticulata*	...	KY076553	KY304063	KY086323
C. gloeosporioides	KARE532	Fresno	*C. reticulata*	...	KY076554	KY304064	KY086324

(*Continued on next page*)

[a] Species in bold denote GenBank accessions.
[b] Isolate codes correspond to Kearney Agricultural Research and Extension Center (KARE), University of California, Riverside (UCR), and Centraalbureau voor Schimmelcultures (CBS).
[c] ITS = internal transcribed spacer; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; TUB2 = beta-tubulin.
[d] Isolates selected for morphological characterization (Table 2).
[e] Isolates used in pathogenicity tests.
[f] Denotes type/ex-type material.

from a single conidium was used to determine morphological and molecular characterizations and is stored in a culture collection maintained by the Eskalen lab at the University of California, Davis, CA.

Morphological characterization. Fungi were initially identified to the genus level based on cultural and microscopic examination (colony color, colony growth pattern, growth rate, and conidia shape). Of those resembling *Colletotrichum* spp., several representative isolates from each orchard were further studied using published descriptions (Damm et al. 2012). To examine spore morphology, isolates were grown on synthetic nutrient-poor agar medium (SNA), oatmeal agar (OA), and/or PDA to induce conidial production and incubated under continuous fluorescent light for about 2 weeks at an average temperature of 23 to 25°C as described by Damm et al. (2012). Conidial masses were scooped with a toothpick and mounted in a drop of lactoglycerol on a microscope slide. The lengths and widths 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 appressorium lengths and widths, were calculated for each isolate using Microsoft Excel (Microsoft Corporation, Redmond, WA).

DNA isolation, amplification, and phylogenetic analysis. Genomic DNA for each isolate was extracted using a slightly modified method of Cenis (1992). Oligonucleotide primers ITS4 and ITS5 (White et al. 1990) were used to amplify the ITS1-5.8S-ITS2 regions of the rDNA locus, 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 (Guerber et al. 2003) were used to amplify a 200-bp intron of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). Polymerase chain reaction (PCR)

Table 1. (*Continued from previous page*)

Species[a]	Isolate[b]	Location	Host	Cultivar	GenBank accession[c]		
					ITS	GAPDH	TUB2
C. gloeosporioides	KARE533	Fresno	*C. reticulata*	...	KY076555	KY304065	KY086325
C. gloeosporioides	KARE538	Fresno	*C. reticulata*	...	KY076556	KY304066	KY086326
C. gloeosporioides	KARE539	Fresno	*C. reticulata*	...	KY076557	KY304067	KY086327
C. gloeosporioides	KARE541	Fresno	*C. reticulata*	...	KY076558	KY304068	KY086328
C. gloeosporioides	KARE543	Fresno	*C. reticulata*	...	KY076559	KY304069	KY086329
C. gloeosporioides	KARE546	Fresno	*C. reticulata*	...	KY076560	KY304070	KY086330
C. gloeosporioides	KARE548	Fresno	*C. reticulata*	...	KY076561	KY304071	KY086331
C. gloeosporioides	KARE549	Fresno	*C. reticulata*	...	KY076562	KY304072	KY086332
C. gloeosporioides	UCR2410	Fresno	*C. sinensis*	Fisher	MK101016	...	...
C. gloeosporioides	UCR2411	Fresno	*C. sinensis*	Fisher	MK101017	...	...
C. gloeosporioides	UCR2412	Fresno	*C. sinensis*	Fisher	MK101018	...	...
C. gloeosporioides	UCR2544	Tulare	Spore trap	...	KY076563	...	...
C. gloeosporioides	UCR2552[de]	Tulare	*C. reticulata*	Clemenules	KY076564	KY304073	KY086333
C. gloeosporioides	UCR2564[d]	Tulare	*C. reticulata*	Clemenules	KY076565	...	...
C. gloeosporioides	UCR2569[d]	Tulare	*C. reticulata*	Clemenules	KY076566	KY304074	KY086334
C. gloeosporioides	UCR2575	Tulare	*C. reticulata*	4B	KY076567	KY304075	KY086335
C. gloeosporioides	UCR3551[d]	Kern	*C. reticulata*	Clemenules	KY076568	KY304076	KY086336
Colletotrichum boninense	CBS112115	Australia	*Leucospermum* sp.	...	JQ005160	JQ005247	JQ005594
C. boninense	CBS123755[f]	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[f]	Germany	*Oncidium* sp.	...	JQ005169	JQ005256	JQ005603
C. oncidii	CBS130242	Germany	*Oncidium* sp.	...	JQ005170	JQ005257	JQ005604
C. torulosum	CBS102667	New Zealand	*Passiflora edulis*	...	JQ005165	JQ005252	JQ005599
C. torulosum	CBS128544[f]	New Zealand	*Solanum melongena*	...	JQ005164	JQ005251	JQ005598
C. brassicicola	CBS101059[f]	New Zealand	*Brassica oleracea* var. *gemmifera*	...	JQ005172	JQ005259	JQ005606
C. beeveri	CBS128527[f]	New Zealand	*Brachyglottis repanda*	...	JQ005171	JQ005258	JQ005605
C. colombiense	CBS129817	Colombia	*P. edulis*	...	JQ005173	JQ005260	JQ005607
C. colombiense	CBS129818[f]	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[f]	Colombia	*Hevea brasiliensis*	...	JQ005222	JQ005309	JQ005656
C. phyllanthi	CBS175.67[f]	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[f]	Brazil	*P. edulis*	...	JQ005235	JQ005322	JQ005669
C. brasiliense	CBS128528	Brazil	*P. edulis*	...	JQ005234	JQ005321	JQ005668
C. parsonsiae	CBS128525[f]	New Zealand	*Parsonsia capsularis*	...	JQ005233	JQ005320	JQ005667
C. hippeastri	CBS125377[f]	China	*Hippeastrum vittatum*	...	JQ005230	JQ005317	JQ005664
C. hippeastri	CBS125376	China	*H. vittatum*	...	JQ005231	JQ005318	JQ005665
C. dacrycarpi	CBS130241[f]	New Zealand	*Dacrycarpus dacrydioides*	...	JQ005236	JQ005323	JQ005670
C. constrictum	CBS128503	New Zealand	*S. betaceum*	...	JQ005237	JQ005324	JQ005671
C. constrictum	CBS128504[f]	New Zealand	*Citrus limon*	...	JQ005238	JQ005325	JQ005672
C. gloeosporioides	CBS112999[f]	Italy	*C. sinensis*	...	JX010152	JX010056	JX010445
Monilochaetes infuscans	CBS869.96	Unknown	Unknown	...	JQ005780	JX546612	JQ005864

was carried out essentially as described by Damm et al. (2012). Reaction mixtures consisted of 1× reaction buffer, 0.2 mM dNTPs, 0.2 μM forward and reverse primers, 2.5 U of standard Taq polymerase (New England Biolabs, MA), and template DNA ranging from 50 to 100 ng/μl. For ITS, 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 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 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) were compared with known sequences in GenBank (Table 1). Separate analyses were run for each gene region dataset in addition to a combined dataset of all three genes after visually examining 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. Two pathogenicity tests were conducted using 2-year-old potted clementine '4B' trees grafted to Carrizo rootstock and maintained in a greenhouse at the University of California, Riverside. In the first pathogenicity test, four trees were stem inoculated by making wounds approximately 6 inches above the soil line using a 5-mm diameter cork-borer to remove bark and expose the cambium. Mycelial plugs were taken from the edge of a 1-week-old colony of *C. gloeosporioides* and *C. karstii* (Table 1) and were placed into the wounds; noninfested PDA plugs were used for controls. Wounds were covered with petroleum jelly and wrapped with Parafilm. This experiment was conducted twice. Plants were maintained in the greenhouse for 10 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 previously described.

For the second pathogenicity test, four 2-year-old potted clementine '4B' trees grafted to Carrizo rootstock and maintained in a greenhouse at the University of California, Riverside were inoculated using a sterile scalpel to remove bark and expose an approximately 5-mm strip of cambium 6 inches from the soil line in four plants. A 20-μl drop of a conidial suspension in sterile water (1×10^6 spores/ml) obtained from fungal cultures grown on PDA for about 1 week at an average temperature of 23 to 25°C was pipetted onto the wound and a sterile 18-gauge needle was inserted through the droplet and into the xylem to facilitate suspension uptake; sterile water was applied to control plants. Resulting wounds were covered with petroleum jelly and wrapped with Parafilm. This experiment was conducted twice. Inoculated plants were maintained in the greenhouse for approximately 15 months (December 2015 to March 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 to fulfill Koch's postulates. Emerging fungal colonies were recorded and identified by sequencing their ITS regions.

Spore trapping. Spore trapping was done using sticky glass microscope slide traps as described by Eskalen and Gubler (2001). Traps were placed in four citrus orchards (two orchards each in Kern and Tulare counties) previously surveyed for approximately two years (2017 to 2018). Ten traps were deployed in each orchard for a total of 40 traps. One spore trap was randomly placed on a branch of a tree and traps were collected and replaced every 2 weeks and shipped to the laboratory for further processing. Once received, slide traps were rinsed with sterile deionized water in 50 ml falcon tubes 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 5 days. Fungal colonies resembling *Colletotrichum* were identified by gross colony morphology as described previously. Colony forming units (CFU) were assumed to be derived from a single spore and were counted and recorded. 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.

Statistical analyses. 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 were combined. Lesion length data were $\log_{10}$ transformed to normalize data; however, data from the initial pathogenicity test could

Table 2. Conidial dimensions of representative *Colletotrichum* spp. from citrus shoot cankers in this study and comparison with previous studies

Species	Isolate	Conidial size (μm mean ± SD)[a]			Appressoria size (μm mean ± SD)[b]
		PDA[c]	SNA	OA	SNA
Colletotrichum karstii	UCR3550	15.3 ± 1.1 × 5.0 ± 0.3	16.1 ± 0.8 × 5.2 ± 0.2	14.8 ± 0.8 × 5.2 ± 0.2	…
C. karstii	UCR3552	14.3 ± 0.8 × 5.2 ± 0.4	14.0 ± 1.3 × 5.6 ± 0.5	12.4 ± 0.8 × 5.8 ± 0.4	8.0 ± 1.6 × 6.1 ± 1.1
C. karstii	UCR3553	13.6 ± 0.8 × 5.9 ± 0.3	13.6 ± 1.2 × 5.6 ± 0.5	14.0 ± 0.6 × 5.8 ± 0.4	8.0 ± 1.6 × 6.1 ± 1.1
C. karstii	UCR1717	13.5 ± 1.1 × 7.5 ± 0.3	…	…	…
C. karstii	UCR1763	13.5 ± 1.1 × 7.5 ± 0.3	…	…	…
C. gloeosporioides	UCR2410	13.5 ± 1.1 × 7.5 ± 0.3	16.3 ± 0.6 × 5.2 ± 0.4	15.6 ± 0.7 × 5.4 ± 0.3	11.9 ± 2.4 × 6.5 ± 1.0
C. gloeosporioides	UCR2411	13.5 ± 1.1 × 7.5 ± 0.3	16.2 ± 1.2 × 5.2 ± 0.5	15.3 ± 0.7 × 5.4 ± 0.4	10.5 ± 1.9 × 7.0 ± 1.3
C. gloeosporioides	UCR2412	13.5 ± 1.1 × 7.5 ± 0.3	15.8 ± 0.9 × 5.2 ± 0.4	15.1 ± 0.3 × 5.4 ± 0.3	11.0 ± 2.2 × 6.7 ± 1.3
C. gloeosporioides	UCR2552	13.5 ± 1.1 × 7.5 ± 0.3	15.5 ± 1.4 × 5.2 ± 0.4	14.4 ± 1.2 × 5.1 ± 0.5	11.7 ± 1.9 × 7.3 ± 1.6
C. gloeosporioides	UCR2564	13.5 ± 1.1 × 7.5 ± 0.3	15.3 ± 1.3 × 4.6 ± 0.5	14.3 ± 1.0 × 4.7 ± 0.3	11.0 ± 2.3 × 6.8 ± 1.5
C. gloeosporioides	UCR2569	13.5 ± 1.1 × 7.5 ± 0.3	15.4 ± 0.8 × 4.7 ± 0.4	15.2 ± 1.9 × 5.1 ± 0.4	11.6 ± 2.8 × 7.2 ± 1.3
C. gloeosporioides	UCR3551	13.5 ± 1.1 × 7.5 ± 0.3	15.5 ± 0.7 × 4.6 ± 0.3	14.3 ± 0.9 × 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.

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 with control plants were evaluated using the steel with control method with confidence level $\alpha = 0.05$. For the second pathogenicity test, mean lesion lengths were compared using a one-way analysis of variance. Mean lesion lengths of fungal inoculated plants compared with control plants were evaluated using Dunnett's method with confidence level $\alpha = 0.05$. Figures were made using SigmaPlot (version 11; Systat Software Inc.).

Results

Field survey and fungal isolation. The most common symptoms observed included twig and shoot dieback, gummosis, and in some cases branch dieback and wood cankers (Fig. 1). All symptoms were found throughout all 13 orchards surveyed. However, the single

Fig. 1. Symptoms of Colletotrichum dieback. **A,** Shoot dieback symptoms on clementine. **B,** Gumming symptoms on an infested shoot. **C,** Branch dieback symptoms on clementine. **D,** Wood discoloration and canker on the wood.

orchard surveyed from Madera Co. had a low incidence of twig and shoot dieback compared with the other 12 orchards surveyed. There were no obvious correlations between symptoms and 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.

A total of 284 symptomatic samples were collected from the 13 orchards. Of the 284 samples, 98 yielded species of *Colletotrichum*; 6, 57, 100, and 18% from Madera, Tulare, Fresno, and Kern counties, respectively. *C. karstii* was recovered from 3, 31, 50, and 12% of samples collected from Madera, Tulare, Fresno, and Kern counties, respectively, whereas *C. gloeosporioides* was recovered from 3, 22, 70, and 10% of samples collected from Madera, Tulare, Fresno, and Kern counties, respectively.

Other fungi isolated from symptomatic tissues were identified using colony characteristics and sequencing the ITS regions of representative isolates as described previously and included *Alternaria* spp., *Penicillium* spp., *Fusarium* sp., *Quambalaria* spp., Botryosphaeriaceae spp., and Diatrypaceae spp. Botryosphaeriaceae spp. were recovered from 27 of the 284 samples collected and Diatrypaceae spp. were recovered from eight of the 284 samples. Both Botryosphaeriaceae and Diatrypaceae spp., known 'canker' pathogens associated with citrus, were never co-isolated with species of *Colletotrichum.*

Morphological characterization. Isolates of *Colletotrichum* from this study could usually be divided into two distinct groups based on colony morphology. However, there were many isolates where the distinction was not obvious, which was the reason molecular tools were used to identify the distinct species. The first group consisted of colonies that were white- to cream-colored, pale orange on reverse, and with numerous conidiomata (orange-colored) developing over time throughout the colony. Conidia were hyaline, straight, and cylindrical with rounded ends. These isolates matched closely published descriptions of *C. karstii* (Damm et al. 2012) (Table 2). The second group consisted of isolates forming cream to gray colonies, with dark gray aerial mycelium developing over time, reverse colony color was cream to mottled 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* (Weir et al. 2012).

Phylogenetic analysis. Individual analysis of the ITS, GAPDH, and TUB2 datasets yielded similar topologies based on all phylogenetic methods used (data not shown). Therefore, only the combined dataset is reported based on maximum parsimony. The combined data set consisted of 1,092 nt, with 225 conserved sites, 851 variable sites, and 290 parsimony-informative sites. Maximum parsimony analysis generated two trees with scores of CI = 0.68, RI = 0.93, and RCI = 0.63. The combined data set (Fig. 2) revealed that isolates from this study grouped into two well-supported clades. The first major clade (91% bootstrap support) consisted of isolates from this study and other vouchered specimens of *C. karstii* that included reference isolate CBS106.91 of *C. karstii*. *C. phyllanthi* and *C. annellatum* were the most closely related species to *C. karstii*. The second major clade (99% bootstrap support) grouped isolates from this study and the type specimen of *C. gloeosporioides* (CBS112999). There were only slight nucleotide differences between species of all isolates used in this study within all three loci (Fig. 2).

Pathogenicity tests. In the first pathogenicity test, internal lesions were produced in stems of the inoculated plants and typical acervuli of *Colletotrichum* were produced near the inoculation site 10 days after inoculation. More than half of the plants inoculated with *C. karstii* and only one plant inoculated with *C. gloeosporioides* produced acervuli. In the majority of cases, gumming was also observed surrounding the point of inoculation for inoculated plants but not for control plants. There was a significant difference ($\chi^2 = 17.627$; $P < 0.001$) in lesion lengths produced between the two fungal treatments (Fig. 3); no internal lesions were observed in control shoots. Lesions produced by both *Colletotrichum* spp. were brown to chocolate-brown and extended from both ends of the inoculation point. A significant difference in lesion lengths was found between the control plants and plants inoculated with *C. karstii* ($P = 0.003$) and *C. gloeosporioides* ($P = 0.015$) and between fungal species ($P = 0.013$). Fungal recovery of isolates from inoculated stems ranged between 25 to 100%; no *Colletotrichum* spp. were recovered from control stems.

In the second pathogenicity test, internal vascular necrosis was observed in plants inoculated with both *C. karstii* and *C. gloeosporioides* (Fig. 4) 15 months after inoculation. Necrotic regions were brown to chocolate-brown and extended from both ends of the inoculation point. No lesions nor vascular streaking was observed in control plants. Unlike the first pathogenicity test, no fruiting bodies were observed at the point of inoculation. A significant difference ($P < 0.001$) was observed between fungal treatments and there was a significant difference in lesion lengths produced by *C. karstii* ($P < 0.001$) and *C. gloeosporioides* ($P = 0.035$) and the control and between fungal species ($P < 0.001$). Fungal recovery from inoculated shoots ranged from 25 to 100% and no *Colletotrichum* spp. were recovered from control plants.

Spore trapping. The majority of fungal spores were trapped during or after precipitation events. In Kern County (Fig. 5A), the majority of spores were trapped during or closely following a precipitation event in March-April 2017 and Feb-March 2018. In Tulare County (Fig. 5B), the majority of spores were trapped during or closely following precipitation events between April-May 2017 and January 2018. Total spore counts between the two orchards trapped in Kern Co. were higher than counts observed in Tulare Co. (>60 spores) during March 2017, April 2017, February 2018, and March 2018. Across all locations, the majority of spores were trapped when average temperatures were between 10 and 25°C.

Discussion

This is the first study to report *C. karstii* as a pathogen of citrus causing twig and shoot dieback with or without gumming and occasionally branch dieback and wood canker in the Central Valley of California. Pathogenicity tests on clementine mandarin confirmed that *C. karstii* is a more aggressive pathogen of citrus in CA than *C. gloeosporioides* based on in planta experiments. Both species were commonly isolated from symptomatic tissues and were often found coinfecting the symptomatic samples but were never found associated with other known canker pathogen species of citrus within Botryosphaeriaceae and Diatrypaceae. Unlike anthracnose, which can cause twig dieback and is associated with *C. gloeosporioides*, this disease is associated with two species of *Colletotrichum* and is not limited to twig dieback alone but is also associated with shoot dieback and in some cases, woody cankers. Taken together, this confirms *C. karstii* as a new pathogen of citrus in California causing a disease distinct from anthracnose that is caused by *C. gloeosporioides*.

The association of *C. karstii* with citrus twig and shoot dieback in California represents a significant finding since this is the first report of *C. karstii* as a pathogen of citrus in the United States. Anthracnose disease of citrus was mainly attributed thus far to *C. gloeosporioides* and *C. acutatum* that are considered mainly as foliar and fruit pathogens (Timmer et al. 2000). Although symptoms of anthracnose caused by *C. gloeosporioides* in citrus include twig dieback, leaf drop, and necrosis on fruits as a postharvest disease, a progression to shoot dieback and association with branch dieback and wood cankers has not been observed (Timmer et al. 2000; 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). Several studies have shown the ability of *Colletotrichum* to cause shoot dieback in olive, red stinkwood, poplars, camellia, coprosma, almond, apple, and persimmon (Adaskaveg and Förster 2000; Forbes and Pearson 1987; Marks et al. 1965; Moral et al. 2009; Mwanza et al. 1999; Sun et al. 2014; Roberts 1915; Zhang 2008).

Recently, Ramos et al. (2016) determined *C. karstii*, in addition to *C. gloeosporioides*, to be commonly associated with twig dieback of lemon in Portugal and found both species capable of causing dieback and branch necrosis of sweet orange and mandarin in the field. Although results from pathogenicity tests in our study did not reproduce

the branch 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* can cause leaf anthracnose in California citrus although it has been isolated from leaf lesions and confirmed to infect leaves of several citrus varieties in other regions (Peng et al. 2012; Ramos et al. 2016). *C. karstii* has been reported also as causing preharvest anthracnose on sweet orange (Aiello et al. 2015), but this symptom was not observed in our study.

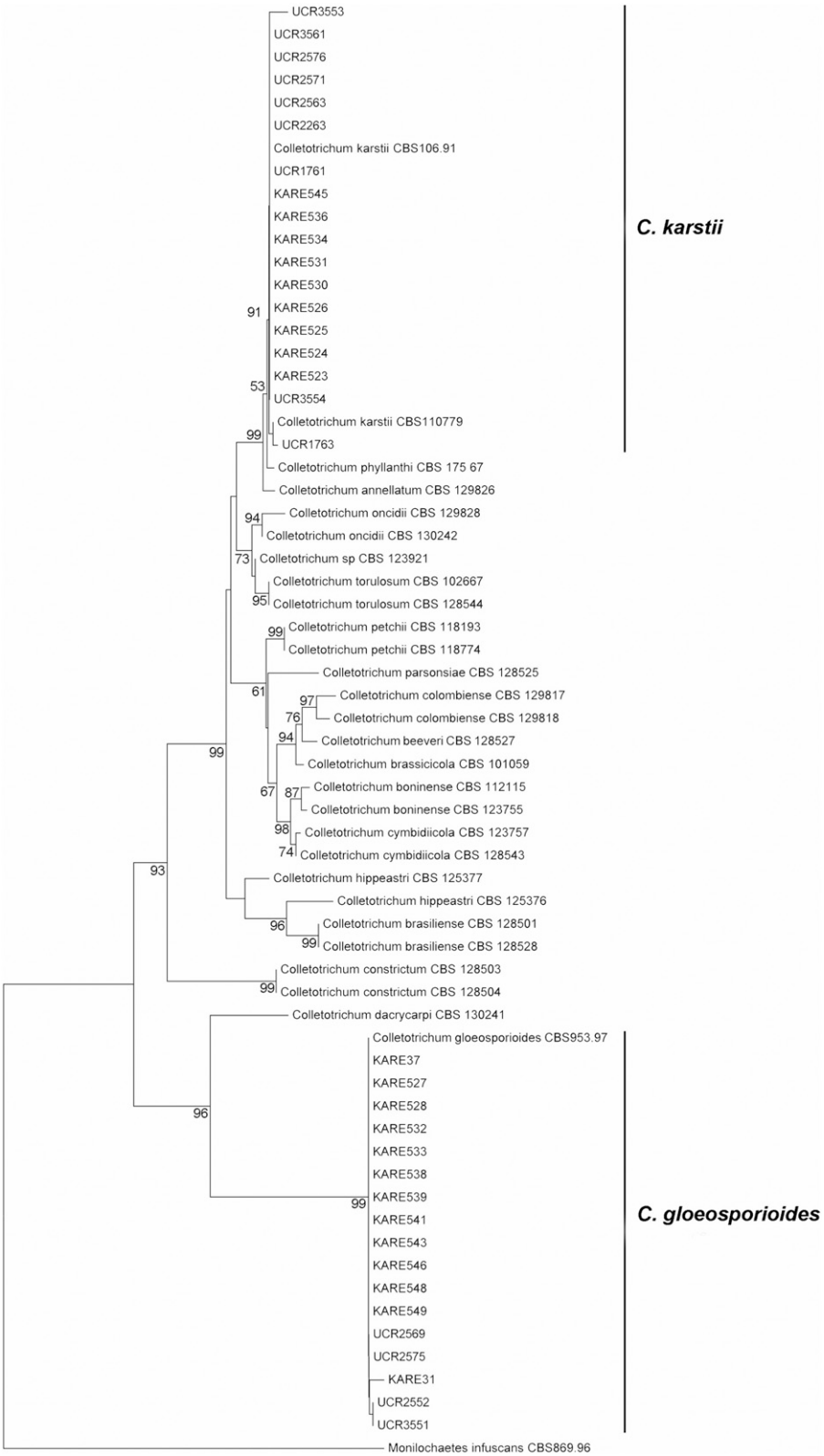

Fig. 2. One of the most parsimonious unrooted trees based on internal transcribed spacer 1, 5.8S ribosomal DNA, ITS2 (ITS); 200 bp intron of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH); and partial β-tubulin gene region (TUB2) sequences for isolates of species of the *Colletotrichum boninense* species complex inferred from maximum parsimony analysis. Numbers on branches are bootstrap values from 1,000 bootstrap replicates.

During this study, *C. karstii* was isolated from citrus wood canker samples that resembled wood canker symptoms typical of those caused by *Botryosphaeria* and its allied members (Adesemoye et al. 2014). 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. Phytotoxins may also contribute to the symptoms observed as toxins produced by *C. acutatum* have been shown to cause wilting and branch dieback in olive (Moral et al. 2009). It is unknown if the *Colletotrichum* spp. presented in this study produce phytotoxins that directly contribute to the cankering and dieback observed. In hosts such as apple and persimmon, anthracnose symptoms can include both shoot dieback and wood cankers (Roberts 1915; Zhang 2008). In these cases, the dieback observed in twigs and shoots can also progress into woody branches if the infection is severe. Pathogenicity results from this study confirmed *C. karstii* as a pathogen of citrus and suggest that this fungus may also 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.

C. karstii is distributed worldwide throughout Africa, Asia, North and South America, Europe, and Oceania and is associated with a number of plant species including agricultural commodities such as apple (Velho et al. 2014), avocado (Velázquez-del Valle et al. 2016), citrus (Guarnaccia et al. 2017; Huang et al. 2013; Peng et al. 2012; Ramos et al. 2016), mango (Lima et al. 2013), olive (Schena et al. 2014), and pistachio (Lichtemberg et al. 2017). It is considered the most common and diverse 'species' in the *C. boninense* complex (Damm et al. 2012). However, *C. karstii* has only been reported from citrus in China, Italy, Malta, New Zealand, Portugal, Spain and South Africa (Aiello et al. 2014; Huang et al. 2013; Peng et al. 2012; Ramos et al. 2016). *C. karstii* is a plurivorous

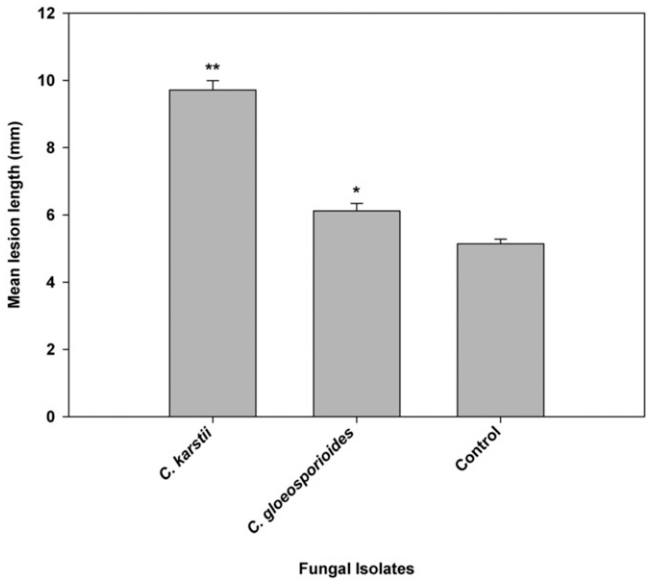

Fig. 3. Pathogenicity of *Colletotrichum* spp. on '4B' clementine after 10 days. Vertical lines represent standard error of the mean. Asterisks (*) denote the following significance: * = $P < 0.05$ and ** = $P < 0.01$.

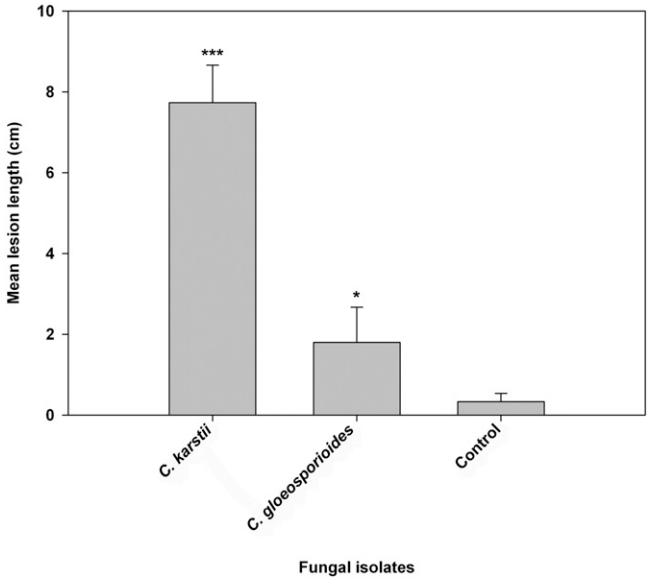

Fig. 4. Pathogenicity of *Colletotrichum* spp. on '4B' clementine after 15 months. Vertical lines represent standard error of the mean. Asterisks (*) denote the following significance: * = $P < 0.05$ and *** = $P < 0.001$.

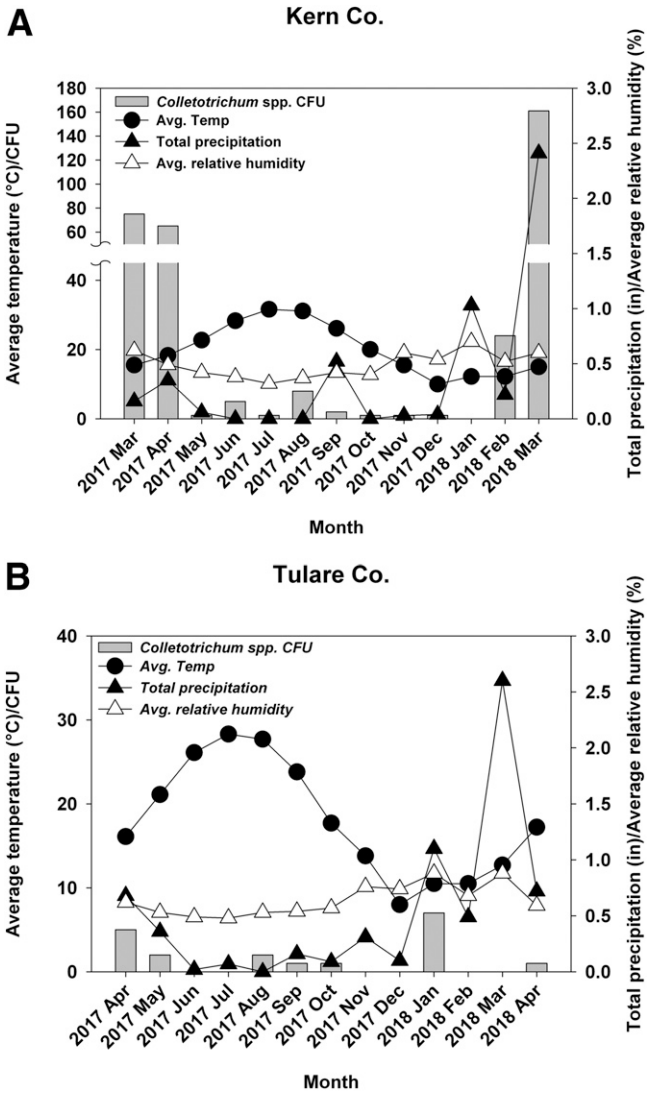

Fig. 5. Monthly spore trap counts with temperature (°C), precipitation (mm), and relative humidity (%) for **A,** Kern and **B,** Tulare counties. Vertical bars represent total colony forming units (CFU) counted from each citrus orchard by month. Lines represent average monthly temperature (°C) and relative humidity (%) and total monthly precipitation (mm).

pathogen and within citrus specifically, it does not appear to be variety specific since it was found on several varieties of citrus during this study as well as other studies (Guarnaccia et al. 2017; Huang et al. 2013; Peng et al. 2012; Ramos et al. 2016). Damm et al. (2012) reported high sequence variability within strains of *C. karstii* that 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 within either species. It is unknown at this time if this lack of genetic variability could be attributed to clonal population structure of both species on citrus in California. Additional studies utilizing additional markers such as microsatellites, AFLP, or RAD-sequencing would help to resolve this aspect of their biology/epidemiology.

Although little is known regarding the epidemiology of *C. karstii* on citrus, several environmental 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 (Dodd et al. 1989; Hunter and Buddenhagen 1972; 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. 5), but *Colletotrichum* spp. were not always correlated with rainfall. Similar results were found with other spore trap studies in California (Eskalen and Gubler 2001; Eskalen et al. 2013; Úrbez-Torres et al. 2010). Wounding is also known to predispose plants to infection by *Colletotrichum* (Lubbe et al. 2006; Muimba-Kankolongo and Bergstrom 1992; Shaw 1995) and typical agricultural practices and the environment in CA citrus groves (pruning, shearing, wind/sand damage) give both of these species the opportunity to colonize citrus trees. 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 suggests that young, tender tissues developing in the late spring are likely necessary for initial pathogen colonization.

The findings of this study have determined *C. karstii* to be a new pathogen of citrus in California associated with a disease which is distinct from citrus anthracnose caused by *C. gloeosporioides*. Additionally, *C. karstii* is a more aggressive pathogen than *C. gloeosporioides* and more remains to be understood regarding the disease cycle of this pathogen, which raises concerns for the management of this new pathosystem. Currently no strategies exist for the management of this emerging disease in citrus. 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 branches and pruning debris during dry periods followed by immediate disposal of infested material, and sanitizing pruning equipment. Chemical management using fungicides is being investigated and these methods may become part of an integrated pest management strategy for *Colletotrichum* diseases of citrus in California.

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