Olive Twig and Branch Dieback: Etiology, Incidence, and Distribution in California

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Abstract

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Eighteen different fungal species were isolated from symptomatic wood of olive trees (*Olea europaea*) affected by twig and branch dieback in California and identified by means of morphological characters and multigene sequence analyses of the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2), a partial sequence of the β-tubulin gene, and part of the translation elongation factor 1-α gene (EF1-α). These species included *Diaporthe viticola*, *Diatrype oregonensis*, *Diatrype stigma*, *Diplodia mutila*, *Dothiorella iberica*, *Lasiodiplodia theobromae*, *Phaeomoniella chlamydospora*, *Phomopsis* sp. group 1, *Phomopsis* sp. group 2, and *Schizophyllum commune*, which are for the first time reported to occur in olive trees; *Eutypa lata*, *Neofusicoccum luteum*, *Neofusicoccum vitifusiforme*, and *Phaeoacremonium aleophilum*, which are for the first time reported to occur in olive trees in the United States; and *Botryosphaeria dothidea*, *Diplodia seriata*, *Neofusicoccum mediterraneum*, and *Trametes versicolor,* which have been previously reported in olive trees in California. Pathogenicity studies conducted in olive cultivars Manzanillo and Sevillano showed *N. mediterraneum* and *Diplodia mutila* to be the most virulent species and *Diatrype stigma* and *D. oregonensis* the least virulent when inoculated in olive branches. Intermediate virulence was shown for the rest of the taxa. This study demystifies the cause of olive twig and branch dieback and elucidates most of the fungal pathogens responsible for this disease in California.

The evergreen tree European olive (*Olea europaea* L.), with over 9.2 million hectares cultivated and more than 19.3 million metric tons harvested in 2009 worldwide, is one of the most extensively planted fruit crops in the world (9). Although the Mediterranean Basin accounts for 95% of the world's olive production, olive trees are also cultivated in several regions of the world with Mediterranean and/or temperate climates, including China, Australia, New Zealand, South Africa, Argentina, Uruguay, Chile, Peru, El Salvador, Mexico, and California in the United States (9).

Olives were introduced into California from northern Mexico by Franciscan padres in the early 1770s, with the first trees being planted at the San Diego de Alcalá Mission, now city of San Diego (7). Nowadays, the California olive industry produces 99% of the olives in the United States and comprises over 21,000 ha of bearing trees. In 2010, California's olive production was over 195,000 metric tons, which represented a crop valued at over $113 million (41). Historically, the California olive industry has been based almost entirely on the production of canned ripe olives, with about 90% of the total production being used for canning packs, including ripe (whole or pitted), green-ripe (whole or pitted), and sliced, chopped, wedged, and broken (all pitted) (7). The introduction of novel super-high-density olive varieties, on the other hand, has recently raised the interest among growers in new plantations for the production of olive oil. As a result, over 7,000 ha of olive trees have been planted in California since 2005, of which about 80% are super-high-density olive tree varieties (50).

Olive trees are known to be drought resistant and hardy, suffering from few major disease problems (22). Among all diseases affecting olives, dalmatian disease caused by *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & De Not., olive anthracnose caused by *Colletotrichum acutatum* J.H. Simmonds and/or *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., olive knot caused by *Pseudomonas syringae* pv. *savastanoi* (Smith) Young, Dye & Wilkie, peacock spot (synonyms: bird's eye spot and olive leaf spot) caused by *Spilocaea oleaginea* (Castagne) S. Hughes, and Verticillium wilt caused by *Verticillium dahliae* Kleb. are probably the most economically important and extensively studied diseases of olives worldwide (13,18,33). Less studied, olive twig and branch dieback symptoms are characterized by dead twigs in the affected parts of the tree, which are generally associated with the decline of entire young stems and/or older branches (25,27). Cross-sections of affected tree parts (stems, branches, and/or trunk) reveal the presence of perennial cankers in woody tissues, which diminishes water and nutrient movement through both xylem and phloem. Characteristic dieback symptoms appear when water and nutrient demand exceeds the conductive capacity of the vascular tissues. Eventually, death of scaffold branches or the entire tree occurs when the growth of the cankers cuts off vascular flow (2). However, only a few studies on the etiology of olive twig and branch dieback can be found throughout the literature. Malathrakis (16) reported olive cankers and consequent dieback associated with the fungus *Phoma incompta* Sacc. & Martelli in Greece in 1979. Between the late 1980s and early 1990s, *Cytospora oleina* Berl. and *Eutypa lata* (Pers.) Tul. & C. Tul. were identified as the causal agents of olive branch dieback in Greece (26,27). During the 2000s, several fungi were associated with branch dieback, stem canker, and/or shoot necrosis of olive trees, including *E. lata* (35), a *Phoma* sp. (24), and several Botryosphaeriaceae species (14,20,25,32). Most recently, *B. dothidea*, *Diplodia seriata* De Not., and *Neofusicoccum mediterraneum* Crous, M.J. Wingf. &

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*The *e*-Xtra logo stands for "electronic extra" and indicates that Figures 2 and 3 appear in color online.

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A.J.L. Phillips were reported to cause branch dieback and necrosis, blight, and eventual death of olive shoots in California (20).

In California, olive twig dieback has long been known to commonly occur in orchards throughout the San Joaquin Valley (30,33). The disease has been historically associated with a *Diplodia* sp., which gave Diplodia dieback its name among California growers (33). However, both fungus and symptoms were found most of the time associated with either sunburned areas or declining olive tree parts affected by Verticillium wilt and/or olive knot (20,30,33), and thus, very little importance was given to olive twig dieback in the state. Nevertheless, in the past few years, branch canker and twig dieback of olive trees has become a major concern among growers from the main olive-producing areas in California, and diseased samples of both young and mature trees have been continuously submitted to our laboratory.

Perennial cankers and consequent dieback are known to cause a direct impact on the health of many economically important woody perennial crops worldwide, reducing both yield and tree longevity (2,8). In contrast, the lack of information currently available on the etiology of olive twig and branch dieback in California makes it difficult at the present time to assess the significance of this disease in the state. Therefore, the objectives of this study were to (i) determine the incidence and geographic distribution of olive twig and branch dieback in California by conducting field surveys, (ii) identify the different fungal species associated with the disease by means of morphological and molecular studies, and (iii) evaluate the pathogenicity of the different fungi in the two most common olive cultivars, Manzanillo and Sevillano, planted in the state.

Materials and Methods

Field surveys and fungal isolations. From October 2008 to September 2009, field surveys were conducted throughout the main olive-production areas in California, including Butte, Fresno, Glenn, Madera, Merced, Napa, Riverside, Sacramento, Santa Bar-

Fig. 1. Geographical distribution of fungal species on olive trees in California associated with olive twig and branch disease.

bara, Solano, Sonoma, Tehama, Tulare, Ventura, and Yolo counties (Fig. 1). In total, 803 samples were collected from 59 mature orchards (15 years old and older). Samples included trunks, branches, and twigs collected from trees showing characteristic dieback symptoms. Samples were collected from the most prevalent olive cultivars grown in California, including Ascolano, Manzanillo, Mission, and Sevillano. Additionally, 61 samples were collected from olive trees located in urban landscape sites in the cities of Davis, Merced, Napa, Sacramento, San Francisco, San Luis Obispo, Santa Barbara, Sonoma, and Temecula (Fig. 1). Diseased samples were first inspected for the presence of fungal fruiting structures (e.g., pycnidia, perithecia, pseudothecia, etc.) in the laboratory using a Leica MZ95 (Leica Microsystems GmbH, Wetzlar, Germany) stereo microscope. Thereafter, the outer bark of the samples was peeled off, and samples (including both sapwood and heartwood) were surface sterilized by submerging them in 0.5% sodium hypochlorite for 5 min. After air drying, sapwood tissue was shaved away to expose the margin between the cankered and healthy tissue; then the exposed wood was sprayed with 95% ethanol and briefly flamed. Small pieces of tissue (approximately 25 mm^2) were placed on 85-mm-diameter petri dishes containing 4% potato dextrose agar (PDA) (Difco, Detroit, MI) amended with tetracycline hydrochloride (0.01%) (Sigma-Aldrich, St. Louis, MO) (PDA-tet). Cultures were incubated at room temperature (24 ± 2°C) until fungal colonies were observed. The most prevalent fungal taxa observed from the symptomatic wood tissue were then individually transferred to fresh PDA-tet. Pure cultures of the different fungi were obtained by hyphal tip from colony margins and placed on fresh PDA. Pure fungal colonies were then incubated at ambient laboratory light and temperature conditions.

Morphological characterization. Fungal species were first identified tentatively to genus based on colony characteristics (color, mycelium growth speed, and type and shape of the colony) after 3 or 4 week's incubation. When available, both pycnidial and conidial characteristics (shape, size, color, presence or absence of septum) from colonies forming in vitro were recorded. For those cultures lacking sporulation, pycnidia production was induced by placing 5-mm-diameter mycelium plugs over double-autoclaved

Table 1. Isolates from olive trees from California included in the phylogenetic analyses

				GenBank accession no.		
Species	**Isolate**	**Cultivar**	**Origin**	**ITS**[y]	**EF1-α**[z]	**β-tubulin**
Botryosphaeria dothidea	UCD30-Oe	Sevillano	Butte Co.	JX515699	JX515747	JX515667
Botryosphaeria dothidea	UCD62-Oe	Mission	Butte Co.	JX515700	JX515748	JX515668
Diaporthe viticola	UCD316-Oe	Sevillano	Napa Co.	JX515701	JX515749	-
Diaporthe viticola	UCD327-Oe	Manzanillo	Napa Co.	JX515702	JX515750	-
Diatrype oregonensis	UCD60-Oe	Mission	Butte Co.	JX515703	JX515751	JX515669
Diatrype stigma	UCD23-Oe	Mission	Butte Co.	JX515704	JX515752	JX515670
Diatrype stigma	UCD24-Oe	Mission	Butte Co.	JX515705	JX515753	JX515671
Diatrype stigma	UCD356-Oe	Sevillano	Riverside Co.	JX515706	-	JX515672
Diatrype stigma	UCD357-Oe	Sevillano	Riverside Co.	JX515707	-	JX515673
Diplodia mutila	UCD127-Oe	Manzanillo	Glenn Co.	JX515745	JX515786	JX515674
Diplodia mutila	UCD147-Oe	Sevillano	Glenn Co.	JX515746	JX515787	JX515675
Diplodia seriata	UCD301-Oe	Manzanillo	Napa Co.	JX515708	JX515754	JX515676
Diplodia seriata	UCD340-Oe	Mission	Napa Co.	JX515709	JX515755	JX515677
Dothiorella iberica	UCD50-Oe	Mission	Butte Co.	JX515710	JX515756	JX515678
Dothiorella iberica	UCD673-Oe	Mission	Yolo Co.	JX515711	JX515757	JX515679
Eutypa lata	UCD143-Oe	Manzanillo	Glenn Co.	JX515712	JX515758	JX515680
Eutypa lata	UCD144-Oe	Manzanillo	Glenn Co.	JX515713	JX515759	JX515681
Eutypa lata	UCD318-Oe	Sevillano	Napa Co.	JX515714	JX515760	JX515682
Eutypa lata	UCD319-Oe	Sevillano	Napa Co.	JX515715	JX515761	JX515683
Lasiodiplodia theobromae	UCD375-Oe	Mission	Riverside Co.	JX515716	JX515762	JX515684
Lasiodiplodia theobromae	UCD527-Oe	Sevillano	Tehama Co.	JX515717	JX515763	JX515685
Neofusicoccum luteum	UCD360-Oe	Mission	Riverside Co.	JX515718	JX515764	JX515686
Neofusicoccum luteum	UCD369-Oe	Manzanillo	Riverside Co.	JX515719	JX515765	JX515687
Neofusicocum mediterraneum	UCD1-Oe	Sevillano	Butte Co.	JX515720	JX515766	JX515688
Neofusicocum mediterraneum	UCD22-Oe	Mission	Butte Co.	JX515721	JX515767	JX515689
Neofusicocum mediterraneum	UCD453-Oe	Manzanillo	Sacramento Co.	JX515722	JX515768	JX515690
Neofusicocum mediterraneum	UCD679-Oe	Mission	Yolo Co.	JX515723	JX515769	JX515691
Neofusicoccum vitifusiforme	UCD622-Oe	Manzanillo	Ventura Co.	JX515724	JX515770	JX515692
Neofusicoccum vitifusiforme	UCD624-Oe	Manzanillo	Ventura Co.	JX515725	JX515771	JX515693
Neofusicoccum vitifusiforme	UCD630-Oe	Sevillano	Ventura Co.	JX515726	JX515772	JX515694
Phaeoacremonium aleophilum	UCD228-Oe	Manzanillo	Napa Co.	JX515727	JX515773	JX515695
Phaeoacremonium aleophilum	UCD468-Oe	Mission	Sonoma Co.	JX515728	JX515774	JX515696
Phaeomoniella chlamydospora	UCD306-Oe	Manzanillo	Napa Co.	JX515729	JX515775	JX515697
Phaeomoniella chlamydospora	UCD471-Oe	Mission	Sonoma Co.	JX515730	JX515776	JX515698
Phomopsis sp.	UCD181-Oe	Manzanillo	Madera Co.	JX515731	JX515777	-
Phomopsis sp.	UCD182-Oe	Manzanillo	Madera Co.	JX515732	JX515778	-
Phomopsis sp.	UCD213-Oe	Sevillano	Madera Co.	JX515733	JX515779	-
Phomopsis sp.	UCD233-Oe	Manzanillo	Merced Co.	JX515734	JX515780	-
Phomopsis sp.	UCD237-Oe	Manzanillo	Merced Co.	JX515735	JX515781	-
Phomopsis sp.	UCD248-Oe	Sevillano	Merced Co.	JX515736	JX515782	-
Phomopsis sp.	UCD278-Oe	Mission	Merced Co.	JX515737	JX515783	-
Phomopsis sp.	UCD580-Oe	Manzanillo	Tulare Co.	JX515738	JX515784	-
Schizophyllum commune	UCD296-Oe	Sevillano	Napa Co.	JX515739	-	-
Schizophyllum commune	UCD304-Oe	Manzanillo	Napa Co.	JX515740	-	-
Schizophyllum commune	UCD309-Oe	Manzanillo	Napa Co.	JX515741	-	-
Trametes versicolor	UCD311-Oe	Manzanillo	Napa Co.	JX515742	-	-
Trametes versicolor	UCD312-Oe	Manzanillo	Napa Co.	JX515743	-	-
Trametes versicolor	UCD313-Oe	Mission	Napa Co.	JX515744	-	-

[y] rDNA Internal Transcribed Spacer region.
[z] Translation Elongation Factor.

pine needles placed on 2% water agar (Difco) as described by Úrbez-Torres et al. (46). Pycnidia were mounted in water, and conidial masses were observed by bright field microscopy using a Leica DMLB (Leica Microsystems GmbH) compound microscope. Images were recorded with a Leica DFC480 digital camera.

DNA extraction, amplification, and phylogenetic analyses. Representative isolates of each fungal taxa identified using morphological characteristics were selected for molecular identification (Table 1). Fungal isolates were cultured on PDA and grown for 2 weeks. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Oligonucleotide primers ITS1 and ITS4, Bt2a and Bt2b, and EF1-728F and EF1-986R were used to amplify the internal transcribed spacer (ITS) ITS1-5.8S-ITS2 ribosomal DNA region, a portion of the beta-tubulin (BT) gene, and part of the translation elongation factor (EF1-α) gene, respectively (5,10,51). ITS, BT, and EF1-α amplification reactions were carried out using a thermal cycler (PTC-100, MJ Research, Watertown, MA) (5,10,51). Amplification products were purified using QIAquick PCR purification Kit (Qiagen Inc.). Both strands of the ITS, BT, and EF1-α amplicons were sequenced using an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, CN) at the Division of Biological Sciences sequencing facility at University of California, Davis. Fungal sequences were edited and assembled using Sequencher version 4.1 (Gene Codes, Ann Arbor, MI) and were aligned using the ClustalW multiple alignment program (34). Manual adjustments of sequence alignment were carried out using BioEdit Sequence Alignment Editor Version 7.0.8. (Ibis Biosciences, Carslbad, CA). Fungal sequences, including those from ex-type specimens of taxa, when possible, were selected from GenBank (Table 2) based on their high similarity to query sequences using MegaBLAST.

Phylogenetic analyses were performed on individual datasets on the fungal families Botryosphaeriaceae (ITS, BT, and EF1-α) and Diatrypaceae (ITS and BT), and on the genera *Diaporthe–Phomopsis* (ITS and EF1-α), *Phaeoacremonium* (ITS and BT), *Phaeomoniella* (ITS and BT), and *Schizophyllum* and *Trametes* (ITS). All tree topologies were visually compared for congruence in order to permit concatenation of data sets. Multigene phylogenetic analyses were then performed on the Botryosphaeriaceae (ITS+BT+EF1-α), Diatrypaceae (ITS+BT), *Diaporthe–Phomopsis* (ITS+EF1-α), *Phaeoacremonium* (ITS+BT), and *Phaeomoniella* (ITS+BT) (single family and/or genera trees based on both individual and multigene datasets not shown). Phylogenetic analysis of the ITS region, including all taxa, and using the Basidiomycetes genera *Schizophyllum* and *Trametes* as an out-group, was performed to illustrate all fungal species identified from olive trees

Table 2. Isolates from GenBank included in the phylogenetic analyses

					GenBank accession no.		
Species	**Isolate**[w]	**Host**	**Origin**	**Collector**	**ITS**[x]	**EF1-α**[y]	**β-tubulin**
Botryosphaeria dothidea	**CMW8000**	*Prunus* sp.	Switzerland	B. Slippers	AY236949	AY236898	AY236927
Botryosphaeria dothidea	UCD1064So	*Vitis vinifera*	California, USA	J.R. Úrbez-Torres	DQ233600	GU294733	DQ233621
Cryptosphaeria pullmanensis	UCD2371NV	*Vitis vinifera*	Nevada, USA	J.R. Úrbez-Torres	GQ293966	n/a[z]	GQ294011
Cryptovalsa ampelina	UCD311Ma	*Vitis vinifera*	California, USA	J.R. Úrbez-Torres	GQ293906	n/a	GQ293975
Diaporthe ambigua	**CBS 114015**	*Pyrus communis*	South Africa	S. Denman	AF230767	GQ250299	n/a
Diaporthe ambigua	CBS 123210	*Foeniculum vulgare*	Portugal	J.M. Santos	EU814479	GQ250300	n/a
Diaporthe ambigua	CBS 123211	*Foeniculum vulgare*	Portugal	J.M. Santos	EU814478	GQ250301	n/a
Diaporthe angelicae	**CBS 111592**	*Heracleum sphondylium*	Austria	W. Jacklitsch	AY196779	GQ250302	n/a
Diaporthe aspalathi	**CBS 117169**	*Aspalathus linearis*	South Africa	J.C.J. van Rensburg	DQ286275	DQ286249	n/a
Diaporthe crotalariae	**CBS 162.33**	*Crotalaria spectabilis*	North America	G.F. Weber	FJ889445	GQ250307	n/a
Diaporthe helianthi	**CBS 592.81**	*Helianthus annuus*	Serbia	M. Muntanola-Cvetkovic	AY705842	GQ250308	n/a
Diaporthe melonis	CBS 507.78	*Cucumis melo*	Texas, USA	I. Beraha & M.J. O'Brein	FJ889447	GQ250314	n/a
Diaporthe neotheicola	**CBS 123208**	*Foeniculum vulgare*	Portugal	A.J.L. Phillips	EU814480	GQ250315	n/a
Diaporthe vaccinii	**CBS 160.32**	*Oxycoccus macrocarpus*	Oregon, USA	H.F. Bain	AY952141	GQ250326	n/a
Diaporthe viticola	**CBS 113201**	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY485750	GQ250327	n/a
Diaporthe viticola	111	*Vitis vinifera*	Portugal	E. Diogo	GQ250200	GQ250329	n/a
Diaporthe viticola	Di-C006/2	*Hydrangea macrophylla*	Portugal	A. Alves	GQ250199	GQ250328	n/a
Diatrype oregonensis	DRO102	*Prunus armeniaca*	California, USA	F.P. Trouillas	GQ293933	n/a	GQ293998
Diatrype oregonensis	CA117	*Vitis vinifera*	California, USA	F.P. Trouillas	GQ293934	n/a	GQ293996
Diatrype sp.	CDB016	*Vitis vinifera*	California, USA	F.P. Trouillas	GQ293950	n/a	GQ294001
Diatrype stigma	CA074	*Vitis vinifera*	California, USA	F.P. Trouillas	GQ293944	n/a	GQ294005
Diatrype stigma	DCHR5	*Vitis vinifera*	California, USA	F.P. Trouillas	GQ293945	n/a	GQ294006
Diatrype stigma	DCASH200	*Quercus* sp.	California, USA	F.P. Trouillas	GQ293947	n/a	GQ294003
Diatrype whitemanensis	DDES500	*Acer macrophyllum*	California, USA	F.P. Trouillas	GQ293952	n/a	GQ294009
Diatrypella verrucaeformis	DCH500	*Quercus* sp.	California, USA	F.P. Trouillas	GQ293926	n/a	GQ293991
Diplodia mutila	**CBS 112553**	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259093	AY573219	DQ458850
Diplodia mutila	UCD288Ma	*Vitis vinifera*	California, USA	J.R. Úrbez-Torres	DQ008313	EU012411	DQ008336
Diplodia seriata	**CBS 112555**	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259094	AY573220	DQ458856
Diplodia seriata	UCD244Ma	*Vitis vinifera*	California, USA	J.R. Úrbez-Torres	DQ008314	EU012406	DQ008337
Dothiorella iberica	**CBS 115041**	*Quercus ilex*	Spain	J. Luque	AY573202	AY573222	EU673096

(*continued on next page*)

[w] Isolate numbers in bold represent ex-type specimens.
[x] rDNA Internal Transcribed Spacer region.
[y] Translation Elongation Factor.
[z] n/a = not available.

in California. Single and multigene phylogenetic analyses were performed with PAUP version 4.0b10 (31) using maximum parsimony (MP) with a heuristic search and 1,000 random addition sequence replicates. Trees were rooted using the midpoint rooting option, and tree bisection-reconnection (TBR) was used as the branch swapping algorithm. Branches of zero length were collapsed, and all multiple, equally parsimonious trees were saved. Alignment gaps were treated as missing data, and all characters were unordered and equally weighted. Measures including tree length, consistency index (CI), and retention index (RI) were calculated. Bootstrap support (BS) was estimated using 1,000 replicates to assess the robustness of each clade. Fungal sequences from olive from California were deposited into GenBank, and representative isolates are maintained in the collection of the Plant Pathology Department at the University of California, Davis (Table 1).

Pathogenicity tests. In order to determine which fungal species isolated from cankers of olive trees were important pathogens and to differentiate them from potential saprophytes, secondary colonizers, and/or weak parasites, one representative isolate of each fungal species identified from symptomatic wood tissue was selected to determine pathogenicity. Pathogenicity tests were conducted on mature Manzanillo and Sevillano olive trees located in an experimental commercial orchard at the University of California Nickels Soil Laboratory Field Station in Arbuckle, CA in October of 2009. Olive branches (2- and 3-year-old) from both cultivars were individually inoculated with fungal species. The distal end of each tree branch was inoculated by placing a 5-mm-diameter mycelium plug from a 7-day-old PDA culture in a wound made with a 5-mm-diameter cork-borer. Wounds were sealed with petroleum jelly and protected with Parafilm. Fungal treatments were compared to control treatments inoculated with noncolonized media plugs, also sealed with petroleum jelly and protected by Parafilm. Ten branches per fungal isolate (1 branch per tree) and per olive cultivar were used, and inoculations were arranged in a completely randomized design. Samples were collected after 6 months of incubation and returned to the laboratory for assessment of canker length. Samples were surface disinfected as previously described. After air drying, samples were split longitudinally through the point of inoculation, and the extent of both acropetal and basipetal vascular discoloration from the point of inoculation was measured. In an attempt to fulfill Koch's postulates, small pieces of necrotic tissue from the edge of each lesion were cut and placed on PDA-tet to recover the inoculated fungus.

Data from the pathogenicity test were analyzed using SAS (Version 9.1.3; SAS Institute, Cary, NC). Homogeneity of variance was tested using Levene's test. Residuals were visually inspected for

Table 2. (*continued from previous page*)

Species	Isolate[w]	Host	Origin	Collector	GenBank accession no. ITS[x]	EF1-α[y]	β-tubulin
Dothiorella sarmentorum	**IMI63581b**	*Ulmus* sp.	United Kingdom	E.A. Ellis	AY573212	AY573235	EU673102
Eutypa lata	UCDE7	*Vitis vinifera*	California, USA	P.E. Rolshausen	DQ006944	n/a	DQ00700
Eutypa lata	UCDE31	*Vitis vinifera*	California, USA	P.E. Rolshausen	DQ006933	n/a	DQ006990
Eutypa lata	UCDE38	*Vitis vinifera*	California, USA	P.E. Rolshausen	DQ006935	n/a	DQ006992
Eutypa lata	UCD2275MO	*Vitis vinifera*	Missouri, USA	J.R. Úrbez-Torres	HQ288221	n/a	HQ288300
Eutypa leptoplaca	D-Rsn-200	*Vitis vinifera*	California, USA	F.P. Trouillas	AY684237	n/a	AY684212
Lasiodiplodia theobromae	CBS 164.96	Fruit along the coral reef coast	Papua New Guinea	n/a	AY640255	AY640258	EU673110
Lasiodiplodia theobromae	CAA006	*Vitis vinifera*	California, USA	T.J. Michailides	DQ458891	DQ458876	DQ458859
Lasiodiplodia theobromae	UCD205Co	*Vitis vinifera*	California, USA	J.R. Úrbez-Torres	DQ008310	EU012398	DQ008333
Neofusicoccum luteum	**CBS 110299**	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259091	AY573217	DQ458848
Neofusicoccum mediterraneum	**CBS 121718**	*Eucalyptus* sp.	Greece	Crous, Wingfield & Phillips	GU251176	GU251308	GU251836
Neofusicoccum mediterraneum	UCD720SJ	*Vitis vinifera*	California, USA	J.R. Úrbez-Torres	GU799452	GU799483	GU799475
Neofusicoccum parvum	**CMW9081**	*Populus nigra*	New Zealand	G.J. Samuels	AY236943	AY236888	AY236917
Neofusicoccum ribis	**CMW7772**	*Ribes* sp.	New York, USA	B. Slippers & G. Hudler	AY236935	AY236877	AY236906
Neofusicoccum vitifusiforme	**STE-U 5252**	*Vitis vinifera*	South Africa	J.M. van Niekerk	AY343383	AY343343	n/a
Neofusicoccum vitifusiforme	UCD2183MO	*Vitis vinifera*	Missouri, USA	K. Striegler & G.M. Leavitt	HQ288214	HQ288258	HQ288293
Phaeoacremonium aleophilum	**CBS 246.91**	*Vitis vinifera*	Serbia	M. Muntanola-Cvetkovic	AF017651	n/a	AF286811
Phaeoacremonium aleophilum	CBS 100397	*Vitis vinifera*	Italy	n/a	AF197981	n/a	AF246806
Phaeoacremonium angustius	**CBS 249.95**	*Vitis vinifera*	USA	P. Larignon	AF197974	n/a	AF246814
Phaeomoniella chlamydospora	**CBS 229.95**	*Vitis vinifera*	Italy	L. Mugnai	AF197973	n/a	AF253968
Phaeomoniella chlamydospora	STE-U 3066	*Vitis vinifera*	South Africa	n/a	AF197986	n/a	AF253969
Phaeomoniella chlamydospora	UCD2548MO	*Vitis vinifera*	Missouri, USA	K. Striegler & G.M. Leavitt	HQ288241	HQ288276	HQ288313
Phomopsis dauci	**CBS 315.49**	*Daucus carota*	Netherlands	J.A. von Arx	FJ889451	GQ250348	n/a
Phomopsis phoenicicola	**CBS 161.64**	*Areca catechu*	India	H.C. Srivastava	FJ889452	GQ250349	n/a
Phomopsis sp.	UCD1685Sl	*Vitis vinifera*	California, USA	J.R. Úrbez-Torres	FJ794470	JX515785	n/a
Phomopsis sp.	CAL-5	*Vitis vinifera*	California, USA	n/a	AY745085	AY745055	n/a
Phomopsis sp.	151	*Vitis vinifera*	Portugal	E. Diogo	GQ250226	GQ250364	n/a
Phomopsis sp.	Ph-AC002	*Acanthus* sp.	Portugal	E. Diogo	GQ250216	GQ250354	n/a
Phomopsis viticola	**CBS 114016**	*Vitis vinifera*	France	P. Larignon	AF230751	GQ250351	n/a
Spencermartinsia viticola	**CBS 117009**	*Vitis vinifera*	Spain	J. Luque	AY905554	AY905559	EU673104

each treatment, and when necessary a log10 transformation was used to improve homogeneity of variance. Difference in length of discoloration caused by each fungal isolate was determined by one-way analyses of variance. Treatment means were compared using Fisher's least significant difference (LSD) test at the 5% significance level. A two-way ANOVA was performed to determine significant differences between Manzanillo and Sevillano olive cultivars.

Results

Field survey and symptoms of the disease. Olive twig and branch dieback symptoms were observed in all 15 counties surveyed and from all of the 59 orchards sampled (Table 3). Branch dieback of olive trees was observed in mature trees throughout California no matter the cultivar surveyed (Ascolano, Manzanillo, Mission, and Sevillano). Additionally, the disease was also observed in landscape olive trees located in the urban areas surveyed.

Symptoms associated with olive twig and branch dieback were characterized by scarce or abundant twig death localized in single or multiple branches, respectively (Fig. 2A to C). Death of twigs was usually associated with cankered stems and/or main branches (Fig. 2D). These cankers often extended through the branches until reaching the trunk causing dieback and eventual death of the branch and/or tree (Fig. 2E). Wedge-shaped cankers were the most common vascular symptom observed when affected branches were cross-sectioned (Fig. 2B and F). However, other vascular symptoms such as dark streaking of the wood and/or light brown tissue were observed in contrast to the healthy yellowish-green tissue (Fig. 2G and H). Cankers were also observed in young trees affecting the crown of the tree (Fig. 2I). Although cankers were found to develop mostly from pruning wounds made in the main branches (Fig. 2J and K), they were also observed to develop from both sunburned areas (Fig. 2L) and olive knot galls (Fig. 2M).

Morphological characterization and fungal incidence. Results from canker isolations along with morphological studies showed species in the families Botryosphaeriaceae Theiss. & P. Syd. and Diatrypaceae Nitschke and the genus *Phomopsis* Sacc. to be the most predominant fungi associated with branch cankers of olive trees in California.

Morphologically, the Botryosphaeriaceae fungal group was characterized by a light-green to dark-olivaceous fast-growing mycelium on PDA-tet (Fig. 3A and B). With age, most of these cultures developed black, globose fruiting bodies (pycnidia), which produced either pigmented or hyaline spores (conidia). Based on comparison with earlier literature (46) and previously identified isolates from grapevines in California, Botryosphaeriaceae fungal cultures with pigmented conidia were tentatively identified as different species including *Lasiodiplodia theobromae*, *Diplodia seriata*, *Diplodia mutila*, and *Dothiorella* species. Fungal cultures with hyaline conidia were tentatively identified as *Botryosphaeria dothidea* or at least three different *Neofusicoccum* species. Botryosphaeriaceae species were the most prevalent fungi isolated from both cankers (43% of the total) and twig dieback (24.2% of the total) and were found in all counties surveyed (Table 3).

The *Diaporthe–Phomopsis* fungal group was characterized by having white to light-gray, slow-growing mycelium (Fig. 3C and D). Fungal colonies were slightly raised and some developed prominent growth rings with margins becoming black with age. Colonies produced dark, eustromatic pycnidia over time. Mucilaginous light-cream-colored cirrhi were observed from pycnidia (Fig. 3D). Cirrhi contained filiform and mostly curved conidia. All these morphological characteristics were consistent with the description of *Diaporthe–Phomopsis* spp. (40,48); however, identification to species level was not possible based only on colony and conidia morphology. *Diaporthe–Phomopsis* spp. were the second most prevalent fungi isolated from both cankers (12.9% of the total) and twig dieback (9.4% of the total) and were most commonly found in olive orchards located throughout the San Joaquin Valley, Central Coast, and southern California (Table 3).

Diatrypaceae species were characterized by having white to white-cream flocculent slow-growing mycelium on PDA-tet (Fig. 3E and F). With age, a light-brown to dark-brown coloration devel-

Table 3. Incidence of fungal species isolated from olive trees in the main olive-production areas of California

				Number (%) samples yielding											
	Total samples[w]			Botryosphaeriaceae		*Diaporthe-Phomopsis*		Diatrypaceae		Basidiomycota	*P.a.-P.m.*[x]	Mix[y]		Others[z]	
District/Co./City*	Or	Ck	Td	Ck	Td	Ck	Td	Ck	Td	Ck	Ck	Ck	Td	Ck	Td
Sacramento Valley	22	201	68	99 (49.3)	18 (26.5)	-	-	26 (12.9)	-	-	-	8 (4)	-	68 (33.8)	50 (73.5)
Butte Co.	6	62	11	20 (32.3)	6 (54.5)	-	-	7 (11.3)	-	-	-	-	-	35 (56.5)	5 (45.5)
Glenn Co.	6	11	37	5 (45.5)	4 (10.8)	-	-	6 (54.5)	-	-	-	-	-	-	33 (89.2)
Sacramento Co.	3	33	10	15 (45.5)	39 (30)	-	-	4 (12.1)	-	-	-	-	-	14 (42.4)	7 (70)
Sacramento	-	5	-	4 (80)	-	-	-	-	-	-	-	1 (20)	-	-	-
Solano Co.	2	25	-	10 (40)	-	-	-	6 (24)	-	-	-	4 (16)	-	5 (20)	-
Tehama Co.	3	25	10	13 (52)	5 (50)	-	-	3 (12)	-	-	-	1 (4)	-	8 (32)	5 (50)
Yolo Co.	2	28	-	22 (78.6)	-	-	-	-	-	-	-	2 (7.1)	-	4 (14.3)	-
Davis*	-	12	-	10 (83.3)	-	-	-	-	-	-	-	-	-	2 (16.7)	-
North Coast	8	107	31	30 (28)	6 (19.4)	5 (4.7)	9 (29)	24 (22.4)	7 (22.6)	18 (16.8)	5 (4.7)	5 (4.7)	-	20 (18.7)	9 (29)
Napa Co.	5	28	31	8 (28.6)	6 (19.4)	1 (3.6)	9 (29)	7 (25)	7 (22.6)	10 (35.7)	-	2 (7.1)	-	-	9 (29)
Napa *	-	7	-	3 (42.9)	-	-	-	2 (28.6)	-	-	-	1 (14.3)	-	1 (14.3)	-
San Francisco *	-	10	-	4 (40)	-	2 (20)	-	-	-	-	-		-	4 (40)	-
Sonoma Co.	3	53	-	10 (18.9)	-	-	-	13 (24.5)	-	8 (15.1)	5 (9.4)	2 (3.8)	-	15 (28.3)	-
Sonoma *	-	9	-	4 (44.4)	-	2 (22.2)	-	2 (22.2)	-	-	1 (11.1)		-	-	-
San Joaquin Valley	18	207	29	91 (44)	7 (24.1)	44 (21.3)	3 (10.3)	-	-	2 (1)	-	25 (12.1)	4 (13.8)	45 (21.7)	15 (51.7)
Fresno Co.	3	24	8	12 (50)	3 (37.5)	5 (20.8)	2 (25)	-	-	2 (8.3)	-	3 (12.5)	2 (25)	2 (8.3)	1 (12.5)
Madera Co.	6	71	-	35 (49.3)	-	19 (26.8)	-	-	-	-	-	5 (7)	-	12 (16.9)	-
Merced Co.	5	44	21	15 (34.1)	4 (19)	3 (6.8)	1 (4.8)	-	-	-	-	4 (9.1)	2 (9.5)	22 (50)	14 (66.7)
Merced*	-	13	-	6 (46.2)	-	3 (23.1)	-	-	-	-	-	2 (15.4)	-	2 (15.4)	-
Tulare Co.	4	55	-	23 (41.8)	-	14 (25.5)	-	-	-	-	-	11 (20)	-	7 (12.7)	-
Central Coast	7	90	-	40 (44.4)	-	22 (24.4)	-	2 (2.2)	-	-	7 (7.8)	12 (13.3)	-	7 (7.8)	-
Santa Barbara Co.	2	28	-	22 (78.6)	-	5 (17.9)	-	-	-	-	-	1 (3.6)	-	-	-
Santa Barbara*	-	4	-	3 (75)	-	1 (25)	-	-	-	-	-		-	-	-
Ventura Co.	5	58	-	15 (25.9)	-	16 (27.6)	-	2 (3.4)	-	-	7 (12.1)	11 (19)	-	7 (12.1)	-
Southern California	4	70	-	30 (42.9)	-	16 (22.9)	-	11 (15.7)	-	-	-	8 (11.4)	-	5 (7.1)	-
Riverside Co.	4	64	-	25 (39.1)	-	16 (25)	-	11 (17.5)	-	-	-	7 (10.9)	-	5 (7.8)	-
Temecula*	-	6	-	5 (83.3)	-	-	-	-	-	-	-	1 (16.7)	-	-	-
Total	59	675	128	290 (43)	31 (24.2)	87 (12.9)	12 (9.4)	63 (9.3)	7 (5.5)	20 (3)	12 (1.8)	58 (8.6)	4 (3.1)	145 (21.5)	74 (57.8)

[w] Or. = Total number of orchards surveyed. Ck. = Perennial canker. Td. = Twig dieback.
[x] *P.a.* = *Phaeomoniella chlamydospora*. *P.m.* = *Phaeoacremonium aleophilum.*
[y] Mix = Number of samples from which two or more fungal species were isolated from the same symptom.
[z] Others = Number of samples from which different fungi were isolated (endophytes, saprophytes and/or unknown fungi).

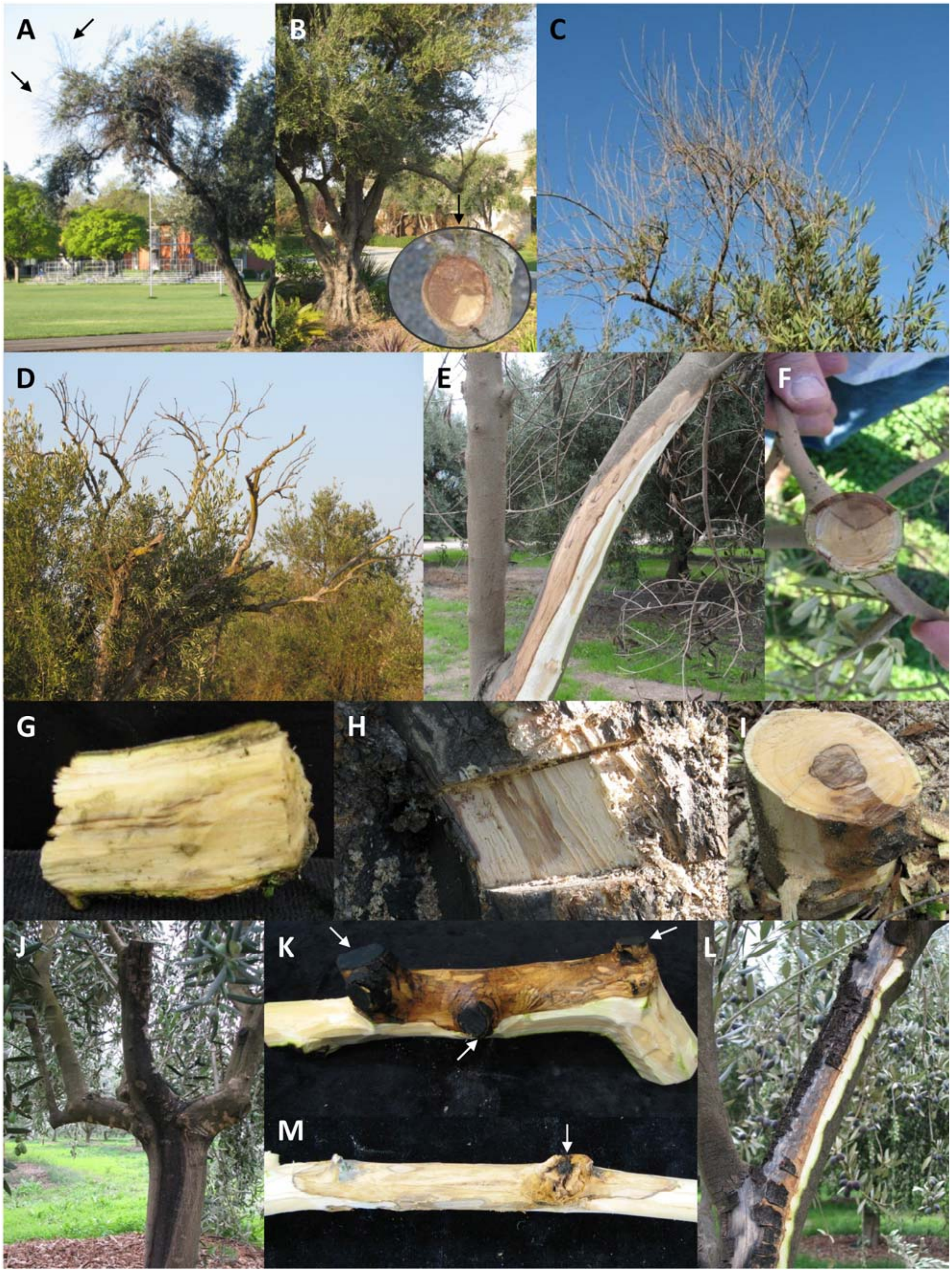

Fig. 2. Olive twig and branch dieback symptoms observed in olives in California orchards. **A,** Black arrows show moderate symptoms of twig dieback in an ornamental olive tree in the city of Davis. **B,** Branch dieback in an ornamental tree located in a private garden in the city of Merced. Cross-section of this branch showed a wedge-shaped canker. **C,** Severe twig dieback observed in a commercial olive orchard. **D,** Severe branch dieback. **E,** Perennial canker in an olive branch showing a well-defined dark line of demarcation between infected and healthy tissues. **F,** Wedge-shaped cankers were the most prevalent vascular symptom observed in symptomatic wood. **G,** Dark streaking of the wood. **H,** Light brown symptomatic wood tissue. **I,** Cankers were also observed at the crown of olive trees. **J,** Canker observed to develop from a large pruning cut. **K,** White arrows show several pruning wounds in an olive branch from where perennial canker developed. **L,** Cankers were also observed to be associated with sunburn areas. **M,** Cankers were also observed to develop from olive knot wounds caused by the bacterium *Pseudomonas syringae* pv. *savastanoi* in both twigs and branches.

Fig. 3. Four-week-old colony morphology on potato dextrose agar of the different fungal genera isolated from olive trees in California. **A,** *Neofusicoccum mediterraneum*. **B,** *Dothiorella iberica*. **C,** *Diaporthe viticola*. **D,** *Phomopsis* sp. group 1. **E,** *Diatrype stigma*. **F,** *Eutypa lata*. **G,** Basidiocarps of *Schizophyllum commune* on an olive tree trunk. **H,** *Schizophyllum commune* colony morphology. **I,** Basidiocarps of *Trametes versicolor* on an olive tree trunk. **J,** *Trametes versicolor* colony morphology. **K,** *Phaeomoniella chlamydospora*. **L,** *Phaeoacremonium aleophilum*. **M,** Canker length caused for each of the fugal species identified in this study. 1) control, 2) *Diatrype stigma* UCD23-Oe, 3) *Diatrype oregonensis* UCD60-Oe, 4) *Eutypa lata* UCD143-Oe, 5) *Schizophyllum commune* UCD296-Oe, 6) *Trametes versicolor* UCD461-Oe, 7) *Diaporthe viticola* UCD316-Oe, 8) *Phomopsis* sp. group 1 UCD181-Oe, 9) *Phaeomoniella chlamydospora* UCD306-Oe, 10) *Phaeoacremonium aleophilum* UCD468-Oe, 11) *Lasiodiplodia theobromae* UCD527-Oe, 12) *Dothiorella iberica* UCD163-Oe, 13) *Botryosphaeria dothidea* UCD62-Oe, 14) *Diplodia seriata* UCD340-Oe, 15) *Diplodia mutila* UCD127-Oe, 16) *Diplodia mutila* UCD127-Oe, 17) *Neofusicoccum vitifusiforme* UCD622-Oe, 18) *Neofusicoccum luteum* UCD369-Oe, and 19) *Neofusicoccum mediterraneum* UCD1-Oe. **N,** The same wedge-shaped cankers observed in symptomatic olive wood in the field were reproduced in the pathogenicity test. **O,** Black arrows show pycnidia of *Neofusicoccum mediterraneum* embedded in the bark of a young olive stem.

oped at the center of the colony on the reverse. Some cultures exhibited slow growth with irregular and lobate colony margins. Conidia produced by pycnidia in the plates were filiform and mostly curved in shape. These characters were consistent with the description of species in the Diatrypaceae family (38). Morphological characteristics allowed us to identify several isolates as *Eutypa lata*; however, identification to species level for the rest of the isolates was not possible based only on colony and conidia morphology. Diatrypaceous isolates were the third most prevalent fungi isolated from both cankers (9.3% of the total) and twig dieback (5.5% of the total) and were primarily found in orchards located throughout the North Coast and the Sacramento Valley (Table 3).

Less common fungi isolated from symptomatic trees were the basidiomycete species *Schizophyllum commune* Fr. and *Trametes versicolor* (L.) Lloyd, which were first identified based on the basidiocarps observed on the trees (Fig. 3G and I). *Schizophyllum commune* basidiocarps were also observed on PDA culture media in vitro (Fig. 3H). Additionally, comparison with previously identified isolates from grapevines available in our collection allowed us to identify the slow-growing mycelia of *Phaeomoniella chlamydospora* Crous & W. Gams and *Phaeoacremonium aleophillum* W. Gams, Crous, M.J. Wingfield & L. Mugnai (Fig. 3K and L), which were sporadically isolated from olive cankers from orchards sampled in both North and Central Coast olive-producing regions (Table 3). Other fungi isolated from both cankers and/or dead twigs were *Alternaria alternata* (Fr.) Keissl., *Alternaria citri* Ellis & N. Pierce, *Alternaria* sp., *Aspergillus* sp., *Biscogniauxia mediterranea* (De Not.) Kuntze, *Camarosporium brabeji* Marincowitz, M.J. Wingf. & Crous, *Coniozyma leucospermi* (Crous & Denman) Crous, *Drechslera erythrospila* (Drechsler) Shoemaker, *Epicoccum nigrum* Link, *Nigrospora oryzae* (Berk. & Broome) Petch, *Paecilomyces sinensis* Q.T. Chen, S.R. Xiao & Z.Y. Shi, *Penicillium cecidicola* Seifert, Hoekstra & Frisvad, *Penicillium* sp., *Phoma macrostoma* Mont., and *Sordaria* sp. These fungi were isolated from 21.5 and 57.8% of branch cankers and twig dieback (Table 3), respectively; however, the occurrence of each species was highly variable. Among them, species of *Aspergillus* and *Alternaria* were the most prevalent. Most of these fungi were identified to species level based on molecular identification of the ITS region of rRNA (ITS1-5.8S-ITS2).

Phylogeny. PCR amplifications of the ITS, BT, and EF1-α regions gave products of approximately 0.6, 0.4, and 0.2 kb, respectively. The Botryosphaeriaceae combined ITS, BT, and EF1-α analysis included 40 taxa and contained 1,240 total characters with 847 being constant, 57 parsimony-uninformative, and 339 parsimony-informative. The heuristic search produced 18 most parsimonious trees with 629 steps each (CI = 0.803, RI = 0.936) (trees not shown). The analysis confirmed the identification of eight different species in the Botryosphaeriaceae from California olive trees, including *Lasiodiplodia theobromae*, *Diplodia mutila*, *Diplodia seriata*, *Dothiorella iberica*, *Botryosphaeria dothidea*, *Neofusicoccum mediterraneum*, *Neofusicoccum vitifusiforme,* and *Neofusicoccum luteum*. Botryosphaeriaceous isolates from olives from California grouped in well-supported clades (>90% bootstrap value) along with ex-type specimens retrieved from GenBank when available and/or with previously identified species in the Botryosphaeriaceae from California from different hosts.

The combined ITS and BT analysis of Diatrypaceae spp., *Ph. chlamydospora* and *Pm. aleophilum* included 34 taxa and contained 940 total characters with 473 being constant, 52 parsimony-uninformative, and 415 parsimony-informative. The heuristic search produced 24 most parsimonious trees with 1,024 steps each (CI = 0.709, RI = 0.866) (trees not shown). The analysis confirmed the identification of three species of Diatrypaceae, *Diatrype oregonensis*, *Diatrype stigma,* and *Eutypa lata*, and corroborated the identification of both *Ph. chlamydospora* and *Pm. aleophilum* from olive in California.

The combined ITS and EF1-α analysis of *Diaporthe* and *Phomopsis* spp. included 30 taxa and contained 883 total characters with 519 being constant, 47 parsimony-uninformative, and 317 parsimony-informative. The heuristic search produced 4 most parsimonious trees with 1,001 steps each (CI = 0.596, RI = 0.771) (trees not shown). The analysis confirmed the identification of three different species, including *Diaporthe viticola* and two nondetermined *Phomopsis* spp., namely, *Phomopsis* sp. group 1 and *Phomopsis* sp. group 2. The combined ITS and EF1-α analysis showed isolates UCD316-Oe and 327-Oe to group in a well-supported clade (100% bootstrap) with different *Diaporthe viticola* isolates from *V. vinifera*, including the ex-type isolate CBS 113201. California olive isolates UCD181-Oe, UCD-182-Oe, and UCD213-Oe, and UCD233-Oe, UCD237-Oe, UCD248-Oe, UCD278-Oe, and UCD580-Oe grouped in two separate well-supported clades (100% bootstrap) and shared identical gene sequences with *Phomopsis* sp. isolates UCD1685Sl and CAL-5 from *V. vinifera* from California, respectively.

California olive isolates UCD296-Oe, UCD297-Oe, UCD298-Oe, and UCD304-Oe and isolates UCD311-O3, UCD312-Oe, UCD313-Oe, and UCD461-Oe shared 99 and 100% similarity with most ITS sequences of *Schizophyllum commune* and *Trametes versicolor* from GenBank, respectively. Figure 4 shows the ITS phylogenetic analysis with all fungal taxa used in this study, including those identified from olive trees in California.

Pathogenicity test. Mean lengths of vascular discoloration caused by the 18 fungal isolates tested and the control in the Manzanillo and Sevillano olive cultivars are shown in Table 4. Six months after inoculation, cankers observed in cross-section from the inoculated branches were very similar in shape (wedge-shaped to round) to those observed and collected from commercial olive orchards (Fig. 3M and N). Canker shape caused by all inoculated species was very similar with the exception of *Pa. chlamydospora* and *Ph. aleophilum*, which caused a necrotic streaking of the vascular system. Although all fungal species tested in the pathogenicity test caused cankers and/or vascular discoloration of the wood significantly different in length ($P < 0.05$) than the control, virulence varied among species (Table 4). The botryosphaeriaceous taxa *N. mediterraneum* and *Diplodia mutila* were shown to be the most virulent species in both Manzanillo (63.1 and 43.6 mm mean lesion, respectively) and Sevillano (74.8 and 76 mm mean lesion, respectively) cultivars (Table 4). *N. vitifusiforme* and *T. versicolor* in both cultivars and *E. lata* in the Sevillano cultivar followed in virulence, causing mean lesions more than 10 mm in length (Table 4). The remaining fungal species caused mean lesions that were between 5 and 10 mm in length. *Diatrype stigma* and *D. oregonensis* were the least virulent species, causing mean lesions less than 4 mm in length (Table 4). Percentages of fungal recovery were high (>70%) for all treatments with the exception of *Pa. chlamydospora*, *D. stigma,* and *D. oregonensis* (50% or lower) (Table 4). Both Manzanillo and Sevillano olive cultivars used in the pathogenicity test were susceptible to infection by all fungal species tested. However, ANOVA revealed that Sevillano was more susceptible to infection caused by *N. mediterraneum*, *Diplodia mutila*, *E. lata*, and *Diaporthe viticola* than Manzanillo. On the other hand, Manzanillo was more susceptible to infection caused by *L. theobromae* and *Pa. chlamydospora* than Sevillano (Table 4).

Discussion

Eighteen fungal species belonging to 12 different genera within eight different families were isolated from symptomatic wood of olive trees affected by twig and branch dieback in California and identified by means of morphological characters and multigene sequence analyses. These species included *Botryosphaeria dothidea*, *Diaporthe viticola*, *Diatrype oregonensis*, *Diatrype stigma*, *Diplodia mutila*, *Diplodia seriata*, *Dothiorella iberica*, *Eutypa lata*, *Lasiodiplodia theobromae*, *Neofusicoccum luteum*, *Neofusicoccum mediterraneum*, *Neofusicoccum vitifusiforme*, *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora*, *Phomopsis* sp. group 1, *Phomopsis* sp. group 2, *Schizophyllum commune*, and *Trametes versicolor*.

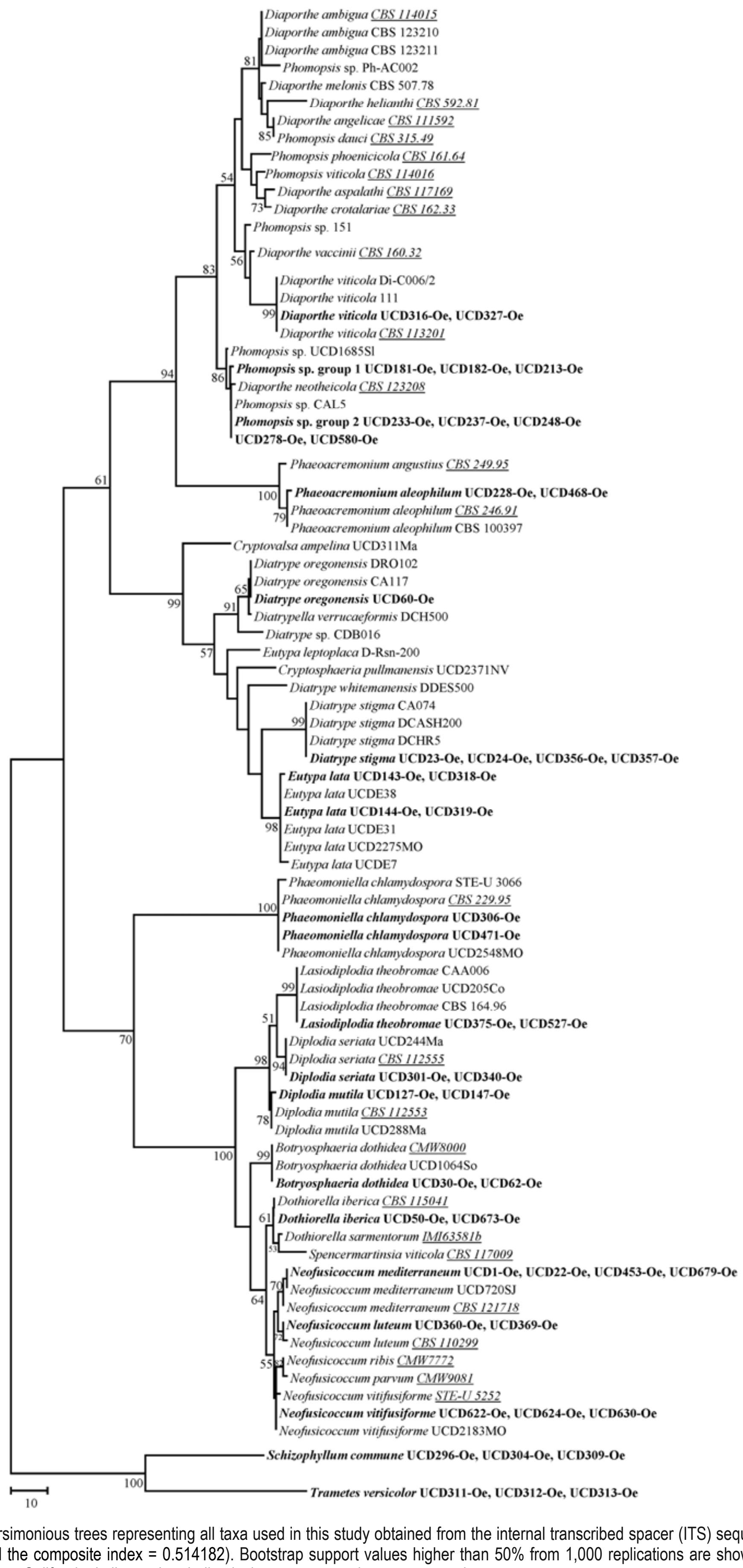

Fig. 4. One of the 62 most parsimonious trees representing all taxa used in this study obtained from the internal transcribed spacer (ITS) sequence data (length = 550, CI = 0.552920, RI = 0.927213, and the composite index = 0.514182). Bootstrap support values higher than 50% from 1,000 replications are shown at the nodes. Bold isolates represent isolates from olive from California. Italics and underline isolates represent the ex-type specimen.

Olive twig and branch dieback symptoms were manifested by defoliation and/or wilting of the leaves in young twigs. Although these symptoms were typically observed to affect only part of the tree, entire trees were occasionally observed to be affected in some of the orchards surveyed. In the field, these symptoms can easily be confused with those resembling Verticillium wilt (13,26), which could explain to some extent why olive twig and branch dieback has remained overlooked and consequently little studied by plant pathologists for many years. Nevertheless, olive branch and twig dieback can be distinguished from Verticillium wilt by the presence of flat to slightly sunken and darker areas along the affected stems, older branches, and/or trunk, which reveal perennial cankers when the bark is removed. Additionally, pycnidia and/or perithecia of some of the fungi associated with olive twig and branch dieback can be found embedded in the bark of the affected areas. Moreover, cankered zones along branches also reveal a well-defined dark line of demarcation between infected and healthy tissues. The dark streaking of the wood symptom observed in olive trees in this study, on the other hand, and from which *Pa. chlamydospora* and *Pm. aleophilum* were isolated, resembles the vascular symptoms associated with Verticillium wilt, caused by *Verticillium dahliae*. In this case, sample collection and fungal identification by either traditional plating and/or molecular techniques would be required in order to properly identify the causal agent.

Perennial cankers and subsequent dieback have been widely studied in many different perennial hosts worldwide. However, to date, only a few studies regarding this problem have been carried out on olive trees (20,25–27,32). Additionally, most of these studies have focused their research only in a single fungal species as the cause of olive dieback (25–27,32). The current study represents the first attempt to elucidate the etiology of olive twig and branch dieback, and contrary to previous studies, it shows that not only one but a complex of several taxonomically unrelated fungal species are involved in this disease.

Among all the fungal taxa isolated from symptomatic wood of olive trees in this study, species of Botryosphaeriaceae were the most prevalent. Botryosphaeriaceae species are primarily known to cause olive fruit rot (6,15,19,20,22), including the dalmatian disease, caused by *B. dothidea* and considered the most important fruit disease of table olives (20). However, several botryosphaeriaceous taxa have also been reported to cause olive branch dieback, including *N. luteum* in New Zealand (32), *N. ribis* and *N. mediterraneum* in Spain (20,25), and *Diplodia seriata* in Croatia (14). Most recently, Moral et al. (20) reported *B. dothidea*, *D. seriata,* and *N. mediterraneum* to cause branch cankers and blighted shoots of olive trees in California. The current study corroborates the presence of these latter species in olive trees in California and adds five new records of Botryosphaeriaceae species in olive trees in America, including *Diplodia mutila*, *Dothiorella iberica*, *L. theobromae*, *N. luteum*, and *N. vitifusiforme*. Additionally, this study reports for the first time *D. mutila*, *D. iberica*, and *L. theobromae* to occur in olive trees. Moreover, complementary to the study conducted by Moral et al. (20) in which only three counties (Glenn, Madera, and Fresno) were surveyed in California, the present study shows Botryosphaeriaceae species to be widespread throughout all olive-growing regions in the state, including ornamental olive trees planted along streets and in both public and private gardens in several California cities. The pathogenicity trial showed the Botryosphaeriaceae species, *N. mediterraneum* and *D. mutila*, to be the most virulent in olive trees among all the fungi in this study. The high virulence of *N. mediterraneum* showed in this study agrees with the results reported by Moral et al. (20) in which this species was shown to be very aggressive when inoculated into olive branches. However, the current study shows that *D. mutila* can be equally or more virulent than *N. mediterraneum* in olive trees. On the other hand, the rest of Botryosphaeriaceae species tested in this study were significantly less virulent, with *D. seriata* and *D. iberica* as the least virulent of the Botryosphaeriaceae. Similarly, *B. dothidea* and *D. seriata* isolates from California have been reported to be either nonpathogenic or weakly virulent when inoculated in olive branches (20) or grapevines (45). The family Botryosphaeriaceae includes several well-known plant pathogens that cause perennial cankers and consequent dieback in a broad range of economically important woody perennial crops and ornamental plants, as well as in both native and introduced forest tree species (8,43). In California, species of Botryosphaeriaceae not only occur in a wide range of different hosts (4) but are also considered one of the major threats to the almond (12), grapevine (43), and pistachio (17) industries, reducing yields and shortening the life span of these crops. The present study adds olive trees as a major host of Botryosphaeriaceae species in

Table 4. Mean lesion length caused by different fungal species isolated from diseased olive trees from California on 2- and/or 3-year-old branches of Manzanillo and Sevillano olive cultivars 6 months after inoculation

		Manzanillo			Sevillano		
Species	Isolate	Lesion length[x] ± SE	Signif. dif.[y]	Samples[z]	Lesion length ± SE	Signif. dif.	Samples
Neofusicoccum mediterraneum	UCD1-Oe	63.1 ± 6.6 a	A	10	74.8 ± 5.2 a	B	10
Diplodia mutila	UCD127-Oe	43.6 ± 6.4 b	A	10	76.0 ± 6.6 a	B	9
Neofusicoccum vitifusiforme	UCD622-Oe	12.4 ± 1.5 c	A	10	10.5 ± 0.8 b	A	10
Ttrametes versicolor	UCD461-Oe	10.1 ± 0.7 d	A	9	10.3 ± 1.2 bc	A	10
Neofusicoccum luteum	UCD369-Oe	9.0 ± 0.9 de	A	10	7.5 ± 1.1 def	A	10
Eutypa lata	UCD143-Oe	8.4 ± 0.3 ef	A	10	10.1 ± 0.9 bcd	B	9
Botryosphaeria dothidea	UCD62-Oe	8.1 ± 0.8 ef	A	10	9.0 ± 1.0 cde	A	10
Lasiodiploida theobromae	UCD527-Oe	7.9 ± 0.7 efg	A	10	6.1 ± 0.4 efg	B	9
Phomopsis sp. group 2	UCD248-Oe	7.7 ± 0.5 efg	A	10	7.3 ± 0.6 def	A	10
Diaporthe viticola	UCD316-Oe	7.2 ± 0.5 fgh	A	10	9.4 ± 0.7 cde	B	9
Diplodia seriata	UCD340-Oe	7.2 ± 0.4 fgh	A	9	8.1 ± 0.8 def	A	10
Phomopsis sp. group 1	UCD181-Oe	6.7 ± 0.7 gh	A	9	6.5 ± 0.6 fg	A	8
Dothiorella iberica	UCD163-Oe	6.1 ± 0.5 hi	A	7	4.3 ± 0.7 hi	A	9
Phaeomoniella chlamydospora	UCD306-Oe	6.0 ± 0.6 hi	A	4	3.1 ± 0.4 ij	B	5
Schizophyllum commune	UCD296-Oe	5.5 ± 0.6 ij	A	10	6.3 ± 0.8 gh	A	8
Phaeoacremonium aleophilum	UCD468-Oe	5.1 ± 0.6 ij	A	8	4.9 ± 0.6 hi	A	8
Diatrype stigma	UCD23-Oe	3.2 ± 0.2 k	A	5	4.3 ± 0.8 hi	A	4
Diatrype oregonensis	UCD60-Oe	2.9 ± 0.6 k	A	3	3.3 ± 0.3 ij	A	5
Control		1.4 ± 0.1 l	A		1.3 ± 0.1 k	A	

[x] Values represent the average in millimeters of acropetal and basipetal extent of vascular discoloration (10 repetitions per isolate) measured from the point of inoculation. SE = Standard error of the mean. Means with the same letter are not significantly different at the 0.05 level.

[y] Significant differences of each isolate among Manzanillo and Sevillano cultivars. Means with the same capital letters in each row are not significantly different at the 0.05 level.

[z] Number of samples from which the fungus was re-isolated out of 10 samples inoculated.

California and highlights the important role that several of these species play on twig and olive branch dieback disease.

Fungal taxa in the genus *Phomopsis* (teleomorph: *Diaporthe* Nitschke) were the second most prevalent fungi isolated from olive cankers and twig dieback in California. The genus *Phomopsis* contains over 900 species, which are known to be cosmopolitan and found as endophytes, parasites, and saprotrophs in a wide range of hosts (8,40). However, no record of either the asexual (*Phomopsis*) or the sexual form (*Diaporthe*) has been reported to occur in olive trees (8). Two different *Phomopsis* spp. were isolated from olive wood in California, which represents the first report of olive trees as host of species belonging to the *Phomopsis* genus. Although combined ITS and EF1-α phylogenetic analyses showed *Phomopsis* isolates from olive in California to share 99% homology with previously identified *Phomopsis* sp. isolates UCD1685Sl (46) and CAL-5 (29) from *V. vinifera* in California, they differed from the rest of *Phomopsis* and/or *Diaporthe* species available in GenBank; and thus, they were designated as *Phomopsis* sp. group 1 and *Phomopsis* sp. group 2. These results indicate both *Phomopsis* spp. isolated from olive and grapevine in California are potential novel species within this genus; however, further morphological and phylogenetic studies, which were beyond the scope of this study, are currently underway to confirm whether or not these two taxa represent novel *Phomopsis* spp. To date, *D. viticola* has only been reported to occur on *Hydrangea macrophylla* and *Vitis vinifera,* and its distribution is restricted to Germany and Portugal (8,28,40). *Diaporthe viticola* is reported for the first time here to occur in olive trees, as well as in a country other than Germany and Portugal. *Phomopsis* sp. group 1, *Phomopsis* sp. group 2, and *D. viticola* were shown to be pathogenic in olive branches; however, all three species were considered to be intermediately virulent based on the extent of vascular wood death they caused.

The diatrypaceous fungi *E. lata*, *D. oregonensis,* and *D. stigma* were the third most prevalent fungi isolated from olive cankers in California. To date, *E. lata*, *D. oregonensis,* and *D. stigma* have been shown to occur in 136, 114, and 10 different plant hosts, respectively (8). However, only *E. lata* has been previously reported in olive trees causing olive dieback in Greece (27) and in Italy (35). Therefore, this is the first report of *E. lata* and *D. oregonensis* and *D. stigma* in olive trees in America and worldwide, respectively. Pathogenicity of *E. lata* in olive trees was demonstrated by Rumbos (27) in the early 1990s, who reported Greek isolates of this species to be highly virulent, causing up to 52 mm canker length 12 months after inoculation. On the other hand, this study showed *E. lata* isolates from California to be moderately virulent when inoculated in olive branches, causing an average length necrosis significantly less severe. The lower virulence observed with California isolates of *E. lata* in olive trees could be due to variability in isolate virulence, type of inoculated tissue, shorter incubation period, age of the host, and/or differences in cultivar susceptibility among many other possible factors. However, whether any of these factors play a direct role in the virulence of *E. lata* in olive remains unclear at this time; and thus, a more complete pathogenicity study using a higher number of both *E. lata* isolates and olive cultivars will be required to further clarify these hypotheses. Contrary to *E. lata*, *D. stigma* and *D. oregonensis* were shown in this study to be the least virulent fungi in olive trees among all fungal species tested. Trouillas and Gubler (37) recently showed grapevine isolates of *D. stigma* and *D. oregonensis* to cause lesions in grapevines that were not significantly different from those observed in the negative controls, suggesting that these fungi may be saprotrophic on this host. The low virulence of both *D. stigma* and *D. oregonensis* observed in this study supports the hypothesis that these diatrypaceous fungi may act as saprotrophs in olive trees.

Although less prevalent, the basidiomycetous fungi *S. commune* and *T. versicolor*, as well as the grape measles (esca) fungi, *Pa. chlamydospora* and *Pm. aleophilum*, were also sporadically isolated from symptomatic wood of olive trees in California. Among these fungi, *T. versicolor* and *Pm. aleophilum* have been previously reported to occur in olive trees in California (1) and in Italy (11), respectively. However, Koch's postulates were not fulfilled. Although moderate and low virulence were shown in this study for *T. versicolor* and *Pm. aleophilum*, respectively, these pathogenicity tests indicated that both fungi can infect and colonize green live olive tissue. *S. commune* and *Pa. chlamydospora*, on the other hand, are reported for the first time here as weak pathogens of olive trees. Additionally, for the first time, this study shows *Pa. chlamydospora* to occur in a host other than *V. vinifera*.

All fungal species identified in this study from olive trees, except the two *Phomopsis* spp., have been widely recognized as important pathogens of grapevines, involved in what is known as the grapevine trunk disease complex worldwide (21,23,37,45,48,49). Results from the field survey conducted in this study showed all these fungi occur in olive orchards either near or adjacent to vineyards throughout California. *E. lata,* for example, was only isolated from symptomatic olive trees in Napa, Sonoma, and Sacramento counties, which are known to be the grapevine-growing regions with the highest rate of Eutypa dieback of grapevines in California (46). Similarly, the botryosphaeriaceous fungi *L. theobromae*, *N. luteum,* and *N. mediterraneum*, which have previously been reported to occur in grapevines only in counties throughout the San Joaquin Valley and Southern California (46,47), were primarily isolated from olive trees from the same geographical regions. Another example is *D. viticola*, a fungal species found almost exclusively on grapevines and associated with Phomopsis cane and leaf spot symptoms (49). In this study, *D. viticola* was only isolated from olive trees in the grape-growing regions of Napa and Sonoma counties where Phomopsis cane and leaf spot disease is commonly observed on grapevines. However, most of the fungal species isolated from olive cankers and known to be highly virulent on grapevines (*E. lata*, *B. dothidea*, *L. theobromae*, *N. luteum*, *Pa. chlamydospora*, and *Pm. aleophilum*) were found to be of intermediate to low virulence when inoculated in olive branches. These results suggest these fungi are better adapted to infect grapevine wood, and possibly only the most virulent isolates of some of these species would be more aggressive in olive trees. Moral et al. (20) showed isolates of *B. dothidea* from infected olive fruit to be nonpathogenic when inoculated on olive branches and thus specialized in which olive tissues are infected. A more thorough pathogenicity study in olive trees using multiple high virulence isolates of each fungal species from grapevines and/or other hosts will be required to confirm this hypothesis.

Likewise, several of the botryosphaeriaceous fungi isolated from olive trees in this study are also known to cause cankers and consequent dieback in other economically important perennial crops in California. For instance, *B. dothidea* causes panicle and shoot blight of pistachio (17). Moreover, *B. dothidea*, *D. seriata,* and *N. mediterraneum* cause cankers and dieback of almond (12). Additionally, *N. mediterraneum* was reported to be highly virulent in walnut trees, causing twig and branch dieback (39). In this study, *B. dothidea* and *N. mediterraneum* were mainly found to occur in olive orchards in both Sacramento (Butte, Glenn, Tehama, and Yolo counties) and San Joaquin valleys (Fresno, Madera, Merced, and Tulare counties), which correspond with over 52, 43, and 42% of the total almond, pistachio, and walnut acres planted in California, respectively (42). Similar to what occurs with grapevines in counties such as Napa and Sonoma, olive trees are often planted near or adjacent to almond, pistachio, and/or walnut orchards throughout both the Sacramento and San Joaquin valleys. This perceived cross infection of multiple hosts by these fungi has been reported to occur in southern Spain, where pistachio and olive trees grow together, sometimes within the same orchard (20), and further support the hypothesis that infected hosts adjacent to olive orchards may serve as sources of inoculum for these pathogens and vice versa (12). Furthermore, in the case of botryosphaeriaceous species, such a proximity of olive trees to almonds, grapevines, pistachios, and/or walnuts throughout these particular growing regions in California and/or other countries could be essential for cross infections, since dissemination of pycnidiospores is primarily water-splashed over relatively short distances (3,44). Consequently, larger buffer zones between olive orchards and these crops may reduce risk of cross infection by botryosphaeriaceous species and/or other fungi with a similar mode of spread. However, increasing zones may not be an economically viable alternative for mature and already well-established olive orchards, since removal of old trees would be necessary; but larger buffer zones could be considered when planting new olive orchards in California.

This study has shown significant information regarding the etiology and importance of olive twig and branch dieback as well as the distribution of the causal agents throughout California. However, it has only focused on the status of this disease in traditional mature and low-density olive orchards throughout the state (200 to 500 trees per ha). Traditional olive orchards for the production of either table olives or olive oil are on the other hand no longer popular when establishing new olive plantings worldwide due to the high cost, primarily as a consequence of labor expenses for harvesting and pruning (36). Consequently, olive growers in California are currently switching to super-high-density olive production farming, characterized by high tree density (1,500 to 2,500 trees per ha) and based on hedgerows, machine harvest, and mechanical pruning (36). Severe mechanical pruning will probably be necessary to preserve low tree size in order to maximize machine harvesting efficiency (36). Mechanical pruning in such a high-density crop will create hundreds of thousands of new pruning wounds every year, which are known to be the main point of entrance for all the fungi identified in this study and responsible for causing olive twig and branch dieback (21,37,43,48). Moreover, mechanical harvest may also result in many injuries to the trees which could be susceptible to infection. Therefore, olive twig and branch dieback incidence is suggested to be higher in super-high-density olive orchards than in traditional plantings, in which pruning is sometimes kept to a minimum. Consequently, research needs to be continued, not only to determine the importance of olive twig and branch dieback in this new olive production system, but also to develop and implement effective management strategies under this new approach to farming olives.

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