Phomopsis Dieback: A Grapevine Trunk Disease Caused by *Phomopsis viticola* in California

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Abstract

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Field surveys recently conducted in California and in other grape-growing regions in the United States showed *Phomopsis viticola* to be one of the most prevalent fungi isolated from grapevine perennial cankers in declining vines. The current study has not only confirmed the presence of *P. viticola* from grapevine cankers in California but also has for the first time revealed the occurrence of *Diaporthe ambigua*, *D. eres*, and *D. neotheicola* in symptomatic grapevine wood in California by means of morphological studies and multi-gene sequence analysis. Pathogenicity trials conducted on mature cordons of *Vitis vinifera* ‘Syrah’ and ‘Red Globe’, as well as on lignified Syrah dormant canes, showed *P. viticola* isolates from California to be capable of causing perennial cankers. Lengths of vascular discoloration caused by *P. viticola* were similar to those caused by *Eutypa lata* and several Botry-

osphaeriaceae spp., which are well-known grapevine trunk disease pathogens. Additionally, a lack of spring growth was commonly observed in dormant canes inoculated with *P. viticola* spore suspensions in two pathogenicity trials. As part of this study, *V. vinifera* ‘Cabernet Sauvignon’ and ‘Zinfandel’ wood was shown to be more susceptible to infection by *P. viticola* than ‘Barbera’, ‘Chardonnay’, ‘Merlot’, and ‘Thompson Seedless’ wood. After more than 40 years overlooking *P. viticola* as a grapevine wood pathogen, this study provides strong evidence of the role of *P. viticola* as a canker-causing organism, and suggests its addition to the fungi involved in the grapevine trunk disease complex. Results from this study suggest *D. ambigua* and *D. neotheicola* to be saprophytes or weak pathogens on grapevine wood.

Phomopsis (Sacc.) Bubák (teleomorph: *Diaporthe* Nitschke) is a cosmopolitan fungal genus that contains both plant pathogens and endophytes described from a wide range of annual and perennial hosts, including economically important crops (18,55). Since *Phomopsis* was established as fungal genus by Saccardo in 1905 (45), over 900 species have been described (44). This large proliferation of species names has primarily been a consequence of the fact that most of the species were characterized by host affiliation (55). Presently, however, it is well-accepted that the same *Phomopsis* species can be found on different hosts and that more than one species can occur on the same host. Consequently, host association is no longer considered relevant in the taxonomy of this genus and its *Diaporthe* teleomorphs (30,43). Moreover, plasticity of morphological characters in these genera makes identification to species difficult (43). Accordingly, *Phomopsis* and *Diaporthe* species are currently identified primarily by molecular phylogenies (12,46,47, 53,61).

Phomopsis viticola (Sacc.) Sacc. is historically known to infect grapevines (*Vitis vinifera* and *Vitis* spp.) causing Phomopsis cane

and leaf spot disease (22). Although *P. viticola* occurs wherever grapes are grown (18), Phomopsis cane and leaf spot is more severe in grape-growing regions characterized by a humid temperate climate through the growing season. Crop losses up to 30% have been reported to be caused by Phomopsis cane and leaf spot (16,37). *P. viticola* can infect all green parts of the grapevine, and thus disease symptoms can be observed on leaves as small pale-green to yellow spots with necrotic centers. Canes show brown to black necrotic irregular-shaped lesions, and clusters show rachis necrosis and brown, shriveled berries close to harvest (22). Phomopsis cane and leaf spot is a well-studied grapevine disease, and much has been written about its etiology (30,31,36,48), epidemiology (16,17,22,33,37), and management (11,15,19,32,37).

However, *P. viticola* has not always been associated exclusively with Phomopsis cane and leaf spot symptoms. In 1909, *P. viticola* (syn. *Fusicoccum viticolum* Reddick) was originally associated with a syndrome named “necrosis of the grapevine” in the state of New York (41), which was renamed dead-arm disease of grapes a few years later (42,49). Although some of the dead-arm disease symptoms first described by Reddick in 1909 (41) were in agreement with what is currently known as Phomopsis cane and leaf spot, the author also associated *P. viticola* with other symptoms, including lack of spring growth, shoot dieback, short shoot internodes, small leaves crimped about the margin, and fruit shrivel in mid- to late-summer, all of which could be observed on one or both cordons. A few years later, Reddick observed that most of the vines affected by the dead-arm disease also presented sectorial necrosis in the vascular system when cross-sections were made in cordons and/or trunks (42). From the early 1920s to the mid-1960s, dead-arm disease was reported to occur in almost all grape-growing regions in the eastern United States and Canada (2,3,5,9,10,65), California (21), Japan (23), and South Africa (13). Proof of pathogenicity of *P. viticola* on grapevine wood was also reported (9,42). However, studies on grapevines worldwide during the 1970s led to the discovery of the diatrypaceous fungus *Eutypa lata* (Pers.) Tul. & C. Tul. (syn. *Eutypa armeniacae* Hansf. & M.V. Carter). This fungus was thus subsequently associated with some of the dead-

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arm disease symptoms such as stunted shoots with small, chlorotic, and distorted leaves, and perennial cankers in the wood (14,25,27,28). Such research caused the scientific community to reconsider the role that *P. viticola* played in this syndrome. By the end of the 1970s, the association of *E. lata* with this symptomatology was evident and proof of pathogenicity was established (28). These studies led scientists to conclude that *E. lata* and not *P. viticola* incited dead-arm disease symptoms of grapevines (29). Since then, *P. viticola* has been strictly considered the causal agent of Phomopsis cane and leaf spot.

Nevertheless, field surveys conducted between 2003 and 2006 in California revealed *P. viticola* to be the third most prevalent fungus isolated from grapevine cankers (7% recovery) after species in the Botryosphaeriaceae and Diatrypaceae families. Moreover, *P. viticola* was the main fungal pathogen isolated from grapevine cankers, with 26% recovery in the southern San Joaquin Valley (59). More recently, studies conducted in grape-growing regions of Arkansas, Missouri, and Texas showed *P. viticola* to be among the most prevalent fungi, isolated from 6 to 15% of grapevine cankers (57,60). Furthermore, *P. viticola* was recently reported from grapevine perennial cankers in grape-growing regions in the northeastern United States (4). The frequent recovery of *P. viticola* from perennial cankers has again raised the question whether this fungus plays a role in grapevine dieback originally reported by Reddick in 1909 (41). This study was undertaken to identify and characterize the morphological features, multi-locus phylogeny, and pathogenicity of the different *Phomopsis* spp. isolated from grapevine cankers in California in order to determine whether or not *P. viticola*

cola should also be considered as a fungal grapevine trunk disease pathogen.

Materials and Methods

Phomopsis isolates. The majority of the isolates used in this study were collected from perennial cankers observed in spur positions, cordons, and/or trunks from grapevines showing characteristic dieback symptoms during field surveys conducted between 2003 and 2006 throughout all grape-growing regions of California (59) (Fig. 1a to c). Additionally, seven isolates obtained between 2006 and 2010 from grapevine cankers and/or vines showing Phomopsis cane and leaf spot symptoms submitted to our laboratory by University of California Cooperative Extension Viticulture Farm Advisors and pest control advisors were also included in the study (Fig. 1d and e). *Phomopsis* isolates from symptomatic grapevine tissue were isolated as described by Úrbez-Torres et al. (59). They were tentatively identified to genus based on both colony and conidium morphological characters, and stored in the Plant Pathology Department fungal collection at the University of California, Davis. Pure cultures from the collection were grown on 85-mm-diameter petri dishes containing 4% potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) amended with tetracycline hydrochloride (0.01%) (Sigma-Aldrich, St. Louis, MO) at room temperature ($24^{\circ}\text{C} \pm 2^{\circ}\text{C}$) until fungal colonies were observed. Colonies were then transferred onto fresh PDA and incubated unsealed under ambient laboratory conditions of light and temperature, followed by identification to species based on morphology and molecular analyses.

DNA extraction, amplification, and phylogenetic analyses. Representative isolates of the different *Phomopsis* spp. previously identified based on morphological characters were selected for molecular identification (Table 1). Total genomic DNA was extracted from 2-week-old PDA cultures using the AquaPure Genomic DNA Kit (Bio-Rad Laboratories, Hercules, CA). Oligonucleotide primers ITS1 and ITS4 and EF1-728F and EF1-986R were used to amplify the ITS1-5.8S-ITS2 gene (ITS) and part of the translation elongation-factor 1- α gene (EF1- α), respectively (6,63). Each polymerase chain reaction (PCR) was comprised of 5 μl of 10 $\times$ PCR buffer containing 15 mM MgCl_2 , 2 μl of 25 mM MgCl_2 , 1 μl of 10 mM dNTPs, 2 μl of 0.5 mM of each primer, 0.25 μl of *Taq* DNA polymerase (*Taq* PCR core kit; Qiagen, Valencia, CA) at 5 units/ μl , and 2 μl of DNA template adjusted with purified water (Water Systems; Millipore, Billerica, MA) to a final volume of 50 μl . PCR reactions were carried out in a thermal cycler (PTC 200; M. J. Research Company, Watertown, MA) following previously described ITS (63) and EF1- α (6) temperature profiles. Amplification products were purified using the QIAquick PCR purification kit (Qiagen). Both strands of the ITS and EF1- α were sequenced using an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, CN) at the Division of Biological Sciences sequencing facility at the University of California, Davis.

Fungal sequences were edited and assembled using Sequencher version 4.1 (Gene Codes, Ann Arbor, MI) and aligned using the software BioEdit Sequence Alignment Editor Version 7.1.3.0 (20). Alignments were checked and adjusted manually where necessary. Sequences of *Phomopsis* and *Diaporthe* spp. from grapevine and other hosts, including ex-type specimens identified in previous studies and available in GenBank, were included in the phylogenetic analyses (Table 2). Separate phylogenetic analyses were performed on individual ITS and EF1- α datasets (individual trees are not illustrated), and tree topologies were compared. Phylogenetic analysis of the ITS and EF1- α combined datasets was inferred in MEGA5 (51) by Neighbor-Joining (NJ) using the Maximum Composite Likelihood method and by Maximum Parsimony (MP) using the heuristic search option with 1,000 random addition sequence replicates and the Close-Neighbor-Interchange (CNI) algorithm. Alignment gaps were treated as missing data and all characters were unordered and equally weighted. Tree length, consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated in the MP method. Bootstrap support was

Fig. 1. Grapevine symptoms associated with *Phomopsis viticola* in California. **a**, A lack of spring growth at the spur position. **b**, Wedge-shape perennial cankers in the cordon. **c**, Black, flask-shape pycnidia of *P. viticola* on the cordon of a declining vine. **d** and **e**, Phomopsis cane and leaf spot symptoms. **d**, White arrows point to small pale-green to yellow spots with necrotic center on leaves. **e**, Black arrows point dark-brown to black necrotic irregular-shaped lesions on shoots. **f**, Vascular discoloration caused by *P. viticola* isolate UCD115Fr on a 'Syrah' dormant cane. **g**, Wedge-shape lesion observed in the cross section.

estimated using 1,000 replicates to assess the robustness of each clade in both phylogenetic methods. *Phomopsis* and *Diaporthe* fungal sequences from grapevine 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).

Inoculum preparation. Three different pathogenicity experiments were conducted using either mycelium plugs or conidial suspensions from each of the isolates selected (Table 1). Conidial suspensions of approximately 1×10^5 conidia/ml were obtained as described by Úrbez-Torres and Gubler (58). Mycelium inoculations were conducted using 5-mm-diameter mycelium plugs harvested from the active colony edge of 2-week-old cultures. Two out of the three experiments (mature wood cordon and 'Syrah' dormant cane inoculations) were part of a broader study conducted to determine the pathogenicity of Botryosphaeriaceae spp. on grapevines (58).

Mature wood cordon inoculations. *P. viticola* isolate UCD1935SB was used in this experiment (Table 1). Pathogenicity tests were independently conducted in a 14-year-old Syrah vineyard located at the University of California, Kearney Agricultural Research and Extension Center in Parlier, CA and in a 7-year-old 'Red Globe' vineyard at the University of California Field Station

in Davis, CA. Vines in both vineyards were cordon trained and spur pruned. Fifteen-day-old mycelium plugs were used to inoculate mature wood cordon tissue. Inoculations were conducted at the end of the cordon between the last two spur positions by drilling a hole 10 to 15 mm deep using a 5-mm-diameter electric drill. Wounds were immediately inoculated by placing a mycelial plug in the hole, sealed with petroleum jelly, and wrapped with Parafilm (Pechiney, Chicago, IL). Noncolonized agar plugs were used as a negative control. Ten cordons (one cordon per vine) were inoculated in each Syrah and Red Globe vineyard in January and February of 2006, respectively, in a completely randomized design. Cordons were collected 24 months after inoculation. Cordons were disinfected, sectioned longitudinally through the point of inoculation, and length of vascular discoloration recorded both upward and downward from the point of inoculation. Koch's postulates were fulfilled by reisolation of the fungus by placing 10 small pieces of symptomatic tissue from the edge of each lesion on PDA-tet. Identification of the reisolated fungus was assessed by morphological comparison to the original isolate UCD1935SB. Both pathogenicity trials also included isolates of the grapevine trunk disease pathogens *E. lata* and Botryosphaeriaceae spp. (58). In order to determine the significance of *P. viticola* as a grapevine trunk disease pathogen, its mean length of vascular discoloration

Table 1. *Diaporthe* and *Phomopsis* species isolated from *Vitis vinifera* wood cankers and *Phomopsis* cane and leaf spot in California vineyards between 2005 and 2010

Species	Isolate number ^t	Origin/ Date collected ^u	Symptom ^v	Cultivar ^w	Collector	GenBank acc. numbers	
						ITS ^x	EF1- α ^y
<i>D. ambigua</i>	UCD1151Na	Napa Co./ 09/2009	Canker in cordon	Cabernet Sauvignon	J.R. Úrbez-Torres	KF017908	KF017890
<i>D. ambigua</i>	UCD2076Te	Riverside Co./ 05/2005	Canker in cordon	Chardonnay	J.R. Úrbez-Torres	KF017909	KF017891
<i>D. ambigua</i>	UCD2078Te	Riverside Co./ 05/2005	Canker in cordon	Chardonnay	J.R. Úrbez-Torres	KF017910	KF017892
<i>D. ambigua</i>	UCD2079Te	Riverside Co./ 05/2005	Canker in cordon	Sangiovese	J.R. Úrbez-Torres	KF017911	KF017893
<i>D. ambigua</i>	UCD2114Te	Riverside Co./ 05/2005	Canker in cordon	Sangiovese	J.R. Úrbez-Torres	KF017912	KF017894
<i>D. eres</i>	UCCE993A	Sonoma Co./ 03/2010	Canker in cordon	Pinot Noir	R. J. Smith	KF017913	KF017895
<i>D. eres</i>	UCCE1004B	Sonoma Co./ 04/2010	Soft-rotted wood	Chardonnay	R. J. Smith	KF017914	KF017896
<i>D. neotheicola</i>	UCCE993B	Sonoma Co./ 03/2010	Canker in cordon	Pinot Noir	R. J. Smith	KF017915	KF017897
<i>D. neotheicola</i>	UCD2327Mar	Marin Co./ 06/2007	Canker in cordon	Pinot Noir	J.R. Úrbez-Torres	KF017916	KF017898
<i>D. neotheicola</i>	UCD2328Mar	Marin Co./ 06/2007	Canker in cordon	Pinot Noir	J.R. Úrbez-Torres	KF017917	KF017899
<i>D. neotheicola</i>	UCD2329Mar	Marin Co./ 06/2007	Canker in cordon	Pinot Noir	J.R. Úrbez-Torres	KF017918	KF017900
<i>P. viticola</i>	UCD115Fr	Fresno Co./ 11/2003	Canker in cordon	Grenache	J.R. Úrbez-Torres	KF017919	KF017901
<i>P. viticola</i>	UCD1935SB	Santa Barbara Co./ 05/2005	Canker in cordon	Chenin Blanc	J.R. Úrbez-Torres	KF017920	GU294704
<i>P. viticola</i>	UCD1938SB	Santa Barbara Co./ 05/2005	Canker in cordon	Chenin Blanc	J.R. Úrbez-Torres	KF017921	GU294705
<i>P. viticola</i>	UCD2009SB	Santa Barbara Co./ 05/2005	Canker in cordon	Chardonnay	J.R. Úrbez-Torres	KF017922	GU294706
<i>P. viticola</i>	UCCE964A	Sonoma Co./ 09/2009	Bleached cane ^z	Zinfandel	R. J. Smith	KF017923	KF017902
<i>P. viticola</i>	UCCE964B	Sonoma Co./ 09/2009	Bleached cane ^z	Zinfandel	R. J. Smith	KF017924	KF017903
<i>P. viticola</i>	UCCE964C	Sonoma Co./ 09/2009	Bleached cane ^z	Zinfandel	R. J. Smith	KF017925	KF017904
<i>P. viticola</i>	UCCE964D	Sonoma Co./ 09/2009	Bleached cane ^z	Zinfandel	R. J. Smith	KF017926	KF017905

^t UCD: University of California Davis, UCCE: University of California Cooperative Extension.

^u Month/year.

^v Grapevine symptom from which the fungus was isolated.

^w Grapevine (*Vitis vinifera*) cultivar from which the fungus was isolated.

^x ITS: internal transcribed spacer.

^y EF1- α : Translation elongation factor 1- α .

^z Grapevine showing Phomopsis cane and leaf spot disease symptoms.

caused in each cultivar was separately compared with those caused by the well-known grapevine canker pathogens *E. lata* and Botryosphaeriaceae species from the same trials and previously published (58).

Syrah dormant cane inoculations. *P. viticola* isolates UCD1935SB, UCD1938SB, and UCD115Fr were used in this experiment (Table 1). The field trial was conducted in the same 14-year-old Syrah vineyard located at Kearney Agricultural Research and Extension Center. Grapevines were pre-pruned to 8- to 10-bud short canes in January 2006, and fresh pruning wounds were immediately inoculated by applying 50 µl of a 1×10^5 conidia/ml suspension from each isolate as described by Úrbez-Torres and Gubler (58). Control treatments were inoculated by applying 50 µl of sterile distilled water. Each treatment was applied onto one 1-year-old dormant cane per grapevine on each of 10 grapevines in a completely randomized design. Treatments also included three isolates of *E. lata* (DHB100, DCA200, and UCD783St) as positive controls (52). *E. lata* dormant cane inoculations were conducted by applying an ascospore suspension from each isolate as described by Trouillas and Gubler (52). Canes were collected 13 months after

inoculation, surface disinfected, and sectioned longitudinally. The extent of vascular discoloration was measured from the point of inoculation as previously described. Koch's postulates were fulfilled by reisolation of the fungus from infected tissue as described above. This pathogenicity trial also included isolates of the grapevine trunk disease pathogens Botryosphaeriaceae spp. (58). As described in the cordon pathogenicity test, the mean length of vascular discoloration caused by the *P. viticola* isolates was compared with those caused by the well-known grapevine canker pathogens *E. lata* and Botryosphaeriaceae spp. from the same trial. Mean lengths of vascular discoloration caused by species of Botryosphaeriaceae from this trial were published in 2009 (58).

Grapevine cultivar susceptibility to infection by *P. viticola* and *Diaporthe* spp. *P. viticola* isolates UCD115Fr, UCCE964A, and UCD1935SB; *D. ambigua* isolates UCD1151Na and UCD2079Te; and *D. neotheicola* isolate UCD2327Mar were used in this study (Table 1). Six different grapevine cultivars were tested, including Barbera, Cabernet Sauvignon, Chardonnay, Merlot, and Zinfandel (cordon trained and spur pruned) located at the Kearney Agricultural Research and Extension Center and Thomp-

Table 2. *Diaporthe* and *Phomopsis* species used in the phylogenetic analyses

Species	Isolate ^w	Host	Origin	Collector	GenBank acc. numbers ^v	
					ITS ^x	EF1- α ^y
<i>D. alleghaniensis</i>	CBS 495.72	<i>Betula alleghaniensis</i>	Ontario, Canada	R.H. Arnold	FJ889444	GQ250298
<i>D. ambigua</i>	CBS 114015	<i>Pyrus communis</i>	South Africa	S. Denman	AF230767	GQ250299
<i>D. ambigua</i>	CBS 117374	<i>Aspalathus linearis</i>	South Africa	J.C.J. van Rensburg	DQ286237	DQ286268
<i>D. ambigua</i>	CBS 123210	<i>Foeniculum vulgare</i>	Portugal	J.M. Santos	EU814479	GQ250300
<i>D. ambigua</i>	CBS 123211	<i>F. vulgare</i>	Portugal	J.M. Santos	EU814478	GQ250301
<i>D. ambigua</i>	CPC 5423	<i>A. linearis</i>	South Africa	J.C.J. van Rensburg	DQ286247	DQ286273
<i>D. angelicae</i>	CBS 111592	<i>Heracleum sphondylium</i>	Austria	W. Jaklitsch	AY196779	GQ250302
<i>D. aspalathi</i>	CBS 117169	<i>A. linearis</i>	South Africa	J.C.J. van Rensburg	DQ286275	DQ286249
<i>D. crotalariae</i>	CBS 162.33	<i>Crotalaria spectabilis</i>	North America	G.F. Weber	FJ889445	GQ250307
<i>D. eres</i>	Pho12	<i>V. vinifera</i>	New York, USA	P. Rolshausen	JN214572	JN192166
<i>D. eres</i>	Pho27	<i>V. vinifera</i>	Connecticut, USA	P. Rolshausen	JN214586	JN192180
<i>D. eres</i>	Pho30	<i>V. vinifera</i>	Quebec, Canada	P. Rolshausen	JN214589	JN192183
<i>D. eres</i>	Pho31	<i>V. vinifera</i>	Quebec, Canada	P. Rolshausen	JN214590	JN192184
<i>D. helianthi</i>	CBS 592.81	<i>Helianthus annuus</i>	Serbia	M. Muntanola-Cvetkovic	AY705842	GQ250308
<i>D. hickoriae</i>	CBS 145.26	<i>Carya glabra</i>	Michigan, USA	L.E. Wehmeyer	FJ889446	GQ250309
<i>D. lusitanicae</i>	CBS 123212	<i>F. vulgare</i>	Portugal	J.M. Santos	EU814477	GQ250310
<i>D. melonis</i>	CBS 507.78	<i>Cucumis melo</i>	Texas, USA	L. Beraha & M.J. O'Brien	FJ889447	GQ250314
<i>D. neotheicola</i>	CBS 123208	<i>F. vulgare</i>	Portugal	A.J.L. Phillips	EU814480	GQ250315
<i>D. neotheicola</i>	CBS 123209	<i>F. vulgare</i>	Portugal	A.J.L. Phillips	GQ250192	GQ250316
<i>D. neotheicola</i>	Ph-C182/1	<i>Acer negundo</i>	Portugal	A.J.L. Phillips	GQ250195	GQ250319
<i>D. perijuncta</i>	CBS 109745	<i>Ulmus glabra</i>	Austria	W. Jaklitsch	AY485785	GQ250323
<i>D. phaseolorum</i>	CBS 127266	<i>H. annuus</i>	Croatia	K. Vrandečić	HM347707	HM347672
<i>D. phaseolorum</i>	PS03	<i>Glycine max</i>	Croatia	K. Vrandečić	HM347702	HM347670
<i>D. stewartii</i>	CBS 193.36	<i>Cosmos bipinnatus</i>	n/a ^z	A.L. Harrison	FJ889448	GQ250324
<i>D. vaccinii</i>	CBS 160.32	<i>Oxycooccus macrocarpus</i>	Oregon, USA	H.F. Bain	AY952141	GQ250326
<i>D. viticola</i>	CBS 113201	<i>V. vinifera</i>	Portugal	A.J.L. Phillips	AY485750	GQ250327
<i>P. amygdali</i>	11A	<i>Prunus dulcis</i>	Portugal	E. Diogo	GQ281792	GQ250339
<i>P. cuppatea</i>	CBS 117499	<i>A. linearis</i>	South Africa	J.C.J. van Rensburg	AY339322	AY339354
<i>P. dauci</i>	CBS 315.49	<i>Daucus carota</i>	Netherlands	J.A. von Arx	FJ889451	GQ250348
<i>P. phoenicicola</i>	CBS 161.64	<i>Areca catechu</i>	India	H.C. Srivastava	FJ889452	GQ250349
<i>P. sclerotoides</i>	CBS 296.67	<i>Cucumis sativus</i>	Netherlands	Van der Kesteren	AF439626	GQ250350
<i>P. viticola</i>	CBS 114016	<i>V. vinifera</i>	France	P. Larignon	AF230751	GQ250351
<i>P. viticola</i>	114	<i>V. vinifera</i>	Portugal	E. Diogo	GQ250214	GQ250352
<i>P. viticola</i>	115	<i>V. vinifera</i>	Portugal	E. Diogo	GQ250215	GQ250353
<i>P. viticola</i>	Pho16	<i>V. vinifera</i>	Massachusetts, USA	P. Rolshausen	JN214575	JN192169
<i>P. viticola</i>	Pho25	<i>V. vinifera</i>	Virginia, USA	P. Rolshausen	JN214584	JN192178
<i>P. viticola</i>	PhoCT1L	<i>V. vinifera</i>	Connecticut, USA	P. Rolshausen	JN214592	JN192186
<i>P. viticola</i>	UCD2236MO	<i>V. vinifera</i>	Missouri, USA	K. Striegler & G.M. Leavitt	HQ288245	HQ288280
<i>P. viticola</i>	UCD2599MO	<i>V. vinifera</i>	Missouri, USA	K. Striegler & W.D. Gubler	HQ288248	HQ288283
<i>P. viticola</i>	UCD2600MO	<i>V. vinifera</i>	Missouri, USA	K. Striegler & W.D. Gubler	HQ288249	HQ288284
<i>P. viticola</i>	UCD2317AR	<i>V. vinifera</i>	Arkansas, USA	K. Striegler & G.M. Leavitt	HQ288247	HQ288282
<i>P. viticola</i>	UCD2403TX	<i>V. vinifera</i>	Texas, USA	P. Adams	FJ790864	GU294711
<i>P. viticola</i>	UCD2408TX	<i>V. vinifera</i>	Texas, USA	P. Adams	FJ790865	KF017906
<i>P. viticola</i>	UCD2471TX	<i>V. vinifera</i>	Texas, USA	P. Adams	FJ790866	KF017907

^v GenBank accession numbers in italics were generated in this study.

^w Isolate numbers in bold represent ex-type specimens. CBS: Centraalbureau voor Schimmelfcultures, Utrecht, Netherlands.

^x ITS: internal transcribed spacer.

^y EF1- α : Translation elongation factor 1- α .

^z n/a: not available.

son Seedless (head trained and cane pruned) located at the University of California Field Station in Davis. All cultivars were pruned to 8- to 10-bud short canes in December 2009, and fresh pruning wounds were immediately inoculated by applying 50 μ l of a 1×10^5 conidia/ml suspension from each isolate as previously described. Control treatments were inoculated by applying 50 μ l of sterile distilled water. Each treatment was applied onto one 1-year-old dormant cane per grapevine on each of 10 grapevines in a completely randomized design. Canes were collected 6 months after inoculation and brought to the laboratory. Length of vascular discoloration was measured and fulfillment of Koch's postulates was conducted as previously described.

Data analyses. Data from the different assays were independently analyzed using analysis of variance (ANOVA) in SAS (Version 9.2; SAS Institute, Cary, NC). For each experiment, homogeneity of variance was tested using Levene's test, residuals were visually inspected, and when necessary the $\log_{10}$ transformation was used to reduce heteroscedasticity. Within each of the three assays, the significance of the difference in length of discoloration caused by each treatment was determined by one-way ANOVA. Tukey's test at the 5% significance level was used to analyze differences in the extent of vascular discoloration between the treatment means. A two-way ANOVA was performed to determine significant differences between Syrah and Red Globe grape cultivars in the cordon pathogenicity test. Length of vascular discoloration caused by both *E. lata* and Botryosphaeriaceae spp. in the mature wood cordon trial and Botryosphaeriaceae spp. in the Syrah dormant cane inoculation trial, previously published (58), were also included in the statistical analyses and compared against *P. viticola*.

Results

Morphological characterization. Based on colony and morphological characteristics of the conidia, the isolates selected in this study were classified into four different groups. The first group, which included the highest number of isolates, was characterized by white to light brown mycelium, becoming dark brown with age (Fig. 2a). Mycelium was slightly raised and slow growing, taking up to 4 weeks to reach 6-cm colony diameter on PDA. The colony of this type of isolate was characterized by forming prominent growth rings with margins becoming black with age (Fig. 2b and c). Colonies produced dark brown, eustromatic pycnidia from which mucilaginous light-cream cirrhi containing both *alpha* (hyaline, ellipsoidal, and unicellular) and *beta* (hyaline, filiform, and slightly curved) conidia were observed (Fig. 2b). These morphological characteristics were similar to previous descriptions of *P. viticola* (9,22,30,39,57,60). A second group of isolates was characterized by white, dense, slightly aerial, fast-growing mycelium (Fig. 2d). With age, sparse, solid-olivaceous dark patches of mycelium could be observed (Fig. 2e). Colonies became dark cream-colored in the central part and dark olivaceous toward the edge (Fig. 2f). Pycnidia were formed on PDA from which cream conidial masses containing hyaline, ellipsoidal, and biguttulate *alpha*-conidia were observed (Fig. 2g). Filiform *beta*-conidia were not observed. The morphological characters of both colony and conidia were in agreement with those described by van Rensburg et al. (62) for *Diaporthe ambigua* Nitschke, and thus they were first tentatively classified as *D. ambigua*. The third group of isolates was characterized by having completely white, slow-growing mycelium on both the surface and the reverse side (Fig. 2h). With age, the center of the colony became dark green, and black pycnidia covered in mycelium developed. Both *alpha*-conidia (hyaline, fusoid, unicellular, and biguttulate) and *beta*-conidia (hyaline, filiform, and aseptate) could be observed in the light cream-colored conidial masses exuded from the pycnidia. The last group of isolates was characterized by having a flat, white, and slow-growing mycelium, which developed forming prominent growth rings. With age, black flask-shaped pycnidia developed from which mucilaginous light cream-colored conidial masses exuded containing both *alpha* (hyaline, fusoid, biguttulate, and

unicellular) and *beta* (hyaline, filiform, aseptate, and curved) conidia (Fig. 2i). Based on these morphological characters, it was not possible to identify isolates from groups three and four to species level, and they were first classified as *Phomopsis* sp. Identification to species level was achieved by ITS and EF1- α sequence analysis.

ITS and EF1- α phylogeny. PCR amplifications of the ITS and EF1- α selected regions gave products of approximately 0.5 and 0.3 kb, respectively. To evaluate the phylogenetic relationships among the different *Phomopsis*/*Diaporthe* isolates obtained from grapevines in California, homologous sequence searches of the obtained ITS and EF1- α sequence data were performed using the BLAST program against the GenBank database. This allowed us to (i) confirm the preliminary morphological identification of *P. viticola* and *D. ambigua*; (ii) identify unidentified isolates from morphological groups 3 and 4 to species level; and (iii) select homologous sequences for the multi-locus phylogenetic analyses (Table 2). A total of 65 taxa were included in the phylogenetic analysis of the combined ITS and EF1- α dataset using Maximum Parsimony (MP) and Neighbor-Joining (NJ). The alignment contained a total of 886 characters including gaps. The MP evolutionary history generated 66 equally parsimonious trees, of which one is shown (Fig. 3). The NJ analysis resulted in a tree with similar topology as the MP tree. Based on the phylogenetic analysis of the combined ITS and EF1- α dataset, *Phomopsis*/*Diaporthe* isolates originating from grapevines in California formed four well-supported clades confirming the morphological classification. The first clade, with bootstrap values of 100% (MP and NJ) and containing isolates from morphological group 1, consists of *P. viticola*. *P. viticola* isolates from California, isolated from both perennial cankers and *Phomopsis* cane and leaf spot symptoms, had nearly identical sequences and grouped together with *P. viticola* isolates from grapevines from Arkansas, Connecticut, Massachusetts, Missouri, Texas, and Virginia in the United States, and Portugal and France, including the ex-type specimen CBS114016 (Fig. 3). The second clade, with bootstrap values of 100% (MP and NJ) and containing isolates

Fig. 2. Colony morphology of *Phomopsis*/*Diaporthe* species identified from grapevine cankers in California. **a to c,** *Phomopsis viticola* isolate UCCE989A. **a,** *P. viticola* was the sole fungus isolated from most of the grapevine perennial cankers collected in the San Joaquin Valley. **b,** Four-week-old light brown to gray *P. viticola* colony. Dark brown, eustromatic pycnidia with mucilaginous light cream-colored cirrhi-containing spores. **c,** Characteristic growth rings with margins becoming black with age could be observed on the back of the plate. **d to g,** *Diaporthe ambigua* isolate UCD2076Te. **d,** Young colony characterized by white fluffy mycelium. **e,** Sparse, solid-olivaceous, dark patches of mycelium developed with age. **f,** Colonies became dark cream in the central part and dark olivaceous towards the margins. **g,** *Diaporthe ambigua* hyaline, ellipsoidal and biguttulate *alpha*-conidia. Scale bar = 10 μ m. **h,** *Diaporthe neotheicola* isolate UCCE993B. **i,** *Diaporthe eres* isolate UCCE993A showing pycnidia with a mucilaginous light-cream cirrhi containing spores.

from morphological group 3, consists of *Diaporthe neotheicola* A.J.L. Phillips & J.M. Santos (Fig. 3). *D. neotheicola* isolates from California, isolated from perennial cankers, grouped together with *D. neotheicola* isolates from fennel (*Foeniculum vulgare* L.) from

Fig. 3. One of 66 equally parsimonious trees resulting from the alignment of 886 characters of the combined ITS rDNA and EF1- α regions. Length = 697; consistency index (CI) = 0.497, retention index (RI) = 0.887, retention composite index (RC) = 0.470. Isolates in bold represent *Phomopsis*/*Diaporthe* isolated from grapevines in California in this study. Isolates in italics and underlined signify holotype cultures linked morphologically to the type material. Bootstrap values with 1,000 replications are shown above branches for maximum Parsimony (MP) (only bootstrap value higher than 50% is represented) and below the branches for neighbor-joining (NJ). NJ optimal branch length sum = 0.992. Branches represented with an asterisk were not present in the NJ tree. Bar represents 10 changes.

Portugal, including the ex-type specimen (Fig. 3). The third clade, with bootstrap values of 99% (MP) and 100% (NJ) and containing isolates from morphological group 4, consists of *Diaporthe eres* Nitschke (Fig. 3). *D. eres* isolates from California, which could not be identified to species based on morphological characters, grouped together with *D. eres* isolates from grapevines in Connecticut and New York in the United States and Québec in Canada (Fig. 3). The fourth clade, with bootstrap values of 100% (MP and NJ) containing isolates from morphological group 2, consists of *D. ambigua* (Fig. 3). *D. ambigua* isolates from grapevines in California, isolated only from perennial cankers, grouped together with *D. ambigua* isolates from non-grapevine hosts in Portugal and South Africa, including the ex-type specimen (Fig. 3).

Pathogenicity studies. *Mature wood cordon inoculations.* The mean extent of vascular discoloration caused by *P. viticola* isolate UCD1935SB at 24 months after inoculation was 54.2 and 49.3 mm in Red Globe and Syrah, respectively (Table 3). A two-way ANOVA revealed no significant differences in the extent of vascular discoloration caused by *P. viticola* isolate UCD1935SB between Red Globe and Syrah ($F = 2.88$, $P = 0.0921$). Reisolation rates of isolate UCD1935SB were 80 and 90% from Red Globe and Syrah, respectively (Table 3). The mean length of vascular discoloration caused by *P. viticola* isolate UCD1935SB in mature wood in both cultivars did not differ statistically from the vascular discoloration caused by the well-documented grapevine canker fungal pathogens *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips and *E. lata* (Table 3). Perennial cankers developed from the point of inoculation with *P. viticola* isolate UCD1935SB in both cultivars by 24 months after inoculation.

Syrah dormant cane inoculations. The mean extent of vascular discoloration caused by *P. viticola* isolates UCD1935SB, UCD1938SB, and UCD115Fr at 13 months after inoculation and reisolation rates are shown in Table 4. The length of vascular discoloration caused by *P. viticola* ranged from 35.9 to 28.4 mm and did not differ statistically from that caused by isolates of *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not. (38.4 mm), *Diplodia mutila* (Fr.) Mont. (38.7 mm), *Diplodia seriata* De Not. (32.3 mm), and *E. lata* (ranged from 49.5 to 39.9 mm) from the same study (Table 4). Vascular discoloration and perennial wedge-shaped cankers had developed from the point of inoculation with the *P. viticola* isolates by 13 months after inoculation (Fig. 1f and g).

Grapevine cultivar susceptibility to infection by P. viticola and Diaporthe spp. The mean extent of vascular discoloration caused by *P. viticola* isolates UCD115Fr, UCCE964A, and UCD1935SB; *D. ambigua* isolates UCD1151Na and UCD2079Te; and *D. neotheicola* isolate UCD2327Mar at 6 months after inoculation on cvs. Barbera, Cabernet Sauvignon, Chardonnay, Merlot, Thompson Seedless, and Zinfandel and their reisolation rates are shown in Table 5. Among all three species tested, *P. viticola* was the most virulent in all six cultivars, with reisolation rates always higher than 60%. *P. viticola* isolates caused longer vascular discoloration than the water-inoculated controls in all six cultivars. Overall, statistical analyses revealed Zinfandel and Cabernet Sauvignon to be the cultivars most susceptible to *P. viticola*. Length of vascular discoloration caused by *P. viticola* did not differ statistically among cvs. Thompson Seedless, Barbera, Chardonnay, and Merlot. Overall, *D. ambigua* and *D. neotheicola* caused smaller lesions than *P. viticola*, which in some cases were not statistically different than the water-inoculated controls. Thompson Seedless and Cabernet Sauvignon were susceptible to infection by *D. ambigua* isolates UCD1151Na and UCD2079Te, respectively. *D. ambigua* reisolation rates ranged between 40 and 70%. Among all three species studied, *D. neotheicola* showed the lowest reisolation rates (20 to 50%) in all cultivars except Thompson Seedless, in which the fungus was reisolated from 80% of the inoculated canes (Table 5).

Discussion

Morphological studies along with sequence analyses of both the ITS and EF1- α regions confirmed the identification of *P. viticola*

from grapevine cankers and *Phomopsis* cane and leaf spot symptoms in California, and also revealed the presence of *D. ambigua*, *D. eres*, and *D. neotheicola* from symptomatic grapevine wood. Although these three *Diaporthe* spp. have long been described as either pathogens or endophytes from different hosts (18), their occurrence on grapevines has only recently been reported (4,64). While *P. viticola* has long been known to occur in California vineyards (21), this is, to our knowledge, the first report of *D. ambigua*, *D. eres*, and *D. neotheicola* on grapevines in the state of California.

Species identification in *Phomopsis/Diaporthe* had historically been based on morphological and cultural characteristics, as well as on host affiliation (30,35,43,47,54,55). These species recognition criteria have long been used on grapevines to identify *P. viticola*, which for many years was the sole *Phomopsis* species reported to occur in this host. However, it is currently well-accepted that the vast majority of the *Phomopsis/Diaporthe* spp. can only be properly identified to species by using DNA sequence analyses (8,12,46,47,53,54). Nevertheless, solely using ITS analysis was not sufficient to separate all *Phomopsis/Diaporthe* isolates from California (single ITS phylogenetic tree not shown). Isolate classification was only possible when either the single EF1- α or combined ITS and EF1- α phylogenies were analyzed. These results are in agreement with the recent suggestion that EF1- α is a better phylogenetic marker in *Diaporthe* and *Phomopsis* than ITS (46). Although Castlebury et al. (8) previously reported the ITS region not to be particularly informative in *Phomopsis*, most of the molecular approaches to identify *Phomopsis/Diaporthe* spp. over the last decade have relied only on ITS sequences, since they are the most common in databases (8,12,24,47,57,61,64). More recently, several studies have shown multi-locus phylogenetic analyses to provide better resolution for *Phomopsis/Diaporthe* spp. than single-gene analyses, and sequences from up to six different loci including ITS, EF1- α , β -tubulin, calmodulin, and mating types (MAT1-1-1 and MAT1-2-1) are currently available in GenBank (46,47,53,54,62).

P. viticola has been long known and studied as a pathogen of grapevines, and pathogenicity studies conducted in the early 1900s and mid-1960s suggested that this fungus was able to infect mature wood and cause perennial cankers (9,10,42). However, all these studies failed in the attempt to reproduce the dead-arm stunted and chlorotic spring growth foliar symptoms thought to be associated with *P. viticola*. Due to these inconclusive results, and after a decade of studies focused on *E. lata* as the sole causal agent of grapevine dieback (14,25,27,28), Moller and Kasimatis (29) concluded in the early 1980s that grapevine cankers and consequent dead-arm symptoms were caused solely by *E. lata* and not *P. viticola*. Consequently, the majority of the studies over the past 30 years have only focused on studying *P. viticola* as the causal agent of *Phomopsis* cane and leaf spot (1,16,17,31–34,36–38,48), and pathogenicity studies of *P. viticola* and other *Phomopsis/Diaporthe* spp. only involved testing on green grapevine tissues (30,31,36,40,48,62).

Therefore, this study represents the first attempt over the past 40 years to re-evaluate the role of *P. viticola* as a grapevine canker-causing agent, as well as to determine susceptibility of different grapevine cultivars to wood infection caused by *P. viticola* and other *Diaporthe* spp. found in grapevines in California.

Results from the grapevine cordon inoculation trial show that *P. viticola* isolates from California can successfully infect and colonize mature grapevine wood and develop perennial cankers beyond the point of inoculation, which confirm the original observations and results reported by Reddick (41,42), Coleman (10), and Chamberlain et al. (9); but are in disagreement with those reported by Moller and Kasimatis (29). Although the lengths of discoloration recorded in the cordon inoculation trial were substantially smaller than those reported by both Reddick (42) and Coleman (10), re-isolation success of *P. viticola* from the cankers developed in the inoculated cordons in this study was much higher than those previously reported (10,29,42). These differences could be a consequence of several factors; however, variability in isolate virulence, mode and time of inoculation, and differences in grapevine cultivar susceptibility have all been shown to play an important role in differences observed among other grapevine trunk pathogens (52,58). Therefore, the use of a nonvirulent isolate could be one of the reasons why the *P. viticola* isolate used by Moller and Kasimatis (29) did not cause symptoms when inoculated into mature wood. Results

Table 4. Mean lesion length caused by *Phomopsis viticola* isolates UCD1935SB, UCD1938SB, UCD115Fr and comparison against other fungal grapevine trunk pathogens on Syrah dormant canes 13 months after inoculation

Species	Isolate	Lesion length ^x $\pm$ SE	R ^y
<i>Neofusicoccum parvum</i> ^z	UCD642So	105.6 $\pm$ 19.6 a	10
<i>Lasiodiplodia theobromae</i> ^z	UCD1814Md	92.5 $\pm$ 14.9 a	10
<i>Botryosphaeria dothidea</i> ^z	UCD1065So	58.8 $\pm$ 14.8 b	10
<i>Eutypa lata</i>	DHB100	49.5 $\pm$ 10.9 bc	9
<i>E. lata</i>	DCA200	40.2 $\pm$ 9.6 bc	8
<i>E. lata</i>	UCD782St	39.9 $\pm$ 5.5 bc	9
<i>Diplodia mutila</i> ^z	UCD1953SB	38.7 $\pm$ 5.8 bc	7
<i>B. dothidea</i> ^z	UCD1181Me	38.4 $\pm$ 8.1 bc	10
<i>Phomopsis viticola</i>	UCD115Fr	35.9 $\pm$ 3.6 bc	9
<i>Dothiorella viticola</i> ^z	UCD1642Yo	33.6 $\pm$ 4.9 bc	7
<i>P. viticola</i>	UCD1938SB	32.7 $\pm$ 3.3 bc	8
<i>Diplodia seriata</i> ^z	UCD244Ma	32.3 $\pm$ 7.2 c	10
<i>P. viticola</i>	UCD1935SB	28.4 $\pm$ 2.9 c	6
Control ^z		13.4 $\pm$ 1.2 d	0

^x Mean lesion length in millimeters. Mean values correspond to the extent of vascular discoloration measured downward 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 according to Tukey's test.

^y R: Number of samples from which the fungus was reisolated out of 10 samples inoculated.

^z Botryosphaeriaceae spp. results obtained in the same trial and published in 2009 (58) are included in the table for comparison against *P. viticola*.

Table 3. Comparison of mean lesion length caused by *Phomopsis viticola* isolate UCD1935SB and other fungal grapevine trunk pathogens on cordons of 'Red Globe' and 'Syrah' 24 months after inoculation

Species	Isolate	Red Globe		Syrah	
		Lesion length (mm) ^x $\pm$ SE	R ^y	Lesion length (mm) ^x $\pm$ SE	R ^y
<i>Lasiodiplodia theobromae</i> ^z	UCD1814Md	80.2 $\pm$ 5.6 a	10	80.4 $\pm$ 6.7 a	10
<i>Neofusicoccum parvum</i> ^z	UCD642So	75.1 $\pm$ 9.7 ab	10	63.8 $\pm$ 5.2 b	10
<i>Eutypa lata</i> ^z	DHB100	70.8 $\pm$ 8.1 ab	8	60.1 $\pm$ 3.2 b	7
<i>Phomopsis viticola</i>	UCD1935SB	54.2 $\pm$ 6.8 bc	8	49.5 $\pm$ 4.3 bc	9
<i>Botryosphaeria dothidea</i> ^z	UCD1181Me	47.8 $\pm$ 4.5 c	7	42.6 $\pm$ 5.9 dc	7
<i>Diplodia mutila</i> ^z	UCD1953SB	33.1 $\pm$ 2.5 dc	6	30.3 $\pm$ 5.8 de	7
Control ^z		26 $\pm$ 1.4 d	0	21.3 $\pm$ 1.2 e	0

^x Mean lesion length in millimeters. Values correspond to the mean of the sum of both upward and downward extent of vascular discoloration 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 according to Tukey's test.

^y R: Number of samples from which the fungus was re-isolated out of 10 samples inoculated.

^z *Eutypa lata* and Botryosphaeriaceae spp. results obtained in the same trial and published in 2009 (58) are included in the table for comparison against *P. viticola*.

Table 5. Mean lesion length caused by *Phomopsis viticola* and *Diaporthe* species in six different *Vitis vinifera* cultivars

Cultivar	Control	<i>Phomopsis viticola</i>						<i>Diaporthe ambigua</i>						<i>Diaporthe neotheicola</i>	
		UCD115Fr		UCCE964A		UCD1935SB		UCD1151Na		UCD2079Te		UCD2327Mar			
		(mm) ^u ± SE ^v	R ^w	(mm) ^u ± SE ^v	R ^w	(mm) ^u ± SE ^v	R ^w	(mm) ^u ± SE ^v	R ^w	(mm) ^u ± SE ^v	R ^w	(mm) ^u ± SE ^v	R ^w	(mm) ^u ± SE ^v	R ^w
Zinfandel	4.1 ± 0.4	42.7 ± 4.7 a ^x	6	28.6 ± 2.6 a ^x	9	21.7 ± 1.6 b ^x	8	8.8 ± 0.5 b	4	12.7 ± 4.7 b	6	8 ± 0.8 b	3		
Cabernet ^y	3.6 ± 0.3	31.9 ± 2.4 b ^x	10	28.8 ± 1.4 a ^x	10	30.7 ± 1.5 a ^x	8	9.3 ± 1.2 b ^x	6	16.8 ± 1.8 a ^x	7	13.2 ± 1.1 a ^x	4		
Thompson ^z	2.9 ± 0.3	19.7 ± 2.3 c ^x	7	15.3 ± 1.6 b ^x	8	14.4 ± 1.5 c ^x	7	17.3 ± 3.6 a ^x	6	10.2 ± 0.9 b ^x	7	14.1 ± 2.7 a ^x	8		
Barbera	3.4 ± 0.4	15.8 ± 1.9 c ^x	7	15.8 ± 1.6 b ^x	8	16.9 ± 1.2 bc ^x	8	9.5 ± 1.1 b ^x	6	7.2 ± 0.6 b ^x	3	6.8 ± 0.6 b	2		
Chardonnay	3.8 ± 0.5	11.9 ± 1.2 c ^x	7	13.8 ± 1.9 b ^x	10	17.5 ± 1.6 bc ^x	10	11.3 ± 0.8 b ^x	4	9.3 ± 0.7 b ^x	6	10.8 ± 1.3 ab ^x	5		
Merlot	3.8 ± 0.4	13.2 ± 1.2 c ^x	10	14.5 ± 1.3 b ^x	10	15.5 ± 2.6 c ^x	10	8.8 ± 0.9 b ^x	5	7.5 ± 0.5 b	4	7.6 ± 0.8 b	3		
Average	3.6 ± 0.4	22.5 ± 2.3	7.8	19.5 ± 1.7	9.3	19.4 ± 1.6	8.5	10.8 ± 1.4	5.1	10.6 ± 1.6	5.5	10.1 ± 1.2	4.1		

^u Mean lesion length in millimeters. Mean values correspond to the extent of vascular discoloration measured downward from the point of inoculation.

^v SE: Standard error of the mean. Means within columns with the same letter are not significantly different at the 0.05 level according to Tukey's test.

^w R: Number of samples from which the fungus was reisolated out of 10 samples inoculated.

^x Significant differences at the 0.05 level based on a Tukey's test between the vascular discolorations means of each treatment and control plants per row.

^y Cabernet Sauvignon.

^z Thompson Seedless.

obtained in the current study from the Syrah dormant cane inoculation trial showed all three different *P. viticola* isolates from California to be pathogenic and to cause longer discoloration than the noninoculated controls. Overall, these results support recent pathogenicity tests performed under controlled environmental conditions (mycelia and/or spore inoculation of dormant potted vines in greenhouse tests) in which *P. viticola* isolates from different grape-growing regions and isolated from perennial cankers from declining vines were shown to cause noticeable vascular discolorations in woody tissue (4,57,60). Additionally, the latter pathogenicity tests showed no differences in susceptibility of dormant grapevine wood to infection caused by *P. viticola* between *V. vinifera* 'Cabernet Sauvignon' and 'Red Globe' (57), among four different interspecific hybrids (60), and between *V. labruscana* 'Concord' and *V. vinifera* 'Chardonnay' (4). Similarly, this study revealed no statistical differences in susceptibility caused by *P. viticola* between Syrah and Red Globe when mature wood was inoculated. In California, the cultivar Thompson Seedless has been historically known as highly susceptible to *Phomopsis* cane and leaf spot (19,26). However, there is very little information regarding the susceptibility of pruning wounds to *P. viticola* infection. Interestingly, Thompson Seedless dormant wood was shown in this study to be not highly susceptible to infection caused by *P. viticola*. Although most of the *P. viticola* isolates obtained from perennial cankers in the San Joaquin Valley were isolated from Thompson Seedless (J. R. Úrbez-Torres, unpublished data), these results may indicate *P. viticola* to be more successful infecting grape tissues such as leaves, green shoots, rachis, and berries, all associated with *Phomopsis* cane and leaf spot, than dormant wood in specific cultivars.

This study reported for the first time the occurrence of *D. ambigua*, *D. eres*, and *D. neotheicola* on grapevines in California. Previous pathogenicity studies conducted in vitro on green shoots showed *D. ambigua* to cause lesions similar to the nonpathogen *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams and the noninoculated controls (61). Although the anamorph of *D. neotheicola*, *Phomopsis theicola* Curzi, was reported from esca-infected grapevines in South Africa, no pathogenicity studies were conducted (64). Consequently, with the current information available and until further pathogenicity studies are completed, *D. ambigua* and *D. neotheicola* are suggested here as saprophytes on grapevine wood. In this study, *D. eres* was isolated and identified after conclusion of the pathogenicity studies. Thus, it could not be included in any of the trials. *D. eres* was recently found associated with grapevine perennial cankers in vineyards in the northeastern United States, and preliminary pathogenicity studies suggested that this species is pathogenic on grapevines but less virulent than *P. viticola* (4).

When *P. viticola* was originally described as the causal agent of the dead-arm disease, it was also thought to be responsible for the foliar symptomatology observed in the declining vines, which included lack of spring growth, bud mortality, shoot dieback, short shoot internodes, small leaves crimped about the margin, and fruit

shriveling (41). However, research conducted since the mid-1970s has clearly shown that symptoms such as shoot dieback, short shoot internodes, and crimping of the leaves are indeed caused by the fungal pathogen *E. lata* (7,28,29,50). In the current study, none of these symptoms were observed in any of the pathogenicity trials conducted, further supporting that *E. lata* and not *P. viticola* is responsible for this symptomatology. On the other hand, lack of spring growth and subsequent bud mortality, originally thought to be caused by *P. viticola* but currently associated primarily with *Botryosphaeria* dieback (56), were recorded in both the Syrah dormant canes and the cultivar susceptibility trials. These results not only support the observations made in the early 1900s (10,41,42), but also those from field surveys recently conducted in different grape-growing areas in the United States, in which *P. viticola* was isolated alone from grapevine cankers from declining vines showing lack of spring growth and bud mortality (57,59,60).

This study has shown *P. viticola* isolates from California to be pathogenic on mature wood and to cause perennial cankers and vascular discoloration similar to those caused by *E. lata* and several *Botryosphaeriaceae* spp., which strongly support the role of *P. viticola* as a grapevine wood pathogen and suggest its addition among the fungi involved in the grapevine trunk disease complex. Future research should focus on investigating the time of pruning wound infection in the different grape-growing regions, in order to develop control strategies that may differ from those currently used to prevent *Phomopsis* cane and leaf spot.

Acknowledgments

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