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RESEARCH PAPERS

Pleurostomophora richardsiae, *Neofusicoccum parvum* and *Phaeoacremonium aleophilum* associated with a decline of olives in southern Italy

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Summary. In a recent survey of olive groves in the Canosa di Puglia, Cerignola and Foggia areas of southern Italy a serious decline of olive trees was seen. The symptoms comprised a general decline of the trees beginning with foliar browning and leaf drop, wilting of apical shoots, die-back of twigs and branches, and brown streaking under the bark of the trunk, branches and twigs. In more advanced stages of the disease necroses and cankers were observed on the bark. The symptoms were similar to those caused by *Verticillium* wilt, but morphological and molecular analyses revealed the presence of *Pleurostomophora richardsiae*, *Phaeoacremonium aleophilum* and various genera and species in the Botryosphaeriaceae. Pathogenicity tests carried out on young shoots showed that *Pl. richardsiae*, *Pm. aleophilum* and *Neofusicoccum parvum* were pathogenic and capable of causing brown wood streaking. Since *Pl. richardsiae* was the most aggressive of these three fungi and frequently it was the only one isolated from diseased trees it was considered to be a primary cause of the decline. To our knowledge, this is the first report of *Pl. richardsiae* as a pathogen of olive trees.

Key words: *Olea europaea*, olive disease, pathogen.

Introduction

The practice of olive cultivation spread rapidly from Asia Minor throughout the Mediterranean region due to its nutritional and health-promoting properties. Several European countries are now among the largest producers of olive oil and table olives. Indeed, Europe is the major olive-producing area in the world, and after Spain, Italy is the second largest producer, with a total crop area of 1,144.42 ha (FAOSTAT, 2012).

Olives are susceptible to different bacterial, viral and fungal pathogens, which can cause severe diseases of the drupes, leaves, wood and roots. Wood decay is caused by several species, e.g., *Fomes fulvus*, *Polyporus oleae*, *Schizophyllum commune*, *Phellinus punctatus* and *Trametes* spp., while *Verticillium dahliae* is the main cause of vascular disease (Jiménez-Díaz *et al*., 1998; Nigro *et al*., 2005). Other fungal species are often associated with olives, such as weakly pathogenic, saprobic and endophytic fungi. Indeed, Carlucci *et al.* (2008) reported that *Lecythophora lignicola*, *Phoma incompta*, *Phoma cava, Pleurostomophora richardsiae, Phaeoacremonium* spp. and species of the Botryosphaeriaceae are all associated with brown streaking in olive xylem, but none was proved to be the cause of this symptom.

During a recent survey of olive orchards in the Canosa di Puglia, Cerignola and Foggia areas of southern Italy, a decline of olive trees was noted.

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The symptoms consisted of a generalised decline of the trees starting with foliar browning and leaf drop, wilting of apical shoots, die-back of twigs and branches, and brown streaking under the bark of the trunk, branches and twigs. In more advanced stages of the disease, necrosis and cankers were observed on the bark.

Initially, these symptoms were thought to be caused by *Verticillium* spp., although brown subcortical streaking, necrosis and cankers are not generally attributed to *Verticillium* wilt (López-Escudero and Mercado-Blanco, 2011). Based on the isolation of other fungi from brown wood streaking in olive trees (Carlucci *et al.*, 2008), attention was focused on other fungi, including *Pl. richardsiae, Phaeoacremonium* spp. and various species in the Botryosphaeriaceae. The aim of the present study was to identify and characterise the main fungi associated with declining olive trees, and to determine their role in the disease.

Materials and methods

Fungal isolations

Samples of parts of the roots and collars, trunks and branches were collected from 42 symptomatic olive trees (cv. Coratina) 18–35-years old. The samples were transported to the laboratory for analysis. Following surface-sterilisation of the samples according to Fisher *et al.* (1992), the bark was removed and small samples (1–3 mm^2) were taken from the immediate sub-cortical tissues with a scalpel. These samples were placed on malt extract agar [2% malt extract (Oxoid Ltd., Basingstoke, UK); 2% agar (Difco, USA)] with 500 mg L^{-1} streptomycin sulphate (Oxoid Ltd.), and incubated at 25°C (±3°C) in the dark. All fungal colonies morphologically similar to *Pleurostomophora* species were grown until they sporulated and then a conidial suspension was spread on agar plates. After 24–36 h of incubation single germinating conidia were transferred to fresh plates of potato dextrose agar (PDA, Oxoid Ltd.). Genera and species in the Botryosphaeriaceae were identified by reference to the keys, descriptions and sequence data provided in Phillips *et al.* (2013). *Phaeoacremonium* species were identified according to Mostert *et al.* 2005. Other fungi were identified based on their micromorphology and cultural characters. Reference strains are maintained in the culture collection of the Department of Science of Agriculture, Food and Environment, of the University of Foggia, Italy. The isolation frequencies per olive plant were calculated as the number of tissue portions infected by a given fungus, divided by the total number of tissue segments incubated, and expressed as percentages.

DNA extraction and microsatellite-primed polymerase chain reaction

Genomic DNA of all *Pleurostomophora* isolates was extracted from 200 μg fresh fungal mycelium that was grown on potato dextrose agar plates for 7 days, according to a new protocol that we optimised here for *Pleurostomophora* strains. The mycelium was scraped off the plates and ground in liquid nitrogen to a fine powder with a pestle and mortar. Then, 250 mg of the ground mycelium was collected in 2 mL microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) in 600 μL DNA extraction buffer [200 mM Tris-HCl, pH 8.0, 200 mM EDTA, pH 8.0, 0.5% (w/v) SDS, pH 7.2, 1.2% (v/v) β-mercaptoethanol], supplemented with 5 μL proteinase K (stock, 20 mg L^{-1}; Oxoid Ltd.), and kept at 65°C for 1 h. Chloroform (600 μL; Oxoid Ltd.) was then added, and the samples were vortexed for 15 s. The samples were centrifuged (10,000 rpm, 18°C, 15 min), and the aqueous phase was transferred to new 2 mL microcentrifuge tubes, with the addition of 3 μL RNAase (stock, 40 mg L^{-1}; Oxoid Ltd.). The samples were left at 37°C for 30 min, and then an equal volume of chloroform was added to each sample, vortexed for 15 s, followed by centrifugation (10,000 rpm, 18°C, 10 min). The aqueous phases were transferred into new 1.5 mL centrifuge tubes (Eppendorf AG), with the addition of 0.5 vol. cold 7.5 M ammonium acetate (Oxoid Ltd.). Following gentle mixing, the samples were left on ice for 30 min, and then centrifuged (13,000 rpm, 2°C, 10 min). The aqueous phase was collected and transferred to 2.0 mL centrifuge tubes, with the addition of 0.7 vol. cold isopropanol (Oxoid Ltd.) and gentle mixing; these were then left at –25°C overnight. After this, the samples were centrifuged (13,000 rpm, 2°C, 25 min), the supernatant discarded, and the pellets washed twice with 700 μL cold 70% ethanol, with centrifugation (13,000 rpm, 2°C, 15 min) and discarding of the supernatants. After the final centrifugation, the pellets were left to dry at room temperature with the tubes open, for 10–15 min. The pellets were then dissolved in 100 μL hot (65°C) TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA),

and left at 55°C for 10 min. Methods for DNA isolation from Botryosphaeriaceae and *Phaeoacremonium* are as described by Phillips *et al.* (2013) and Mostert *et al.* (2005), respectively.

Microsatellite-primed polymerase chain reaction (PCR) profiles were generated for 38 *Pleurostomophora* isolates with M13 primers (Meyer *et al.*, 1993) according to Santos and Phillips (2009). The isolates were clustered into a consensus dendrogram on the basis of their profiles, with the dendrogram built using the BioNumerics software, version 5.1 (Applied Maths, Kortrijk, Belgium), using Pearson's correlation coefficients and the unweighted pair group method with arithmetic mean. The reproducibility levels were calculated as the means of the reproducibilities obtained for the M13 primer. For this purpose, 10% of the isolates were chosen at random and their profiles were analysed again.

ITS Amplification, sequencing and phylogenetic analysis

The 5.8S rDNA gene and flanking internal transcribed spacers 1 and 2 of 15 *Pleurostomophora* isolates as representative were amplified with the primers ITS1 and ITS4 (White *et al.*, 1990). PCR reactions were performed according to Carlucci *et al.* (2012). The amplified PCR fragments were purified with NucleoSpin extract II purification kits (Macherey-Nagel) before both strands were sequenced by PRIMM srl. In addition, part of the translation elongation factor 1-α (EF 1-α) was amplified and sequenced for the Botryosphaeriaceae according to Phillips *et al.* (2013) and partial sequences of the β-tubulin and actin gene were generated for *Phaeoacremonium* as detailed by Mostert *et al.* (2005).

Nucleotide sequences were edited with BioEdit, version 7.0.9 (http://www.mbio.ncsu.edu/BioEdit) and aligned with additional sequences retrieved from GenBank (www.ncbi.nlm.gov), using ClustalX, v. 1.83 (Thompson *et al.*, 1997). Phylogenetic analyses were performed using PAUP version 4.0b10 (Swofford, 2003) for neighbour joining analysis and for maximum parsimony. The Kimura 2-parameter substitution model (Kimura, 1980) was used for the distance analysis. All of the characters were unordered and of equal weight. Bootstrap values were obtained from 1000 neighbour-joining bootstrap replicates. For the parsimony analyses, alignment gaps were treated as missing data, and all characters were unordered and of equal weight. Maximum-parsimony analysis was performed using the heuristic search option, with random addition of sequences (1000 replications), and tree bisection–reconnection as the branch-swapping algorithm, with the MULTREES options on. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated with 1000 bootstrap replications (Hillis and Bull, 1993). Tree length, consistency index, retention index and rescaled consistency index were calculated, and the resulting trees were visualised with TreeView, version 1.6.6 (Page, 1996). New sequences were lodged in GenBank, and the alignment and phylogenetic tree were deposited in TreeBASE (S15002). The tree was rooted to *Diaporthe ambigua* (AJ458389).

Morphology

Colony morphology of the *Pleurostomophora* isolates was determined in cultures incubated on malt extract agar at 25°C (±3°C) in the dark for 3 weeks, according to Vijaykrishna *et al.* (2004). Microscopic characteristics of these strains were determined according to Carlucci *et al.* (2012). Conidial shape and size were measured from 100% lactic acid mounts, using a Leica application suite measurement module (Leica Microsystem GmbH, Wetzlar, Germany). Photomicrographs were recorded with a Leica DFC320 digital camera on a Leica DMR microscope fitted with Nomarski differential interference contrast optics. Conidial dimensions were measured with the ×100 objective and at least 30 conidia were measured for each isolate. The means, standard deviation (SD) and 95% confidence intervals were calculated.

Pathogenicity tests

Two isolates of *Pm. aleophilum* (Pal4, Pal9), two isolates of *Neofusicoccum parvum* (Bot84, Bot88), and two isolates of *Pl. richardsiae* (PLEU9, PLEU27) were included in the pathogenicity tests. The pathogenicity tests were carried out in June 2013 on green shoots (0.5–1.5 cm diam., 30–50 cm long) of 18-year-old olive trees cv. Coratina grown in an open field in an olive grove in Canosa di Puglia. The shoots were inoculated on wounds made at the internodes. Agar plugs (0.3–0.5 cm diam.) were taken from 10-day-old cultures grown on potato dextrose agar at 23°C (±2°C),

and inserted under the bark. After inoculation, the wounds were wrapped with wet sterile cottonwool and sealed with Parafilm. The controls were inoculated with sterile agar plugs. Each experiment included 20 replicates per treatment. The shoots were examined at 25 days after inoculation when the length of wood discoloration that occurred both under the bark and in the inner wood were measured. The mean, standard deviation, and maximum and minimum lengths of wood discoloration were determined. Isolations were made from all of the inoculated shoots, the isolates were identified as described initially, to fulfill Koch's postulates. One-way analysis of variance was performed using Statistica, version 6 (StatSoft, Hamburg, Germany), to evaluate the differences in the extension of sub-cortical discoloration induced by the fungal isolates. Duncan's tests were used for comparisons of treatment means, at $P<0.01$.

Results

Isolations

Cankers and longitudinal brown streaking were seen under the bark of the trunk and branches of declining trees (Figure 1). The mycoflora isolated from the olive plants that showed the disease symptoms was very variable and is reported in Table 1. *Acremonium* spp., *Aspergillus* spp. and *Penicillium* spp. were isolated more frequently than other fungi (Table 1), although they are considered to be saprobic. *Phaeoacremonium* spp. were isolated from the trunks and branches, with isolation frequencies that ranged from 1.8% to 4.4%. Fungi belonging to the Botryosphaeriaceae (i.e., *Botryosphaeria dothidea*, *Diplodia mutila*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum luteum*, *Neofusicoccum parvum*) were isolated with isolation frequencies ranging from 0.4% to 5.8%. *Pleurostomophora richardsiae* was isolated more frequently from trunks and branches, with isolation frequency ranging from 18.3% to 23.4%, respectively. This fungus was also isolated from the roots, although with a relatively low isolation frequency of 1.7%. *Phaeoacremonium* and Botryosphaeriaceae spp. were mainly isolated from older trees (more than 25 years old), while *Pl. richardsiae* was isolated mainly from younger trees (18–22 years old) (data not shown).

Other fungi, identified from their micromorphological and cultural features, were infrequently isolated from a few olive plants, such as *Alternaria alternata*, *Aureobasidium oleae*, *Cylindrocarpon destructans*, *Cytospora oleina*, *Diaporthe* spp., *Epicoccum nigrum*, *Phoma incompta*, *Fomitiporia mediterranea*, *Lecythopho-*

Table 1. Fungi isolated from the cankers and sub-cortical brown streaking of wilted olive plants.

Fungus	Fungal isolation frequency (%)			
	Root/ collar	Trunk	Branches	Total
Acremonium spp.	20.5	11.8	8.4	14.2
Alternaria alternata	2.1	4.8	3.0	3.3
Aspergillus spp.	14.1	6.1	5.4	8.9
Aureobasidium oleae	0.0	1.3	5.4	1.9
Botryosphaeria dothidea	0,6	2,4	1,9	1.6
Cylindrocarpon destructans	5.1	0.0	0.0	1.9
Cytospora oleina	0.0	1.3	0.6	0.6
Diaporthe spp.	0.0	3.9	1.2	1.7
Diplodia mutila	0.0	0.7	0.5	0.4
Diplodia seriata	1.1	3.3	3.1	2.4
Epicoccum nigrum	8.1	0.9	0.0	3.3
Fomitiporia mediterranea	0.0	1.7	0.0	0.3
Lasiodipodia theobromae	0.7	2.0	2.0	1.5
Lecythophora lignicola	0.4	0.4	1.2	1.1
Microsphaeropsis olivacea	0.0	0.4	0.0	0.2
Neofusicoccum luteum	0.0	0.8	1.0	0.5
Neofusicoccum parvum	1.9	7.8	8.3	5.8
Penicillium spp.	1.7	4.8	3.6	5.4
Phaeoacremonium aleophilum	0.0	4.4	1.8	2.1
Phoma incompta	0.0	0.4	0.6	0.3
Pleurostomophora richardsiae	1.7	18.3	23.4	13.5
Verticillium spp.	0.9	0.0	0.0	0.3
Bacteria	28.6	12.2	21.6	20.9
No fungi	6.8	9.6	17.2	7.9
Total	100.0	100.0	100.0	100.0
Number of plants analyzed	42			

Figure 1. Disease symptoms observed on olive trees. (a) Wilt and die-back. (b–d) Sub-cortical browning as longitudinal streaking from young (12 years old) and adult (26 years old) trees. (e) Cankers and brown streaking on branches. White arrow, region from which *Pl. richardsiae* was frequently isolated. (f, g) Microscopic features of *Pl. richardsiae*. (f) Conidiophores and conidiogenous cells. (g) Brown, globose, and hyaline, cylindrical conidia. Scale bar in f, g = 10 μm.

Table 2. Fungal isolates used in the phylogenetic study

Species	Isolate number[a]	Host	Locality	Collector	GenBank ITS
Pl. ochracea	**CBS 131321**	Human yellow-grain mycetoma	Khartoum, Sudan	N.A. Mhmoud and A. Fahal	JX073270
Pl. ootheca	**CBS 115329**	Degrading wood	British Columbia	L. Mostert	HQ878590
Pl. repens	**CBS 294.39**	Pine lumber	Florida, USA	R.W. Davidson	AF083195
Pl. richardsiae	PLEU3	Olive cv. Coratina	Foggia, Italy	A. Carlucci	KF751176
	PLEU4	Olive cv. Coratina	Canosa di Puglia, Italy	F. Lops	-
	PLEU5	Olive cv. Coratina	Cerignola, Italy	A. Carlucci	KF751177
	PLEU6	Olive cv. Coratina	Cerignola, Italy	A. Carlucci	-
	PLEU7	Olive cv. Coratina	Canosa di Puglia, Italy	F. Lops	KF751178
	PLEU8	Olive cv. Coratina	San Severo, Italy	M.L. Raimondo	KF751179
	PLEU9	Olive cv. Coratina	Canosa di Puglia, Italy	F. Lops	-
	PLEU11	Olive cv. Coratina	Foggia, Italy	F. Lops	-
	PLEU21	Olive cv. Coratina	Cerignola, Italy	A. Carlucci	-
	PLEU22	Olive cv. Coratina	Cerignola, Italy	A. Carlucci	KF751180
	PLEU24	Olive cv. Coratina	Cerignola, Italy	A. Carlucci	-
	PLEU25	Olive cv. Coratina	Canosa di Puglia, Italy	F. Lops	KF751181
	PLEU26	Olive cv. Coratina	Foggia, Italy	F. Lops	KF751182
	PLEU27	Olive cv. Coratina	Cerignola, Italy	A. Carlucci	-
	PLEU29	Olive cv. Coratina	Torremaggiore, Italy	M.L. Raimondo	KF751183
	CBS 270.33	-	Sweden	E. Melin	AY729811
Pm. aleophilum	**CBS 246.91**	Root and stem	Yugoslavia	M. Muntañola-Cvetkovic	AF017651
Pm. angustius	CBS 101737	*Vitis vinifera*	France	P. Larignon	AF197976
Pm. inflatipes	**CBS 391.71**	*Quercus virginiana*	Texas	R.S. Halliwell	AF197990
Pm. mortoniae	**CBS 101585**	Root stock	California	L. Morton	AF295328
	CBS 211.97	*Fraxinus excelsior*	Sweden	J. Stenlid	AF295329
Pm. parasiticum	**CBS 860.73**	-	USA	Z. Yan	U31841
Pm. rubrigenum	CBS 498.97	Decorticated wood	Puerto Rico	S. Huhndorf	AF197988
Pm. viticola	LCP 933886	*Vitis vinifera*	France	P. Larignon	AF118137
D. ambigua	CMW5287	*Malus domestica*	Pretoria, South Africa	N. Moleleki	AJ458389

[a] Ex-type isolates are shown in bold.

ra lignicola, *Microsphaeropsis olivacea*, and *Verticillium* spp. Because of their low frequency and inconsistent isolation frequency these were not considered to be the cause of the disease symptoms observed.

Molecular identification and phylogenetic analysis

Identities of the Botryosphaeriaceae and *Phaeoacremonium* species (Table 1) was confirmed by BLAST searches in GenBank. Since the association of *Pl.*

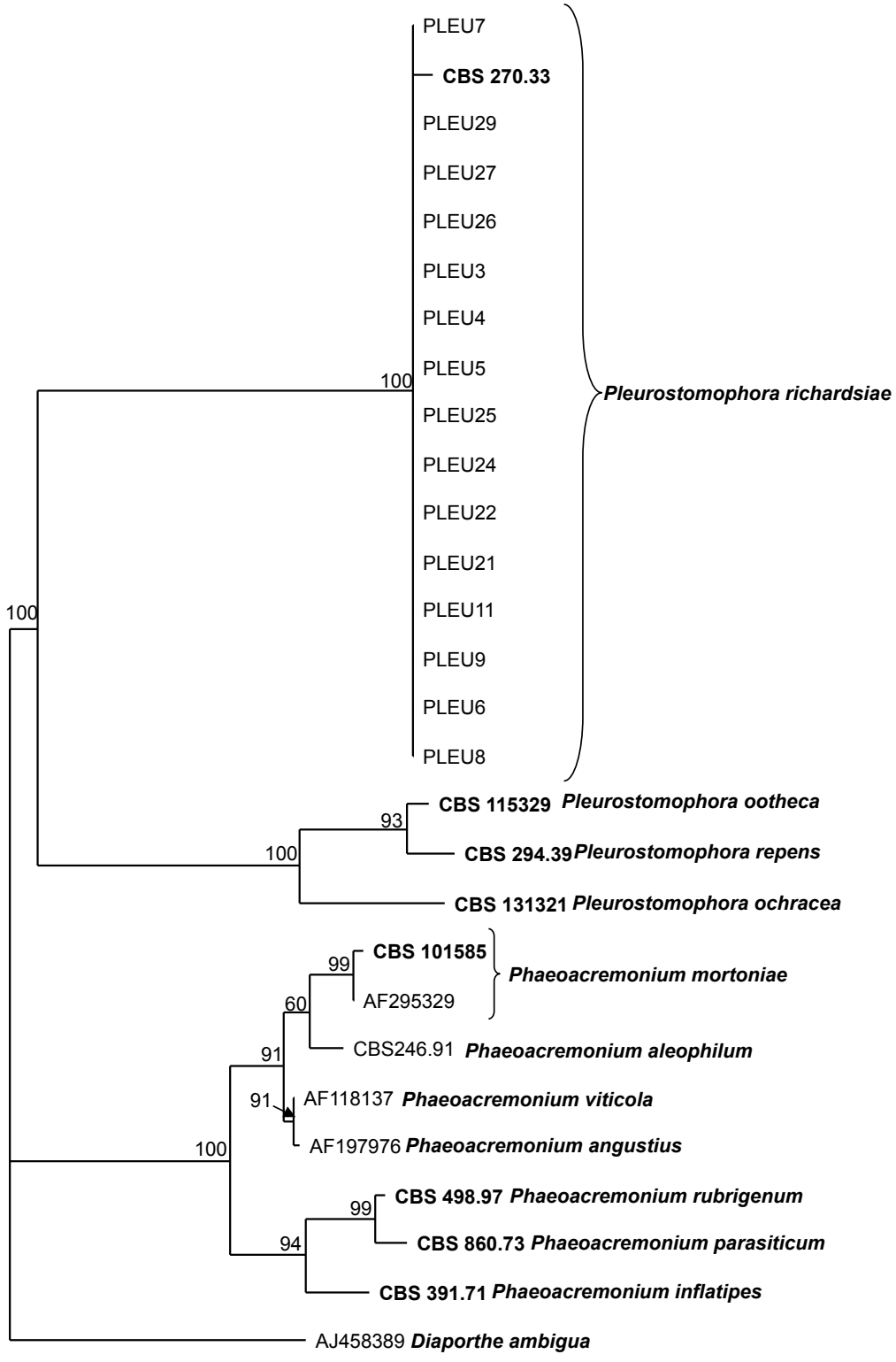

Figure 2. One of four most parsimonious trees obtained from heuristic searches of ITS sequence data. Bootstrap support values from 1000 replicates are shown at the nodes. The tree is rooted to *Diaporthe ambigua*.

richardsiae with declining olive trees was unusual, a more intensive phylogenetic study was undertaken. The microsatellite-primed PCR dendrogram of 38 isolates generated a single clade (data not shown), from which 15 isolates were chosen for sequencing the ITS (Table 2). The ITS dataset consisted of 27 ingroup isolates and 1 outgroup isolate (Table 2). After alignment, and following the exclusion of incomplete portions at each end, the dataset consisted of 498 characters. Of these 498 characters 305 were constant, while 54 were variable and parsimony-uninformative. Maximum parsimony analysis of the remaining 139 parsimony-informative characters resulted in four most parsimonious trees, one of which is shown in Figure 2 (tree length = 325; consistency index = 0.825, retention index = 0.930 and homoplasy index = 0.175). The neighbour joining analysis produced a tree with similar topology to the maximum parsimony tree. The 15 *Pleurostomophora* isolates sequenced in this study clustered in the same clade with the ex-type isolate of *Pl. richardsiae* (CBS 270.33, GenBank AY729811).

Morphology

The *Pl. richardsiae* strains produced two different types of conidia, namely, brown globose conidia 1.5 μm diam., and hyaline, allantoid to cylindrical conidia (6–6.5 × 2 μm) (Figure 1). The micromorphology agreed in all ways with the descriptions provided by Schol-Schwarz (1970), Domsch *et al.* (1980), De Hoog *et al.* (2000) and Vijaykrishma *et al.* (2004).

Pathogenicity tests

The results of pathogenicity tests were all determined at 25 days from inoculation. All three species tested in the inoculation experiment produced brown streaking in the wood. The most aggressive fungus was *Pl. richardsiae*, at 25 days after inoculation the two isolates caused significantly longer regions of brown streaking (averages of 3.3 and 3.7 cm) than the other two species tested, and resulted in death of all young shoots (Table 3). Although inoculation with the two *N. parvum* isolates resulted in death of all the shoots, the length of the brown wood streaking was significantly less (averages of 1.7 and 2.3 cm) than in shoots inoculated with *Pl. richardsiae*. The isolates of *Pm. aleophilum* caused brown streaking 0.7–1.0 cm in length, but did not kill the shoots. Furthermore, symptoms induced by the two *Pm. aleophilum* isolates were found in only 23% and 28% of inoculated plants, whereas *Pl. richardsiae* and *N. parvum* induced brown wood streaking in all the inoculated shoots. All fungi were re-isolated from symptomatic tissues, thus fulfilling Koch's postulates (Table 3).

Table 3. Pathogenicity assays carried out with two isolates each of *Pm. aleophilum, Pl. richardsiae* and *N. parvum* inoculated on young olive shoots (cv. Coratina).

Fungal species	Isolate ID	Brown streaking length (cm) after 25 days			Re-isolation	
		Mean	SD	Max-Min[a]	(%)	Dead shoot
Control	H_2O d	0.0 A[b]	-	-	-	No
Pm. aleophilum	PAL4	0.7 AB	0.5	1.3–0.0	23	Yes
	PAL9	1.0 BC	0.7	2.2–0.0	28	Yes
N. parvum	BOT84	1.7 CD	0.7	2.6–0.2	100	Yes
	BOT88	2.3 D	0.7	3.5–1.3	100	Yes
Pl. richardsiae	PLEU9	3.3 E	1.5	6.6–0.6	100	Yes
	PLEU27	3.7 E	1.5	6.7–1.4	100	Yes

[a] Maximum and minimum values detected on the basis of 20 observations.
[b] Values followed by a different capital letter in each column are significantly different according to Duncan's test ($P > 0.01$).

Discussion

Although a range of fungi were isolated from the wood of declining olive trees, most were infrequent or inconsistently associated with the symptoms. However, three species (*N. parvum*, *Pl. richardsiae* and *Pm. aleophilum*) were consistently isolated from diseased trees and at moderate to high frequencies. Thus, these three species were considered to be potential pathogens and possible causes of the disease, and were selected for further studies on their role in the disease.

The results of the pathogenicity tests demonstrated that *N. parvum*, *Pl. richardsiae* and *Pm. aleophilum* are pathogenic on olives. However, only *N. parvum* and *Pl. richardsiae* induced symptoms in all inoculated shoots, and infections by both of them resulted in death of the shoots. This suggests that both species are likely causes of the decline and brown wood streaking of olive trees. Of the two, *Pl. richardsiae* was considered to be the most aggressive since it caused significantly longer regions of brown wood streaking than *N. parvum*. Furthermore, it was frequently the only fungus isolated from symptomatic younger trees, while *N. parvum* was never found alone but was always associated with *Pl. richardsiae*.

Various species of the Botryosphaeriaceae were isolated from diseased trees. Some are known to be pathogens responsible for brown wood streaking in several hosts including olive, grapevine, apricot, peach, and oak (von Arx, 1987; Denman *et al*., 2000; Phillips *et al*. 2002; Alves *et al*., 2004; Niekerk *et al*., 2004; Damm *et al*., 2007; Burruano *et al*. 2008; Lazzizera *et al*. 2008; Phillips *et al.* 2008; Úrbez-Torres, 2011). On olives, species of the *Botryosphaeriaceae* are primarily known to cause fruit rot (Chattaoui et al., 2011; Lazzizera *et al*. 2008; Moral et al., 2008; Phillips et al., 2005), but some have been reported associated with branch dieback, cankers and blighted shoots of worldwide (Taylor *et al*., 2001; Moral *et al*., 2010; Romero *et al*., 2005; Kaliterna *et al*., 2012). However, this is the first time that *N. parvum* has been associated with decline of olive trees in southern Italy or anywhere else in the world.

Neofusicoccum parvum is well known as a pathogen of woody hosts and is regarded as a serious pathogen on some including grapevines, pine and *Eucalyptus* (Golzar and Burgess, 2011; Iturritxa *et al*., 2011; Úrbez-Torres, 2011; Úrbez-Torres *et al*., 2013). Since it was always associated with *Pl. richardsiae* infections in the present study, and never found on its own, *N. parvum* was not considered to be the primary cause of olive decline and brown wood streaking. Nevertheless, it is clearly pathogenic on olives and would likely contribute to the severity of the decline

Phaeoacremonium aleophilum was also isolated from olive trunks and branches, although at relatively low frequencies. Since it caused relatively mild symptoms consisting of small regions of brown wood streaking in only some of the inoculated shoots, this species was considered to play an insignificant role in the decline disease.

Pleurostomophora richardsiae was considered to be the main agent responsible for wilt of apical foliage, brown streaking under the bark, and cankers of trunks and branches. It was always isolated from symptomatic wood tissues of all diseased plants, and frequently it was the only fungus isolated from younger (18–22 old years) diseased olive trees. Botryosphaeriaceae and *Phaeoacremonium* species, some of which are already known as pathogens of olives (Carlucci *et al*., 2008; Lazzizera *et al*., 2008), were isolated more frequently from symptomatic tissues of older plants (more of 25 old years). These species were never isolated alone but were always associated with other fungi including *Pl. richardsiae*.

Pleurostomophora was introduced by Vijaykrishna *et al.* (2004) to accommodate the phialophora-like anamorphs of *Pleurostoma* species. Four species are currently recognised, namely *Pl. richardsiae*, *Pl. repens, Pl. ootheca* (Vijaykrishna *et al.,* 2004) and *Pl. ochracea* (Mhmoud *et al*., 2012). *Pleurostomophora richardsiae* (syn. *Phialophora richardsiae*) is also known to be a human pathogen (Uberti-Foppa *et al.* 1995; Ikai *et al.* 1988; Pitrak *et al.* 1988; De Hoog *et al.* 2000) and the cause of subcutaneous phaeohyphomycotic cysts after traumatic implantation (Guého *et al.* 1989). More recently it was reported as a pathogen of grapevines by Eskalen *et al.* (2004) and Rolshausen *et al.* (2010) in California (USA), and by Halleen *et al.* (2007). *Pleurostomophora ochracea* is known to be the cause of human eumycetoma (Mhmoud *et al*., 2012), while *Pl. repens* and *Pl. ootheca* have only been associated with pine lumber and degrading wood, respectively (Vijaykrishna *et al.*, 2004).

Eskalen *et al.* (2004) and Rolshausen *et al.* (2010) demonstrated in pathogenicity tests that *Pl. richardsiae* can infect pruning wounds of grapevine, although other fungi such as Botryosphaeriaceae species and *Phaeomoniella chlamydospora* were more aggressive. Halleen *et al.* (2004) consider *Pl. richardsiae* to be a

vascular pathogen of grapevines, since it was able to cause vascular discoloration similar to Petri disease when it was inoculated into grapevines. White *et al.* (2011) reported that *Pl. richardsiae* was not found abundantly in their studies carried out on diseased grapevines, maybe because the vines were very old. Indeed, they isolated from grapevine higher rates of Basidiomycete fungi, which are known agents of white rot, and *Pa. chlamydospora*, the fungus responsible for internal symptoms such as black and brown wood streaking.

To our knowledge, this is the first report of brown wood streaking and decline of olive trees caused by *Pl. richardsiae*. Future studies should be aimed at determining the incidence of the disease and the pathogen in more regions and countries where olives are grown. The interaction of *Pl. richardsiae* with other fungi associated with these symptoms would be another area worthy of study.

Literature cited

Alves A., J. Luque, A. Phillips, 2004. *Botryosphaeria corticola*, sp. nov. on *Quercus* species, with notes and description of *Botryosphaeria stevensii* and its anamorph, *Diplodia mutila*. *Mycologia* 96, 598–613.

Arx J.A.,von, 1987. *Plant Pathogenic Fungi*. J. Cramer, Berlin, Germany.

Burruano S., V. Mondello, G. Conigliaro, A. Alfonzo, A. Spagnolo, L. Mugnai, 2008. Grapevine decline in Italy caused by *Lasiodiplodia theobromae*. *Phytopathologia Mediterranea* 47, 132–136.

Carlucci A., F. Lops, M.L. Raimondo, V. Gentile, L. Colatruglio, M. Mucci and S. Frisullo, 2008. Comportamento patogenetico di alcuni isolati fungini associati a striature brune del legno dell'olivo [Behaviour of some pathogenic fungal isolates associated with brown streaks of olive wood]. *Petria* 18, 15–26.

Carlucci A., M.L. Raimondo, J. Santos, A.J.L. and Phillips, 2012. *Plectosphaerella* species associated with root and collar rots of horticultural crops in southern Italy. *Persoonia* 28, 34–48.

Chattaoui M., A. Rhouma, S. Krid, M. Ali Triki, J. Moral, M. Msallem, A. Trapero, 2011. First report of fruit rot of olives caused by *Botryosphaeria dothidea* in Tunisia. *Plant Disease* 95, 770.

Damm U., P.W. Crous and P.H. Fourie, 2007. *Botryosphaeriaceae* as potential pathogens of *Prunus* species in South Africa, with descriptions of *Diplodia africana* and *Lasiodiplodia plurivora* sp. nov. *Mycologia* 99, 664–680.

De Hoog G.S., J. Guarro, J. Gené and M.J. Figueras, 2000. *Atlas of Clinical Fungi*. Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

Denman S., P.W. Crous, J.E. Taylor, I. Pascoe and M.J. Wingfield, 2000. An overview of the taxonomic history of *Botryosphaeria,* and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. *Studies in Mycology* 45, 129–140.

Domsch K.H., W. Gams and T.H. Anderson, 2007. *Compendium of Soil Fungi*. 2nd edition. IHW-Verlag, Eching, Germany.

Eskalen A., S.R. Latham and W.D. Gubler, 2004. Pathogenicity of *Phialophora* sp. on grapevines in California. *Phytopathologia Mediterranea* 94, S151.

FAOSTAT, 2012. Food and Agriculture Organization, Statistical Database (http://www.faostat.fao.org).

Fisher P.J., O. Petrini, L.E., Petrini and E. Descals, 1992. A preliminary study of fungi inhabiting xylem and whole stems of *Olea europaea*. *Sydowia* 44, 117–121.

Golzar H. and T.I. Burgess, 2011. *Neofusicoccum parvum*, a causal agent associated with cankers and decline of Norfolk Island pine in Australia**.** *Australasian Plant Pathology*, 40, 484–489.

Guého E., A. Bonnefoy, J. Luboinski, J.C. Petit and G.S. de Hoog, 1989. Subcutaneous granuloma caused by *Phialophora richardsiae*: case report and review of the literature. *Mycoses* 32, 219–223.

Halleen F., L. Mostert and P.W. Crous, 2007. Pathogenicity testing of lesser known vascular fungi of grapevines. *Australasian Plant Pathology* 36, 277–285.

Hillis D.M. and J.J. Bull, 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* 4, 182–192.

Ikai K., H. Tomono and S. Watanabe, 1988. Phaeohyphomycosis caused by *Phialophora richardsiae*. *Journal of the American Academy of Dermatology* 19, 478–481.

Iturritxa E., B. Slippers, N. Mesanza, M.J. Wingfield, 2011. First report of *Neofusicoccum parvum* causing canker and die-back of *Eucalyptus* in Spain. *Australasian Plant Disease Notes* 6, 57–59.

Jiménez-Díaz R.M., E.C.Tjamos and M.Cirulli, 1998. Verticillium wilt of major tree hosts. Olive. *In*: Hiemstra J.A., Harris D.C. (eds.). *A Compendium of Verticillium Wilts in Tree Species*, pp. 13–16. Ponsen and Looijen, Wageningen, The Netherlands.

Kaliterna K., I. Ivic, D. Bencic, A. Mesic, 2012. First report of *Diplodia seriata* as causal agent of olive dieback in Croatia. *Plant Disease* 96, 290.

Kimura M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16, 111–120.

Lazzizera C., S. Frisullo, A. Alves, A.J.L. Phillips, 2008. Morphology, Phylogeny and Pathogenicity of *Botryosphaeria* and *Neofusicoccum* species associated with drupe rot of olives in Southern Italy. *Plant Pathology* 57, 948–956.

López-Escudero F.J., Mercado-Blanco J., 2011. *Verticillium* wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. *Plant Soil* 344, 1–50.

Meyer W., T.G. Mitchell, E.Z. Freedman and R. Vilgalys, 1993. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. *Journal of Clinical Microbiology* 31, 2274–2280.

Mhmoud N.A, S.A. Ahmed, A.H. Fahal, G.S. de Hoog, A.H.G.G. van den Ende, W.W.J. van de Sande, 2012. *Pleu-*

rostomophora ochracea, a novel agent of human eumycetoma with yellow grains. *Journal Clinical Microbiology* 50, 2987–2994.

Moral J., F. Luque and A. Trapero, 2008. First report of *Diplodia seriata*, the anamorph of "*Botryosphaeria*" *obtusa*, causing fruit rot of olive in Spain. *Plant Disease* 92, 311.

Moral J., C. Muñoz-Díez, N. González, A.Trapero, T. Michailides, 2010. Characterization and pathogenicity of Botryosphaeriaceae species collected from olive and other hosts in Spain and California. *Phytopathology* 100, 1340–1351.

Mostert L., Groenewald J.Z., Summerbell R.C., Robert V., Sutton D.A., Padhye A.A., Crous P.W, 2005. Species of *Phaeoacremonium* associated with infections in humans and environmental reservoirs in infected woody plants. *Journal of Clinical Microbiology* 43, 1752–1767.

Niekerk van J.M., P.W. Crous, J.Z. Groenewald, P.H. Fourie, F. Halleen, 2004. DNA phylogeny, morphology and pathogenicity of *Botryosphaeria* species on grapevines. *Mycologia* 96, 781–798.

Nigro F., P. Gallone, G. Romanazzi, L. Schena, A. Ippolito and M.G. Salerno, 2005. Incidence of Verticillium wilt on olive in Apulia and genetic diversity of *Verticillium dahliae* isolates from infected trees. *Journal of Plant Pathology* 87, 13–23.

Page R.D., 1996. TreeView: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12, 357–358.

Phillips A.J.L, F. Fonseca, V. Povoa, R. Castilho, G. Nolasco, 2002. A reassessment of the anamorphic fungus *Fusicoccum luteum* and description of its teleomorph *Botryosphaeria lutea* sp. nov. *Sydowia* 54, 59–77.

Phillips A.J.L., A. Alves, J. Abdollahzadeh, B. Slippers, M.J. Wingfield, J.Z. Groenewald, P.W. Crous, 2013. The *Botryosphaeriaceae*: Genera and species known from culture. *Studies in Mycology* 76, 51–167.

Phillips A.J.L., A. Alves, S.R. Pennycook, P.R. Johnston, A. Ramaley, A. Akulov, P.W. Crous, 2008. Resolving the phylogenetic and taxonomic status of dark-spored teleomorph genera in the *Botryosphaeriaceae. Persoonia* 21, 29–55.

Phillips A.J.L., I.C. Rumbos, A. Alves and A. Correia, 2005. Morphology and phylogeny of *Botryosphaeria dothidea* causing fruit rot of olive. *Mycopathologia* 159, 433–439.

Pitrak D.L., E.W. Koneman, R.C. Estupinan and J. Jackson, 1988. *Phialophora richardsiae* infection in human. *Reviews of Infectious Diseases* 10, 1195–1203.

Rolshausen P.E., J.R. Úrbez-Torres, S. Rooney-Latham, A. Eskalen, R.J. Smith and W.D. Gubler, 2010. Evaluation of pruning wound susceptibility and protection against fungi associated with grapevine trunk diseases. *American Journal of Enology and Viticulture* 61, 113–119.

Romero M.A., M.E. Sánchez, A. Trapero, 2005. First report of *Botryosphaeria ribis* as a branch dieback pathogen of olive trees in Spain. *Plant Disease* 89, 208.

Santos J.M. and A.J.L. Phillips, 2009. Resolving the complex of *Phomopsis* species and their *Diaporthe* teleomorphs on *Foeniculum vulgare*. *Fungal Diversity* 34, 111–125.

Schol-Schwarz M.B., 1970. Revision of the genus *Phialophora*. *Persoonia* 6, 59–94.

Smith H., M.J. Wingfield, P.W. Crous and T.A. Coutinho, 1996. *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. in South Africa. *South African Journal of Botany* 62, 86–88.

Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts, USA.

Taylor R.K., C.N. Hale, W.F.T. Hartill, 2001. A stem canker disease of olive (*Olea europaea*) in New Zealand. *New Zealand Journal of Crop and Horticultural Sciences* 29, 219–228.

Thompson J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins, 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876–4882.

Uberti-Foppa C., L. Fumagalli, N. Gianotti, A.M. Viviani, R. Vaiani and E. Guého, 1995. First case of osteomyelitis due to *Phialophora richardisiae* in a patient with HIV infection. *AIDS* 9, 975–976.

Úrbez-Torres J.R., 2006. Identification and Distribution of *Botryosphaeria* spp. associated with grapevine cankers in California. *Plant Disease* 90, 1503–1490.

Úrbez-Torres J.R., 2011. The status of *Botryosphaeriaceae* species infecting grapevines. *Phytopathologia Mediterranea* 50, S5–S45.

Vijaykrishna D., L. Mostert, R. Jeewon, W. Gams, K.D. Hyde and P.W. Crous, 2004. *Pleurostomophora*, an anamorph of *Pleurostoma* (*Calosphaeriales*), a new anamorph genus morphologically similar to *Phialophora*. *Studies in Mycology* 50, 387–395.

Úrbez-Torres J.R., F. Peduto, P.M. Vossen, W.H. Krueger, W.D. Gubler, 2013. Olive twig and branch dieback: Etiology, incidence, and distribution in California. *Plant Disease* 97, 231–244.

White T.J., T. Bruns, S. Lee and J. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols, a Guide to Methods and Applications* (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). Academic Press Inc., New York, NY, USA, 315–322.

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