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RESEARCH PAPERS

Pleurostomophora richardsiae associated with trunk diseases of grapevines in southern Italy

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Summary. *Pleurostomophora richardsiae* (Nannf. apud Melin & Nannf.) L. Mostert, W. Gams & Crous was previously known mainly as a human pathogen. However, more recently this fungus has been isolated from wood tissue of grapevines that show Petri and esca disease symptoms in California (USA) and South Africa. During an assessment carried out in southern Italy, the abundant presence of this fungus was demonstrated by morphological, cultural and molecular means. *Pleurostomophora richardsiae* was isolated from sub-cortical wood patches and streaking of trunks and cordons of grapevine cultivars that showed decline and dieback symptoms. To understand its putative pathogenic role, pathogenicity tests were conducted in greenhouse experiments, where young grapevine plants of two cultivars were artificially inoculated with two isolates each of *Pl. richardsiae, Lasiodiplodia theobromae* and *Phaeoacremonium aleophilum*. Within 130 d, all three fungi produced brown streaking in both grapevine cultivars. The *L. theobromae* and *Pl. richardsiae* isolates were the most aggressive. Although the *Pm. aleophilum* isolates were pathogenic, they induced less severe wood streaking than the other two fungi. Therefore, *Pl. richardsiae* is considered a fungal pathogen of grapevine. All three fungal species were re-isolated from discolored tissue of all inoculated shoots, thus fulfilling Koch's postulates.

Key words: *Vitis vinifera,* wood browning, phylogeny, pathogenicity.

Introduction

Grapevine diseases that affect the trunks, cordons and branches of grapevine plants can cause serious economic losses worldwide. The esca disease complex is one of the most common diseases of vineyards, and it is widespread in Europe and worldwide in grape-growing countries, such as in France (Larignon and Dubos, 1997), Germany (Fischer and Kassemeyer, 2003), Greece (Rumbos and Rumbou, 2001), Italy (Mugnai *et al.*, 1999), Portugal (Sofia *et al.*, 2006), Spain (Armengol *et al.*, 2001), the United States of America (Gubler *et al.*, 2004), Australia (Pascoe and Cottral, 2000), and South Africa (White *et al.*, 2011). Esca is a disease associated with different vascular and foliar symptoms, such as tracheomycosis and leaf tiger-stripes due to *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* (Crous *et al.*, 1996; Armengol *et al.*, 2001; Rumbos and Rumbou, 2001; Martin and Cobos, 2007), and wood decay or white rot due to basidiomycetes (Fischer, 2006), including *Fomitiporia mediterranea* (the most common causal agent in Europe) (Armengol *et al.*, 2001; Fischer, 2002; Fischer and Kassemeyer, 2003; Martin and Cobos, 2007); *Fomitiporia polymorpha* (North America) (Fischer and Binder, 2004; Fischer, 2006); *Fomitiporia australiensis* (Australia) (Fischer *et al.*, 2005); *Fomitiporia capensis* (South Africa) (Cloete *et al.*, 2014); *Phellinus* sp. (Europe and North America) (Larignon and Dubos, 1997; Fischer and Binder, 2004); and *Stereum hirsutum* (Crous *et al.*, 1996; Mugnai *et al.*, 1996; Larignon

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and Dubos, 1997). Other grapevine trunk diseases with symptoms of vascular discoloration of wood and perennial cankers have been often observed on grapevines (Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Sofia *et al.*, 2006), caused by fungal pathogens such as *Phomopsis viticola* (van Niekerk *et al.*, 2005), *Eutypa lata* (Larignon and Dubos, 1997), and several Botryosphaeriaceae spp. (Phillips, 2002; van Niekerk *et al.*, 2004; Úrbez-Torres, 2011). Additionally, species of *Cylindrocarpon* and *Campylocarpon* (Halleen *et al.*, 2004), *Cylindrocladiella* (Jones *et al.*, 2012), and *Ilyonectria* (Cabral *et al.*, 2011), known to be responsible for black-foot disease, are frequently associated with grapevine decline. Gramaje *et al.* (2011) and Navarrete *et al.* (2011) reported that *Cadophora melinii* and *C. luteo-olivacea* were also associated with grapevine trunk diseases. Eskalen *et al.* (2004), Rolshausen *et al.* (2010), and White *et al.* (2011) concluded that *Pleurostomophora richardsiae* is a vascular pathogen of grapevine in California (USA) and South Africa.

In Italy, the severity of grapevine diseases is particularly significant in older vineyards, because they can cause yield reductions and/or death of the plants (Mugnai *et al.*, 1999). To date, the fungal pathogens recorded are those responsible for esca disease, which are *Phaeoacremonium* spp. (Tegli *et al.*, 2000; Essakhi *et al.*, 2008; Raimondo *et al.*, 2014), *Ph. chlamydospora* (Mugnai *et al.*, 1999), and *F. mediterranea* (Cortesi *et al.*, 2000; Ciccarone *et al.*, 2004), and those responsible for wood diseases, such as several species of Botryosphaeriaceae (Cristinzio, 1978; Burruano *et al.*, 2008; Carlucci *et al.*, 2009; Romanazzi *et al.*, 2009; Linaldeddu *et al.*, 2010; Spagnolo *et al.*, 2011; Mondello *et al.*, 2013), *E. lata* (Minervini and Bisiach, 1995), *Phomopsis viticola* (Mostert *et al.*, 2001; De Guido *et al.*, 2003), and *Cylindrocarpon* spp. (Grasso and Magnano di San Lio, 1975).

During an assessment carried out from 2012 to 2013 in several vineyards in the Apulia region (southern Italy), the main internal and external disease symptoms that occurred across the many grapevine cultivars were different, and included plant decline, trunk dieback and cankers, foliage discoloration, defoliation, and wilt. Among the fungal agents encountered, a large number of isolates resembling *Pl. richardsiae* was collected. Apart from the reports by Eskalen *et al.* (2004), Rolshausen *et al.* (2010), and White *et al.* (2011), little information is available on *Pl. richardsiae* and its pathogenicity on grapevine. Furthermore, its involvement in esca is not clear. Therefore, the present study was carried out to confirm the identity of the *Pl. richardsiae* isolates, assess the pathogenicity of this species and determine its role in grapevine decline, dieback and wood discoloration in the Apulia region.

Materials and methods

Isolations

From December 2012 to December 2013, 124 samples of grapevines were collected from 16 vineyards. These were taken from six different grapevine cultivars: 'Bombino bianco', 'Trebbiano', 'Italia', 'Lambrusco', 'Sangiovese' and 'Montepulciano', from plants that were between 12 and 28 years old (Table 1). The samples consisted of parts of trunks, cordons, canes or shoots of grapevines that showed decline, dieback and various esca symptoms of foliar discoloration, defoliation, and wilt, as well as sub-cortical brown streaking, central brown necrosis, and/or white rot.

Samples were transported to the laboratory for analysis. Following surface sterilisation of the symptomatic trunks and cordons (Fisher *et al.*, 1992), the bark of each sample was removed with a sterile scalpel and thin wood sections (1 to 3 mm thick) were cut from sub-cortical tissues (Figure 1). Small wood portions were placed on malt extract agar [MEA: 2% malt extract (Oxoid Ltd); 2% agar (Difco)] supplemented with 500 mg L^{-1} streptomycin sulphate (Oxoid Ltd), and incubated at 25°C (±3°C) in the dark. After 5–7 d of incubation, conidia were spread over plates of potato dextrose agar (PDA, Oxoid Ltd.) and after incubating overnight, single germinating conidia were transferred to fresh PDA plates. The monoconidial reference strains are maintained in the culture collection of the Department of Sciences, Agriculture, Food and Environment of the University of Foggia, Italy.

The isolation frequency (IF; %) per grapevine cultivar was calculated as the number of tissue portions infected by a given fungus, divided by the total number of tissue segments incubated. The affected plant percentage (APP; %) per grapevine cultivar was also calculated as the number of plants infected by a given fungus divided by the total number of symptomatic plants sampled.

Identifications and molecular characterisation

Genomic DNA of all of the isolates was extracted as described by Carlucci *et al.* (2013). Genera and

species in the Botryosphaeriaceae were identified by reference to the keys, description and sequence data provided in Phillips *et al.* (2013). *Diaporthe* isolates were identified according to Santos and Phillips (2009). *Fomitiporia* isolates were identified according to Fischer (2006). *Phaeoacremonium* isolates were identified by reference to the keys, description and sequence data provided in Mostert *et al.* (2006). *Eutypa lata*, *Epicoccum nigrum*, *Penicillium* spp. and *Aspergillus* spp. were identified based on their micromorphology and culture characters.

The *Pleurostomophora* isolates (435) were initially characterised on the basis of their microsatellite-primed PCR (MSP-PCR) profiles, as described by Santos and Phillips (2009) using the primer M13 (5′– GAG GGT GGC GGT TCT– 3′) (Meyer *et al.*,

Table 1. Isolation frequency (and affected plant percentage) of fungi isolated from grapevine plants of six cultivars.

Fungi isolated	Isolation frequency[a] (affected plant percentage[b])					
	Grapevine cultivar with white berries (age, years)			Grapevine cultivar with red berries (age, years)		
	Bombino bianco (14)	Trebbiano (21)	Italia (17)	Sangiovese (20)	Lambrusco (12)	Montepulciano (28)
Botryosphaeria dothidea	6.8 (10.3)	6.4 (13.5)	5.5 (13.8)	3.4 (7.9)	8.5 (13.5)	4.8 (9.4)
Diplodia seriata	19.4 (70.1)	22.8 (73.1)	22.5 (59.7)	29.3 (73.4)	28.4 (72.4)	28.3 (85.5)
Diplodia corticola	5.3 (19.1)	4.6 (14.4)	3.1 (8.2)	1.9 (41.8)	4.1 (10,4)	0.9 (2.7)
Dothiorella iberica	2.7 (6.2)	3.5 (4.1)	4.9 (8.0)	3.2 (6.1)	1.7 (2.9)	4.3 (6.9)
Dothiorella sarmentorum	1.2 (2.7)	1.4 (1.7)	0.8 (1.3)	1.7 (3.2)	1.2 (2.0)	0.9 (1.4)
Lasiodiplodia theobromae	4.3 (7.5)	4.8 (6.5)	8.2 (13.5)	4.3 (6.9)	5.9 (7.4)	6.4 (5.7)
Neofusicoccum parvum	6.6 (11.8)	6.3 (8.5)	8.5 (11.8)	1.9 (5.2)	4.7 (10.3)	5.7 (9.7)
Botryosphaeriaceae spp.	**46.3 (100)**	**49.8 (92.8)**	**53.5 (100)**	**45.7 (100)**	**54.5 (92.5)**	**51.3 (100)**
Pleurostomophora richardsiae	32.6 (85.6)	36.4 (100)	39.1 (100)	35.3 (87.5)	37.2 (87.5)	30.7 (87.5)
Diaporthe spp.	0.5 (1.2)	0.9 (1.5)	0.3 (1.1)	2.3 (1.8)	0.9 (1.2)	–
Eutypa lata	–	–	–	2.7 (2.1)	1.1 (1.4)	1.4 (1.5)
Fomitiporia mediterranea	–	1.6 (1.8)	2.2 (3.8)	–	1.7 (2.5)	2.4 (2.2)
Phaeoacremonium aleophilum	1.7 (2.5)	0.9 (1.1)	0.3 (1.1)	1.3 (1.1)	–	2.2 (2.1)
Epicoccum nigrum	1.5	–	–	3.5	–	3.1
Penicillium spp.	6.4	3.1	–	2.3	1.5	1.6
Aspergillus spp.	7.5	5.1	1.4	4.7	1.6	2.4
No growth or Bacteria	3.5	2.2	3.2	2.2	1.5	4.9
Vineyards monitored (No.)	3	2	2	3	3	3
Plants analysed (No.)	21	16	17	24	27	19

[a] Isolation Frequency (IF; %) was calculated as the number of tissue portions infected by a given fungus, divided by the total number of tissue segments incubated.

[b] Affected plant percentage (APP; %) per grapevine cultivar was also calculated, as the number of plants infected by a given fungus, divided by the total number of symptomatic plants sampled.

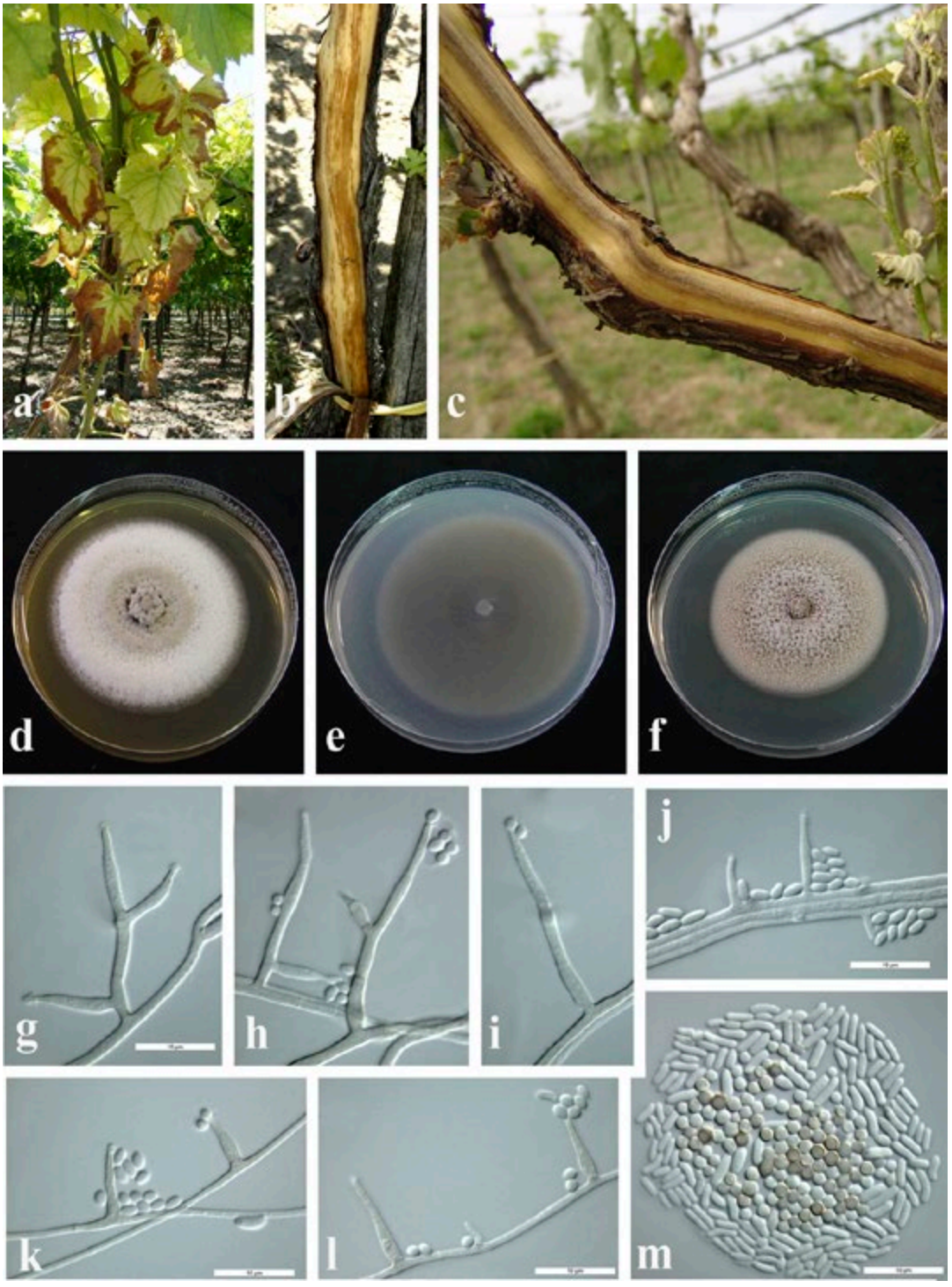

Figure 1. (a–c) Disease symptoms observed on the grapevine plants. (a) Foliar symptoms of discoloration and marginal necrosis. (b, c) Sub-cortical brown streaking on trunk and young cordons. (d–f) Twenty-one-day-old colonies on MEA (d), OA (e) and PDA (f) at 25°C. (g) Branched conidiophores. (h, i) Single conidiophores. (j) Mycelia occurring in bundles of up to three, with type I and type II phialides. (k, l) Type II phialides. (m) Brown, sub-globose, and hyaline, allantoid and oblong-ellipsoidal conidia.

1993). DNA banding patterns were analysed with BioNumerics software, version 5.1 (Applied Maths), using Pearson's correlation coefficients and the unweighted pair group method with arithmetic mean. The reproducibility levels were calculated by comparing the banding profiles obtained for the M13 primer. For this purpose, from any cluster, a number of strains that represented about 10% was chosen at random, and their profiles were re-analysed.

DNA amplification and sequencing

The 5.8S rDNA gene and flanking internal transcribed spacers 1 and 2 (ITS) of 30 representative *Pleurostomophora*, Botryosphaeriaceae, *Diaporthe* and *Fomitiporia* strains were amplified with primers ITS1 and ITS4 (White *et al.*, 1990). In addition, part of the translation elongation factor-1-α (EF 1-α) was amplified with primers EF1-688F and EF1-1251R (Alves *et al.*, 2008) for the Botryosphaeriaceae. For the *Phaeoacremonium* strains, the β-tubulin (TUB) and actin genes (ACT) were amplified, respectively, using the primers T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995), and ACT-512F and ACT-783R (Carbone and Kohn, 1999). The amplified PCR fragments were purified using purification kits (NucleoSpin extract II; Macherey-Nagel) before DNA sequencing. Both strands of the PCR products were sequenced by PRIMM srl service. The nucleotide sequences were edited with BioEdit, version 7.0.9. Identifications of all sequences were made by BLAST searches in GenBank and, where possible, compared with sequences from ex-type isolates. In addition, the ITS sequences of *Pleurostomophora* strains were aligned with additional sequences retrieved from GenBank, using ClustalX, version 1.83 (Thompson *et al.*, 1997) for phylogenetic study.

Phylogenetic analyses of the *Pleurostomophora* dataset were performed with PAUP, version 4.0b10 (Swofford, 2003) for neighbour joining and maximum parsimony (MP) analyses. (NJ) analysis used the Kimura-2 parameter substitution model (Kimura, 1980). All characters were unordered and of equal weight. Bootstrap values were obtained from 1000 replicates. For the MP analysis, alignment gaps were treated as missing data and all characters were unordered and of equal weight. MP 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 using 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 (www.treebase.org). *Diaporthe ambigua* (AJ458389) was used as the outgroup in the phylogenetic analyses.

Morphology

The *Pleurostomophora* isolates were morphologically characterised on MEA (Vijaykrishna *et al.*, 2004), PDA, and oatmeal agar (OA; 30 g oats, 8 g Oxoid agar, 1000 mL water), incubated at 25 ± 2°C in the dark for 16 d. Colony morphology and colour were assessed on MEA, PDA and OA at 25 ± 2°C after 21 d using the colour charts of Rayner (1970). Cardinal temperatures for growth were determined on MEA incubated in the dark at temperatures ranging from 5°C to 40°C, at 5°C intervals and including 37°C. Radial growth was measured after 8 d at 25 ± 2°C, on MEA plates.

Micromorphological characterisation was performed according to Carlucci *et al.* (2012). Dimensions and morphology of conidiophore structures and the size, phialide type and shape, bundles, conidial shape and size were measured from 100% lactic acid mounts by 30 measurements (×1000 magnification), using a Leica Application Suite (LAS) measurement module (Leica Microsystem GmbH). Phialide type and shape were determined according to Mostert *et al.* (2006). Photomicrographs were recorded with a Leica DFC320 digital camera on a Leica DMR microscope fitted with Normaski differential interference contrast optics. The 5th and 95th percentiles were calculated for all of the measurements, and extremes are presented (in parentheses).

Pathogenicity tests

To assess the pathogenic ability of *Pl. richardsiae*, pathogenicity tests were performed with two isolates (PLEU188; PLEU420). For comparative purposes,

two isolates each of *Pm. aleophilum* (Pm5; Pm37) and *Lasiodiplodia theobromae* (Botryosphaeriaceae) (Bot97; Bot1278) were included. *Phaeoacremonium aleophilum* was chosen because it is one of main fungal agents involved in the esca complex, while *L. theobromae* is one of the more virulent members of the Botryosphaeriaceae on grapevines (Úrbez-Torres, 2011). Pathogenicity tests were carried out in May 2013, on green shoots (0.5–1.5 cm diam.) of 5-year-old plants of grapevine cvs. 'Lambrusco' (red berries) and 'Trebbiano' (white berries) grown in pots in a greenhouse.

Agar plugs (0.5–1.5 cm diam.) taken from 10-d-old fungal cultures grown on PDA at 23 ± 2°C were inserted into wounds (1.0–2.0 cm long) made with a sterile scalpel under the bark of the shoot surfaces. After inoculation, the wounds were wrapped with wet sterile cotton wool and sealed with Parafilm. The controls were inoculated with sterile agar plugs. Each treatment included 20 replicates. The shoots were examined 70 d (end of July 2013) and 130 d (end of September 2013) after inoculation, and the length of the necrotic lesion was measured. Isolations were made from the inoculated shoots and the resulting isolates were identified.

To test if the data followed normal distributions, the Shapiro-Wilk test (W test) was used. Homogeneity of variance of datasets on the base of two cultivars and of samplings at 70 and 130 d after inoculation was assessed using the Levene test. A factorial ANOVA analysis was performed separately for each fungal species on the basis of two cultivars and of samplings at 70 and 130 d after inoculation, in order to determine the significance of any differences in lesion lengths caused by the isolates for the same fungal species, differences due to grapevine cultivar, and to detect any interaction between these factors (isolate × cultivar). A one-way analysis of variance (ANOVA) was performed using Statistica, version 6 (StatSoft), to assess the significance of differences in the extent of vascular discoloration induced by *Pm. aleophilum*, *Pl. richardsiae* and *L. theobromae* in both cultivars. Duncan's test was used for comparison of treatment means at $P<0.05$.

Results

Isolates

The mycobiota isolated from the symptomatic grapevine samples is outlined in Table 1. About 50% of the fungi isolated from sub-cortical tissues of the plants from the grapevine cultivars belonged to the Botryosphaeriaceae (*Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum* spp.), with isolation frequencies (IFs) ranging from 45.7% on 'Sangiovese' to 54.5% on 'Lambrusco'. The affected plant percentages (APPs) for the Botryosphaeriaceae species were high, with values ranging from 92.5% to 100%. The most common species isolated was *Diplodia seriata* with isolation frequencies ranging from 19.4% on 'Bombino bianco' to 29.3% on 'Sangiovese', while *B. dothidea*, *D. corticola*, *Do. iberica*, *Do. sarmentorum*, *L. theobromae* and *N. parvum* were isolated with IFs less than 10% (Table 1).

Pleurostomophora richardsiae was abundantly isolated from all of the grapevine cultivars, with IFs ranging from 30.7% on 'Montepulciano' to 39.1% on 'Italia'. APPs were similar to those of the Botryosphaeriaceae species, ranging from 85.6% to 100% (Table 1). The ranges of the IFs and APPs of *Pm. aleophilum* and *F. mediterranea* from sub-cortical tissues were less than those for the above fungi. IFs ranged from 0.3% on 'Italia' to 2.4% on 'Montepulciano', while APPs ranged from 1.1% to 3.8%. Other fungi that are frequently reported as associated with grapevine plants, such as *Diaporthe* spp. and *E. lata*, were isolated from relatively few plants. IFs ranged from 1.1% on 'Lambrusco' to 2.7% on 'Sangiovese'. *Epicoccum nigrum*, *Penicillium* spp. and *Aspergillus* spp. were isolated from these grapevine plant samples with IFs ranging from 1.4% on 'Italia' to 7.5% on 'Bombino bianco'. Since the IFs of these fungi were so small, they were not considered to play any significant role in the disease and thus were not investigated further.

Molecular identification and phylogenetic analysis

The MSP-PCR dendrogram generated one cluster (Figure 2), from which five isolates from each grapevine cultivar were chosen as representative for sequencing, for a total of 30 isolates (Table 2). The ITS dataset consisted of 44 taxa, including the outgroup (*Diaporthe ambigua*) (Table 2). After alignment, and following the exclusion of incomplete portions at either end, the dataset consisted of 518 characters of which 316 were constant, while 58 were variable and parsimony uninformative. MP analysis of the remaining 144 parsimony-informative characters resulted in three most parsimonious trees, one of which is shown in Figure 3, (TreeBase S16188). All of the trees retained showed the following values: tree

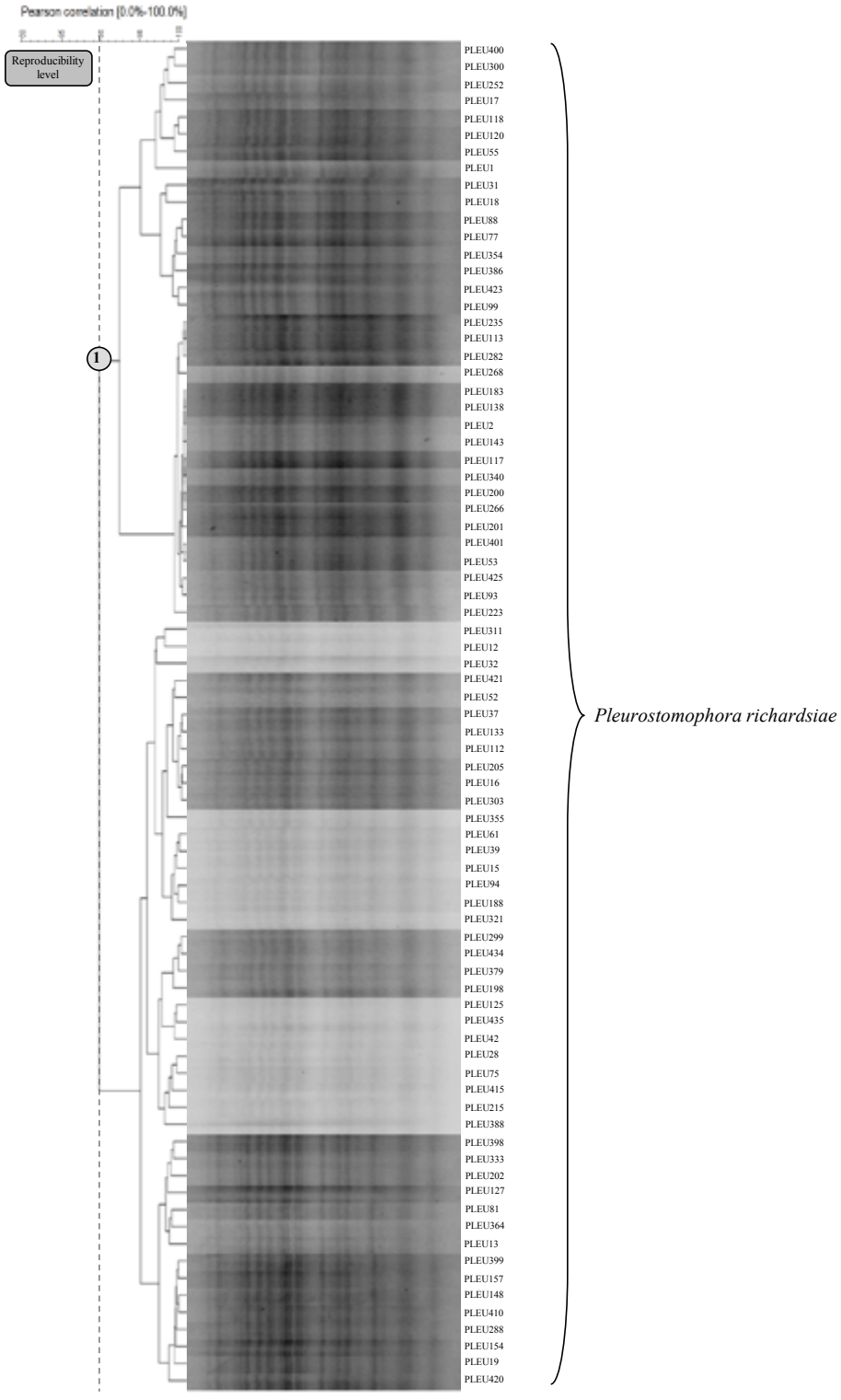

Figure 2. Consensus cladogram from MSP-PCR profiles obtained with primer M13. The vertical dashed line corresponds to the reproducibility level (90%) from which one cluster of isolates is inferred (indicated by numbered circle).

Table 2. Sources of the fungal species and GenBank accession numbers used in the phylogenetic analyses.

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	PLEU12	Grapevine cv. Bombino bianco	Cerignola, Italy	A. Carlucci	–
	PLEU16	Grapevine cv. Bombino bianco	Cerignola, Italy	F. Lops	KF573184
	PLEU252	Grapevine cv. Bombino bianco	Canosa di Puglia, Italy	A. Carlucci	KM252714
	PLEU340	Grapevine cv. Bombino bianco	Canosa di Puglia, Italy	F. Lops	–
	PLEU401	Grapevine cv. Bombino bianco	San Severo, Italy	M.L. Raimondo	–
	PLEU2	Grapevine cv. Trebbiano	Foggia, Italy	M.L. Raimondo	–
	PLEU15	Grapevine cv. Trebbiano	San Severo, Italy	M.L. Raimondo	–
	PLEU188	Grapevine cv. Trebbiano	San Severo, Italy	M.L. Raimondo	KM252715
	PLEU268	Grapevine cv. Trebbiano	Cerignola, Italy	A. Carlucci	KM252716
	PLEU355	Grapevine cv. Trebbiano	Cerignola, Italy	A. Carlucci	–
	PLEU28	Grapevine cv. Italia	Foggia, Italy	A. Carlucci	KF573187
	PLEU112	Grapevine cv. Italia	Cerignola, Italy	F. Lops	–
	PLEU299	Grapevine cv. Italia	Cerignola, Italy	A. Carlucci	–
	PLEU379	Grapevine cv. Italia	Trinitapoli, Italy	A. Carlucci	KM252717
	PLEU435	Grapevine cv. Italia	Canosa di Puglia, Italy	F. Lops	–
	PLEU1	Grapevine cv. San Giovese	Foggia, Italy	A. Carlucci	KF573182
	PLEU18	Grapevine cv. San Giovese	Cerignola, Italy	A. Carlucci	KF573185
	PLEU99	Grapevine cv. San Giovese	Cerignola, Italy	A. Carlucci	–
	PLEU202	Grapevine cv. San Giovese	San Severo, Italy	M.L. Raimondo	–
	PLEU388	Grapevine cv. San Giovese	Torremaggiore, Italy	M.L. Raimondo	–
	PLEU13	Grapevine cv. Lambrusco	Cerignola, Italy	A. Carlucci	KF573183
	PLEU154	Grapevine cv. Lambrusco	Cerignola, Italy	A. Carlucci	–
	PLEU215	Grapevine cv. Lambrusco	San Ferdinando, Italy	A. Carlucci	–

Species	Isolate number[a]	Host	Locality	Collector	GenBank ITS
	PLEU364	Grapevine cv. Lambrusco	Trinitapoli, Italy	F. Lops	–
	PLEU420	Grapevine cv. Lambrusco	Canosa di Puglia, Italy	A. Carlucci	KM252718
	PLEU19	Grapevine cv. Montepulciano	San Severo, Italy	M.L. Raimondo	KF573186
	PLEU77	Grapevine cv. Montepulciano	Foggia, Italy	M.L. Raimondo	–
	PLEU148	Grapevine cv. Montepulciano	Canosa di Puglia, Italy	F. Lops	–
	PLEU223	Grapevine cv. Montepulciano	Cerignola, Italy	A. Carlucci	KM252719
	PLEU410	Grapevine cv. Montepulciano	Cerignola, Italy	A. Carlucci	–
	CBS 270.33	–	Sweden	E. Melin	AY729811
	CBS 117177	*Vitis vinifera*	Paarl, South Africa	L. Mostert	EF042106
Pm. aleophilum	**CBS 246.91**	Root and stem	Yugoslavia	M. Muntañola-Cvetković	AF017651
Pm. angustius	CBS 101737	*Vitis vinifera*	France	P. Larignon	AF197976
Pm. inflatipes	**CBS 391.71**	*Quercus virginiana*, Stem	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.

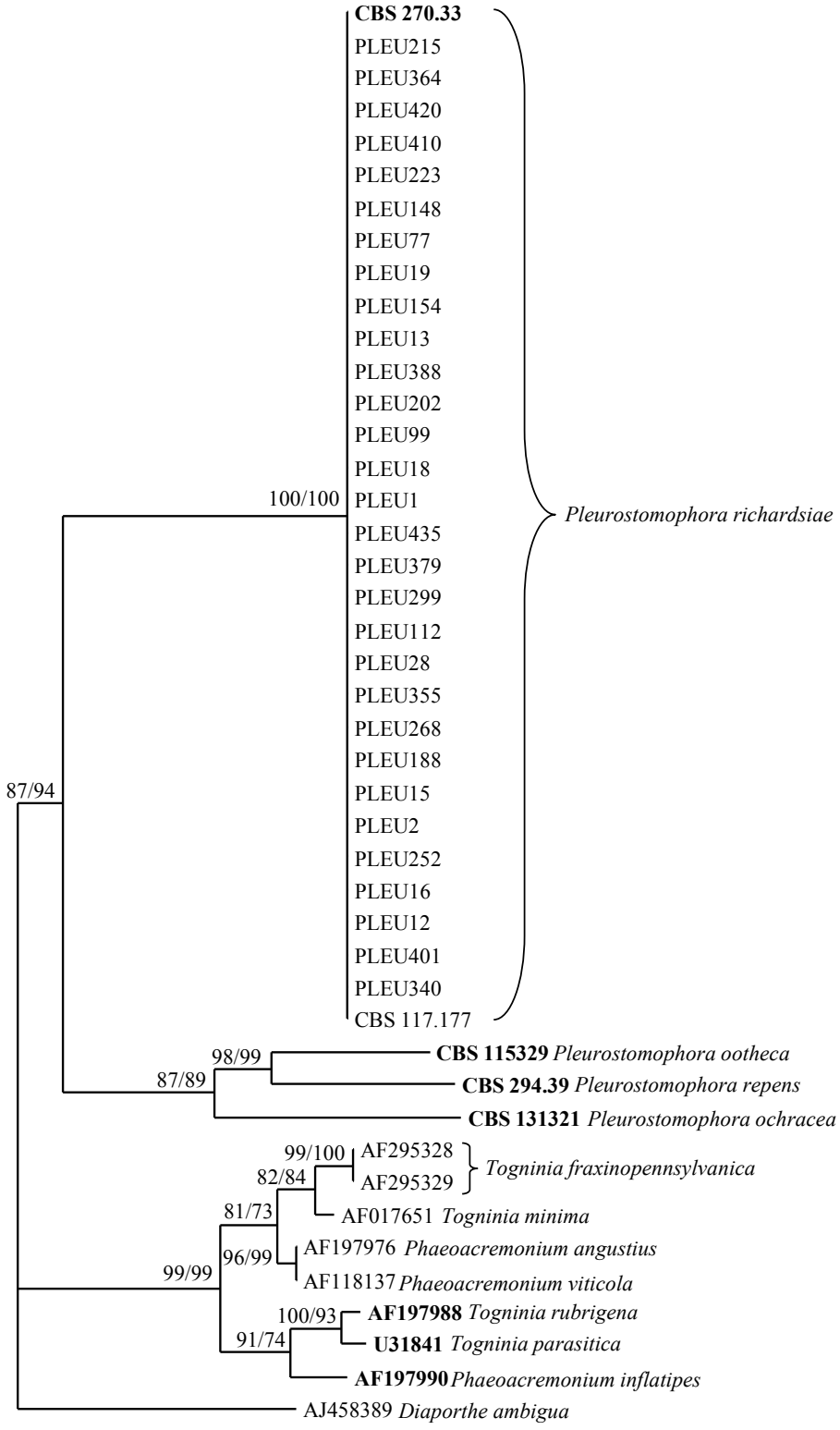

Figure 3. One of the most parsimonious trees obtained from alignment of the ITS sequence data with bootstrap support values valid for NJ/MP. *Ex-type* isolates are in bold face. *Diaporthe ambigua* was used as an outgroup.

length 348, consistency index 0.816, retention index 0.923; rescaled consistency index 0.753, and homoplasy index 0.184. Topology of the NJ tree was similar to that of the MP tree. The 30 *Pleurostomophora* isolates sequenced in this study clustered in the same clade as the ex-type isolate of *Pl. richardsiae* (CBS 270.33; GenBank AY729811).

Morphology

To date, a detailed morphological description of *Pl. richardsiae* is not available, and the last one by De Hoog *et al.* (2000) was referred to as *Phialophora richardsiae*. Therefore we provide a full description of this species.

Colonies on MEA reaching 53 to 55 mm diam. after 8 d at 25 ± 2°C. After 21 d, colonies on MEA cottony, with entire margins, tilleul buff (17‴f) to white towards the periphery, both above and reverse; colonies on OA flat, with entire margin, tawny-olive (17‴i), both above and reverse; colonies on PDA cottony, with entire margin, tilleul buff (17‴f) to fawn (13‴), both above and reverse. Minimum temperature for growth 10°C, optimum 25°C, and maximum 35°C. *Mycelium* composed of branched septate, sometimes fasciculate, hyphae. *Conidiophores* (23–)25–34.5(–37) (av. 29.9) µm × (1.3–)1.5–1.8(–2) (av. 1.7) µm, mostly short and unbranched, erect, up to three-septate, ending in a single terminal phialide, sometimes with a single lateral phialide, occasionally branched, (21–)23–26(–28) (av. 24.7) µm × (1–)1.3–1.5 (av. 1.4) µm. *Phialides* terminal or lateral, mostly monophialidic, hyaline with collarettes 1–1.5 µm long, 0.5–0.7 µm wide; type I phialides (sensu Mostert et al., 2006) were mostly cylindrical, elongated, occasionally swollen at the bases, (5–) 6–7.5 (–8) (av. 6.8) µm × (1.8–)2–2.4(–2.7) (av. 2.1) µm; type II phialides were predominant, elongate-ampulliform and constricted at the bases to subulate, tapering towards the apices, (11.5–)13–15(–17) (av. 14.1) µm × (1.5–)2–2.3(–3) (av. 2.1) µm; type III phialides not seen. *Conidia* either brown and sub-globose, (1.0–)1.5 (av. 1.4) µm diam., L/W = 1.0, or hyaline, reniform to allantoid, (5–)5.8–6.2(–7) (av. 6.0) µm × (1.5–)2.0(–2.5) (av. 2.0) µm, L/W = 3.1, or oblong-ellipsoidal, (7–)8–9(–11.5) (av. 8.4) µm × (1.5–)2.0–2.5(–3) (av. 2.3) µm, L/W = 3.7.

Pathogenicity tests

According to the Shapiro-Wilk test, data from the pathogenicity tests of 70 and 130 d after inoculations followed normal distributions with W values of 0.93 for 70 d (P<0.00001) and 0.96 for 130 d (P<0.00001). The Levene test revealed that homogeneity of variance was significant for both grapevine cultivars. The F values related to 'Trebbiano' were 11.02 (P<0.001) at 70 d and 7.14 (P<0.001) at 130 d after inoculation, while those related to 'Lambrusco' were 9.92 (P<0.001) at 70 d and 6.88 (P<0.001) at 130 d after inoculation.

Factorial ANOVA analysis demonstrated that no significant differences could be detected between the grapevine cultivars inoculated with *Pm. aleophilum* (70 d, $F_{1,1} = 0.33$, $P = 0.57$; 130 d, $F_{1,1} = 0.08$, $P = 0.77$), *Pl. richardsiae* (70 d, $F_{1,1} = 0.57$, $P = 0.45$; 130 d, $F_{1,1} = 0.28$, $P = 0.60$) and *L. theobromae* (70 d, $F_{1,1} = 0.75$, $P = 0.39$; 130 d, $F_{1,1} = 0.26$, $P = 0.61$). Significant differences between the *Pm. aleophilum* isolates were detected in sampling at 70 d after inoculation ($F_{1,1} = 21.5$, P<0.001), while in sampling at 130 d, no significant differences were detected ($F_{1,1} = 1.33$, $P = 0.25$). Significant differences between the *L. theobromae* isolates were detected in both samplings (70 d, $F_{1,1} = 13.61$, P<0.001; 130 d, $F_{1,1} = 10.11$, $P = 0.0021$). No significant differences between the *Pl. richardsiae* isolates were detected in both samplings (70 d, $F_{1,1} = 0.18$, $P = 0.67$; 130 d, $F_{1,1} = 0.001$, $P = 0.99$). Interactions of cultivars with *Pm. aleophilum* isolates were significant ($F_{1,1} = 4.26$, $P = 0.04$) in sampling at 130 d after inoculation, with *Pl. richardsiae* isolates ($F_{1,1} = 6.83$, $P = 0.011$) in sampling at 130 d after inoculation, while no significant differences were detected with *L. theobromae* isolates in both samplings.

Mean lengths of the extent of vascular discolorations caused by *Pm. aleophilum*, *Pl. richardsiae* and *L. theobromae* (one-way analysis of variance) are reported in Table 3. All of the fungal species produced brown wood discolorations on shoots of both grapevine cultivars. *Lasidiplodia theobromae* was the most aggressive species on both grapevine cultivars. Seventy days after inoculation, *L. theobromae* produced the strongest brown wood discoloration, ranging from a mean length of 34.1 cm (on cv. 'Trebbiano') to 35.7 cm (on 'Lambrusco'). After 130 d, the pathogen had caused discolorations of up to mean length 69.2 cm on 'Lambrusco'. *Pleurostomophora richardsiae* was also aggressive on both grapevine cultivars; after 70 and 130 d, respectively, it produced discolorations of mean length 26.7 to 48.2 cm, close to the extent of those caused by *L. theobromae*.

Phaeoacremonium aleophilum produced brown wood discolorations on both grapevine cultivars but

Table 3. Brown streaking lengths (Mean, SD, Max, Min) in grapevine stems from pathogenicity essays carried out with *Lasiodiplodia theobromae*, *Pleurostomophora richardsiae* and *Phaeoacremonium aleophilum*.

Fungal species	Isolate ID	Brown streaking length (cm)						Re-isolation	
		After 70 days			After 130 days			(%)	Dead shoot
		Mean	SD	Max-Min[a]	Mean	SD	Max-Min[a]		
Symptoms that occurred on 'Trebbiano'									
Control	H_2O d	0.3 A	–	–	0.4 A	–	–	–	No
Pm. aleophilum	Pm5	7.5 B	2.3	11.4–3.6	31.1 B	3.8	36.5–24.5	48	Yes[b]
	Pm37	10.1 B	2.4	14.1–5.9	30.4 B	2.7	34.9–25.6	72	Yes
Pl. richardsiae	PLEU188	25.8 D	3.3	31.8–18.5	44.5 C	5.6	55.3–31.3	88	Yes
	PLEU420	26.4 D	3.6	32.7–19.5	47.6 D	5.1	56.3–33.5	94	Yes
L. theobromae	BOT1278	29.8 E	6.2	40.2–18.3	51.7 E	6.2	60.1–36.8	100	Yes
	BOT97	34.1 F	5.6	43.5–23.6	56.0 F	7.1	67.8–37.1	98	Yes
Symptoms that occurred on 'Lambrusco'									
Control	H_2O d	0.4 A	–	–	0.4 A	–	–	–	No
Pm. aleophilum	Pm5	7.8 B	2.6	13.8–3.9	29.7 B	3.3	35.4–23.6	69	Yes
	Pm37	10.4 C	2.6	17.7–7.2	32.1 B	3.4	38.6–26.5	84	Yes
Pl. richardsiae	PLEU188	26.7 D	3.5	31.7–19.5	45.2 C	4.2	58.4–39.5	92	Yes
	PLEU420	26.7 D	3.1	31.8–18.3	48.2 D	5.8	57.2–32.8	98	Yes
L. theobromae	BOT1278	30.5 E	5.6	39.5–21.5	52.2 E	6.4	65.4–38.6	90	Yes
	BOT97	35.7 F	5.5	44.1–24.5	57.0 F	6.0	69.2–42.7	100	Yes

[a] Maximum and minimum values detected on the basis of 20 observations.
[b] Dead, no resumption of shoot was observed up to the end of June 2013.
Values followed by a different capital letter in each column are significantly different according to Duncan's tests ($P<0.05$).

the discolorations were shorter than those caused by *L. theobromae* and *Pl. richardsiae*, of mean length between 29.7 cm and 32.1 cm at 130 d after inoculation. The fungal species were re-isolated from discolored tissue of all inoculated shoots, fulfilling Koch's postulates (Table 3).

Discussion

The fungi isolated during the assessments carried out in Apulia can cause wood disease such as vascular discoloration and cankers on grapevine trunks and branches. *Pleurostomophora richardsiae* was isolated frequently from wood showing brown sub-cortical patches and streaking. It was isolated often as pure cultures from xylem tissue of young plants that showed general decline symptoms, and from xylem of the older plants that showed both canker and esca symptoms. *Pleurostomophora richardsiae* was always isolated in association with *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia* or *Neofusicoccum* species, and sometimes also with *Phaeoacremonium* spp. and *Fomitiporia mediterranea*.

In the pathogenicity tests, *L. theobromae, Pl. richardsiae* and *Pm. aleophilum* were pathogenic on both grapevine cultivars tested. *Lasiodiplodia theobromae* and *Pl. richardsiae* were the most aggressive, as they caused longer brown wood discolorations than *Pm.*

aleophilum. As the APPs for *Pl. richardsiae* were high and similar to those for *L. theobromae*, we consider *Pl. richardsiae* to be a serious pathogen of grapevine in southern Italy. In a similar way, Halleen *et al.* (2007) considered this species to be a serious threat to South African grapevines. We worked under artificial conditions, so these effects still need to be confirmed in field experiments over long periods, where they might be influenced by environmental variables. However, artificial inoculation allowed us to directly demonstrate that *Pl. richardsiae* can infect the wood tissues of grapevines in the same way as the other fungi examined. We conclude that *Pl. richardsiae* can cause sub-cortical patches that evolve into longitudinal brown streaking of the wood in younger grapevines, which later extends both longitudinally and through the cross-section of the wood, often in association with other fungi.

Pleurostomophora richardsiae was recombined from *Phialophora richardsiae* by Vijaykrishna *et al.* (2004), who used molecular tools, and at the same time stated that *Pl. richardsiae* differs morphologically from the type *Pl. ootheca* and also from *Pl. repens*. Previously, *Pl. richardsiae* was known as the causal agent of a subcutaneous phaeohyphomycotic cyst after traumatic implantation (Guého *et al.*, 1989), and as a human pathogen (Pitrak *et al.*, 1988; De Hoog *et al.*, 2000). More recently, Eskalen *et al.* (2004) reported a fungus from grapevine roots and trunks with vascular discoloration due to general physical stress that they attributed to a *Phialophora* sp. Subsequently, Rolshausen *et al.* (2010) demonstrated that *Pl. richardsiae* can infect and colonise pruning wounds of grapevines after artificial inoculation, and thus cause vascular discoloration and trunk disease. The symptoms observed by Eskalen *et al.*, (2004) were similar to those observed in the present study, although we identified *Pl. richardsiae* as a causal agent from plants that showed the tiger leaf symptoms that are characteristic of esca, and from plants that showed foliar chlorosis with necrotic margins, but no wood discoloration. It is likely that the *Phialophora* sp. reported by Eskalen *et al.* (2004) is *Pl. richardsiae*, although it was neither possible to compare the morphological features nor the ITS sequences, because they are not available. In addition, White *et al.* (2011) confirmed that *Pl. richardsiae* is involved in the esca disease complex, although it was hard to understand the individual behaviour of the fungus, because different fungal species were isolated from symptomatic wood tissue. On the basis of results obtained from our study, several different species of fungi were isolated from individual grapevine samples, but *Pl. richardsiae* was the most frequently isolated species. This indicates that it could be a pathogen of grapevines and this was confirmed by the pathogenicity tests carried out in this study. Therefore, we consider that *Pl. richardsiae* is a pathogen causing trunk disease together with *N. parvum* and *L. theobromae*, known trunk disease pathogens of grapevine. In Italy, this is the first report of brown sub-cortical patches and streaking on grapevine wood caused by *Pl. richardsiae*. Recently, and for the first time, Carlucci *et al.* (2013) associated this fungus with brown wood streaking and decline of olive trees in southern Italy and worldwide. Further studies are necessary to confirm the role of *Pl. richardsiae* in grapevine trunk diseases worldwide, and to investigate its host range.

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