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RESEARCH PAPERS - 10TH SPECIAL ISSUE ON GRAPEVINE TRUNK DISEASES

Fungi associated with grapevine trunk diseases in nursery-produced *Vitis vinifera* plants

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Summary. Grapevine trunk diseases (GTDs) are one of the most important groups of fungal diseases affecting grapevine plants worldwide. One of the main causes of GTDs infection occur during nursery plant production processes. The phytosanitary status was determined for 150 young grapevine plants (two varieties grafted onto different rootstocks) that were produced in three European nurseries. Some plants were analyzed upon submission, while others were assessed after up to 12 months growth in a greenhouse. Fungal species associated with GTDs were identified and characterized from the scions, graft unions, rootstocks, or roots. A total of 449 fungal isolates associated with GTDs were obtained, and 20 species were identified by morphological characteristics and DNA analyses. Five species were involved in Botryosphaeria dieback, six in black foot disease, six in Diaporthe dieback, and three in Petri disease. Incidence of GTDs on grapevine plants was between 81 and 100%, with different diseases varying between rootstocks and grapevine varieties. Isolates of other fungal genera not involved in the GTD complex were also detected, including *Colletotrichum*, *Fusarium*, and *Rhizoctonia*. The high presence of GTDs during nursery production of grapevine plants raises the need to implement effective control methods that could prevent the spread of these diseases to vineyards.

Key words: Botryosphaeriaceae, *Cylindrocarpon*, *Diaporthe*, trunk diseases, Petri disease, young vine decline.

Introduction

Grapevine trunk diseases (GTDs) are one of the most destructive groups of fungal diseases affecting *Vitis vinifera* worldwide (Larignon and Dubos 1997; Agustí-Brisach *et al*., 2013a; Gramaje *et al*., 2018). Several trunk disease pathogens cause premature decline and dieback of grapevines. These pathogens include: the causal agents of Petri disease; species of the Botryosphaeriaceae which cause Botryosphaeria dieback (Úrbez-Torres, 2011); *Phomopsis viticola* (*Diaporthe ampelina*) which is the leading cause of Diaporthe dieback (Fourie and Halleen 2004; Úrbez-Torres *et al*., 2013); and *Cylindrocarpon*-like species which cause black foot disease (Halleen *et al*., 2004; Agustí-Brisach and Armengol, 2013).

Several authors have reported decreases in survival rates of grafted grapevines affected by GTDs grown in nurseries and young vineyards (Halleen *et al*., 2003, 2004; Gramaje *et al*., 2009; Rego *et al*., 2009; Agustí-Brisach *et al*., 2011; Gramaje and Armengol, 2011; Cabral *et al*., 2012a; Gramaje *et al*., 2018). These decreases are probably due to the banning of sodium arsenite for disease control (Mugnai *et al*., 1999; Bertsch *et al*., 2013; Gramaje *et al*., 2018). The external symptoms of the diseases in young grapevines include stunted growth, reduced vigour, delayed or absent sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins, bud mortality, fruit rotting, cane bleaching, failure of the graft unions, wilting, and dieback. All of these symptoms may be accompanied by sunken necrotic root lesions and reductions in the root biomass and root hairs (Gramaje and Armengol, 2011). The decline of young vines due to fungal infections has mainly been attributed to *Cylindrocarpon*-like species, Petri disease

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fungi, and Botryosphaeriaceae species acting alone or, more frequently in combination (Giménez-Jaime *et al*., 2006; Rego *et al*., 2009; Probst *et al*., 2012; Carlucci *et al*., 2017). Analysis of canes of mother-plants of rootstocks and scions prior to grafting has also shown that they were mainly infected with Botryosphaeriaceae species (Rego *et al*., 2009, Billones-Baaijens *et al*., 2013a, 2013b, 2015) and *Diaporthe ampelina* (Rego *et al*., 2009).

Traditional propagation techniques used in viticulture can have significant effects on the quality of the vines produced, and apparently healthy grafted plants also contain fungi that cause GTDs (Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013b; Carlucci *et al*., 2017). The aim of the research described in the present paper was to determine the phytosanitary status of young grapevine plants produced in three grapevine nurseries, and to identify and characterize the fungi associated with GTDs.

Materials and methods

Plant material

The phytosanitary status was examined of 150 young grapevine plants [62 of *Vitis vinifera* 'Albariño'; 22 grafted onto rootstock 196-17 Castel (196-17C); 40 onto rootstock 110 Richter (110 R); 88 of *V. vinifera* 'Savagnin' grafted onto rootstock Fercal 242]. The plants were produced in three different commercial nurseries, two in Spain (nurseries 1 and 2) and one in France (nursery 3). All grafted vines holding plant passports had good external condition upon submission. The study of 'Albariño' variety plants grafted onto 196-17C rootstock (from nursery 2) was conducted upon arrival to the laboratory. 'Albariño' plants grafted onto 110R rootstock (nursery 1) and 'Savagnin' plants (nursery 3) were transplanted into 6 L capacity pots containing a mixture of commercial substrate and sand (1:1). These plants were grown in a greenhouse for 12 months ('Albariño' plants on 110R rootstock) or 6 months ('Savagnin' plants), with manual watering each week.

Fungal isolations

To isolate the fungi associated with GTDs, each plant was cut into four pieces of approx. 3–5 cm: scion, graft union, rootstock, and roots (where fragment "roots" comprised both the base of the rootstock and the roots). The potted plants were carefully removed from their containers, and their roots were washed to eliminate any residual substrate. Wood pieces were peeled, and all pieces were rinsed for 1 min. in 1% sodium hypochlorite solution, and then rinsed twice (5 min. each) with distilled water. Each plant fragment was left to dry on non-sterile paper towels for at least 2 h. The pieces were then each cut into several small pieces and included in a 2% malt extract agar (MEA, Difco, Becton Dickinson) medium supplemented with 0.5 g L^{-1} of streptomycin sulfate (MEAs) under sterile conditions. Approximately ten to 14 wood pieces from each section were placed in two MEAs Petri dishes (five to seven pieces per dish), which were sealed with Parafilm® (Bemis Co.), and then incubated in the dark at 24ºC for 1 month. The dishes were examined daily under the microscope to check for fungal growth.

Disease assessments

Incidence of GTDs was calculated as the number of plants affected by any GTD divided by the total number of plants and the incidence of each disease and plant parts was calculated in the same way, considering one or more species causing one type of GTD as one. Associations between GTD and nursery, or between GTD and grapevine plant parts, were assessed using two simple correspondence analyses, conducted using IBM SPSS Statistics v. 22.0 (SPSS Inc.).

Fungal identification

Morphological identification

Cultures grown in MEAs were initially classified based on the macroscopic mycelium characteristics, including colony shape, texture, colour and growth rate, as well as on microscopic features, including shape and colour of mycelia, and colour, shape and size of the conidia. Isolates of fungi associated with GTDs were subcultured on potato dextrose agar (PDA) (Difco) by hyphal-tip subculturing, and were then incubated in the dark at 24ºC. Morphological features were analyzed with a Nikon Eclipse E600 microscope, and measurements were made with a Nikon digital camera DXM1200 and the measurement module of NIS-Elements. The *Cylindrocarpon*-like isolates were grown on Spezieller Nährstoffarmer agar (SNA) (Nirenberg, 1976) in Petri dishes also containing four pieces (1 cm^2) of filter paper (Alaniz *et al*., 2007), in

order to enhance sporulation. The Botryosphaericeae spp. isolates were subcultured on pine needle agar (PNA) to promote the production of pycnidia (Phillips *et al*., 2013). The SNA and PNA cultures were incubated at 25ºC under NUV + fluorescent illumination with a 12-h photoperiod. Cardinal temperatures for growth of isolates were determined on PDA incubated in the dark at temperatures from 5 to 40°C (at 5ºC intervals), with four replicate plates of selected cultures at each temperature. Radial colony growth of the isolates was measured by taking two colony diameter measurements perpendicular to each other.

Molecular identification and characterization

A total of 276 isolates were analyzed using amplification and sequencing with different molecular markers. This was carried out for 86 *Cylindrocarpon*-like isolates, 130 Botryosphaeriaceae isolates, 30 *Diaporthe* isolates, and 30 isolates associated to Petri disease fungi. Genomic DNA was extracted from 2 to 7 d old pure colonies of GTD-associated fungi grown in PDA, using the commercial kit E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek), and following the short protocol recommended by the manufacturer. For preliminary molecular identifications, the internal transcribed spacer region and intervening 5.8S nrRNA gene (ITS) was amplified for all isolates, with the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White *et al*., 1990). Six additional loci were amplified and sequenced according to each GTD-associated fungal group, using the primers and PCR protocols listed in Table 1.

The selected gene regions were amplified in a SureCycler 8800 thermal cycler (Agilent Technologies), by adding 1 µL of template DNA (10–20 ng) into a PuReTaq Ready-To-Go PCR Beads (GE Healthcare) tube containing 0.5 µL each of forward and reverse primers (0.2 mM), and adjusting to a final reaction

Table 1. Loci and primer pairs used to amplify selected gene regions for the main groups of grapevine trunk disease fungi obtained in this study.

Fungi	Loci[a]	Primer pairs	References
Cylindrocarpon-like	*his3*	CYLH3F, CYLH3R	Crous *et al*., 2004
	ITS	ITS4, ITS5	White *et al*., 1990
	LSU	LR0R, LR5	Rehner and Samuels, 1994; Vilgalys and Hester, 1990
	tef1	EF1-728F, EF1-986R	Carbone and Kohn, 1999
		CylEF-1, CylEF-R2	Groenewald, unpublished; Crous *et al*., 2004
	tub2	T1, CYLTUB1R	O'Donnell and Cigelnik, 1997; Crous *et al*., 2004
Botryosphaeriaceae	ITS	ITS1, ITS4	White *et al*., 1990
	LSU	LR0R, LR5	Rehner and Samuels, 1994; Vilgalys and Hester, 1990
	rpb2	RPB2Bot6F, RPB2Bot7R	Sakalidis *et al*., 2011
	tef1	EF1-728F, EF1-986R	Carbone and Kohn, 1999
	tub2	Bt2a, bt2b	Glass and Donaldson, 1995
Diaporthe	*cmdA*	CAL-228F, CAL-737R	Carbone and Kohn, 1999
		CL1, CL2A	O'Donnell *et al*., 2000
	his3	CYLH3F, H3-1B	Crous *et al*., 2004; Glass and Donaldson, 1995
	ITS	ITS1, ITS4	White *et al*., 1990
	tef1	EF1-728F, EF1-986R	Carbone and Kohn, 1999
	tub2	Bt2a, Bt2b	Glass and Donaldson, 1995

[a] *cmdA*: calmodulin; *his3*: histone H3; ITS: the internal transcribed spacer region and intervening 5.8S nrRNA; LSU: 28S large subunit; *rpb2*: RNA polymerase II second largest subunit; *tef1*: translation elongation factor 1-alpha; *tub2*: β-tubulin.

volume of 25 μL with nuclease-free water. PCR products were separated by electrophoresis in 2% (w/v) agarose gels in TBE 0.5X, stained with Midori Green (NIPPON Genetics Europe), and then examined under UV light. PCR products were then purified with the Illustra ExoProStar 1-Step kit (GE Healthcare Life Sciences). Amplicons were sequenced in forward and reverse directions using both PCR primers with the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems), in an ABI Prism 3500 Genetic Analyzer (Applied Biosystems). Nucleotide arrangements at ambiguous positions were clarified using the forward and reverse sequences. Consensus sequences were assembled with the MEGA v.6 software (Tamura *et al*., 2013), and were compared with homologous sequences using the BLASTn search of the NCBIs Genbank nucleotide database.

Phylogenetic analyses included representative sequences of GTD-causing fungi obtained during this study (six *Cylindrocarpon*-like, nine Botryosphaeriaceae spp., and six *Diaporthe* isolates), and related sequences retrieved from GenBank, including, where possible, sequences from ex-type specimens selected for their high similarity to isolate sequences, using MegaBLAST (Table 2).

Different gene regions were aligned using the MAFFT v.7 online interface (https://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley, 2013), and manually corrected where necessary. Unreliable alignment regions were filtered using the GUIDANCE2 online server (Sela *et al*., 2015). Congruence between the different datasets was tested using a 70% reciprocal bootstrap criterion on each individual locus for each group of GTD-causing fungi (Mason-Gamer and Kellogg, 1996). Selected genes were then combined to infer multigene analyses. The combined dataset of five loci (ITS, LSU, *tef1*, *tub2*, *his3*) was used to obtain the multilocus phylogeny of *Cylindrocarpon*-like fungi, along with five loci (ITS, *tef1*, *tub2*, *his3*, *cmdA*) for *Diaporthe* spp., and four for (ITS, *tef1*, *tub2*, *rpb2*) the Botryosphaeriaceae isolates.

Phylogenetic analyses were based on the maximum likelihood (ML) of all individual loci, and on both the ML and Bayesian inference (BI) in the case of the multilocus analyses. Substitution models for each sequence dataset were inferred with MrModeltest2 v. 2.3 (Nylander 2004). Both analyses were performed in the CIPRES Science Gateway web server (www.phylo.org) (Miller *et al*., 2010). ML trees were obtained using the RAxML-HPC Black Box tool v. 8.2.10 (Stamatakis *et al*., 2008). A general time reversible (GTR) model was applied, with a gamma-distributed rate variation including 1,000 bootstrap replicates. Bayesian analyses were inferred with MrBayes in XSEDE V. 3.2.6 (Ronquist and Huelsenbeck, 2003).

The phylogenetic trees and data files were viewed with the MEGA v. 6 and FigTree v. 1.3.1 software (Rambaut and Drummond, 2010). *Campylocarpon fasciculare* (CBS 112613), was used as the outgroup to infer the phylogenies of the *Cylindrocarpon*-like species, *Diaporthella corylina* (CBS 121124) for *Diaporthe*, and *Saccharata proteae* (CBS 115206) for Botryosphaeriaceae species.

Results

A total of 2155 fungal isolates were obtained from all the sampled plants. Of these, 449 were classified as fungi associated with GTDs, 117 associated to Black foot disease (BFD), 147 to Botryosphaeria dieback (BD), 30 to Petri disease (PD), 126 to Diaporthe dieback (DD), and 29 to pestalotioid fungi (PF). Ninety-three percent of the plants were infected by at least one grapevine trunk pathogen (82% of 'Albariño' 196-17C plants, 92 % of the 'Savagnin' Fercal 242 plants, and 100 % of the 'Albariño' 110R plants) (Table 3). Most of the affected vines presented more than one GTD per plant (40% were infected by two, 31% by three and 9% by four GTDs). Fungi associated to BFD were the most prevalent in 'Albariño' plants, regardless of the rootstock type, affecting up to 77% of these plants. Botryosphaeria dieback fungi were the most detected fungi in the 'Savagnin' plants (66%). Diaporthe dieback was the second most common disease detected in 'Savagnin' and 'Albariño'110R plants, with incidence ranging from 59 to 73%. Petri disease and pestalotioid fungi were the least prevalent taxa, accounting for only 13 to 41% and 0 to 20% of the infections, respectively (Table 3). *Cylindrocarpon*-like fungi were isolated principally from the bases of the rootstocks and the roots, whereas fungi associated with BD and DD were mainly isolated from the graft unions and the rootstocks. Using Chi-square analyses, significant differences were detected for GTD incidence between the three studied nurseries and between GTDs and the different analyzed plant parts ($P<0.001$). Correspondence analyses showed that there was a relationship among nurseries and each GTD, except for BFD, the common disease in all three nurseries (Figure 1). Regarding the different analyzed

Table 2. Fungal isolates, and GenBank accession numbers of sequences used in phylogenetic analyses.

Species	Isolate[a]	Genbank accession number[b]						
		ITS	LSU	*tub2*	*tef1*	*his3*	*cmdA*	*rpb2*
Black foot agents								
Campylocarpon fasciculare	CBS 112613^T; CPC 3970	AY677301	HM364313	AY677221	JF735691	JF735502	–	–
"Cylindrocarpon" sp. 1	**EFA 443; CECT 20995**	*MF440368*	*MF467250*	*MF797791*	*MH070095*	*MF471471*	–	–
Dactylonectria alcacerensis	CBS 129087^T; Cy159	JF735333	KM231629	AM419111	JF735819	JF735630	–	–
Dactylonectria anthuriicola	CBS 564.95^T	JF735302	KM515897	JF735430	JF735768	JF735579	–	–
Dactylonectria estremocensis	CBS 129085^T; Cy145	JF735320	KM231630	JF735448	JF735806	JF735617	–	–
Dactylonectria hordeicola	CBS 162.89^T	AM419060	KM515898	AM419084	JF735799	JF735610	–	–
Dactylonectria macrodidyma	CBS 112615^T; CPC 3976	AY677290	KM515900	AY677233	JF735836	JF735647	–	–
Dactylonectria macrodidyma	**EFA 446**	*MF44037*	*MF467253*	*MF797794*	*MH070098*	*MF471474*	–	–
Dactylonectria novozelandica	CBS 112608; CPC 3987	AY677288	KM515901	AY677235	JF735821	JF735632	–	–
Dactylonectria pauciseptata	CBS 120171^T	EF607089	KM515903	EF607066	JF735776	JF735587	–	–
Dactylonectria pauciseptata	**EFA 445**	*MF440370*	*MF467252*	*MF797793*	*MH070097*	*MF471473*	–	–
Dactylonectria torresensis	CBS 129086^T; Cy218	JF735362	KM231631	JF735492	JF735870	JF735681	–	–
Dactylonectria torresensis	**EFA 447**	*MF440372*	*MF467254*	*MF797795*	*MH070099*	*MF471475*	–	–
Dactylonectria vitis	CBS 129082^T; Cy233	JF735303	KM515907	JF735431	JF735769	JF735580	–	–
Ilyonectria cyclaminicola	CBS 302.93	JF735304	KM515913	JF735432	JF735770	JF735581	–	–
Ilyonectria capensis	CBS 132815^T; CPC 20695	JX231151	KM515908	JX231103	JX231119	JX231135	–	–
Ilyonectria coprosmae	CBS 119606	JF735260	KM515910	JF735373	JF735694	JF735505	–	–
Ilyonectria europaea	CBS 537.92	EF607079	KM515914	EF607064	JF735757	JF735568	–	–
Ilyonectria leucospermi	CBS 132809^T; CPC 20701	JX231161	KM515917	JX231113	JX231129	JX231145	–	–
Ilyonectria liliigena	CBS 732.74	JF735298	KM515920	JF735426	JF735763	JF735574	–	–
Ilyonectria liriodendri	CBS 117527; Cy76	DQ178165	KM515922	DQ178172	JF735698	JF735509	–	–
Ilyonectria liriodendri	**EFA 448**	*MF440373*	*MF467255*	*MF797796*	*MH070100*	*MF471476*	–	–
Ilyonectria lusitanica	CBS 129080^T; Cy197	JF735296	KM515923	JF735423	JF735759	JF735570	–	–
Ilyonectria robusta	**EFA 184**	*MF440364*	*MF467246*	*MF797787*	*MH070091*	*MF471467*	–	–
Ilyonectria robusta	CBS 308.35^T	JF735264	KM515928	JF735377	JF735707	JF735518	–	–

(Continued)

Table 2. (Continued).

Species	Isolate[a]	Genbank accession number[b]						
		ITS	LSU	*tub2*	*tef1*	*his3*	*cmdA*	*rpb2*
Ilyonectria rufa	CBS 640.77	JF735277	KM515931	JF735399	JF735731	JF735542	–	–
Neonectria ditissima	CBS 226.31	JF735309	AY677330	DQ789869	JF735783	JF735594	–	–
Neonectria lugdunensis	CBS 125485; DAOM 235831	KM231762	KM231625	KM232019	KM231887	KM231482	–	–
Neonectria obtusispora	CBS 183.36; IMI 113895	AM419061	KM515943	AM419085	JF735796	JF735607	–	–
Neonectria ramulariae	CBS 151.29; IMI 113894	JF735313	HM042436	JF735438	JF735791	JF735602	–	–
Neonectrtia major	CBS 240.29[T]; IMI 113909	JF735308	KM515942	DQ789872	JF735782	JF735593	–	–
Botryosphaeria dieback agents								
Botryosphaeria dothidea	**EFA 466**	*MG547967*	*MH071397*	*MH118935*	*MH118926*	–	–	*MH118944*
Botryosphaeria dothidea	CBS 100564	KX464085	KX464242	KX464781	KX464555	–	–	KX463951
Diplodia mutila	CBS 112553	AY259093	AY928049	DQ458850	AY573219	–	–	–
Diplodia mutila	**EFA 467**	*MG547968*	*MH071398*	*MH118936*	*MH118927*	–	–	*MH118945*
Diplodia seriata	CBS 112555[ET]	AY259094	AY928050	DQ458856	AY573220	–	–	–
Diplodia seriata	**EFA 468**	*MG547969*	*MH071399*	*MH118937*	*MH118928*	–	–	*MH118946*
Neofusicoccum algeriense	CBS 137504[T]	KJ657702	–	–	KJ657715	–	–	–
Neofusicoccum algeriense	ALG9	KJ657704	–	–	KJ657721	–	–	–
Neofusicoccum andinum	CBS 117453[T]; CMW 13455	AY693976	DQ377914	KX464923	AY693977	–	–	KX464002
Neofusicoccum andinum	CBS 117452; CMW 13446	DQ306263	KX464373	KX464922	DQ306264	–	–	KX464001
Neofusicoccum australe	CBS 139662[T]; CMW 6837	AY339262	–	AY339254	AY339270	–	–	EU339573
Neofusicoccum cryptoaustrale	CBS 122813[T]; CMW 23785	FJ752742	KX464416	FJ752756	FJ752713	–	–	KX464014
Neofusicoccum italicum	MFLUCC 15-0900[T]	KY856755	–	–	KY856754	–	–	–
Neofusicoccum kwambonambiense	CBS 123639[T]; CMW 14023	EU821900	KX464422	EU821840	EU821870	–	–	EU821930
Neofusicoccum kwambonambiense	CBS 123640; CMW 14205	EU821901	KX464423	EU821841	EU821871	–	–	EU821931
Neofusicoccum luteum	CBS 562.92[T]	KX464170	KX464430	KX464968	KX464690	–	–	KX464020
Neofusicoccum luteum	**EFA 469**	*MG547970*	*MH071400*	*MH118938*	*MH118929*	–	–	*MH118947*
Neofusicoccum parvum	**EFA 470**	*MG547971*	*MH071401*	*MH118939*	*MH118930*	–	–	*MH118948*
Neofusicoccum parvum	CBS 138823[T]; CMW 9081	AY236943	AY928045	AY236917	AY236888	–	–	EU821963

(Continued)

Table 2. (Continued).

Species	Isolate[a]	Genbank accession number[b]						
		ITS	LSU	*tub2*	*tef1*	*his3*	*cmdA*	*rpb2*
***Neofusicoccum* sp. 1**	**EFA 436; CECT 20988**	*MG547965*	*MH071395*	*MH118933*	*MH118924*	–	–	*MH118942*
***Neofusicoccum* sp. 1**	**EFA 471**	*MG547972*	*MH071402*	*MH118940*	*MH118931*	–	–	*MH118949*
***Neofusicoccum* sp. 1**	**EFA 472**	*MG547973*	*MH071403*	*MH118941*	*MH118932*	–	–	*MH118950*
***Neofusicoccum* sp. 2**	**EFA 437; CECT 20989**	*MG547966*	*MH071396*	*MH118934*	*MH118925*	–	–	*MH118943*
Neofusicoccum stellenboschiana	CBS 110864[T]; STE-U 4598	AY343407	KX464513	KX465047	AY343348	–	–	KX464042
Neofusicoccum stellenboschiana	CBS 121116; STE-U 6071	EF445356	KX464515	KX465049	EF445387	–	–	KX464044
Saccharata proteae	CBS 115206; CPC 4378	KF766226	DQ377882	KF531790	KF766438	–	–	GU357753
Diaporthe dieback agents								
Diaporthe ampelina	CBS 111888; STE-U 2673; CPC 2673	KC343016	–	KC343984	KC343742	KC343500	KC343258	–
Diaporthe ampelina	CBS 267.80	KC343018	–	KC343986	KC343744	KC343502	KC343260	–
Diaporthe ampelina	**EFA 460**	*MH050429*	–	*MH051284*	*MH051278*	*MH025965*	*MH051290*	–
Diaporthe angelicae	CBS 111592[ET]	KC343027	–	KC343995	KC343753	KC343511	KC343269	–
Diaporthe angelicae	CBS 501.90	KC343030	–	KC343998	KC343756	KC343514	KC343272	–
Diaporthe australafricana	CBS 111886 [T]; STE-U 2676; CPC 2676	KC343038	–	KC344006	KC343764	KC343522	KC343280	–
Diaporthe australafricana	CBS 113487; STE-U 2655; CPC 2655	KC343039	–	KC344007	KC343765	KC343523	KC343281	–
Diaporthe chamaeropis	CBS 454.81	KC343048	–	KC344016	KC343774	KC343532	KC343290	–
Diaporthe chamaeropis	CBS 753.70	KC343049	–	KC344017	KC343775	KC343533	KC343291	–
Diaporthe endophytica	CBS 133811[T]; LGMF916;CPC 20292	KC343065	–	KC344033	KC343791	KC343549	KC343307	–
Diaporthe endophytica	LGMF911; CPC 20287	KC343066	–	KC344034	KC343792	KC343550	KC343308	–
Diaporthe eres	CBS 287.74	KC343084	–	KC344052	KC343810	KC343568	KC343326	–
Diaporthe eres	CBS 101742	KC343073	–	KC344041	KC343799	KC343557	KC343315	–
Diaporthe eres	**EFA 464**	*MH050433*	–	*MH051288*	*MH051282*	*MH025969*	*MH051294*	–
Diaporthe foeniculina	CBS 123208[T]	KC343104	–	KC344072	KC343830	KC343588	KC343346	–

(Continued)

Table 2. (Continued).

Species	Isolate[a]	Genbank accession number[b]						
		ITS	LSU	*tub2*	*tef1*	*his3*	*cmdA*	*rpb2*
Diaporthe foeniculina	CBS 123209[T]	KC343105	–	KC344073	KC343831	KC343589	KC343347	–
Diaporthe foeniculina	**EFA 465**	*MH050434*	–	*MH051289*	*MH051283*	*MH025970*	*MH051295*	–
Diaporthe impulsa	CBS 114434	KC343121	–	KC344089	KC343847	KC343605	KC343363	–
Diaporthe impulsa	CBS 141.27	KC343122	–	KC344090	KC343848	KC343606	KC343364	–
Diaporthe novem	CBS 127269	KC343155	–	KC344123	KC343881	KC343639	KC343397	–
Diaporthe novem	CBS 127270	KC343156	–	KC344124	KC343882	KC343640	KC343398	–
Diaporthe novem	**EFA 461**	*MH050430*	–	*MH051285*	*MH051279*	*MH025966*	*MH051291*	–
Diaporthe phaseolorum	CBS 113425	KC343174	–	KC344142	KC343900	KC343658	KC343416	–
Diaporthe phaseolorum	CBS 116019	KC343175	–	KC344143	KC343901	KC343659	KC343417	–
Diaporthe phaseolorum	**EFA 462**	*MH050431*	–	*MH051286*	*MH051280*	*MH025967*	*MH051292*	–
Diaporthe rudis	CBS 266.85	KC343237	–	KC344205	KC343963	KC343721	KC343479	–
Diaporthe rudis	CBS 113201[ET]	KC343234	–	KC344202	KC343960	KC343718	KC343476	–
Diaporthe rudis	**EFA 463**	*MH050432*	–	*MH051287*	*MH051281*	*MH025968*	*MH051293*	–
Diaporthella corylina	CBS 121124	KC343004	–	KC343972	KC343730	KC343488	KC343246	–

[a] ALG: Personal culture collection A. Berraf-Tebbal; CBS: Westerdijk Fungal Biodiversity Centre, Utrecht, The Netherlands; CECT: Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; CMW: Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CPC: Collection Pedro Crous, housed at CBS; Cy: Cylindrocarpon collection housed at Laboratório de Patologia Vegetal "Veríssimo de Almeida" - ISA, Lisbon, Portugal; DAOM; Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada; EFA: Estación Fitopatolóxica Areeiro, Deputación de Pontevedra, Pontevedra, Spain; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K; LGMF: culture collection of Laboratory of Genetics of Microorganisms, Federal University of Parana, Curitiba, Brazil; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Ria, Thailand; STE-U: Culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa. [T] and [ET] indicate ex-type and ex-epitype strains, respectively. Species and isolate names obtained in this study are shown in bold, and corresponding GenBank accession numbers in italics.

[b] ITS: internal transcribed spacer regions of the nrDNA and intervening 5.8S nrDNA; LSU: 28S large subunite; *tub2*: partial beta-tubulin gene; *tef1*: partial translation elongation factor 1-alpha gene; *his3*: partial histone H3 gene; *cmdA*: partial calmodulin gene; *rpb2*: RNA polymerase II second largest subunit.

Table 3. Incidence of grapevine trunk diseases (%) in plants of 'Albariño' and 'Savagnin' grapevine varieties from three nurseries, grafted onto 110R, 196-17 or Fercal 242 rootstocks, expressed by, plant parts and nursery.

Nursery	Cultivar/ Rootstock	No.	Disease[1]	% of GTD affected plants (No. of plants)	% of GTD affected plants part (No. of plants)			
					Scion	Graft union	Rootstock	Rootstock base/roots
1	'Albariño'	40	BFD	77.5 (31)	5 (2)	2.5 (1)	-	75 (30)
			BD	52.7 (21)	5 (2)	35 (14)	35 (14)	15 (6)
	110 Richter		PD	15 (6)	7.5 (3)	-	5 (2)	5 (2)
			DD	72.5 (29)	20 (8)	50 (20)	37.5 (15)	15 (6)
			PF	20 (8)	5 (2)	7.5 (3)	12.5 (5)	-
			GTD	100 (40)	35 (14)	77.5 (31)	62.5(25)	82.5(33)
2	'Albariño'	22	BFD	77.3 (17)	-	-	13.6 (3)	68.2 (15)
			BD	18.2 (4)	-	4.5 (1)	-	13.6 (3)
	196-17 Castel		PD	40.9 (9)	4.5 (1)	22.7 (5)	9.1 (2)	4.5 (1)
			DD	22.7 (5)	4.5 (1)	13.6 (3)	-	4.5 (1)
			PF	0 (0)	-	-	-	-
			GTD	81.8 (18)	9.1 (2)	40.9 (9)	22.7 (5)	72.7 (16)
3	'Savagnin'	88	BFD	55.7 (49)	2.3 (2)	-	8 (7)	52.3 (46)
			BD	65.9 (58)	29.5 (26)	43.2 (38)	35.2 (31)	5.7 (5)
	Fercal 242		PD	12.5 (11)	1.1 (1)	5.7 (5)	4.5 (4)	2.3 (2)
			DD	59.1 (52)	22.7 (20)	19.3 (17)	35.2 (31)	3.4 (3)
			PF	19.3 (17)	11.4 (10)	1.1 (1)	5.7 (5)	3.4 (3)
			GTD	92 (81)	47.7 (42)	63.6 (56)	59.1 (52)	58 (51)

[1] BFD = Black foot disease, BD = Botryosphaeria Dieback, PD = Petri Disease, DD = Diaporthe Dieback and PF = Pestalotioid fungi.

plant parts, there was an obvious tissue association between BFD and the rootstock bases and roots, between PD and BD and the graft unions, and between DD and the rootstocks (Figure 2).

Isolates of the GTD fungi were identified to family or genus based on colony and conidium morphological characters. Two hundred and seventy six isolates (86 *Cylindrocarpon*-like, 130 Botryosphaeriaceae, 30 *Diaporthe* and 30 Petri disease isolates) were also identified using molecular techniques.

BLAST comparisons and phylogenetic analyses followed by checks of morphological features identified 20 fungal species: six *Cylindrocarpon*-like species (*Dactylonectria hordeicola, D. macrodidyma, D. pauciseptata, D. torresensis*, *Ilyonectria liriodendri, and I. robusta*), five Botryosphaeriaceae species (*Botryosphaeria dothidea*, *Diplodia mutila*, *D. seriata, Neofusicoccum luteum*, and *N. parvum*), three Petri disease fungi (*Cadophora luteo-olivacea*, *Phaeoacremonium minimum*, and *Phaeomoniella chlamydospora*), and six *Diaporthe* spp. (*D. ampelina*, *D. eres*, *D. foeniculina*, *D. novem*, *D. phaseolorum,* and *D. rudis*) (Table 4). In all cases, the BLAST searches yielded 99-100 % identity with sequences available at the GenBank database.

Comparisons of the 70% reciprocal bootstrap tree topologies of the individual loci showed no incongruences for the *cmdA*, *his3*, ITS, *tef1* and *tub2* gene regions in the *Cylindrocarpon* and *Diaporthe* phylogenies. However, the LSU gene region revealed a conflicting tree topology in the *Cylindrocarpon* phylogeny, and *tef1* and *rpb2* loci revealed a conflicting topology in the Botryosphaeriaceae phylogeny, compared to the

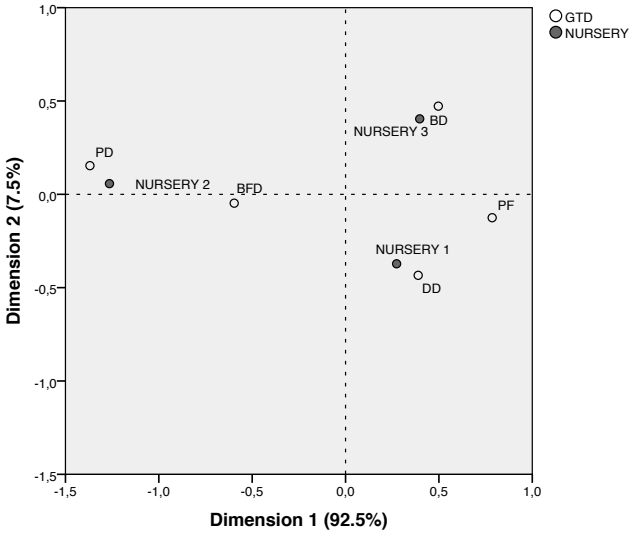

Figure 1. Correspondence analysis biplot between GTDs and the three nurseries. BFD = Black foot disease, BD = Botryosphaeria dieback, PD = Petri disease, DD = Diaporthe dieback, and PF = Pestalotioid fungi.

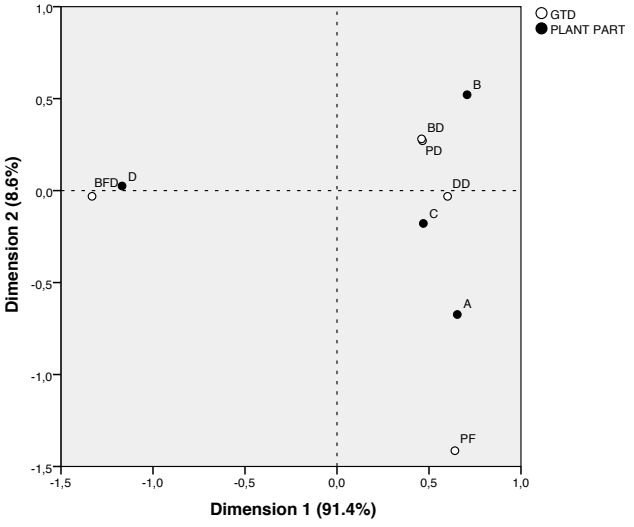

Figure 2. Correspondence analysis biplot between GTDs and the analyzed plant parts (A: scion; B: graft union; C: rootstock; D: roots). BFD = Black foot disease, BD = Botryosphaeria dieback, PD = Petri disease, DD = Diaporthe dieback, and PF = Pestalotioid fungi.

other gene regions. However, as these conflicts only involved the placement of single species, this was ignored and the selected gene regions were combined, following the argument of Cunningham (1997) that combining incongruent partitions increases phylogenetic accuracy.

The *Cylindrocarpon*-like combined analysis of the five genes (ITS, LSU, *his3*, *tef1*, *tub2*) clustered the representative *Cylindrocarpon*-like isolates into six clades and allowed the identification of the isolate EFA 443 as *D. hordeicola*, based on its clustering in a well-supported group formed by the CBS 162.89 ex-type strain retrieved from GenBank (bootstrap support (BS) = 100%, Bayesian posterior probability (BPP) = 1.0) (Figure 3). Nevertheless, since the CBS 162.89 ex-type is a sterile culture, its morphological characteristics could not be compared.

The EFA 443 isolate grew on PDA between 5ºC and 30ºC, reaching optimal growth at 20ºC, and was characterized by felty, ochre-sienna mycelia with a whitish, irregular colony margin, differing completely from other *Cylindrocarpon*-like colonies examined. No sporodochia were observed. The microconidia (0–1 septate) were hyaline, cylindrical and straight, and most had visible hila; 0- septate (9.5–) 15.4 ± 2.5 (–20.4) × (3.6–) 4.5 ± 0.5 (–5.6) µm, with length to width ratios of 2.6 to 4.2; 1- septate (13.5–) 18.8 ± 2.7 (–26.3) × (3.6–) 5.0 ± 0.5 (–5.8) µm, with length to width ratios of 2.8 to 5.0. The macroconidia 1 (–3)-septate were hyaline, straight or slightly curved, and cylindrical, but slightly wider towards the obtuse tips and more rounded at the bases, mostly with central hila: 1 septate- (21.1–) 26.6 ± 2.6 (–32.1) × (4.5–) 5.3 ± 0.4 (–6.1) µm, with length to width ratios of 4.1 to 6.3; 2 septate- (20.1–) 28.8 ± 4.0 (–35.5) × (4.4–) 5.6 ± 0.5 (–6.5) µm, with length to width ratios of 4.0 to 6.6; 3 septate- (26.2–) 36.1 ± 4.7 (–44.8) × (5.1–) 5.9 ± 0.5 (–6.9) µm, with length to width ratios of 4.4 to 8.0 (n = 30 observations per structure). Chlamydospores were rarely observed, and were either globose or subglobose (9.5–13.4) × (8.1–12.7) µm (n = 10), smooth but seemingly rough, intercalary or terminal, forming single structures or chains, hyaline, and brownish (Figure 4). This isolate was deposited in the Spanish Type Culture Collection (*Colección Española de Cultivos Tipo*, CECT) with culture code CECT 20995.

The phylogenetic analysis of the selected Botryosphaeriaceae isolates, based on ITS, *rpb2*, *tef1*, and *tub2* sequences grouped them in seven different clades (Figure 5). The identification of four *Neofusicoccum* isolates (namely EFA 436, 437, 471 and 472) was not conclusive. They were clustered into a distinct, but not well-supported, clade, closely grouped with *N.*

Table 4. Isolates of grapevine trunk disease fungi obtained from plants of grapevine varieties 'Albariño' or 'Savagnin' grafted on 110R, 196-17 or Fercal 242 rootstocks.

Species isolated	Number of isolates by variety or plant parts[1]											
	'Albariño' /110R				'Albariño' /196-17C				'Savagnin' / Fercal 242			
	A	B	C	D	A	B	C	D	A	B	C	D
Dactylonectria hordeicola												1[a]
Dactylonectria macrodidyma				8								4
Dactylonectria pauciseptata				17					1			4
Dactylonectria torresensis				3				6			2	17
Ilyonectria liriodendri	2	1		5			2	9				3
Ilyonectria robusta												1
"Cylindrocarpon" sp.[2]				2			1	3	1		5	19
Black Foot Disease total	**2**	**1**		**35**			**3**	**18**	**2**		**7**	**49**
Botryosphaeria dothidea	1	7	7	5					8	13	16	3
Diplodia mutila		1								1		
Diplodia seriata		1	1					1	2	4		
Neofusicoccum luteum									2	1		
Neofusicocccum parvum	1	4	4	1		1		2	12	14	9	2
Neofusicoccum sp. 1		1[b]	2[c]	1[d]								
Neofusicoccum sp. 2		1[e]	1									
Botryosphaeriaceae [2]									2	7	8	
Botryosphaeria Dieback total	**2**	**15**	**15**	**7**		**1**		**3**	**26**	**40**	**33**	**5**
Cadophora luteo-olivacea	1		2	2	1	4	2	1	1	4	1	
Phaeoacremonium minimum									1			
Phaeomoniella chlamydospora	2		1			1				1	3	2
Petri Disease total	**3**		**3**	**2**	**1**	**5**	**2**	**1**	**2**	**5**	**4**	**2**
Diaporthe ampelina		1			1[f]	1				1	3	
Diaporthe eres		4[g]	2									
Diaporthe foeniculina		1	1[h]	1								
Diaporthe novem		1				1[i]				1		3
Diaporthe phaseolorum		1		1[j]								
Diaporthe rudis		2[k]				1			2			1
Diaporthe sp.[2]	8	10	12	4				1	19	15	27	
Diaporthe Dieback total	**8**	**20**	**15**	**6**	**1**	**3**		**1**	**21**	**17**	**30**	**4**
Pestalotiopsis sp.[2]	2	3	5						10	1	5	2
Truncatella sp.[2]												1
Pestalotiod Fungi total	**2**	**3**	**5**						**10**	**1**	**5**	**3**
Total				**145**				**39**				**265**

[1] A: Scion; B: Graft-union; C: Rootstock; D: Rootstock base and roots. [2:] Isolates identified only by morphological analysis to genus or family level.[a] EFA 443; [b] EFA 436; [c] EFA 471; [d] EFA 472; [e] EFA 437, [f] EFA 460; [g] EFA 464; [h] EFA 465; [i] EFA 461; [j] EFA 462 [k] EFA 463.

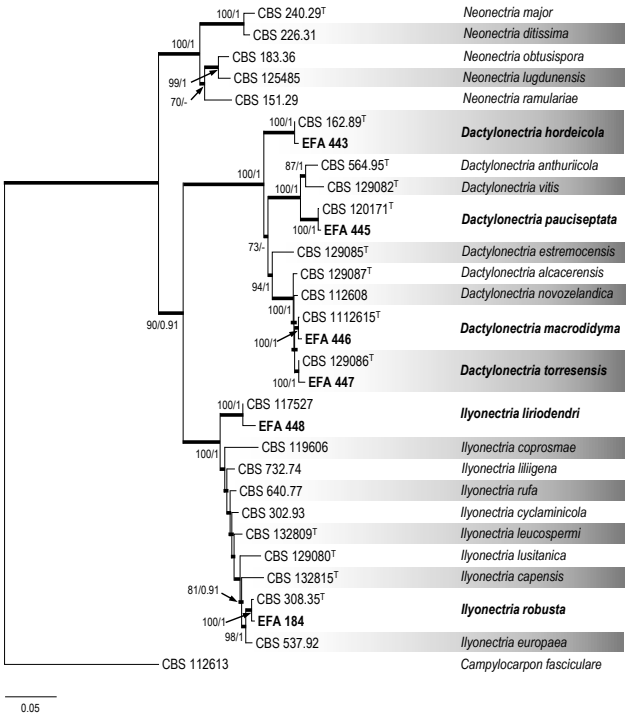

Figure 3. Phylogram generated from Maximum Likelihood analysis of *Cylindrocarpon* species isolated in this study and their closely related species based on combined ITS, LSU, *his3*, *tef1* and *tub2* sequence data. Thickened branches are those present in the maximum likelihood and Bayesian inference trees. Bayesian posterior probabilities ≥0.90 and bootstrap support values for ML ≥70% are indicated at the nodes. The tree was rooted to *Campylocarpon fasciculare* (CBS 122613). Isolates obtained in this study are in bold and ex-type reference strains are indicated with[T]. The scale bar represents the expected changes per site.

algeriense and *N. italicum* (BS <70%, BPP) <0.9) (Figure 3). The morphology of these isolates growing on PNA was characterized by dark and septate conidia as *N. italicum*, and spermatia as *N. algeriense*, which matches the findings described, respectively, by Marin-Felix *et al*., (2017) and Berraf-Tebbal *et al*., (2014). The isolate EFA 437 formed a highly supported and separate group comprised by the ex-type strain of *N. australe* (CMW 6837) (BS >70 %, BPP >0.9). However, the morphological characteristics of EFA 437 did not match those of the CMW 6837 ex-type (Phillips *et a*l., 2013), as two-septate conidia and spermatia were observed. The isolates EFA 436 and EFA 437 were deposited in CECT with culture codes, respectively, CECT 20988 and CECT 20989.

The combined dataset of multigene phylogenetic analysis of ITS, *cmdA, his3*, *tef1*, and *tub2* loci enabled the classification of six representative *Diaporthe* isolates (namely EFA 460, 461, 462, 463, 464 and 465) into six well-supported clades (BS = 100%; BPP = 1.0), with each isolate corresponding to a separate species: *Diaporthe ampelina*, *D. eres, D. foeniculina, D. novem, D. phaseolorum*, and *D. rudis* (Figure 6).

The remaining 1,706 isolates belonged to 28 different genera. Six genera were common in the analyzed plants and are considered to be grapevine pathogens, including *Aspergillus, Botrytis, Colletotrichum, Fusarium, Phoma*, and *Rhizoctonia*. The prevalence of *Fusarium* fungi, of which five species were identified (*F. avenaceum, F. lateritium, F. oxysporum, F. proliferatum*, and *F. solani*), was very high in all *V. vinifera* plants, affecting 92 to 98%.

Discussion

Based on their morphological characteristics, sequencing and phylogenetic analysis, 20 different species of fungi were identified associated with GTDs on 150 young nursery-produced plants of *Vitis vinifera* (varieties 'Albariño' and 'Savagnin'). The incidence of this complex disease was very high, with 93% of the analyzed plants affected by at least one GTD-associated fungus. Previous studies carried out in Spanish nurseries and young vineyards have reported incidence rates of GTDs of 31% to 48.8% (Aroca *et al*., 2006), 9.5% to 73.8 % (Aroca *et al*., 2010), 75.8 % (Gramaje *et al*., 2009), and 76.4% (Giménez-Jaime *et al*., 2006). Young vineyards may become infected by the planting material used, either systemically in plants due to infected mother vines, through contamination during the propagation processes (Gramaje and Armengol, 2011) or through annual pruning wounds providing many infection sites each growing season (Gramaje *et al.* 2018).

Black foot fungi were the most frequently isolated from 'Albariño' grafted rootstocks. This disease is considered to be the most significant phytosanitary problem in nurseries (Gramaje and Armengol, 2011; Carlucci *et al*., 2017). BFD was first associated with *Cylindrocarpon obtusisporum* and *C. destructans* (Halleen *et al*., 2004; Agustí-Brisach and Armengol 2013); however, up to 24 other species of the genera *Campylocarpon, Cylindrocladiella, Dactylonectria, Ilyonectria, Neonectria* and *Thelonectria* have also been reported to cause BFD (Gramaje *et al*., 2018). In the present

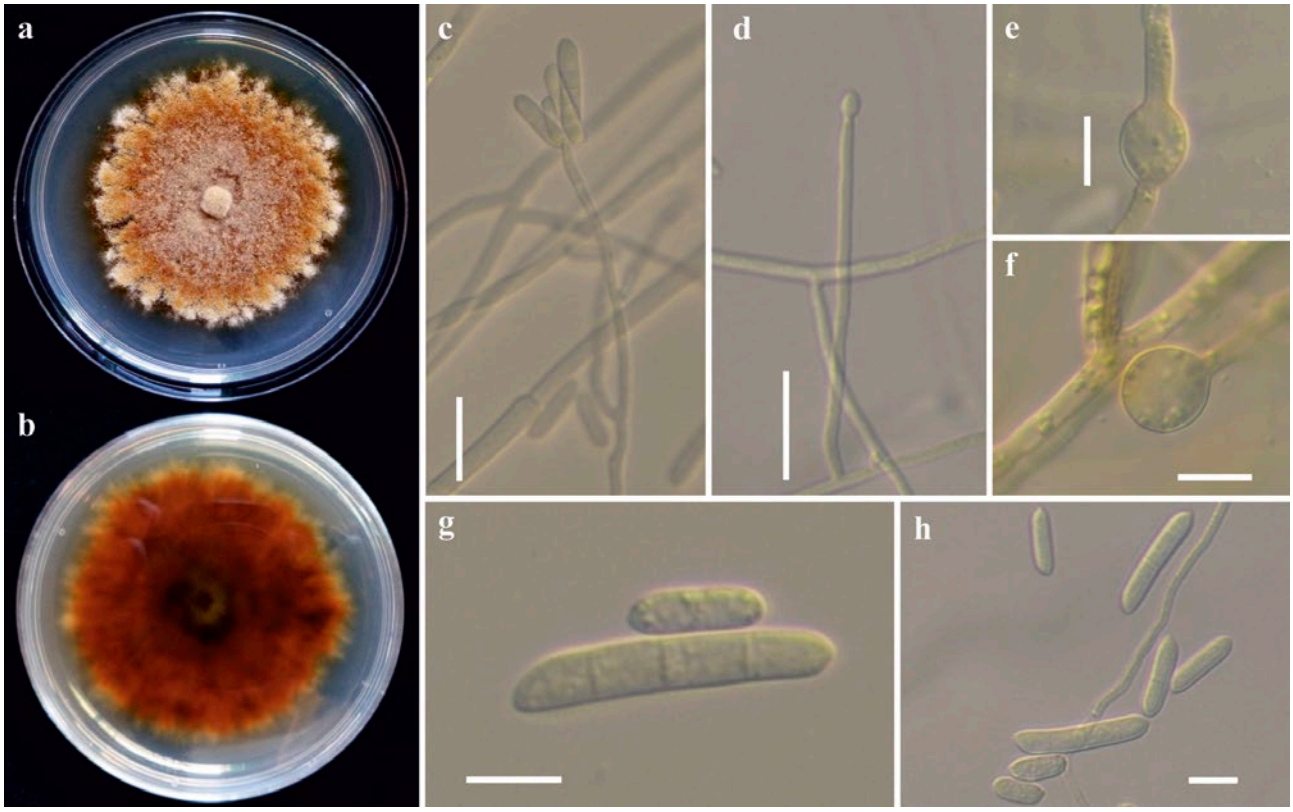

Figure 4. *Dactylonectria hordeicola.* (a-b): upper and lower sides of 20-d-old colony on PDA plate at 24ºC. (c-d): conidiophores and phialides (bars =20 μm). (e-f): chlamydospores (bar = 10 μm). (g-h): microconidia and macroconidia (bar = 10 μm).

study, five species of fungi associated with BFD were identified, namely; *D. macrodidyma, D. pauciseptata, D. torresensis, I. liriodendri,* and *I. robusta*. These species were also detected by Úrbez Torres *et al,*. (2014) as the causes of BFD in a field survey of young vineyards in British Columbia. Spanish research reported the prevalence of the *D. macrodidyma*-complex associated with BFD in Spain (Alaniz *et al.* 2011; Agustí-Brisach *et al.* 2013a). Nevertheless, in the present study incidence of BFD pathogens was different in each of the three nurseries, and *Dactylonectria pauciseptata* and *I. liriodendri* were the most frequently isolated species from the two surveyed Spanish nurseries while *D. torresensis* was the most common fungus from the French nursery. These three species are also considered to be the prevalent causes of BFD (Cabral *et al*., 2012b). One isolate obtained from the base of Fercal 242 rootstock, namely EFA 443, was identified as *Dactylonectria hordeicola*. This species was identified based on phylogenetic analyses of the CBS 162.89 ex-type sterile culture (Lombard *et al*., 2014). To our knowledge, this is the first morphological description of *D. hordeicola*. Further pathogenicity tests should be performed to confirm this specie as a BFD-causing pathogen in grapevine.

Botryosphaeria dieback was the most prevalent disease among the 'Savagnin' grafted plants, affecting up to 66% of the analyzed specimens. Surveys carried out in other nurseries have shown that grapevine infections by Botryosphaeriaceae fungi may originate from the propagation nurseries (Rumbos and Rumbou, 2001; Aroca *et al*., 2006; Giménez-Jaime *et al*., 2006; Billones-Baaijens *et al*., 2013a; 2013b; 2015). To date, 26 Botryosphaeriaceae taxa from the genera *Botryosphaeria, Diplodia, Dothiorella, Lasiodiplodia, Neofusicoccum, Neoscytalidium* and *Phaeobotryosphaeria*, have been identified as associated with the Botryosphaeria dieback in grapevines (Gramaje *et al*., 2018). In the present study, Botryosphaeriaceae species were the most frequently detected GTD-causing fungi. The

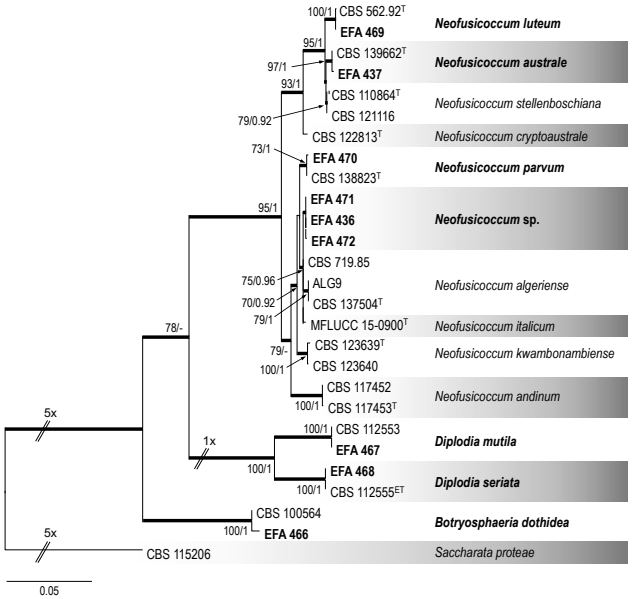

Figure 5. Phylogram generated from Maximum Likelihood analyses of Botryosphaeriaceae species isolated in this study and their closely related species based on combined ITS, LSU, *his3*, *tef1* and *tub2* sequence data. Thickened branches are those present in both the maximum likelihood and Bayesian inference trees. Bayesian posterior probabilities ≥0.90 and bootstrap support values for ML ≥70% are indicated at the nodes. The tree was rooted to *Saccharata proteae* (CBS 115206). Isolates generated in this study are in bold, ex-type reference strains are indicated with^{T} and ex-epitype reference strains with^{ET}. The scale bar represents the expected changes per site.

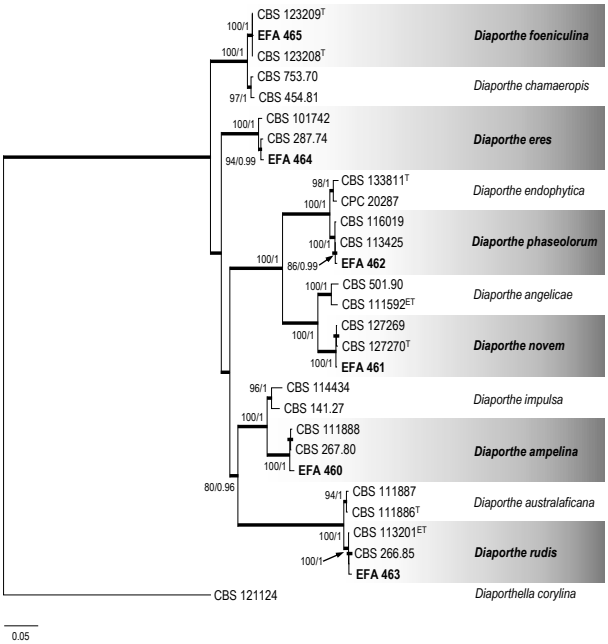

Figure 6. Phylogram generated from Maximum Likelihood analysis of *Diaporthe* species isolated in this study and their closely related species based on combined ITS, *cmdA*, *his3*, *tef1* and *tub2* sequence data. Thickened branches are those present in both the maximum likelihood and Bayesian inference trees. Bayesian posterior probabilities ≥0.90 and bootstrap support values for ML ≥70% are indicated at the nodes. The tree was rooted to *Diaporthella corylina* (CBS 121124). Isolates obtained in this study are in bold, ex-type reference strains are indicated with^{T} and ex-epitype reference strains with^{ET}. The scale bar represents the expected changes per site.

five species identified, *B. dothidea, D. mutila, D. seriata, N. luteum,* and *N. parvum*, matched those detected in Portuguese vineyards (Phillips, 2002).

The results of morphological and phylogenetic analyses of the isolates of *Neofusicoccum* sp. 1 were inconclusive. However, BLAST search comparisons of isolates EFA 436, EFA 471, and EFA 472 yielded 99% homology with sequences of *N. italicum* (KY856755) and *N. algeriense* (KX505906). The multigene analysis of combined ITS, *rpb2*, *tef1*, and *tub2* sequence data was unable to distinguish our isolates from those of *N. parvum, N. algeriense*, or *N. italicum*, thus comprising an unresolved clade. In addition, single phylogenies obtained from each individual locus revealed inconsistencies among each locus and the combined loci. Similar results were obtained by Lopes *et al*., (2017), in phylogenetic analyses of *Neofusicoccum* species based on MAT genes and the combination of genes ITS, *tef1*, and *tub2*. According to the authors, by applying the principle of phylogenetic species recognition (Taylor *et al*., 2000), *N. algeriense* is phylogenetically indistinguishable from *N. parvum*. Therefore, both would represent a single species. Additionally, *Neofusicoccum italicum* Dissan. & K.D. Hyde, was recently described in Marin-Felix *et al*., (2017), exclusively on the basis of ITS and *tef1* sequences of a single isolate (ex-type MFLUCC 15-0900). The morphological characteristics of the *Neofusicoccum* sp. 1 isolates in our study matched those of *N. algeriense*, although fewer dark and septate conidia were observed in comparison with the original description of *N. italicum* (Marin-Felix *et al*., 2017), and these differed from those observed in *N. parvum* (ex-type CMW 9081) by Phillips *et al*.,

(2013). For the EFA 437 isolate, in spite of the phylogenetic analyses clustering the isolate in a well-supported clade with *N. australe*, its few morphological characteristics differed from those of the CMW 6837 ex-type (Phillips *et al*., 2013), as two-septate conidia and spermatia were observed. Hence, further investigation is warranted to identify these isolates.

Fungi associated with Petri Disease were detected in 18% of analyzed plants, less frequent than for the other diseases. PD is also considered an important trunk fungal disease affecting young grapevines (Mugnai *et al*., 1999; Mostert *et al*., 2006; Agustí-Brisach *et al*., 2011; Gramaje and Armengol, 2011), which causes significant losses in newly planted vineyards (Mostert *et al*., 2006). The disease can be spread by planting infected plants (Aroca *et al.* 2006). The following PD-causing species have been identified to date: *Phaeomoniella chlamydospora*, *Pleurostoma richardsiae*, 29 species of *Phaeoacremonium*, and six *Cadophora* spp. (Gramaje *et al*., 2018). In our study three species were detected: *C. luteo-olivacea, P. minimum*, and *P. chlamydospora*, the three main species related to this disease (Gramaje *et al*., 2018). Unless *P. minimum* is considered to be the most common and widely distributed species affecting grapevines (Gramaje *et al*., 2015), we only identified this fungus in one plant. PD detection could be underestimated probably due to the high incidence of other grapevine trunk diseases and endophytes growing in the same sections with high growth rates, like Botryosphaeriaceae, *Diaporthe*, *Fusarium* or *Trichoderma* spp.

Diaporthe dieback was the second most important GTD after BFD in nursery 1 and BD in nursery 3. As the others, this disease is often detected in propagation material and young vines (Fourie and Halleen, 2004; Rego *et al*., 2009; Aroca *et al*., 2010; Moreno-Sanz *et al*., 2013), and for many years, was mostly associated with *Diaporthe ampelina*. Recent studies have identified eleven new species as grapevine wood pathogens (Gramaje *et al*., 2018; Guarnaccia *et al*., 2018). In the present study, six species were identified amongst 30 isolates, including; *Diaporthe ampelina, D. eres, D. foeniculina, D. novem, D. phaseolorum* and *D. rudis*, showing high diversity within this genus in grapevine. The taxa *Diaporthe ampelina, D. eres*, and *D. rudis* have specifically been reported as GTD-associated species in Spain (Sánchez-Torres *et al*., 2008; Guarnaccia *et al*., 2018). *Diaporthe novem* and *D. phaseolorum* have also been detected in grapevines (Larignon, 2016), although no infections by these fungi have been identified to date in Spain. Guarnaccia *et al.* (2018) recently suggested that *D. foeniculina* isolates could be misidentified, as this species is closely related to *D. baccae*, which has also been detected in grapevines (Úrbez-Torres *et al*., 2013; Guarnaccia *et al*., 2018). Further research is required to verify the identity of the isolates of *D. foeniculina*, and other species of the *Diaporthe* not included in this study.

In the last five years, an increasing number of studies have reported pestalotioid fungi in grapevines (Arzanlou *et al*., 2013; Maharachchikumbura *et al*., 2016; Lawrence *et al*., 2018). These fungi have been isolated from wedge-shape cankers and their association with dark streaking of the wood, light-brown discoloration and central necrosis in diseased grapevines (Úrbez-Torres *et al*., 2012). Recent results suggest that pestalotioid fungi are involved in the grapevine trunk-disease complex (Lawrence *et al*., 2018). To our knowledge, at least four genera have been included in this group, including *Neopestalotiopsis*, *Pestalotiopsis*, *Truncatella* and *Seimatosporium*, and two of these have been reported in the present study.

Molecular and morphological analyses allowed the identification of fungal isolates to species level for most of the GTD isolates. However, BLAST searches and morphological examination were unable to identify 11 GTD isolates, so it was necessary to perform multi-locus phylogeny to determine their identity. Previous studies have demonstrated that sequence alignments with four loci allow separation and renaming sterile fungi as new species (Lombard *et al*., 2014), to identify cryptic species within *Neofusicoccum* (Sakalidis *et al*., 2011), whereas five loci are necessary to give successful classification of *Diaporthe* species (Santos *et al*., 2017).

Other potential pathogens, including *Colletotrichum, Fusarium*, or *Rhizoctonia*, were also isolated from the grapevine plants. *Fusarium* spp. were the most prevalent fungi isolated from the grapevine tissues. The pathogenic nature of this genus remains unclear; some authors have suggested that *Fusarium oxysporum* may be an aggressive grapevine pathogen (Highet and Nair, 1995; Brum *et al*., 2012), while other studies report that the presence of *Fusarium* spp. is normal in vines and regard these fungi as common endophytes (Rumbos and Rumbou, 2001; Moreno-Sanz *et al*., 2013; Bruez *et al*., 2014). In a recent study, pathogenicity tests with several species of *Fusarium* present in vines revealed similar damage to that resulting from BFD-causing fungi (Úrbez-Torres *et al*., 2017).

Results obtained in the present study confirm that grapevine trunk diseases are significant threats to nursery plants, and through them to newly established grapevines. Preventive measures should therefore be implemented during nursery propagation processes to guarantee the use of healthy rootstock plants, and reduce the propagation of these diseases in vineyards. Integrated disease management programmes, including physical, fungicide, biological, and other control strategies, has been suggested as the most effective strategy to reduce infections by fungal trunk pathogens in nurseries (Halleen and Fourie, 2016; Gramaje *et al*., 2018).

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