Occurrence and Diversity of Black-Foot Disease Fungi in Symptomless Grapevine Nursery Stock in Spain

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Abstract

In this study, 3,426 grafted grapevines ready to be planted from 15 grapevine nursery fields in Northern Spain were inspected from 2016 to 2018 for black-foot causing pathogens. In all, 1,427 isolates of black-foot pathogens were collected from the asymptomatic inner tissues of surface sterilized secondary roots and characterized based on morphological features and DNA sequence data of the nuclear ribosomal DNA-internal transcribed spacer region, histone H3, translation elongation factor 1- α and β -tubulin genes. Eleven species belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Thelonectria* were identified, including *Dactylonectria alcacerensis*, *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *D. torresensis*, *Ilyonectria liriodendri*, *I. pseudodestructans*, *I. robusta*, *Neonectria quercicola*, *Neonectria* sp. 1, and *Thelonectria olida*. In addition, two species are newly

described, namely *D. riojana* and *I. vivaria*. Twenty-four isolates representing 13 black-foot species were inoculated onto grapevine seedlings cultivar ‘Tempranillo’. The pathogenicity tests detected diversity in virulence among fungal species and between isolates within each species. The most virulent species was *D. novozelandica* isolate BV-0760, followed by *D. alcacerensis* isolate BV-1240 and *I. vivaria* sp. nov. isolate BV-2305. This study improves our knowledge on the etiology and virulence of black-foot disease pathogens, and opens up new perspectives in the study of the endophytic phase of these pathogens in grapevines.

Keywords: *Cylindrocarpon*-like fungi, endophyte, Nectriaceae, systematics, *Vitis vinifera*, tree fruits, etiology, pathogen diversity

Endophytes are defined as organisms living in the internal tissues of plants that exhibit no visible symptoms as a result of the colonization (Hallmann et al. 1997). This endophytic relationship is often unnoticed due to the lack of symptoms in the plant and is usually only discovered by examining internal tissues with a microscope, by aseptic isolations of endophytes from plants, or from polymerase chain reaction (PCR)-based methods of DNA extracted from surface-sterilized plant tissues (Stone et al. 2000). Many fungal endophytes have been sought and characterized for their ability to produce biologically active secondary metabolites with potential applications in agriculture, medicine, and other areas (Wang and Dai 2011).

In grapevine, numerous studies have been conducted to analyze the endophytic fungal communities in different plant organs (Bruez et al. 2014, 2016; Casieri et al. 2009; Cosoveanu et al. 2014; Dissanayake et al. 2018; González and Tello 2011; Hofstetter et al. 2012; Kraus et al. 2019; Martini et al. 2009; Pinto et al. 2014). However, special

focus has been given on the fungal microbiome on its woody tissues, due to the problems arising from grapevine trunk diseases (GTDs) (Bruez et al. 2014, 2016; Casieri et al. 2009; Hofstetter et al. 2012; Kraus et al. 2019). These diseases, namely esca, eutypa, and Phomopsis and Botryosphaeria diebacks, as well as black-foot and Petri diseases, are some of the most destructive fungal diseases of grapevine in grape growing areas of the world. Management of GTDs has been intensively studied for decades with some great advances made in the understanding of the causal pathogens, their epidemiology, impact, and control (Gramaje et al. 2018).

Black-foot disease is a soil-borne disease caused by a broad range of *Cylindrocarpon*-like asexual morphs belonging to the genera *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Thelonectria* (Gramaje et al. 2018). Among these, *Dactylonectria torresensis* is the most frequent species in Italy (Carlucci et al. 2017), Portugal (Reis et al. 2013), and Spain (Berlanas et al. 2017). Internal symptoms of black-foot diseased vines usually range from black, sunken, necrotic lesions on roots to reddish brown discoloration in the base of the rootstock trunk (Fourie and Halleen 2001). Foliar symptoms associated with black-foot disease are practically indistinguishable from those observed in grapevines affected by Petri disease and include delayed bud-break, chlorotic foliage with necrotic margins, overall stunting, and wilting of leaves or entire shoots (Agustí-Brisach and Armengol 2013). They also resemble symptomatology associated with abiotic disorders such as spring frost, winter damage, nutrient deficiency, and/or water stress (Gramaje et al. 2018).

Black-foot disease is particularly important in grapevine nurseries and new plantations. *Cylindrocarpon*-like asexual morphs produce conidia, and some species also produce chlamydospores in culture. The conidia are spread in soil water, and the chlamydospores can allow these pathogens to survive in the soil for extended periods of time (Petit et al. 2011). Infection can occur through the small wounds made when roots break off during the planting process, through the incomplete callusing of the lower trunk, or through wounds made in the grapevine propagation process, such as disbudding wounds, from which the infection progresses downward to the base of the

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trunk (Halleen et al. 2006a). Black-foot disease pathogens have also been detected, identified, and quantified in soil samples by PCR-based methods (Agustí-Brisach et al. 2014; Cardoso et al. 2013; Damm and Fourie 2005; Langenhoven et al. 2018; Probst et al. 2010; Úrbez-Torres et al. 2015) or by dilution plating technique together with the use of a semiselective medium (Berlanas et al. 2017).

Much of the current knowledge on black-foot disease pathogens of grapevine has been derived from research based on populations isolated from vines displaying foliar or internal wood disease symptoms. Recent research in South Africa has also suggested that black-foot causing fungi could be latent in visually healthy grapevine nursery stock (Langenhoven et al. 2018). In Spain, grapevine nursery surveys had been previously conducted for the detection of black-foot pathogens (Agustí-Brisach et al. 2013; Alaniz et al. 2007); however, this research focused on these organisms as plant pathogens causing necrotic symptoms in grapevine tissues. Therefore, the objectives of this study were to (i) conduct extensive surveys in grapevine nurseries and identify the fungal species associated with black-foot disease from asymptomatic endorhizosphere tissues of externally asymptomatic plants based on morphological and molecular methods, and (ii) determine the pathogenicity of these fungal endophytic species on grapevine.

Materials and Methods

Nursery survey and fungal isolation. Isolates used in this study were collected from externally symptomless grafted plants of 110 Richter rootstock ready to be planted from 15 nursery fields in Northern Spain from 2016 to 2018 (Table 1). Fungal isolations were made from the asymptomatic endorhizosphere tissue of secondary roots. For this purpose, sections of externally symptomless roots (2 cm long and 1 to 3 mm diameter) were cut, washed under running tap water, surface sterilized in 33% sodium hypochlorite (commercial 40 g Cl/liter) for 1 min, and rinsed twice with sterile distilled water. The bark was carefully peeled out and the endorhizosphere tissue was plated on malt extract agar (MEA) (Conda Laboratories, Madrid, Spain) supplemented with 0.35 g liter⁻¹ streptomycin sulfate (MEAS) (Sigma-Aldrich Laboratories, St. Louis, MO), and incubated at 25°C in darkness for 12 days. Colonies resembling black-foot disease pathogens were subcultured on potato dextrose agar (PDA) (Conda Laboratories). All isolates were single-spored in order to obtain pure cultures and stored in filter paper at -20°C. The incidence was determined as the mean percentage of grafted plants that were infected by black-foot fungi. The disease severity in infected grafted plants was determined as the mean percentage of root segments (10 segments per plant) that were colonized by these fungi.

Morphological identification and characterization. Cultures were grown on PDA, oatmeal agar (OA) (Sigma-Aldrich Laboratories), and synthetic nutrient agar (SNA) (Sifin Diagnostics, Berlin, Germany) with or without two 1 cm² pieces of sterile filter paper on the medium surface (Crous et al. 2009). Plates were incubated at 25°C under near-UV light with a 12-h photoperiod. To induce perithecia of new species, heterothallic and homothallic crosses were performed according to Cabral et al. (2012a). The length and width of 40 conidia were measured at ×1,000 magnification with a compound Nikon Eclipse Ni-e microscope. Minimum, maximum, mean, and standard deviation were calculated from the measurements. Conidial color, shape, and the presence of septa were also recorded. Colony morphological characters were observed and colony colors were determined with the color chart of Rayner (1970).

Molecular characterization and phylogenetic analyses. Total genomic DNA was extracted from fresh mycelium after 10 days of incubation on PDA using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Norcross, GA) following the manufacturer's instructions. The identification of black-foot pathogens was made by sequencing part of the histone H3 (*his3*) gene. In addition, the internal transcribed spacer and intervening 5.8S gene (ITS) region, partial regions of the translation elongation factor 1- α (*tef1*), and the β -tubulin (*tub2*) genes were also sequenced. Each 25- μ l reaction volume contained

12.5 μ l of FastGene Ready Mix (NIPPON Genetics Europe, Dueren, Germany), 1 μ l of each primer, 1 μ l of DNA template (10 ng/ μ l), and 9.5 μ l sterile distilled water, and PCR was performed using a C1000 touch thermal cycler (Bio-Rad Laboratories, Hercules, CA). For *his3*, ITS, and *tub2* genes, amplification conditions included an initial denaturation step of 3 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 1 min. A final extension was performed at 72°C for 1 min. For *tef1* gene, amplification conditions were as follows: initial denaturation step of 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and elongation at 72°C for 45 s. A final extension was performed at 72°C for 10 min. Primers used were CYLHEF and CYLH3R (Crous et al. 2004b) for *his3*, ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) for ITS, CylIEF-1 (5'-ATGGGTAAGGAVGAVAAGAC-3'; J. Z. Groenewald, *unpublished*) and CylIEF-R2 (Crous et al. 2004b) for *tef1*, and T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) for *tub2*. PCR products were visualized in 1% agarose gels (agarose D-1 Low EEO, Conda Laboratories). PCR products were sequenced in both directions by Eurofins GATC Biotech (Cologne, Germany). Sequences were edited and aligned using the program MEGA v. 6 (Tamura et al. 2013). The alignments for each locus were combined in a single file using GenTools (<https://github.com/aberlanas/gen-tools>).

Fungal sequences from grapevines from Spain were aligned with published GenBank sequences, including ex-type specimens from grapevines and other hosts for comparison using MAFFT sequence alignment program v. 6 (Katoh and Toh 2008) (Supplementary Table S1). Alignments were corrected visually and manually edited in Sequence Alignment Editor v. 2.0a11 (Rambaut 2002). Firstly, phylogenetic analyses were conducted on the *his3*, ITS, *tef1*, and *tub2* single-locus alignments for representative isolates obtained in this study. In addition, a multilocus phylogenetic analysis was performed on the combined *his3*, ITS, *tef1*, and *tub2* datasets. A partition homogeneity test was conducted in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003). The congruence among the *his3*, ITS, *tef1*, and *tub2* datasets were tested at 1,000 replicates.

Maximum Likelihood (ML) and Bayesian analyses were performed on the concatenated alignment, while individual gene alignments were only subjected to ML analyses. ML analyses were performed in MEGA v. 6 (Tamura et al. 2013) using the best fit model as estimated with the Bayesian information criterion in jModelTest 2.1.10 (Darriba et al. 2012). Branch support was calculated from 1,000 bootstrap replicates for the single and concatenated datasets. The Bayesian analysis of the combined four-loci was performed with MrBayes v. 3.2.1 (Ronquist et al. 2012) based on the results of the jModelTest. The Markov Chain Monte Carlo sampling (MCMC) analysis of four chains started in parallel from a random tree topology (Rodríguez et al. 1990). The number of generations was set at 10 M and the run was stopped automatically when the average standard deviation of split frequencies fell below 0.01. Trees were saved each 1,000 generations. Burn-in was set at 25%, after which the likelihood values were stationary and the remaining trees were used to calculate posterior probabilities. *Campylocarpon fasciculare* (CBS 112613) and *Ca. pseudofasciculare* (CBS 112679) were used as outgroups in phylogenetic analyses.

Representative black-foot disease isolate sequences obtained in this study were submitted to GenBank (Supplementary Table S2), and the alignments in TreeBASE under the study number 24347 (<https://treebase.org>), and taxonomic novelties in MycoBank (www.Mycobank.org) (Crous et al. 2004a).

Effect of temperature on mycelial growth. Twenty-four fungal isolates, belonging to 13 different species, were randomly selected for temperature-growth assay (Table 2). Five-mm diameter mycelial plugs were transferred to the center of PDA plates and incubated in darkness from 5 to 35°C at 5°C intervals. Colony diameter was recorded after 10 days in two orthogonal directions. Four replicate plates per isolate were used and the experiment was conducted twice. Regression curves were fitted to the values of temperature versus

radial growth in millimeters per day for each isolate. The optimum temperature for radial growth and the maximum daily radial growth were calculated in the fitted equation for each isolate. Data were analyzed using the Kruskal-Wallis test, and mean ranks of isolates were compared at $P = 0.05$ using Dunn's test.

Pathogenicity tests. Two-month-old grapevine seedlings (cv. Tempranillo) were used for pathogenicity tests. Seedlings were planted individually in 250 cm³ plastic pots containing sterilized peat moss and inoculated when one to two leaves had emerged by immersing the roots in a 10⁵ ml⁻¹ conidial suspension for 1 h (Martínez-Diz et al. 2018). The viability of the conidia was tested by spreading 150 µl of each conidial suspension diluted to 100

conidia ml⁻¹ onto PDA plates and counting the resulting colonies after 2–3 days incubation at 25°C in the dark. Twenty-four fungal isolates, belonging to 13 different species, were used (Table 3). Six seedlings per isolate were inoculated, and control plants were inoculated with sterile distilled water. The experiment was repeated twice. Seedlings were maintained in a growth chamber at 25°C in a completely randomized design. Plants were inspected every morning from day 1 to day 61 for the development of foliar symptoms. However, due to the lack of specificity of foliar symptoms in grapevine seedlings, as well as the short time interval from foliar symptom development to plant death, the following parameters were considered: the mean time from inoculation in which the plant stayed alive

Table 1. Location of the field nurseries surveyed and the incidence and severity of black-foot disease and fungal species isolated

Location	Year of sampling	Plants analyzed	Incidence (%) ^x	Severity (%) ^y	Fungal species (no. of isolates) ^z
Larraga, Navarra	2016	10	60.0	13.3	<i>Dactylonectria torresensis</i> (8)
Larraga, Navarra	2016	10	90.0	11.1	<i>D. torresensis</i> (10)
Mendavia, Navarra	2016	5	80.0	10.0	<i>D. torresensis</i> (4)
Mendavia, Navarra	2017	598	23.4	15.8	<i>D. macrodidyma</i> (25) <i>D. novozelandica</i> (26) <i>D. torresensis</i> (168) <i>Ilyonectria liriiodendri</i> (1) <i>Thelonectria olida</i> (1)
Mendavia, Navarra	2017	614	23.0	13.7	<i>D. macrodidyma</i> (21) <i>D. novozelandica</i> (12) <i>D. torresensis</i> (154) <i>I. liriiodendri</i> (5) <i>I. robusta</i> (1)
Larraga, Navarra	2017	40	12.5	10.0	<i>D. macrodidyma</i> (2) <i>D. torresensis</i> (3)
Larraga, Navarra	2017	120	60.8	13.3	<i>D. alcacerensis</i> (8) <i>D. torresensis</i> (89)
Larraga, Navarra	2017	220	14.1	11.9	<i>D. alcacerensis</i> (1) <i>D. macrodidyma</i> (2) <i>D. torresensis</i> (33)
O Barco de Valdeorras, Galicia	2018	50	26.0	14.6	<i>I. liriiodendri</i> (1) <i>D. macrodidyma</i> (5) <i>D. pauciseptata</i> (3) <i>D. torresensis</i> (8) <i>I. liriiodendri</i> (2)
O Barco de Valdeorras, Galicia	2018	243	4.9	16.7	<i>I. robusta</i> (1) <i>D. alcacerensis</i> (2) <i>D. torresensis</i> (6) <i>I. liriiodendri</i> (12)
Larraga, Navarra	2018	136	8.8	10.8	<i>D. torresensis</i> (13)
Larraga, Navarra	2018	120	60.0	16.1	<i>D. alcacerensis</i> (25) <i>D. torresensis</i> (91)
Logroño, La Rioja	2018	260	19.2	14.0	<i>D. alcacerensis</i> (4) <i>D. novozelandica</i> (6) <i>D. torresensis</i> (54) <i>D. riojana</i> sp. nov. (2) <i>I. liriiodendri</i> (3)
Larraga, Navarra	2018	960	51.8	12.3	<i>D. alcacerensis</i> (2) <i>D. torresensis</i> (425) <i>I. liriiodendri</i> (132) <i>I. pseudodestructans</i> (11) <i>I. robusta</i> (27) <i>I. vivaria</i> sp. nov. (3) <i>Neonectria quercicola</i> (2) <i>N. sp. 1</i> (1) <i>T. olida</i> (3)
Logroño, La Rioja	2018	40	12.5	18.0	<i>D. novozelandica</i> (2) <i>D. torresensis</i> (5) <i>N. quercicola</i> (2)

^x Disease incidence of black-foot pathogens was determined as the percentage of grafted plants that were infected by these fungi.

^y Disease severity in infected grafted plants was determined as the percentage of root segments (10 segments per plant) that were colonized by black-foot fungi.

^z Identification of black-foot pathogens was first confirmed by sequencing part of the *his3* gene. (*D.*): *Dactylonectria*; (*I.*): *Ilyonectria*; (*T.*): *Thelonectria*, (*N.*): *Neonectria*.

(MTA) and the percentage of dead plants. Two months after inoculation, all plants were uprooted and washed free of soil. Roots, crown, and stem were aseptically plated on MEAS to reisolate the black-foot disease fungal species as described above.

Statistical analysis. Homogeneity of variance across treatments was evaluated using Levene's test prior to the analysis of variance (ANOVA) (Box et al. 1978). The percentage of dead plants and the MTA data were subjected to ANOVA factorial design with the

Table 2. Temperature-growth relationship for black-foot disease associated fungal isolates^w

Species	Isolate	Adjusted model ^x				Temperature (°C)			Growth (mm/day) ^z
		R ²	a	b	c	Minimum	Maximum	Optimal ^y	
<i>Dactylonectria alcacerensis</i>	BV-1240	0.71	-0.0157	0.6266	-3.2011	10	25	19.9 ab	3.0 bc
<i>D. alcacerensis</i>	BV-1469	0.82	-0.0124	0.4985	-2.4377	10	30	20.1 ab	2.6 bcd
<i>D. macrodidyma</i>	BV-1366	0.80	-0.0154	0.6179	-3.0275	10	30	20.1 ab	3.2 bc
<i>D. macrodidyma</i>	BV-0535	0.79	-0.0141	0.5684	-2.7668	10	30	20.2 ab	3.0 bc
<i>D. novozelandica</i>	BV-0760	0.78	-0.0148	0.5834	-2.6809	10	30	19.8 ab	3.1 bc
<i>D. novozelandica</i>	BV-1369	0.75	-0.0177	0.7107	-3.3549	10	30	20.1 ab	3.8 bc
<i>D. pauciseptata</i>	BV-1354	0.83	-0.0169	0.6898	-3.2981	5	30	20.4 ab	3.7 bc
<i>D. pauciseptata</i>	BV-1360	0.73	-0.0215	0.8871	-4.7891	10	30	20.6 a	4.4 ab
<i>D. riojana</i> sp. nov.	BV-1396	0.92	-0.0107	0.4272	-2.0464	10	30	20.0 ab	2.2 d
<i>D. riojana</i> sp. nov.	BV-1397	0.93	-0.0109	0.4351	-2.068	10	30	20.0 ab	2.3 cd
<i>D. torresensis</i>	BV-0901	0.78	-0.0135	0.5373	-2.6696	10	30	19.9 ab	2.7 bcd
<i>D. torresensis</i>	BV-0666	0.76	-0.0181	0.7081	-3.2878	10	30	19.6 ab	3.6 bc
<i>Ilyonectria liriiodendri</i>	BV-1591	0.81	-0.0138	0.5362	-2.1454	5	30	19.4 ab	3.1 bc
<i>I. liriiodendri</i>	BV-1642	0.80	-0.0173	0.668	-2.5882	5	30	19.3 ab	3.9 bc
<i>I. pseudodestructans</i>	BV-2142	0.84	-0.0099	0.3956	-1.8645	10	30	20.0 ab	2.1 d
<i>I. pseudodestructans</i>	BV-2609	0.71	-0.0158	0.6301	-3.2205	10	30	19.9 ab	3.0 bc
<i>I. robusta</i>	BV-1593	0.80	-0.0191	0.7121	-2.4659	5	25	18.6 b	4.2 ab
<i>I. robusta</i>	BV-1654	0.80	-0.0245	0.9523	-4.1827	10	30	19.4 ab	5.1 a
<i>I. vivaria</i> sp. nov.	BV-1924	0.78	-0.0173	0.6657	-2.5358	5	35	19.4 ab	3.9 bc
<i>I. vivaria</i> sp. nov.	BV-2305	0.77	-0.0184	0.7116	-3.0192	5	30	19.3 ab	3.9 bc
<i>Neonectria quercicola</i>	BV-2137	0.82	-0.0191	0.7274	-2.6044	5	35	19.0 ab	4.3 ab
<i>N. quercicola</i>	BV-1661	0.85	-0.0194	0.7427	-2.6372	5	30	19.1 ab	4.5 ab
<i>Neonectria</i> sp. 1	BV-2682	0.78	-0.0149	0.5603	-1.7684	5	30	18.8 b	3.5 bc
<i>Thelonectria olida</i>	BV-0537	0.76	-0.0133	0.5287	-2.5958	5	30	19.9 ab	2.7 bcd

^w Data are the mean of eight replicates for each isolate. For each column, means with the same letter are not significantly different ($P = 0.05$) according to the Kruskal-Wallis test and the Dunn's test for multiple comparisons of mean ranks.

^x Mycelial growth on potato dextrose agar at 5 to 35°C was adjusted to a second-degree polynomial model: $Y = aT^2 + bT + c$, in which Y = mycelial growth (mm/day); a , b , and c are the regression coefficients; and R^2 = coefficient of determination.

^y Optimal temperature estimated by the adjusted model.

^z Maximum growth rate estimated by the adjusted model.

Table 3. Pathogenicity of black-foot pathogens on seedlings of *Vitis vinifera* cv. Tempranillo^y

Genera	Fungal species	Isolate	% of dead plants	MTA (days) ^z	% of isolation ($n = 12$)
<i>Dactylonectria</i>	<i>Dactylonectria novozelandica</i>	BV-0760	100 a	12.9 f	100
	<i>D. alcacerensis</i>	BV-1240	75.0 b	41.2 e	100
	<i>D. macrodidyma</i>	BV-1366	66.7 bc	56.4 ab	100
	<i>D. macrodidyma</i>	BV-0535	41.7 cd	54.9 abc	83.3
	<i>D. pauciseptata</i>	BV-1360	41.7 cd	47.3 cde	100
	<i>D. riojana</i> sp. nov.	BV-1397	41.7 cd	57.5 ab	100
	<i>D. alcacerensis</i>	BV-1469	33.3 cd	49.5 bcde	75.0
	<i>D. riojana</i> sp. nov.	BV-1396	33.3 cd	49.8 bcde	100
	<i>D. torresensis</i>	BV-0901	25.0 cde	58.2 ab	100
	<i>D. novozelandica</i>	BV-1369	16.7 cdef	57.4 ab	90.9
	<i>D. pauciseptata</i>	BV-1354	8.3 ef	55.4 abc	83.3
	<i>D. torresensis</i>	BV-0666	8.3 ef	56.3 ab	100
	<i>Ilyonectria vivaria</i> sp. nov.	BV-1924	50.0 c	53.3 abcd	100
	<i>I. vivaria</i> sp. nov.	BV-2305	50.0 c	49.8 bcde	41.7
	<i>I. liriiodendri</i>	BV-1591	25.0 cde	60.3 a	100
<i>Ilyonectria</i>	<i>I. pseudodestructans</i>	BV-2142	25.0 cde	56.2 ab	83.3
	<i>I. liriiodendri</i>	BV-1642	16.7 cde	54.5 abc	91.7
	<i>I. robusta</i>	BV-1654	16.7 cde	59.3 a	83.3
	<i>I. pseudodestructans</i>	BV-2609	8.3 ef	59.0 a	75
	<i>I. robusta</i>	BV-1593	8.3 ef	60.5 a	66.7
	<i>Neonectria</i> sp. 1	BV-2682	25.0 cde	55.3 abc	0
	<i>N. quercicola</i>	BV-2137	8.3 ef	60.4 a	50
	<i>N. quercicola</i>	BV-1661	0.0 f	61.0 a	100
<i>Thelonectria</i>	<i>Thelonectria olida</i>	BV-0537	58.3 c	45.4 de	100
	Control		10.4 ef	59.4 a	0

^y For each column, means with the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

^z MTA: Mean time from inoculation in which the plant stayed alive.

following factors: experiment (performed twice), pathogen and isolate nested in pathogen (two isolates for each pathogen species except for *Neonectria* sp. 1 and *Thelonectria olida*). Means were compared by Duncan's multiple range test at $P = 0.05$ using IBM SPSS Statistics 19.0.

Results

Incidence of black-foot disease pathogens. Based on colony morphology, conidial characteristics, and phylogenetic analyses (see below), 1,427 isolates associated with 13 species belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Thelonectria* were identified. *Dactylonectria torresensis* was isolated from grafted plants in all grapevine nursery fields, and accounted for 75.05% of all isolates (Table 1). The remaining isolates were identified as *D. macrodidyma* (3.85%), *D. novozelandica* (3.22%), *D. alcacerensis* (2.94%), *D. pauciseptata* (0.21%), *Dactylonectria* sp. (0.14%), *Ilyonectria liriiodendri* (10.93%), *I. robusta* (2.03%), *I. pseudodestructans* (0.8%), *I. sp.* (0.21%), *Neonectria* sp. 1 (0.06%), *N. quercicola* (0.28%), and *T. olida* (0.28%). In all nursery fields surveyed, incidence ranged from 4.9 to 90%, while disease severity ranged from 10 to 18%.

Morphological characterization. The color of the colonies ranged from pale buff to chestnut or cinnamon to vinaceous on PDA, and from buff to chestnut or brown to cinnamon on OA. Based on the microscopic observations, almost all the isolates produced macroconidia, microconidia, and chlamydospores which matched descriptions for *Cylindrocarpum*-like asexual morphs. Based on the morphological features, it was possible to separate the isolates into three different groups among black-foot disease isolates. The first consisted of isolates showing 5-septate, cylindrical, and generally straight macroconidia, and microconidia were ellipsoidal to oblong, and no chlamydospores were observed. These characteristics matched those described earlier for the genus *Thelonectria* (Chaverri et al. 2011). The second group of isolates had 5-septate, fusiform, and curved macroconidia. Microconidia and chlamydospores were uncommon. These matched descriptions for the genus *Neonectria* (Chaverri et al. 2011). The third consisted of isolates showing 1 to 3-septate, cylindrical, and generally straight macroconidia. Microconidia were ellipsoidal to ovoid. These characteristics matched those described earlier for the genera *Dactylonectria* (Lombard et al. 2014) or *Ilyonectria* (Chaverri et al. 2011). According to Lombard et al. (2014), *Dactylonectria* can be distinguished from *Ilyonectria* by the characteristics of the sexual morph as well as the absence of chlamydospore production in culture.

Molecular characterization and phylogenetic analyses. The AIC best-fit nucleotide substitution model identified by jModelTest was Hasegawa-Kishino-Yano model (HKY) with gamma distributed with invariant sites rates (G+I) for *his3* and ITS and HKY+G for *tef1* and *tub2*. In the *his3*, *tub2*, and *tef1* single-locus alignments, three major clades belonging to the genera *Ilyonectria*, *Neonectria*, and *Dactylonectria* were obtained (Fig. 1). Looking at individual gene trees obtained using the model proposed by jModelTest, the *his3* tree enables the separation of all species with high bootstrap values (Fig. 1). For *tub2*, all species could be resolved, except *Dactylonectria novozelandica* (Supplementary Fig. S1). Individual *tef1* gene phylogeny revealed a lack of resolution for some species, including *I. lusitanica*/*I. europaea*, *I. rufal*/*I. panacis*, *I. crassal*/*I. pseudodestructans*/*I. protearum*, and *D. vitis*/*D. hispanica* (Supplementary Fig. S2). The ITS tree does not resolve any species, as nucleotide sequences were indistinguishable for this region.

The partition homogeneity test ($P = 0.112$) led us to combine the *his3*, ITS, *tef1*, and *tub2* datasets (Fig. 2). The ML tree and the Bayesian consensus tree had similar topology, and therefore only the ML tree is presented with bootstrap support values (BS) and posterior probability values (PP). The four loci alignment contained 91 taxa (including the two outgroups). The ML tree and the Bayesian consensus tree obtained with the four-loci alignment confirmed the existence of two novel taxa within our set of isolates.

Taxonomy. Based on the phenotypical characters previously recorded along with the phylogenetic analysis, one species each of *Ilyonectria* and *Dactylonectria* are described (Figs. 3 and 4). No perithecia were observed in the heterothallic or homothallic crosses performed.

Dactylonectria riojana C. Berlanas, M. Andrés-Sodupe, S. Ojeda & D. Gramaje, sp. nov. MycoBank MB 829945 (Fig. 3). Etymology: Name refers to the Spanish region of La Rioja, where the fungus was isolated.

Diagnosis. Morphologically, *D. riojana* can be distinguished by its shorter conidiophores when compared with *D. novozelandica*, *D. torresensis*, *D. alcacerensis*, and *D. macrodidyma*. Nineteen polymorphisms can distinguish *D. riojana* from *D. alcacerensis*: two in *tub2* locus, 11 in *his3* locus, four in *tef1* locus, and two in ITS. Seventeen polymorphisms can distinguish *D. riojana* from *D. torresensis*: one in *tub2* locus, four in *his3* locus, 10 in *tef1* locus and two in ITS. Eleven polymorphisms can distinguish *D. riojana* from *D. novozelandica*: seven in *his3* locus, two in *tef1* locus, and two in ITS. Twenty-three polymorphisms can distinguish *D. riojana* from *D. macrodidyma*: three in *tub2* locus, 13 in *his3* locus, and seven in *tef1* locus.

Typus. Spain: La Rioja, Logroño, on roots of 110 Richter grapevine rootstock (*Vitis berlandieri* × *Vitis rupestris*), 2018, C. Berlanas (CBS H-23883 – holotype; CBS 145413 = BV-1396 – ex-type culture).

Conidiophores simple. Complex conidiophores not observed. Simple conidiophores arising laterally or terminally from aerial mycelium, solitary to aggregated, unbranched or some branched with up to three phialides, 1- to 2-septate, 35 to 65 µm long; phialides monophialidic, more or less cylindrical, tapering slightly toward the apex, 12 to 29 µm long, 2.0 to 2.5 µm wide at the base, 2.5 to 3.5 µm at the widest point, 1.5 to 3 µm near the aperture.

Macroconidia (1 to) 3-septate, straight, cylindrical, but may narrow toward the tip, more or less broadly rounded, and the base appearing acute due to the presence of the hilum; 1-septate (25–) 30 to 35 (–47) × (6.5–) 7 to 8 (–8.5) µm (av. 33 × 8 µm), L/W ratio (3–) 3.8 to 4.5 (–6) (av. 4.2), 2-septate (30–) 35 to 39 (–51) × (6–) 7 to 8.5 (–9.5) µm (av. 39 × 8 µm), L/W ratio (3–) 4 to 5 (–7) (av. 4.5), and 3-septate (36–) 44 to 48 (–55) × (7–) 7.5 to 8.2 (–9) µm (av. 45 × 8 µm), L/W ratio (4–) 4.5 to 6 (–7) (av. 5.5).

Microconidia 0–1 septate, aseptate microconidia subglobose to oval, (9–) 10 to 13.5 (–16.5) × (5–) 6 to 6.5 (–7) (av. 12 × 6 µm), L/W ratio (1.5–) 1.8 to 2.2 (–2.4) (av. 2); 1-septate, mostly subcylindrical, (12.5–) 18 to 21 (–23.5) × (6–) 7 to 8 (–8.5) (av. 20.3 × 7 µm), L/W ratio (2–) 2.5 to 2.8 (–3.2) (av. 2.7). Chlamydospores rarely observed.

Culture characteristics. Mycelium felty with average density (OA) or strong density (PDA). Surface on OA straw to buff; margin buff. Surface on PDA honey to buff; margin buff. Zonation absent, transparency homogeneous and margin uneven (OA) and even (PDA). Reverse similar to surface, except in color, buff to sepia on OA and sepia to chestnut on PDA. Colonies reaching a radius of 26.8 mm after 7 days at 25°C. Minimum temperature for growth 10°C, optimum 20°C, maximum 30°C.

Host and distribution. *Vitis berlandieri* × *Vitis rupestris* (roots of 110 Richter grapevine rootstock) (Spain, La Rioja).

Notes. *D. riojana* is closely related to *D. novozelandica*, *D. torresensis*, *D. alcacerensis*, and *D. macrodidyma*. The morphology of these species is very similar, but *D. riojana* can be distinguished by its shorter conidiophores and the inability to grow at 5°C. No chlamydospores were observed in *D. riojana*, while these structures rarely occur in *D. novozelandica*, *D. torresensis*, and *D. alcacerensis*, or sometimes occur in *D. macrodidyma*.

Ilyonectria vivaria C. Berlanas, B. López-Manzanares, R. Bujanda & D. Gramaje, sp. nov. MycoBank MB 829951 (Fig. 4). Etymology: Latin, from vivarium, meaning nursery. In reference to the environment it was frequently isolated from.

Diagnosis. Morphologically, *I. vivaria* can be distinguished by its slower growth at 25°C and the ability to grow at 35°C when compared with *I. mors-panacis*. Seventy-six polymorphisms can distinguish *I. vivaria* from *I. mors-panacis*: 17 in *tub2* locus, 32 in *his3* locus, 25 in *tef1* locus, and two in ITS.

Typus. Spain: Navarra, Larraga, on roots of 110 Richter grapevine rootstock (*Vitis berlandieri* × *Vitis rupestris*), 2018, C. Berlanas (CBS H-23884 – holotype; CBS 145414 = BV-2305 – ex-type culture).

Conidiophores simple or complex. Simple conidiophores arising laterally or terminally from aerial mycelium, solitary to aggregated, unbranched or some branched with up to four phialides, 1- to

4-septate, 42 to 165 µm long; phialides monophialidic, more or less cylindrical, tapering toward the apex, 21 to 47 µm long, 2.0 to 2.5 µm wide at the base, 2.5 to 3.0 µm at the widest point, 1.5 to 2.5 µm near the aperture.

Complex conidiophores aggregated in small sporodochia, repeatedly and irregularly branched; phialides cylindrical, but tapering toward the apex, 10 to 25 µm long, 1.5 to 2.5 µm wide at the

base, 2.0 to 2.5 µm at the widest point, and 1.5 to 2.0 µm wide at the apex.

Macroconidia (1 to) 3-septate, straight, cylindrical, but may narrow toward the tip, more or less broadly rounded, and base sometimes with a visible, centrally hilum; 1-septate (22–) 27 to 34 (–41) × (6–) 6.5 to 8.0 (–9) µm (av. 31 × 7.5 µm), L/W ratio (3–) 3.5 to 4.2 (–6) (av. 4), 2-septate (26–) 33 to 38 (–47) × (6–) 7.0 to 8.0

Fig. 1. Maximum likelihood phylogeny of *Cylindrocarpon*-like asexual morphs as estimated from the alignment of the histone H3 gene sequences. Maximum likelihood bootstrap percentages are indicated at the nodes. Support values less than 70% bootstrap are omitted. The tree was rooted to *Campylocarpon fasciculare* (CBS 112679) and *Ca. pseudofasciculare* (CBS 112613). The scale bar indicates 0.05 expected changes per site. (T): ex-type cultures. Tentative new species are indicated in red.

(–9) μm (av. $35 \times 7.5 \mu\text{m}$), L/W ratio (3–) 4 to 5.5 (–7) (av. 4.5), and 3-septate (33–) 45 to 51 (–56) $\times$ (7–) 7.4 to 8 (–8.5) μm (av. $47 \times 7.5 \mu\text{m}$), L/W ratio (4–) 4.5 to 6.5 (–7) (av. 5).

Microconidia 0–1 septate, aseptate microconidia ellipsoidal to oval, (8–) 9 to 11.5 (–13.5) $\times$ (4.5–) 5.5 to 6.5 (–7) (av. $10.5 \times 6 \mu\text{m}$), L/W ratio (1.5–) 1.5 to 2 (–2.2) (av. 1.7); 1-septate, mostly

ellipsoidal (12–) 15 to 21 (–18.5) $\times$ (5.5–) 7 to 8 (–8.5) (av. $18 \times 6.5 \mu\text{m}$), L/W ratio (1.5) 2.5 to 3 (–3.3) (av. 2.8). Chlamydospores globose to cylindrical, 10 to 17 $\times$ 10 to 20 μm diam., smooth, but often appearing rough due to deposits, thick-walled, terminal on short lateral branches, rarely intercalary, single, in chains or in clumps, hyaline, becoming medium brown.

Fig. 2. Maximum likelihood phylogeny of *Cyliodrocarpon*-like asexual morphs as estimated from concatenated alignments of four gene dataset (ITS, *tub2*, *his3*, and *tef1*). Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes (ML/PP). Support values less than 70% bootstrap or 0.80 posterior probability are omitted or indicated with ‘–’. The tree was rooted to *Campylocarpon fasciculare* (CBS 112613) and *Ca. pseudofasciculare* (CBS 112679). The scale bar indicates 0.05 expected changes per site. (T): ex-type cultures. New species are indicated in red.

Culture characteristics. Mycelium cottony to felty with average density in both OA and PDA. Surface on OA honey to buff; margin buff. Surface on PDA buff, to honey to isabelline toward the center; margin buff. Zonation concentric (PDA) or absent (OA), transparency homogeneous and margin uneven

(OA) and even (PDA). Reverse similar to surface, except in color, buff to cinnamon on OA and chestnut to cinnamon on PDA. Colonies reaching a radius of 27.3 mm after 7 days at 25°C. Minimum temperature for growth 5°C, optimum 19.4°C, maximum 35°C.

Fig. 3. *Dactylonectria riojana* (ex-type culture BV-1396). Ten-day-old colonies grown at 20°C in darkness on potato dextrose agar (PDA) (A) and oatmeal agar (OA) (B). C–D, Simple, sparsely branched conidiophore of the aerial mycelium. E–H, Micro- and macroconidia. Scale bars: C–F = 10 μ m; G = 20 μ m; H = 50 μ m.

Fig. 4. *Ilyonectria vivaria* (ex-type culture BV-2305). Ten-day-old colonies grown at 20°C in darkness on potato dextrose agar (PDA) (A) and oatmeal agar (OA) (B). C–F, Simple, sparsely branched conidiophore of the aerial mycelium. G, Chlamydospores in mycelium. H–I, Micro- and macroconidia. Scale bars: C–I = 10 μ m.

Host and distribution. *Vitis berlandieri* × *Vitis rupestris* (roots of 110 Richter grapevine rootstock) (Spain, Navarra).

Notes. *I. vivaria* is closely related to *I. mors-panacis* based on the *his3* sequence alignment. The morphology of both species is very similar, but *I. vivaria* can be distinguished by its slower growth after 7 days at 25°C and the ability to grow at 35°C.

Effect of temperature on mycelial growth. Analyses of variance indicated no significant differences of the mycelial growth between experiments ($P = 0.4115$), so data from both experiments were averaged. Most of the isolates studied failed to grow on PDA at 35°C, with the exception of *N. quercicola* isolate BV-2137 and *I. vivaria* sp. nov. isolate BV-1924, while some of them grew at 5°C (*D. pauciseptata* isolate BV-1354, *I. liriodendri* isolates, *I. robusta* isolate BV-1593, *I. vivaria* sp. nov. isolates, *N. sp. 1* isolate BV-2682, *N. quercicola* isolates, and *T. olida* isolate). Optimal temperatures for mycelial growth ranged from 18.6 to 20.6°C (Table 2). Significant differences were found in the optimal temperature between *D. pauciseptata* isolate BV-1360, and *Neonectria* sp. 1 isolate BV-2682 and *I. robusta* isolate BV-1593 ($P < 0.05$), but it was not possible to statistically differentiate the remaining isolates, because of the overlap in the optimal temperature measurements among them (Table 2). According to the Kruskal-Wallis test, maximum growth rates of isolates differed significantly ($P < 0.05$). The relationship between growth rate and temperature for all isolates was best described by a second-degree polynomial ($Y = aT^2 + bT + cT$). The three regression coefficients were highly significant in all cases ($P < 0.01$). The coefficient of determination (R^2) ranged from 0.71 to 0.93. Isolates with maximum growth rate ≤ 3 mm/day included *D. alcacerensis* isolates, *D. macrodidyma* isolate BV-0535, *D. riojana* sp. nov. isolates, *D. torresensis* isolate BV-0901, *I. pseudodestructans* isolates, and *T. olida* isolate. Isolates with maximum growth rate between 3 and 5 mm/day included *D. macrodidyma* isolate BV-1366, *D. novozelandica* isolates, *D. pauciseptata* isolates, *D. torresensis* isolate BV-0666, *I. liriodendri* isolates, *I. robusta* isolate BV-1593, *I. vivaria* sp. nov. isolates, *N. quercicola* isolates, and *N. sp. 1* isolate. The only species that grew more than 5 mm/day was *I. robusta* (isolate BV-1654).

Pathogenicity tests. In all fungi, the viability of conidia was at least 90%. For each species, there were differences between pathogen isolates when evaluating the percent of dead plants and the MTA ($P < 0.05$), so the data of isolates within species were not combined. Grapevine seedlings did not show any foliar symptoms during the experiment; however, the susceptibility to black-foot disease fungi measured as the percent of dead plants and the MTA varied among fungal isolates (Table 3). *D. novozelandica* isolate BV-0760 was the most virulent species followed by *D. alcacerensis* isolate BV-1240, *D. macrodidyma* isolate BV-1366, and *I. vivaria* sp. nov. isolates when measuring the percent of dead plants. Grapevine seedlings inoculated with *D. novozelandica* isolate BV-0760 had significantly less MTA than those inoculated with other fungal species. *D. alcacerensis* isolates, *D. pauciseptata* isolate BV-1360, *D. riojana* sp. nov. isolate BV-1396, *I. vivaria* sp. nov. isolate BV-2305, and *T. olida* differed significantly with respect to the control when measuring the MTA. Percentage of reisolation ranged from 41.7 to 100%. *Neonectria* sp. 1 was not successfully isolated from any inoculated seedling. No black-foot disease pathogens were isolated from the control treatment (Table 3).

Discussion

This study is the first comprehensive effort to characterize a group of fungi associated with GTDs isolated from visually symptomless vines and asymptomatic internal wood tissue. Our results demonstrate that black-foot disease fungi can live as latent pathogens within grapevine and might become pathogenic under specific conditions. The pathogenicity of black-foot disease fungi on grapevines have been so far demonstrated in tests performed under field (Sieberhagen 2016) and controlled conditions (Alaniz et al. 2010; Brown et al. 2013; Cabral et al. 2012b; Gubler et al. 2004; Jaspers et al. 2007; Probst et al. 2019). Recent studies reported the sporadic occurrence of other trunk disease fungi in grapevine as latent pathogens

(Eichmeier et al. 2018; Hofstetter et al. 2012), without any disease symptoms ever becoming evident. Most of these studies employed molecular tools to analyze the grapevine fungal microbiome without the need to cultivate the fungal isolates. However, functions such as mutualism or pathogenicity in fungi can rarely be predicted by using these tools (Brader et al. 2017).

Several factors have been reported to be determinants in triggering pathogenicity in an endophyte that was previously asymptomatic, such as the host genotype, changes in plant gene expression, nutrient status, habitat or the locally occurring abiotic stress that might reduce host fitness, resulting in distortion of this delicate balance and thus influencing the symptomatology in plants (Johnson and Oelmüller 2009). Abiotic stress factors in grapevine nursery fields and new plantations include J-rooting, waterlogging, water stress, winter-kill, nutrition deficiency, soil compaction, and/or overcropping (Gramaje et al. 2018).

In the scientific literature, observations of black-foot disease fungi as endophytes colonizing asymptomatic vines (Langenhoven et al. 2018) or other plant species (Agustí-Brisach et al. 2011; Bonito et al. 2016; Langenhoven et al. 2018) have been documented. Many of these asymptomatic plants are cereals and brassicaceous crops, used in crop rotations in grapevine nurseries (Langenhoven et al. 2018), and weeds, which may be present in field nurseries and established vineyards along with cultivated crops (Agustí-Brisach et al. 2011; Langenhoven et al. 2018). We therefore suggest that these endophytic associations among black-foot disease fungi and symptomless hosts are not unusual relationships in nature. The fact that the black-foot disease fungi can be endophytes on weeds or other hosts has important implications, such as symptomless plants inadvertently serving as sources of hidden diversity of black-foot species, or serving as inoculum reservoirs. Because most research conducted on fungal trunk diseases has focused on pathogens infecting important agricultural commodities such as grapevine, almond, or olive (Gramaje et al. 2016), we still lack a thorough understanding of the true nature of the associations of these fungi with other plants and their environment. In addition, the effects of weeds and/or other symptomless hosts on the genetic composition of black-foot disease pathogen populations have seldom been explored and thus remain poorly understood.

A wide diversity of black-foot disease pathogens were identified in this study, bringing the total number of fungal species associated with this disease isolated from grapevines in Spain to 17. These results confirm the richness of black-foot species in the Iberian Peninsula, with 14 species reported in Portugal (Cabral et al. 2012a, 2012c; Reis et al. 2013), compared with other countries in which black-foot disease is also prevalent, such as South Africa with 8 species (Halleen et al. 2004, 2006b; Langenhoven et al. 2018), New Zealand with 7 species (Bleach et al. 2006; Pathrose 2012), and Canada (Úrbez-Torres et al. 2014) and Italy (Carlucci et al. 2017) with 5 species each. Our findings also confirm *Dactylonectria* as a genus commonly associated with infections in grapevine as reported previously by Lombard et al. (2014), and *D. torresensis* being the most prevalent black-foot species in Spain. This study represents the first report of *I. pseudodestructans* and *N. quercicola* on grapevine in this country. Hosts and distribution of *I. pseudodestructans* include Kentucky bluegrass (Canada), *Quercus* sp. (Austria), and grapevine (Portugal). To our knowledge, *N. quercicola* has been reported only in holm oak root seedlings showing decline aerial symptoms in forest nurseries in Spain (Mora-Sala et al. 2018).

Two novel species are newly described, namely *D. riojana* and *I. vivaria*. Morphological characteristics have been reported to play a major role in the description of fungal species (Taylor et al. 2000). However, in our study, the use of such characters alone to delimit the new species was insufficient, thus highlighting the usefulness of DNA sequences for such purpose. The *his3* region has previously shown to be the most informative locus for the correct identification of black-foot disease fungi (Cabral et al. 2012a). In our study, the use of the *his3*, *tub2*, and *tefl* alone allowed us to describe these two novel species. However, the ITS tree could not resolve any species, as nucleotide sequences were indistinguishable

for this region. These results are in agreement with previous studies aimed to characterize *Cylindrocarpon*-like fungi by using individual ITS dataset (Cabral et al. 2012c; Úrbez-Torres et al. 2014). Despite the ineffectiveness in resolving these fungal species, the ITS region has been widely used in multilocus sequence analyses of *Cylindrocarpon*-like fungi (Cabral et al. 2012a; Carlucci et al. 2017; Mora-Sala et al. 2018; Parkinson et al. 2017; Úrbez-Torres et al. 2014). The multilocus sequence analysis using *his3*, ITS, *tef1*, and *tub2* regions confirmed the level of polymorphism that enabled the fungal description.

Different inoculation methods, such as watering (Alaniz et al. 2009) or soaking (Martínez-Diz et al. 2018) the roots of grapevine seedlings in conidial suspensions, soaking bases of grapevine rootstock cuttings (Alaniz et al. 2010; Cabral et al. 2012b; Probst et al. 2019) or roots (Pathrose et al. 2014) in conidial suspensions, and vacuum-inoculation of conidial suspensions throughout the vascular system of rootstock cuttings (Sieberhagen 2016) have been used for virulence screening of black-foot disease fungi. Results of our study show that soaking roots of grapevine seedlings is a rapid and effective technique for evaluating virulence of *Cylindrocarpon*-like asexual morph isolates since most of the fungi collected from asymptomatic vines were capable of colonizing roots and causing disease under controlled conditions. Virulence varied among species and between isolates within each fungal species. High degree of virulence variability was also noticed in the 14 *D. macrodidyma* isolates (Alaniz et al. 2009) and 17 *I. liriodendri* isolates (Pathrose et al. 2014) collected in Spain and New Zealand, respectively. Subsequent taxonomic studies suggested that the *D. macrodidyma* isolates used by Alaniz et al. (2009) might represent separate species within the *D. macrodidyma*-species complex (Cabral et al. 2012b). In our study, *D. novozelandica* isolate BV-0760 was considered the most virulent species, followed by *D. alcacerensis* isolate BV-1240 and *I. vivaria* sp. nov. isolate BV-2305. Cabral et al. (2012b) performed pathogenicity tests with 60 isolates belonging to 14 black-foot species collected from grapevine and other hosts and concluded that *I. lusitanica*, *D. estremocensis*, and *I. europaea* were more virulent to grapevine than to the species previously accepted as the main causal agents of black-foot disease, namely *D. macrodidyma* and *I. liriodendri*.

Pathogenicity in fungal trunk pathogens of grapevine is a complex phenomenon. The combination of many factors, such as pathogen and host genotypes and abiotic and other environmental stresses, as well as microbial interactions, seems to determine the outcome of the reaction of grapevine to the pathogen. The present study improves our knowledge on the etiology and virulence of black-foot disease pathogens, and opens up new perspectives in the study of the endophytic role of these pathogens on grapevines. The future research on black-foot disease needs to investigate (i) how these pathogens colonize the endorhizosphere and establish themselves inside, and (ii) what triggers latent black-foot disease fungi to transition from an endophyte to a pathogen, and cause disease symptoms in grapevine.

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