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Black foot in nursery grapevines in Uruguay caused by *Dactylonectria* and *Ilyonectria*

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Summary. Black foot is a serious soilborne fungal disease causing decline of young grapevines. Affected plants show brown to dark streaks developing from the rootstock bases, wood necroses at trunk bases, sunken necrotic lesions on roots, and reduced root biomass. Several fungi, commonly known as *Cylindrocarpon*-like asexual morphs, have been associated with black foot. Nursery vines are infected during rooting in propagation processes, which is important for dissemination of the pathogens. Species associated with black foot in nursery vines produced in Uruguay were characterized by molecular, phenotypical and pathogenicity studies. From 2017 to 2019, 181 rooted vines grafted onto '1103P', 'SO4', '101-14', '3309C' or 'Gravesac' rootstocks were sampled, and 71 *Cylindrocarpon*-like fungal isolates were recovered from rootstock tissues (basal ends and roots). Based on multi-gene phylogenetic analyses of HIS3, TEF and TUB2, and supported by phenotypical characterization, five species of *Dactylonectria* and *Ilyonoectria* were identified, with *D. macrodidyma* being the most prevalent followed by *D. novozelandica*, *D. torresensis*, *D. palmicola* and *I. liriodendri*. Four *Ilyonectria* isolates could not be identified to species level. Isolate pathogenicity was assessed using healthy rooted 'Gravesac' plants. After three months, isolates of all species infected the plants, causing necrotic lesions on roots and reducing root biomass. On average, 39% of ready-to-plant nursery vines were affected by black foot, emphasizing the need to develop integrated management to reduce black foot incidence in Uruguayan grapevine nurseries, based on studies under local conditions.

Keywords. *Cylindrocarpon*-like fungi, grapevine trunk disease, *Vitis vinifera*.

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INTRODUCTION

Black foot is a serious soilborne fungal disease affecting nursery grapevine plants and young vineyards (Halleen *et al*., 2006a; Alaniz *et al*., 2007; Gramaje and Armengol, 2011; Agustí-Brisach and Armengol, 2013). This disease, considered a major cause of young vine decline (Gramaje and Armengol, 2011), occurs in the main grape-growing countries worldwide, including Portugal (Rego *et al*., 2000), South Africa (Fourie and Halleen, 2001), New Zealand (Halleen *et al*., 2004), France (Halleen *et al*., 2004), United States (Petit and Gubler, 2005), Spain (Alaniz *et al*., 2007), Australia (Whitelaw-Weckert *et*

al., 2007), Uruguay (Abreo *et al*., 2010), Canada (Petit *et al*., 2011), Turkey (Özben *et al*., 2012), Iran (Mohammadi *et al*., 2013), Brazil (dos Santos *et al*., 2014), Italy (Carlucci *et al*., 2017), Czech Republic (Pečenka *et al*., 2018), Algeria (Aigoun-Mouhous *et al*., 2019), China (Ye *et al*., 2021), and Argentina (Longone *et al*., 2022).

Grapevines affected by black foot pathogens show reduced root biomass, sunken necrotic root lesions, dark brown to black streaks that develop from rootstock bases, and wood necroses at trunk bases (Rego *et al*., 2000; Halleen *et al*., 2006a; Alaniz *et al*., 2007; Agustí-Brisach and Armengol, 2013). Foliar symptoms usually appear during the first 3 to 5 years after planting, and involve delayed and failed bud-break, reduced vigour, shortened internodes, chlorotic foliage with necrotic margins, wilting and usually plant death (Halleen *et al*., 2006a; Agustí-Brisach and Armengol, 2013). Death occurs quickly when young vines are infected, while as vines age, a more gradual decline occurs (Gubler *et al*., 2004).

Black foot was first known to be caused by "*Cylindrocarpon*" species, but in the last decade, this genus has undergone extensive taxonomic revision (Chaverri *et al*., 2011; Cabral *et al*., 2012a, 2012b; Lombard *et al*., 2014). Currently, more than 30 fungal species of *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, *Pleiocarpon,* and *Thelonectria,* commonly known as *Cylindrocarpon*-like asexual morphs, are associated with black foot (Agustí-Brisach and Armengol, 2013; Lombard *et al*., 2014; Carlucci *et al*., 2017; Aigoun-Mouhous *et al*., 2019). Among these, *D. torresensis* has been reported as the prevalent species in several countries (Reis *et al*., 2013; Berlanas *et al*., 2017; Carlucci *et al*., 2017; Aigoun-Mouhous *et al*., 2019; Akgül *et al*., 2022).

Although the epidemiology of black foot has not been completely clarified, it is well known that black foot pathogens can produce abundant conidia which are dispersed by free water in the soil (Petit *et al*., 2011) and can infect grapevines through natural openings or wounds in trunk bases and roots (Agustí-Brisach and Armengol, 2013). Some species are also able to produce chlamydospores which allow long-term survival in soil (Halleen *et al*., 2004). In addition, several weeds are hosts of black foot pathogens, and these hosts can be inoculum sources for grapevine infections (Agustí-Brisach *et al*., 2011).

Black foot pathogens have been frequently isolated from nursery grapevine plants, indicating that these plants play important roles in the spread of this disease (Halleen *et al*., 2006a; Abreo *et al*., 2010; Agustí-Brisach *et al*., 2013; Cardoso *et al*., 2013; Carlucci *et al*., 2017; Pintos *et al*., 2018; Aigoun-Mouhous *et al*., 2019; Berlanas *et al*., 2020; Akgül *et al*., 2022). Several studies have focused on determining when infection occurs during propagation processes, and there is consensus that the nursery rooting phase is where black foot pathogen infections increase significantly (Halleen *et al*., 2003; Agustí-Brisach *et al*., 2013; Carbone *et al*., 2022). These pathogens can infect nursery plants from the incomplete callus zones or from wounds on roots during rooting in soil, as demonstrated by Probst *et al*. (2019).

A recent study in Uruguay revealed that many plants produced at a local nursery were infected with black foot pathogens (Carbone *et al*., 2022). The present study focused on molecular and phenotypic characterization of the pathogens associated with black foot in nursery vines and assessed the pathogenicity of identified species after inoculation of rooted grapevine rootstocks.

MATERIALS AND METHODS

Sampling material and fungal isolations

During 2017, 2018 and 2019, a total of 181 1-year-old ready-to-plant grapevine plants, grafted onto rootstocks of '1103P' (*Vitis berlandieri* × *V. rupestris*), 'SO4' (*V. riparia* × *V. berlandieri*), '101-14' (*V. riparia* × *V. rupestris*), '3309C' (*V. riparia* × *V. rupestris*), or 'Gravesac' ('161-49' (*V. berlandieri* × *V. riparia*) × '3309C'), were randomly selected from the main commercial grapevine nursery in Uruguay. Fifty-two plants were sampled in 2017, 83 in 2018, and 46 were sampled in 2019. The nursery is located in Canelones (34°34'48.45"S; 56°17'50.17"W), the traditional grape-growing region of Uruguay. To isolate black foot pathogens, the basal part and roots of each plant were separated and then surface sterilized by soaking each portion in 95% ethanol for 1 s followed by flaming (Delgado *et al*., 2016). Cross and longitudinal cuts were then made at the basal portion of the rootstock to reveal internal black foot symptoms. Seven pieces of wood, approx. 5 mm in length, were taken from the margin between necrotic and apparently healthy tissues, using a sterile scalpel, including the basal part of the rootstock and roots. In 2017 and 2019, the wood pieces were selected equally from the basal parts of the rootstocks and roots, while in 2018, the pieces were selected predominantly from the basal parts of the rootstocks. The small pieces of wood were plated onto potato dextrose agar (PDA) (Oxoid Ltd.) supplemented with 0.4 g L^{-1} of streptomycin sulphate (PDAS) (Sigma-Aldrich). The plates were incubated for 5 to 21 d at 25°C in the dark and examined daily for fungal growth. Fungal colonies resembling black foot pathogens, i.e., with aerial and cottony mycelia ranging from white to yellow or light to dark brown, and with mac-

roconidia and microconidia (Halleen *et al*., 2004; 2006b; Cabral *et al*., 2012a), were subculture onto fresh PDA plates to obtain pure cultures. Single conidium isolates were obtained (Carlucci *et al*., 2017), and were stored in colonized sterile filter papers at -20°C. Representative isolates were deposited in the fungal culture collection of the Department of Plant Protection, Faculty of Agronomy, University of the Republic, Uruguay.

Molecular identification of isolates

Total genomic DNA was extracted from 1-week old pure cultures grown on PDA at 25°C in the dark, using the commercial Quick-DNA™ Fungal/Bacterial Miniprep Kit (ZymoResearch), following the manufacturer´s instructions. Primary identification of black foot pathogens was conducted by sequencing part of the histone H3 gene (HIS3) and comparing the sequences with those deposited in the GenBank, using the BLAST source (https://blast.ncbi.nlm.nih.gov/Blast.cgihttps://blast.ncbi.nlm.nih.gov/Blast.cgihttps://blast.ncbi.nlm.nih.gov/Blast.cgi). To confirm the isolate identity, partial regions of the translation elongation factor 1-α (TEF) and the beta-tubulin (TUB2) genes were also sequenced, and a multilocus phylogenetic analysis was performed on the three combined gene regions (Cabral *et al*., 2012a; 2012b; Berlanas *et al*., 2020). The primers used were CYLH3F and CYLH3R for HIS3 (Crous *et al*., 2004), CylEF-1 (5'-ATGGGTAAGGAVGAVAAG AC-3'; J. Z. Groenewald, unpublished) and CylEF-R2 (Crous *et al*., 2004) for TEF, and T1 (O'Donnell and Cigelnik, 1997) and BT2b (Glass and Donaldson, 1995) for TUB2.

Polymerase chain reaction (PCR) amplifications were performed on a MultiGene™ Mini (Labnet International Inc.). Each PCR reaction contained 1× PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.4 µM of each primer, 1 U of DNA polymerase (Bioron), and 1 µL of template DNA. The PCR reaction was adjusted to a final volume of 20 µL with MQ water. The PCR conditions consist of an initial step at 94°C for 3 min followed by 34 cycles for TUB2 and TEF regions, and 40 cycles for HIS3 gene, of denaturation at 94°C for 30 s, annealing at 58°C for TUB2 and TEF and 55°C for HIS3, for 30 s, and elongation at 72°C for 45 s. A final extension was performed at 72°C for 10 min. PCR products were visualized in 1.5% agarose gels stained with GelRedTM, through a transilluminator under UV light. A GeneRuler 100-bp DNA ladder plus (Thermo) was used as a molecular weight marker. PCR products were purified and sequenced by Macrogen Inc., Seoul, Korea.

For each fungus genus, sequences of each gene region were aligned using the ClustalW program, available within MEGA 11.0.11 (https://www.megasoftware.net/), and were manually edited when necessary. Related sequences and sequences of the phylogenetically closest species obtained from GenBank, including ex-type isolates, were incorporated to the alignments (Supplementary Table 1). Multilocus alignments were carried out using Sequence Matrix v.1.8 (http://www.ggvaidya.com/taxondna/). Multilocus phylogenetic analyses were constructed using Bayesian inference (BI) and Maximum likelihood (ML) methods. BI and ML analyses were inferred with, respectively, MrBayes v3.2.7a and RAxML v8.2.12 programs, implemented in CIPRES Science Gateway v3.3 (http://www.phylo.org/). For BI analysis, best-fit models of nucleotide substitution were selected for each gene according to the Akaike information criterion (AIC), using the jModelTest2 v2.1.6 tool (Darriba *et al*., 2012) implemented in CIPRES Science Gateway v3.3. Four Markov chain Monte Carlo (MCMC) chains were run simultaneously, starting from a random tree to 10 million of generations. Trees were sampled every 1000 generations, and the first 2500 were discarded as the burn-in phase of each analysis. Posterior probabilities were determined from a majority-rule consensus tree generated from the remaining 7500 trees. For the ML analysis, the Generalized Time Reversible (GTR) model, with gamma correction (G) nucleotide substitution, and 1000 bootstrap iterations, were indicated. The other parameters were used as default settings. Sequences obtained in this study were submitted to the GenBank database (Supplementary Table 2).

Morphological characterization of isolates

A sub-sample of nine representative isolates belonging to *Dactylonectria* and *Ilyonectria*, identified in this study using molecular analyses, was selected for phenotypical characterization (Supplementary Table 2). Cultures were grown on PDA and incubated at 25°C in darkness. Ten days later, colony morphological characteristics were observed, and colony colour (Rayner, 1970) was determined. Lengths and widths of 50 conidia per isolate, including macro- and microconidia, were measured at 400× magnification, using a digital camera (Microscope eye-piece camera, AM-4023X, Taiwan) incorporated into the microscope. Conidium colour, shape, and number of septate, and presence of chlamydospores, were recorded.

Pathogenicity tests

The nine isolates selected for morphological characterization were used to determine their pathogenicity on

rooted grapevine plants. Dormant cuttings (0.3 m long) of 'Gravesac' rootstock were surface disinfected according to Akgül *et al.* (2022), placed in a plastic box containing sterilized growth substrate, irrigated, and kept in an acclimatized room (25°C, 85% relative humidity, 12 h photoperiod) for 1 month to induce root formation. The isolates were grown on PDA at 25°C in the dark for 2 weeks and were then liquefied in distilled water (one plate in 150 mL of distilled water). Root tips of the rooted cuttings were slightly cut and were then inoculated by immersing the roots in the culture suspension for 30 s (one liquified plate of one isolate per plant). The inoculated plants were then individually planted in a 2 L capacity pot containing commercial plant growth substrate. Six plants per isolate were inoculated, and six plants were treated with distilled water as controls. The plants were irrigated with tap water and maintained in greenhouse conditions (at 20±2°C), in a completely randomized experimental design.

Three months after inoculation the plants were uprooted, and their roots were carefully washed with tap water and dried in an air-circulated oven at 65°C for 48 h to constant weight, and root dry weights were recorded. Root dry weight data were analysed for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. Data were subjected to statistical analyses by performing analysis of variance (ANOVA) and means comparison by Duncan test at $P = 0.05$, using InfoStat/E version 2020 (http://www.infostat.com.ar). In addition, Koch´s postulates were completed by re-isolation of the inoculated fungi. For this, roots were separated, washed with tap water, and surface sterilized (as above). Small pieces of the roots were then cut, plated onto PDAS, and incubated in the same conditions as indicated above. Isolates identity was determined by morphological characteristics.

RESULTS

Fungal isolations

A total of 71 isolates resembling *Cylindrocarpon*-like asexual morphs were obtained from ready-to-plant grafted vines with characteristic black foot vascular symptoms. The symptoms consisted of wood necroses at the trunk bases, dark brown streaks developing from the bases of the plants, and sunken necrotic lesions on the roots (Figure 1). Based on the isolation frequency, incidence of black foot was 60% in 2017, 15% in 2018 and 61% in 2019.

Figure 1. Internal symptoms of black foot in ready-to-plant nursery grapevine plants. Wood necrosis and dark brown streaks developing from the base of the plant (a) and necrosis in roots (b).

Molecular identification of isolates

BLAST search of the partial HIS3 gene region placed 58 isolates in *Dactylonectria* and 13 within *Ilyonectria*. Subsequently, phylogeny of the individual data sets from the HIS3, TUB2 and TEF gene regions showed no significant conflicts in tree topology, so the trees were combined. The *Dactylonectria* dataset contained 96 taxa (58 from this study and two outgroups) and 1856 characters including gaps (TUB2 = 1-579; TEF = 580-1387; HIS3 = 1388-1856), of which 564 were parsimony informative. The *Ilyonectria* dataset consisted of 52 taxa (13 from this study and two outgroups) and 1882 characters including gaps (TUB2 = 1-567; HIS3 = 568-1038; TEF = 1039-1882), of which 526 were parsimony informative.

The AIC best-fit evolutionary models of nucleotide substitution used for the Bayesian Inference analysis for the *Dactylonectria* dataset were GTR with gamma distributed with invariant sites rates (G+I) for HIS3, and GTR+G for TUB2 and TEF. For *Ilyonectria*, the best-fit nucleotide substitution models were GTR+G+I for HIS3, Hasegawa-Kishino-Yano (HKY) model +G for TUB2, and GTR+G for TEF. In both genera, the topologies of the BI and ML consensus trees were similar, so only the BI trees with posterior probability values and bootstrap support values are presented.

Phylogenetic analyses allowed identification of four species among the 58 *Dactylonectria* isolates, with *D. macrodidyma* being the prevalent species (n = 31), followed by *D. novozelandica* (n = 14), *D. torresensis* (n = 10), and *D. palmicola* (n = 3) (Figure 2). Within the *Ilyonectria* group, the analyses identified nine isolates as *I. liriodendri,* and the remaining four isolates were grouped in a separate clade with the unidentified *Ilyonectria* strain STEU 8918 from South Africa. The node support value for this clade was 0.75 according to the BI method, while with ML method this node was not formed (Figure 3).

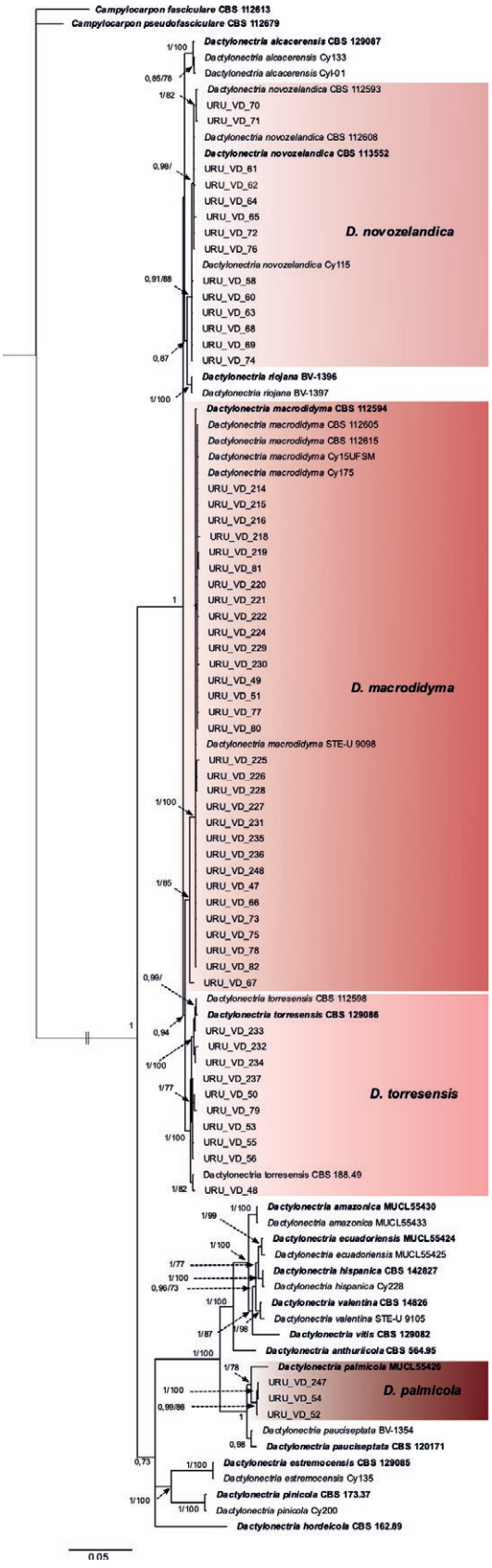

Figure 2. Bayesian inference phylogenetic tree built using the concatenated sequences of the HIS3, TEF and TUB2 genomic regions of 58 *Dactylonectria* isolates from Uruguayan nursery grapevines, and sequences retrieved from the GenBank (ex-type indicated in bold font). *Campylocarpon fasciculare* CBS 112613 and *Campylocarpon pseudofasciculare* CBS 112679 were used as the outgroups. Posterior probability and maximum likelihood bootstrap support values greater than, respectively, 0.70 and 70 are shown at the nodes before and after each branch. The scripts indicate that the nodes do not exist in the maximum likelihood tree. Double hash marks indicate branch lengths shortened at least 2-fold to facilitate visualization. The scale bar represents the estimated number of substitutions per site.

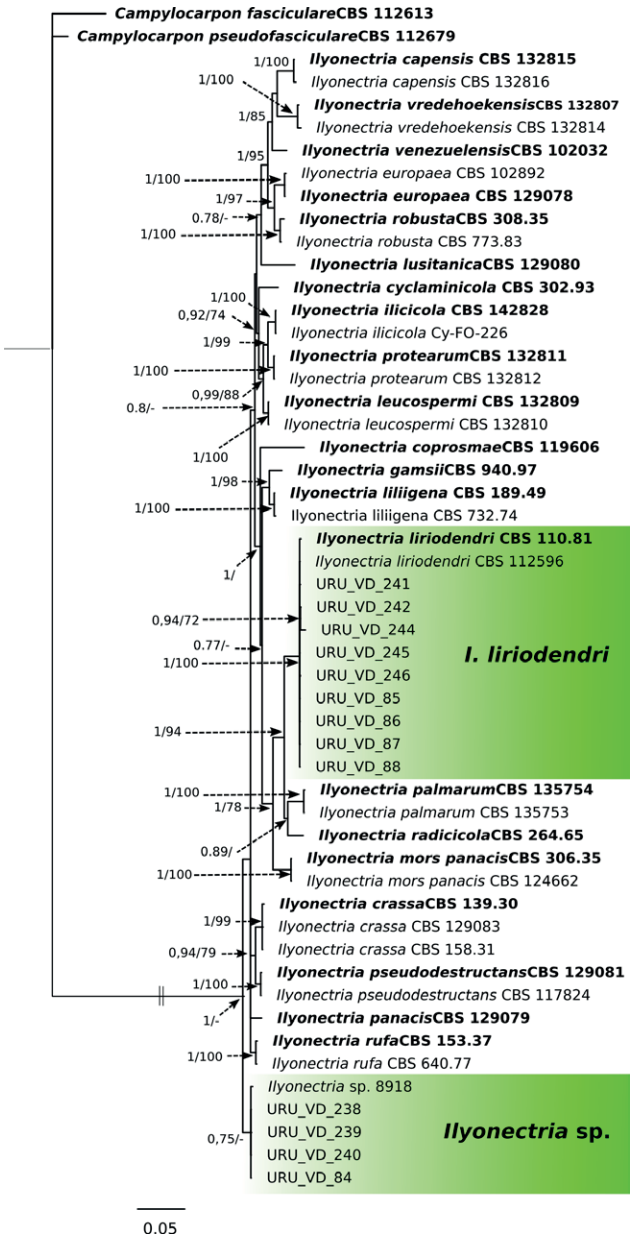

Figure 3. Bayesian inference phylogenetic tree built using the concatenated sequences of the HIS3, TEF and TUB2 genomic regions of 13 *Ilyonectria* isolates obtained from Uruguayan nursery grapevines, and sequences retrieved from the GenBank (ex-type indicated in bold font). *Campylocarpon fasciculare* CBS 112613 and *Campylocarpon pseudofasciculare* CBS 112679 were used as outgroups. Posterior probability and maximum likelihood bootstrap support values greater than 0.70 and 70 are shown at the nodes, respectively, before and after each bar. The scripts indicate that the nodes do not exist in the maximum likelihood tree. Double hash marks indicate branch lengths shortened at least 2-fold to facilitate visualization. The scale bar represents the estimated number of substitutions per site.

Morphological characterization of isolates

All the *Dactylonectria* and *Ilyonectria* isolates had aerial and cottony mycelia, and their colonies were white to yellow or light to dark brown on PDA. Conidiophores were simple or complex, sporodochial, and produced microconidia and macroconidia (Figure 4, Table 1). Macroconidia were predominantly straight, occasionally slightly curved and typically cylindrical, for the *D. macrodidyma*, *D. novozelandica*, *D. torresensis, D. palmicola,* and *I. liriodendri* isolates. Microconidia were generally ellipsoidal to ovoidal and straight. In addition, the isolates URU-VD-80 and URU-VD-242 produced chlamydospores, which is consistent with the characteristics expected for *D. macrodidyma* and *I. liriodendri* (Halleen *et al*., 2006b). The isolate URU-VD-84, identified as *Ilyonectria* sp., also had simple or complex conidiophores, produced sporodochia, and microconidia that were 0-1 septate, ellipsoidal to ovoidal and slightly curved, and macroconidia that were 1-3 septate (predominantly 1-septate), cylindrical and usually slightly curved, and produced chlamydospores (Figure 4, Table 1).

Pathogenicity tests

All the evaluated isolates were pathogenic on 'Gravesac' rootstock cuttings. At 3 months after inoculation, significant reductions (P = 0.0193) of mean root biomass were recorded from the inoculation treatments compared with the control treatment (Table 2). The inoculated plants had sunken necrotic lesions on roots and more brownish roots than the non-inoculated plants. Mean root dry weights ranged from 1.14 g to 1.76 g in inoculated plants and was 2.03 g for the non-inoculated controls. According to root dry weight reduction, both *D. macrodidyma* isolates, URU-VD-80 and URU-VD-231, were the most virulent, causing, respectively, 44% and 42% reductions compared with the non-inoculated control. The isolates of *I. liriodendri* (URU-VD-242), *D. torresensis* (URU-VD-234) and *D. novozelandica* (URU-VD-71) were the least virulent (causing, respectively, 20%, 18% and 13% reductions in root dry weight), but the mean root weights from these isolates were not significantly different (P > 0.05) from the control treatment. The other evaluated isolates showed intermediate behaviour. In addition, all the inoculated fungi were re-isolated from inoculated plants, with re-isolation rates ranging from 22% to 100%, whereas no pathogens were re-isolated from the non-inoculated controls (Table 2).

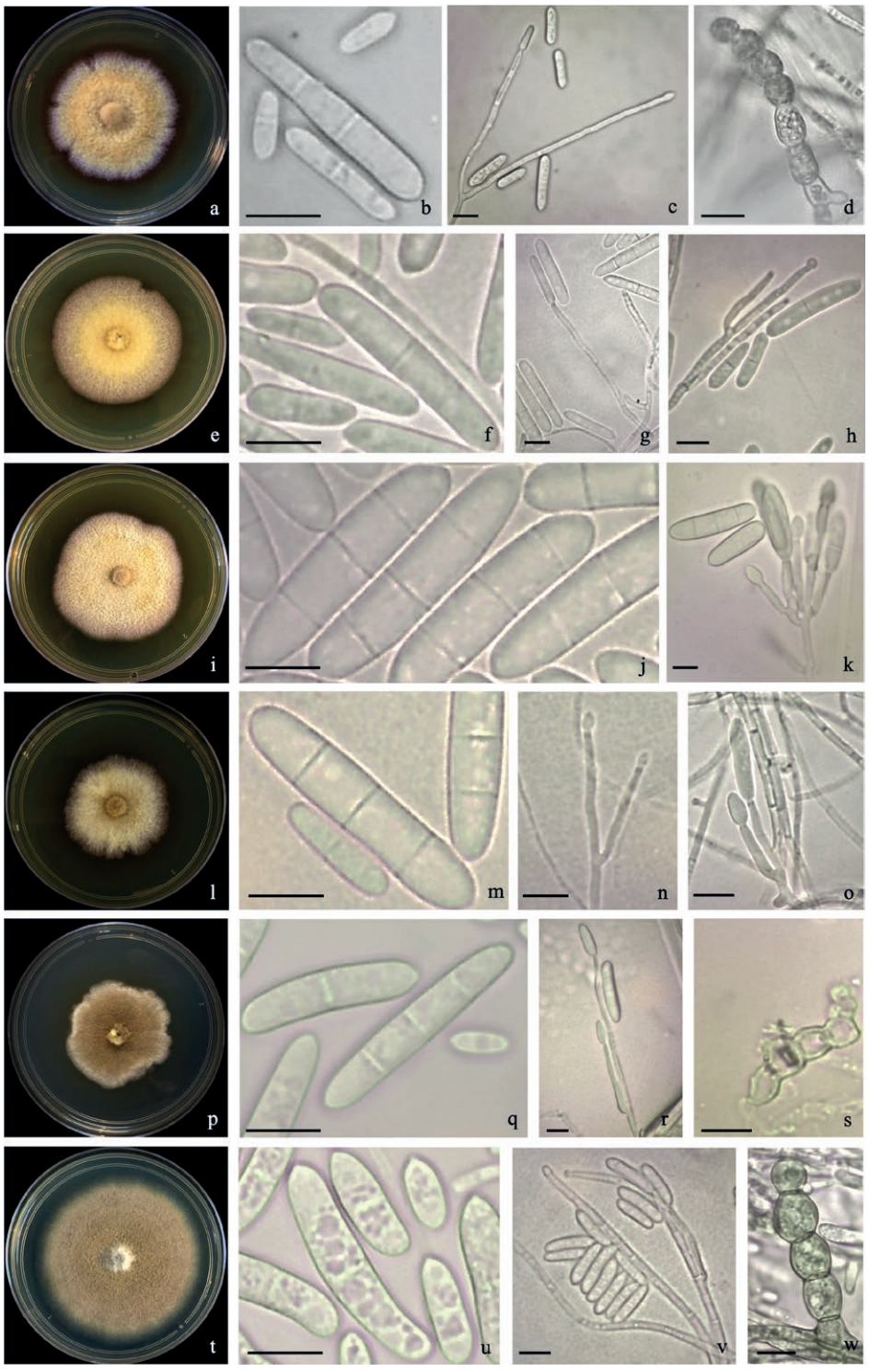

Figure 4. Morphological characteristics of *Dactylonectria* and *Ilyonectria* species isolated from grapevine nursery plants in Uruguay. Ten-d-old colonies on PDA at 25°C, of *D. macrodidyma* (a), *D. novozelandica* (**e**), *D. palmicola* (**i**), *D. torresensis* (l), *I. liriodendri* (p), and *Ilyonectria* sp. (t). Macro- and microconidia (b), simple conidiophore (**c**) and a chain of chlamydospores (d) of *D. macrodidyma*. Macro- and microconidia (f) and simple conidiophores (g and h) of *D. novozelandica*. Macroconidia (**j**) and a complex sporodochial conidiophore (k) of *D. palmicola*. Macro- and microconidia (m) and simple conidiophores (n and o) of *D. torresensis*. Macro- and microconidia (q), simple conidiophore (r) and a chain of chlamydospores (**s**) of *I. liriodendri*. Macro- and microconidia (u), simple conidiophore (v) and chain of chlamydospores (w) of *Ilyonectria* sp. Bars = 10 µm.

Table 1. Mean dimensions of aseptate and septate conidia of nine representative *Dactylonectria* and *Ilyonectria* isolates obtained in this study.

Species	Isolate	Microconidia				Macroconidia					
		Aseptate		One-septate		One-septate		Two-septate		Three-septate	
		Length	Width	Length	Width	Length	Width	Length	Width	Length	Width
Dactylonectria macrodidyma	URU-VD-80	10.32 ± 1.08	3.38 ± 0.34	14.11 ± 1.43	3.89 ± 0.27	22.08 ± 1.99	4.29 ± 0.28	24.17 ± 2.34	4.35 ± 0.40	-	-
D. macrodidyma	URU-VD-231	10.37 ± 1.38	3.42 ± 0.37	13.62 ± 1.89	3.71 ± 0.32	23.53 ± 2.69	5.05 ± 0.69	28.77 ± 1.76	5.98 ± 0.74	31.48 ± 0.82	5.11 ± 0.74
D. novozelandica	URU-VD-64	10.64 ± 0.61	3.29 ± 0.22	14.61 ± 2.22	3.56 ± 0.39	27.52 ±1.65	4.74 ± 0.37	31.17 ± 2.94	5.41 ± 0.77	33.25 ± 2.01	5.56 ± 0.43
D. novozelandica	URU-VD-71	10.95 ± 1.18	3.01 ±0.47	14.89 ±2.03	3.66 ± 0.33	27.07 ±1.67	4.62 ± 0.42	29.02 ± 1.66	5.07 ± 0.43	31.45 ± 2.50	5.19 ± 0.48
D. torresensis	URU-VD-79	11.30 ± 1.37	3.84 ± 0.55	15.01 ± 2.14	4.26 ± 0.57	26.76 ± 3.00	5.92 ± 0.47	29.56 ± 2.10	5.79 ± 0.38	30.04 ± 0.04	5.87 ± 0.55
D. torresensis	URU-VD-234	12.00 ± 1.87	4.05 ± 0.49	14.65 ± 2.04	4.65 ± 0.30	29.35 ± 4.04	6.23 ± 0.58	32.42 ± 2.45	7.03 ± 0.81	34.47 ± 4.12	7.18 ± 0.70
D. palmicola	URU-VD-54	12.79 ± 3.13	5.30 ± 1.46	-	-	37.22 ± 2.14	8.30 ± 0.82	36.44 ± 2.09	8.15 ± 0.60	37.37 ± 2.27	8.30 ± 0.70
Ilyonectria liriodendri	URU-VD-242	9.39 ± 1.60	3.75 ± 0.43	13.02 ± 2.53	3.66 ± 0.48	22.70 ± 1.92	4.42 ± 0.75	27.01 ± 2.35	4.59 ± 0.35	29.13 ± 3.04	4.95 ± 0.50
Ilyonectria sp.	URU-VD-84	10.79 ± 1.65	4.07 ± 0.40	17.41 ± 2.35	4.59 ± 0.45	24.88 ± 2.24	5.03 ± 0.45	27.59 ± 1.88	5.60 ± 0.81	29.11	4.84

Table 2. Mean root dry weights and proportions of inoculated fungus re-isolations for selected *Dactylonectria* and *Ilyonectria* isolates inoculated on rooted grapevine cuttings of 'Gravesac' rootstock.

Fungal species	Isolate	Dry root weights (g)[a]	Re-isolation (%)
Dactylonectria macrodidyma	URU-VD-80	1.14 a	80
D. macrodidyma	URU-VD-231	1.17 a	100
D. torresensis	URU-VD-79	1.21 ab	86
Ilyonectria sp.	URU-VD-84	1.32 ab	22
D. palmicola	URU-VD-54	1.37 ab	57
D. novozelandica	URU-VD-64	1.47 ab	100
I. liriodendri	URU-VD-242	1.62 abc	83
D. torresensis	URU-VD-234	1.66 abc	25
D. novozelandica	URU-VD-71	1.76 bc	83
Non-inoculated control		2.03 c	0

[a] Data are the mean of six replicates for each isolate. Means with same letter are not significantly different (P = 0.05) according to Duncan test.

DISCUSSION

Based on phylogenetic analyses, morphological studies and pathogenicity tests, the present study has identified five species of *Dactylonectria* and *Ilyonectria* causing black foot in locally produced nursery grapevines in Uruguay. The species identified were *D. macrodidyma* (31 isolates), *D. novozelandica* (14 isolates), *D. torresensis* (ten isolates), *I. liriodendri* (nine isolates) and *D. palmicola* (three isolates), while four *Ilyonectria* isolates could not be identified to species level.

All isolates were recovered from nursery grapevine plants showing typical black foot symptoms (wood necrosis at the trunk bases, dark brown streaks developing from the bases of plants, and sunken necrotic lesions on roots), as previously described by Halleen *et al*. (2006a) and Agustí-Brisach and Armengol (2013). Incidence of black foot based on proportions of pathogen isolations, was approx. 60% in 2017 and 2019, but substantially less (15%) in 2018. This difference was probably because isolations in 2018 were predominantly from the basal parts of the rootstocks. Probst *et al*. (2019) demonstrated that both *D. macrodidyma* and *I. liriodendri* can infect grapevines through wounded roots and callused basal ends. Results obtained in the present study indicate that under Uruguayan nursery production conditions, wounded roots are the main pathway of infection for these pathogens, rather than the basal callus tissues of the plants.

The multilocus phylogenetic approach allowed identification of black foot pathogens at species level, and morphological characterization supported these results. Col-

ony morphology and characteristics (shape and size) of macro- and microconidia, as well as production of chlamydospores by some isolates, were consistent with those expected for the identified species (Halleen *et al*., 2004; 2006b; Cabral *et al*., 2012a; Gordillo and Decock, 2017).

Dactylonectria macrodidyma was the prevalent species found causing black foot in the nursery plants analysed. This species was first described associated with grapevine in South Africa as *C. macrodidyma* by Halleen *et al*. (2004), and subsequently named as *D. macrodidyma* by Lombard *et al*. (2014). This species has been associated with black foot disease in several other countries, including New Zealand (Halleen *et al*., 2004; Probst *et al*., 2019), Chile (Auger *et al*., 2007), Spain (Alaniz *et al*., 2007), Switzerland (Hofstetter *et al*., 2009), Canada (Petit *et al*., 2011; Úrbez-Torres *et al*., 2014), Portugal (Cabral *et al*., 2012a; Reis *et al*., 2013), Turkey (Özben *et al*., 2012; Akgül *et al*., 2022), Brazil (dos Santos *et al*., 2014), Algeria (Aigoun-Mouhous *et al*., 2019), China (Ye *et al*., 2021) and Argentina (Longone *et al*., 2022). In a previous study in Uruguay, Abreo *et al*. (2010) found *D. macrodidyma* as the prevalent species causing black foot on symptomatic plants collected from commercial vineyards.

The second most common pathogen found was *D. novozelandica*, which was first described in grapevine as *I. novozelandica* by Cabral *et al*. (2012a), and then renamed *D. novozelandica* by Lombard *et al*. (2014). This species has been associated with black foot in New Zealand (Cabral *et al*., 2012a), Peru (Alvarez *et al*., 2012), South Africa (Cabral *et al*., 2012a), United States (Cabral *et al*., 2012a), Portugal (Reis *et al*., 2013), Spain (Agustí-Brisach *et al*., 2013), Algeria (Aigoun-Mouhous *et al*., 2019) and Turkey (Akgül *et al*., 2022). In Uruguay, *D. novozelandica* has been found causing crown and root necrosis on strawberry (Vigliecca *et al*., 2022).

Dactylonectria torresensis was first described by Cabral *et al*. (2012a) as *I. torresensis* in grapevine in Portugal and was then reclassified as *D. torresensis* by Lombard *et al*. (2014). This species has been reported as the prevalent cause of black foot in Portugal (Reis *et al*., 2013), Italy (Carlucci *et al*., 2017), Spain (Berlanas *et al*., 2017), Algeria (Aigoun-Mouhous *et al*., 2019) and Turkey (Akgül *et al*., 2022). In addition, the fungus has been associated with black foot in Australia, New Zealand, South Africa, United States (Cabral *et al*., 2012a), Canada (Úrbez-Torres *et al*., 2014), and China (Ye *et al*., 2021).

Dactylonectria palmicola was the fourth *Dactylonectria* species found associated with black foot in this study. This species was described by Gordillo and Decock (2017) in *Euterpe precatoria* in the Amazon rainforest of Ecuador. The present study is the first record of *D. palmicola* causing black foot on grapevine. The isolates URU-VD-52 and URU-VD-54 identified as *D. palmicola* in the present study, were previously misidentified as *D. pauciseptata* when phylogenetic analysis was performed using only the HIS3 gene region (Carbone *et al*., 2022). Although the HIS3 region has been demonstrated to be the most robust locus for identification of black foot pathogens (Cabral *et al*., 2012a), the present results suggest that multilocus sequence analysis, including HIS3, TUB2 and TEF, is essential to ensure correct identification of closely related fungi causing black foot.

Within *Ilyonectria, I. liriodendri* was the prevalent species found in the present study. This pathogen was first described in grapevine by Halleen *et al*. (2006b) as *C. liriodendri*, and subsequently classified as *I. liriodendri* by Chaverri *et al*. (2011). This species has been reported on grapevine in South Africa (Halleen *et al*., 2006b), Australia (Whitelaw-Weckert *et al*., 2007), Spain (Alaniz *et al*., 2007), Switzerland (Casieri *et al*., 2009), Iran (Mohammadi *et al*., 2009), Brazil (Russi *et al*., 2010), United States (Petit *et al*., 2011), Canada (Petit *et al*., 2011; Úrbez-Torres *et al*., 2014), Portugal (Reis *et al*., 2013), New Zealand (Pathrose *et al*., 2014), Italy (Carlucci *et al*., 2017), Argentina (Longone *et al*., 2022) and Turkey (Akgül *et al*., 2022). In Uruguay, *I. liriodendri* was first reported affecting symptomatic plants in commercial vineyards by Abreo *et al*. (2010).

Isolates classified as *Ilyonectria* sp. in the present study were grouped with the unidentified South African *Ilyonectria* strain STEU 8918, but with a low support value (0.75) in the BI analysis, and absence of this clade in the ML analysis. For this South African isolate, which was obtained by van der Merwe (2019) from the crown of a nectarine nursery tree, only the HIS3 gene region is available on the GenBank database. This probably explains the low support of the clade in the BI phylogenetic tree and absence of this clade in the ML tree. The pathogenicity tests conducted in the present study confirmed pathogenicity of this species to grapevine, which emphasizes the importance of prescriptive description of this new species.

Results obtained here have shown that all inoculated *Dactylonectria* and *Ilyonectria* isolates infected rooted 'Gravesac' rootstocks, but virulence varied among species and between isolates within species, which is in accordance with previous studies (Probst *et al*., 2019; Aigoun-Mouhous *et al*., 2019; Berlanas *et al*., 2020; Akgül *et al*., 2022). After 3 months from inoculation, the maximum reduction in root dry weight was 44%, and no plant death was recorded. A longer post inoculation period may have resulted in greater reductions of root biomass, because development of black foot symptoms

is usually slow (Whitelaw-Weckert, *et al*. 2007), and may have allowed all isolates to significantly reduce root dry weights compared to the control treatment. Whitelaw-Weckert *et al*. (2007) were unable to detect consistent black foot symptoms after 18 months from inoculation with *C. liriodendri* of 1-year-old rooted *V. vinifera* plants, although they confirmed pathogenicity by re-isolation of this fungus, as occurred in the present study.

Dactylonectria macrodidyma was the most aggressive species in the present study. Both isolates of this fungus caused the lowest root dry weights. This result agrees with those of Ye *et al*. (2021) in China, where *D. macrodidyma* was the most aggressive pathogen compared with *D. torresensis*, *D. alcacerensis*, *Cylindrocladiella lageniformis* and *Neonectria* sp. In contrast, in research conducted in Algeria by Aigoun-Mouhous *et al*. (2019), an isolate of *D. torresensis* was the most virulent, followed by isolates of *D. novozelandica* and *D. macrodidyma*. Pathogenicity tests recently conducted in Turkey on '1103P' rootstock cuttings showed that *D. novozelandica* was the most virulent species compared with *Cylindrodendrum alicantinum*, *Cylindrocladiella peruviana*, *D. macrodidyma*, *D. torresensis*, *I. liriodendri* and *I. robusta* (Akgül *et al*., 2022). This is in accordance with the study by Berlanas *et al*. (2020) in Spain, where a strain of *D. novozelandica* was found to be the most virulent compared with several black foot fungi, including *D. macrodidyma*, *D. torresensis* and *I. liriodendri*, inoculated on *V. vinifera* 'Tempranillo'. In contrast, Probst *et al*. (2019) testing different inoculation methods and propagule types in New Zealand, observed that *I. liriodendri* was generally more pathogenic than *D. macrodidyma*.

In conclusion, the present study has shown that about 39% of analysed ready-to-plant nursery vines were infected by black foot pathogens. This high proportion is likely to compromise the longevity of new vineyards. This result emphasizes the need to implement integrated management strategies to reduce black foot incidence in Uruguayan grapevine nurseries. Physical practices such as hot-water treatments have shown promising results for controlling black foot in several countries, but with unacceptable levels of disease control (Gramaje and Armengol, 2011). Nevertheless, this technology should be evaluated in each grapevine region. Use of antagonist microorganisms is currently a major objective of the research to prevent grapevine trunk diseases, but the results remain unconvincing (Martínez-Diz *et al*., 2021). Other practices, such as biofumigation with *Brassica* spp., have been shown to reduce soilborne pathogen inoculum levels and help prevent infection by black foot pathogens in young plants (Berlanas *et al*., 2018). Appropriate and environmentally-friendly chemical controls can also be considered, while local regulations allow the use of promising chemical active ingredients. Taking this into account, future studies should focus on evaluating different practices to avoid or reduce fungal infections by black foot pathogens in nursery grapevines under local conditions.

AUTHOR CONTRIBUTIONS

MJC was responsible for performing the assays, data analyses and drafted the manuscript of this work. RR assisted in experimental assays, data analyses, and made contributions to the manuscript. PM contributed to the interpretation of the results and made critical revisions to the manuscript. SA supervised the assays, analysis and interpretation of the results and performed critical revisions of the manuscript. All the authors approved the final manuscript.

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