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RESEARCH PAPER

Unveiling inoculum sources of black foot pathogens in a commercial grapevine nursery

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Summary. Black foot of grapevine is an important disease caused primarily by *Ilyonectria* spp. and "*Cylindrocarpon*" *pauciseptatum*. These pathogens affect grapevine nurseries and young vineyards, causing the decline and death of plants. In the nursery, the primary infections of the grafted cuttings are mainly attributed to soil-borne inoculum, which could infect the roots and the basal end of rootstocks during the rooting stage. The aim of this research was to detect other possible sources of inoculum throughout the different nursery stages by classical and molecular techniques (nested-PCR and multiplex nested-PCR). Results revealed the presence of the *I. liriodendri* and/or *I. macrodidyma* complex in grapevine rootstock and scion cuttings, cutting tools, water from hydration tanks, well water, callusing medium, one indoor air sample and soils collected from mother fields and nurseries. "*Cylindrocarpon*" *pauciseptatum* was only detected in the callusing medium, nursery soils, rooted-graftlings and on the root pruning machine. Forty four isolates obtained from soils (mother fields and nurseries) and rooted graftlings (six grapevine cultivar/rootstock combinations) were sequenced for part of histone H3 gene to resolve the species. While *I. liriodendri*, *I. macrodidyma* and *I. torresensis* were identified from soil samples, from rooted graftlings it was also possible to detect *I. liriodendri*, *I. macrodidyma*, *I. novozelandica*, *I. torresensis*, *Ilyonectria* sp. 2, *"C." pauciseptatum* and four *Ilyonectria* isolates which are close to *I. cyclaminicola*. The results demonstrated that, in addition to nursery soils, mother field soils, rootstock and scion cuttings, water from wells and hydration tanks, callusing media, cutting tools and indoor air should be considered as potential sources of inoculum for black foot pathogens.

Key words: *Ilyonectria* spp., inoculum sources, black foot disease, *Vitis vinifera*.

Introduction

Black foot of grapevine is a major disease associated with the decline of young vineyards, mostly affecting vines up to 10 years old (Sweetingham, 1983; Larignon, 1999; Fourie and Halleen, 2001). Symptoms include poor root growth, necrotic roots, dark brown discolouration of the basal end of the rootstock, delayed bud break, weak shoot growth and decline and death of affected vines. Internal necroses of wood are revealed in cross-sections of the affected areas, whereas in longitudinal section they appear as dark vascular streaking (Rego, 1994; Larignon, 1999; Gubler *et al*., 2004; Halleen *et al*., 2006b). The disease also affects nursery plants and young vines shortly after transplantation. Although the failure and decline of recently planted vineyards could be attributed to several factors including abiotic stresses, fungi associated with black foot and Petri diseases are considered major agents of young vine failure (Oliveira *et al*., 2004; Gramaje and Armengol, 2011).

Black foot disease is caused by a complex of fungi, being *Ilyonectria liriodendri* and *Ilyonectria macrodidyma* (Halleen *et al*., 2004; Petit and Gubler, 2005; Halleen *et al*., 2006a; Alaniz *et al*., 2007) the most widely and frequently reported. *Campylocarpon fasciculare*, *Campylocarpon pseudofasciculare* (Halleen *et*

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al., 2004; Abreo *et al*., 2010; Alaniz *et al*., 2011) and "*Cylindrocarpon*" *pauciseptatum* (Schroers *et al*., 2008; Martin *et al*., 2011) are also reported to be involved in the disease, but so far these pathogens have a limited geographical distribution.

Diversity within *I. macrodidyma* was found by Alaniz *et al*. (2008) through ISSR (Inter-simple sequence repeat), further verifying that some groups of *I. macrodidyma* showed to be more virulent than *I. liriodendri*. Also, a high level of genetic diversity was revealed among isolates of *I. macrodidyma,* based on a multi-gene analysis (β-tubulin, histone H3, translation elongation factor 1-alpha, and the internal transcribed spacers on both sides of the 5.8S nuclear ribosomal RNA gene) and morphology (Cabral *et al*., 2012b). These findings enabled the description of four new species, *I. estremocensis, I. alcacerensis, I. novozelandica* and *I. torresensis*, which were previously included in *I. macrodidyma* complex. Also, from the disentanglement of the *Cylindrocarpon* root rot pathogen complex, the species *I. europaea*, *I. lusitanica*, *I. pseudodestructans* and *I. robusta* (Cabral *et al*., 2012c) were revealed to induce black foot symptoms on grapevine (Cabral *et al*., 2012a).

In addition to black foot pathogens, grapevine propagation material is frequently infected by other fungi, namely *Phaemoniella chlamydospora* and *Phaeoacremonium* spp. the causal agents of Petri disease (Rego *et al*., 2000; Gubler *et al*., 2004; Gramaje and Armengol, 2011). Studies on this disease have revealed that rootstock mother plants and the propagation process of grapevine plants should be considered as important sources of inoculum (Whiteman *et al*., 2004; Damm and Fourie, 2005; Retief *et al*., 2006; Whiteman *et al*., 2007; Vigues *et al*., 2008; Pollastro *et al*., 2009; Aroca *et al*., 2010; Gramaje and Armengol, 2011).

Infection of propagating material by black foot pathogens has mainly been attributed to soil-borne inoculum, which is thought to infect the roots and the basal end of rootstocks, during the rooting stage in nursery fields (Rego *et al*., 2001; Halleen *et al*., 2003; Gubler *et al*., 2004; Oliveira *et al*., 2004; Halleen *et al*., 2007). Prior to planting, the infrequent occurrence of black foot pathogens in canes or cuttings of rootstock mother plants has been reported (Rego *et al*., 2001; Halleen *et al*., 2003; Fourie and Halleen, 2004). By contrast, Botryosphaeriaceae and *Phomopsis* spp. are among the most frequently isolated fungi from this plant material (Rego *et al*., 2001; Fourie and Halleen, 2004; Aroca *et al*., 2010), and contribute to the increased susceptibility of rootstock cuttings or grafted plants to black foot pathogens during the rooting process in nursery (Oliveira *et al*., 2004).

Potential inoculum sources for trunk diseases during the grapevine propagation process were recently reviewed by Gramaje and Armengol (2011). However, in addition to soil, vineyard weeds (Agustí-Brisach *et al*., 2011) and, to a lesser extent, rootstock mother plants, little is known so far about other hypothetical sources of black foot inoculum.

The main purpose of this study was to detect potential sources of inoculum for *Ilyonectria* spp. and "*Cylindrocarpon*" *pauciseptatum* at each stage of the propagation process using both classical and molecular approaches, to gain a clearer understanding in the disease cycle of black foot of grapevine.

Materials and methods

Sampling and fungal isolation

The grapevine propagation process in a commercial nursery, located in the "Oeste" region of Portugal, was followed during the 2010–2011 season. The process began in late autumn with the pruning of rootstock and scion mother vines. After harvesting, the dormant canes were cut in the warehouse (30 cm rootstock cuttings and one-bud scions) and stored in cold chambers at 2–4°C and 90% relative humidity until late winter. Then, the rootstock and scion cuttings were drenched in hydration tanks containing a water soluble copper-based product (150 mL hL^{1}, TaloServ, Cultaza Lda, Portugal), for at least 1 h, according to standard practice in the nursery. Following hydration, the cuttings were bench-grafted, using omega-cut grafting machines and the graft union protected with melted wax, usually containing growth regulators. Grafted vines were placed in callusing boxes filled with callusing medium, usually peat and incubated at 30–32°C and 90% of relative humidity for 10 to 12 days to promote callusing of the graft union. After the warm incubation period the callusing boxes were held at ambient temperature for a week. The callused graftlings were then removed from callused boxes and dipped again in melted wax. The graftlings were then planted in field nursery for rooting and grown on until they became dormant in the autumn-early winter. After uprooting, the plants were prepared for sale by root pruning, dipping in wax and packaging.

Samples of plant material, indoor-air (cold-chambers and warehouses), well water, water from hydration tanks, callusing medium, soil samples (from mother vine fields, nursery fields and plots in rotation) and rooted graftlings were randomly taken at different stages of the propagation process and analysed.

Unless otherwise stated, fungi were isolated and cultured on Petri dishes containing potato dextrose agar (PDA, Difco, USA) supplemented with chloramphenicol (250 mg L^{-1}) (PDAC), followed by incubation in darkness at 20 ± 1ºC. Plates were routinely examined for fungal growth and fungi were preliminary identified to the genus level on the basis of colony appearance. Colonies of *Ilyonectria*-like fungi and those resembling "*C.*" *pauciseptatum* were obtained from single-spores, sub-cultured on PDA and incubated in darkness at 20 ± 1ºC, until required for analysis.

Plant material included grapevine rootstock and scion cuttings (30 cuttings of each), grafted-cuttings taken from callusing boxes (50 plants) and rooted graftlings (110 plants), collected from different root field nurseries (Table 1).

Small pieces of wood were taken from the basal end of cuttings and from two different regions of grafted-cuttings and rooted graftlings (bottom of rootstock and scion), in order to isolate fungi associated with black foot disease. The wood fragments were surface disinfected by immersion in a NaOCl solution (0.35% w/w active chlorine) for 2 min, rinsed in sterile distilled water for 30 s and plated on Petri dishes containing potato dextrose agar (PDA, Difco, USA) supplemented with chloramphenicol (250 mg L^{-1}) (PDAC), followed by incubation in darkness at 20 ± 1ºC. Plates were routinely examined for fungal growth and fungi were preliminary identified to the genus level, on the basis of colony appearance. Colonies of *Ilyonectria*-like fungi and those resembling "*C.*" *pauciseptatum* were obtained from single-spores, sub-cultured on PDA and incubated in darkness at 20 ± 1ºC.

Soil was sampled in a total of 22 fields including rootstock and scion mother vine fields, grapevine nurseries and plots in rotation (Table 2). Soil samples close to roots were collected to a depth of 20 cm from 12 different locations in each field. Samples of callusing medium, were taken before and afte ruse. All soil and callusing-medium samples were air-dried and sieved (2 mm mesh size). Then, 10 g of each sample were diluted in 90 mL of sterile distilled water with two drops of Tween 80® and shaken for 30 min at 180 rpm, using an agitator (Agitorb 160 E, Aralab, Portugal) and diluted in 10 fold series up to 10^{-3} dilution. From each dilution, an aliquot of 0.5 mL was plated onto 15 mL of PDAC and incubated as previously described. Indoor air from eight cold-chambers and three warehouses (in a total of 77 samples), was monitored by trapping airborne fungal propagules on PDAC plates, left open for 30 min. every 15 days over a four month period.

Six water samples (250 mL each) from the scion and rootstock hydration tanks and from well water used to fill these tanks were collected. Thirty-one cutting tools (pruning shears used to cut scions and rootstocks, pruning shears used to prune rooted graftlings, blades from omega-cut grafting machines and root pruning guillotine) were washed with sterile water, which was collected in sterile tubes and maintained at approximately 4ºC, until use. From each sample, one aliquot of 0.5 mL was plated on PDAC and incubated as described.

DNA extraction and purification

DNA from plant materials was extracted according to the protocol of Cenis (1992), modified by Nascimento *et al.* (2001). DNA extraction from soil and callusing medium followed the SDS protocol (Method 2) described by Damm and Fourie (2005) modified with substitution of the 0.5 g acid-washed sand by 0.5 g glass beads, acid washed ≤106 µm (Sigma-Aldrich, G4649). Before extraction, the soil samples were air-dried and passed through a 2 mm mesh sieve, whereas the callusing medium was ground into fine power in the presence of liquid nitrogen.

Aliquots of 50 mL from the water samples were centrifuged in Falcon tubes for 10 min at 9,000 rpm and the supernatant discarded. Each pellet was resuspended in 2 mL TE and shaken for 5 min. Then, 0.5 mL of each sample was used for DNA extraction using the same protocol referred for soils and callusing medium.

In order to remove PCR inhibitors the DNA was loaded on a pre-prepared polyvinylpolypyrrolidone column as described by Damm and Fourie (2005), incubated for 5 min and centrifuged through the column for 5 min at 720 *g*.

Nested-PCR

The fungus-specific primer ITS1F (Gardes and Bruns, 1993) and the universal primer ITS4 (White

Table 1. Detection of *Ilyonectria liriodendri*, *I. macrodidyma* complex and *"Cylindrocarpon" pauciseptatum* in samples from different grapevine propagation materials.

Type of material	Grapevine cultivar / Rootstock	Isolation area	No. plants tested positive / Total		Species or species complex by Multiplex Nested PCR[c]
			Microbial isolation[a]	Nested PCR[b]	
Cuttings	Aragonez		3/10	2/9	*I. liriodendri; I. macrodidyma* complex
	Aragonez		1/10	6/10	*I. liriodendri; I. macrodidyma* complex
	Touriga Nacional		4/10	5/10	*I. liriodendri; I. macrodidyma* complex
	1103P		4/10	7/10	nd
	1103P		1/10	9/10	*I. liriodendri; I. macrodidyma* complex
	110R		4/10	6/10	*I. liriodendri; I. macrodidyma* complex
Callused graftlings	Caladoc/110R	Rootstock	0/10	7/10	*I. liriodendri; I. macrodidyma* complex
		Scion	0/10	4/8	*I. liriodendri; I. macrodidyma* complex
	Gouveio/1103P	Rootstock	1/10	8/10	*I. liriodendri; I. macrodidyma* complex
		Scion	0/10	8/9	*I. liriodendri; I. macrodidyma* complex
	Fernão Pires/1103P	Rootstock	3/10	5/10	*I. liriodendri; I. macrodidyma* complex
		Scion	0/10	10/10	*I. liriodendri; I. macrodidyma* complex
	Moscatel Roxo/110R	Rootstock	1/10	9/10	*I. liriodendri; I. macrodidyma* complex
		Scion	0/10	4/10	*I. liriodendri; I. macrodidyma* complex
	Touriga Nacional/110R	Rootstock	0/10	10/10	*I. liriodendri; I. macrodidyma* complex
		Scion	1/10	nd	nd
Rooted graftlings	Alicante Bouschet/1103P	Rootstock	3/10	10/10	*I. liriodendri; I. macrodidyma* complex
		Scion	1/10	7/10	*I. liriodendri; I. macrodidyma* complex
	Aragonez/99R	Rootstock	1/10	2/10	*I. liriodendri I. macrodidyma* complex
		Scion	1/10	1/9	*I. liriodendri*
	Caladoc/110R	Rootstock	3/10	3/10	*I. macrodidyma* complex*; "C." pauciseptatum*
		Scion	0/10	1/10	*I. liriodendri; I. macrodidyma* complex
	Fernão Pires/1103P	Rootstock	7/10	8/10	*I. liriodendri; I. macrodidyma* complex*; "C." pauciseptatum*
		Scion	1/10	3/9	*I. liriodendri; I. macrodidyma* complex
	Gouveio/1103P	Rootstock	3/10	1/10	*I. liriodendri; I. macrodidyma* complex*; "C." pauciseptatum*
		Scion	1/10	2/10	*I. liriodendri*
	Jaen/99R	Rootstock	2/10	10/10	*I. liriodendri; I. macrodidyma* complex*; "C." pauciseptatum*

(*Continued*)

Table 1. *Continues.*

Type of material	Grapevine cultivar / Rootstock	Isolation area	No. plants tested positive / Total		Species or species complex by Multiplex Nested PCR[c]
			Microbial isolation[a]	Nested PCR[b]	
		Scion	3/10	5/10	*I. liriodendri; I. macrodidyma* complex
	Moscatel Roxo/110R	Rootstock	3/10	5/10	*I. liriodendri; I. macrodidyma* complex
		Scion	1/10	1/8	*I. liriodendri; I. macrodidyma* complex
	Rabigato/110R	Rootstock	3/10	10/10	*I. liriodendri; I. macrodidyma* complex
		Scion	2/10	10/10	*I. liriodendri; I. macrodidyma* complex
	Touriga Franca/1103P	Rootstock	3/10	7/10	*I. liriodendri; I. macrodidyma* complex
		Scion	0/10	6/10	*I. liriodendri; I. macrodidyma* complex
	Touriga Nacional/110R	Rootstock	2/10	3/10	*I. macrodidyma* complex
		Scion	0/10	1/8	*I. liriodendri; I. macrodidyma* complex
	Touriga Nacional cl16/1103P	Rootstock	1/10	6/10	*I. liriodendri; I. macrodidyma* complex
		Scion	1/10	nd	nd

[a] Isolations made on potato dextrose agar supplemented with chloramphenicol 250 mg L^{-1}.
[b] First-round amplification with ITS1F/ITS4 primers; second-round amplification with Dest1/Dest4 (Hamelin *et al*., 1996).
[c] First-round amplification with ITS1F/ITS4 primers; second-round amplification with Lir1/Lir2, Mac1/MaPa2 and Paul1/MaPa2 primer sets to amplify respectively, *Ilyonectria liriodendri*, *I. macrodidyma* complex and "*Cylindrocarpon*" *pauciseptatum* (Alaniz *et al*., 2009).

et al., 1990) were used in a first-stage amplification, followed by a second-stage amplification with the primers Dest1 and Dest4 (Hamelin *et al*., 1996) to detect *Ilyonectria* spp. Amplification conditions were as described by Nascimento *et al*. (2001).

PCR products were separated by electrophoresis in 1.2% agarose gel in 0.5× TBE buffer (Tris-Borate-EDTA). Gels were stained with ethidium bromide and photographed under UV.

Multiplex nested-PCR

In order to identify *I. liriodendri, I. macrodidyma* complex and "*C.*" *pauciseptatum*, a combination of nested-PCR with a multiplex approach was used according to Alaniz *et al*. (2009). After the first PCR reaction with the ITS1F/ITS4 primers, the secondary PCR reaction was carried out by using three sets of primers, Lir1/Lir2,l Mac1/MaPa2 and Paul1/MaPa2, to amplify respectively *I. liriodendri* (253 bp fragment), *I. macrodidyma* complex (387 bp fragment) and "*C.*"*pauciseptatum* (117 bp fragment).

The PCR products were separated and stained as previously described for nested-PCR.

Histone H3 sequencing

A collection of 44 *Ilyonectria* spp. isolates was obtained from soil samples (mother fields and nurseries) and rooted graftlings (six scion/rootstock combinations) in this study (Table 3). These isolates were sequenced for part of histone H3 gene (previously shown to be a very informative locus; Cabral *et al*., 2012c) in order to identify the species involved. For each isolate, genomic DNA was isolated from mycelium following the protocol of Cenis (1992), modified by Nascimento *et al*. (2001).

Sequencing of part of the histone H3 gene was

Table 2. Detection of *Ilyonectria liriodendri*, *I. macrodidyma* complex and "*Cylindrocarpon*" *pauciseptatum* in samples from water, callusing medium, indoor air, cutting tools and mother field and nursery soils.

Sample	Type of sample	No. samples tested positive / Total		Species or species complex by Multiplex Nested PCR[c]
		Microbial isolation[a]	Nested PCR[b]	
Water	Well water	0/4	4/4	*I. liriodendri*, *I. macrodidyma* complex
	Hydration tank (rootstock cuttings)	0/1	1/1	*I. liriodendri*, *I. macrodidyma* complex
	Hydration tank (scion cuttings)	0/1	1/1	*I. liriodendri*
Callusing medium	Before use	0/1	1/1	*I. liriodendri*, *"C." pauciseptatum*
	After use	0/1	1/1	*I. liriodendri*, *I. macrodidyma* complex, *"C." pauciseptatum*
Indoor air	Warehouses	0/21	nd	nd
	Cold-chambers	1/56	1/56	*I. liriodendri*
Cutting tools	Rootstocks	0/10	5/9	*I. liriodendri*
	Scions	0/2	2/2	nd
	Graftlings	0/8	6/8	*I. liriodendri*, *I. macrodidyma* complex
	Grafting machines	0/10	3/5	*I. liriodendri*
	Guillotine (roots of graftlings)	1/1	1/1	*I. liriodendri*, *I. macrodidyma* complex, *"C." pauciseptatum*
Mother field soils	110R rootstock	0/1	1/1	*I. macrodidyma* complex
	1103P rootstock	1/2	2/2	*I. macrodidyma* complex
	Aragonez cultivar	1/1	1/1	*I. liriodendri*, *I. macrodidyma* complex
	Touriga Nacional cultivar	1/1	1/1	*I. liriodendri*
Nursery soils	Before planting	4/6	6/6	*I. liriodendri*, *I. macrodidyma* complex
	During planting	0/2	2/2	*I. liriodendri*, *I. macrodidyma* complex, *"C." pauciseptatum*
	Immediately after uprooting	5/9	9/9	*I. liriodendri*, *I. macrodidyma* complex, *"C." pauciseptatum*

[a] Isolations made on potato dextrose agar supplemented with chloramphenicol 250 mg L^{-1}.
[b] First-round amplification with ITS1F/ITS4 primers; second-round amplification with Dest1/Dest4 (Hamelin *et al*., 1996).
[c] First-round amplification with ITS1F/ITS4 primers; second-round amplification with Lir1/Lir2, Mac1/MaPa2, and Paul1/MaPa2 primer sets to amplify respectively *Ilyonectria liriodendri*, *I. macrodidyma* complex and "*Cylindrocarpon*" *pauciseptatum* (Alaniz *et al*., 2009).

performed after PCR amplification as described by Cabral *et al*. (2012b), with modifications: Taq DNA Polymerase was from Dream Taq (Fermentas, Vilnius, Lithuania) with 1× buffer containing 2 mM $MgCl_2$; annealing temperature was increased to 58ºC. Sequencing reactions were performed by the company STAB VIDA Lda (Portugal). Sequences were assembled and edited to resolve ambiguities, using the SeqMan module of the Lasergene software package (DNAStar, Madison, WI, USA). Consensus sequences for all isolates were compiled into a single file (Fasta format) and aligned using MEGA version 5 (Tamura *et al*., 2011). Following manual adjustment of the alignment by eye where necessary, the

Table 3. List of isolates obtained in this study.

Strain number	Isolated from	Species	Accession number
CyMC 1	Mother field soil, Touriga Nacional cultivar	*I. macrodidyma*	KC576843
CyMC 3	Mother field soil, Touriga Nacional cultivar	*I. macrodidyma*	KC576844
CyMC 4	Mother field soil, Aragonez cultivar	*I. macrodidyma*	KC119412
CyMC 5	Mother field soil, 1103P rootstock	*I. torresensis*	KC119413
CyMC 7	Mother field soil, 1103P rootstock	*I. torresensis*	KC119414
CyMC 9	Mother field soil, 1103P rootstock	*I. torresensis*	KC576845
CyMC 10	Mother field soil, 1103P rootstock	*I. macrodidyma*	KC576846
CyMC 14	Mother field soil, 1103P rootstock	*I. torresensis*	KC119415
CyMC 17	Mother field soil, 1103P rootstock	*I. torresensis*	KC119416
CyMC 19	Mother field soil, 1103P rootstock	*I. macrodidyma*	KC119417
CyMC 69	Mother field soil, 1103P rootstock	*I. liriodendri*	KC119418
CyMC 20	Mother field soil, 1103P rootstock	*I. torresensis*	KC576847
CyMC 22	Mother field soil, 1103P rootstock	*I. torresensis*	KC576848
CyMC 28	Nursery soil imediately after uprooting	*I. torresensis*	KC576849
CyMC 29	Nursery soil imediately after uprooting	*I. torresensis*	KC576850
CyMC 30	Nursery soil imediately after uprooting	*I. macrodidyma*	KC119419
CyMC 31	Nursery soil imediately after uprooting	*I. macrodidyma*	KC576851
CyMC 47	Nursery soil imediately after uprooting	*I. torresensis*	KC119420
CyMC 34	Rooted graftlings, Alicante Bouschet × 1103P isolated from scion	*I. macrodidyma*	KC576852
CyMC 35/	Rooted graftlings, Alicante Bouschet × 1103P isolated from scion	*I. macrodidyma*	KC119421
CyMC 36	Rooted graftlings, Alicante Bouschet × 1103P isolated from rootstock	*I. liriodendri*	KC576853
CyMC 37	Rooted graftlings, Alicante Bouschet × 1103P isolated from rootstock	*I. liriodendri*	KC119422
CyMC 38	Rooted graftlings, Alicante Bouschet × 1103P isolated from rootstock	*I. liriodendri*	KC576854
CyMC 39	Rooted graftlings, Alicante Bouschet × 1103P isolated from rootstock	*Ilyonectria* sp. 2 Cabral *et al*., 2012a	KC119423
CyMC 45	Rooted graftlings, Alicante Bouschet × 1103P isolated from rootstock	*I. novozelandica*	KC119424
CyMC 40	Rooted graftlings, Fernão Pires × 1103P isolated from rootstock	*Ilyonectria* sp.	KC119425
CyMC 41	Rooted graftlings, Fernão Pires × 1103P isolated from rootstock	*I. macrodidyma*	KC576855
CyMC 42	Rooted graftlings, Fernão Pires × 1103P isolated from rootstock	*I. torresensis*	KC576856
CyMC 43	Rooted graftlings, Fernão Pires × 1103P isolated from rootstock	*"C." pauciseptatum*	KC119426
CyMC 44	Rooted graftlings, Fernão Pires × 1103P isolated from rootstock	*"C." pauciseptatum*	KC119427
CyMC 46	Rooted graftlings, Fernão Pires × 1103P isolated from rootstock	*I. macrodidyma*	KC119428
CyMC 48	Rooted graftlings, Jaen × 99R isolated from rootstock	*Ilyonectria* sp.	KC119429
CyMC 49	Rooted graftlings, Jaen × 99R isolated from rootstock	*I. liriodendri*	KC576857

(*Continued*)

Table 3. *Continues.*

Strain number	Isolated from	Species	Accession number
CyMC 50	Rooted graftlings, Jaen × 99R isolated from rootstock	*Ilyonectria* sp.	KC119430
CyMC 53	Rooted graftlings, Jaen × 99R isolated from rootstock	*I. liriodendri*	KC576858
CyMC 54	Rooted graftlings, Jaen × 99R isolated from rootstock	*I. liriodendri*	KC119431
CyMC 56	Rooted graftlings, Jaen × 99R isolated from rootstock	*I. liriodendri*	KC576859
CyMC 58	Rooted graftlings, Jaen × 99R isolated from rootstock	*Ilyonectria* sp.	KC119432
CyMC 61	Rooted graftlings, Moscatel roxo × 110R isolated from rootstock	*I. torresensis*	KC119433
CyMC 62	Rooted graftlings, Moscatel roxo × 110R isolated from rootstock	*I. macrodidyma*	KC119434
CyMC 63	Rooted graftlings, Rabigato × 110R isolated from rootstock	*I. liriodendri*	KC119435
CyMC 64	Rooted graftlings, Rabigato × 110R isolated from rootstock	*I. liriodendri*	KC119436
CyMC 65	Rooted graftlings, Touriga Nacional cl 16 × 1103P isolated from rootstock	*I. novozelandica*	KC119437
CyMC 66	Rooted graftlings, Touriga Nacional cl 16 × 1103P isolated from rootstock	*I. novozelandica*	KC576860

alignment, which included other sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/) was subjected to phylogenetic analyses consisting of Maximum Parsimony performed in MEGA version 5 using the heuristic search option, where gaps and missing data were treated as complete deletions. The robustness of the topology was evaluated by 1000 bootstrap replications (Hillis and Bull, 1993). Measures for the maximum parsimony as tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were also calculated. Sequences were deposited in GenBank with accessions KC119412 to KC119437 and KC576843 to KC576860.

Results

Sampling of plant material

Results from sampling of grapevine propagation material carried out by microbiological methods revealed the presence of *Ilyonectria*-like fungi either in rootstock or scion cuttings (Table 1). In callused graftlings of different scion/rootstock combinations, there was a generally lower incidence of *Ilyonectria*-like fungi than in material collected from mother plants. Finally, samples of dormant-field finished plants (rooted graftlings) revealed the presence of *Ilyonectria*-like fungi and/or "*C.*" *pauciseptatum* predominantly in the base of the rootstock but also in the scion. For the scion/rootstock combinations, which were evaluated during callusing and after the rooting stage, *Ilyonectria*-like fungi incidence was, on average higher for rooted graftlings than for callused graftlings (Table 1).

With few exceptions, results from nested-PCR allowed the detection of *Ilyonectria*-like fungi in a higher number of samples than were detected by microbiological methods. The greatest discrepancy was in the results for the callusing graftlings. Using PCR based methods black foot associated pathogens were identified in the majority of the samples, mainly on the rootstock with incidence values ranging from 50 to 100%, whereas the incidence ranged from 0 to 30% when microbiological methods were used. Also noticeable were the values for *Ilyonectria*-like fungi incidence in the scion (40 to 100%). For example, in the combination "Fernão Pires" × 1103 P the scion was infected in all plants (100%), while the causative agents of black foot disease were found at the base of the rootstock in only 50% of samples (Table 1).

Results from the use of the three sets of specific primers Lir1/Lir2, Mac1/MaPa2 and Paul1/MaPa2 showed that the majority of the planting material was infected simultaneously with *I. liriodendri* and fungi encompassed by the complex *I. macrodidyma* (Table 1, Figure 1). Single infections by *I. macrodidyma* fungi were mostly found in rootstocks, whereas

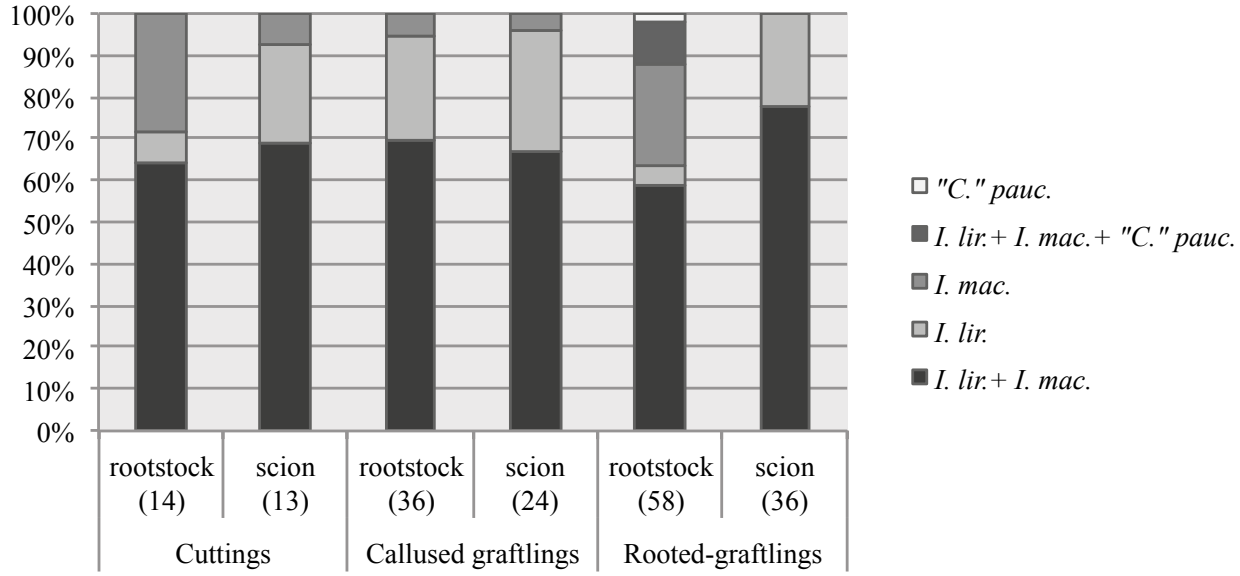

Figure 1. Proportion of *Ilyonectria liriodendri*, *I. macrodidyma* complex and "*Cylindrocarpon*" *pauciseptatum*, alone or in combination, on different grapevine propagation materials, by multiplex nested PCR (sample size in brackets).

I. liriodendri alone was mainly detected in the scion. In callused material, the proportion of each fungus, alone or in combination, was similar for both the rootstock and the scion. However, "*C.*" *pauciseptatum*, was only found on the rootstock in rooted graftlings, alone or in combination with *Ilyonectria* spp. (Figure 1).

Soils and callusing media

Ilyonectria-like fungi and/or "*C.*" *pauciseptatum* were found in the 22 soils analysed by multiplex nested PCR (Table 2), but when the soil-dilution plating method was used these fungi were only detected in 12 soils. Alone or in combination, fungi in the *I. macrodidyma* complex were predominant, being present in 21 out of 22 soils analysed, whereas *I. liriodendri* and "*C.*" *pauciseptatum* were detected in 11 and four soil samples, respectively (data not shown). In the rootstock mother field soils, only fungi in the *I. macrodidyma* complex were detected in contrast to the results obtained from the grapevine scion mother–fields, where *I. liriodendri* was found, alone or in combination, with the *I. macrodidyma* complex (Table 2).

In soils intended to be used for field nursery planting, black foot pathogens were detected in only four out of six soil samples, using microbiological methods. However, when PCR-based methods were applied, all samples tested positive for those pathogens, revealing that fungi in the *I. macrodidyma* complex were present in all soils, either alone (one sample) or in combination with *I. liriodendri* (Figure 2). During the rooting stage, soil samples collected from two grapevine nurseries yielded *I. macrodidyma* fungi combined with either *I. liriodendri* or "*C.*" *pauciseptatum*. None of these pathogens was detected by microbiological methods from these soil samples (Figure 2; Table 2).

After the rooting stage, five out of nine samples tested positive using microbiological methods, contrasting with nine positive samples, by PCR-based methods. The *I. macrodidyma* complex was present in all positive soils; alone (55%) or in combination with *I. liriodendri*, "*C.*" *pauciseptatum* or *I. liriodendri* and "*C.*" *pauciseptatum* (Figure 2).

Both *I. liriodendri* and "*C.*" *pauciseptatum* were detected in the callusing-medium, before use, while after the callusing stage the *I. macrodidyma* complex was also present. The detection of any of these species in the callusing media was achieved only by molecular approaches (Table 2).

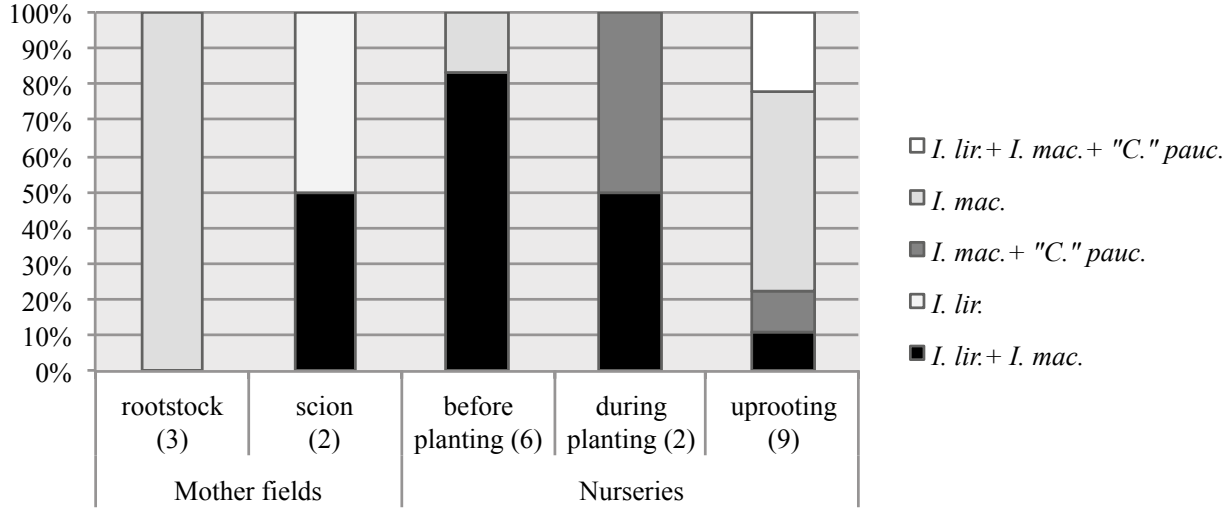

Figure 2. Proportion of *Ilyonectria liriodendri* (*I. lir.)*, *I. macrodidyma* complex (*I. mac.*) and "*Cylindrocarpon*" *pauciseptatum* ("*C." pauc.*), alone or in combination, in the different soils sampled, assessed by multiplex nested PCR (sample size in brackets).

Indoor air, water and cutting tools

The indoor air was monitored fortnightly for four months in three warehouses and eight cold-chambers. Air samples were generally negative for black foot fungi, but *I. liriodendri* was detected in one air sample collected inside a cold-chamber where scion and rootstock cuttings were stored (Table 2).

Ilyonectria-like fungi were detected by nested-PCR in well water used to fill the hydration tanks and water from the hydration tanks after scion or rootstock cuttings were soaked. Further analysis by multiplex nested-PCR revealed the presence of *I. liriodendri* in all water samples, whilst *I. macrodidyma* complex was absent from the tank used for scion hydration. The dilution-plating method did not detect pathogens associated with black foot from water samples.

Only the sample collected from the guillotine used for root pruning (finished vines) tested positive for black foot pathogens using microbiological methods. However, further analyses using molecular approaches revealed the presence of *I. liriodendri*, *I. macrodidyma* complex and *"C." pauciseptatum* (Table 2). On other cutting tools, including the grafting machines, *I. liriodendri* was the predominant fungus. The *I. macrodidyma* complex combined with *I. liriodendri* was found on cutting tools used to prune graftlings, but not on other cutting tools.

Sequencing of Histone H3 gene

Amplification products of approximately 450 bp were obtained for part of the histone H3 gene for the isolates with accession numbers in Table 3. The manually adjusted alignment contained 79 taxa (including the two outgroups) and 538 characters including alignment gaps. Of the 406 characters used in the analysis, 118 were parsimony-informative, 22 were variable and parsimony-uninformative, and 278 were constant. Parsimony analysis of the alignment yielded 276 most parsimonious trees (TL = 274 steps; CI = 0.603; RI = 0.946; RC = 0.583), one of which is shown in Figure 3.

Results showed that, from 44 *Ilyonectria* spp. isolates obtained from soils and rooted graftlings (six scion/rootstock/combinations), 13 isolates grouped into *I. liriodendri*, 12 in *I. macrodidyma*, 13 in *I. torresensis*, three in *I. novozelandica*, two in "*C.*" *pauciseptatum* and one in *Ilyonectria* sp. 2, while none were assigned to *I. alcacerensis* or *I. estremocensis*. The isolates CyMC48, CyMC50 and CyMC58 cluster together (but with a five base difference) with the isolate CBS302.83 (*I. cyclaminicola*) and most likely they are *I. cyclaminicola*. The isolate CyMC40 is clearly different from CBS302.83 with 31 bp difference (Figure 3). These four *Ilyonectria* isolates need further characterization to better clarify their taxonomic position.

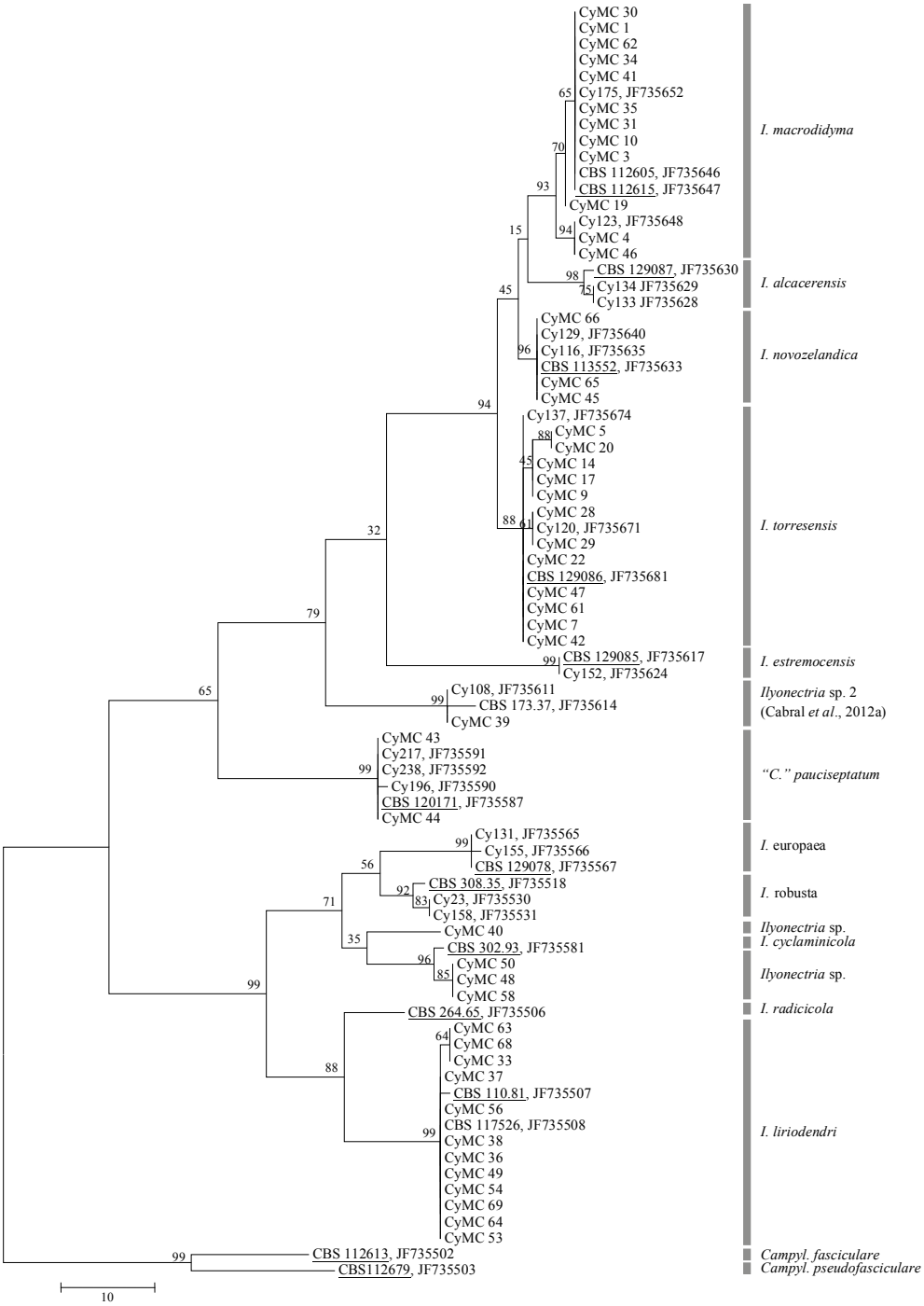

Figure 3. The first of 276 equally most parsimonious trees obtained from part of Histone H3 sequence alignment of *Ilyonectria* isolates and relatives with a heuristic search using MEGA version5. The tree was rooted using *Campylocarpon* isolates as outgroup sequences and bootstrap support values are indicated near the nodes. Ex-type strains are underlined. Isolates obtained in this study start with CyMC, sequences download from GenBank have the accession number after the isolate number. Scale bar shows 10 changes.

In soil samples (18 isolates) a majority of isolates clustered with *I. torresensis* (10 isolates) and *I. macrodidyma* (7 isolates), along with one *I. liriodendri* isolate. By contrast, among 26 isolates obtained from rooted graftlings, nine were identified as *I. liriodendri*, five as *I. macrodidyma*, three as *I. novozelandica*, four as an unidentified *Ilyonectria* sp., two as "*C.*" *pauciseptatum*, two as *I. torresensis* and one *Ilyonectria* sp. 2.

Discussion

Our study provides evidence that in addition to soil and grapevine rootstock cuttings, scion cuttings are frequently infected with *I. liriodendri* and the *I. macrodidyma* complex, the most widespread causative agents of black foot disease worldwide.

Ilyonectria fungi, along with "*C.*" *pauciseptatum*, are known to be soil inhabitants, infecting the underground parts of grapevines, mainly the roots and the basal end of the rootstocks (Rego *et al*., 2000; Halleen *et al*., 2006b). Although these pathogens have rarely been isolated from canes of rootstock mother plants (Rego *et al*., 2001; Fourie and Halleen, 2002; Pinto *et al*., 2005). It is however, accepted that they could easily infect the canes, since in most nurseries the rootstock mother plants are trained to sprawl on the soil (Gramaje and Armengol, 2011). The scion mother vines are not similarly trained, and contact of the canes with the soil is only sporadic and short-term. Therefore, it is intriguing that higher incidences of *Ilyonectria* spp. are being detected with on canes of scion mother vines and thus it follows that the hypothesis of aerial dissemination of some of these fungi should not be ruled out. As far as we know, perithecia of both *I. liriodendri* (Halleen *et al*., 2006a) and fungi in the *I. macrodidyma* complex (Halleen *et al*., 2004; Chaverri *et al*., 2011) have not been found under natural conditions on grapevine; and the teleomorph of "*C.*" *pauciseptatum* remains unknown (Schroers *et al*., 2008). However, perithecia of *Neonectria radicicola* (purported teleomorph of "*Cylindrocarpon*" *destructans,* which was believed in the past to be the causative agent of black foot of grapevine) have been observed on grapevine cuttings, after artificial inoculation (Larignon, 1999). It is not possible to determine the true identity of this pathogen, but it was probably either *I. liriodendri* or *I. macrodidyma*. If perithecial formation (and ascospore production) was observed in artificially inoculated plants, it is reasonable to expect that a similar phenomenon might occur in nature, but remain unnoticed. The minute size of *Ilyonectria* fruiting bodies and imprecise knowledge of where on the vine they could be formed may explain why they have not yet been observed.

In the present study, mixed infections caused by *I. liriodendri* and *I. macrodidyma* complex were the most consistently detected in all plant material "*C.*" *pauciseptatum* was only detected on rooted graftlings (rootstock part), and although unexpected, "*C.*" *pauciseptatum* was detected in peat used for the callusing process, both before and after use. However, the pathogen was never detected at the callusing stage, thus indicating that infection occurs later, during the rooting stage in the nursery.

In the commercial nursery surveyed in this study, the dormant canes were harvested in the field, cut in the warehouses and immediately stored in cold chambers. Despite the attempts to detect airborne inoculum inside the warehouses and cold chambers over different sampling periods (21 and 56 air samples were collected, respectively), only one sample taken from a cold-chamber tested positive for *I. liriodendri*. Although scarcely representative, the trapped spores were viable, since they were detected by isolating on culture medium.

Rootstock and scion cuttings were hydrated in water containing a copper-based product. However, the effectiveness of this product was not evaluated against *Ilyonectria* spp. The water used to fill the tanks also tested positive for *I. liriodendri* and *I. macrodidyma* complex, and water samples collected from the tanks after cutting hydration proved again that at least one pathogen was present. Therefore, even healthy rootstock or scion cuttings could be infected by black foot pathogens during this hydration/disinfection procedure. It is possible that as a result of the copper-based product added to the soaking water, the amount of surface inoculum on the cuttings could be reduced, but infection of clean material during hydration is still a risk. These findings are of major importance; however they should be interpreted carefully since no water sample yielded positive results for black foot pathogens by microbiological isolation revealing that the inoculum detected by molecular methods was non-culturable.

Ilyonectria liriodendri and/or *I. macrodidyma* complex were frequently detected on cutting tools, including grafting machines, by molecular methods,

however culturable propagules were only found on blades of the guillotine used to prune the roots of finished plants (after the rooting stage in field nursery). *"C." pauciseptatum* was detected in addition to *I. liriodendri* and the *I. macrodidyma* complex on this machine by nested-multiplex PCR, providing additional evidence that to date, the occurrence of this pathogen appears to be restricted to soils and rootstocks.

Eleven samples (ten plants each) of finished plants (rooted graftlings) were also analysed and results revealed the presence of black foot pathogens in all samples, irrespective of the analytical method used. In addition to the basal region of the rootstock, it was possible to isolate the black foot pathogens from eight of the scions varieties. The cultural practices in these nursery fields do not include covering the grafting unions with soil to prevent drying, as reported by Halleen *et al*. (2003, 2006b). Thus the infection of the scion varieties was most probably due to the use of infected scions for grafting, although aerial dissemination of spores in the field cannot be excluded.

Species of *Ilyonectria* (Chaverri *et al*., 2011), as well as "*C*." *pauciseptatum* (Schroers *et al*., 2008) typically form resistance structures, chlamydospores, which provide the principal means of long-term survival for these pathogens in the soil. In Portugal, a four year rotation cycle is presently mandatory for grapevine nurseries. Grapevines are only planted the first year, followed by three years of other crops. Despite compliance with this current legal and regulatory obligation, our results demonstrate that after the rotation cycle, before or immediately nursery planting, viable propagules of black foot pathogens are still present in the soil. As a consequence, even healthy graftlings rooted in these nurseries could be infected. For socio-economic reasons, it is not practical to extend of the actual rotation cycle, but more comprehensive studies to determine which crops should be included in the rotation are of outmost importance.

Recently Cabral *et al*. (2012b) showed that *I. macrodidyma* was a species complex encompassing six monophyletic species, *I. alcacerensis*, *I. estremocensis*, *I. novozelandica*, *I. torresensis* and two referred as *Ilyonectria* sp. 1 and sp. 2. Results from sequencing part of the histone H3 gene showed that *I. macrodidyma* (*sensu stricto*) was the only species present on the soil of scion mother plants, whereas on the soils of rootstock mother plants *I. torresensis* was dominant, although *I. macrodidyma* and *I. liriodendri* also exist. From five nursery soils, after 1-yr of rooting stage, only *I. macrodidyma* (*sensu stricto*) and *I. torresensis* were found, with predominance for *I. torresensis*. However, when rooted-graftlings were analysed, the richness of species found was much greater; *I. liriodendri*, *I. macrodidyma*, *I. torresensis*, *I. novozelandica*, *Ilyonectria* sp. 2, *"C." pauciseptatum* and four *Ilyonectria* isolates which are close to *I. cyclaminicola* were detected. These last *Ilyonectria* isolates along with *I. novozelandica* are reported for the first time in grapevines in Portugal. Overall, these results do not identify when the plant materials were infected by soil-borne inoculum, or if they were already infected when they were planted. To determine precisely when specific plant material is infected in the nursery and by which *Ilyonectria* species, a more directed survey at all stages of production from the mother plants to the finished rooted graftlings is needed.

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