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Research Papers

Virulence and cross-infection potential of *Ilyonectria* spp. to grapevine

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Summary. Black foot is an important disease of grapevines, affecting vines in nurseries as well as in young plantations. In recent years the disease has increased in incidence and severity throughout the world. Black foot is associated with at least two *Campylocarpon* and 12 *Ilyonectria* species, most of which have only recently been described. The recognition of previously unknown species, together with published reports of variability in virulence between and within species identified as *I. macrodidyma* and *I. liriodendri*, underlined the need to compare the virulence of isolates from these complexes. A further objective of this work was to determine the cross-infection potential of isolates of these species from other hosts to grapevine. Results from this study revealed recently described species such as *I. lusitanica*, *I. estremocensis* and *I. europaea* to be more virulent to grapevine than the species previously accepted as the main causal agents of black foot, such as *I. liriodendri* and *I. macrodidyma*. Furthermore, these results also provided support for isolates obtained from non-grapevine hosts to be as virulent to grapevines as isolates obtained from grapevine, underlying the cross-infection potential of these pathogens.

Key words: black foot disease; Cylindrocarpon root rot; pathogenicity; *Vitis vinifera*.

Introduction

Black foot is an important disease of grapevines in most countries throughout the world. In recent years the disease has been reported with an increased incidence and severity, affecting both nurseries and young plantations, causing typical darkening of the basal end of rootstock plants (Halleen *et al*., 2004; Oliveira *et al*., 2004). Declining plants are frequently found in infected vineyards, showing slow growth, reduced vigour, retarded sprouting, shortened internodes, sparse and chlorotic foliage (Rego *et al*., 2000; Halleen *et al*., 2006a), resulting frequently in plant death and forcing growers to replant considerable areas.

Black foot is caused by several *Cylindrocarpon*-like species residing in the genera *Campylocarpon* and *Ilyonectria*. Two species of *Campylocarpon* have been reported, namely *Campyl. fasciculare* Schroers, Halleen & Crous and *Campyl. pseudofasciculare* Halleen, Schroers & Crous (Halleen *et al*., 2004), although these have thus far only been reported from South Africa (Halleen *et al*., 2004) and Uruguay (Abreo *et al*., 2010). The genus *Ilyonectria* was recently established within what was formerly regarded as *Neonectria s. lat*., accommodating well-known pathogens such as *Ilyonectria liriodendri* (Halleen, Rego & Crous)

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P. Chaverri & C. Salgado and *I. macrodidyma* (Halleen, Schroers & Crous) P. Chaverri & C. Salgado (Chaverri *et al*., 2011). In fact, *I. liriodendri* and *I. macrodidyma* are the species most commonly reported from affected grapevines (Petit and Gubler, 2005; Halleen *et al*., 2006b; Alaniz *et al*., 2007). Recent studies have shown, however, that many of these records actually represent some newly described species (Cabral *et al*., 2012a, 2012b). These include *I. alcacerensis* A. Cabral, Oliveira & Crous, *I. estremocensis* A. Cabral, Nascimento & Crous, *I. novozelandica* A. Cabral & Crous and *I. torresensis* A. Cabral, Rego & Crous which were described from within the *I. macrodidyma* species complex (Cabral *et al*., 2012b), and *I. europeaea* A. Cabral, Rego & Crous, *I. lusitanica* A. Cabral, Rego & Crous, *I. pseudodestructans* A. Cabral, Rego & Crous, *I. robusta* (A.A. Hildebr.) A. Cabral & Crous and *I. vitis* A. Cabral, Rego & Crous, which emerged from the *I. radicicola* (Gerlach & L. Nilsson) P. Chaverri & C. Salgado species complex (Cabral *et al*., 2012a). *Ilyonectria torresensis* was found to be associated with *Vitis vinifera, Abies nordmanniana, Fragaria* sp. and *Quercus* sp. in countries throughout the world. In contrast, *I. alcacerensis* has thus far only been reported from *V. vinifera* in the Iberian Peninsula. *Ilyonectria novozelandica* was associated with *V. vinifera* in New Zealand, South Africa and the USA, but also reported on *Festuca duriuscula* in Portugal. *Ilyonectria estremocensis* was isolated from *V. vinifera* in Portugal and *Picea glauca* in Canada (Cabral *et al*., 2012b). *Ilyonectria europaea*, *I. pseudodestructans* and *I. robusta* were found on *V. vinifera* in Portugal and on other host plants in different parts of the world, while *I. lusitanica* and *I. vitis* were thus far exclusively reported from grapevines (Cabral *et al*., 2012a). Besides these, "*Cylindrocarpon*" *pauciseptatum* Schroers & Crous was associated with diseased roots of *Vitis* spp. in New Zealand and Slovenia (Schroers *et al*., 2008), in Uruguay (Abreo *et al*., 2010), in Portugal (Cabral *et al*., 2012a) and in Spain (Martín *et al*., 2011).

Ilyonectria macrodidyma was reported as more virulent to grapevines than *I. liriodendri*, although variation in virulence among groups of *I. macrodidyma* was also found (Alaniz *et al*., 2009). However, no other comparative virulence studies have been reported among the pathogens causing black foot disease of grapevine. This is becoming particularly relevant, as at least 12 species are currently recognised to be associated with this disease. Moreover, most of these species are not exclusive to grapevine, and infect several other hosts, underlining the cross-infection potential of isolates from other hosts to grapevines. Therefore, the objective of this work was to compare the virulence of isolates from different species associated with black foot disease of grapevines, as well as to test the pathogenicity of isolates from other hosts to grapevine.

Materials and methods

A total of 60 isolates were analysed, 36 of which are from grapevines (Table 1). The other hosts include *Olea europaea* (five isolates) and *Quercus* spp. (five isolates), among others. Species covered in this study include "*C.*" *pauciseptatum* (three isolates from grapevine and one from *Olea europaea*), *I. alcacerensis* (two isolates from grapevine), *I. estremocensis* (four isolates from grapevine), *I. europaea* (two isolates from grapevine, one from *Actinidia chinensis* and one from *Aesculus hippocastanum*), *I. liriodendri* (four isolates from grapevine, one from *Liriodendron tulipifera*, one from *Malus domestica,* and one from *Quercus suber*), *I. lusitanica* (one isolate from grapevine), *I. macrodidyma* (three isolates from grapevine and two from *Olea europaea*), *I. novozelandica* (five isolates from grapevine and one from *Festuca duriuscula*), *I. pseudodestructans* (two isolates from grapevine and two from *Quercus* sp.), *I. robusta* (three isolates from grapevine, two from *Quercus* spp., two from *Panax quinquefolium*, one from *Prunus cerasus*, one from *Tilia petiolaris*, one from *Thymus* sp. and one from an aquarium with *Anodonta*), *I. vitis* (one isolate from grapevine), *I. torresensis* (four isolates from grapevine, one from *Fragaria* x *ananassa* and one from *Olea europaea*), an *I. estremocensis*-like undescribed species, here referred as *llyonectria* sp2 (L. Mostert, personal communication; two isolates from grapevine, one from *Pinus laricio* and one from an unknown host) and an *I. venezuelensis*-like undescribed species, here referred as *llyonectria* sp1 (Cabral *et al*., unpublished data).

Cuttings of the susceptible rootstock 1103P (Alaniz *et al*., 2010) were rooted for 1.5 to 2 months at 20°C in a rooting bench containing perlite and sand. Irrigation was carried out by overhead nebulisation for 5 s every 10 min.

After the rooting period, plants were removed from the bench and the roots were slightly pruned. The wounded cuttings were dip-inoculated by immersing the roots and the basal end of the cuttings in a 10^6 mL^{-1} conidial suspension (for each isolate listed

Table 1. List of isolates studied with collection details.

Strain code [a]	Collected/isolated by year	Host/substrate	Location
"Cylindrocarpon" pauciseptatum			
OL-CM3	C. Rego, 2008	*Olea europaea*	Portugal, Campo Maior
Cy196	N. Cruz, 2005	*Vitis vinifera*, basal end of 4-year-old plant; scion Alvarinho; rootstock 196-17	Portugal, Melgaço/Monção
Cy217	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Gouveio	Portugal, Torres Vedras
Cy238	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Petit Verdot; rootstock 110R	Portugal, Vidigueira
Ilyonectria vitis			
CBS 129082; Cy233	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Touriga Nacional; rootstock 110R	Portugal, Vidigueira
Ilyonectria alcacerensis			
Cy134; IAFM Cy20-1	J. Armengol	*Vitis vinifera*	Spain, Ciudad Real, Villarubia de los Ojos
CBS 129087; Cy159	A. Cabral & H. Oliveira, 2004	*Vitis vinifera*, basal end of a 3-year-old plant with root discolouration and decline symptoms; scion Sangiovese; rootstock 1103P	Portugal, Alcácer do Sal, Torrão
Ilyonectria sp2			
CBS 173.37; IMI 090176	T.R. Peace, 1937	*Pinus laricio*, associated with dieback	UK, England, Devon, Haldon
Cy108	C. Rego, 1999	*Vitis vinifera*, basal end of a 4-year-old plant showing decline symptoms; scion Aragonez; rootstock SO4	Portugal, Nelas
Cy200	N. Cruz, 2005	*Vitis vinifera*, basal end of a 16-year-old plant; scion Alvarinho; rootstock 196-17	Portugal, Melgaço
CBS 159.34; IMI 113891	H.W. Wollenweber, 1934	-	Germany
Ilyonectria estremocensis			
Cy135	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz
CBS 129085; Cy145	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, basal end of a 1.5-year-old plant showing decline symptoms; scion Aragonez; rootstock 3309C	Portugal, Estremoz
Cy152	C. Rego & T. Nascimento, 2003	*Vitis vinifera*, asymptomatic 1.5-year-old plant; scion Aragonez; rootstock 3309C	Portugal, Estremoz
Cy243	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old-plant; scion Touriga Nacioal; rootstock 110R	Portugal, Vidigueira
Ilyonectria macrodidyma			
OL_CM4	C. Rego, 2008	*Olea europaea*	Portugal, Campo Maior
OL_CM6	C. Rego, 2008	*Olea europaea*	Portugal, Campo Maior
Cy128	W.D. Gubler	*Vitis vinifera*	USA, California
Cy175	C. Rego, 2004	*Vitis vinifera*, basal discolouration; scion Touriga Nacional; rootstock 1103P	Portugal, Torre de Moncorvo
Cy250	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Chardonnay rootstock 110R	Portugal, Vidigueira

(*Continued*)

Table 1. *Continues.*

Strain code [a]	Collected/isolated by year	Host/substrate	Location
CBS 112615	F. Halleen, 2000	*Vitis vinifera*, roots, asymptomatic nursery plant; scion Sultana; rootstock 143-B Mgt	South Africa, Western Cape, Malmesbury, Jakkalsfontein
Ilyonectria novazelandica			
Cy230	F. Caetano, 2005	*Festuca duriuscula*	Portugal, Lisbon
CBS 113552	R. Bonfiglioli, 2003	*Vitis* sp. decline of nursery plants dead rootstocks	New Zealand, Candy P New Ground
Cy129	W.D. Gubler	*Vitis vinifera*	USA, California
Cy130	W.D. Gubler	*Vitis vinifera*	USA, California
CBS 112593	F. Halleen, 2000	*Vitis vinifera*, roots of asymptomatic nursery plant; scion Pinotage; rootstock 101-14 Mgt	South Africa, Western Cape, Wellington, Voorgroenberg
Ilyonectria torresensis			
Cy222	L. Leandro	*Fragaria* x *ananassa*	USA, North Carolina, Asheville
HC7	C. Rego, 2007	*Olea europaea*	Portugal, Avis
Cy118	W.D. Gubler	*Vitis vinifera*	USA, California
Cy214	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Grenache	Portugal, Torres Vedras
CBS 129086; Cy218	A. Cabral, 2007	*Vitis vinifera*, asymptomatic; scion Chenin	Portugal, Torres Vedras
Cy260	C. Rego, 2008	*Vitis vinifera*, basal end of 2-year-old plant; scion Cabernet Sauvignon; rootstock 110R	Portugal, Vidigueira
Ilyonectria europaea			
Cy131	P. Lecomte & S. Chamont, 2000	*Actinidia chinensis* 'Hayward', internal lesion of stem	France, St. Chicq-du-Gaue
CBS 537.92	V. Demoulin, 1992	*Aesculus hippocastanum*, wood	Belgium, Liège
CBS 129078; Cy241	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Petit Verdot; rootstock 110R	Portugal, Vidigueira
Cy155	C. Rego & H. Oliveira, 2004	*Vitis vinifera*, 2-year-old, with decline symptoms, scion Alfrocheiro; rootstock SO4	Portugal, Alter do Chão
Ilyonectria liriodendri			
CBS 110.81; IMI 303645	J.D. MacDonald & E. Butler, 1978	*Liriodendron tulipifera*, root	USA, California, Yolo Co., Davis
Cy164	C. Rego, 1997	*Malus domestica*; cultivar Lysgolden; rootstock MM106	Portugal, Porto de Mós, Valbom
Cy232	L. Inácio & J. Henriques, 2007	*Quercus suber*, stem	Portugal, Macedo de Cavaleiros
Cy5	C. Rego, 1992	Vitis vinifera, 4-year-old, with decline symptoms; scion Boal Branco; rootstock 99R	Portugal, Torres Vedras, Dois Portos

(*Continued*)

Table 1. *Continues.*

Strain code [a]	Collected/isolated by year	Host/substrate	Location
CBS 117526; Cy68	C. Rego, 1999	*Vitis vinifera*, asymptomatic rootstocks; rootstock 99 R, clone 179F	Portugal, Ribatejo e Oeste
Cy190	N. Cruz, 2005	*Vitis vinifera*, basal end of a 6-year-old plant; scion Alvarinho; rootstock 196-17	Portugal, Monção, Cortes
Cy253	C. Rego, 2008	*Vitis vinifera*, basal end of a 2-year-old plant; scion Petit Verdot; rootstock 110R	Portugal, Vidigueira
Ilyonectria lusitanica			
CBS 129080; Cy197	N. Cruz, 2005	*Vitis vinifera*, below grafting zone, 6-year-old; scion Alvarinho; rootstock 196-17	Portugal, Melgaço, Alvaredo
Ilyonectria sp1			
OL2	C. Rego & H. Oliveira, 2007	*Olea europaea*	Portugal, Évora
Ilyonectria pseudodestructans			
CBS 117824	E. Halmschlager, 1993	*Quercus* sp., root	Austria, Patzmannsdorf
CBS 117812	E. Halmschlager, 1993	*Quercus* sp., root	Austria, Patzmannsdorf
CBS 129081; Cy20	C. Rego, 1996	*Vitis vinifera*, 4-year-old, with decline symptoms, scion Malvasia Fina; rootstock 1103P	Portugal, Gouveia, São Paio
Cy22	C. Rego, 1996	*Vitis vinifera*, 5-year-old, with decline symptoms, scion Aragonez; rootstock 99R	Portugal, Viseu, Silgueiros
Ilyonectria robusta			
CD1666	R. D. Reeleder, 1998	*Panax quinquefolium*	Canada, Nova Scotia
CBS 308.35	A.A. Hildebrand	*Panax quinquefolium*	Canada, Ontario
CPC 13532; DAOM 139398	-	*Prunus cerasus* cultivar Montmorency	Canada, Ontario
CBS 117813	E. Halmschlager, 1993	*Quercus robur*, root	Austria, Niederweiden
CBS 117815	E. Halmschlager, 1993	*Quercus* sp., root	Austria, Patzmannsdorf
Cy231	F. Caetano, 2005	*Thymus* sp.	Portugal, Lisbon
CBS 605.92	R. Schröer, 1992	*Tilia petiolaris*, root	Germany, Hamburg
Cy23	C. Rego, 1997	*Vitis* sp. rootstock 99R clone 179F in nursery	Portugal, Ribatejo e Oeste
Cy158	C. Rego & T. Nascimento, 2004	*Vitis vinifera*, 1-year-old, died before sprouting; scion Alicante Bouschet; rootstock 1103P	Portugal, Lamego, Cambres
CBS 129084; Cy192	N. Cruz, 2005	*Vitis vinifera*, basal end of 25-year-old plant; scion Alicante; rootstock 196-17	Portugal, Monção
CBS 773.83	J. Hemelraad	Water, in aquarium with *Anodonta*	Netherlands, Utrecht

[a] CBS: CBS-KNAW Fungal Biodiversity Centre (Centraalbureau voor Schimmelcultures), Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; Cy,OL and HC: Cylindrocarpon collection housed at Laboratório de Patologia Vegetal "Veríssimo de Almeida" - ISA, Lisbon, Portugal; DAOM: Agriculture and Agri-Food Canada National Mycological Herbarium, Canada; IAFM: Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Spain; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.

in Table 1), for 60 min. Conidia were harvested by flooding 14 d old potato-dextrose agar (PDA, Difco, USA) cultures with sterile distilled water, and dislodged with a sterile glass rod. The spores and mycelium were then filtered through a double layer of cheesecloth, and the conidial concentration estimated using a haemocytometer, which was then adjusted with sterile distilled water. After inoculation, the rooted cuttings were planted in 1 L bags containing a mixture of soil, peat and sand (2:1:1, v/v/v), and maintained in a greenhouse at 24±5°C (day) and 18°C (night) with approximately a 12 h photoperiod. For negative control plants, sterile distilled water was used instead of conidial suspension.

The plants were grown on the greenhouse for 4.5 months and, following this period, results were evaluated for each isolate (10–12 plants per isolate, including the control), and compared to the control. The parameters analysed were focused on the loss of root (number and root dry weight, and the length of the longest root) and shoot (number of shoot nodes and the length and shoot dry weight; usually a single shoot was formed) biomass and on the intensity of wood colonisation by the pathogens (percentage of reisolation). For the latter, 10 pieces of wood from the basal end of each rootstock plant (at least 2 cm above the bottom) were excised, disinfected (for 1 min in a NaClO solution with 0.35% w/w as active chlorine), rinsed with distilled water and placed in Petri dishes containing PDA amended with 250 mg L^{-1} chloramphenicol (BioChemica, AppiChem, Germany). The dishes were incubated at 20°C for up to 2 weeks and observed for the presence of *Ilyonectria* colonies, which was confirmed through morphological appearance of colonies and conidial characteristics. The percentage of reisolation was calculated as the proportion of wood pieces from which *Ilyonectria* colonies were recovered, versus the total number of pieces of wood for each plant.

All data were subjected to a one-way ANOVA and means compared using the Tukey's test at a 5% significance level (STATISTICA 8.0). Before analysis, arcsine-square root transformation was performed for data expressed as percentage.

To confirm results from this experiment, data from a subsequent, smaller experiment were used for comparison under the same conditions as stated above. Isolates tested were from the following species: *I. estremocensis* (isolates Cy135, Cy144, Cy145, Cy152 and Cy153 from grapevine), *I. europaea* (isolate Cy131 from *Actinidia chinensis*), *I. liriodendri* (isolates Cy5, Cy68 and Cy76 from grapevine, Cy164 from *Malus domestica* and Cy232 from *Quercus suber*), *I. novozelandica* (isolate Cy230 from *Festuca* sp.), *I. pseudodestructans* (isolates Cy20 and Cy22 from grapevine, and CBS 117812 from *Quercus* sp.), *I. robusta* (CBS 117818 from *Quercus* sp. and Cy231 from *Thymus* sp.), *I. torresensis* (isolates OL1 from *Olea europaea*, Cy96 from *Quercus* sp. and Cy221 and Cy222 from *Fragaria* x *ananassa*) and *Ilyonectria* sp1 (isolate OL2 from *Olea europaea*).

Results

At the end of the first experiment, root rot symptoms were visible in inoculated plants, in contrast to the uninoculated control plants. Symptoms included root lesions, vascular discolouration, and necrosis in the basal plant tissues, although the quantification of these lesions and discolouration was not possible. Symptoms related to reduced vigour were more readily quantifiable. In general, inoculated plants had shorter shoots with less nodes, as well as less and shorter roots, although significant differences were found among isolates (Table 2).

The percentage of reisolation ranged from a minimum of 18.6% for isolate OL2 (*Ilyonectria* sp1, from *Olea europaea*) to a maximum of 96.5% for isolate CBS 537.92 (*I. europaea*, from *Aesculus hippocastanum*). Control plants had 0% reisolation, differing significantly from all tested isolates except OL2 and Cy230. This trait had the fewest homogeneous groups among all the traits studied.

The average number of roots in the control plants was 36.3, which did not differ significantly from the maximum value recorded from inoculated plants (35.8 for isolate CBS 112615; *I. macrodidyma* from grapevine). The minimum value for NR was 19.2 for isolate CBS 117526 (*I. liriodendri*, from grapevine), which represents a 47% reduction in the number of roots.

The root dry weight ranged from a maximum of 4.50 g for isolate OL2 (which did not differ significantly from the control plants; 4.08 g) to a minimum of 0.49 for Cy243 (*I. estremocensis*, from grapevine; 88% reduction from control).

The length of the longest root for the control plants was 49.6 cm, with all inoculated plants showing a significant reduction from that value, ranging from a minimum reduction of 23% for isolate OL2 to

Table 2. Comparison (average and homogeneous groups) among *Ilyonectria* spp. isolates for their effect on the frequency of reisolation (%RI), on the number of roots (NR), length of the longest root (LR, cm) root dry weight (RDW, g), number of shoot nodes (NSN), shoot length (SL, cm) and shoot dry weight (SDW, g) of grapevine rootstock 1103P.

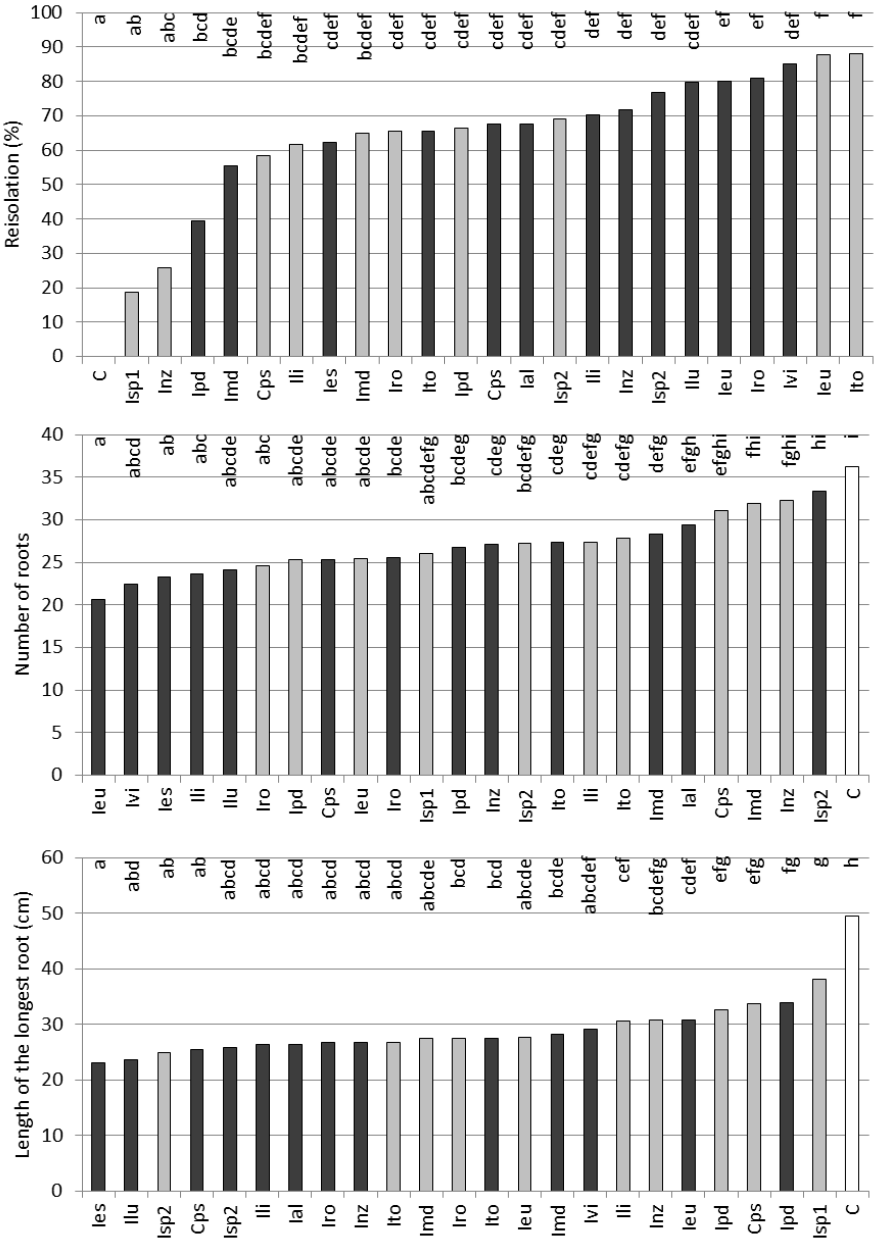
Species	Strain code	Host/substrate	%RI [a]	NR	LR	RDW	NSN	SL	SDW
Control			0 a	36.3 t	49.6 s	4.08 tuv	15.9 g	52.2 o	0.95 g-k
"Cylindrocarpon" pauciseptatum	OL-CM3	*Olea europaea*	56 b-g	31.3 m-t	33.8 o-r	4.25 uv	12.5 c-f	35.8 j-n	0.67 b-j
	Cy196	*Vitis vinifera*	90 efg	23.7 a-k	22.4 abc	1.63 b-h	10.5 a-f	33.0 f,h-m	0.53 a-e
	Cy217	*Vitis vinifera*	52 b-g	29.6 i-s	30.1 d-p	3.82 r-v	11.4 a-f	30.1 b-l	0.72 b-j
	Cy238	*Vitis vinifera*	56 b-g	22.8 a-i	23.7 a-f	0.99 abc	10.8 a-f	22.4 abc	0.51 a-d
Ilyonectria vitis	Cy233	*Vitis vinifera*	85 d-g	22.4 a-h	29.1 c-p	2.98 k-s	13.2 efg	36.1 j-n	0.76 b-k
Ilyonectria alcacerensis	Cy134	*Vitis vinifera*	51 b-g	33.2 q-t	27.7 b-o	2.10 d-n	10.3 a-f	30.3 c-l	0.74 b-j
	CBS 129087	*Vitis vinifera*	83 d-g	25.2 a-m	25.0 b-i	2.00 c-l	11.6 a-f	27.2 b-i	0.68 b-j
Ilyonectria sp2	CBS 173.37	*Pinus laricio*	68 b-g	25.0 a-n	24.9 b-j	1.92 b-l	10.0 a-f	31.8 d-m	0.53 a-e
	Cy108	*Vitis vinifera*	76 c-g	33.8 rst	26.0 b-j,l	2.06 d-l,n	10.7 a-f	40.0 mn	0.93 g-k
	Cy200	*Vitis vinifera*	78 c-g	32.9 p-t	25.6 b-j	2.71 i-q	12.8 efg	43.0 n	0.99 i,k
	CBS 159.34	Unknown	70 b-g	29.0 g-r	25.0 b-i	1.98 c-l	10.0 a-f	31.5 d-l	0.57 a-f
Ilyonectria estremocensis	Cy135	*Vitis vinifera*	63 b-g	25.7 a-n	23.2 a-d	1.57 b-g	11.2 a-f	34.8 h-n	0.67 b-j
	CBS 129085	*Vitis vinifera*	71 b-g	27.1 d-q	26.0 b-l,n	1.68 b-j	10.3 a-f	31.7 e-l	0.71 b-j
	Cy152	*Vitis vinifera*	63 b-g	20.3 abc,e	25.4 b-j	1.49 a-f	9.8 a-f	30.0 b-l	0.61 b-g
	Cy243	*Vitis vinifera*	53 b-g	20.3 abc,e	16.8 a	0.49 a	8.9 abc	18.1 a	0.22 a
Ilyonectria macrodidyma	OL_CM4	*Olea europaea*	57 b-g	30.3 k-t	24.8 b-h	1.40 a-e	10.5 a-f	35.3 i-n	0.82 d-k
	OL_CM6	*Olea europaea*	74 b-g	33.8 rst	30.3 e-p	3.36 q-u	10.2 a-f	35.5 i-n	0.92 f-k
	Cy128	*Vitis vinifera*	61 b-g	20.9 abc,ef	22.0 abc	1.63 b-h	9.8 a-f	28.8 b-k	0.56 a-e
	Cy175	*Vitis vinifera*	70 b-g	26.4 c-o	28.2 b-o	2.20 d-n	11.0 a-f	34.2 h-m	0.79 c-k
	Cy250	*Vitis vinifera*	33 bcd	30.0 j-s	29.8 d-p	2.14 d-n	10.2 a-f	23.3 a-d	0.50 a-d
	CBS 112615	*Vitis vinifera*	58 b-g	35.8 st	32.7 k,m-r	1.98 c-l	10.0 a-f	29.0 b-k	0.77 c-k
Ilyonectria novazelandica	Cy230	*Festuca duriuscula*	26 abc	32.3 o-t	30.7 e-p	2.39 e-q	10.6 a-f	31.4 d-l	0.84 d-k
	CBS 113552	*Vitis* sp.	71 b-g	24.4 a-l	27.7 b-o	2.11 d-n	10.4 a-f	35.2 i-n	1.08 k
	Cy129	*Vitis vinifera*	75 c-g	25.2 a-m	26.2 b-l	1.97 c-k	12.1 b-f	35.6 i-n	0.70 b-j
	Cy130	*Vitis vinifera*	72 b-g	27.0 d,g-p	24.3 b-e	1.75 b-h	11.7 b-f	33.1 h-m	0.65 b-h
	CBS 112593	*Vitis vinifera*	69 b-g	32.1 n-t	29.9 d-p	2.63 h-q	12.4 c-f	35.5 i-n	0.97 h-k
Ilyonectria torresensis	Cy222	*Fragaria* x *ananassa*	92 efg	27.5 d,g-r	28.6 b-o	2.28 d-p	11.6 a-f	37.7 lmn	0.95 g-k
	HC7	*Olea europaea*	84 d-g	28.3 g-r	25.2 b-j	2.63 h-q	11.5 a-f	35.7 j-n	0.77 b-k
	Cy118	*Vitis vinifera*	70 b-g	25.0 a-l	25.4 b-j	1.71 b-i	10.9 a-f	36.3 j-n	0.63 b-h,j
	Cy214	*Vitis vinifera*	76 b-g	27.4 d,f-r	27.7 b-o	3.08 m-t	9.8 a-f	27.9 b-k	0.78 b-k
	CBS 129086	*Vitis vinifera*	73 b-g	25.3 a-m	28.8 c-o	3.25 o-u	8.1 a	23.4 a-e	0.70 b-j

(*Continued*)

Table 2. *Continues.*

Species	Strain code	Host/substrate	%RI [a]	NR	LR	RDW	NSN	SL	SDW
	Cy260	*Vitis vinifera*	37 b-e	30.7 l-t	28.0 b-o	3.03 k-t	11.0 a-f	28.5 b-k	0.71 b-j
Ilyonectria europaea	Cy131	*Actinidia chinensis*	73 b-g	22.6 a-h	31.0 e-r	2.19 d-o	10.8 a-f	32.3 f-m	0.71 b-j
	CBS 537.92	*Aesculus hippocastanum*	96 g	28.1 g-r	24.8 b-h	2.73 i-q	9.6 a-f	26.6 a-h	0.61 b-g
	CBS 129078	*Vitis vinifera*	76 c-g	21.3 a-f	30.6 f-p	2.03 d-l	11.1 a-f	28.0 b-j	0.71 b-j
	Cy155	*Vitis vinifera*	85 d-g	19.8 abc	31.0 e-r	2.21 d-o	11.1 a-f	26.6 a-h	0.62 b-h
Ilyonectria liriodendri	CBS 110.81	*Liriodendron tulipifera*	75 b-g	27.5 d-r	23.8 a-h	0.77 ab	10.0 a-f	36.7 j-n	0.73 b-k
	Cy164	*Malus domestica*	51 b-g	29.0 h-r	33.0 m,o-r	2.90 k-r	13.0 fg	37.1 lmn	0.63 b-g
	Cy232	*Quercus suber*	64 b-g	25.2 a-n	33.3 m-r	2.77 j-r	11.3 a-f	32.7 f,h-m	0.74 b-k
	Cy5	*Vitis vinifera*	51 b-g	23.6 a-j	33.0 k-r	3.10 m,o-t	11.6 a-f	33.0 f,h-m	0.77 b-k
	CBS 117526	*Vitis vinifera*	88 efg	19.2 a	38.0 qr	1.99 c-l	11.3 a-f	31.8 e-m	0.66 b-j
	Cy190	*Vitis vinifera*	75 b-g	25.4 a-m	24.9 b-h	1.65 b-h	10.7 a-f	32.2 f,h-m	0.76 b-k
	Cy253	*Vitis vinifera*	67 b-g	26.0 a-o	32.4 j-r	3.97 s-v	11.0 a-f	26.1 a-h	0.61 b-h
Ilyonectria lusitanica	Cy197	*Vitis vinifera*	80 c-g	24.1 a-l	23.7 a-f	1.28 a-d	8.9 a-d	24.3 a-g	0.41 ab
Ilyonectria sp1	OL2	*Olea europaea*	19 ab	26.1 b-o	38.2 r	4.50 v	8.8 ab	21.8 ab	0.45 abc
Ilyonectria pseudodestructans	CBS 117824	*Quercus* sp.	83 d-g	20.0 ab	31.2 h-r	1.34 a-d	9.8 a-f	30.8 c-l	0.88 e-k
	CBS 117812	*Quercus* sp.	48 b-f	30.6 l-t	34.1 o-r	3.06 m,o-s	11.5 a-f	30.5 c-l	0.75 b-k
	CBS 129081	*Vitis vinifera*	53 b-g	28.2 g-r	36.2 pqr	3.35 p-u	11.3 a-f	33.8 h-m	0.97 g-k
	Cy22	*Vitis vinifera*	32 bcd	26.3 b-o	31.8 i-r	1.49 a-f	10.3 a-f	32.5 f,h-m	0.67 b-j
Ilyonectria robusta	CD1666	*Panax quinquefolium*	42 b-e	21.3 a-f	26.7 b-n	1.53 b-f	9.7 a-e	23.8 a-e,g	0.54 a-d
	CBS 308.35	*Panax quinquefolium*	74 b-g	25.7 a-o	24.0 a-g	1.45 a-f	11.1 a-f	34.5 h-m	0.62 b-g
	CPC 13532	*Prunus cerasus*	77 b-g	22.2 a-g	32.8 k-r	3.44 q-v	12.5 c-g	34.9 h-n	0.99 ijk
	CBS 117813	*Quercus robur*	72 b-g	23.1 a-i	30.8 e-q	2.60 g-q	11.4 a-f	34.9 h-n	0.74 b-k
	CBS 117815	*Quercus* sp.	75 b-g	27.5 d,g-r	21.4 ab	2.47 f-q	12.6 d-g	36.5 k-n	0.64 b-j
	Cy231	*Thymus* sp.	96 fg	26.6 b-q	27.0 b-o	3.40 p-v	11.6 a-f	35.7 h-n	0.60 a-j
	CBS 605.92	*Tilia petiolaris*	50 b-g	24.3 a-l	28.0 b-o	1.49 a-f	10.2 a-f	31.5 e-l	0.91 f-k
	Cy23	*Vitis vinifera*	92 efg	20.0 a-f	20.7 ab	1.52 a-h	10.1 a-f	32.1 e-m	0.65 b-j
	Cy158	*Vitis vinifera*	80 c-g	25.9 a-o	26.2 b-n	2.01 c-l,n	11.3 a-f	34.8 h-n	0.71 b-j
	CBS 129084	*Vitis vinifera*	71 b-g	28.8 g-r	31.0 g-q	1.96 c-l	11.5 a-f	34.7 h-m	0.78 b-k
	CBS 773.83	water	40 b-e	28.0 g-r	28.6 b-o	3.00 l-s	10.9 a-f	31.8 e-m	0.73 b-j

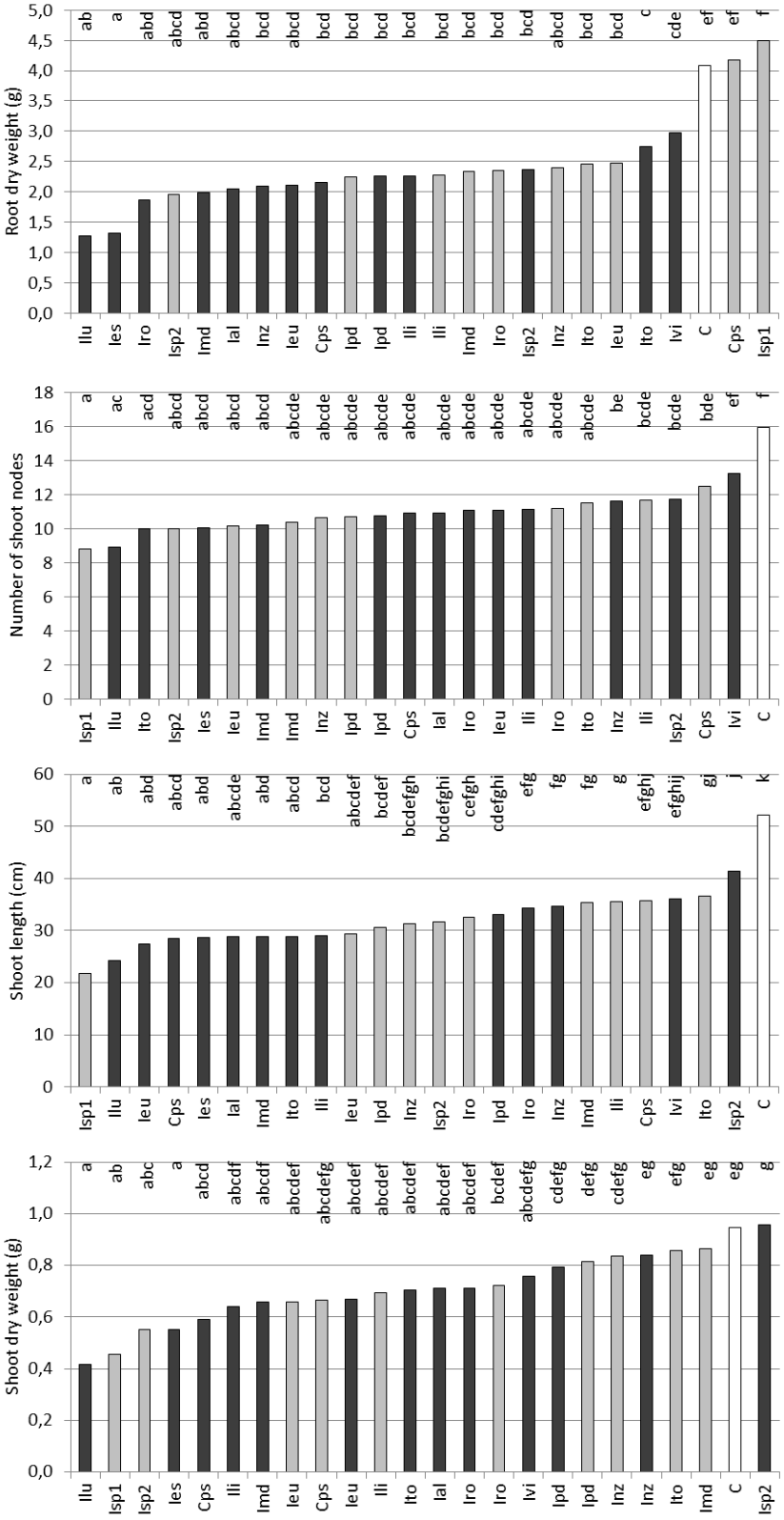
[a] In each column, values followed by the same letter do not differ statistically according to Tukey's test (P=0.05)



(*Continued*)

Figure 1. Comparison among *Ilyonectria* spp. isolates from grapevines (black bars) and from other hosts (grey bars) for their effect on grapevine rootstock 1103P in: frequency of reisolation; number of roots; length of the longest root; root dry weight; number of shoot nodes; shoot length; shoot dry weight. Bars affected by the same letter do not differ statistically according to Tukey's test (P=0.05). C, Control (white bars); Cps, "*C.*" *pauciseptatum* (average of three isolates from grapevine and one isolate from another host); Ial, *I. alcacerensis* (two isolates from grapevines); Ies, *I. estremocensis* (four isolates from grapevines); Ieu, *I. europaea* (two isolates from grapevines and two from other hosts); Ili, *I. liriodendri* (four isolates from grapevines and three from other hosts); Ilu, *I. lusitanica* (one isolate from grapevines); Imd, *I. macrodidyma* (four isolates from grapevines and two from other hosts); Inz, *I. novozelandica* (four isolates from grapevines and one from another host); Iro, *I. robusta* (three isolates from grapevines and eight from other hosts); Isp1, *Ilyonectria* sp1 (one isolate from olive); Ipd, *I. pseudodestructans* (two isolates from grapevines and two from other hosts); Isp2, *Ilyonectria* sp2 (two isolates from grapevines and two from other hosts); Ito, *I. torresensis* (four isolates from grapevines and two from other hosts); Ivi, *I. vitis* (one isolate).

(*Continues*)


Root dry weight (g)
Number of shoot nodes
Shoot length (cm)
Shoot dry weight (g)

a maximum of 66% (16.8 cm) for isolate Cy243.

The average number of shoot nodes in the control plants was 15.9, ranging for the inoculated plants from 13.2 nodes for isolate Cy233 (*I. vitis*, from grapevine), which did not differ significantly from the control, to 8.1 nodes for isolate CBS 129086 (*I. torresensis*, from grapevine), which represents a 49% reduction.

The average shoot length was 52.2 cm in the control plants, ranging from 43.0 cm for isolate Cy200 (*Ilyonectria* sp2, from grapevine; 43.0 cm, 18% reduction) to 18.1 cm for isolate Cy243 (18.1 cm, 65% reduction).

The shoot dry weight ranged from a maximum of 1.08 g for isolate CBS 113552 (*I. novozelandica*, from grapevine), which did not differ significantly from the control (0.95 g), to a minimum of 0.22 g (Cy243), which represents a 80% reduction.

Considering the isolates obtained from grapevine separate from the isolates from other hosts, significant differences were observed among species and to the control (Figure 1). The percentage of reisolation ranged between 39.4% for *I. pseudodestructans* and 85.0% for *I. vitis* for grapevine isolates, all of them differing significantly from the control. Results for isolates from other hosts ranged between 88.1% for *I. torresensis* and 18.6% for *Ilyonectria* sp1 (which did not differ from the control, along with *I. novozelandica*; the latter was the single species with significant differences among isolates from grapevine and other hosts).

For grapevine isolates, the number of roots ranged between a maximum of 33.3 for *Ilyonectria* sp2 (the single species that did not differ significantly from the control; 36.3) and a minimum of 20.7 for *I. europaea*, representing a 43% reduction in the number of roots. Among the isolates from other hosts, "*C.*" *pauciseptatum*, *I. macrodidyma* and *I. novozelandica* did not differ statistically from the control (non-grapevine isolates from "*C.*" *pauciseptatum* and *I. novozelandica* differed significantly from grapevine isolates), while inoculations with *I. robusta* resulted in the lowest number of roots (a 32% reduction).

The length of the longest root was significantly lower for all samples when compared to the control, ranging between a maximum of 33.8 cm for *I. pseudodestructans* (a 32% reduction from the control) and a minimum of 22.9 cm for *I. estremocensis* (54% reduction) for grapevine isolates, and between 38.2 cm for *Ilyonectria* sp1 and 24.9 cm for *Ilyonectria* sp2 for isolates from other hosts. Significant differences were recorded, however, for "*C.*" *pauciseptatum* inoculations between grapevine (25.4 cm) and non-grapevine isolates (33.7 cm).

For grapevine isolates, the root dry weight of inoculated plants ranged between a maximum of 2.98 g for *I. vitis* (the single species that does not differ significantly from the control; 4.08 g) and a minimum of 1.28 g for *I. lusitanica* (a 69% reduction from the control). Among non-grapevine isolates, "*C.*" *pauciseptatum* and *Ilyonectria* sp1 did not differ statistically from the control (and "*C.*" *pauciseptatum* non-grapevine isolates differed significantly from grapevine isolates), while inoculations with *Ilyonectria* sp2 resulted in a root dry weight of 1.95 g (a 52% reduction).

Similarly, the number of shoot nodes ranged between a maximum of 13.2 for plants inoculated with *I. vitis* (the single species that did not differ significantly from the control; 15.9) and a minimum of 8.9 for plants inoculated with *I. lusitanica* (a 44% reduction from the control) for grapevine isolates, and between 12.5 for "*C.*" *pauciseptatum* and 8.8 for *Ilyonectria* sp1 among isolates from other hosts. For each species, no significant differences were found between grapevine and non-grapevine isolates.

Shoot length was significantly shorter than that of the control for all samples, ranging between a maximum of 41.4 cm for *Ilyonectria* sp2 (a 21% reduction from the control) and a minimum of 24.3 for *I. lusitanica* (53% reduction) for grapevine isolates, and between 36.7 cm for *I. torresensis* and 21.8 cm for *Ilyonectria* sp1 for isolates from other hosts. Non-grapevine isolates had significantly higher values than grapevine isolates for several species, such as "*C.*" *pauciseptatum*, *I. liriodendri*, *I. macrodidyma* and *I. torresensis*, while the opposite was recorded for *Ilyonectria* sp2.

The shoot dry weight ranged between a maximum of 0.96 g for *Ilyonectria* sp2 and a minimum of 0.41 g for *I. lusitanica* (a 57% reduction from the control) for grapevine isolates ("*C.*" *pauciseptatum*, *I. estremocensis*, *I. liriodendri*, *I. lusitanica* and *I. macrodidyma* were significantly lower than the control) and of 0.45 g for *Ilyonectria* sp1 for non-grapevine isolates. Differences between grapevine and non-grapevine isolates were only recorded for *Ilyonectria* sp2 (0.96 g and 0.55 g, respectively).

Inoculated plants in the second experiment also revealed typical black foot symptoms, with significant reductions in root and shoot biomass as com-

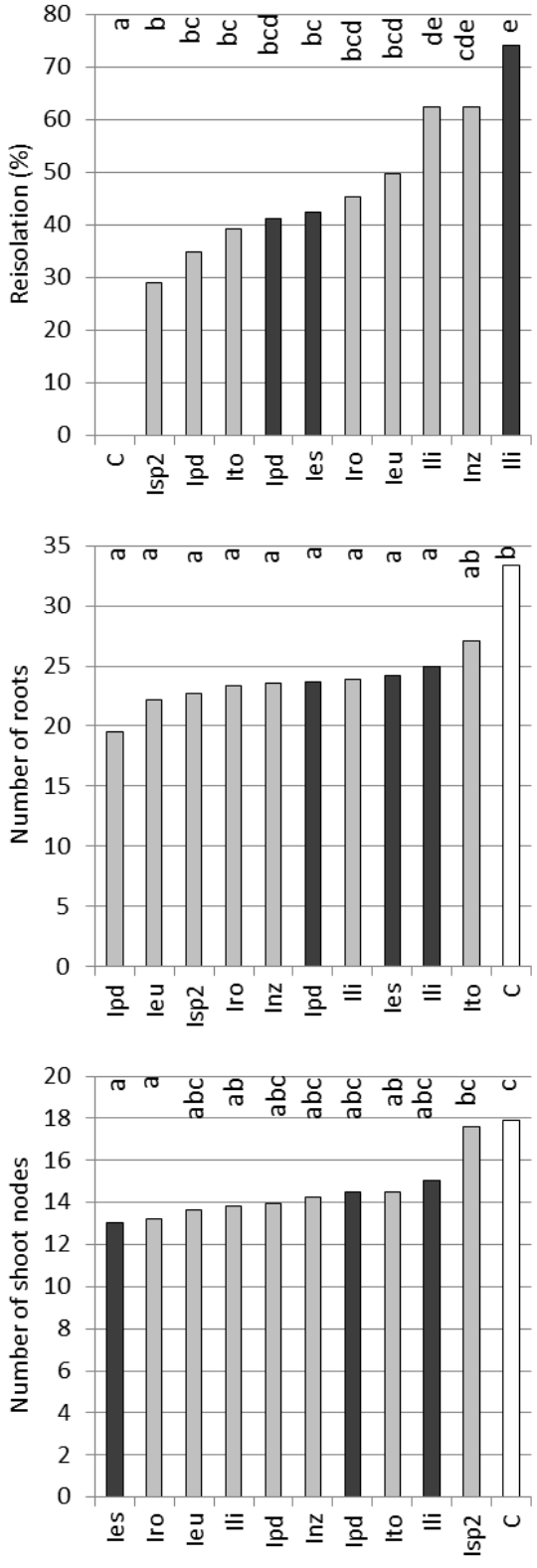


Figure 2. Comparison among *Ilyonectria* spp. isolates from grapevines (black bars) and from other hosts (grey bars) for their effect on grapevine rootstock 1103P in: frequency of reisolation; number of roots; root dry weight; number of shoot nodes; shoot length. Bars affected by the same letter do not differ statistically according to Tukey's test (P=0.05). C, Control (white bars); Ies, *I. estremocensis* (five isolates from grapevines); Ieu, *I. europaea* (one isolate from another host); Ili, *I. liriodendri* (three isolates from grapevines and two from other hosts); Inz, *I. novozelandica* (one isolate from another host); Ipd, *I. pseudodestructans* (two isolates from grapevine); Iro, *I. robusta* (three isolates from other hosts); Isp1, *Ilyonectria* sp1 (one isolate from olive); Ipd, *I. pseudodestructans* (two isolates from grapevines and two from other hosts); Isp2, *llyonectria* sp2 (one isolate from another host); Ito, *I. torresensis* (four isolates from other hosts).

pared to the control plants (Figure 2). Considering the species for which grapevine isolates were analysed, *I. estremocensis* was slightly more virulent than *I. liriodendri* and *I. pseudodestructans*, particularly in parameters concerning the aerial plant part, although the frequency of reisolation was significantly lower than that of *I. liriodendri*. Furthermore, results confirmed most non-grapevine isolates to be as virulent as grapevine isolates.

Discussion

Black foot disease symptoms recorded at the end of the experiments were associated with a reduction in plant growth and vigour, less shoot internodes and roots, and shorter and thinner shoots. These are illustrated by a reduction in the number of roots (up to 47%), shoot nodes (up to 49%), shoot length (up to 65%), length of the longest root (up to 66%), shoot dry weight (up to 80%), and root dry weight (up to 88%).

Frequency of reisolation was the least informative character, only separating the control plants and the isolates OL2 (*llyonectria* sp1) from *Olea europaea* and Cy230 (*I. novozelandica*) from *Festuca duriuscula*, from the remaining isolates. Traits related to the roots were slightly more informative than those related to the aerial plant parts, thus corroborating results from Alaniz *et al*. (2010).

In general, grapevine isolates from the species *I. lusitanica*, *I. estremocensis*, *I. europaea* and *"C." pauciseptatum* were the most virulent, while those from species such as *I. novozelandica*, *I. pseudodestructans*, *I. vitis* and *llyonectria* sp2 were the least virulent, with intermediate results for *I. robusta*, *I. liriodendri*, *I. macrodidyma*, *I. torresensis* and *I. alcacerensis*. For some species however, differences were recorded between characters related to the roots and to the aerial plant parts. Symptoms related to inoculations by *I. lusitanica*, *I. estremocensis* and *"C." pauciseptatum* isolates were equally prominent based on root and aerial part parameters. In contrast, symptoms caused by *I. europaea*, *I. novozelandica* and *I. robusta* isolates were more prominent on roots than on aerial parts, while symptoms of *I. torresensis* and *I. macrodidyma* were more noticeable on aerial plant parts. However, the effect of these pathogens in the aerial parts should be interpreted while taking into consideration that only ungrafted rootstocks were studied here. Experiments using grafted plants would be necessary to reach conclusions on the effect of these pathogens on the aerial parts of grapevine plants. In spite of this, the results obtained here reveal that different *Ilyonectria* species and "*C.*" *pauciseptatum* induce diverse levels of severity on the aerial plant parts. This observation may be relevant in infected fields of rootstock mother-plants, because, most likely, the canes will be shorter, thinner and of poorer quality, thus compromising the later success of cuttings made from such vines.

A comparison among all isolates revealed isolates Cy243 (*I. estremocensis*), Cy197 (*I. lusitanica*), Cy23 (*I. robusta*), Cy238 ("*C.*" *pauciseptatum*) and Cy128 (*I. macrodidyma*), all from grapevines, to be the most virulent, while the least virulent were isolates OL-CM3 ("*C.*" *pauciseptatum*) from *Olea europaea*, Cy200 (*llyonectria sp2*) from grapevine, CBS 129081 (*I. pseudodestructans*) from grapevine, CBS 112593 (*I. novozelandica*) and Cy164 (*I. liriodendri*) from *Malus domestica*. Virulence to the roots varied among isolates, which in turn exhibit different effects on the aerial parts. Isolates Cy23 (*I. robusta*), Cy128 (*I. macrodidyma*), Cy152 (*I. estremocensis*), Cy196 ("*C.*" *pauciseptatum*), CBS 110.81 (*I. liriodendri*, from *Liriodendron tulipifera*) or CBS 117824 (*I. pseudodestructans*, from *Quercus* sp.) showed high virulence in roots, but limited effects on the aerial parts. On the contrary, isolates CBS 129086 (*I. torresensis*), Cy250 (*I. macrodidyma*), CBS 537.92 (*I. europaea*, from *Aesculus hippocastanum*), CBS 159.34, and particularly isolate OL2 (*llyonectria* sp1, from *Olea europaea*) had low reisolation frequency and caused little effect on roots, but a very prominent effect on the above ground parts of inoculated plants. When isolate OL2 was inoculated on olive plants, it was found to be highly virulent (Cabral *et al*., unpubl. data), inducing not only aerial symptoms but also root and crown necroses. This indicates that *llyonectria* sp1 may be more host-specialized than the other species studied here, suggesting that although there are taxa with wide host ranges, host specialisation also occurs in some species of *llyonectria*. However, the unexpected pattern of symptoms produced by OL2 or other isolates, suggests that further work is required to fully elucidate the grapevine-*Ilyonectria* pathosystem. To date little information exists on the mechanisms of host infection and root colonization, as well as the concomitant mechanisms of host-defense response. In apple trees for example, it was hypothesized that the most virulent "Cylindrocarpon" isolates do not proliferate extensively within the host

tissue, but rather cause damage to the host by the secretion of cell wall degrading enzymes or toxins (Tewoldemedhin *et al*., 2011).

For each fungal species, comparisons between grapevine and non-grapevine isolates could not suggest specific trends, with the notable exception of isolates from *Olea europaea* (and to some extent from *Festuca duriuscula*), which were always less virulent than grapevine isolates from the same species ("*C.*" *pauciseptatum*, *I. macrodidyma* and *I. torresensis*). However, frequency of reisolation did not differ significantly to that of other isolates, suggesting that these isolates are fully capable of infecting and colonizing the inoculated plants. The capacity of isolates from hosts such as *Actinidia chinensis*, *Fragaria* x *ananassa*, *Malus domestica* and *Quercus* spp. to be as virulent as the grapevine isolates, including isolates from some of the most virulent species, such as *I. europaea*, raises the importance of the cross-infection potential of isolates from other hosts to grapevine, particularly for plants that are likely to precede grapevine in cultivation, either in a vineyard or nursery. In fact, a recent study addressing apple replant disease (Tewoldemedhin *et al*., 2011) revealed the involvement of species also pathogenic to grapevine in the present study, such as "*C.*" *pauciseptatum*, *I. macrodidyma* and *I. liriodendri*, supporting their polyphagous nature.

Furthermore, many isolates of the *I. macrodidyma* species complex were obtained from roots of several monocotyledons and dicotyledons weed families sampled in Spanish vineyards. When inoculated on grapevines, these isolates were able to induce typical black foot disease symptoms (Agustí-Brisach *et al.*, 2011). In addition to the hosts referred to above, therefore, weeds may represent an important inoculum source of *I. macrodidyma* s. lat. in vineyards.

Besides the importance of cross-infection potential as well as indications of host specificity, the present study also revealed that grapevine isolates from newly described species such as *I. lusitanica*, *I. estremocensis* and *I. europaea* are more virulent to grapevine than the species previously accepted to represent the main causal agents of black foot, such as *I. liriodendri* and *I. macrodidyma*.

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