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Plant health and food safety

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8th Special issue dedicated to grapevine trunk diseases

Guest editor: Josep Armengol

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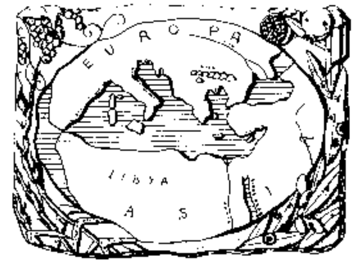

PHYTOPATHOLOGIA MEDITERRANEA

Plant health and food safety

The international journal edited by the Mediterranean Phytopathological Union
founded by A. Ciccarone and G. Goidànich

Phytopathologia Mediterranea is an international journal edited by the Mediterranean Phytopathological Union The journal's mission is the promotion of plant health for Mediterranean crops, climate and regions, safe food production, and the transfer of knowledge on diseases and their sustainable management.
The journal deals with all areas of plant pathology, including epidemiology, disease control, biochemical and physiological aspects, and utilization of molecular technologies. All types of plant pathogens are covered, including fungi, nematodes, protozoa, bacteria, phytoplasmas, viruses, and viroids. Papers on mycotoxins, biological and integrated management of plant diseases, and the use of natural substances in disease and weed control are also strongly encouraged. The journal focuses on pathology of Mediterranean crops grown throughout the world.
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REVIEW

Black-foot disease of grapevine: an update on taxonomy, epidemiology and management strategies

Carlos AGUSTÍ-BRISACH and Josep ARMENGOL

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Summary. Black-foot is one of the most destructive grapevine trunk diseases in nurseries and young vineyards, causing necrotic root lesions, wood necrosis of the rootstock base, and a gradual decline and death of grapevines. Causal agents of the disease are included into the genera *Campylocarpon*, "*Cylindrocarpon*", *Cylindrocladiella* and *Ilyonectria*. Recent taxonomical studies of *Neonectria* and related genera with "*Cylindrocarpon*"-like anamorphs based on morphological and phylogenetic studies, divided *Neonectria* into five genera. Thus, the current taxonomical position and classification of the causal agents of black-foot disease, mainly "*Cylindrocarpon*"/*Ilyonectria*, comprises one of the main topics of this review. The review also provides an update on geographical distribution, epidemiology and management strategies of the disease.

Key words: *Campylocarpon*, "*Cylindrocarpon*", *Cylindrocladiella*, *Ilyonectria*, *Vitis vinifera*.

Introduction

Black-foot disease of grapevines is a serious disease in most wine and grape-producing regions of the world, particularly in nurseries and young vineyards (Halleen *et al.*, 2006a). The causal agents are included into the genera *Campylocarpon*, "*Cylindrocarpon*", *Cylindrocladiella* and *Ilyonectria* (Crous *et al*., 1993; Halleen *et al*. 2004; Halleen *et al*., 2006b; Schroers *et al*., 2008; Chaverri *et al*., 2011; Cabral *et al*., 2012a, c; Lombard *et al*., 2012). This disease was first described in 1961 (Grasso and Magnano Di San Lio, 1975), and over the last decade, its incidence has increased significantly in most grapevine production areas of the world (Halleen *et al*., 2006a; Alaniz *et al*., 2007).

Although these pathogens usually manifest on mature grapevines, they have also been frequently isolated from symptomatic or asymptomatic rootstock mother-plants, rooted rootstock cuttings, bench-graft and young grafted vines in different grapevine production areas around the world, being considered the most common pathogenic fungi associated with young nursery vines (Rumbos and Rumbou, 2001; Halleen *et al*., 2003; Fourie and Halleen, 2004; Oliveira *et al*., 2004; Aroca *et al*., 2006; Dubrovsky and Fabritius, 2007; Halleen *et al*., 2007). Moreover, it is well known that these pathogens are common in the soil causing infection of grafted vines after some months of growth in nursery soils (Halleen *et al*. 2003, 2007; Chaverri *et al*., 2011).

Characteristic symptoms of black-foot disease include a reduction in root biomass and root hairs with sunken and necrotic root lesions (Rego *et al*., 2000; Halleen *et al*., 2006a; Alaniz *et al*., 2007, 2009; Abreo *et al*., 2010). In some cases the rootstock diameter of older vines is thinner below the second tier. To compensate for the loss of functional roots, a second crown of horizontally growing roots is sometimes formed close to the soil surface. Removal of rootstock bark reveals black discoloration and necrosis of wood tissue which develops from the base of the rootstock (Figures 1A, 1B). The pith is also compacted and dis-

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Figure 1. A, black discoloration and necrosis of wood tissue which develops from the base of the rootstock, characteristic of black-foot disease; B, longitudinal section of a rootstock showing dark-brown to black discoloration; C, Un-sprouted grapevine propagation material in a grapevine nursery; D, grapevine plants showing stunded growth, reduced vigour and retarded sprouting in a young plantation.

colored (Scheck *et al*., 1998b; Larignon, 1999; Fourie and Halleen, 2001; Halleen *et al*., 2006a).

External symptoms show reduced vigour with small-sized trunks, shortened internodes, uneven wood maturity, sparse foliage, and small leaves with interveinal chlorosis and necrosis (Figures 1C, 1D). Field symptoms of black-foot disease affected vines are frequently indistinguishable from those of caused by Petri disease (Scheck *et al*., 1998b; Rego *et al*., 2000; Halleen *et al*., 2006a; Alaniz *et al*., 2007, 2009; Abreo *et al*., 2010). When young vines are infected, death occurs quickly, nevertheless as the vine ages, infection results in a more gradual decline and death might only occur after a year (Gubler *et al*., 2004). Disease symptoms on mature vines (5 years and older) are noticed early in the growing season. Affected vines achieve poor new growth, fail to form shoots after winter dormancy, and die by mid-summer. Often shoots also dry and die during the summer. Vines with reduced vegetative growth also die during the subsequent dormant winter period (Halleen *et al*., 2006a).

Causal agents

Taxonomy and distribution

The common name black-foot disease was proposed by Scheck *et al*. (1998b), to designate the disease caused by "*Cylindrocarpon*" *destructans* (Zinnsm.)

Scholten and "*C.*" *obtusisporum* (Cooke & Harkn.) Wollenw., which were the two species traditionally reported as the causal agents of basal rot or root necrosis on grapevines. Nevertheless, this disease was already named as "pied noir" in French language since 1969, because of the presence of black necrosis on the base of diseased rootstocks (Badour, 1969).

The first report of "*C.*" *destructans* on grapevine was made in France in 1961 (Maluta and Larignon, 1991). Since then, it has been isolated from diseased vines in Italy (Grasso, 1984), Portugal (Rego, 1994), California (Scheck *et al*., 1998b), Argentina (Gatica *et al*., 2001), Germany (Fischer and Kassemeyer, 2003), Pennsylvania (Gugino and Travis, 2003), New Zealand and South Africa (Halleen *et al*., 2004), Brazil (Garrido *et al*., 2004) and Canada (Petit *et al*., 2011). "*Cylindrocarpon*" *obtusisporum*, has also been reported to produce black-foot symptoms on grapevine in Sicily (Grasso and Magnano di San Lio, 1975) and California (Scheck *et al*., 1998a).

The generic name "*Cylindrocarpon"* was introduced in 1913 by Wollenweber for anamorphs belonging to *Nectria* section *Willkommiotes* Wollenw. This section included species without chlamydospores. Few years later, in 1917, Wollenweber expanded the concept of "*Cylindrocarpon*" to include species forming mycelial chlamydospores in culture, being "*C.*" *destructans* the most important member of this group (Brayford, 1993). In 1966, Booth split the genus into four groups based on the presence or absence of microconidia and chlamydospores: (i) "*Cylindrocarpon*" *magnusianum* (Sacc.) Wollenw., which was the anamorph of the type species of *Neonectria*, (ii) "*C.*" *cylindroides* Wollenw., which was the type species of the genus "*Cylindrocarpon*", (iii) "*C.*" *destructans*, which was the anamorph of *Neonectria radicicola*, and (iv) members of "*Cylindrocarpon*" species predominantly connected with teleomorphs of the '*Nectria*' *mammoidea* group (Brayford, 1993; Halleen *et al*., 2006a). "*Cylindrocarpon*" *obtusisporum* was originally described from the USA (California) as occurring on *Acacia* sp., where it was observed to form macroconidia and chlamydospores (Booth, 1966). "*Cylindrocarpon*" *obtusisporum* strains identified by Booth (1966) originated from a broad range of host plants in Europe, New Zealand, North America, and, at least partly, formed microconidia.

Traditionally, representatives of all '*Nectria*' groups with "*Cylindrocarpon*" anamorphs were transferred into *Neonectria* (Rossman *et al*., 1999; Mantiri *et al*., 2001; Brayford *et al*., 2004). Mantiri *et al*. (2001) and Brayford *et al*. (2004) analyzed mitochondrial small subunit (SSU) ribosomal DNA (rDNA) sequence data of some of the species and concluded that the *Neonectria*/"*Cylindrocarpon*" species grouped together by this reclassification were monophyletic. However, these authors also found that this overall *Neonectria*/"*Cylindrocarpon*" clade included distinct subclades corresponding to at least three of the four groups delineated by Booth (1966). Significant molecular variation among taxa with "*Cylindrocarpon*"-like anamorphs was found by Seifert *et al*. (2003) in a study on fungi causing root rot of ginseng (*Panax quinquefolius* L.) and other hosts. The dendrograms in this study, based on partial β-tubulin gene (TUB), and nuclear ribosomal internal transcribed spacer (ITS) region sequences, suggested that subclades including (i) *Neon*. *radicicola*, which consisted of numerous phylogenetically distinct units, (ii) *Neon*. *macroconidialis* (Samuels & Brayford) Seifert, and (iii) a subclade comprising two distinct isolates, one from *V. vinifera* in Ontario, Canada and the other from *Picea* sp. in Quebec, Canada, were monophyletic. Other "*Cylindrocarpon*" species appeared to be excluded from this monophyletic group (Halleen *et al*., 2006a).

Significant variation in cultural and morphological characters was observed among "*Cylindrocarpon*" strain isolates from grapevines in nurseries and vineyards of South Africa, New Zealand, Australia and France, which were morphologically and phylogenetically characterized by Halleen *et al*., (2004). Thus, these authors described a novel species, "*C.*" *macrodidymum* Schroers, Halleen & Crous, also associated with black-foot disease of grapevines. Since then, this species has been reported in California (Petit and Gubler, 2005), Portugal (Rego *et al*., 2005), Chile (Auger *et al*., 2007), Spain (Alaniz *et al*., 2007), Uruguay (Abreo *et al*., 2010), northeastern United States and southeastern Canada (Petit *et al*., 2011) and Turkey (Özben *et al*., 2012).

"*Cylindrocarpon*" *obtusisporum* and "*C.*" *macrodidymum* had been considered as two different species associated with black-foot disease of grapevines. Nevertheless, Halleen *et al*. (2004) suggested the possibility that Grasso and Magnano di San Lio (1975) and Scheck *et al*. (1998a) misidentified "*C.*" *obtusisporum* and that it was in fact "*C.*" *macrodidymum.* In this sense, Halleen *et al*. (2004) indicated that macroconidia of "*C.*" *macrodidymum* measure [(26–)34–36–38(–45)×(4–)5.5–6–6.5(–8) μm], whereas those of

the type of "C." *obtusisporum* measure (30–35×4–5 μm) (Cooke, 1884). However, the shape of the macroconidia distinguishes "C." *macrodidymum* from the type of "C." *obtusisporum*, which Cooke (1884) described as having conidia with obtuse ends. Booth (1966) described macroconidia of similar shape in "C." *obtusisporum*. According to Booth, however, 2–3-septate macroconidia of "C." *obtusisporum* measure (34–50×6–7.5 μm). "*Cylindrocarpon*" *obtusisporum* isolates obtained from California formed perithecia when cross-inoculated with "C." *macrodidymum*, giving further evidence to support the misidentification theory. This was also confirmed by sequence comparisons (Halleen *et al*., 2006a). In 2005, Petit and Gubler confirmed the presence of "C." *macrodidymum* in the USA, and concluded that black-foot disease in California is caused by "C." *macrodidymum* and "C." *destructans* (Petit and Gubler, 2005).

Moreover, Halleen *et al*., (2004) established a new genus, *Campylocarpon* Halleen, Schroers & Crous, which is "*Cylindrocarpon*"-like in morphology, associated with black-foot disease of grapevines. Species of this genus and members of the former "*Nectria*" *mammoidea* group, are excluded from *Neonectria*/"*Cylindrocarpon"*, because phylogenetic analyses revealed that these species are phylogenetically not closely related to *Neonectria*/"*Cylindrocarpon"* genera (Halleen *et al*., 2004; Schroers *et al*., 2008). From this genus, two species were included as the causal agents of black-foot disease: *Campylocarpon fasciculare* Schroers, Halleen & Crous, which has been reported in South Africa (Halleen *et al*., 2004), Brazil (Correia *et al*., 2012), and Spain (Alaniz *et al*., 2011b) and *Campyl. pseudofasciculare* Halleen, Schroers & Crous, which has been reported in South Africa (Halleen *et al*., 2004), Uruguay (Abreo *et al*., 2010), Brazil (Correia *et al*., 2012) and Perú (Álvarez *et al*., 2012).

As highlighted before, "C." *destructans* was originally identified as the causal agent of black-foot disease (Maluta and Larignon, 1991), but the status of "C." *destructans* as the causal agent of the disease was since then questioned. In fact, Halleen *et al*. (2006b), compared "C." *destructans* strains isolated from diseased grapevines in France, New Zealand, Portugal and South Africa with "C." *destructans*-like anamorphs obtained from various herbaceous or woody hosts. DNA analyses of their ITS and TUB showed that these isolates were genetically identical with "C." *liriodendri* J.D. MacDon. & E.E. Butler, which was first associated with root rot of tulip poplar (*Liriodendron tulipifera* L.) in California by MacDonald and Butler (1981). Thus, because these species had identical sequences, "C." *destructans* isolates collected from asymptomatic or diseased grapevines affected by black-foot disease were renamed as "C." *liriodendri*, associating "C." *destructans* only with root rot on other herbaceous or woody hosts (Halleen *et al*., 2006b). In addition, in order to clarify the taxonomy of "C." *destructans* causing black-foot in California, Petit and Gubler (2007) also compared "C." *destructans* isolates obtained from grapevines in California with "C." *liriodendri* isolates from South Africa. All of them were identical, and consequently "C." *destructans* isolates were also renamed as "C." *liriodendri*. This species has been later reported as a black-foot pathogen of grapevine in Australia (Whitelaw-Weckert *et al*., 2007), Spain (Alaniz *et al*., 2007), Brazil (Russi *et al*., 2010), Iran (Mohammadi *et al*., 2009), Switzerland (Casieri *et al*., 2009), Uruguay (Abreo *et al*., 2010) and northeastern United States and southeastern Canada (Petit *et al*., 2011). The teleomorphs of "C." *liriodendri* and "C." *macrodidymum* were described as *Neonectria liriodendri* Halleen, Rego & Crous and *N. macrodidyma* Halleen, Schroers & Crous (Halleen *et al*., 2004, 2006b).

In 2008, a new species associated with black-foot disease of grapevines, "C." *pauciseptatum* Schroers & Crous, was described in New Zealand and Slovenia (Schroers *et al*., 2008). To date, this species has been isolated from affected grapevines in Uruguay (Abreo *et al*., 2010), Canada (O'Gorman and Haag, 2011), Spain (Martin *et al*., 2011) and Portugal (Cabral *et al*., 2012a). Phylogenetic studies carried out in New Zealand and Slovenia by Schroers *et al*. (2008), indicated that "C." *pauciseptatum* is the closest phylogenetic sister-taxon of "C." *macrodidymum* and both species are closely related to the "C." *destructans*-complex, which also includes "C." *liriodendri*.

Thus, at this moment, "C." *destructans*, "C." *liriodendri*, "C." *macrodidymum*, "C." *obtusisporum*, "C." *pauciseptatum*, *Campyl. fasciculare* and *Campyl. pseudofasciculare* were considered as the main species associated with young vines showing symptoms of black-foot disease in most of grapevine producing areas worldwide. In addition, other "*Cylindrocarpon*" species have been associated occasionally with black-foot disease of grapevine: *"Cylindrocarpon" didymum* (Harting) Wollenw. in Canada (Petit *et al*., 2011), "C." *olidum* (Wollenw.) Wollenw. in Spain (De Francisco

et al., 2009) and "*C*." *olidum* var. *crassum* Gerlach in Uruguay (Abreo *et al*., 2010).

Chaverri *et al*. (2011) performed a phylogenetic study of *Neonectria*, "*Cylindrocarpon*" and related genera with "*Cylindrocarpon*"-like anamorphs. Morphological and molecular phylogenetic analyses data accumulated over several years have indicated that *Neonectria sensu stricto* and "*Cylindrocarpon*" *sensu stricto* are phylogenetically congeneric, while *Neonectria sensu lato* and "*Cylindrocarpon*" *sensu lato* do not form a monophyletic group, suggesting that *Neonectria*/"*Cylindrocarpon*" represents more than one genus. Thus, based on results of the phylogenetic study, these authors divided *Neonectria* into five genera based on a combination of characters linked to perithecial anatomy and conidial septation: *Neonectria*/"*Cylindrocarpon*" *sensu stricto* (Booth's groups 1 and 4), *Rugonectria*, *Thelonectria* (group 2), *Ilyonectria* (group 3) and anamorph genus *Campylocarpon*. According to this, only *Neonectria* has "*Cylindrocarpon*" anamorphs, while the remaining genera have "*Cylindrocarpon*"-like anamorphs, and since then are referred to as "*Cylindrocarpon*". Consequently, "*C." liriodendri* and "*C*." *macrodidymum* were included into *Ilyonectria* genus, with *I. radicicola* as the type species, and re-identified as *Ilyonectria liriodendri* (Halleen, Rego & Crous) Chaverri & Salgado and *I. macrodidyma* (Halleen, Schroers & Crous) P. Chaverri & Salgado, respectively (Chaverri *et al*., 2011).

Moreover, Cabral *et al*. (2012a) were able to delineate 12 new taxa in the *I. radicicola*-complex, previously known as the "*C*." *destructans*-complex, by using a multi-gene DNA analysis supported by morphological characters. Other *Ilyonectria* species within *I. radicicola*-complex have been also found associated with black-foot disease of grapevine: *Ilyonectria europaea* A. Cabral, Rego & Crous, *I. lusitanica* A. Cabral, Rego & Crous, *I. pseudodestructans* A. Cabral, Rego & Crous and *I. robusta* (A.A. Hildebr.) A. Cabral, Rego & Crous, reported in Portugal (Cabral *et al*., 2012a, 2012c). Another *Ilyonectria* spp., *I. vitis* has also been described in Portugal (Cabral *et al*., 2012a), and isolates belonging to *Neonectria mammoidea* group have also been associated with the disease in Canada (Petit *et al*., 2011). Soon thereafter, following this study, Cabral *et al*. (2012c), demonstrated the existence of polymorphism into *I. macrodidyma*-complex. This hypothesis was in agreement with the results obtained by Alaniz *et al*. (2009), who already detected relevant genetic diversity in "*C*." *macrodidymum* by using inter-simple sequence repeat (ISSR) technique. However, previous phylogenetic analysis showed low variation in the large subunit (LSU) ribosomal DNA (rDNA), TUB and ITS sequences of "*C*." *macrodidymum* isolates obtained from grapevine in different countries (Halleen *et al*, 2004; Petit and Gubler, 2005; Alaniz *et al*., 2007). Thus, in order to clarify this hypothesis, Cabral *et al*. (2012c) performed a phylogenetic study of *I. macrodidyma*-complex by using ITS, TUB, histone H3 gene (HIS) and translation elongation factor 1-α (TEF) sequence analysis. Consequently, six new species of *Ilyonectria* (*I. alcacerensis* A. Cabral, Oliveira & Crous, *I. estremocensis* A. Cabral, Nascimento & Crous, *I. novozelandica* A. Cabral & Crous, *I. torresensis* A. Cabral, Rego & Crous, and *Ilyonectria* sp. 1, *I*. sp. 2,) and *I. macrodidyma*, which are morphologically rather similar, were recognised into the *I. macrodidyma*-complex. All these species have been reported in Portugal, with the exception of *I. novozelandica* which has been reported in South Africa, USA, New Zealand (Cabral *et al*., 2012a, 2012c). Recently, *I. alcacerensis*, *I. macrodidyma*, *I. novozelandica*, and *I. torresensis* have also been found on grapevines in Spain (Agustí-Brisach *et al*., 2013a, 2013b).

Regarding, "*C*." *pauciseptatum*, it is not clear in which genera it has to be included, although it is very similar in morphology to *I. anthuriicola* A. Cabral & Crous (Cabral *et al*., 2012a).

Finally, another genus, *Cylindrocladiella* Boesew., which is also *Cylindrocarpon*-like in morphology, has recently been associated with black-foot disease of grapevines (Van Coller *et al*., 2005; Agustí-Brisach *et al*., 2012; Jones *et al*., 2012). This genus was established by Boesewinkel (1982) to accommodate five *Cylindrocladium*-like species producing small and cylindrical conidia. This decision was based on the fact that species of *Cylindrocladiella* had different conidiophores branching patterns, conidial shapes, dimensions, cultural characteristics and teleomorphs from those of *Cylindrocladium* (Van Coller *et al*., 2005; Lombard *et al*. 2012). Since then, several taxonomic studies of these fungi have relied on morphologically and to lesser extent on DNA sequence comparisons of the ITS and TUB gene regions, recognizing nine species of *Cylindrocladiella* (Crous and Wingfield, 1993; Victor *et al*., 1998; Van Coller *et al*., 2005). Lombard *et al*. (2012), have just described 18 new *Cylindrocladiella* species based on morphological and phylogenetic

studies employing ITS, TUB, HIS and TEF gene regions. Nevertheless, only two species into this genus have been found associated with black-foot disease on grapevines: *Cylindrocladiella parva* (P.J. Anderson) Boesew., which has been reported in South Africa (Van Coller *et al*., 2005), New Zealand (Jones *et al*., 2012) and Spain (Agustí-Brisach *et al*., 2012) and *Cyl. peruviana* (Bat., J.L. Bezerra & M.P. Herrera) Boesew., which has been reported in South Africa (Van Coller *et al*., 2005), Perú (Álvarez *et al*., 2012) and Spain (Agustí-Brisach *et al*., 2012).

A list of all "*Cylindrocarpon*"/*Ilyonectria*, *Campylocarpon* and *Cylindrocladiella* species, which have been reported associated with black-foot disease of grapevine and their geographical distribution, is presented in Table 1.

Morphological and cultural characterization

"*Cylindrocarpon*"/*Ilyonectria*, *Campylocarpon* and *Cylindrocladiella* species have characteristic distinctive morphological and cultural patterns (Figures 2 and 3; Table 2). The anamorphs of "*Cylindrocarpon*"/*Ilyonectria* produce abundant microconidia and chlamydospores. Macro- and microconidia apparently are produced from the same conidiophores which are 40–160 μm long, generally simple, unbranched or sparsely branched, irregularly or verticillately branched, rarely densely branched, and with cylindrical phialides. Macroconidia are straight or curved, hyaline, 1–3-septate, rarely > 3-septate [25–50(–55)×5–7.5 μm], generally with a prominent basal or lateral abscission scar or hilum. Microconidia are ellipsoidal to ovoid, hyaline, 0–1-septate, with a lateral or basal hilum [3–15×2.5–5(–6) μm] (Figures 2A, 2B). Chlamydospores are abundant, generally intercalary, globose, single or in chains, becoming brownish. In addition, colony morphology on PDA is very heterogeneous (Figure 2C). Aerial mycelium is floccose to felted, and the colour varies from white to yellow or light to dark brown. The margin of the colony can be entire, slightly lobulated, or lobulated (Figures 3A–3H) (Booth, 1966; Samuels and Brayford, 1990; Chaveri *et al*., 2011).

Table 1. Fungal species which have been reported associated with black-foot disease of grapevines and their geographical distribution.

Species	Distribution
Campylocarpon fasciculare Schroers, Halleen & Crous	South Africa (Halleen *et al*., 2004), Spain (Alaniz *et al*., 2011b) and Brazil (Correia *et al*., 2012).
Campylocarpon pseudofasciculare Halleen, Schroers & Crous	South Africa (Halleen *et al*., 2004), Uruguay (Abreo *et al*., 2010), Brazil (Correia *et al*., 2012) and Perú (Álvarez *et al*., 2012)
"Cylindrocarpon" destructans (Zinssm.) Scholten	France (Maluta and Larignon, 1991), Italy (Grasso, 1984), Argentina (Gatica *et al*., 2001), Germany (Fischer and Kassemeyer, 2003), Pennsylvania (Gugino and Travis, 2003), Brazil (Garrido *et al*., 2004) and Canada (Petit *et al*., 2011)
"Cylindrocarpon" didymum (Harting) Wollenw.	Canada (Petit *et al*., 2011)
"*Cylindrocarpon*" *obtusisporum* (Cooke & Harkn.) Wollenw.	Sicily (Grasso and Magnano di San Lio, 1975) and California (Scheck *et al*., 1998a)
"*Cylindrocarpon*" *olidum* (Wollenw.) Wollenw.	Spain (De Francisco *et al*., 2009)
"*Cylindrocarpon*" *olidum* var. *crassum* Gerlach	Uruguay (Abreo *et al*., 2010)
"Cylindrocarpon" pauciseptatum Schroers & Crous	New Zealand and Slovenia (Schroers *et al*., 2008), Uruguay (Abreo *et al*., 2010) Canada (O'Gorman and Haag, 2011), Spain (Martin *et al*., 2011) and Portugal (Cabral *et al*., 2012a)
Cylindrocladiella parva (P.J. Anderson) Boesew.	South Africa (Van Coller *et al*., 2005), New Zealand (Jones *et al*., 2012) and Spain (Agustí-Brisach *et al*., 2012)

(*Continued*)

Table 1. *Continues.*

Species	Distribution
Cylindrocladiella peruviana (Bat., J.L. Bezerra & M.P. Herrera) Boesew.	South Africa (Van Coller *et al*., 2005), Spain (Agustí-Brisach *et al*., 2012) and Perú (Álvarez *et al*., 2012)
Ilyonectria alcacerensis A. Cabral, Oliveira & Crous	Portugal (Cabral *et al*., 2012c) and Spain (Agustí-Brisach *et al*., 2013b)
Ilyonectria estremocensis A. Cabral & Crous	Portugal (Cabral *et al*., 2012c)
Ilyonectria europaea A. Cabral, Rego & Crous	Portugal (Cabral *et al*., 2012a)
Ilyonectria liriodendri (Halleen, Rego & Crous) Chaverri & Salgado	France, New Zealand, Portugal and South Africa (Halleen *et al*., 2006b), Australia (Whitelaw-Weckert *et al*., 2007), California (Petit and Gubler, 2007), Spain (Alaniz *et al*., 2007), Iran (Mohammadi *et al*., 2009), Switzerland (Casieri *et al*., 2009), Brazil (Russi *et al*., 2010), Uruguay (Abreo *et al*., 2010), northeastern United States and southeastern Canada (Petit *et al*., 2011),
Ilyonectria lusitanica A. Cabral, Rego & Crous	Portugal (Cabral *et al*., 2012a)
Ilyonectria macrodidyma (Halleen, Schroers & Crous) P. Chaverri & C. Salgado	Australia, France, New Zealand and South Africa (Halleen *et al*., 2004), California (Petit and Gubler, 2005), Chile (Auger *et al*., 2007), Uruguay (Abreo *et al*., 2010), northeastern United States and southeastern Canada (Petit *et al*., 2011), Portugal (Cabral *et al*., 2012c), Turkey (Özben *et al*., 2012) and Spain (Agustí-Brisach *et al*., 2013b).
Ilyonectria novozelandica A. Cabral, Nascimento & Crous	South Africa, USA and New Zealand (Cabral *et al*., 2012c) and Spain (Agustí-Brisach *et al*., 2013a, b)
Ilyonectria pseudodestructans A. Cabral, Rego & Crous	Portugal (Cabral *et al*., 2012a)
Ilyonectria robusta (A.A. Hildebr.) A. Cabral, Rego & Crous	Portugal (Cabral *et al*., 2012a)
Ilyonectria torresensis A. Cabral, Rego & Crous	Australia, Canada, New Zealand, Portugal, South Africa, Spain and USA (Cabral *et al*., 2012c; Agustí-Brisach *et al*., 2013a, b)
Ilyonectria vitis A. Cabral, Rego & Crous	Portugal (Cabral *et al*., 2012a)
Ilyonectria sp. 2 (Cabral *et al*., 2012c)	Portugal (Cabral *et al*., 2012c)
Isolates belonging to *Neonectria mammoidea* group	Canada (Petit *et al*., 2011)

Campylocarpon is similar to "*Cylindrocarpon*"/*Ilyonectria*, although *Campylocarpon* spp. produce macroconidia mostly curved, while microconidia are absent and chlamydospores are rare or also absent. Conidiophores appear arising laterally from single or fasciculate aerial hyphae or from creeping substrate hyphae, singly or in loose or dense aggregates (Figure 2D). Conidial heads form pionnotes-like aggregates. Conidiophore show a stipe base to 16 µm wide, which bear several phialides or a penicillus of irregular branches with terminal branches bearing 1 or several phialides. Macroconidia are as in *Ilyonectria*, but typically curved, and with up to 6 septa, [(24–)35–60(–62)×6.5–9 µm], apical cell obtuse, basal cell obtuse or with inconspicuous hilum (Figure 2E). Regarding colony morphology on PDA, aerial mycelium is abundant, covering the whole or sectors of the colony, white to off-white or slightly brownish, thickly cottony to felty, intermingled with or giving rise to erect white or brown hyphal strands. This strands sometimes are partly covered by off-white slime (Figures 3I, 3J) (Halleen *et al*., 2004; Chaverri *et al*., 2011).

Cylindrocladiella species produce hyaline, single, subverticillate, as well as penicilliate conidiophores,

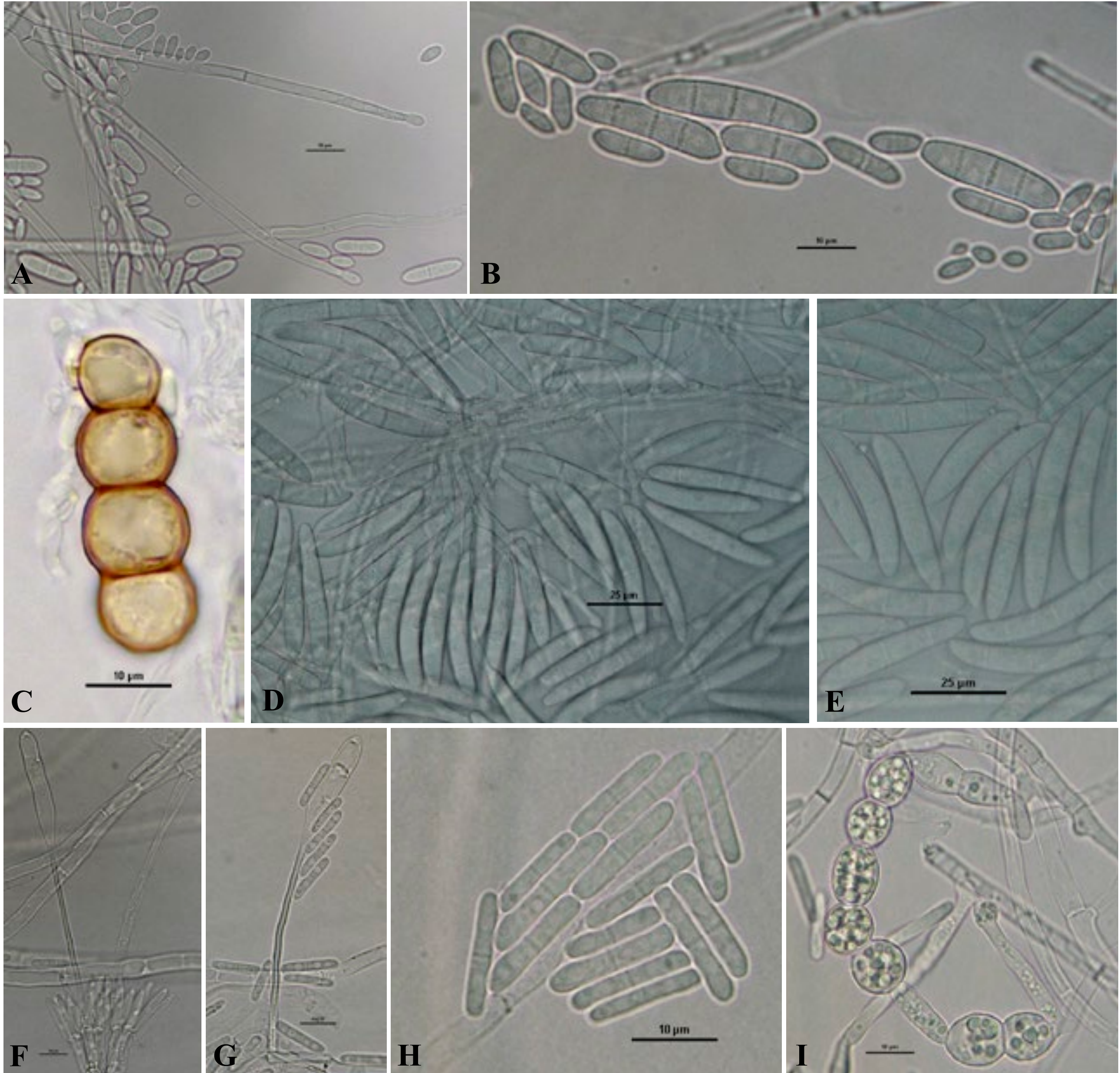

Figure 2. A, Conidiophores of *I. liriodendri*; B, Macro- and microconidia of *Ilyonectria liriodendri*; C, Chlamydospores in chains of "*Cylindrocarpon*" *pauciseptatum*; D, Conidiophores of *Campyl. fasciculare*; E, Macroconidia of *Campylocarpon fasciculare*; F, Penicillate conidiophores of *Cylindrocladiella parva*; G, Terminal vesicles of *Cyl. parva*; H, Conidia of *Cyl. parva*; H, Chlamydospores in chains of *Cyl. parva*. Scale bars: a–c, f–i = 10 µm; d–e = 25 µm.

with primary and secondary branches. The phialides are terminal, hyaline, in whorls of 2–4, with or without obvious collarets. In general, stipe is centrally arranged on conidiophores, with a single basal septum, terminating in a thin-walled, hyaline vesicle of characteristic shape (Figures 2F, 2G). Conidia are cylindrical, rounded at both ends, straight, hyaline, (0)–1-septate, [(9–)11–13(–15)×2–4 µm], sometimes

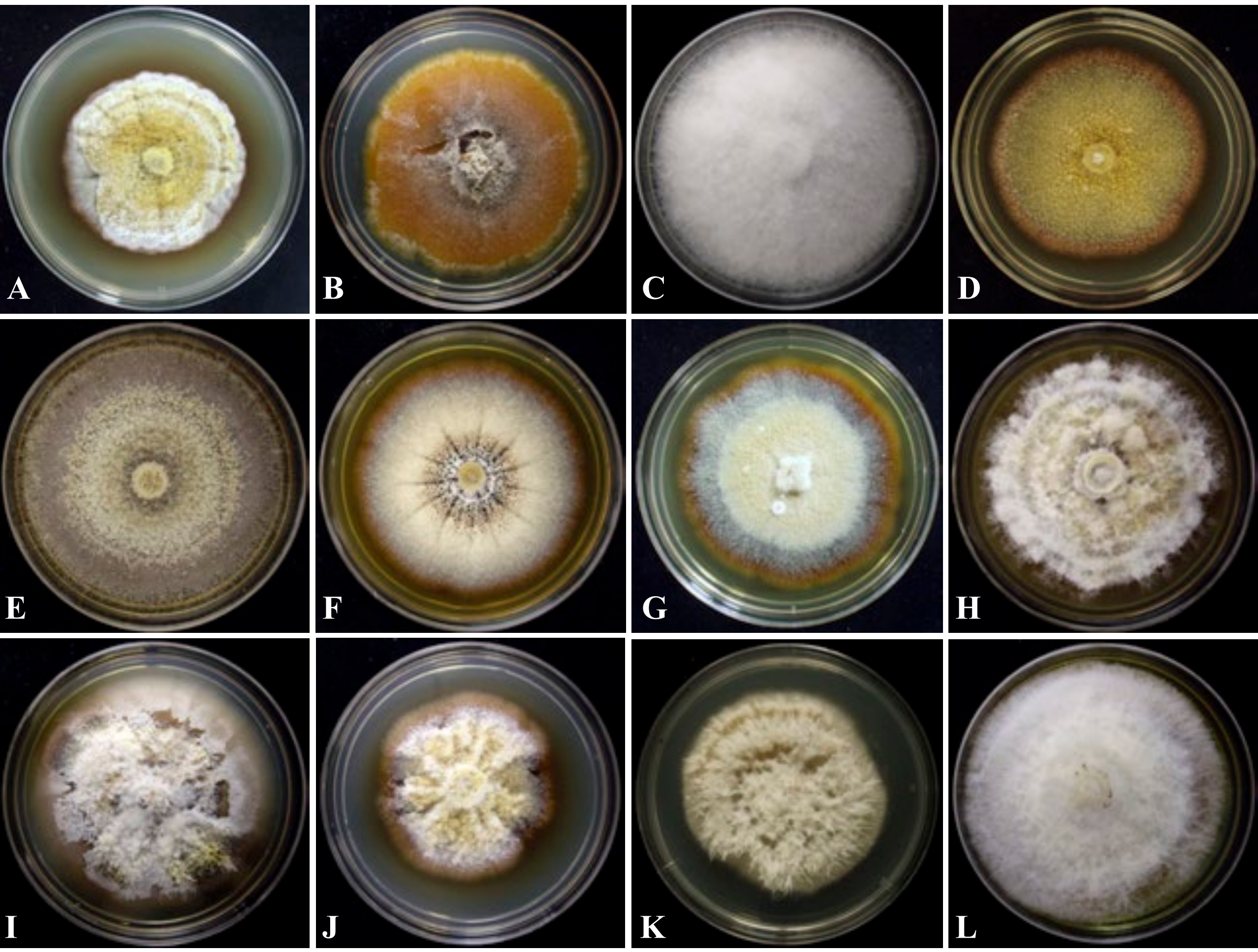

Figure 3. Colonies of black-foot pathogens grown on PDA. A, "*Cylindrocarpon*" *destructans* (CBS 301.93); B, "*C.*" *pauciseptatum*; C, "*C.*" *obtusisporum*; D, *Ilyonectria alcacerensis*; E, *I. liriodendri*; F, *I. novozelandica*; G, *I. macrodidyma*; H, *I. torresensis*; I, *Campylocarpon fasciculare*; J, *Campyl. pseudofasciculare*; K, *Cylindrocladiella parva*; L, *Cyl. peruviana*.

becoming swollen at one end with age (Figure 2H). Chlamydospores are abundant or moderate, more frequently arranged in chains than clusters (Figure 2I). Aerial mycelium ranges from dark to light brown (Figures 3K, 3L) (Crous and Wingfield, 1993; Lombard *et al*., 2012).

Epidemiology

Campylocarpon, "*Cylindrocarpon*", *Cylindrocladiella* and *Ilyonectria* species are generally regarded as pathogens and / or saprobes of a wide range of angiosperm and gymnosperm hosts and substrates in temperate, sub-tropical and tropical regions worldwide (Victor *et al*., 1998; Chaverri et *al*., 2011; Lombard *et al*., 2012). In addition to grapevine, they have also been associated with root rot diseases of other economically important hosts (Chaverri *et al*., 2011; Lombard *et al*., 2012), such as: *Actinidia chinensis* Planch. (Erper *et al*., 2011), *Liriodendron tulipifera* L. (MacDonald and Butler, 1981), *Olea europaea* L. (Úrbez-Torres *et al*., 2012), *Panax quinquefolius* L. (Rahman and Punja, 2005), *Persea americana* Mill. (Vitale *et al*., 2012), *Pinus radiata* D. Don (Agustí-Brisach *et al*., 2011b) or *Pinus sylvestris* L. (Menkis and Burokiene, 2012). Lombard *et al*. (2013) have just reported black foot rot disease associated with the cultivation of *Proteaceae* cut flowers in South Africa, and described four new *Ilyonec-*

Table 2. Summary of distinctive morphological and cultural features of "*Cylindrocarpon*"/*Ilyonectria*, *Campylocarpon* and *Cylindrocladiella* genera associated with black-foot disease of grapevines.

Characteristics	"*Cylindrocarpon*"/*Ilyonectria*	*Campylocarpon*	*Cylindrocladiella*
Conidiophores			
	40–160 μm long, generally simple, unbranched or sparsely branched, irregularly or verticillately branched, rarely densely branched, and with cylindrical phialides	Appear arising laterally from single or fasciculate aerial hyphae or from creeping substrate hyphae, singly or in loose or dense aggregates	Hyaline, single, subverticillate, as well as penicillate, with primary and secondary branches
Conidia			
Microconidia	Abundant, ellipsoidal to ovoid, hyaline, 0–1-septate, with a lateral or basal hilum	Absent	Cylindrical, rounded at both ends, straight, hyaline, (0)–1-septate, sometimes becoming swollen at one end with age
Macroconidia	Straight or curved, hyaline, 1–3-septate, rarely > 3-septate, generally with a prominent basal or lateral abscission scar or hilum	Mostly curved, hyaline, with up to 6- septate, apical cell obtuse, basal cell obtuse or with inconspicuous hilum	Absent
Chlamydospores	Abundant, generally intercalary, globose, single or in chains, becoming brownish	Rare or also absent	Abundant or moderate, more frequently arranged in chains than clusters
Colony color	White to yellow or light to dark brown	White to off-white or slightly brownish	Dark to light brown

tria spp. included into the *I. radicicola*-complex: *I. capensis* L. Lombard & Crous, *I. leucospermi* L. Lombard & Crous, *I. protearum* L. Lombard & Crous and *I. vredehoekensis* L. Lombard & Crous.

In grapevine, black-foot pathogens are frequently isolated from rootstock mother-plants, rooted rootstock cuttings, bench-grafts and young grafted vines (Rego *et al*., 2001a; Fourie and Halleen, 2002, 2004; Halleen *et al*., 2004; Oliveira *et al*., 2004; Petit and Gubler, 2005). During the last decade, several surveys of young vineyards have been carried out in different grapevine growing areas worldwide in which black-foot pathogens were isolated from plants used in new plantings (Armengol *et al*., 2001; Fourie and Halleen, 2001; Rego *et al*., 2001b; Rumbos and Rumbou, 2001; Petit and Gubler, 2005; Aroca *et al*., 2006; Giménez-Jaime *et al*. 2006; Alaniz *et al*., 2007; Mohammadi *et al*., 2009; Abreo *et al*., 2010). Recently, studies carried out in commercial grapevine nurseries in Spain by Agustí-Brisach *et al*. (2013a), have demonstrated that inoculum of *Ilyonectria* spp. is also present at the different stages of the grapevine nursery propagation process and suggest that infections caused by these pathogens can also occur during this process. Moreover, these authors confirmed that during the rooting phase in nursery fields the number of plants infected with black-foot pathogens increases markedly (Agustí-Brisach *et al*., 2013a). These results are in agreement with those obtained by Cardoso *et al*. (2012), who detected inoculum sources of black-foot pathogens in a commercial grapevine nursery in Portugal. All together these results suggest that new plants are infected during the propagation process in nurseries and that even the planting material used in the propagation process might be infected with these pathogens (Rego *et al*., 2000; Halleen *et al*., 2003; Aroca *et al*., 2010; Cardoso *et al*., 2012; Agustí-Brisach *et al*., 2013a).

Regarding the role of soil from nursery fields and vineyards as another important source of inoculum, Halleen *et al*. (2003), concluded that black-foot pathogens from soils infected grafted grapevines

once planted in open-rooted nurseries, whereas these pathogens rarely occurred in rootstock propagation material prior to planting. During the grapevine propagation process, at the time of planting, the susceptible basal ends (especially the pith area) of most of the nursery cuttings are partly or even fully exposed, and the young callus roots also break during the planting process, resulting in small wounds susceptible to infection by soilborne pathogens. The occurrence of black-foot pathogens in the graft union might be explained by the nursery practice of covering graft unions with soil for a period of approximately 5 weeks to prevent drying of the callus tissue. Recently, Agustí-Brisach *et al*. (2011a, 2013b, 2013c) have confirmed the presence of black-foot pathogens in soils from rootstock mother fields, nursery fields and vineyards by fungal isolation from roots of grapevine seedlings used as bait plants, multiplex-nested PCR and qPCR, and fungal isolation from roots of weeds collected from the three types of soil.

Campylocarpon, "*Cylindrocarpon*", *Cylindrocladiella* and *Ilyonectria* species are known to be saprobes in soil, which can occur on dead plant substrata, or act as weak pathogens of plants infecting wounds of roots and stems of various hosts through wounds and/or natural openings (Fourie and Halleen, 2006; Halleen *et al*., 2006a, 2007; Schroers *et al*., 2008; Probst *et al*., 2012). Furthermore, the production of chlamydospores in most species of these genera may allow them to survive for extended periods in soil (Halleen *et al*., 2004). However, very little information is currently available regarding the survival of these pathogens, and the role of chlamydospores during subsequent infections (Halleen *et al.,* 2006a). The effect of temperature, pH and water potential (Ψ_s) on mycelial growth, sporulation and chlamydospore production of *I. liriodendri*, *I. macrodidyma*-complex and "*C*". *pauciseptatum* isolated from grapevines was studied by Agustí-Brisach and Armengol (2012). Three isolates per species were incubated on potato dextrose agar (PDA) under different temperature, pH, and Ψ_s conditions. All isolates were able to grow over a range of temperatures from 5 to 30ºC, with an optimum temperature between 20 to 25ºC, but they did not grow at 35ºC. Active mycelial growth was observed in a broad range of pHs, from 4 to 8. Regarding the effect of Ψ_s, in general, mycelial growth was higher on amended media at -0.5, -1.0 or/and -2.0 MPa compared with that obtained on nonamended PDA (-0.3 MPa), and it was reduced at Ψ_s values lower than -2.0 MPa. Regarding the sporulation, most of the isolates were able to sporulate at all temperatures, pH, and Ψ_s, showing a broad range of variation. In all studied conditions, *I. liriodendri* was found as the species with the highest capacity of sporulation. In general, chlamydospore production was not much affected by temperature, pH and Ψ_s values tested. Chlamydospores were observed in PDA cultures of all isolates at all pH values studied, while just some isolates did not produced them at 5 and 10ºC or -4.0 and/or -5.0 MPa.

Disease risk may be increased by the stresses imposed on young grapevines in nurseries and vineyards. Environmental factors and vineyard management practices, including poor drainage, soil compaction and inadequate planting holes, which cause poor root development, as well as poor nutrition, heavy cropping of young vines and effects of pests and pathogens could be considered as stress factors (Probst *et al*., 2012). High temperatures during summer also play an important role in symptom expression, since the compromised root and vascular system of diseased plants would not be able to supply enough water to compensate for the high transpiration rate during periods of high temperatures (Larignon, 1999). The processes of nursery propagation and vineyard establishment include many practices that cause stress on young vines. During the grapevine propagation process, wounds produced during cutting and bench-grafting, the early development of roots and shoots in the nursery field, uprooting and trimming, extended cold storage and excessive time in containers prior to establishment in the vineyard are all traumatic to the young plants. In addition, after planting out in the field, these vines are again stressed by the need to develop roots and shoots in an environment that is often selected to limit shoot growth (Probst *et al*., 2012). Research studies carried out in New Zealand by Brown *et al*. (2012), confirmed that stress factors such as defoliation can contribute to black-foot disease severity in young vines.

There are few reports about virulence diversity of "*Cylindrocarpon*"/*Ilyonectria* spp. to grapevine. Alaniz *et al*. (2009) detected virulence diversity in *I. macrodidyma*-complex showing that the isolates belonging to ISSR groups G6 and G7 were significantly more virulent than other isolates of *I. macrodidyma*-complex (ISSR groups G3, G4 and G5) and *I. liriodendri* (ISSR groups G1 and G2). Recently, research stud-

ies carried out by Cabral *et al*. (2012b), in which they compared the virulence of *Ilyonectria* spp. isolates, revealed that described species such as *I. lusitanica*, *I. estremocensis* and *I. europaea* are more virulent to grapevine than the species previously accepted as the main causal agents of black-foot, such as *I. liriodendri* and *I. macrodidyma*.

Black-foot pathogens are often part of disease complexes with other fungi or nematodes (Brayford, 1993). In the case of declining grapevines, they are often isolated in association with other fungi such as Petri disease pathogens, Botryosphaeriaceae, *Phomopsis* spp., *Pythium* spp. or *Phytophthora* spp. (Halleen *et al*., 2007).

Management strategies

Presently, no curative control measures are available to eradicate black-foot pathogens in nurseries as well as in vineyards (Oliveira *et al*., 2004; Halleen *et al*., 2007). During the last years, research has been specially focused in the development of procedures and chemical products to prevent or reduce black-foot disease infection of grapevine woody tissues during the propagation process with promising results including, the use of hot-water treatments, biological control, applications of chitosan, use of arbuscular mycorrhizal (AM) fungi or fungicides (Alaniz *et al*., 2011a).

In vineyards, management strategies recommended for prevention and disease management mainly involve the prevention and/or correction of predisposing stress situations (Halleen *et al*., 2007). In nurseries, where there are many opportunities for infection by black-foot pathogens during the propagation process, there have recently been advances in the development of procedures and products to prevent or reduce the infection of woody tissue by these pathogens. Thus, good hygiene and wound protection are of the utmost importance in order to obtain a healthy vine, which is fundamental to the successful beginning and sustainability of all grape vineyards (Gramaje and Armengol, 2011).

In this context, a sanitation program is required to improve the quality of grapevine planting material. Chemical, physical, and biological control, and other management strategies have to be used to decrease the incidence and severity of black-foot pathogens during the nursery propagation process as well as during the growing season in vineyards.

Chemical control

Studies carried out in Portugal by Rego *et al*. (2006) indicated that the fungicides benomyl, prochloraz and the mixtures of carbendazim with flusilazole and cyprodinil with fludioxonil inhibited mycelial growth of "*C.*" *destructans in vitro*, whereas tebuconazole and difenoconazole were less effective. *In vivo* studies on potted grapevines proved that benomyl, tebuconazole, and the mixtures of carbendazim with flusilazole and cyprodinil with fludioxonil significantly improved plant growth and decreased disease incidence compared with non-treated vines. In a later study carried in a commercial nursery, these authors found that fludioxonil and the mixtures of cyprodinil with fludioxonil and pyraclostrobin with metiram reduced the incidence and severity of black-foot pathogens on grapevine plants grown in a commercial field with grapevine cultivation history (Rego *et al*., 2009)

In studies performed in semi-commercial nursery trials in South Africa, grapevine rootstock and scion cuttings were soaked in some chemical products prior to cold storage, prior to grafting and prior to planting in field nurseries. Natural infection levels in basal stem and graft unions of uprooted nursery grapevines were evaluated eight months after planting. Among the different products tested, benomyl didecyldimethylammonium chloride and captan were consistently the best treatments as growth parameters were not negatively influenced and pathogen incidences in basal ends and graft unions of uprooted plants were reduced (Fourie and Halleen, 2006).

In a later study carried out also in South Africa, Halleen *et al*. (2007) evaluated various chemical preplanting treatments for prevention of infection by black-foot and Petri disease pathogens. A total of 13 fungicides were evaluated *in vitro* against "*C.*" *liriodendri*, "*C.*" *macrodidymum*, *Campyl*. *fasciculare* and *Campyl*. *pseudofasciculare*. Results indicated that benomyl, flusilazole, imazalil and prochloraz were effective in reducing mycelial growth of black-foot pathogens. Nevertheless, only benomyl and imazalil showed some effect to control these pathogens in semi-commercial field trials. However, the results were inconsistent, perhaps because of generally low and varying infection levels in the roots and rootstocks, respectively

Recently, Alaniz *et al*. (2011a) conducted a pot assay with several fungicides in order to determine their potential to prevent infections caused by "*C.*" *liriodendri* and "*C.*" *macrodidymum* during the rooting

phase in the grapevine propagation process. Results showed that captan, carbendazim, copper oxychloride, didecyldimethylammonium chloride, hydroxyquinoline sulfate, and prochloraz decreased the root disease severity values in both species compared with the control treatment; but only captan, carbendazim, and didecyldimethylammonium chloride presented a percentage of reisolation values significantly different from the control treatment in the case of the cuttings inoculated with "*C.*" *liriodendri*, and prochloraz in the case of those inoculated with "*C.*" *macrodidymum.*

Hot-water treatment

The use of hot water treatment (HWT) has been reported as a promising method for the control of black-foot disease pathogens in grapevine propagation material. HWT of rootstock cuttings prior to grafting or HWT of dormant nursery plants after uprooting has been strongly recommended for their effectiveness in reducing infection levels in nursery plants (Gramaje and Armengol, 2011).

Halleen *et al*. (2007), evaluated the effect of HWT at 50ºC for 30 min on dormant nursery grapevines after uprooting. In this study, no black-foot pathogens were isolated from rootstock and roots of plants which were subjected to HWT, whereas these pathogens were isolated from 16.8% of rootstocks and from 4.1% of roots from control plants. Gramaje *et al*. (2010) evaluated the effect of HWT *in vitro* on conidial and mycelial growth of "*C.*" *liriodendri* and "*C.*" *macrodidymum* at a range of temperature from 41 to 49ºC for 30, 45 or 60 min. Conidial germination was inhibited by treatments above 45ºC for 45 min, while treatments above 48ºC for 45 min were necessary to inhibit the mycelial growth. These results suggest that standard HWT protocols at 50ºC for 30 min may be sufficient to control black-foot pathogens in grapevine propagation material.

Biological control

The potential use of biocontrol agents as wound protectants and growth stimulants in grapevine nurseries has also been reported. Research studies conducted in a semi-commercial nursery trial in South Africa, showed the growth stimulating attributes of commercial products of *Trichoderma*, as well as the positive effect on natural infection by grapevine trunk pathogens. Although *Trichoderma* treatments notably reduced the incidence of these pathogens in roots of nursery grapevines, low levels of them were recorded (Fourie *et al*., 2001). Fourie and Halleen (2006), performed soak-treatments of propagation material by using products containing *T. harzianum* Rifai obtaining inconsistent results. Then, Halleen *et al*. (2007) evaluated the effect of products containing *T. harzianum* in soil as a potential biological control agent of grapevine trunk diseases, showing that the incidence of black-foot pathogens in nursery grapevines was not reduced by the effect of *T. harzianum*. These authors pointed out that, in general, the growth stimulating effect due to *Trichoderma*, which significantly improved root development, would possibly make plants more tolerant to black-foot disease when subjected to stress. However, the potential use of *Trichoderma* as biocontrol agent should be studied further to develop application methods that may ensure a more consistent efficacy (Fourie and Halleen, 2006; Halleen *et al*., 2007).

Other management strategies

Given the difficulty of controlling grapevine trunk pathogens using the measures previously described, other management strategies such as host resistance, biofumigation or mycorrhizal colonization have been studied as alternatives to control black-foot disease on grapevines

In a research study carried out in California, it was noted that the rootstocks *Vitis riparia* 039-16 and Freedom had a good degree of resistance to this disease (Gubler *et al*., 2004). However, in a later study, Jaspers *et al*. (2007) evaluated the susceptibility of the more commonly planted grapevine rootstocks in New Zealand such as Riparia Glorie, Schwarzman, K5BB, 140-R, 3309C and 420A, under greenhouse conditions showing that all rootstock varieties included in the study were susceptible to black-foot pathogens to some degree. These findings were in agreement with those obtained by Alaniz *et al*. (2010), who evaluated the susceptibility of the grapevine rootstocks most commonly used in Spain (110-R, 1103-P, 140-R, 161-49C, 196-17C, Fercal and SO4) to "*C.*" *liriodendri* and "*C.*" *macrodidymum* and found that all rootstocks inoculated were affected by the disease, being the rootstock 110-R the most susceptible to both pathogens.

Green crops of *Brassica* species such as mustard (*B. juncea* [L.]) Coss.) and rape (*B. napus* L.) incorpo-

rated into the soil release volatile isothiocyanates, which are known to suppress pathogenic fungal species. Thus, the potential of the biofumigation using these crops have been evaluated in nursery fields and vineyards as a possible alternative for methyl bromide and metham sodium for the control of black-foot pathogens (Stephens *et al*., 1999; Bleach *et al*., 2010). Studies conducted by Stephens *et al*. (1999) showed that this biofumigant did not reduce the percentage of root or stem tissue containing this pathogen at harvest. However, in a New Zealand experiment which crops of mustard, rape and oats (*Avena sativa* L.) were grown in a vineyard previously infested by black-foot pathogens, showed that biofumigation using mustard was the most effective, reducing disease incidence in rootstocks (Bleach *et al*., 2010). It appeared that mustard meal incorporated into infested soil was as good as growing the plants and incorporating the plant into the soil. These findings indicated that biofumigation using mustard may be highly effective for reducing soilborne black-foot pathogens inoculum and the incidence of the disease (Bleach *et al*., 2010). Consequently, this may give a valuable control tool for growers who replant into a pathogen-contaminated site after the removal of infected plants in an established vineyard.

Compost also is known to suppress pathogenic fungal species. In fact, Gugino and Travis (2003) evaluated the efficacy of several types of compost on the suppression of "*C.*" *destructans*. In this study, the population of "*C.*" *destructans* was monitored over time in soilless mixes amended with 0, 10, 25 and 50% compost using serial soil dilution plating. The preliminary results indicated an increasing reduction in the "*C.*" *destructans* population as the amount of compost increased from 0 to 50%. Moreover, several microorganisms were isolated from these composts also demonstrating antagonism toward "*C.*" *destructans in vitro*.

Regarding the use of the endomycorrhizal symbiosis as alternative control measure, Petit and Gubler (2006) indicated that grapevines inoculated with an arbuscular-mycorrhizal (AM) fungus, *Glomus intraradices* N.C. Schenck & G.S. Sm., were less susceptible to black-foot disease than nonmycorrhizal plants. Even though "*C.*" *macrodidymum* was consistently recovered from both mycorrhizal and nonmycorrhizal plants, disease severity was significantly lower when vines were preinoculated with *G. intraradices*. These findings were in agreement with those obtained by Bleach *et al*. (2008), who evaluated the impact of *G. mosseae* (T.H. Nicolson & Gerd.) Gerd. & Trappe and *Acaulospora laevis* Gerd. & Trappe on grapevine establishment in soils infested with black-foot pathogens. In this study, the AM associations also improve health and growth of young grapevine plants. Although the mechanisms by which AM fungi protect plants against soilborne pathogens is poorly understood, it is often hypothesized that they include improving nutrition of the host, competition for infection sites and changes to root ultrastructure. Results from this study suggest that preplant applications of AM fungi may help prevent black-foot disease in the nursery and in the vineyard (Petit and Gubler, 2006; Bleach *et al*., 2008).

The use of chitosan which is a high molecular-weight polymer that is non-toxic and biodegradable has also been evaluated as another control measure for grapevine trunk pathogens. In a research study carried out in Portugal, Nascimento *et al*. (2007) explored the *in vitro* and *in vivo* fungicidal effect of chitosan on some of the most important grapevine wood fungi. The results showed that chitosan was effective in reducing mycelial growth of all fungi and significantly improved plant growth and decrease diseased incidence compared with untreated plants. Moreover, the effect of chitosan against "*C.*" *liriodendri* was similar to that achieved with some selected fungicides such as tebuconazole and mixtures of carbendazim with flusilazole, and cyprodinil with fludioxonil.

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RESEARCH PAPER

Overview of grapevine trunk diseases in France in the 2000s

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Summary. The National Grapevine Trunk Disease Survey was conducted in France from 2003 to 2008 to monitor grapevine trunk diseases (GTDs), eutypa dieback and esca/black dead arm (BDA). Data collected from seven regions, 329 vineyards and 12 cultivars were analysed. There were great variations amongst regions in the incidence of GTDs. For esca/BDA, two groups were distinguished: vineyards in Jura and Charentes had greater incidence (93–95%) than those of Bordeaux, Alsace and Bourgogne (54–82%). Incidence increased in Charentes over the 6-year survey, with the highest values being recorded during the last 2 years. For eutypa dieback, all vineyards of Charentes were affected, with 17 to 25% of vines expressing symptoms; for the other regions, 52 to 80% of vineyards were affected, with incidences below 3%. Cultivars Savagnin and Trousseau in Jura were especially affected by esca/BDA. Instead, Ugni Blanc in Charentes was most affected by eutypa dieback. One cultivar could be significantly more affected in one region than in another. The global health status of the vineyards was also investigated. *(i)* For four regions, 82% (Jura) to 87% (Alsace) of the grapevines were healthy, but this percentage decreased steadily (67%) in Charentes. *(ii)* Plants infected by GTDs were 32 and 18% in Jura and Charentes respectively, and only 2.9% in the Bourgogne region. (iii) The unproductive plants, *i.e.* dead, missing, replanted or restored, represented a significant part of the losses (6.6% in Charentes to 9.9% in Jura). The extension of GTDs is discussed with regard to the abiotic and biotic factors that may favour the diseases.

Key words: grapevine cultivar, esca, eutypa dieback, black dead arm, botryosphaeria dieback.

Introduction

Grapevine trunk diseases (GTDs), include three main diseases affecting grapevine wood: eutypa dieback, esca decline and botryosphaeria dieback, which are widespread in the main vine-growing regions of the world (Scheck *et al.,* 1998; Mugnai *et al*., 1999; Armengol *et al.,* 2001; Rumbos and Rumbou, 2001; Edwards and Pascoe, 2004; Gimenez-Jaime *et al*., 2006). An increase in the incidence of GTDs over the last 10–15 years has been reported worldwide (Carter, 1991; Chiarappa, 2000; Graniti *et al*., 2000; Surico *et al.,* 2000; Reisenzein *et al.,* 2000; Úrbez-Torres *et al*., 2006, 2008, 2009; Sosnowski *et al*., 2007; Bertsch *et al*., 2012). In France, until the late nineties, the most common trunk diseases were eutypa and esca, at the

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turn of the century, a third and new disease caused by Botryosphaeriaceous species was identified in Bordeaux (Larignon *et al.*, 2001) and in other French vine-growing areas (Panon, 2000). This disease is associated with *Diplodia seriata* ("*Botryosphaeria obtusa*") and *Botryosphaeria dothidea* and was referred to as "black dead arm" (BDA) adopting the same name previously used in Hungary by Lehoczky (1974) for cankers caused on grapevine by other Botryosphaeriaceae species, namely "*Botryosphaeria stevensii*".

In the same years in many vinegrowing countries in the world an increasing incidence of wood cankers caused by various Botryosphaeriaceae species was recorded, referred to as botryosphaeria dieback (Úrbez-Torres, 2011).

Because of the threat that these diseases cause to vineyards, numerous studies were carried out during the last decade to identify the causal fungi and their interactions with the vine in order to develop disease management strategies (Larignon *et al.*, 2009; Bertsch *et al.*, 2012). In regard to aetiology, the symptoms that occur in the trunk, leaves and berries have been extensively described, revealing that eutypa dieback symptoms differ markedly from those of esca and BDA, with differentiation between the latter two often proving difficult. Lecomte *et al.* (2012) reported that foliar symptoms of esca showed transitory phases that overlapped with some BDA descriptions. For esca, due to the complexity of the symptoms, changes in the definition of this disease have been reported in the literature over the last ten years (Surico *et al.*, 2008; Bertsch *et al.*, 2012). Two major simplifications in disease terminology were proposed to replace the five initial terms used for esca: brown wood streaking, Petri disease, young esca, esca and esca proper (Mugnai *et al.*, 1999). The first simplification was made by Surico (2009), who suggested that the term "young esca" be replaced by "grapevine leaf stripe disease" (GLSD), so that the term "esca" only included white rot (esca) and esca proper. For the three tracheomycotic syndromes, brown wood streaking, Petri disease and GLSD, the term phaeotracheomycotic complex was also proposed, as the same fungi are involved in the three symptomatically different diseases. The second simplification was proposed by Lecomte *et al.* (2012) when they chose not to separate esca symptoms into mild or apoplectic forms, but to use a classification based on a gradual scale of severity, starting from some leaves showing only discolorations up to complete vine wilting.

Eutypa lata, was identified long ago (Carter, 1988) as the main agent of the eutypa dieback, even if, more recently, other species of the diatrypaceae (Trouillas *et al.*, 2010; Trouillas and Gubler, 2010) were found to be involved. In the botryosphaeria dieback, according to Úrbez-Torres (2011), 21 different Botryosphaeriaceae species are associated with this disease. Some of them are described to produce in France, beside the typical wood cankers and dieback, also foliar symptoms that resemble closely the grapevine leaf stripe disease, or esca tiger-stripe symptom. On the other hand esca was defined as a complex of diseases, as it involves fungi belonging to various species and families. Generally, the grapevine leaf stripe disease is thought to result mainly from the pathogenic activity of vascular pathogens, e.g. *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum*, while the wood white decay is mainly caused in Europe by *Fomitiporia mediterranea.* In the present paper the term esca/BDA will be used overall, as a clear distinction between the two tiger stripe symptoms, grapevine leaf stripe disease and BDA, is really difficult in field surveys; hence the authors propose that the GLSD vascular agents and the BDA agents, both producing toxic metabolites, can have a relevant synergistic role in the leaf symptoms formation (Andolfi *et al.*, 2011). The involvement of other microorganisms is still a matter of speculation and recent studies indicate that a diverse microflora colonise the wood of esca-diseased grapevines (Bruez *et al.*, 2011; Maher *et al.*, 2012), but its exact role remains to be determined. It is surely clearly ackwnowledged that the different pathogens can coexist in the same vine (Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Bruez, 2013). Finally, the origin of the various terms that define esca should be associated with the microbial species that colonize the wood of grapevines at a specific time. The activity and succession of fungal microflora that occur within the wood may lead to the complexity of the symptoms, thus generating the various terms used in the literature.

The current epidemic spread of esca dates back to the early 1990s (Mugnai *et al.*, 1999), according to various European surveys of this disease (Reisenzein *et al.*, 2000; Surico *et al.*, 2000) while BDA was reported in the early XXI century (Larignon *et al.*, 2001; Lecomte *et al.*, 2005). Survey results provided vinegrowers and scientists with information on the incidence and evolution of these diseases. The National Grapevine Trunk Diseases Survey was established in

France to monitor the evolution and determine the importance of eutypa dieback and esca/BDA during the period from 2003 to 2008. The survey was conducted in 11 French regions, on 27 varieties of vines and for more than 600 vineyards. A preliminary survey provided information on 256 individual vineyards and, at that time, the mean incidences for eutypa dieback and esca/BDA were 2.23 and 3.25%, respectively (Fussler *et al.*, 2008).

The objective of this study was to assess the status of GTDs in France by comparing disease incidence between regions and cultivars.

Materials and methods

The survey of the French vineyards

The survey of grapevine trunk diseases was done in 329 vineyards randomly chosen across 7 regions of France. In each vineyard, 300 grapevines were assessed in ten randomly chosen groups of 30 vines. Each year during the survey, observations were made on the same grapevines. The grapevines were monitored at two periods of the growing season: in June (at the end of flowering and at the beginning of fruit setting) to assess foliar symptoms of eutypa dieback and in August–September (berry ripening) of esca/BDA. At the same time, dead, missing, replanted, restored and healthy (without GTD symptoms) plants were counted: they represented the unproductive grapevines. The survey was done by employees of FranceAgriMer, the French Plant Protection Agency (French Ministry of Agriculture), Institut National de la Recherche Agronomique (INRA), Institut Français de la Vigne et du Vin (IFV) and vinegrowers associations of each region.

Vineyards were monitored in the regions of Alsace, Bordeaux, Bourgogne, Charentes, Jura, Centre and over Provence-Alpes Côte d'Azur (PACA) over the 6-year period. Some were not monitored one year (Jura in 2003, Alsace in 2006) or two years (Centre in 2007 and 2008). In all, 12 cultivars were included, which generally differed between regions (Table 1).

For each vineyard, the information collected consisted of age, cultivar and rootstocks. Note that, for each cultivar the age value corresponds to the mean of the ages of the vineyards in 2008, in brackets are the minimum and maximum ages of the vineyards (Table 1).

Statistical analyses

The frequency of eutypa dieback and of esca/BDA corresponds to the percentage of affected vineyards. The incidence of eutypa dieback and of esca/BDA represents the percentage of affected vines. For the incidence, the binomial confidence intervals have been calculated by using the package Hmisc of the R software (version 2.14.2) with the command "bioconf" (binomial confidence intervals are calculated because normal estimates are meaningless below 10%).

The plant mortality, i.e. the percentage of dead and missing grapevines, and the replanted and restored plants were also counted. For each result the standard deviation has been estimated.

The non-parametric Kruskal-Wallis test has been used to compare the data obtained with the Cabernet-Sauvignon and Sauvignon cultivars when they were planted in various regions (variances were not equal according to the Levene's test). The pairwise Wilcoxon test has been used after the Kruskal-Wallis test to confirm the results.

Contingency tables allowed us to determine if, for the same cultivar, a relation exists between the rates of esca/BDA and of eutypa dieback. Chi-square test for independence was performed using the package Rcmdr of the R software package (2.14.2).

For eutypa dieback or esca/BDA symptom incidence, three separate bifactorial analyses were performed: a Cultivar × Year of Survey analysis of variance, a Cultivar × Region analysis of variance and a Region × Age analysis of covariance. This design was necessary because most cultivars were peculiar to a region only and the two factors couldn't be jointly analyzed. The percentages of esca/BDA and eutypiose symptomatic expression were transformed into logits to account for the binomial nature of the data.

Results

Comparing frequency and incidence of grapevine trunk diseases between regions

In Charentes, eutypa dieback was recorded in all 27 vineyards surveyed (Figure 1a). For the Alsace, Bordeaux and Bourgogne regions, frequencies varied between 42 and 80% (Figure 1a). In Jura, eutypa dieback was observed in 8% of vineyards in 2004 and 32–38% of vineyards in the years 2005–2007.

Table 1. French vinegrowing regions, cultivars (age, rootstocks) and vineyards used for the survey done by the National Grapevine Trunk Disease Survey (2003–2008).

Vine growing regions	Cultivars	Age of cultivars in 2008	Rootstocks	Number of vineyards monitored for eutypa dieback and esca/BDA	Years					
					2003	2004	2005	2006	2007	2008
Alsace	Pinot Auxerrois	27.9 (16–40)	3309/S04/Teleki8	24	X	X	X		X	X
	Gewurztraminer	25.42 (7–58)	3309/420A/161-49/S04/34EM/Fercal/41B	24	X	X	X		X	X
	Riesling	27.4 (15–55)	3309/S04/161-49	26	X	X	X		X	X
Bordeaux	Cabernet-Sauvignon	24.5 (11–47)	3309/S04/101-14/420A/Riparia	17	X	X	X	X	X	X
	Merlot	24.7 (12–47)	3309/S04/161-14/420A/Riparia	17	X	X	X	X	X	X
	Sauvignon	20.1 (7–31)	3309/S04/101-14/196-17	17	X	X	X	X	X	X
Bourgogne	Chardonnay, 21	41.9 (21–78)	3309/S04/161-14/SBB/5C	28	X	X	X	X	X	X
	Chardonnay, 89	25.8 (16–49)	S04/41B/Teleki	23	X	X	X	X	X	X
	Pinot noir	42.5 (21–78)	3309/S04/161-49/SBB	40	X	X	X	X	X	X
	Sauvignon	26 (20–38)	3309/S04/41B	13	X	X	X	X	X	X
Centre	Sauvignon	23.4 (16–45)	3309/S04/Riparia/SBB	16	X	X	X	X		
Charentes	Ugni blanc	26.8 (10–38)	Paulsen/Rupestris/Fercal/RSB/161-49/41B/R140	27	X	X	X	X	X	X
Jura	Poulsard	26.7 (15–38)	3309/S04/101-14	25		X	X	X	X	X
	Trousseau	22.5 (15–43)	3309/S04/101-14	25		X	X	X	X	X
	Savagnin	23.9 (14–51)	3309/S04	23		X	X	X	X	X
Provence-Alpes Côte d'Azur	Cabernet-Sauvignon	24.1 (15–29)	S04/R110/Paulsen	10	X	X	X	X	X	X

The incidences of eutypa dieback in the vineyards of Charentes varied from 17 to 26% between years (Figure 2a). From 2003 to 2008, there was a tendency for the incidence to decrease. The three highest percentages (above 21%) were recorded in 2003–2005 and the three lowest (below 20%) in 2006–2008. For the Alsace and Bordeaux vineyards, the incidence of eutypa dieback was always below 4%, the lowest value was obtained the first year of the survey (2003) and the two highest in 2007 or 2008 (Figure 2a). In the Bourgogne vineyards, the incidence was less than 1% in 2004, 2006 and 2007; the maximum was 1.95%

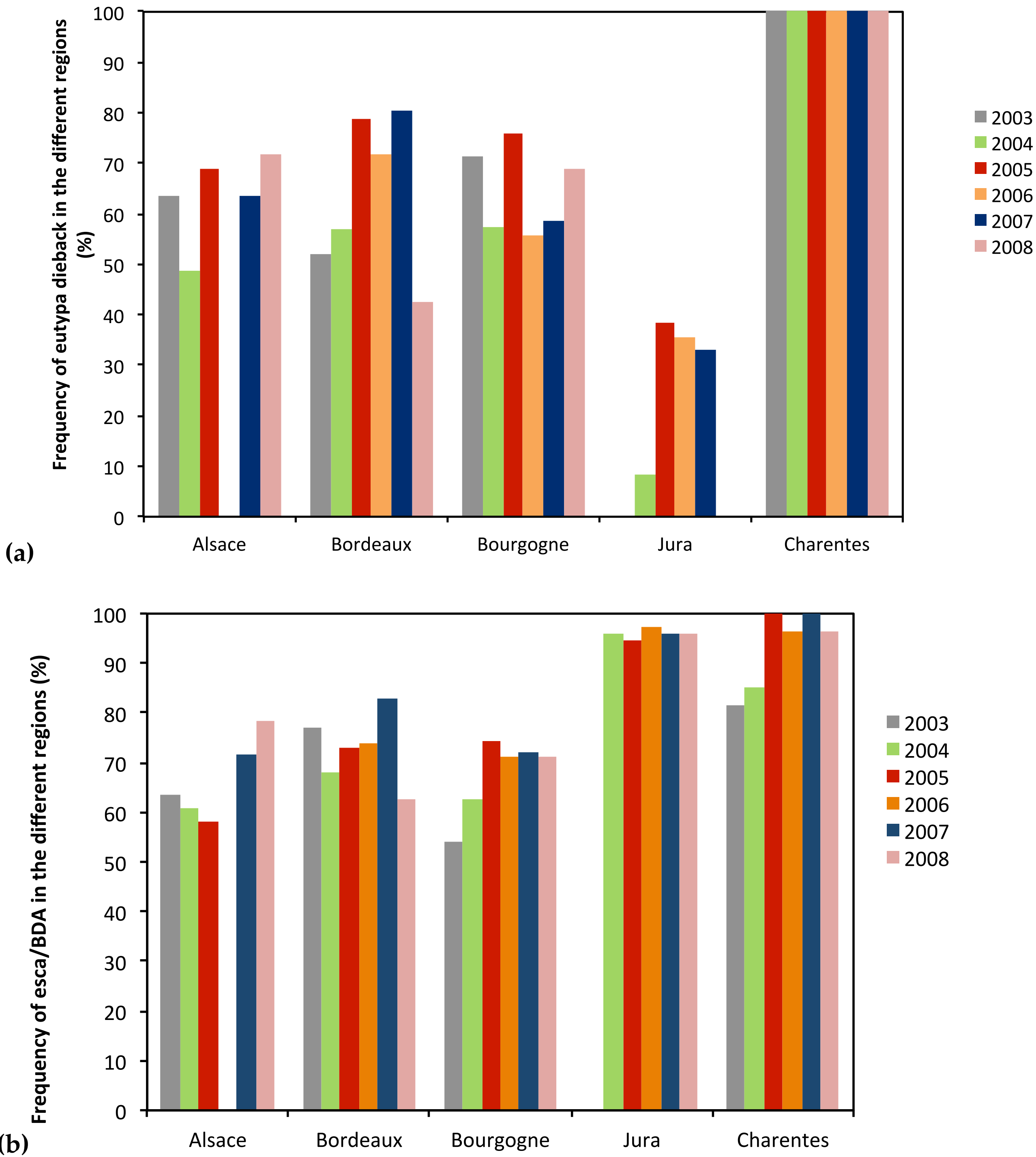


Figure 1. Frequency of eutypa dieback (a) and of esca/BDA (b) in the five regions, Alsace, Bordeaux, Bourgogne, Jura and Charentes surveyed over the six-year period.

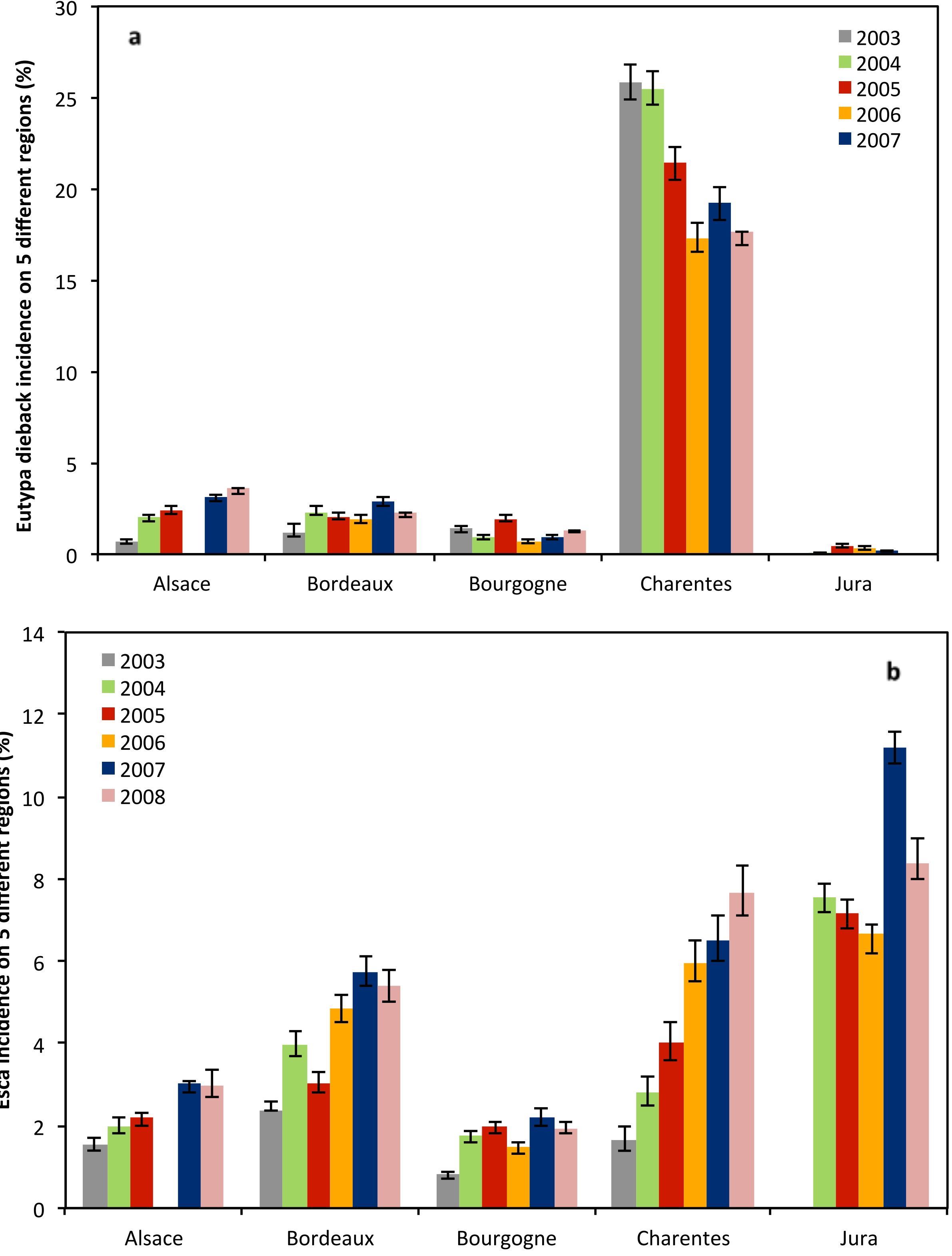


Figure. 2. Eutypa dieback (a) and esca/BDA (b) incidences in the five regions: Alsace, Bordeaux, Bourgogne, Jura and Charentes surveyed over the six-year period. Confidence intervals are indicated on the bars.

in 2005. For the Jura region, the incidence of eutypa dieback in the vineyards was always below 0.5%.

The Jura and Charentes regions had higher frequency and incidence of esca/BDA compared with that of the Alsace, Bordeaux and Bourgogne regions (Figures 1b and 2b). In the Jura vineyards, the frequency was greater than 95% and the incidence varied from 7 to 11%. In Charentes, the frequency of vineyards with esca/BDA was around 81–85% the first two years of the survey, with all vineyards affected in 2005 and in the following years frequency varied from 96 to 100%. The incidence of esca/BDA in vineyards increased with years in Charentes from 2 to 8%.

For the three other regions, the mean frequencies of esca/BDA were relatively similar; 66% in Alsace, 73% in Bordeaux and 68% in Bourgogne. For all five regions, the lowest values of incidence were obtained in 2003 and the highest in 2007 or 2008.

Comparing incidence of grapevine trunk diseases between grapevine cultivars

In the Charentes region, the incidence of eutypa dieback in cv. Ugni Blanc vines (22%) was significantly higher than the other cultivars in other regions in the survey; Cabernet-Sauvignon and Sauvignon (2.5–3%) and all others were below 1% (Figure 3a).

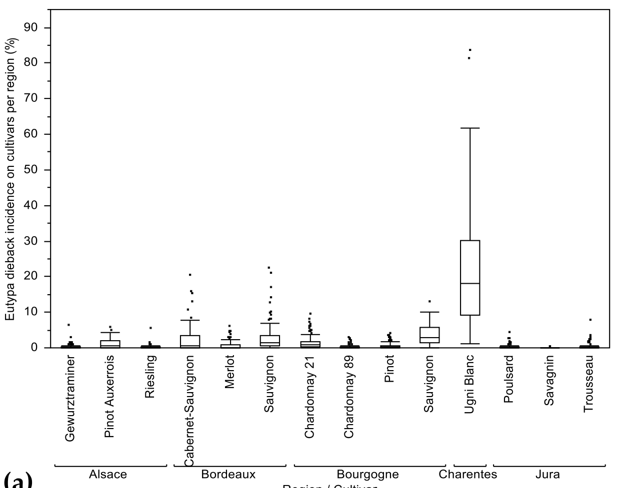
The greatest incidence of Esca/BDA was recorded in cv. Savagnin and Trousseau (10%) in the Jura region (Figure 3b). In contrast, less than 2% of vines of cv. Pinot and Chardonnay in Bourgogne and in cv. Merlot in Bordeaux showed disease. Four percent of Chardonnay vines, planted in the department of the Yonne ("Chardonnay 89" on Figures 3a and 3b) in Bourgogne were recorded with esca/BDA symptoms, as compared with 1% of cv. Chardonnay vines, which were planted in the department of the Côte d'Or ("Chardonnay 21" on Figures 3a and 3b) in the same region. Except for the cv. Sauvignon, from the Bordeaux vineyards, that had 7% of vines attacked, for the other cultivars from the other regions, 3 to 6% of vines expressed esca/BDA foliar symptoms.

The incidence of esca/BDA was significantly greater on Cabernet-Sauvignon grapevines in Provence-Alpes Côte d'Azur than in Bordeaux in 2003–2006 (Kruskal-Wallis test, $P<0.001$) but not in 2007 ($P=0.191$) and 2008 ($P=0.597$) (Figure 4).

When the esca/BDA incidences on Sauvignon cultivar planted in the Bordeaux, Bourgogne and Centre regions are compared over the 6-year survey, no significant differences were observed (Figure 5).

Assessment of grapevine losses from 2003 to 2008

The percentage of healthy plants in Charentes was 67% compared with 89% for Bourgogne, 87% in



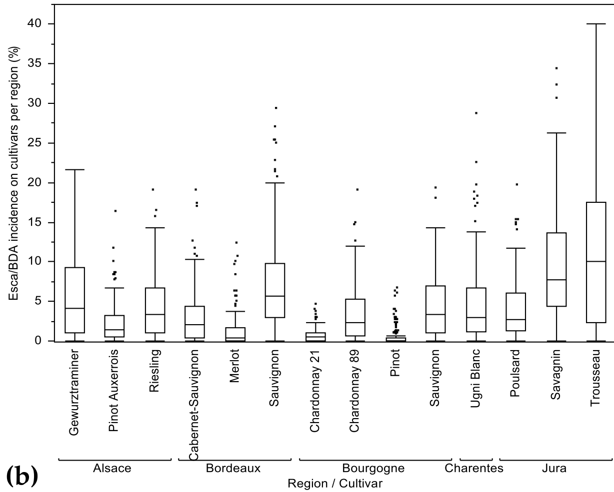


Figure 3. Eutypa dieback (a) and esca/BDA (b) incidences of different cultivars planted in the five regions: Alsace, Bordeaux, Bourgogne, Charentes and Jura surveyed over the six-year period. Chardonnay 21 and 89 are planted in the Côte d'Or and Yonne departments of France respectively. Data are expressed as a box-and-whisker plot showing median, interquartile range (IQR) and extreme values.

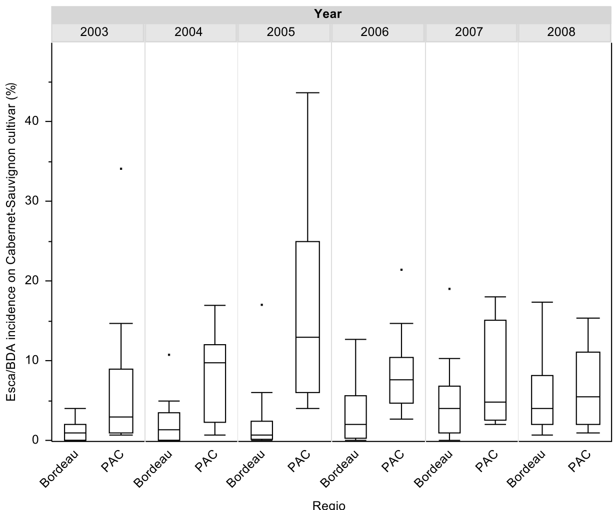


Figure 4. Esca/BDA incidence on Cabernet-Sauvignon cultivar planted in the Bordeaux and Provence-Alpes Côte d'Azur regions (PACA) regions. Data are expressed as a box-and-whisker plot showing median, inter-quartile range (IQR) and extreme values.

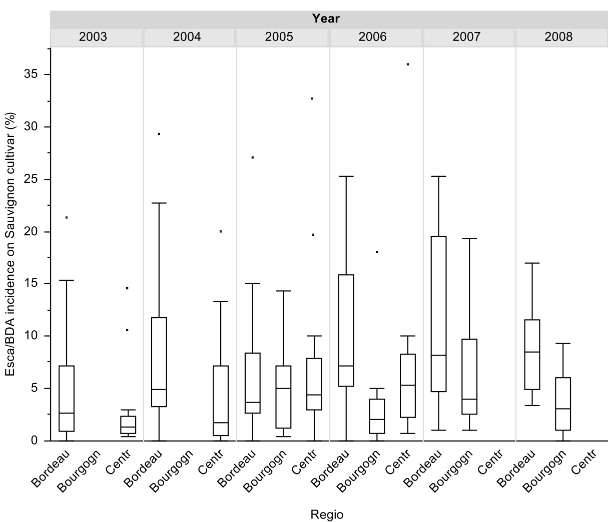


Figure 5. Esca/BDA incidence on Sauvignon cultivar planted in the Bordeaux, Bourgogne and Centre regions. Data are expressed as a box-and-whisker plot showing median, inter-quartile range (IQR) and extreme values.

Alsace, 86% in Bordeaux and 82% in Jura. In Alsace, dead and missing vines accounted for 0.6 and 1.2% of vines, respectively. For the other regions, the incidence of dead plants was relatively similar, from 1.1% in Bourgogne to 1.7% in Charentes. The same trend was observed for the missing vines, the values varied from 2.5% in Bourgogne to 3% in Jura. Replanted vines were most numerous in Jura (5%), Alsace (4.9%) and Bourgogne (4.6%), the lowest percentage was in Charentes with 1.2%. The highest incidence of restored plants was in Alsace (1.4%) and Charentes (0.9%) with only 0.05% of vines restored in Bourgogne (Table 2a).

Regarding the two GTDs surveyed over the six-year period, 21% of the vines expressed symptoms of eutypa dieback in Charentes, in the 4 other regions that value was reduced to 1–3% in Bourgogne, Bordeaux and Alsace and was only 0.3% in Jura. For esca/BDA, 8% of vines expressed leaf symptoms in Jura, that percentage was reduced by half in Charentes and Bordeaux (4–5%), with the lowest value obtained in Bourgogne (1.7%).

Table 2b shows the percentages of unproductive plants, they were relatively similar for all five regions since they ranged from 7% in Charentes to 10% in Jura. For vines affected by GTDs, the highest values were in Charentes (26%) and Jura (9%), while only 3% of the plants were diseased in Bourgogne.

The total percentage of unproductive plants and GTDs affected grapevines across all regions ranged from 33% in Charentes to 11% in Bourgogne (Table 2b).

Link between the diseases and various parameters

For each cultivar, the relations between esca/BDA and eutypa dieback incidences are shown in Table 3. Among the 12 cultivars, a significant relation between the two diseases was only observed for the cultivars Trousseau (Jura region: $0.05>P>0.01$) and Sauvignon (Bordeaux region: $0.05>P>0.01$).

The analyses of variance and covariance (Table 4) showed that for esca/BDA and eutypa dieback expression of the symptoms, there are differences between the regions, between the year and between the cultivars. The age of the vineyards is not a variable associated with the percentage of symptomatic expression of eutypa dieback and esca/BDA ($P>0.05$). The effect of the cultivars is the strongest one ($P<10^{-15}$), followed by that of the region ($P<10^{-9}$) for both diseases. The effect of the year of survey was very significant for eutypa dieback ($P=0.0016$) but only marginally for esca/BDA ($P=0.036$).

Table 2a. Percentage of grapevines that were: healthy, affected by grapevine trunk diseases (eutypa dieback or esca/BDA), dead, missing, replanted, restored in five French regions (surveyed from 2003 to 2008).

Region	Healthy plants	Dead plants	Missing plants	Replanted	Restored plants	Eutypa dieback affected plants	Esca/BDA affected plants
Alsace	87.2 ± 3	0.6 ± 0.4	1.2 ± 0.8	4.9 ± 1.4	1.4 ± 1.8	2.3 ± 0,3	2.3 ± 1.6
Bordeaux	85.7 ± 5.6	1.2 ± 0.9	3.0 ± 2.4	2.9 ± 1.4	0.8 ± 2.1	2.1 ± 1.2	4.2 ± 1.7
Bourgogne	88.8 ± 5	1.1 ± 0.6	2.5 ± 2.8	4.6 ± 8.4	0.1 ± 0.1	1.2 ± 0.6	1.7 ± 0.9
Charentes	67.4 ± 9.3	1.7 ± 0.5	2.8 ± 0.5	1.2 ± 0.9	0.9 ± 1.8	21.2 ± 5.9	4.8 ± 1.8
Jura	82.2 ± 5.5	1.0 ± 0.9	3.0 ± 1.1	5.0 ± 1.8	0.2 ± 0.4	0.3 ± 0,2	8.2 ± 3.0

Table 2b. Percentage of grapevines that were unproductive (dead, missing, replanted, restored are together) or affected by grapevine trunk diseases (eutypa dieback and esca/BDA) in five French regions (surveyed from 2003 to 2008).

Region	Unproductive plants	GTDs affected plants	Total
Alsace	8.1	4.6	12.8
Bordeaux	7.9	6.3	14.3
Bourgogne	8.4	2.9	11.2
Charentes	6.6	26.0	32.6
Jura	9.9	8.5	18.4

When the two variables, region and cultivar, are studied jointly to compare the rate of attack, the results show that the esca attack rate differs depending on the two variables. For eutypiose, the attack rate differs only depending on the variety.

The LSD test results confirmed the differences obtained by the ANCOVA and ANOVA tests.

Discussion

These results show that the GTDs, eutypa dieback and esca/BDA, are present in all French vineyard regions in the survey. However, depending on the region, there were variations in the frequency and incidence of GTDs in the vineyards. Esca/BDA frequency was greater in Jura and Charentes, than in Bordeaux, Alsace and Bourgogne. Generally, from one year to another, the incidence did not fluctuate, except in the Charentes region where a constant increase occurred over the 6 years. For all regions, the highest frequencies were recorded during the last two years, 2007 and 2008. Does that mark the beginning of an epidemic that will develop slowly but steadily over the years? Data obtained in 2009 to 2012 from another survey performed in some French regions (Grosman and Doublet, 2012), i.e. increase of esca/BDA incidence, suggest that this hypothesis is possible. However, the increase observed in the present study seems to be lower than that reported by Reisenzein *et al.* (2000) in Austria. They indicated that the number of vines with external symptoms increased by an average annual rate of 2.7% in 6 years, but in certain vineyards the increase was between 2% and 20%.

Charentes was the only region with significant levels of eutypa dieback, with all its vineyards having from 17 to 25% incidence of symptoms for all 6 years. In the Alsace, Bordeaux and Bourgogne regions, the frequencies fluctuated over the years (from 52 to 80%) but were always lower than in Charentes. However this could also be due to a particular susceptibility of cv. Ugni Blanc, unique to this region. Indeed, our results show that the effect of the cultivar is stronger than that of the region. Unfortunately, most of the cultivars are peculiar to only one of the regions surveyed and the relative influence of the two could only be measured for Cabernet-Sauvignon and Sauvignon Blanc. The incidences were relatively low, as generally less than 3% of the grapevines expressed symptoms. Jura seems relatively free of this disease

Table 3. Correlation between esca/BDA and eutypa dieback using a contingency table chi-squared test.

Region	Cultivar	Data Chi-2 test	Data table
Alsace	Auxerrois	1.79	3.84
	Riesling	0.08	3.84
	Gewurztraminer	0.21	3.84
Bordeaux	Sauvignon	4.17	3.84[a]
	Merlot	1.8	3.84
	Cabernet-Sauvignon	0.003	3.84
Bourgogne	Chardonnay 21	0.7	3.84
	Chardonnay 89	0.002	3.84
	Pinot	0.65	3.84
	Sauvignon	0.04	3.84
Charentes	Ugni blanc	0.001	3.84
Jura	Poulsard	3.3	3.84
	Savagnin	0.13	3.84
	Trousseau	5.66	3.84[a]

[a] Link between esca /BDA and eutypa dieback.

since the frequency was particularly low (8 to 38%), and no more than 0.46% of grapevines expressed symptoms of eutypa dieback. So these data suggest that, unlike esca, eutypa dieback was not increasing in France. To support that observation, even in the most affected region, Charentes, the lowest values of grapevines expressing eutypa dieback were only obtained over the last three years of the survey (2006 to 2008). However, as symptoms of eutypa dieback may fluctuate considerably, we suggest that the disease needs to be surveyed over a longer period.

To obtain an overview of the situation of GTDs in France, it should be borne in mind that, from one year to another, the same grapevines do not necessarily express foliar symptoms. So, when a period of several years is considered, the number of "foliar symptomatic vines at least once" is far more significant. For instance, Surico *et al.* (2000) indicated that, in a 6-year survey in Italy, the annual incidence of esca affected vines ranged from 11 to 19%. But they found numerous different sequences of symptom expression of vines over that period, with a cumu-

Table 4. Correlations for eutypa dieback or esca/BDA symptoms between regions and age of grapevines, cultivars and year and between the cultivar and the region were calculated by analysis of covariance (ANCOVA). The values with an asterisk are significantly different at $\alpha = 0.05$.

P-values for the esca/BDA attack data	
	ANCOVA
Region	1.63E-10 *
Age	0.7119

P-values for the eutypa dieback attack data	
	ANCOVA
Region	4.36E-10 *
Age	0.9847

P-values for the esca/BDA attack data	
	ANCOVA
Cultivars	2.00E-16 *
Year	0.036 *

P-values for the eutypa dieback attack data	
	ANCOVA
Cultivar	2.00E-16 *
Year	0.0016 *

P-values for the esca/BDA attack data	
	ANOVA
Cultivars	2.00E-16 *
Region	0,001 *

P-values for the eutypa dieback attack data	
	ANOVA
Cultivar	2.00E-16 *
Region	0,331

lative incidence of around 50%. In another Italian survey, Surico *et al.* (2006) again reported an increase in esca over time in three vineyards in Siena (50% over 11 years), Ravenna (30% over 6 years) and Florence (51% over 5 years). In France, Grosman and Doublet (2012) reported that in a 10-year survey in Bourgogne in a vineyard with low incidence (3–4% each year), a total of 21% of vines expressed esca/BDA at least once over that period. The expression of foliar symptoms is not neutral, as shown by Guérin-Dubrana *et al.* (2013, this Journal issue) showed that mortality due to esca is consistently associated with the foliar symptom expression the year before grapevine death. For eutypa dieback, symptoms are also a risk factor for death, greater or equal to that of esca. Only two cultivars out of twelve showed a link between eutypa dieback and esca/BDA. So, it does not seem that the two diseases were prominent at the same time within a vineyard.

The present study showed that the trends in the symptomatic expression of GTDs were relatively similar within each of the five geographic regions, but frequencies and incidences differed greatly between regions. We identified several factors that may be responsible for such differences: the cultivars, regions and year of survey. Our study clearly shows that some of the cultivars were affected more than others by esca/BDA, i.e. Savagnin, Trousseau, or eutypa dieback, i.e. Ugni Blanc. Although this factor cannot be separated from the region, its influence was strong. We observed that Cabernet-Sauvignon can be significantly more affected in a certain French region, i.e. Aquitaine, than in another, i.e. Provence-Alpes Côte d'Azur. However, for the cultivar Sauvignon, we did not observe differences between the Bordeaux, Centre or the Bourgogne regions.

For eutypa dieback, Sosnowski *et al.* (2007) reported that differences also occurred. They reported that foliar symptoms caused by *E. lata* on the cultivar Shiraz varied from year to year in South Australia in a 6-year survey and that, although trends were similar for vineyards within geographical regions, differences were observed between the two regions in which the experiments were conducted. All these results indicate that besides "varietal susceptibility", other factors, such as climate and soil, may be involved.

For other countries, White *et al.* (2011) reported that esca was found on a range of grapevine cultivars planted in South Africa, with some of them being the same as those surveyed in France, *i.e.* Chardonnay, Cabernet-Sauvignon, Merlot. However, as the extent of the disease was not mentioned, it was not possible to compare them. A study of the susceptibility of one cultivar planted in different countries, for instance, Cabernet-Sauvignon in France, Italy and the USA, Riesling in France and Austria (Reisenzein *et al.*, 2000), would certainly be useful in characterising the factors that are the more representative of each area. It would subsequently help to determine the main factors that favour GTDs.

We found that regions and the year of survey have an indirect effect on the disease. Each region has its own different climate: Alsace, for instance, has a continental climate and Bordeaux an oceanic one. Although we have not investigated this point, climate certainly has an influence on the development of GTDs. Surico *et al.* (2006) suggested that climate changes have exacerbated the esca problem. The intensity and variations of rainfalls over the years have a strong influence on the disease. Marchi *et al*. (2006) reported that, throughout a growing season, the number of grapevines that remained asymptomatic was inversely related to the rainfall in May–June or in summer. Chronic esca expression is associated with hot periods in summer following rainfalls whereas, during hot, dry summer periods, severe esca (apoplexy) was more common (Surico *et al*., 2000).

Another important point is temperature, which may differ from one year to another. Lecomte *et al.* (2012) observed that, whatever the vineyard or year of survey in the Bordeaux region, the appearance of esca-leaf symptoms increased regularly from the beginning of June until the end of July. Afterwards, the rate of leaf symptom occurrence decreased, with certain symptoms remaining visible until September. Such an evolution of leaf symptoms could be associated with the progressive increase of mean temperatures in early summer.

As regards eutypa dieback, it is well known that the susceptibility of grapevine wounds to infection by *E. lata* spores is favoured by cool winter conditions (Munkvold and Marois, 1995; Chapuis *et al.*, 1998). Sosnowski *et al.* (2007) carried out a 6-year study in South Australia, to study the influence of climate on foliar symptoms of eutypa dieback on grapevines, cv. Shiraz. They found that various parameters, such as winter rainfall 18 months earlier, increased temperatures in spring, and very high and very low rainfalls in October may have had an influence on the disease. These results led the authors

to set up a model predicting the incidence of foliar symptoms of eutypa dieback.

One of the main results from this study is the status of vines growing in the five French regions we surveyed. It was found that 82% (Jura) to 87% (Alsace) of vines were healthy, but only 67% of vines were healthy in Charentes. These relatively low values of healthy plants should be regarded as the consequence of two kinds of aspects. Firstly plants were affected by GTDs in 18 and 32% in Jura and Charentes, respectively, and only 3% in Bourgogne. Secondly, unproductive plants, i.e. dead, missing, replanted or restored grapevines, represented a significant part of losses in Charentes (6.6%) and Jura (9.9%). In addition to the yield losses, decrease in wine quality (Dubos and Larignon, 1987; Lorrain *et al.*, 2012) and death of vines caused by GTDs, another part of the losses is due to these diseases, because vinegrowers currently uproot affected plants, replanting or restoring them whenever possible. When grapevines are replanted, there is a waiting period of at least three years before the first harvest. Although the costs associated with trunk diseases are often difficult to quantify, in South Australia, eutypa dieback has been estimated to cause yield losses of at least 860 and 740 kg ha^{-1} for the Shiraz and Cabernet-Sauvignon varieties (Wicks and Davies, 1999). For Shiraz alone, production losses in Australia were equivalent to 20 million Australian dollars. In California, economic losses of up to US$260 million per annum have been attributed to the same disease (Siebert, 2001). In France, relevant assessments have not been made, but it was estimated that GTDs induced losses of around 6–7 million euros per year in the Loir-et-Cher alone (P. Martin-Lalande, personal communication). Hofstetter *et al.* (2012) estimated that the worldwide annual financial cost of the replacement of death plants due to GTDs is in excess of 1.132 billion euros. It is thus clear that these diseases are a major threat for the wine-producing regions everywhere.

To conclude, the present work indicates that GTDs are of major concern for the sustainability of certain French vineyards and vine cultivars. We have mentioned some factors that seem to be of major importance in the development of these diseases. Further studies have to be undertaken to determine accurately their impact on the development of GTDs; presumably, also other factors are involved (Lecomte *et al.*, 2011). Surico *et al.* (2004) identified the use of poor quality planting material and the changes in the use of fungicides as some of the possible causes for the upsurge of esca in Tuscany, Italy. According to Peros *et al.* (2008) the use of good quality planting material and the choice of agronomic practices that favour grapevine longevity appear to be useful strategies to reduce the incidence of esca. The challenge is big one, at the same time, GTDs have created a great apprehension in viticulture, as shown by recent demonstrations in France (M. Bessard, personal communication). One French deputy recently spoke of the "new phylloxera" of the 21st century in his declaration on GTDs at the National Assembly and vinegrowers called for rapid control solutions. However, because grapevine trunk diseases involve a range of biotic and abiotic factors, finding solutions would require managing most of the above-mentioned factors. A systemic approach would seemingly be needed to resolve this complex issue.

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RESEARCH PAPER

Statistical analysis of grapevine mortality associated with esca or Eutypa dieback foliar expression

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Summary. Esca and Eutypa dieback are two major wood diseases of grapevine in France. Their widespread distribution in vineyards leads to vine decline and to a loss in productivity. However, little is known either about the temporal dynamics of these diseases at plant level, and equally, the relationships between foliar expression of the diseases and vine death is relatively unknown too. To investigate this last question, the vines of six vineyards cv. Cabernet Sauvignon in the Bordeaux region were surveyed, by recording foliar symptoms, dead arms and dead plants from 2004 to 2010. In 2008, 2009 and 2010, approximately five percent of the asymptomatic vines died but the percentage of dead vines which had previously expressed esca foliar symptoms was higher, and varied between vineyards. A logistic regression model was used to determine the previous years of symptomatic expression associated with vine mortality. The mortality of esca is always associated with the foliar symptom expression of the year preceding vine death. One or two other earlier years of expression frequently represented additional risk factors. The Eutypa dieback symptom was also a risk factor of death, superior or equal to that of esca. The study of the internal necroses of vines expressing esca or Eutypa dieback is discussed in the light of these statistical results.

Key words: grapevine, trunk diseases, epidemiology, logistic regression.

Introduction

Esca and Eutypa dieback, considered worldwide as two major wood diseases of grapevine (*Vitis vinifera* L.), cause a decrease in productivity and a reduction in vine lifetime (Munkvold *et al*., 1994; Mugnai *et al*., 1999; Creaser and Wicks, 2001). In France, following the banning of sodium arsenite, a large survey aimed at assessing the evolution of grapevine trunk diseases over a six-year period (2003 to 2008) showed that about 11% of vineyard areas were unproductive, due to vine decline and death (Kobès *et al*., 2005) associated with the presence of esca and Eutypa dieback. Both diseases were characterized by development of these pathogens in the woody tissues of the vines and by external symptoms on leaves and berries.

Eutypa dieback, also called dying-arm disease, is caused by an ascomycetous fungus, *Eutypa lata* Tul. & C. Tul., which penetrates into the plant via pruning wounds and then infects the woody tissues (Moller and Kasimatis, 1978). Several years after infection, typical wedge-shaped necroses can be observed in cross-sectioned cordons and trunks, which are associated with external cankers. These symptoms may also be associated with death of the spurs, cordons or entire grapevines. *Eutypa lata* produces various phytotoxins, some of which are transported by the transpiration stream towards the aerial part of the vine where the foliar symptoms can be seen (Moly-

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neux *et al*., 2002; Andolfi *et al*., 2011). After bud break, symptoms of stunted new shoots, which often have small, deformed chlorotic leaves and small fruit clusters can be observed.

The etiology of esca is more complex than that of Eutypa dieback. The term esca is generally used to designate a disease complex (Mugnai *et al*., 1999; Graniti *et al*., 2000; Surico *et al*., 2006). Presence of the vascular pathogens: *Phaeomoniella chlamydospora* (W. Gams, Crous & M.J. Wingf. & L. Mugnai) Crous & W. Gams, *Phaeoacremonium* spp. (mainly *P. aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai) (Surico, 2009*)* can cause typical foliar tiger stripe symptoms, recently termed "grape leaf stripe disease (GLSD)". The wood decay aspect of esca which is visible as a white rot inside the infected trunks and cordons is caused by the basidiomycete fungus, *Fomitiporia mediterranea* M. Fischer, in European countries (Fischer, 2006). In adult vines expressing typical tiger stripe symptoms on leaves, white rot in the wood tissues of the vines, is often associated with various other necroses (Mugnai *et al*., 1999; Surico *et al*., 2006; Calzarano and Di Marco, 2007; White *et al*., 2011). When GLSD is associated with wood decay, it represents the chronic form of "esca proper" (Graniti *et al*., 2000). Apoplexy, the sudden wilt of vines that occurs in summer, is generally considered as the severe (or acute) form of esca and terminates in partial or total vine death (Mugnai *et al*., 1999; Surico *et al*., 2006). This symptom can also be associated with both wedge-shaped necroses in the trunk or arm, and with a large proportion of non-functional tissue (Luque *et al*. *al*., 2012). Apoplexy is assumed to be caused, at least in part, by toxins of fungal origin (Letousey *et al*., 2010). In this paper, the term chronic esca to indicate GLSD or esca proper are used, without checking all the vines showing symptoms for the presence of white rot. The term of acute esca (apoplexy form) is used for the total wilt of vines recorded in summer. The term Eutypa dieback was used when the typical foliar symptoms were recorded, including stunting.

Even if trunk wood diseases constitute a major threat for vineyards, numerous key questions concerning the epidemiology of these diseases still remain to be investigated. For instance, there is no answers to relatively basic questions such as: how long can the infected vines survive? How is it possible to increase vine lifetime? It is, therefore, essential to carry out studies on the temporal dynamics of trunk wood diseases, including the ultimate stage, that of vine death. The two main obstacles to that come from the slow and cryptic development of internal necroses in the wood tissues, and the erratic foliar expression of the diseases. Discontinuity in symptom expression is a characteristic of both esca and Eutypa dieback. For esca, vineyard surveys over several years have shown that the symptoms fluctuate from one year to another, and that plants that express foliar symptoms one year do not necessarily express those symptoms the following year (Hewitt, 1957; Mugnai *et al*., 1999; Surico *et al*., 2000; Redondo *et al*., 2001; Di Marco *et al*., 2011; Marchi *et al*., 2006). For instance, Marchi *et al*. (2006) showed that 27% of vines that had previously expressed esca foliar symptoms did not express symptoms in any of the following five years. Several authors also reported discontinuity in foliar expression for Eutypa dieback (Le Gall, 1992; Creaser and Wicks, 2001; Dumot *et al*., 2004; Sosnowski *et al*., 2007). Even though the reasons for such fluctuations have not been fully established, several hypotheses can be advanced. Péros *et al.* (1999) suggested that variations in symptoms observed in the vineyard could be partly explained by the great genetic diversity in *E. lata* population added to its interaction with fungal communities in wood necroses. Foliar symptom fluctuation could also be attributed to particular interactions between toxic metabolites produced by the involved fungi, and/or to abiotic factors such as rainfall, temperature and grapevine management. According to Surico *et al*. (2000), Marchi *et al*. (2006) and Sosnowski *et al*. (2007), climatic factors presumably influence foliar expression for esca and Eutypa dieback. Marchi *et al*. (2006) showed that the number of vines that displayed foliar symptoms of esca throughout a growing season was directly related to the rainfall in May–June or in summer, depending on the vineyards. Surico *et al*. (2000) observed that cool and rainy summers favoured the expression of chronic esca, but that acute esca *i.e.* apoplexy, was more common during the hot summer period.

Currently, there are very few statistical studies about the relationships between esca or *Eutypa* dieback and mortality, either at field or plant level (Fussler *et al*., 2008; Péros *et al*., 2008). For six years, Surico *et al*. (2000) monitored esca and dead vines in vineyards planted with different cultivars. Before vine death, the plant varied in its annual frequency of symptom expression, which led the authors to conclude that the occurrence of external symptoms in

a given year was very difficult to predict. Although the vine dying process is complex, one way of studying trunk diseases could be to focus on the temporal relationship between esca or Eutypa dieback foliar symptoms and vine mortality at plant level. In this case, individual vine-death can be viewed as a rare, stochastic event, and the logistic models presented here offer particularly appropriate tools to determine the risk of vine death, since they are based on the occurrence of the symptoms over a period of several years preceding vine death. This approach could be a first step in encouraging the development of trunk disease-predicting models in order to improve short- and long-term vineyard management strategies. To achieve this, Stefanini *et al*. (2000), used data recorded in a field in Italy and applied a logistic multimonial model to evaluate the conditional probability that a vine would show symptoms or die, based on the presence of symptoms in the previous year and on the presence of symptoms in the neighbourhood.

The objectives of the present paper are to study the relationships between esca foliar or Eutypa dieback foliar expression and vine mortality in six vineyards, planted with cv. Cabernet Sauvignon, located in different areas of the Bordeaux region. Individual vine recordings over seven years were used to determine the percentage of vine death, whether the vines had previously expressed foliar symptoms or not. As a first step in our modelling approach, logistic regression analysis is used to determine the probability of death based on previously occurring foliar symptoms. A sample of vines that had previously expressed esca or Eutypa dieback was used to verify and to quantify internal necroses. The statistical results are discussed in the light of necrosis analysis.

Materials and methods

Vineyard survey

Esca, Eutypa dieback and mortality were monitored in six different vineyards, belonging to several owners in the Bordeaux region, for seven consecutive years, from 2004 to 2010. The vineyards were selected since they did not show symptoms of the other main causes of dieback: viral diseases and *Armillaria* diseases. All vineyards were planted with the cultivar Cabernet Sauvignon (*Vitis vinifera* L) between 1986 and 1989 and were trained in accordance with the Guyot method. The vines were grafted onto various rootstocks (101-14 Mgt, 3309 Couderc, and SO4) depending on the vineyard (Table 1). In May/June of each year, about 2,000 contiguous vines from each vineyard were individually surveyed for typical foliar symptoms of Eutypa dieback, including the stunting of new shoots with chlorotic, small and cupped leaves, and vines or cordons with no vegetation were recorded as dead vines. At the end of August, the vines expressing chronic esca and those expressing acute esca symptoms were also recorded.

Data management and analysis

For each year, observations from each individual recorded plant were classified into eight categories: chronic esca, acute esca, Eutypa dieback, chronic or acute esca and Eutypa dieback, dead cordon, dead vine, asymptomatic adult vine and asymptomatic young vine. The data were used to calculate the annual disease incidence of chronic esca, defined as the ratio between the number of vines exhibiting chronic esca symptoms in a given year, and the number of vines alive the year before in the vineyard, multi-

Table 1. Characteristics of the six monitored vineyards located in the Bordeaux region (France).

Vineyard	Region	Commune	Year of planting	Rootstock	Vine number
1	Pessac Léognan	Martillac	1989	101-14	2000
2	Graves	Castres	1989	3309 Couderc	2366
3	Pessac Léognan	Gradignan	1989	3309 Couderc	2040
4	Libournais	Galgon	1987	3309 Couderc	1993
5	Graves	Beautiran	1989	-	2028
6	Entre-deux-mers	Espiet	1986	SO4	2050

plied by 100. The annual incidence of other disorders was also calculated: acute esca, Eutypa dieback, and newly dead vines (cordons or entire vines). The Spearman correlation test was applied to study the strength of relationship between esca incidence in year n and death incidence in year n+1.

The percentage of dead vines (cordons or entire vines) was calculated in each of six vineyards, for 2008, 2009 and 2010, using numbers of vines that had previously expressed esca symptoms (chronic or acute) at least once, from 2004 to 2007, to 2008 and to 2009, respectively. A similar procedure was applied for previously asymptomatic vines for the same respective periods. The same method was also used to determine the percentage of dead vines for vines expressing Eutypa dieback symptoms in each of three vineyards that showed the highest level of Eutypa dieback incidence. Logistic regression analysis was used to determine vine death risk in each vineyard for each of the three years 2008, 2009 and 2010 on the basis of previously occurring foliar symptoms (esca and Eutypa dieback) for the four previous years, from 2004 to 2007, from 2005 to 2008, from 2006 to 2009, respectively. As esca and Eutypa dieback foliar symptoms were always recorded on adult vines, the data set contained observations that were restricted to adult plants, in order to homogenize the set. To determine vine death risk in a vineyard in 2008, according to esca symptoms recorded in the preceding years, the observations were classified annually during the 2004-2007 period, as either asymptomatic or esca symptomatic (chronic or acute form). Other observations, including symptomatic Eutypa dieback or dead vines for any year before death, were removed from the data set. For 2008, each observation was classified as either of two categories, living or dead (cordon or entire vine). In order to compare results for each of the three years (2008, 2009 2010), death risk was modeled using the data set which included symptoms for the four preceding years. The data set was prepared for each year of death and each vineyard. Data management was also carried out to determine vine death risk according to Eutypa dieback symptoms in the three vineyards showing the highest level of Eutypa dieback incidence.

For each year and each vineyard, multiple binary logistic regression was used to identify the years in which foliar symptoms were significantly associated with vine death. This form of regression is used when the dependent variable Y is dichotomous (usually Y encodes the presence/absence of an event such as vine death in the given year in this case) and the independent variables (also called covariates) $X_1,\ldots,X_k$ are of any type (Hosmer *et al*., 1989). The method proposes a model for the probability of occurrence of an event occurring given the values of the covariates:

$$P(Y=1|x_1\cdots x_k)=\frac{e^{(\beta_0+\beta_1 x_1+\cdots+\beta_k x_k)}}{1+e^{(\beta_0+\beta_1 x_1+\cdots+\beta_k x_k)}}.$$

Coefficient β_0 is the intercept and e^{β_0} estimates the probability of death without symptoms when there is globally weak prevalence (in this case, with approximately 5% of dead vines). The impact of the covariate x_j on the probability of occurrence is measured through e^{β_j}, which is an odds ratio (OR). In the context of weak global incidence, the OR could be interpreted as a relative risk. In our study, when an OR for the variable "Symptom in a given year X (S0X)" was equal to two, this meant that the probability of death occurring was double that of vines without such symptoms. An OR significantly greater than one indicated that symptoms in a given year represented a risk factor. The interaction between years was also tested. Interaction terms between the statistically significant variables ''Symptom in a given year'' were added to the main effects model and were retained if they were statistically significant ($P<0.05$). For a significant interaction between the variable S0X (variable « Symptom in the year X ») and S0Y (variable « Symptom in the year Y »), the corresponding OR was the product of the ORs of S0X, S0y and S0XxS0Y. In order to select the covariates and then their interactions, a manual backward method was used.

Necrosis analysis

Twenty scions of vines expressing chronic esca symptoms or Eutypa dieback foliar symptoms in 2004 were collected in February 2005 from the surveyed vineyards, in order to quantify cases of internal necrosis. The trunks and cordons were cut into sections and analysed in accordance with the method of Maher *et al*. (2012). Three types of necrosis were considered: (i) sectorial brown necrosis, (ii) total necrosis, that included sectorial and central necroses, and (iii) white rot, characterised by yellow or white soft rot. The relative area covered by these necroses was calculated by dividing the necrotic area by the total area of the wood section. The altered

perimeter, corresponding to the external perimeter of sectorial necrosis or total necrosis, was also measured. The relative perimeter length was calculated by dividing the altered perimeter by the total perimeter of the wood section. The Wilcoxon rank test was used to compare internal necroses in the cordon alone and in the whole scion between symptomatic and asymptomatic grapevines and between the two types of symptoms (esca and Eutypa dieback). That non-parametric statistical test is used to compare two independent samples. All statistical analyses were performed with R 2.8.1 software (R Development Core Team, 2008).

Results

The annual incidence of disease symptoms and dead vines over time

The annual esca (acute and chronic form) incidence varied from 1.4% to 16.8% depending on the vineyard and the year (Figure 1). The chronic form, observed more frequently than the acute form, reached its highest level in 2007 in all six vineyards. For most of the vineyards, the lowest incidence for both forms was observed in 2005. The incidence of Eutypa dieback also varied between 0 and 14.4% according to the vineyard and the year. It was highest in vineyards 4, 5 and 6, reaching greater values than those of esca in vineyard 6. The incidence of Eutypa dieback, like that of esca, was also highest in 2007, except for vineyard 5. The proportion of plants expressing both types of symptoms for a given year was very low, varying from 0 to 2.3% (data not shown). The incidence of partially or totally dead vines differed according to the vineyard and the year, not always showing proportionally similar numbers of vines expressing esca or Eutypa dieback. The incidence of dead vines was usually less than that of vines expressing esca and / or Eutypa dieback, but increased during the last three years (2008, 2009 and 2010) in vineyards 4 and 6. For all six vineyards, the incidence of newly dead vines increased in the last three years, with a peak in vine mortality in 2008 being preceded by a peak of esca symptoms in 2007. The Spearman rank correlation test indicated a significant correlation ($P<0.01$) for the incidence of esca symptoms in year n and the incidence of dead vines in year n+1 ($r = 0.78$). The percentage of partially dead vines (vines with one dead cordon) was higher than that of totally dead vine.

Mortality according to symptom expression

Using data from the seven-year individual vine recordings, the incidence of dead vines and dead cordons in 2008, 2009 and 2010 was determined according to whether or not esca symptoms had been expressed at least once in the previous four, five or six years respectively (Figure 2). The average incidence of dead vines among the vines that had previously expressed esca varied from 7.5 to 41.5% (Figure 2A), in the different vineyards. The lowest percentage was found for vineyard 1 and the highest for vineyard 4. For all the vineyards, the percentage of dead vines in 2008 that had previously expressed esca was higher than or equal to, death in the other years.

Although the dead vine incidence among previously asymptomatic vines was lower than that among vines previously expressing esca symptoms, it reached 9% of the asymptomatic vines in vineyard 6 in 2010 (Figure 2B). Similar variations were observed between vineyards; when the dead vine incidence for vines that had previously expressed symptoms was high, the asymptomatic dead vine incidence was also high.

In three of the six vineyards with a high incidence of Eutypa dieback, the percentage of dead vines among the vines expressing Eutypa dieback symptoms varied between 9.4 and 45.5%, depending on the vineyard (Figure 3A), with the lowest incidence of dead vines being found in vineyard 5. The percentage of dead vines in 2008 that had previously expressed Eutypa dieback symptoms was higher than in the other years. The dead vine incidence among previously asymptomatic vines was lower than that among vines previously expressing Eutypa dieback symptoms and, just like esca, it also reached 9% of the asymptomatic vines in vineyard 6 in 2010 (Figure 3B). Vineyard 5 was characterized by low incidence of dead vines among vines that had previously expressed foliar symptoms and by low dead vine incidence for asymptomatic vines.

Previous years of symptomatic expression associated with mortality

Esca foliar symptoms

The multiple binary logistic regression model was used to select, from among the four variables for a previous year of esca foliar expression (acute or chronic), those associated with mortality in 2008,

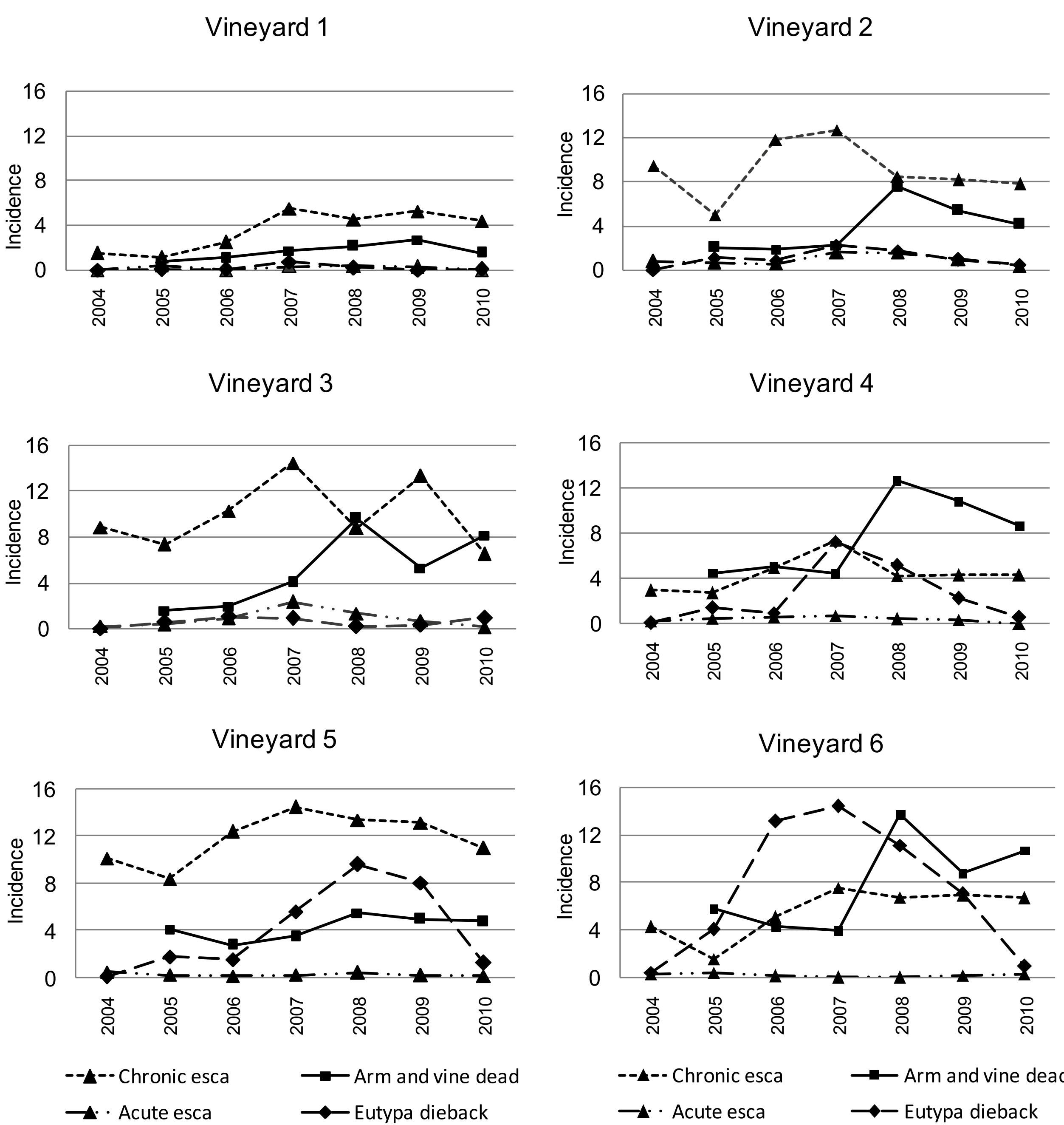


Figure 1. Annual incidence of chronic and acute esca of grapevines, Eutypa dieback foliar symptoms, and dead vines (cordon or trunk) from 2004 to 2010 in the 6 surveyed vineyards.

2009 or 2010 for the six studied vineyards. The results are presented in Table 2.

The value in the Odds Ratio column for the intercept does not indicate an OR, but gives the probability of death for a vine without symptoms in the previous four years, and corresponds to the death incidence shown in Figure 2B. Although the probability of death for vines that had not expressed

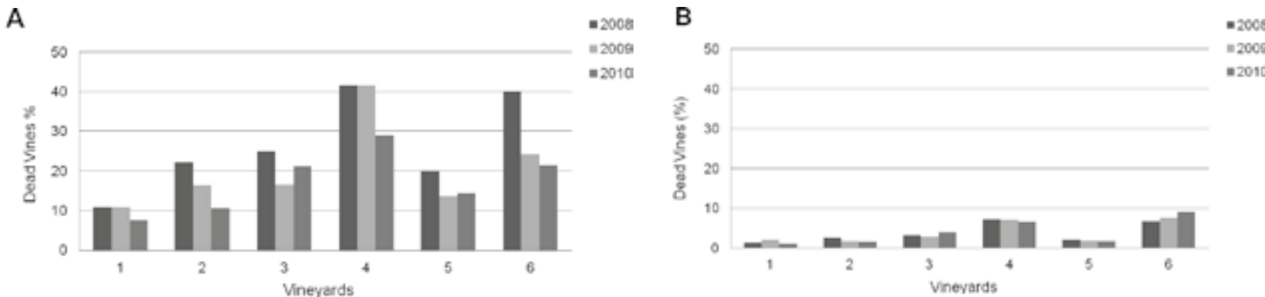
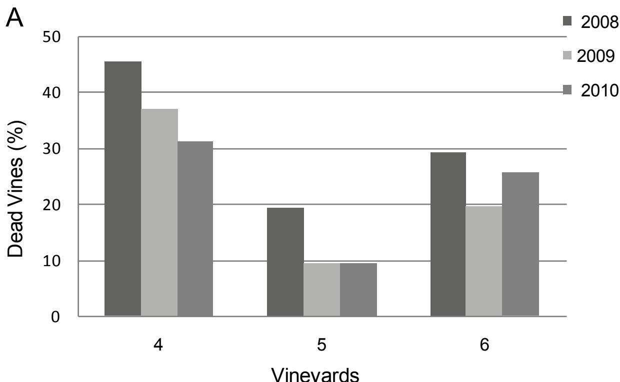


Figure 2. Percentage of dead vines in 2008, 2009 and 2010 among vines either expressing esca symptoms previously (A), or among asymptomatic vines previously (B), in all six vineyards.



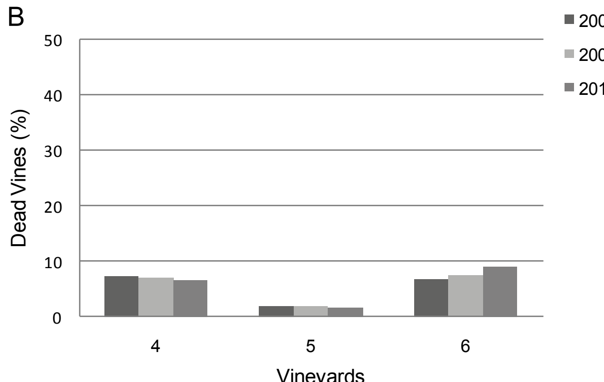


Figure 3. Percentage of dead vines in 2008, 2009 and 2010 among vines either expressing Eutypa dieback foliar symptoms previously (A), or among asymptomatic vines previously (B), in all three vineyards.

symptoms was relatively constant for all three years, most differences were associated with the particular vineyards. The number of selected variables varied between the models from one to four. The presence of symptoms in one of the previous years always increased the probability of death; the year of esca foliar expression preceding vine death was always selected except for the death risk model in 2010 in vineyard 6 and the death risk model in 2009 in vineyard 1 (Table 2). The OR concerned, which varied between 3.3 and 11.3, was generally highest, in comparison with the other selected variables, except for the death risk model in 2008 in vineyard 6.

The variables that were selected for certain models, and which indicated an interaction between two years of foliar expression, had an OR estimate of less than one. This means that the probability of death occurring for vines expressing foliar symptoms for those two years did not show any increase attributable to symptom accumulation. Since, the regression model used esca chronic symptoms (not the acute form) occuring in the years preceding vine death as explanatory variables, and as those results were similar to those presented in Table 2, they have not been presented here.

Eutypa dieback symptom

The results from multiple logistic regressions that were computed with data from the three vineyards showing the highest level of Eutypa dieback incidence, with dead vines as the variable of interest and the symptoms of Eutypa dieback as the explanatory variable are shown in Table 3.

The models are more parsimonious than those used for esca, since only one or two explanatory variables for the previous year of Eutypa dieback foliar expression were selected, except in the case of vineyard 6, for the death year 2008. The OR varied from 2.2 to 11.8. Once again, OR was highest for the variable year preceding death, except in the case of vineyard 5, for vine death in 2010, with the highest OR

Table 2. Odds ratio (Estimate and 95% Confidence Intervals) from the best-fit binary regression logistic model that predicted the risk of vine death in 2008, 2009 or 2010 based on esca symptoms (chronic or acute form) in the previous four years (i.e. S07 = symptoms in 2007) for the six vineyards. *P<0.01, **P<0.001, ***P<0.0001.

Vineyard	Year of vine death	Variable	Significance	Odds ratio	
				Estimate	95% CI
1	2008	Intercept	***	0.014[a]	*0.008-0.020*
		S06	**	5.802	2.025-16.583
		S07	**	4.658	1.753-11.141
	2009	Intercept	***	0.022	*0.015-0.030*
		S05	***	9.011	3.207-23.299
		S07	***	4.598	2.150-9.166
	2010	Intercept	***	0.010	*0.006-0.016*
		S09	***	11.308	5.263-23.782
2	2008	Intercept	***	0.031	*0.023-0.040*
		S04	***	3.787	2.453-5.819
		S06	***	2.569	1.663-3.947
		S07	***	3.847	2.520-5.866
	2009	Intercept	***	0.018	*0.012-0.025*
		S06	***	4.032	1.944-8.120
		S07	***	5.801	2.916-11.281
		S08	***	9.622	4.802-18.719
		S06×S08	*	0.368	0.137-1.001
		S07×S08	**	0.211	0.080-0.563
	2010	Intercept	***	0.016	*0.011-0.024*
		S06	**	2.459	1.420-4.230
		S08	***	7.700	3.847-15.047
		S09	***	8.523	4.454-16.140
		S08×S09	***	0.101	0.038-0.268
3	2008	Intercept	***	0.035	*0.0260-0.046*
		S04	***	3.784	2.400-5.919
		S05	***	2.769	1.696-4.466
		S06	***	2.184	1.393-3.390
		S07	***	4.234	2.824-6.345
	2009	Intercept	***	0.033	*0.024-0.043*
		S06	*	2.026	1.197-3.596
		S07	***	3.194	1.912-5.284
		S08	***	3.337	1.974-5.582
	2010	Intercept	***	0.043	0.032-0.056
		S07	***	5.819	3.117-10.439
		S09	***	6.165	3.927-9.631
		S07×S09	***	0.237	0.110-0.524
4	2008	Intercept		0.075	*0.060-0.092*
		S04	***	3.532	1.626-7.666
		S05	**	2.591	1.250-5.349
		S06	**	2.355	1.310-4.161
		S07	***	5.985	3.845-9.256
	2009	Intercept	***	0.073	*0.058-0.091*
		S06	***	6.498	3.371-12.427
		S07	***	4.868	2.495-9.190
		S08	***	8.903	4.528-17.144
		S07×S08	**	0.218	0.069-0.701
	2010	Intercept	***	0.072	*0.057-0.090*
		S06	*	2.750	1.203-5.960
		S07	***	2.817	1.506-5.093
		S09	***	4.716	2.597-8.378
5	2008	Intercept	***	0.019	*0.012-0.029*
		S06	***	10.676	3.984-25.807
		S07	***	10.108	5.425-18.939
		S06×S07	*	0.253	0.085-0.811
	2009	Intercept	***	0.021	*0.013-0.032*
		S05	*	2.944	1.174-6.898
		S06	***	2.743	1.301-5.597
		S08	***	5.622	3.011-10.537
	2010	Intercept	***	0.022	*0.013-0.034*
		S08	*	6.170	2.312-14.871
		S09	***	7.010	3.257-14.724
		S08×S09	***	0.224	0.071-0.764

(*Continued*)

Table 2. *Continues.*

Vineyard	Year of vine death	Variable	Significance	Odds ratio	
				Estimate	95% CI
6	2008	Intercept	***	0.072	0.055-0.092
		S04	***	5.708	2.723-12.028
		S05	*	3.123	1.053-9.125
		S06	***	4.584	2.402-8.654
		S07	***	3.273	1.917-5.473
	2009	Intercept	***	0.079	*0.006-0.101*
		S06	***	2.782	1.067-6.599
		S08	***	4.332	2.416-7.561
	2010	Intercept	***	0.094	*0.073-0.119*
		S06	***	3.780	2.242-6.244

[a] Intercept estimate means probability of death but without previous symptoms.

Table 3. Odds ratio (Estimate and 95% Confidence Intervals) for the best-fit binary regression logistic model that predicted the risk of vine death in 2008, 2009 or 2010 based on Eutypa dieback symptoms in the previous four years (i.e. S07 = symptoms in 2007) for the three vineyards. **P*<0.01, ***P* <0.001, ****P*<0.0001.

Vineyard	Year of vine death	Variables	Significance	Odds ratio	
				Estimate	95% CI
4	2008	Intercept	***	0.074[a]	*0.059-0.091*
		S07	***	11.417	7.445-17.525
	2009	Intercept	***	*0.073*	*0.057-0.091*
		S07	***	3.822	1.994-7.111
		S08	***	5.604	2.946-10.470
	2010	Intercept	***	*0.072*	*0.056-0.090*
		S07	***	4.547	2.280-8.647
		S09	***	7.708	2.748-21.408
5	2008	Intercept	***	*0.021*	*0.014-0.032*
		S07	***	11.804	5.857-23.346
	2009	Intercept	***	*0.011*	*0.006-0.020*
		S05	*	8.610	1.154-48.307
		S08	***	9.056	3.726-20.190
	2010	Intercept	***	*0.016*	*0.009-0.027*
		S08	***	7.828	2.841-19.952
		S09	**	5.806	1.807-16.054
		S08×S09	*	0.151	0.030-0.788
6	2008	Intercept	***	*0.068*	*0.051-0.088*
		S05	**	2.860	1.348-5.960
		S06	***	4.304	2.238-7.952
		S07	***	5.035	3.003-8.307
		S07×S06	*	0.321	0.136-0.776
	2009	Intercept	***	*0.076*	*0.058-0.097*
		S07	**	2.170	1.271-3.637
		S08	***	3.014	1.791-5.005
	2010	Intercept	***	*0.104*	*0.081-0.131*
		S07	***	2.768	1.727-4.370
		S09	***	3.937	2.395-6.394

[a] Intercept estimate means probability of death without previous symptoms.

being found for the variable S08 (symptoms in 2008). The model also selected the variable indicating interaction between the years of foliar expression, with OR being estimated at less than one.

Necrosis in vines that expressed esca symptoms or Eutypa dieback

The relative area covered by total necrosis was significantly (*P*<0.001) higher in vines expressing Eu-

Table 4. Percentage of the total cross-sectional area of wood corresponding to each type of necrosis, in the cordon alone and in the whole scion of the vine expressing chronic esca foliar symptoms or Eutypa dieback symptoms. Values are means, Minimum and Maximum values of 10 vines with *P* values (Wilcoxon test , *P<0.01, **P<0.001, ***P<0.0001).

Type of necrosis	Position of the necrosis	No. of vines						*P* value	
		Chronic esca foliar symptom			Eutypa dieback				
		Mean	Min	Max	Mean	Min	Max		
Total necrosis	Cordon	25.7	1.2	71.7	54.8	37.5	73.9	0.0005	***
	Scion	20.5	6.0	38.5	40.4	26.0	64.0	0.0007	***
Sectorial brown necrosis	Cordon	20	0	55.3	51.1	35.5	66	0.0002	***
	Scion	14.7	0	36.1	37.1	26.6	60.3	0.001	**
Altered perimeter	Cordon	34.0	8.2	84.3	60.1	40.0	100	0.006	**
	Scion	25.4	5.5	46.2	42.4	22.9	64.8	0.02	*
White rot	Cordon	3.3	0	46.3	1.5	0	10.4	0.09	
	Scion	4.4	0.04	19.8	1.2	0	7.2	0.04	*

typa dieback foliar symptoms than in vines expressing esca foliar symptoms (Table 4). In both types of diseased vines, cordons showed the highest amount of necrosis and percentage of altered perimeter. In vine cordons expressing Eutypa dieback, over half of the wood tissue was affected by necroses, mainly sectorial brown necrosis. Consequently, the percentage of altered perimeter was greater in vines showing Eutypa dieback symptoms than vines showing esca symptoms. Sectorial brown necrosis was always found in vines expressing Eutypa dieback, but this was not the case for esca vines. Of all vines showing esca symptoms, white rot affected 3.3% and 4.4% of the cross-section surface in cordon and total scion, respectively. The relative area covered by white rot was significantly (P<0.05) lower in Eutypa dieback vines than in esca vines.

Discussion

The logistic models developed to compare the risk of vine death in six vineyards in 2008, 2009 and 2010 revealed great differences in the incidence of esca, Eutypa dieback and dead vines. Within each vineyard, a relative constancy of foliar expression was noticed, despite the annual fluctuation of esca incidence over the seven years. Each vineyard was characterized by its specific sanitary status in response to such multiple factors as wood pathogens, rootstock, climatic and soil factors and vineyard management practices over time (Mugnai *et al.*, 1999, Surico *et al.*, 2006). High rates of esca symptoms occurring in vineyards during the same year could be associated with similar climatic conditions occurring in all the vineyards in the same region. Indeed, a strong positive correlation was found within each vineyard between the sum of rain over the period May-August, and the incidence of vines expressing esca (chronic and acute) (data not shown). These results corroborated those of Marchi *et al.* (2006) who reported that variations in esca-foliar expressions were related to rainfall in spring and summer. As with esca, high rates of foliar symptoms of Eutypa dieback were noticed for the same year and were equally associated with the same climatic conditions.

The high correlation between the incidence of esca in year n, and the percentage of dead vines in year n+1, was consistent with the results of logistic regression. The models showed that the occurrence of foliar symptoms in one of the previous years always increased the probability of vine or cordon death. Stefanini *et al.* (2000) also estimated a greater probability of death for a vine that had expressed esca symptom the year preceding death compared with a vine that had been asymptomatic. They showed that

the variable 'sanitary state in the close vicinity" did not improve the conditional probability of the occurence of death, contrary to the occurrence of esca symptom expression. Generally, the models developed in this study also selected one or two earlier years of expression. The results of the logistic model explaining that death depended on the expression of esca symptoms was the same whether the explanatory variables used concerned either the chronic form of esca or the two forms (acute and chronic). This can be attributed to the low incidence of acute esca vines in each of the studied vineyards. Acute esca occurs when particular climatic conditions are met, which was not the case during the present survey period. It should be noted that sometimes OR confidence intervals were not precise, particularly when the corresponding incidence of symptomatic vines was low, since it was not possible to obtain accurate values in this case.

In this study, mortality risk estimated for vines that had expressed foliar symptoms at the field level proved consistent with the results obtained by Péros *et al*. (2008) and Fussler *et al*. (2008).

When Eutypa dieback symptoms were used as the explanatory variables to determine the vine death risk, the number of selected variables in the model was generally lower (one or two) than for esca (two or three). These results suggest that vine death was more rapid when it expressed Eutypa dieback than when it expressed esca. This could be due to the necrosis results since the amount of necrosis and the percentage of altered perimeter in cordon and in total scion were significantly higher in vines showing Eutypa dieback symptoms than in those showing esca foliar symptoms. Two hypotheses can be advanced. First, the higher level of non-functional tissue stops the xylem flow towards the buds during the year after symptom expression. Second, a higher level of greater fungi-toxin production occurs in Eutypa dieback than in esca, with toxins being transported by the sap towards the herbaceous parts of the plant (Andolfi *et al*. 2011). The metabolites produced by *Eutypa lata* also react at the point of production by disrupting the vascular tissue and inhibiting the transport of nutrients (Mahoney *et al*., 2005). Therefore, the toxins can act either at bud burst stage or before, thereby preventing vegetation growth. Whether these toxins are only produced by *E. lata* is still the subject of debate, since Botryosphaeriacaea spp. can also be isolated from the sectorial necroses of vines expressing Eutypa dieback (Luque *et al*., 2009; Lecomte, personal communication).

This study also showed that vine death occurred even if the vines had not expressed foliar symptoms over a period of four to six years. The death of asymptomatic vines could also be related to grapevine cankers caused by different species of Botryosphaeriaceae or Diatrypaceae (Trouillas *et al*., 2010; Úrbez-Torres *et al.,* 2011). One of the surveyed vineyards showed large sectorial necroses, in cross-sectioned cordons, associated with high isolation levels for *E. lata* and Botryosphaeriaceae species (data not shown) without previous foliar symptoms of Eutypa dieback or esca. In addition, individual vineyards showed that when high dead vine incidence occurred among vines that had previously expressed symptoms, it also occurred among asymptomatic vines. These findings suggest that, in both cases, the causes of vine death are similar, but need further elucidation by comparing the wood microflora of previously symptomatic or asymptomatic dead vines. Moreover, these findings could also mean a strong vineyard effect. Each particular vineyard is characterized by its particular environmental (biotic and abiotic) and cultural practices, such as its pruning system which is thought to be a risk factor for esca and *Eutypa lata* (Dumot *et al*., 2004; Surico *et al*., 2006). Studies are currently being undertaken to identify the different factors associated with esca disease and vine death.

The occurrence of white rot in all vine samples having previously expressed foliar symptoms of are indicative of the disease "esca proper". However, white rot was also found in vines that expressed foliar symptoms of Eutypa dieback. These observations show once again the difficulty of characterizing diseases by symptoms when different internal diseases are co-occurring in the same vine (Mugnai *et al*., 1999; Luque *et al*., 2009; White *et al*., 2011).

The comparison of the relative risk of vine death after disease expression between vineyards showed different risks of vine death. Surprisingly, the incidence of vine death was sometimes very low despite the high incidence of esca. This meant that a large number of vines were able to recover after symptom expression, raising the question of the capacity of vine resilience or else the hidden development of the disease, defined by Marchi *et al*. (2006). This capacity to recover after esca symptoms was particularly marked in vineyard 5 which showed a high inci-

dence of esca expression combined with a low mortality. An analysis of the cultural factors should be carried out to highlight the causes associated with that situation. More generally, further studies will be necessary to identify the different factors involved in grapevine trunk diseases explaining differences between vineyards and annual fluctuation of their expression. Other factors, such as vine physiology and climatic conditions, should be taken into account in order to provide an improved understanding and explanation of the risk of vine death.

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RESEARCH PAPER

In vitro cultures of *Vitis vinifera* L. cv. Chardonnay synthesize the phytoalexin nerolidol upon infection by *Phaeoacremonium parasiticum*

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Summary. This study investigated terpene synthase (TPS) activity and terpene antifungal metabolites in calluses and cell suspension cultures of *Vitis vinifera* cv. Chardonnay infected with *Phaecremonium parasiticum*, one of the fungi associated with the grapevine diseases known as “hoja de malvón” and young vine decline. The highest TPS activity, assessed as tritiated farnesyl pyrophosphate ([$^{1-3}$H]-FPP) transformed into hexane-soluble radioactive products, was observed in both inoculated calluses and cell suspension cultures (CSC). When tested in inoculated cell suspension cultures the TPS activity was maximal at 8 h after [$^{1-3}$H]-FPP application and then declined; this was associated with a temporary increase of the sesquiterpene nerolidol. Grape calluses produced: α-pinene, nerolidol and squalene whether or not they were inoculated with *Pm. parasiticum.* As fungal amount raised the relative concentration of α-pinene and nerolidol increased in respect to squalene in calluses. The TPS activity and nerolidol and α-pinene accumulation was correlated with the increase in the amount of inoculated fungus. Of the mentioned metabolites mainly squalene was identified from extracts of fungal cultures. The results suggest that the response of grapevine tissues to *Pm. parasiticum* is dependent on the pathogen concentration and is characterized by increasing TPS activity through *de novo* synthesis.

Key words: grapevine, grapevine trunk diseases, terpene, terpene synthase activity.

Introduction

The grapevine trunk diseases known as “hoja de malvón” (which literally means “geranium like vine leaf”) and “young vine decline” have been observed by Argentinean grape growers since the beginning of the last century. These diseases became important in the last two decades when serious damages and important losses of vine yield were found in many wine regions of Argentina (Gatica *et al*., 2000). They are associated with an array of causal agents identified as a fungi complex where *Phaeoacremonium parasiticum*, is one of the most prevalent (Gatica *et al*., 2000 and 2001; Dupont *et al*., 2002; Gatica *et al*., 2004; Lupo *et al*., 2006). *Phaeoacremonium* spp. are plant pathogens localized in vascular tissues that cause stunted growth, wilting and dieback of various woody hosts, especially grapevines. *Pm. parasiticum* was first isolated from

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diseased grapevine in Mendoza, Argentina (Dupont *et al*., 2002), and subsequently reported in grapevines of Australia, Iran, South Africa and USA (Mostert *et al*., 2006), Chile (Auger *et al*., 2005), Spain (Aroca *et al*., 2006) and Peru (Romero Rivas *et al*., 2009).

Plants have developed different strategies to defend themselves against biotic stress. Among them are the low molecular defensive compounds phytoalexins, produced by secondary plant metabolism (Croteau *et al*., 2000). Knowledge of biosynthesis of phenylpropanoid and terpenoid phytoalexins in different plant tissues provides insights into the mechanisms utilized by plants in regulating these biochemical pathways leading to defense towards pests and diseases (Del Río *et al*., 2001; Amalfitano *et al*., 2009; Degenhardt *et al*., 2009). In this regard, however, there is no information about metabolites effective against *Pm. parasiticum* and little is known about terpenic phytoalexins useful against other xylophagous fungi of woody plants.

It is well known that terpenes are the largest group of plant natural products, comprising at least 30,000 compounds (Croteau *et al*., 2000), with the widest assortment of structural types. Terpenoids form an ample range of structurally related cyclic and acyclic mono-, sesqui-, and diterpenes synthesized by a specific family of enzymes, the terpene synthases (TPS) (Back and Chappell, 1995; Tholl, 2006). These catalysts convert the acyclic prenyl diphosphate and squalene into a multitude of cyclic and acyclic forms, which constitute an essential part of direct and indirect defense systems in ecological interaction against pathogens (Vögelli and Chappell, 1988; Bianchini *et al*., 1999). Studies in several plants show an increase of one type of TPS activity, that of sesquiterpene synthase, and the subsequent accumulation of sesquiterpenes as plant response to pathogen attack (Chappell and Nable, 1987; Choong, 2008). So far there are no effective and environmentally friendly control methods against grapevine trunk diseases, and wine manufacturers' and consumers' negative perception of treatment with synthetic chemicals makes it important to devise alternative methods of control consistent with international regulations controlling organic agriculture (http://www.nal.usda.gov/afsic/pubs/OAP/OAPGuide2.shtml).

On the other hand, attempts to investigate plant defense responses are complicated because the interactions are frequently localized to a few cells and thus the amount of biological material available to the sample is limiting. Also, when investigating an intact host-pathogen system it is often difficult to discern if a response is derived from the plant or from the pathogen. In this way, calluses and cell suspension cultures allow both to analyze cell responses against the pathogen and to compare metabolic changes individually.

Based on above, we hypothesized that grapevine modifies the terpene pathway as defensive responses against *Pm. parasiticum* through production of terpenic phytoalexins. The objectives of this study were to investigate TPS activity and to identify terpene-like antifungal metabolites in *Vitis vinifera* L. cv. Chardonnay calluses and cell suspension cultures exposed to *Pm. parasiticum*.

Materials and methods

Fungal strain

The *Phaeoacremonium parasiticum* used in this work was isolated from wood tissues of diseased grapevines cultivated in Mendoza, Argentina. The fungus was identified according to morphological and molecular characters (Dupont *et al*., 2002) and maintained in glycerol/water solution (10%, v:v), at -20º C in the EEA INTA-Mendoza's fungal collection. For the experiments, *Pm. parasiticum* were maintained on Petri dishes on semi-solid malt extract agar (OXOID Ltd., Basingstoke, Hampshire, England), in a growth chamber at 25 ± 2º C in darkness until the dish surface was partially covered with the fungus. From there, conidial suspensions were prepared in sterile distilled water, and adjusted to 10^7, 10^8 and 10^9 conidia mL^{-1} using a Neubauer counting chamber.

Generation of calluses and cell suspension cultures from *in vitro* plants

Autumn collected vine cuttings (25 cm long) from a vineyard of *Vitis vinifera* L. cv. Chardonnay selected after virus-free indexation were kept in a cold chamber at 5ºC in darkness for 3 weeks. After bud break the cuttings were treated with 100 mg L^{-1} indole-3-butyric acid (IBA, Sigma-Aldrich, St. Louis, MO, USA) for 24–31 h and kept in darkness for root promotion. In order to promote further root and shoot development the cuttings were moved into 2 L pots filled with a mixture of grape compost: sand: vermicompost (7:2:1), disinfected with 500 mg L^{-1} of

streptomycin sulphate, watered weekly with nutrient solution and maintained in a greenhouse with 23 ± 3°C, 40/70 ± 10% RH, and a 16 h photoperiod. After 90 d the plants were dissected in uninodal segments in a laminar flow hood. The explants obtained were surface-sterilized with 70% ethanol 3 min and 0.6% sodium hypochlorite solution (containing Tween-20) for 15 min, and then rinsed 3 times with sterile distilled water. After that, the explants were transferred into glass tubes containing 20 mL of semi-solid Murashige and Skoog culture medium at pH 6.5 (MS; 1962) with 3% sucrose and 0.8% bacteriological agar (OXOID Ltd., Basingstoke, Hampshire, England). The *in vitro* plants were obtained from two consecutive generations of 30 d apical bud explants as follows. Selected apical bud explants were put into glass jars containing 60 mL of half-diluted macronutrients and micronutrients and complete vitamins and iron modified MS medium, plus 10 μg L^{-1} biotin and 5 μg L^{-1} naphthalene acetic acid (NAA, Sigma-Aldrich Inc, St Louis, MO, USA), adjusted to pH 6.5. The culture conditions were maintained at 25 ± 2ºC with 16 h photoperiod of 120 μE m^{-2} s^{-1} provided by white fluorescent lamps. The plants were then used for obtaining calluses and cell suspension cultures.

Apical buds, petioles, and first and second expanded leaves from the *in vitro* plants were collected after 15 d. The leaves were sliced into 3 × 4 mm square pieces, discarding their margins. The explants were placed in glass tubes containing 3 mL of MS medium pH 6.5 with 3% sucrose and 0.8% agar. To improve the calluses quality three combinations of both plant growth regulators, NAA and BA (6-benzyladenine, Sigma-Aldrich Inc, St Louis, MO, USA): 1:1 mg L^{-1}, 5:1 mg L^{-1}, and 2.5:0.5 mg L^{-1}, were assessed. The tubes were sealed with plastic film and maintained in growth chamber at 25 ± 2º C in darkness. After 30 d calluses were evaluated for formation and friability, and the dry weight (DW) was obtained after drying at 60°C until a constant weight was achieved.

Yellowish calluses originated from petioles and subcultured twice (35 d each subculture) in MS medium with NAA and BA 5:1 mg L^{-1} (see above), were transferred to 250 mL flasks containing 70 mL of pH 6.5 MS medium to initiate a set of cell suspension cultures. The cell suspension cultures were maintained and sub-cultured with the MS medium supplemented with NAA and BA, as previously explained, for calluses induction and sub-culture. Then the cultures were incubated in darkness on a rotary shaker at 100 rpm at 25 ± 2ºC to improve quality of the suspensions. The resulting suspension culture consisted of homogeneous single cells and small cell aggregates and therefore were considered of good quality. The viability and the quality of the cell suspensions were monitored by microscopic observations and incubated in semi-solid media in controlled conditions, in order to detect contamination.

Experiments with calluses

Calluses from petioles obtained after two subcultures of 35 d each in MS medium with NAA and BA 5:1 mg L^{-1} (see above) were inoculated with 2 μL of 10^7, 10^8, 10^9 conidia mL^{-1} and 10 μL of 10^9 conidia mL^{-1} suspension of *Pm. parasiticum* and incubated 48 h in a growth chamber at 25 ± 2°C in darkness. As control, a set of three calluses were treated with sterile distilled water without the fungus. After this time each callus was fed with 1 μM of radioactive *trans, trans*-farnesyl pyro-phosphate [$^{1-3}$H]-FPP (20.5 Ci/mmol, Perkin Elmer, Boston, MA, USA) and 10 μM of farnesyl pyro-phosphate (FPP) triammonium salts, (Sigma-Aldrich, St. Louis, MO, USA) and 72 h later processed for TPS activity and for gas chromatography coupled to electron impact mass spectrometry (GC-EIMS) analysis of metabolites.

Experiments with cell suspension cultures

Cell suspension cultures maintained in MS medium with NAA and BA 5:1 mg L^{-1} (see above) and in the rapid phase of growth (approximately 12 d after subculture) were used in all the experiments. Treatment with the elicitor was initiated by adding 1 mL of 10^6 conidia mL^{-1} of *Pm. parasiticum* into the cell suspension cultures on an orbital shaker (160 rpm) at 25 ± 2ºC in darkness. The controls consisted of sterile distilled water without the fungus and three replicates were performed. After 48 h the cell cultures were fed with 1 μM of [$^{1-3}$H]-FPP and 1 μM of FPP as a carrier and at intervals of 2, 8, 72 and 96 h the cell cultures were processed for TPS activity and for GC-EIMS analysis of metabolites.

Terpene synthase activity measurements

Calluses and culture suspensions were homogenized in ice-cold mortar with pestle and 800 μL of 1 M potassium phosphate buffer (pH 6.5–7), 20%

(w/v) glycerol, 10 mM sodium metabisulfite, 10 mM ascorbic acid, 15 mM $MgCl_2$, 0.5% PVP (polyvinyl polypyrrolidone, MW 40.000, Sigma Chem Co, St Louis, MO, USA) and 1.47 mM 2-mercapthoethanol. Then, each total protein homogenate was centrifuged for 10 min at 12,000 *g*. After that, the extracts were partitioned into 200 μL of *n*-hexane and treated with 5 mg of silica gel powder (240–300 Mesh, Sigma-Aldrich) to remove any contaminating FPP or farnesol generated by phosphatase activity. An aliquot of 50 μL radioactive *n*-hexane was then poured with 4 mL of Fluka cocktail (Sigma-Aldrich) and radioactivity was measured by using a Tricarb liquid scintillation analyzer (Perkin Elmer, Chicago, IL, USA). TPS activity was expressed as nmol [$^{1-3}$H]-FPP consumed per mg of protein^{-1}h^{-1} according to Vögeli and Chapell (1988). The protein concentration of the extract was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin (Bio-Rad Laboratories, Philadelphia, PA, USA) as standard. Aliquots of 50–100 μL of the remaining organic phase were used for further GC-EIMS analysis and product identification.

Terpene determination

The hexane phase from calluses and cell suspensions was analyzed by GC-EIMS in order to identify terpene-like products and to estimate their relative concentrations. Two μL of each sample were injected in split-splitless mode in a GC-EIMS system (Clarus 500, Perkin Elmer). The GC column was a Perkin-Elmer Elite-5MS, cross-linked methyl silicone capillary column (0.25 mm internal diameter, 30 m long and 0.25 μm film thickness) eluted with He (0.7 mL min^{-1}). The oven temperature program was 45ºC for 1 min, followed by an increase of 2ºC min^{-1} to 130º C, 20º C min^{-1} to 250º C and held at 250º C for 10 min. The ionization potential was 70 eV and a range of 30 to 400 atomic mass units was scanned. Terpene products from the assays were identified according to Gil *et al.* 2012; by comparison of retention times and full scan mass spectra with a set of authentic standards, nerolidol, pinene, and squalene (Sigma-Aldrich Steinheim, Switzerland) and by comparison with those standards of the National Institute of Standards Technology (NIST) library. To estimate the concentration of the different metabolites 50 ng μL^{-1} of *n*-hexadecane (Supelco, Bellefonte, PA, USA) as an internal standard was added to each sample.

Antifungal activity bioassays

For evaluation of antifungal activity, 4 discs of filter paper (0.5 cm in diameter) were impregnated with 5 μL each of pure authentic nerolidol (97% Sigma-Aldrich, Steinheim, Switzerland) and were equidistantly distributed on semi-solid malt extract agar dishes with *Pm. parasiticum* grown for 8 days. All cultures were incubated at 25 ± 2° C until the mycelium of fungi reached the edges of the control prepared without nerolidol, and the antifungal index was calculated (Bittner *et al*., 2009; Park *et al*., 2009). Each treatment had 5 replications. The antifungal index was calculated as follows:

Antifungal index (%) = (1 - Ds / Dc) × 100; where, Ds is the diameter of the hyphal growth in the Petri dish treated with nerolidol, and Dc is that of the control.

Statistical analysis

The normal data were performed by analysis of variance (ANOVA) and multiple range tests at 95% confidence level. The software Statgraphics Centurion XV version 15.0.10 (Stat point Technologies Inc., Warrenton, VA, USA) was used.

Results

Generation of calluses from *in vitro* plants

The calluses formed in most of the different conditions from 57 to 100%, except for the first leaf callus with the combination of NAA-BA 1:1 mg L^{-1} where calluses formation decay to 28%. The calluses friability was low in the NAA-BA 1:1 mg L^{-1} combination, and the treatments with NAA-BA 5:1 mg L^{-1} showed the best calluses development, greater friability and higher DW. Regarding the explant selection it was observed that calluses from petioles and apical buds had the highest friability and DW (Table 1).

Terpene synthase activity measurements on calluses and cell suspension cultures

Increases of TPS activity was found in calluses inoculated with the highest conidial suspension of *Pm. parasiticum* (Figure 1). The major TPS activity analyzed as [$^{1-3}$H]-FPP consumed (nmoles mg protein^{-1} h^{-1}) was found in the hexane-soluble fraction of calluses inoculated with 2 μL and 10 μL of 10^9 conidia mL^{-1}

Table 1. Calluses formation from apical buds, first and second leaf, and petiole, in three combinations of plant regulator concentrations, NAA-BA 1:1, NAA-BA 5:1 and NAA-BA 2.5:0.5 mg L^{-1}.

Concentration [a]	Origin	Callus		
		Formation (%)	DW [b] (mg)	Friability (%)
1:1	Apical bud	71	16.5	40
	First leaf	28	4.5	50
	Second leaf	86	10.5	33
	Petiole	71	29.4	100
5:1	Apical bud	71	37.2	100
	First leaf	86	15.6	33
	Second leaf	100	13.6	86
	Petiole	100	32.1	100
2.5:0.5	Apical bud	57	36.9	100
	First leaf	86	12.0	33
	Second leaf	86	29.0	83
	Petiole	100	41.4	86

[a] Plant regulator concentrations, NAA-BA 1:1, NAA-BA 5:1 and NAA-BA 2.5:0.5 mg L^{-1}.
[b] DW, callus dry weight.

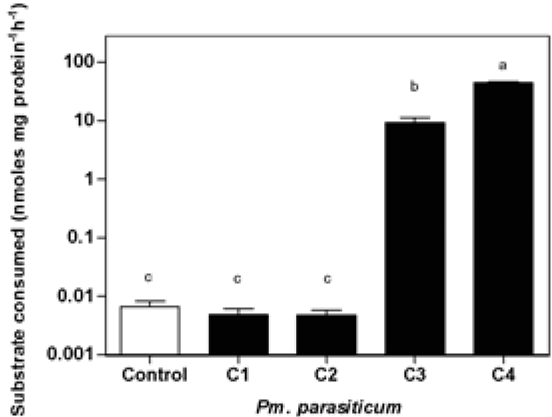


Figure 1. TPS activity expressed as nmoles of [1-3H]-FPP consumed per mg $protein^{-1}$ h^{-1} in calluses inoculated with 2 μL 10^7 (C1), 10^8 (C2) and 10^9 (C3) or 10 μL 10^9 (C4) conidia mL^{-1} and in control.

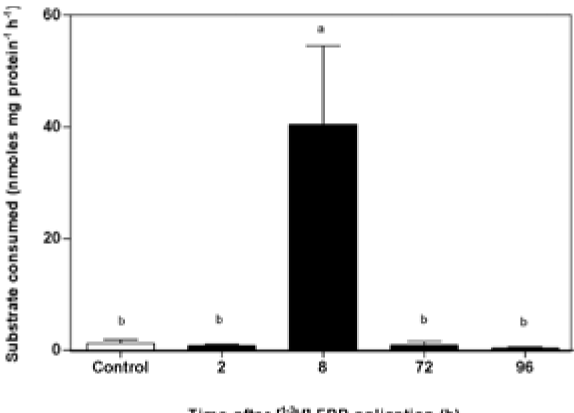
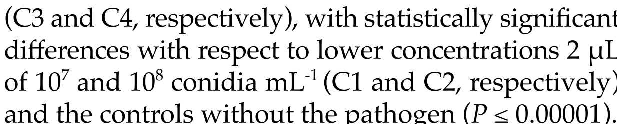


Figure 2. TPS activity expressed as nmoles of [1-3H]-FPP consumed per mg $protein^{-1}$ h^{-1} of cell suspension cultures at 2, 8, 72 and 96 h post application of FPP radioactive, and previously inoculated with 1 mL of 10^6 conidia mL^{-1} of *Pm. parasiticum*.



(C3 and C4, respectively), with statistically significant differences with respect to lower concentrations 2 μL of 10^7 and 10^8 conidia mL^{-1} (C1 and C2, respectively) and the controls without the pathogen ($P \leq 0.00001$).

Figure 2 shows the results of the time-course experiments with cell suspension cultures incubated 48 h with *Pm. parasiticum* as elicitor and the control

without the elicitor, followed by the addition of the radioactive FPP. TPS activity, measured at intervals of 2, 8, 72 and 96 h after [$^{1-3}$H]-FPP application, was maximal at 8 h post radioactive FPP application and then declined progressively at 72 and 96 h showing not statistically different between the TPS activity from cell suspension cultures incubated with *Pm. parasiticum* as elicitor and the control without the elicitor. This remarkable increase in activity in cell suspension cultures infected with the pathogen in a specific time, strongly suggest *de novo* synthesis of TPS (P = 0.0018).

Terpenes determinations on calluses and cell suspension cultures

When the hexane control fractions of grape calluses, were analyzed by GC-EIMS, the most important compound identified was the triterpene squalene (Figure 3A, s). Meanwhile the sesquiterpene nerolidol, the monoterpene α-pinene and also the triterpene squalene (Figure 4A, n, p and s, respectively) were observed in calluses inoculated with the fungus. Moreover, along with the increases in fungal amount it was observed an increase in the relative concentration of α-pinene (from 30 to 383 ng mg^{-1} FW) and nerolidol (from 70 to 915 ng mg^{-1} FW) respect to squalene (from 10 to 49 ng mg^{-1} FW). The highest relative concentration of nerolidol and α-pinene was found in calluses inoculated with 10 μL of 10^9 conidia mL^{-1} (Table 2), while in the control without the pathogen the terpene squalene prevailed (Table 2). Also, was notable the amount of nerolidol in the inoculated cell suspension cultures 8 h post-application of radioactive FPP, was far and away the most abundant compound found, with an estimated amount of 1800 ng mg^{-1} DW (Figure 5A, n).

When the fungal culture was controlled throughout the growth phase and the TPS and terpene metabolites were analyzed, neither significant TPS activity nor terpenes presumably involved in plant defense were found, except for squalene (13 ng mg^{-1} FW).

Antifungal activity bioassays

The mycelial growth of *Pm. parasiticum*, was inhibited at 54% by pure nerolidol at a concentration

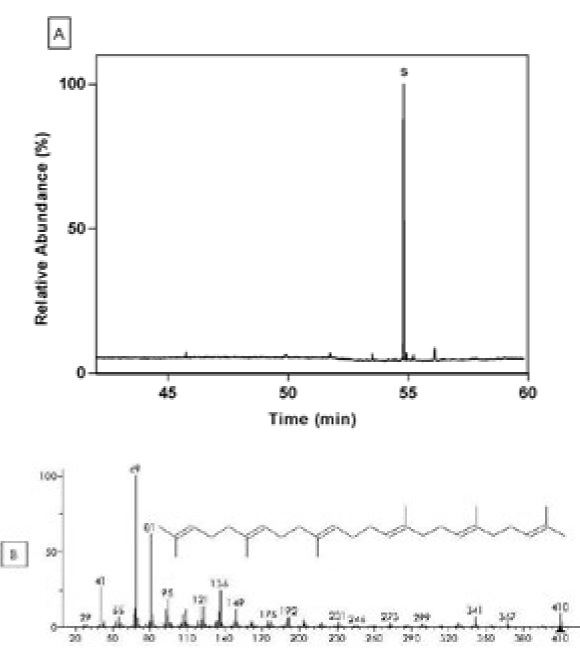


Figure 3. (A) Total Ion Chromatogram (TIC) from extracts of calluses without elicitor. The peak at 54.81 min (s) corresponds to squalene. (B) MS spectra of squalene.

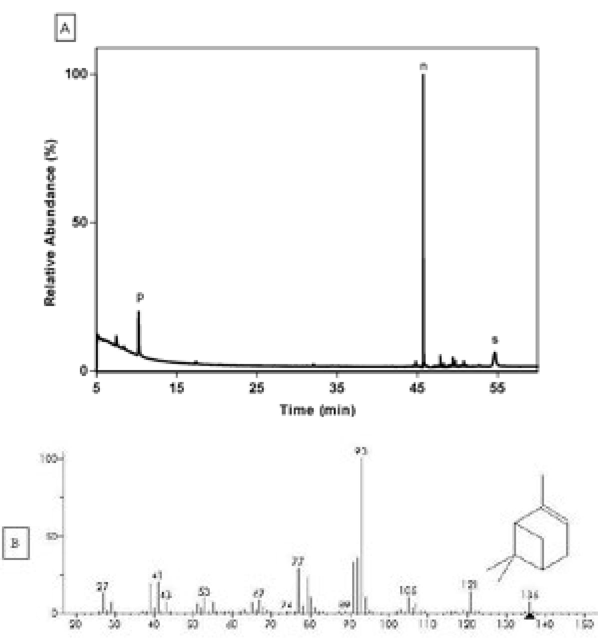


Figure 4. (A) TIC from calluses inoculated with 10 μL of 10^9 conidia mL^{-1}. The peaks in TIC correspond to α-pinene (10.28 min, p), nerolidol (45.74 min, n), and squalene (54.65 min, s). (B) MS spectra of α-pinene.

Table 2. Relative quantities of squalene, α-pinene and nerolidol calculated respect to a known amount of *n*-hexadecane (ng mg^{-1}) by GC-MS, in samples of calluses non inoculated (control) and inoculated with different concentrations of *Pm. parasiticum*.

Compounds	Control [a]	*Pm. parasiticum* [b]			
		C1	C2	C3	C4
Squalene	694	10	10	37	49
α-pinene	27	30	96	211	383
Nerolidol	51	70	255	478	915

Squalene, α-pinene and nerolidol expressed in ng mg^{-1}, calculated as the relationship among the total ion chromatographic peak area respect to a known amount of *n*-hexadecane in:
[a] Calluses non inoculated (control).
[b] Inoculated with 2 μL of 10^7 (C1), 10^8 (C2) and 10^9 (C3), and 10 μL of 10^9 (C4) conidia mL^{-1} of *Pm. parasiticum*.

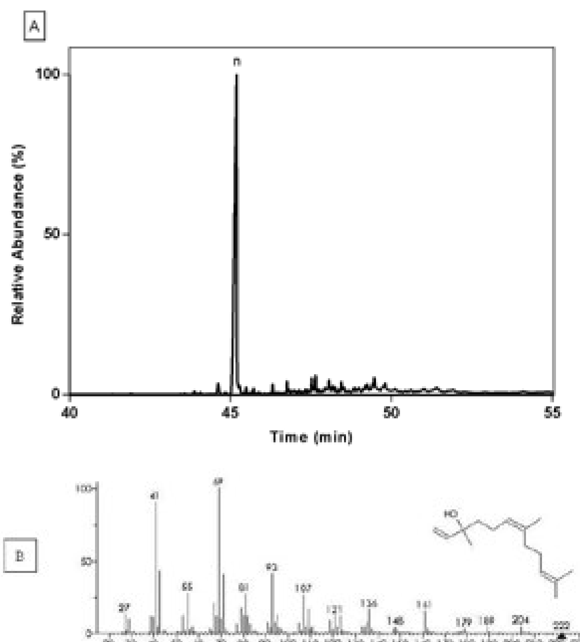


Figure 5. (A) TIC of cell suspension cultures inoculated with *Pm. parasiticum* and analyzed 8 h after application of [$^{1-3}$H]-FPP; the peak at 45.20 min was identified as nerolidol (n). (B) MS spectra of nerolidol.

of 4.2048 μg $disc^{-1}$ (Figure 6). Even though studies have been made of this sesquiterpene in other interactions, this is the first report of antifungal activity of nerolidol in a grapevine pathogen.

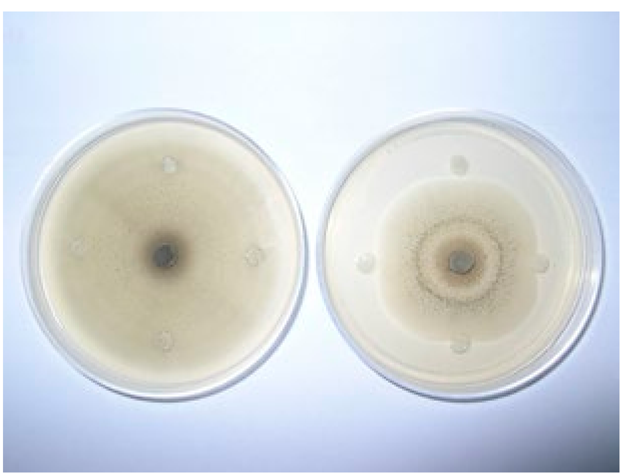


Figure 6. Effect of nerolidol on *Pm. parasiticum* growth in a Petri dish with 15 mL of malt extract agar (right) and *Pm. parasiticum* growth without nerolidol (left).

Discussion

The results from this study support the proposed hypothesis, that is, calluses and cell suspension cultures respond to the elicitor *Pm. parasiticum* by increasing TPS activity and synthesizing specific terpenoids compounds.

The experiment with inoculated calluses showed that the fungal concentration is important in eliciting defensive responses by increasing TPS activity (according to the conidial dilution). This agrees with other reports in which it was observed that the accumulation of capsidiol was proportional to the amount of elicitor added (Chappell *et al*., 1987). Also, by studying the activity of TPS over time with radio-labeled substrate on infected cell suspension cultures, it was found that 2 h after feeding with radio-labeled FPP substrate the TPS activity was scarce, while high activity was registered after 8 h and then declined again, suggesting that the cells respond against the pathogen through *de novo* TPS synthesis. In the studied calluses and cell suspension cultures inoculated with the pathogen, there was a clear correlation between TPS activity and a certain types of terpenes presumably involved in plant defense. Dudley (1986) and Croteau *et al*. (1987) have suggested the accumulation of mono-, sesqui-, and diterpenes in plant tissues challenged by pathogens correlated with an induction of the respective cyclase-like enzyme activities. Also Kapper

et al. (2005) showed that transgenic plants constitutively emit compounds of the complex herbivore-induced volatiles from the sesquiterpene nerolidol, the first intermediate in the route to nonatriene. Our experiments show the presence of nerolidol in calluses and in cell cultures inoculated with the pathogen, while α-pinene was identified in minor amount in calluses only. Regardless of these differences observed amongst the biological systems used, the induction of the TPS activity and the subsequent increase in terpenes against fungus attack were transient since it was found in a given time in the CSC. This is the first report of these compounds being associated with grape-pathogen interaction, although they have been related as phytoalexins in other plant species. In fact, significant increases in the levels of the monoterpenic olefins α-pinene, β-γ-pinene, δ-carene, and δ-phellandrene, which are thought to be toxic to fungus, were found in stem tissue of *Pinus contorta* infected with *C. clavigera* (Croteau *et al.*, 1987). As well, Bayonove (1989) observed that *Botrytis cinerea* would affect the biosynthesis of monoterpenes in different grape varieties.

The results obtained with calluses and cell suspension cultures on different experiments, indicate that the terpene compounds characterized by GC-EIMS after the host-pathogen interaction are exclusively synthesized by the plant tissues as defensive mechanisms to the pathogen attack. That is, the vine would be responding to the pathogen attack altering the pattern of terpene biosynthesis by inducing the synthesis of specific mono- and sesquiterpenes. In parallel, a drastic decrease of squalene concentration, key triterpene in the synthesis of sterols, was found, re-enforcing the idea of remarkable changes in the secondary metabolism of infected plants.

We have observed the effect of nerolidol on *Pm. parasiticum* grown in agar medium, and it is noticeable that the standard nerolidol retard the fungal growth by 54%. Therefore, the antifungal activity of terpenes like citral, eugenol, nerolidol and α-terpineol against *Trichophyton mentagrophytes* may act by affecting its normal metabolism (Park *et al*., 2009).

In conclusion, in our study we found that nerolidol possess antifungal activity that inhibits the growth of *Pm. parasiticum.* Due to its antifungal property, and considering that the USA's Environmental Protection Agency qualifies nerolidol as an innocuous natural volatile compound, its use may be advisable for the treatment of "hoja de malvón" and "young vine decline" diseases as an organic, environmentally-friendly antifungal ingredient.

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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 58°C. 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

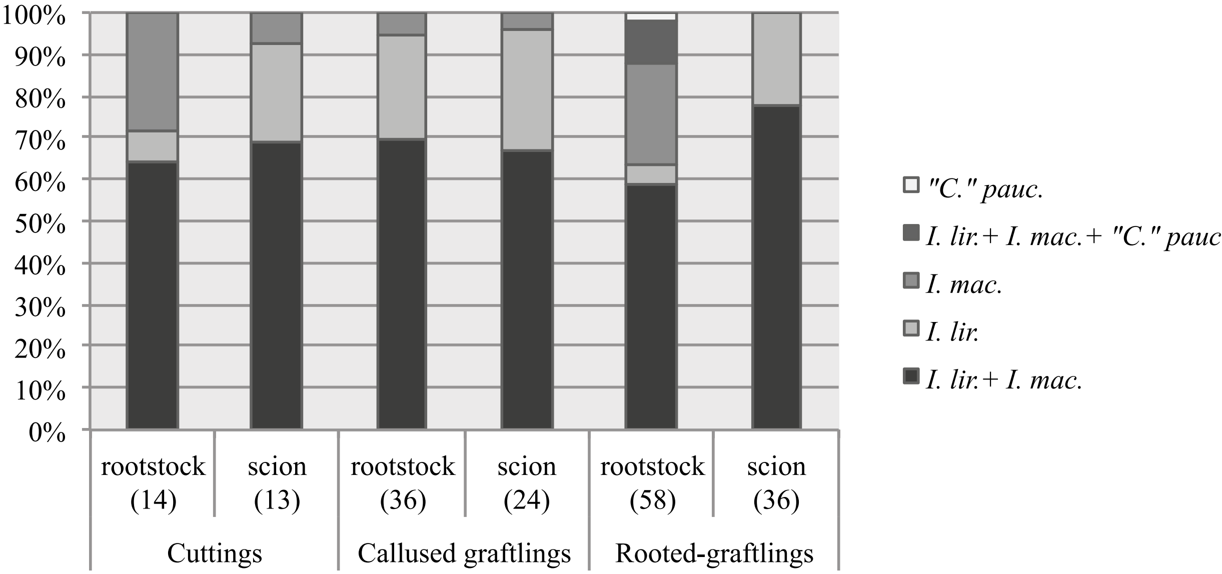

Figure 1. Proportion of *Ilyonectria liriodendri*, *I. macrodidyma* complex and "*Cylindrocarpon*" *pauciseptatum*, alone or in combination, on different grapevine propagation materials, by multiplex nested PCR (sample size in brackets).

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).

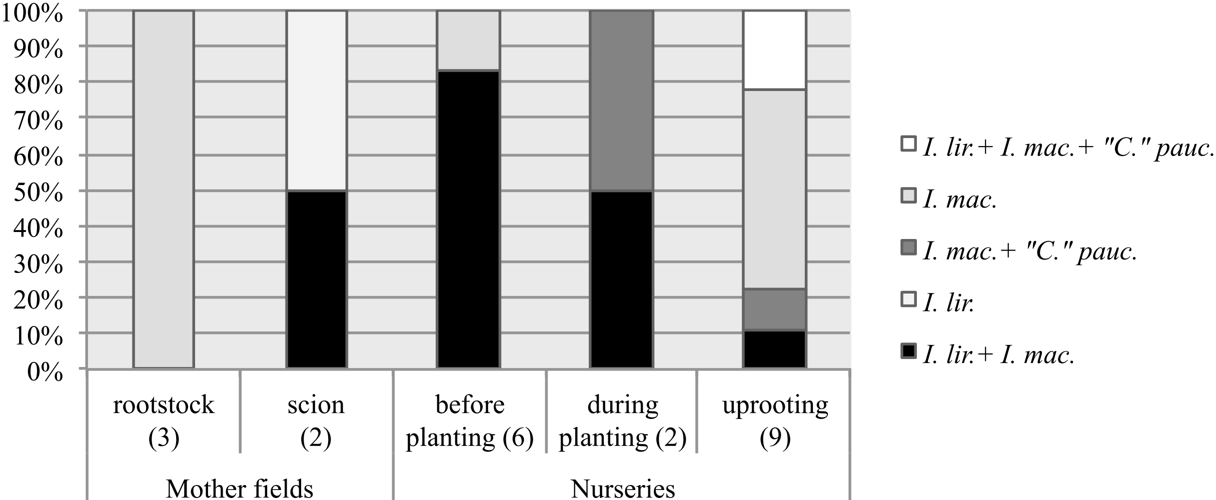

Figure 2. Proportion of *Ilyonectria liriodendri* (*I. lir.*), *I. macrodidyma* complex (*I. mac.*) and "*Cylindrocarpon*" *pauciseptatum* ("*C.*" *pauc.*), alone or in combination, in the different soils sampled, assessed by multiplex nested PCR (sample size in brackets).

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.

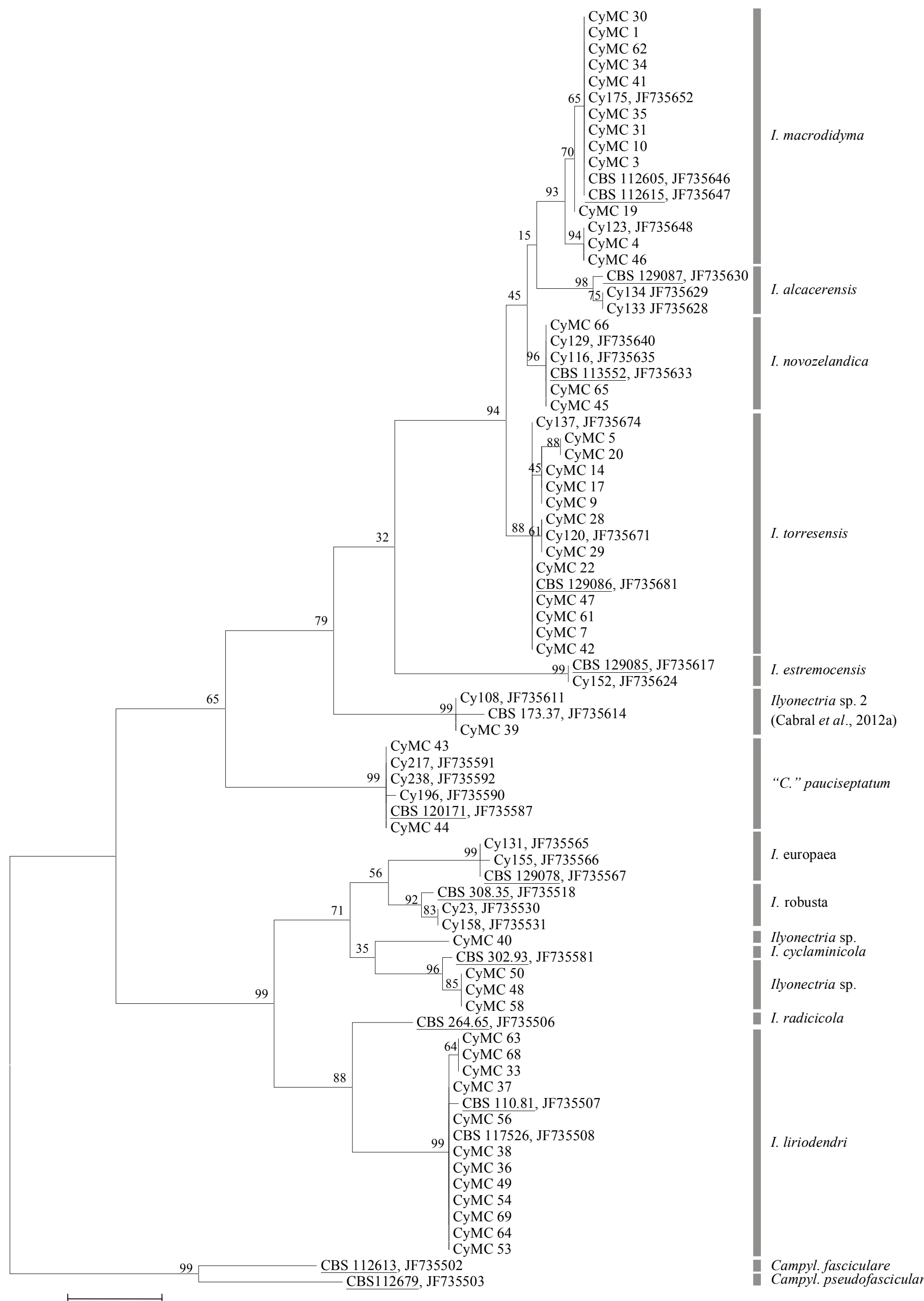

Figure 3. The first of 276 equally most parsimonious trees obtained from part of Histone H3 sequence alignment of *Ilyonectria* isolates and relatives with a heuristic search using MEGA version5. The tree was rooted using *Campylocarpon* isolates as outgroup sequences and bootstrap support values are 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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RESEARCH PAPER

Histopathological study of response of *Vitis vinifera* cv. Cabernet Sauvignon to bark and wood injury with and without inoculation by *Phaeomoniella chlamydospora*

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Summary. *Phaeomoniella chlamydospora* (*Pch*) is one of the main causal agents of tracheomycosis in grapevine. We characterize how this fungus affects the response of *Vitis vinifera* cv. Cabernet Sauvignon to bark and xylem-tissue wounding after six weeks post-treatment. A histological investigation shows that, in xylem tissue, cell-wall modifications in response to wounding are related to suberin deposits rather than to lignin-induced wall thickening. The xylem response does not appear to be disturbed by *Pch* infection. Therefore, cell-wall modification strongly inhibits the development of wound-closure tissue (WCT) but does not prevent the differentiation of the necrophylactic periderm. Hyphae localization in tissue surrounding the wound or inoculation sites indicates that *Pch* colonizes all cell types, such as vascular tissues, paratracheal parenchyma cells, fibers and rays. The results also suggest that efficient compartmentalization separating fascicular xylem portions is assured by thick suberized cell walls bordering the ray parenchyma.

Key words: Grapevine vine-trunk disease, *Phaeomoniella chlamydospora*, CODIT, compartmentalization, bark healing.

Introduction

Grapevine trunk diseases constitute real threats to the worldwide grape and wine industry. Of the numerous grapevine wood pathogens identified to date, *Phaeomoniella chlamydospora (Pch)* is one of the most commonly isolated and also one of the most virulent (Mugnai *et al*., 1999; Eskalen and Gubler, 2001; Marchi *et al*., 2001; Laveau *et al*., 2009; Luque *et al*., 2009; Kuntzmann *et al*., 2010). It is ascomycetous fungus responsible for tracheomycosis in grapevine (Surico, 2009). Infection can lead to different symptoms depending on the level of infection and the age of the vine plant (Surico, 2009). In young vines, *Pch* infection can lead to young-vine decline also called Petri disease. Decline caused by *Pch* in young vines is associated with dark exudates (named black goo) running out of xylem vessels. These black spots appear as wood streaking in longitudinal sections. In young and old vines alike, *Pch* is isolated from vine wood with tiger-striped leaf symptoms associated with the esca syndrome (Mugnai *et al*., 1999; Marchi *et al*., 2001). Vines with esca disease can exhibit a wide variety of symptoms in both trunks and arms, such as hard central brown discoloration, hard V-shaped discoloration, black spot (mostly organized in a concentric manner), or white soft rot (Mugnai *et al*., 1999; Luque *et al*., 2009; Kuntzmann *et al*., 2010). These various symptoms can be explained by the combination of fungal pathogens usually found in the wood of mature vines. These pathogens include the ascomycota *Eutypa lata, Phaeoacremonium* spp.,

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Botryosphaeriaceae spp., and other basidiomycetous fungi (e.g., *Fomitiporia mediterranea*) (Mugnai *et al*., 1999; Luque *et al*., 2009; Kuntzmann *et al*., 2010). From among this variety of symptoms, *Pch* is isolated mainly from black spots and central brown discolorations (Mugnai *et al*., 1999; Luque *et al*., 2009; Kuntzmann *et al*., 2010). In addition, studies based on the characterization of wood microbiota as well as pathogenicity tests indicate that *Pch* may produce sectorial necrosis and canker (Laveau *et al*., 2009; Kuntzmann *et al*., 2010).

Pathogens infecting plants must overcome the defense mechanisms developed by their host. These defense mechanisms can be pre-existing (e.g., physical or chemical barriers such as the cuticle or constitutive antifungal compounds, respectively) or induced. In woody tissues, compartmentalization of pathogens involves anatomical modifications at the junction of healthy and infected tissues, which constitutes a delayed defense mechanism. In pre-existing woody tissues, an area called the reaction zone (RZ) (where the active response of the host occurs) appears after the injury (Pearce, 1996). Another boundary, called the barrier zone (BZ), forms at the time of injury in the plane of the vascular cambium. According to Shigo and the model of compartmentalization of decay in trees (CODIT), four different boundaries can be distinguished in injured tree wood (Shigo and Marx, 1977). The least-effective barrier is the occlusion of xylem vessels by tyloses and gummosis. The next-more effective barrier consists of the last layers of cells produced by the vascular cambium in the annual rings. A still more effective barrier is the rays, but this does not provide a continuous barrier because rays only extend over a limited longitudinal portion of perennial tissues. In the CODIT model, these first three barriers are considered part of the RZ. The fourth barrier constitutes the BZ and seems to be the most impervious and durable barrier with regards to limiting the spread of pathogens. The BZ effectively protects the youngest wood tissue and the vascular cambium (Pearce, 1996). In the RZ and BZ, cell walls may thicken because of lignin or suberin and phenolic compounds may accumulate in cell lumen (Pearce, 1996). It is also known that the ways in which wood responds to injury are likely to vary between woody-plant species (Pearce, 1996; Deflorio *et al*., 2009). The RZ and BZ do not form in a specific manner because they can be produced in response to both infection and wound (Pearce, 1996).

Another fundamental aspect of wound response is the compartmentalization process that occurs in the bark and the development of WCT. After wounding, a ligno-suberized layer of cells usually develops at the periphery on the phloem tissue (Biggs, 1984; Biggs *et al*., 1984). This ligno-suberized layer seems necessary for the regeneration of a new periderm, also called the necrophylactic periderm (NPd). The initiation of WCT begins by the proliferation of a mass of undifferentiated cells (callus) that originates in the vascular cambium. When the callus grows and covers the wound, a new vascular cambium and a new periderm regenerate near the vascular-cambium surface, and the callus gradually becomes differentiated (Armstrong *et al*., 1981). The healing process is considered complete when the continuity of the vascular cambium is restored.

The anatomical features of wood and tissue where suberized cell walls form in response to injury vary from one tree species to another (Biggs, 1987). Even if grapevine is a woody plant, it cannot be considered a tree. Vines are woody plants adapted to climbing existing natural supports to reach the canopy. Therefore, they allocate fewer resources than they otherwise would to building a self-supporting structure and so have a much smaller radial-growth rate than ordinary trees (Carlquist, 1985). The anatomy of cane evolved to efficiently transport water through a plant with a higher leaf/xylem surface ratio than trees (Carlquist, 1985). Today, the compartmentalization process in the woody tissue of vines remains poorly understood.

Characterization of the biochemical activity of enzyme secreted by *Pch* indicates that this fungus poorly metabolizes lignified cell walls (Del Rio *et al*., 2004; Valtaud *et al*., 2009). However, toxins secreted by *Pch*, especially the polypeptide fraction, strongly affect physiological cell processes, which leads to reduced plant responses, cell death, and tissue necrosis (Abou-Mansour *et al*., 2004; Luini *et al*., 2010). In the field of grapevine trunk diseases, compartmentalization of pathogens and pathogen routes in the woody tissue is poorly understood. A genetically modified strain of *Pch* that constitutively expresses DsRed fluorescent protein seems mainly restricted to the lumen of the xylem vessels and, to a lesser extent, to intracellular spaces in cells around the vessels (Mutawila *et al*., 2011). In the same study, other fungi expressing this kind of fluorescent reporter were not detected in the woody tissue, although they could

be isolated by microbiological methods. It thus appears that the fluorescent-reporter approach can lead to a less-demonstrative visualization of pathogens because of the loss of the fluorescence signal in fungal hyphae or because of a high autofluorescence background in woody tissue. The location of fungal hyphae (obtained by transmission electron microscopy) indicates that *Pch* may colonize the intracellular space of the xylem fibers (Valtaud *et al*., 2009).

The objective of the current study is to characterize, at the histological level, the effects of *Pch* inoculation on the response to injury of bark and xylem in grapevine. To achieve this goal, we first characterized the response to injury and then characterized the response in inoculated plants. In addition, after quenching the autofluorescence of the woody tissue, we located the fungal hyphae at the tissue level through an epifluorescent strategy involving Calcofluor White stain.

Materials and methods

Plant material

Fifteen canes of the *Vitis vinifera* cv. Cabernet Sauvignon clone were harvested and cut into 10-cm-average lengths to obtain cuttings with a single internode and with homogenous size and diameter. Cuttings were incubated in a PDA medium at 26°C for 16 h days and 8 h nights in plastic boxes containing humid sterile glass wool until buds broke and the first roots appeared. Plants were potted in a sterile substrate consisting of universal peat, river sand, and perlite (1:1:1). Throughout the entire experiment, plants were grown in a Mylar box chamber for 16 h days and 8 h nights.

Fungal material and preparation of conidia solution

Phaeomoniella chlamydospora isolate CBS 239.74 was grown in a PDA medium at 26°C in the dark. A margin of two-week-old culture (a cube averaging $5 \times 5 \times 5$ mm^3) was harvested with a sterile scalpel and introduced into 1 mL of sterile 10 mM phosphate buffer (pH = 7). The solution was gently mixed, following which a plug of solid medium was removed from the solution. The solution was centrifuged at 1000 rpm and the excess water was removed. Conidia were washed with a sterile 10 mM phosphate buffer, centrifuged, and gently suspended by pipetting into a 500 μL phosphate buffer solution. Conidia were counted with a Malassez counting cell and the solution molarity was adjusted to obtain 1000 conidia in a 50 μL drop.

Plant treatment

A total of 18 plants were used in this study. Three replications were performed, with each replicate consisting of three control and three inoculated plants. One-month-old bark cuttings were surface cleaned with a 70% ethanol solution and the young trunk was wounded with a 3-mm-diameter cooled sterile drill 40 mm under the center off the upper node. The drill wound was perpendicular to the longitudinal axis of the bud. Plants were lain down and inoculated with 50 μL of fresh conidia solution (1000 conidia) or 50 μL of 10 mM phosphate buffer (control). When the drop of solution was totally absorbed by the plant tissue, the wound was covered with parafilm. Plants were grown as described above and were analyzed six weeks after treatment.

Histological methods

A stem segment averaging 30 mm in length was removed using a razor blade. At the center of each stem was a wound or inoculation site. The stems were dehydrated by exposure at 4°C to ethanol solutions (25, 50, 80%; three times 30 min). Samples were conserved in the 80% ethanol solution at 4°C for a maximum of 2 weeks. For histological preparation, samples were rehydrated by exposure to ethanol-water solutions (50, 25%, water) and fixed on aluminum plates with cyanocrylate glue. Samples were cut into 30- and 200-μm-thick sections with a vibratome Leica VT 100S equipped with a sapphire knife. We characterized the plant's reaction by optical microscopy and epifluorescence of the 30 *μm* sections.

Samples were stained with safranine O-Astra Blue (SfrO-AB) to differentiate cellulosic lignified cell walls (stained in red) from nonlignified cell walls (stained in blue) (Srebotnik and Messner, 1994). The lignin and suberin zones in the woody tissue were investigated by using phloroglucinol-HCl (Phl-HCl) (Biggs, 1987). Phl-HCl reacts specifically with lignin and, according to Biggs, also quenches lignin self-fluorescence, which makes it possible to detect suberin by illuminating the sample with ultraviolet

(UV) light. The 200-µm-thick sections were used for fungal localization. These sections were bleached in sodium hypochlorite (2.5% active chlorine) until tissue coloration was weak or extinct (approximately 5 min), and then rinsed three times in deionized water for 5 min each time. To stain fungal hyphae, sections were covered for 2 min with a drop of Calcofluor White M2R, then briefly rinsed in water and mounted with water between slide and cover slip.

Epifluorescence analysis of the sections was done with a Leica DM IRBE microscope equipped with a 100 W HBO mercury-vapor lamp. To both analyze the plant response and localize the fungus in plant tissue, we used a di amido phenyl indol (DAPI) filter (excitation at 340–380 nm, emission is at 425 nm). A Leica DM6000 equipped with a confocal head (AOBS TCS SP2) was used for confocal imaging. To distinguish fungal hyphae, micrographs were acquired for two emission and two excitation wavelengths (excitation at 405 nm, emission at 420–480 nm; shown in blue) from cell-wall signals (excitation at 488 nm, emission at 520–600 nm; shown in red).

Results

Characterization of response to injury in wood of vine cuttings

Six weeks after treatment, longitudinal sections revealed a thin layer on the xylem tissue surrounding the wound (Figures 1A and 1B). Some control plants developed short brown discolorations in the xylem tissue up to the wound (Figure 1B). All nine control plants developed WCT that entirely covered the wound (data not shown).

In the cross sections, the histological structure of WCT is distinguishable from other tissue (Figure 1C). A thick layer of phellem developed at the abaxial surface of the WCT formed from each margin of the wound (Figure 1C). More specifically, the newly formed bark was composed of a periderm layer and a differentiated phloem (Figure 1C). The Vascular cambium separated the phloem from the newly formed xylem (Figure 1C), which was mainly composed of fibers and ray parenchyma and had only a limited number of vessels of small diameter. From the base to the end of the WCT, both bark and xylem tissues were progressively less organized. At the adaxial face of the WCT, the periderm extended from the base of the wood wound to form a continuous structure with the periderm of the abaxial face (Figure 1C). Under the periderm, only a mass of connective tissue composed of undifferentiated cells was observed. These cells reacted to the Phl-HCl test in purple, indicating that they harbored a thin wall that can be weakly lignified with Guaiacyl monomers (Figure 1D).

In the injured xylem, a RZ appears in the tissue around the wound. This RZ is characterized by a more intense coloration of the cell wall by safranine O (Figure 1C) and is restricted at the NPd-xylem junction. The RZ was not distinguishable using the Phl-HCl reaction, which indicates that the lignin content may not have changed or may have undergone only limited change (Figure 1D). After Phl-HCl treatment, UV illumination of the RZ resulted in emission of residual autofluorescence, which indicates that the staining variations resulting from SfrO-AB treatment may have resulted from suberin deposits (Figure 1E). Suberin deposits occur in fibers, large pluriseriate rays, mature tyloses, and paratracheal parenchyma (Figure 1E). No deposits were observed in the necrotic brown areas in the rays. The strongest remaining autofluorescence signal comes from periderms and from cells bordering the rays and fibers at the periphery of the RZ. Although cells displaying this signal were usually organized in one- to two-seriate continuous layers at ray borders, some gaps consisting of a group of two to four cells also appeared along the ray boundary (Figure 1I). In the transversal plane crossing the middle of the wound, tyloses led to vessel occlusions. The tyloses formed mostly in vessels surrounding the zone where reacting fibers and rays were detected, but not within this zone itself (Figure 1E). Some vessel occlusions in the RZ also appeared up to the wound, but with no obvious organized distribution.

Coming out of the NPd-xylem junction, two boundaries appear that are parallel to the plane formed by the vascular cambium (Figure 1FI). The cells of these two layers displayed walls stained deep red with SfrO-AB (Figure 1FG). The wounds made for the experiment led to the first layer (which was at the end of the growth ring of the previous year) and the second layer, both of which were then matched with the BZ (Figure 1G). The growth-ring boundary is formed by a band of one to four flattened thick-walled cells. Their walls react strongly to Phl-HCl staining, producing a deep-purple coloration that contrasts with the woody surrounding

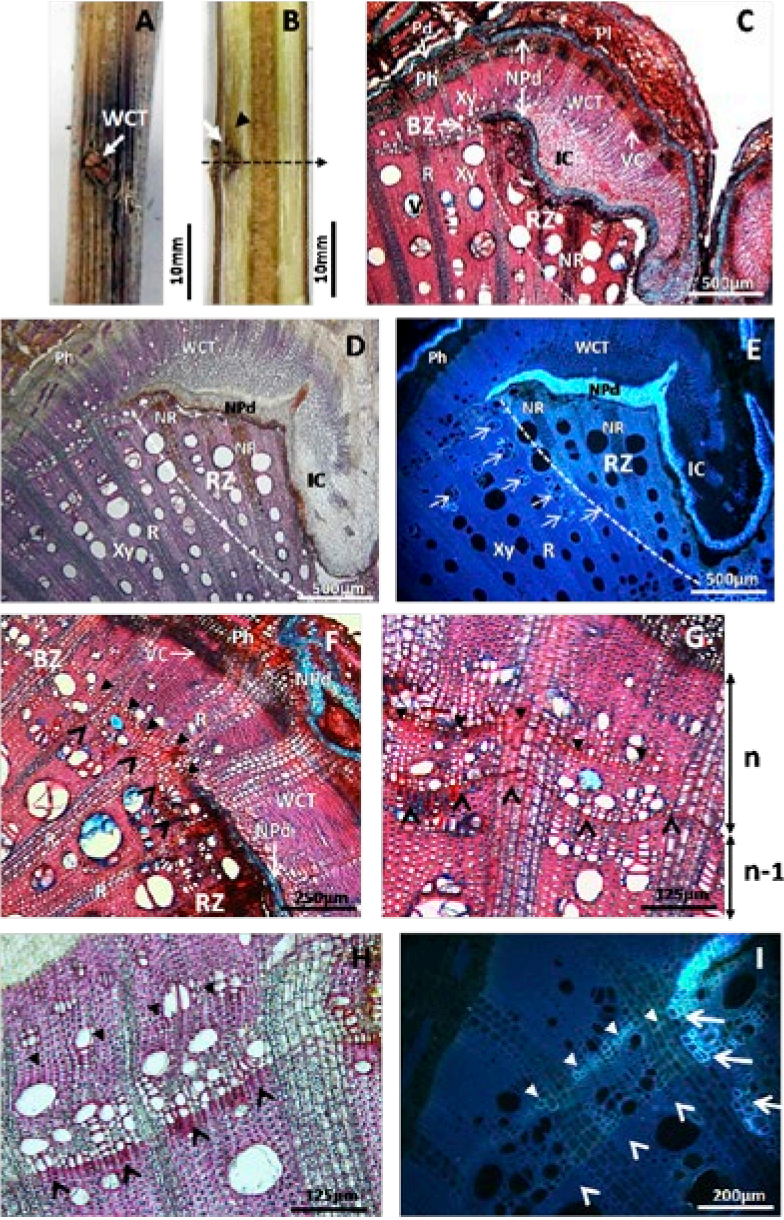

Figure 1. *Vitis vinifera* cv. Cabernet Sauvignon response to injury six weeks after wounding. (A, B) Photographs of phenotype observed in control plant from (A) outer view and (B) in longitudinal section. (A) WCT indicates wound closure tissue. (B) White arrow indicates WCT, black arrow head indicates the brown wood occurring up to the wound in control plants, and dotted arrow indicates the section shown in panels (C–I). (C–E) Large-field micrographs (X5) of (C) injured tissue stained with SfrO-AB, (D) Phl-HCl observed with bright field, and (E) under UV illumination. Dotted lines indicate boundaries of the RZ. (E) Arrows indicate xylem vessels occluded with tyloses. (F–I) Closer view of (F, G) junction between necrophylactic periderm (NPd) and xylem tissue under SfrO-AB staining, (H) Phl-HCl observed with bright field, and (I) under UV illumination. V-shaped arrow heads indicate barrier at the boundary between tissue formed a year prior to this experiment [n-1 in panel (G)] and tissue formed during experiment [n in panel (G)]. Arrow head indicates the barrier formed in response to injury. (I) Arrows indicate the layer of cell exhibiting a strong primary autofluorescence at the border of rays encircling the RZ. The notation IC stands for undifferentiated cells, NR is for necrotic ray, NPd is for necrophylactic periderm, Pd is for periderm, Pl is for phellem, Ph is for phloem, BZ is for barrier zone, RZ is for reaction zone, V is for vessels, Vc is for vascular cambium, WCT is for wound closure tissue, and Xy is for xylem.

tissue (Figure 1H). Under UV illumination, no residual autofluorescence originated from the cell walls in this layer, which indicates an absence of suberin deposits (Figure 1I). Weak residual autofluorescence also came from a thin layer of one to three thin-walled cells composed of primary cells that formed on the annual rings. In the layer consecutive to the drill wound, flat cells were also observed immediately centripetal of the vascular-cambium position at the time of wound (Figure 1H). These cells either do not react to Phl-HCl or do so only in a weak and discontinuous manner. Ultraviolet illumination reveals that the walls of these cells constitute a continuous suberized barrier in fibers and rays (Figure 1I).

Six weeks after wounding, several anatomical changes occurred in response to injury, including the development of WCT, the deposition of suberin in fibers and ray cells in the RZ, and the occlusion by tyloses of vessels surrounding the RZ.

Effect of *Pch* infection on responses of bark and wood injury

Plants inoculated with *Pch* developed black streaking (about 5 mm in length) and brown discoloration in the xylem up and down the wound (Figure 2A). Macroscopically, no WCT was noticed for all the nine plants inoculated with *Pch* (Figure 2A).Nevertheless, NPd was seen at the wound margin (Figures 2B–2D). This NPd separates the living phloem from the dead necrotic phloem (Figure 2C). Under this periderm, WCT consisted of a smaller number of undifferentiated cells. More precisely, the new xylem and phloem tissue formed after the wounding indicates that the vascular cambium remained active at the margin of the wound. Howeversignificantly less xylem tissues are formed ($n = 9$, $P = 0.002$) than in the control plants (Figures 1C and 2B; data not shown). In woody tissue, the RZ appeared the same as that in control plants, both in terms of constitution and

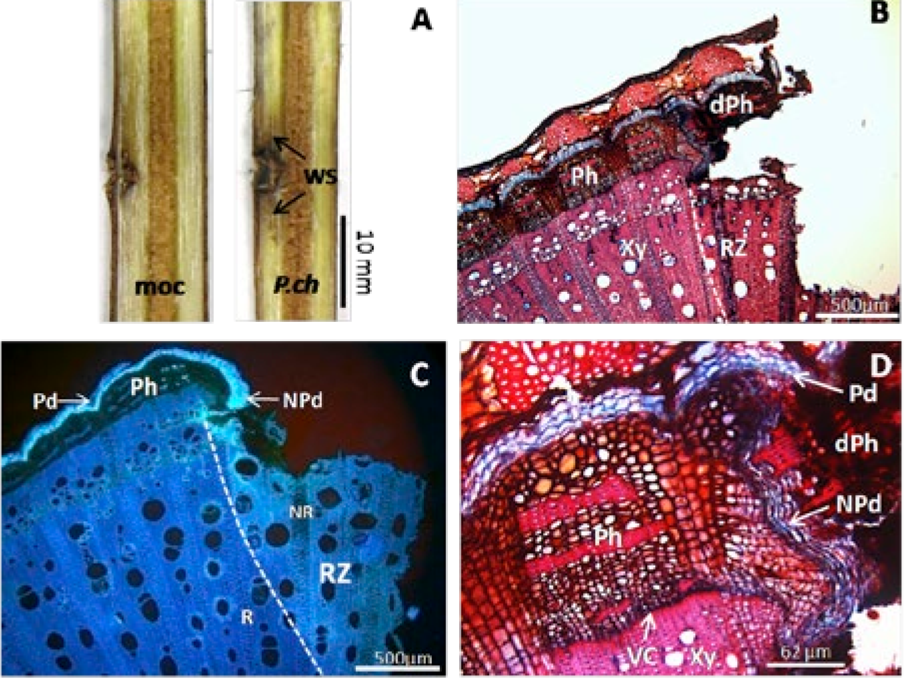

Figure 2. Effect of *Pch* on *Vitis vinifera* cv. Cabernet Sauvignon response to wounding. (A) Photographs of macroscopic phenotype observed in moc (control) and *Pch*-inoculated plants. WS indicates wood streaking in xylem on each side of the wound. (B, C) Large-field micrographs of (B) injured tissue stained with SfrO-AB and (C) Phl-HCl observed under UV illumination. (D) Closer view of bark structure at the border of the wound. The notation dPh stands for dead phloem, NR is for necrotic ray, NPd is for necrophylactic periderm, Pd is for periderm, Ph is for phloem, RZ is for reaction zone, V is for vessels, and Xy is for xylem.

of extension (Figures 1C, 1E, 2B, 2D). In addition, at the RZ periphery, the occlusion of vessels was not affected (Figures 3A–3D).

Thus, *Pch* infection affects the response to injury mainly by inhibiting wood-wound development. Any change in the response of the pre-existing woody tissue is either limited or cannot be distinguished using our approach.

Localization of fungal hyphae in the woody tissue

To localize fungal hyphae in woody samples, the autofluorescence background of woody tissue was quenched and samples were stained with Calcofluor White M2R afterwards (Figures 3 and 4). This approach allows *Pch* propagules in grapevine wood to be clearly detected under UV illumination (Figures 3 and 4). In control plants, we detected on rare occasions the hypha structure of unidentified endophyte fungi in the cell lumina of xylem fibers and vessels. These endophytic fungi were always restricted to a limited area containing a loose network of hyphae (Figures 3A and 3B).

An extended cluster of dense hypha network appeared in the xylem tissue up to the inoculation site of samples from plants inoculated with *Pch*, (Figures 3C–3E). Hypha structures in this cluster were always located in cell lumina and were never detected in cell walls. The overall hypha network seems to be restricted to an area that matches the RZ (Figure 3C). In infected tissue, hypha structures develop in fibers, paratracheal parenchyma cells, rays, and xylem vessels both open and occluded by tyloses (Figures 3D–3F and 4D). Fungal hyphae were also observed in piths, in the surrounding xylem tissue, and in the dead phloem tissue (data not shown). We also found infected vessels in the neighborhood of infected fibers but separated from healthy xylem tissue (Figure 3D).

At the junction of NPd and pre-existing xylem tissue (i.e., xylem tissue that existed before the wound), the dense network of hyphae was limited by the layer of cells bordering the ray (Figures 1I and 4A–4C). These cells had thickened suberized walls and appeared to form a periderm-like continuity in the ray (Figures 1I and 4A–4B). We found no fungal structure in these modified ray cells, as was the case for NPd and newly formed xylem tissue (Figures 4A–4C).

Discussion

In this study, anatomical changes appearing after injury in the xylem of *Vitis vinifera* consist mainly of suberin rather than lignin deposits at the cell-wall level. A feature of grapevine wood is the absence of apotracheal parenchyma cells in the fascicular portion of xylem. In this case, the conjunctive tissue between vessels mainly consists of fibers, even if tracheids rarely appear (Schoch *et al*., 2004). This feature may help to increase stem stiffness by requiring only a minimum amount of conjunctive tissue to avoid damaging the fascicular portion under twisting (Carlquist, 1985). In angiosperm tree species, postinjury modifications in the xylem mainly occur in apotracheal parenchyma cells and in rays but are absent from fiber, which, in tree wood, are nonliving cells (Biggs, 1987; Pearce, 2000). This response can lead to the deposit of lignin or suberin in tree wood. However, in grapevine wood, it seems that fibers react by modifying their wall, thereby playing the role in the compartmentalization process of the apotracheal parenchyma cells of tree wood. This feature may also confer to grapevine wood the ability to build continuous suberized layers such as walls 2, 3, and 4 of the CODIT model. Deposition of suberin may help responsive tissues to conserve its mechanical properties, thereby preventing stems from cracking at the wound (or healing) site.

The vessel occlusions observed in our experiments were mainly consisted in tyloses. Woody-plant species that respond to wounding by depositing suberin tend to occlude their vessels with tyloses rather than gels (Pearce, 1996). In grapevine wood, the type of occlusion also depends on the season (Sun *et al*., 2008). Because this species occludes its vessels with tyloses in summer and gel in winter, our results may be attributed to the temperate conditions of our trials. Suberin deposits also occur in tyloses. Although this phenomenon is known in woody species (Rioux *et al*., 1995), it has never been reported before in grapevine wood. It is interesting that only a very small number of vessel occlusions appear in portions of the xylem where fibers and rays react. In contrast, vessels fully occluded with tyloses appear in the RZ periphery and are organized all around the RZ, which corresponds strongly with wall 3 of the CODIT model (Figures 1E and 3A). Although the injury response in wood might restrict microbe invasion, it must first maintain the water status of the plant by limiting water loss through the wound.

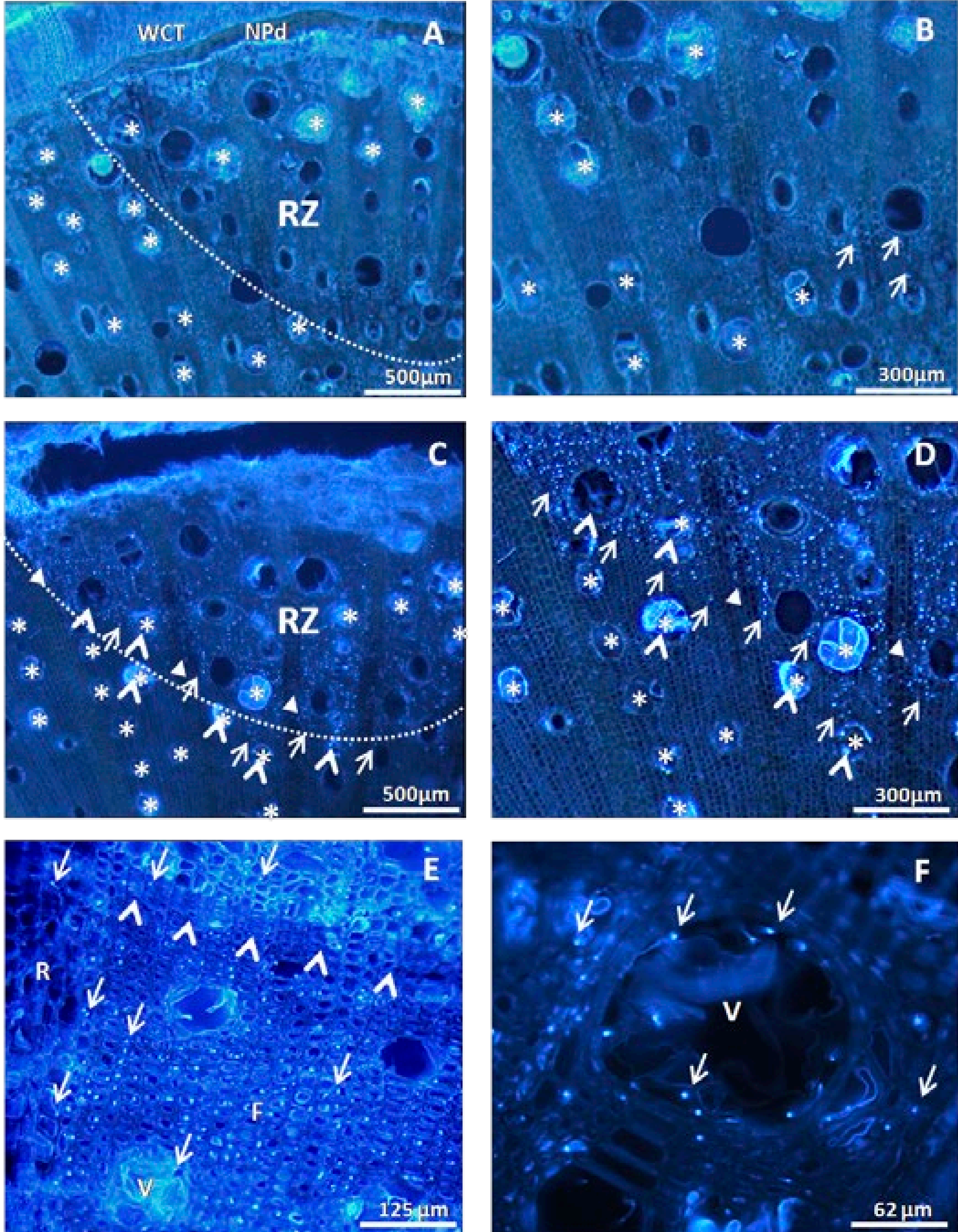

Figure 3. Localization of *Pch* hypha in xylem. (A–F) Epifluorescence micrographs of sections stained with Calcofluor White M2R (UV illumination). (A–D) Cross sections of (A, B) wound of control plant and (C, D) of plant inoculated with *Pch*. Black stars indicate vessels fully occluded with tyloses, arrows indicate fungal hyphae in fibers, arrow heads indicate fungal hyphae in rays, V-shaped arrow heads indicate vessels where fungal hyphae occurs. (A, C) RZ boundaries are indicated by dotted lines. (E) Micrograph showing the dense network of hyphae in infected xylem. Arrow heads indicate the layer of cells delimiting the growth ring, and arrows indicate different xylem-cell types where hyphae are seen. (F) Close view of fungal hyphae (arrows) in xylem vessels occluded with tyloses. The notation F stands for fibers, NPd is for necrophylactic periderm, R is for ray, RZ is for reaction zone, V is for vessel; and WCT is for wound closure tissue.

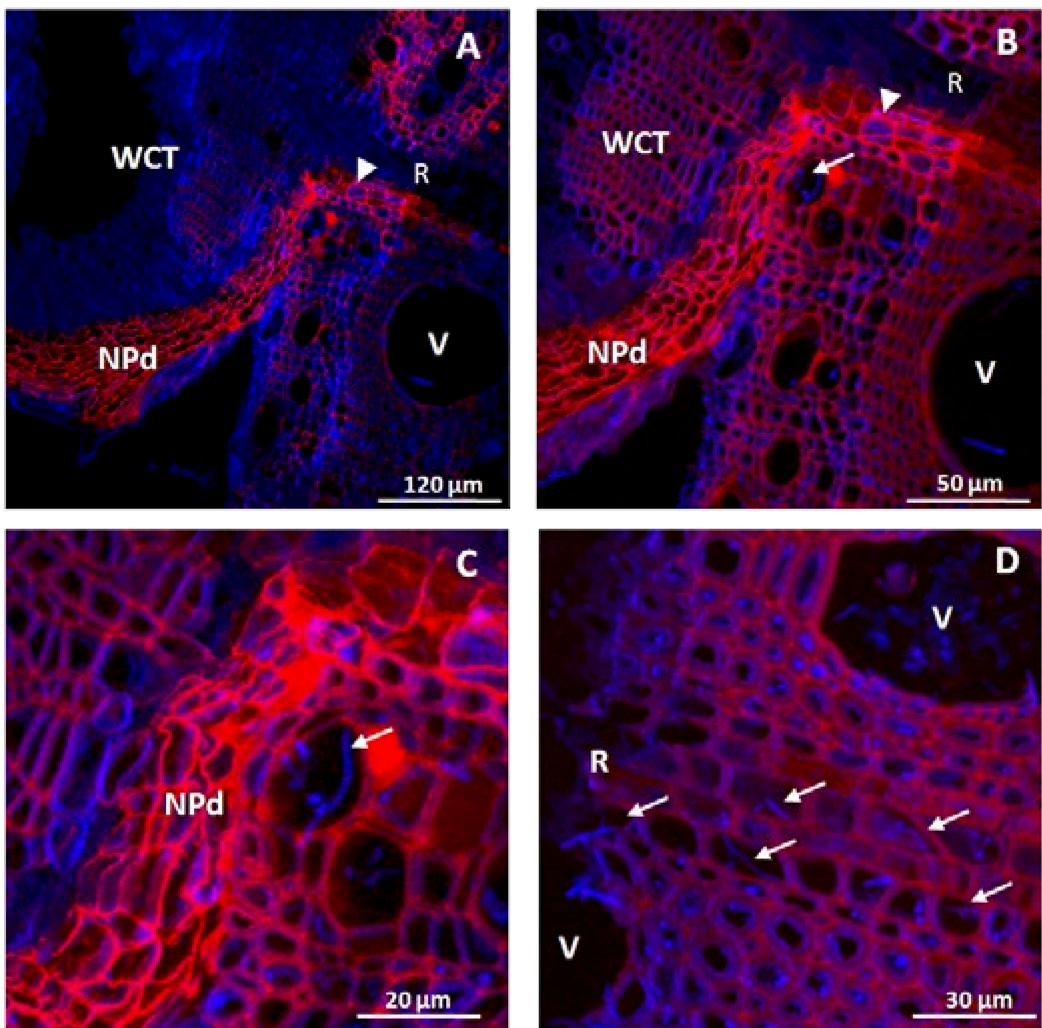

Figure 4. (A–C) Localization of *Pch* hyphae at junction of necrophylactic periderm and xylem tissue. Layout pictures of confocal micrographs obtained with filter Aand filter B. Arrows indicate fungal hyphae near the junction, and arrow head indicates cell layer bordering the ray. (D) Close view of infected xylem. Arrows indicate hyphae progressing in a ray. Note the continuity of hypha in the vessel close to the ray in the bottom-right corner. The notation NPd stands for necrophylactic periderm, R is for ray, V is for vessel, and WCT is for wound closure tissue.

To efficiently prevent water loss around the injured area, plasmodesma closure may accompany the development of the suberized layers in fibers. This process may thus prevent symplastic transport of nutrients and limit the flow of carbon sources required for the synthesis of a significant amount of occluding material in the large vessels of grapevine wood. In this context, the massive water intake around injured tissue must be suppressed by occluding the vessels exiting the RZ.

This study shows that inoculation with 1000 conidia of *Pch* is sufficient to stop callus growth but not tissue differentiation, as for the NPd. The colonized-plug method used in others studies (Laveau *et al*., 2009; Luque *et al*., 2009) may lead to over infection of tissues and enhanced fungus aggressivity. The infection method used in the present study allows the compartmentalization process to be initiated in the xylem and bark. Several phytotoxic compounds have been purified from medium-cultured *Pch*

(Abou-Mansour *et al*., 2004; Bruno and Sparapano, 2007; Luini *et al*., 2010). *In vitro* tests indicate that these toxins reduce callus growth, alter grapevine-cell physiology, and inhibit induced defense mechanisms (Abou-Mansour *et al*., 2004; Luini *et al*., 2010). We find no *Pch* hypha in the newly formed tissue or across the rays limiting the infected xylem portion (Figures 4A–4C). In this context, the thick suberized layers observed here (NPd and cells bordering the rays at the RZ periphery) may provide impervious barriers that efficiently prevent the spread of *Pch* in the newly formed xylem tissue and the adjacent portion of the fascicular xylem. It has often been observed that the layer bordering rays may not provide a continuous suberized barrier along the rays. Thus, we hypothesize that the gaps observed along rays may contribute to a failure in *Pch* compartmentalization by wall 3 of the CODIT model. Inhibition of callus growth and reduction of cambial activity then imply that toxins may circulate (most probably through the symplastic pathway) from infected to surrounding healthy tissue. Assuming that toxins produced by *Pch* are strong necrosis inducers and that these toxins act far from their production site, it appears that *Pch* might behave as a necrotroph pathogen in grapevine wood.

The response to *Pch* infection of occluding xylem vessels with tyloses and gel is documented (Del Rio *et al*., 2001; Mutawila *et al*., 2011). An *in vivo* study showed that *Pch* is able to degrade pectin and use it as a sole source of carbon (Marchi *et al*., 2001). Because pectin polymers are one of the main components of tyloses and gels in occluded vessels, it is not surprising that vessel occlusions (CODIT model wall 1) do not provide an efficient mechanism to restrict the spread of decay. This statement is supported by the fact that networks of *Pch* propagules appear in occluded vessels. *Pch* may spread more quickly in plants through vessels than by other xylem cells, but it may not be restricted strictly to vessels. The colonization of other xylem areas surrounding the vessels (i.e., fibers, tracheids, and rays) could be a second step of the infection process, allowing the slow spread of *Pch* from the infected growth ring to cells of the healthy outer ring, and finally to the vascular cambium. As found here, the cells bordering rays may be one of the most strategic zones in the compartmentalization process in grapevine wood, especially in mature grapevine wood, where large vessels are mostly in contact with surrounding rays (Schoch *et al*., 2004). The black spots from which *Pch* can be isolated are mainly organized in a concentric manner and often in a line that follows portions of a growth-ring-like boundary (CODIT model wall 4) (Mugnai *et al*., 1999; Luque *et al*., 2009). We found that the BZ (CODIT model wall 4), which developed six weeks after injury, consisted only of a thin layer of one to two suberized cells, which contrasts with the thick lignified layer found in tree species (Pearce, 1996). Fully understanding how native cell layers (and eventually modified cell layers) restrict the centrifugal spread of *Pch* in fascicular xylem portions and in rays requires further investigations.

The goals of this study were to characterize the reactions of grapevine woody stem to bark and wood injury and to determine how these responses are affected by *Pch* infection. This approach provides a simple and convenient method to more deeply understand plant-pathogens interactions in the field of grapevine trunk disease. Fully understanding how native (limits of annual rings: CODIT model wall 2) and modified cell layers (BZ: CODIT model wall 4) restrict the centrifugal spread of *Pch* in fascicular xylem portions and in rays requires further investigations.

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RESEARCH PAPER

Contribution for a better understanding of grapevine fungal trunk diseases in the Portuguese Dão wine region

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Summary. Esca and Petri disease, two of the most important fungal trunk diseases of grapevine, are responsible for significant losses by causing premature decline and dieback in vineyards worldwide. The Portuguese Dão wine region is no exception. Local winegrowers' knowledge on Grapevine Trunk Diseases (GTD) in general and of esca and Petri disease in particular is incomplete. The real scope of those problems has been based largely on individual perceptions rather than on a methodical evaluation of the situation. In order to get a full picture of the diseases impact, a leaflet with color pictures was produced and issued to winegrowers, accompanied by a simple questionnaire. The results of this survey represent a first indication of the extent of grapevine trunk diseases in the Dão wine region, specifically its economic impact and relevance to the local wine industry. In conjunction with the survey, several samples of wood collected from esca and Petri disease symptomatic vines, identified during the survey throughout the entire region, were processed and a collection of isolates of *Phaeomoniella chlamydospora* obtained. To determine the intra-specific variability among these isolates, morphological, cultural and molecular characteristics were evaluated. A protocol to study the pathogenicity with *P. chlamydospora* was conducted, consisting on the inoculation of cv. Touriga Nacional's spurs with a previously studied *P. chlamydospora* isolate.

Key words: *Vitis vinifera*, *Phaeomoniella chlamydospora*, Touriga Nacional, variability, pathogenicity.

Introduction

The Dão wine region has a total area of 376,000 ha, from which less than 20,000 ha are dedicated to grapevine (Figure 1). Wine production there might go back to Roman times (Loureiro and Cardoso, 1993). However the oldest signs of wine production date from the VI century CE, including wine presses carved on solid granite (Falcão, 2012), found throughout the entire region and some are still in use. The Dão region is distinguished by its complex orographic features, soils that are typically low-pH sandy granite with low levels of organic matter, and traditional cultivars, most of them of Portuguese origin. Grown throughout Portugal, cv. Touriga Nacional may originate from this region and due to a clonal selection program (Faustino 2011), is starting to internationalize specifically in Australia (Robinson, 1996; Ambrosi *et al*., 1997). This unique region remains a reservoir for Portuguese grape cultivar diversity thanks to specific grapevine preservation projects. In addition, the region hosts an important R&D facility – Centro de Estudos Vitivinícolas do Dão – one of the most important producers of Portuguese grapevine cultivars.

Research to improve knowledge about Grapevine Trunk Diseases (GTD) is taking place around the world. In France, there has been intense work on the evaluation of GTD with the establishment in 2003 of the "Observatoire Nationale des Maladies du Bois de la Vigne", that produces annual reports on GTD surveillance (MAAPAR, 2004). In Portugal lo-

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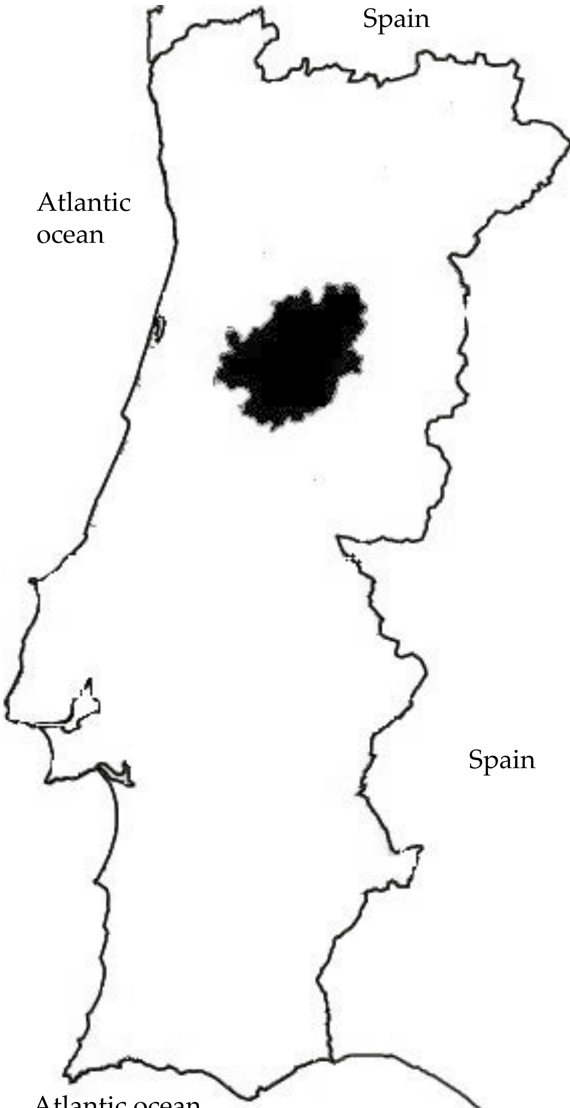

Figure 1. Map of Portugal with the Dão wine region in black.

cal growers are often incapable of understanding the causes for economic losses due to grapevine decline and mortality in their vineyards, which are often due to GTD. The observed symptoms are often confused with other diseases (e.g. viruses), occasional plagues (e.g. *Empoasca* leafhoppers), nutritional and water deficits, thereby misleading them on management solutions. The most common GTD in the Dão, leading to substantial economic losses are esca, Phomopsis cane and leafspot, black dead arm (BDA) and young grapevine declines. Typical foliar symptoms of esca - tiger stripped leaves (Mugnai *et al*., 1999) - are obvious, but foliar symptoms of BDA, disease associated with several species of Botryosphaeriaceae fungi, might resemble those of esca appearing earlier in the season (Larignon *et al*., 2009). BDA is also associated with wood necrosis, being able to infect both young and mature tissues as well as green shoots causing cankers, vascular discoloration, and / or otherwise dark streaking of the wood (Úrbez-Torres and Gubler, 2009). BDA symptoms in wood may be misattributed to *Eutypa* spp., and on herbaceous organs may be confused with Phomopsis cane and leafspot. This panoply of very similar symptoms, sometimes over the same organs, baffles the winegrower.

In previous related studies done at the Dão wine region, *Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams was often isolated both from black and red-brown wood discolouration patches found inside esca affected grapevines and from field spore traps (Sofia *et al.*, 2006). In the present work, the first objective was to increase the knowledge of winegrowers on GTD, while evaluating its frequency in the Dão wine region, centered on a survey among local winegrowers. The results of the survey will, in a near future, be integrated with field data to provide a more accurate picture of the Dão's GTD situation. In order to determine the intra-specific variability among *P. chlamydospora* isolates from Dão wine region, morphological, cultural and molecular characteristics have been evaluated. Finally, an experiment, using a studied isolate of *P. chlamydospora* was conducted on cv. Touriga Nacional, one of the most important of the Dão wine region, to validate a pathogenicity test procedure for grapevine infection with this fungus, which can become an useful tool to help detecting pathogenic variability within the collected isolates of *P. chlamydospora*, or to help on detection of different cultivars' susceptibility to *P. chlamydospora*.

Materials and methods

Leaflet and survey

A four page color leaflet was produced with the key symptoms associated with the main GTD commonly found in the Dão wine region – esca, Phomopsis cane and leafspot, BDA and young grapevine declines - to promote the growers knowledge on GTD (Figure 2). Simultaneously, local growers were invit-

Figure 2. Leaflet produced for divulgation of the main symptoms of grapevine trunk diseases affecting the Dão wine region (English version).

ed to fulfill an easy three step questionnaire (Figure 3) where the first step was a question acknowledging the existence of any of the four GTD on their vineyards; the second step was also a question meant to evaluate the frequency of the disease(s) based on three numerical boundary categories (level 1: few vines affected; level 2: some vines affected; level 3: many vines affected) and the third step concerned the location of the vineyard within the region.

Fungal isolates

Eighteen of the vineyards identified during the survey were studied, and the frequency of GTD evaluated, in order to determine the accuracy of the answers given and also to collect some wood samples from esca and Petri disease symptomatic grapevines. From these samples, cross sections were cut from the trunk, and typical dark brown to black discolored fragments, usually associated to *P. chlamydospora* were extracted. They were surface disinfected by immersion in an 8% solution of NaOCl for 1 min., rinsed with sterile distilled water (SDW), dried with filter paper and placed in Petri dishes containing potato dextrose agar (PDA; Difco, Beckton, Dickinson and Co, Sparks, MD, USA) amended with 250 mg L^{-1} of chloramphenicol (BioChemica, AppliChem, Germany). Inoculated plates were incubated in the dark for eight days, at 25 ± 1°C. After this period, suspected colonies of *P. chlamydospora* were transferred to PDA in order to get pure cultures.

Inquiry on the situation of grapevine trunk diseases on Dão wine region

1. Please, take a look at the leaflet, and if you've found any of the present symptoms on your vine , please check the corresponding box

Disease	Do you find it on your Vineyard?		
	Yes	No	Don't know
Esca			
Phomopsis cane and leaf spot			
Black Dead Arm			
Young Vine Decline			

2. If you have answered positively to question number one, please check, in the table below, the corresponding box, in order to evaluate the frequency of the diseases over your grapevine (1- affects few vines; 2- affects some vines; 3- affects many vines).

Disease	Frequency		
	1	2	3
Esca			
Phomopsis cane and leaf spot			
Black Dead Arm			
Young Vine Decline			

Name of the vineyard
Locality:
Freguesia (Parish):
Concelho (County):

Thank you for your collaboration!

Figure 3. Questionnaire used to evaluate the situation of grapevine trunk diseases in the Dão wine region (English version).

Phenotypic characterization

A collection of twenty *P. chlamydospora* isolates obtained from different locations and different scion/rootstock combinations was used: 17 isolates were collected within the Dão wine region plus three studied and classified isolates obtained from Vidigueira, Alentejo (Ph19), from Arruda dos Vinhos, Extremadura (Ph24) and from a Dão's wine region (Ph30) (Table 1).

All isolates were grown in triplicate on PDA, at 25 ± 1°C, in the darkness for 15 days and phenotypic features (texture, colour, growing margin zonation and hyphal morphology) were described according to Crous and Gams (2000) and González and Tello (2011).

Daily growth and colony mean diameters were obtained after 25 days by measuring two perpendicular diameters for each colony and calculating mean diameters. For each isolate, six repetitions were taken. The number of conidia produced was evaluated according to the method described by Whiting *et al*. (2001).

Molecular characterization

For each isolate, DNA was extracted from mycelium grown on potato dextrose broth (PDB; Difco) using the protocol of Cenis (1992) adapted by Nascimento *et al*. (2001). To study the genetic diversity among *P. chlamydospora* isolates the inter-simple sequence repeat (ISSR) analysis was used. The ISSR primers $(AG)_8YT$ (Fang and Rose, 1997), $(CAG)_5$ (Rodriguez and Yoder, 1991), $HVH(TG)_7$ (Gilbert *et al*., 1999) and MR (5′-GAGGGTGGCG-

Table 1. *Phaeomoniella chlamydospora* isolates studied.

Isolate	Year of isolation	Geographical origin		Host scion/rootstock combination
		Location	Wine region	
Ph19[a]	2008	Vidigueira	Alentejo	Petit Verdot/400VO
Ph24[a]	2011	Arruda dos Vinhos	Estremadura	Touriga Nacional/-
Ph26	2011	Lousã	Dão	Cerceal/-
Ph27	2011	Nelas	Dão	Jaen/-
Ph28	2011	Mangualde	Dão	Jaen/-
Ph29	2012	Mangualde	Dão	Touriga Nacional/-
Ph30[a]	2012	Nelas	Dão	Jaen/S04
Ph31	2012	Nelas	Dão	Tinta Roriz/S04
Ph32	2012	Nelas	Dão	Alfrocheiro/-
Ph33	2012	Seia	Dão	Jaen/-
Ph34	2012	Tondela	Dão	Aragonês/-
Ph35	2012	Mangualde	Dão	Touriga Nacional/-
Ph36	2012	Mangualde	Dão	Encruzado/-
Ph37	2012	Gouveia	Dão	Gouveio/-
Ph38	2012	Nelas	Dão	Touriga Nacional/-
Ph39	2012	Gouveia	Dão	Jaen/-
Ph40	2012	São Martinho da Cortiça	Dão	Baga/-
Ph41	2012	Viseu	Dão	Encruzado/-
Ph42	2012	Mangualde	Dão	Jaen/-
Ph43	2012	Viseu	Dão	Jaen/-

[a] Isolates formerly identified and characterized.

GTTCT-3′) (Bridge *et al*., 1997) were used. Each PCR reaction contained 1× PCR buffer, 3 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 0.8 U of DreamTaq DNA Polymerase (MBI Fermentas, Vilnius, Lithuania), and 3 μL of diluted template DNA in a final volume of 20 μL. Amplifications were performed in a ″Biometra T-Gradient″, with an initial step of 4 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C $(CAG)_5$ and MR or 52°C $(AG)_8$YT and HVH$(TG)_7$ for 45 s, and an elongation at 72°C for 2 min. A final extension was performed at 72°C for 10 min (Talhinhas *et al*., 2003). Reactions without DNA were used as negative controls, and each reaction was repeated at least once. Amplification products were separated by electrophoresis in 2% agarose gels in 0.5 × TBE buffer at 40 V for 19 h. A GeneRuler™ 100 bp Plus DNA Ladder (MBI Fermentas) was used as a molecular weight marker. Gels were stained with ethidium bromide and visualized under UV light, followed by digital image capturing using an UVIdoc system (UVItec Limited, Cambridge, UK). The banding patterns were analyzed with GelCompar II Version 5.10 software package (Applied Maths, Saint-Martens-Latem, Belgium). DNA bands detected by the software were verified by visual examination to correct unsatisfactory detection, and the presence (1) or absence (0) of bands was recorded in a binary matrix.

Genetic similarities were calculated using the Dice coefficient and dendrograms obtained by clustering according to the unweighted pair-group method using arithmetic averages (UPGMA). The robustness of the branches was assessed by bootstrap analysis with 2,000 replicates.

Pathogenicity experiment

A pathogenicity test was carried out in a vineyard of cv. Touriga Nacional, five years old, trained on bilateral cordon with six spurs. During winter, two spurs in each vine were left with three buds and were inoculated immediately after pruning by depositing a droplet of 40 μL of a 10^5 spores mL^{-1} conidial suspension of strain Ph19. Conidial suspension was obtained by plunging a 3 mm diameter micelial disk of the isolate in a 250 mL Erlenmeyer flask containing PDB and placed at 20°C, under darkness, in a reciprocal shaker (90 strokes min^{-1}), for 15 days. The inoculation site was sealed during one week with Parafilm (Parafilm® "M", Pechiney Plastic Packaging, Menasha, USA). Thirty repetitions were made. Control canes were similarly treated but SDW was used instead of inoculum.

Eight months after the inoculation, brown internal discolorations were visible along a longitudinal cut of last year inoculated spurs. Parameters evaluated were thickness (cm) and length from cut to the base (cm) of inoculated canes and length of necrosis (cm). Four pieces of wood were excised from the border of the necrosis and reisolation procedures were performed as described before. The percentage of reisolation was calculated as the proportion of wood pieces from which *P. chlamydospora* colonies were recovered, versus the total number of pieces of wood plated for each plant. Data obtained were compared using an unpaired T-test, type 2. Calculations were performed with the Statistica 6.1 software package (Statsoft Inc., Tulsa, OK, USA).

Results

Results of the GTD survey

During the 2011/2012 survey a total of 62 questionnaires were considered completely fulfilled and validated. It was clear from results that esca was the most well-known GTD of the four explained on the leaflet, with positive identification of its presence in more than 88% of the vineyards (Table 2). Merely 12% of the inquired winegrowers answered that they had never noticed the disease on their vineyards. Concerning the frequency of esca, level 1 of disease frequency was recorded in 80% of the vineyards, level 2 in 16% and level 3 only in 5% of the vineyards (Table 3).

The second most recognizable disease among the inquired was Phomopsis cane and leafspot with 82% of the fulfilled forms confirming its presence. Only 16% of the inquired winegrowers answered that they had never noticed the disease on their vineyards and 2% did not know the disease (Table 2). Regarding frequency of Phomopsis cane and leafspot, 46% of the inquired winegrowers considered it present although affecting a scarce number of vines (level 1),

Table 2. Survey on the situation of grapevine trunk diseases in Dão wine region.

Disease	Do you find it in your vineyard? (%)[a]		
	Yes	No	Don't know
Esca	88	12	0
Phomopsis cane and leafspot	82	16	2
Black dead arm	58	34	8
Young vine decline	30	60	10

[a] Results of a total of 62 questionnaires considered completely fulfilled and validated.

Table 3. Frequency of grapevine trunk diseases in Dão wine region: level 1 - affects few vines; level 2 - affects some vines; level 3 - affects many vines.

Disease	Frequency (%)[a]		
	Level 1	Level 2	Level 3
Esca	80	16	5
Phomopsis cane and leafspot	46	41	12
Black dead arm	72	24	3
Young vine decline	87	13	0

[a] For each of the diseases, frequency was determined considering only the number of questionnaires that acknowledged the existence of the disease. Figures rounded to the next integer.

41% considered it was affecting an important number of plants (level 2) and 12% considered it a serious problem (level 3) (Table 3).

The third identifiable disease for the inquired was BDA with 58% of the fulfilled forms confirming it presence; 34% of the inquired winegrowers answered that they had never noticed the disease on their vineyards and 8% did not know the disease (Table 2). Concerning the frequency of BDA, 72% of the surveyed winegrowers considered it present on their vineyards, but affecting a small number of plants (level 1), for 24% it was affecting some of plants (level 2) and only 3% considered it a severe problem for their vineyards (level 3) (Table 3).

Finally, for young vine decline, 30% of the winegrowers recognized its presence on their vineyards, while 60% never acknowledged the disease on their vineyards and 10% where not familiar with the disease (Table 2). In relation to frequency of young vine decline, 87% of the inquired considered that it was affecting a negligible number of plants (level 1) and 13% considered it present in some plants (level 2) (Table 3).

Phenotypic characterization of the obtained isolates

After 25 days of growth, *P. chlamydospora* isolates produced the characteristic colonies with a felty texture and an absent zonation. However, it was noticeable that the morphology of the colonies was found to be variable among the 20 isolates under study leading to the establishment of four morphological groups (Table 4). Group I shared colony characters such as an olive-grey color, an even growing margin and the existence of predominant filamentous somatic hyphae in PDA. Colonies of group II exhibited olive-grey to white color towards the edge, an even growing margin, producing filamentous, aerial somatic mycelium. Isolates from group III had olive-grey to white color towards the edge, an uneven growing margin and they produced filamentous, aerial somatic mycelium. Finally, group IV had an olive-grey color with the pigment concentrically distributed; an even growing margin and it produced filamentous, aerial somatic mycelium. Mycelial growth rates did not differ significantly among *P. chlamydospora* isolates, and not even within the four mentioned groups.

Phaeomoniella chlamydospora isolates produced the characteristic conidia and chlamydospora-like structures of such species. Sporulation rates of the different isolates (Table 5) showed a broad range of variation (from 2.0 to 14.6 × 10^6 conidia mL^{-1}). Daily growth rate at 25°C ranged from 0.70 to 1.40 mm and the growth diameter at 25°C, after 25 days varied from 17.30 to 34.30 mm.

Molecular characterization

The four ISSR primers tested were able to generate amplification products for all isolates of *P. chlamydospora*. A consensus dendrogram was generated from analysis of all markers (Figure 4). The isolates studied were clustered with *P. chlamydospora* isolates Ph19 and Ph24 with about 82% similarity. This confirms that all the isolates collected in Dão wine region belong to the same species. *Phaeomoniella chlamydospora* isolates were clustered into two groups supported by low bootstrap values, 48% and 54% respectively. The similarity level between groups, around 87%, indicates a low intra-specific genetic diversity. No relationship was found between ISSR band patterns and origin or scion/rootstock combination of isolates and the different groups formed.

Pathogenicity experiment

No significant differences were found among thickness and length (from cut to the base) of the control and inoculated spurs (Table 6). Although *P. chlamydospora* was not recovered from the control spurs, some necroses were noticeable on the tissues of those plants (Table 6). Significant statistic differences were found in the extension of necrosis among control and inoculated canes. It ranged from 2.47 cm in controls to 8.95 cm in inoculated canes. The reisolation percentage reached 72.4% of the canes inoculated.

Discussion

After one year of public education with the GTD leaflet, it was our perception, based on observation of cultural practices like flagging, removing and destruction of symptomatic vines, or in the number of questions on the subject, that winegrowers within the Dão wine region have improved their knowledge on GTD symptoms and general management of the diseases.

Previous works, based on a survey on grapevine trunk diseases (Tomaz *et al.*, 1989), considered esca

Table 4. Distribution of the twenty *Phaeomoniella chlamydospora* isolates among the four morphological groups according to several phenotypic characteristics for each group (after 15 days at 25ºC in PDA).

Group	Isolates	Phenotype in PDA culture	Texture	Colour	Growing margin	Zonation	Hyphal morphology
I	Ph19, Ph24, Ph26, Ph28, Ph33, Ph34, Ph37, Ph40, Ph41		Felty	Olive-grey	Even	Absent	Filamentous somatic hyphae predominant
II	Ph27, Ph32, Ph35, Ph36, Ph39		Felty	Olive-grey to white towards the edge	Even	Absent	Filamentous somatic hyphae predominant, aerial mycelium scanty
III	Ph38, Ph42, Ph43		Felty	Olive-grey to white towards the edge	Uneven	Absent	Filamentous somatic hyphae predominant, aerial mycelium scanty
IV	Ph29, Ph30, Ph31		Felty	Olive-grey; pigment distributed concentrically	Even	Absent	Filamentous somatic hyphae predominant

Table 5. Mean, maximum and minimum values of the phenotypic variables studied for all the *Phaeomoniella chlamydospora* isolates.

Phenotypic variable	Mean[a]	Maximum	Minimum
Sporulation ($\times 10^6$ conidia mL^{-1})	5.9	14.6	2.0
Daily growth rate (mm) at 25°C	1.2	1.4	0.7
Growth (mm) at 25°C, after 25 d	30.0	34.3	17.3

[a] Mean of two independent sets of six replicates for each isolate.

as the main GTD in Dão, having also pointed out for that region the importance of Phomopsis cane and leafspot caused by *Phomopsis viticola* (Sacc.) Sacc. Also, a new emerging disease, designated as european excoriose, caused by *Macrophoma flaccida* (Viala & Ravaz) Cavara (*Fusicoccum aesculli* Corda) was also identified in the area (Tomaz and Rego, 1990).

The survey has provided an overview of the phytosanitary status of grapevines within the Dão wine region especially concerning GTD. The occur-

Table 6. Results of pathogenicity experiment carried out in a vineyard of cv. Touriga Nacional.

Treatments	Thickness (cm)	Length from cut to the base (cm)	Length of necrosis (cm)	Reisolation (%)
Control	3.06a[a]	23.60a	2.47a	0.00a
Inoculated	3.10a	23.70a	8.95b	72.41b

[a] Mean values followed by the same letter are not statistically different at the level 5% .

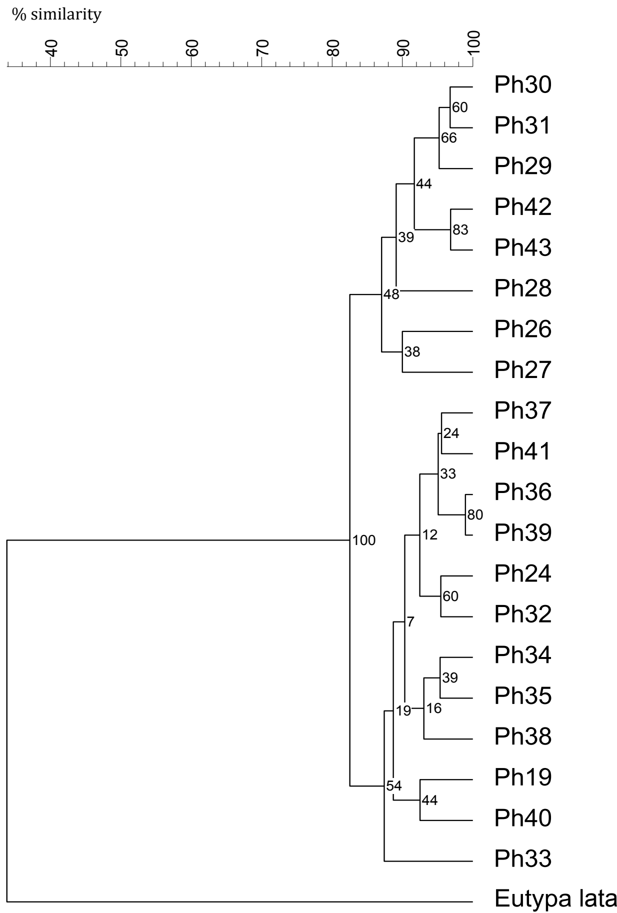

Figure 4. UPGMA cluster analysis based on Dice coefficient of ISSR fingerprints from a collection of isolates of *Phaeomoniella chlamydospora* with the primers $(AG)_8YT$, $(CAG)_5$, $HVH(TG)_7$ and MR. The numbers at the nodes represent bootstrap support values (2,000 replicates). An *Eutypa lata* isolate was used as outgroup.

rence of these fungal diseases in Dão's vineyards is confirmed, although its frequency and incidence in the vineyards is not high enough to become a matter of urgent concern. Esca and Phomopsis cane and leafspot are better known due to published research (Tomaz *et al*., 1989; Tomaz and Rego, 1990). However, BDA caused by Botryosphaeriaceous fungi is not as well known. The abandon of viticulture and the relatively few new plantations registered in this wine region recently (Falcão, 2012; IVV, 2012), has reduced the potential appearance of young plants showing young vine decline symptoms.

Worldwide, *P. chlamydospora* has been regarded as the most important fungus associated with esca and Petri disease (Ridgway *et al*., 2005; Tello *et al*., 2010) together with *Phaeoacremonium* spp. In Portugal, several studies have been focused on *P. chlamydospora* isolates (Chicau *et al*., 2000; Rego *et al*., 2000; Cruz *et al*., 2005; Santos *et al*., 2006). In the Dão wine region, this fungus is usually isolated from esca symptomatic grapevines (Sofia *et al*., 2006). Nevertheless, there is a lack of information about phenotypical and molecular variability of such species.

In our study, analysis of phenotypic characteristics showed that variation among morphological features in culture, such as texture or zonation, concerning *P. chlamydospora* isolates was low. This pattern was consistent with previous studies (Dupont *et al*., 1998; Whiting *et al*., 2005; Tello *et al*., 2010) in which homogeneity was recorded. However, features like colony colour, growing margin or hyphal morphology were found to be variable, allowing the recognition of four groups of *P. chlamydospora* isolates. Within the four recognized phenotypic groups, the variation of phenotypic characteristics was found to be independent of *P. chlamydospora* isolates geographical origin or scion/rootstock combination. The sporulation and the daily growth rate at 25°C of *P. chlamydospora* isolates were similar to the obtained by Tello *et al*. (2010).

In the ISSR primers analysis, *P. chlamydospora* isolates clustered into two groups although no bootstrap support was found for such grouping. Similar results were previously obtained by Tegli *et al*. (2000) and Mostert *et al*. (2006).

The lack of diversity established among the studied isolates might be justified by the short period of time in which the isolates where obtained and by the genetic structure of the population based in asexual reproduction in natural ecosystems (Tegli *et al*., 2000; Pottinger *et al*., 2002; Mostert *et al*., 2006). Moreover, ISSR tools did not detect a significant genetic variability which confirms that sexual reproduction does not occur. Further research based on an enlarged collection of isolates and in other molecular tools is needed to confirm the low genetic diversity within *P. chlamydospora* population in Dão region.

Concerning the results obtained in the pathogenic experiment, the use of the necrosis length in inoculated plants, as a measure of disease severity (Adalat *et al*., 2000; Halleen *et al*., 2007; Laveau *et al*., 2009; Gramaje *et al*., 2010), proved to be an accurate method to evaluate pathogen virulence. The high values obtained on the length of the necrosis formed, agrees with the conclusions of Laveau *et al*. (2009) in which *P. chlamydospora* is considered one of the most aggressive pathogens associated with esca. Taking in account that in Dão region, canes are usually pruned to one to two bud spurs on cordons, the extension of the obtained necrosis and the high frequency of reisolation of *P. chlamydospora* strengthens the idea that recently pruned canes may be a potential entrance for *P. chlamydospora* to the main structure of grapevine, in a short period of time. No esca or Petri disease typical foliar symptoms were observed in inoculated vines. This observation is in accordance with results previously reported by Halleen *et al*. (2007) and Gramaje *et al*. (2010). The inoculation method tested in this pathogenicity test proved to be a successful, simple and practical method to infect plants in field experiments. The known feasibility and simplicity of this method means it will be used in further studies of cultivars' susceptibility to all *P. chlamydospora* isolates obtained, in order to add other criteria for separation of *P. chlamydospora*'s strains. Thus, performing a pathogenicity test with the entire collection could lead to a characterization of hypovirulent or less pathogenic isolates and to a correlation of these data with phenotypic features, geographical origin or ISSR clustering.

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RESEARCH PAPER

Diversity of *Ilyonectria* species in a young vineyard affected by black foot disease

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Summary. Fungi of the *Ilyonectria* genus are the main causal agents of black foot disease of grapevine. These pathogens cause necrosis in the basal end of the rootstock, leading to the early decline and the death of vines in nurseries and young vineyards. In the present study a collection of isolates of the genus *Ilyonectria* obtained from a vineyard located in the Alentejo region, Portugal, was characterised. This vineyard was established with planting material originating from three different nurseries. To assess the inter- and intra-specific variability among isolates, morphological, cultural and biomolecular characteristics were evaluated. Morpho-cultural and molecular data (RAPD and ISSR markers and histone H3 nucleotide sequence) identified *I. estremocensis*, *I. europaea*, *I. liriodendri*, *I. macrodidyma*, *I. torresensis*, *I. vitis* and "*Cylindrocarpon*" *pauciseptatum*. *Ilyonectria torresensis* was the most common species found in the survey, representing more than 50% of the isolates.

Key words: black foot disease, "*Cylindrocarpon*" species, young vineyards, *Vitis vinifera*.

Introduction

Black foot of grapevine is an important disease caused primarily by fungi of the *Ilyonectria* genus. These pathogens cause necrosis at the basal end of the rootstock, leading to the early decline and the death of vines in nurseries and young vineyards (Halleen *et al*., 2004; Oliveira *et al*., 2004). This disease was first described in 1961 (Grasso and Magnano Di San Lio, 1975) and over the last decade its incidence has increased significantly in different grapevine growing areas around the world (Rego *et al*., 2000; Halleen *et al*., 2004; Petit and Gubler, 2005; Alaniz *et al*., 2007). Vines affected by black foot disease show sunken necrotic root lesions and reduced root biomass. By removing the bark, black discoloration and necrosis of wood tissues can be observed extending from the base of the rootstock. Other symptoms include reduced vigour, shortened internodes, sparse foliage and small leaves with interveinal chlorosis and necrosis frequently leading to the death of the plants (Grasso, 1984; Maluta and Larignon, 1991; Scheck *et al*., 1998; Rego *et al*., 2000; Halleen *et al*., 2006a; Alaniz *et al*., 2007).

The genus *Ilyonectria* represents one of several newly established genera of fungi with *Cylindrocarpon*-like anamorphs (Chaverri *et al*., 2011). Previously, Booth (1966) had segregated the genus *Cylindrocarpon* into four groups, based on the presence or absence of microconidia and chlamydospores. Most of the teleomorphs of *Cylindrocarpon* (groups 1, 2 and 4; Booth, 1966) have been classified into the genus *Neonectria*. Recently, Chaverri *et al*. (2011), based on molecular phylogenetic analyses and morphological characters, demonstrated that *Neonectria* comprises at least four different genera: *Neonectria*/*Cylindrocarpon sensu stricto* (Booth's groups 1 and 4), *Rugonectria*, *Thelonectria* (group 2) and *Ilyonectria* (group 3).

Black foot disease of grapevine has been associated with four causal agents, *Ilyonectria liriodendri*

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and *Ilyonectria macrodydima* (Halleen *et al*., 2004; 2006b) and two *Campylocarpon* species, *Campylocarpon fasciculare* and *Campylocarpon pseudofasciculare* (Halleen *et al*., 2004). A fifth species, "*Cylindrocarpon*" *pauciseptatum*, associated with diseased roots of *Vitis* spp. in Slovenia and New Zealand was reported by Schroers *et al.* (2008). The role of "*C.*" *pauciseptatum* as a black foot pathogen was hypothesized by Alaniz *et al.* (2009a) and later confirmed by Cabral *et al.* (2012b). Since its first association with black foot disease, "*C.*" *pauciseptatum* has also been reported in Uruguay (Abreo *et al.*, 2010), Spain (Martín *et al.*, 2011) and Portugal (Cabral *et al*., 2012a).

Since *I. macrodidyma* was first reported as a new species (Halleen *et al*., 2004), several additional reports have associated this pathogen with grapevine black foot disease (Petit and Gubler, 2005; Rego *et al*., 2006; Alaniz *et al*., 2007; Abreo *et al*., 2010; Cabral *et al*., 2012c), which appears to be more virulent than *I. liriodendri* (Alaniz *et al*., 2009b). However, diversity among groups of *I. macrodidyma* was detected by Inter-Simple Sequence Repeat (ISSR) markers and pathogenicity tests (Alaniz *et al*., 2009b). Later, Cabral *et al.* (2012c) recognised six new species within *I. macrodidyma* (*I. alcacerensis*, *I. estremocensis*, *I. novozelandica*, *I. torresensis*, *Ilyonectria* sp1 and *Ilyonectria* sp2), thus demonstrating that *I. macrodidyma* was a species complex. Further, *I. europaea*, *I. lusitanica*, *I. pseudodestructans*, *I. robusta* and *I. vitis*, formerly included in the *I. radicicola* complex (Cabral *et al*., 2012a), were found to be associated with black foot of grapevine (Cabral *et al*., 2012b).

With the recent changes in the taxonomy of the causal agents of the grapevine black foot disease, namely in the *I. radicicola* and *I. macrodydima* complexes, the aim of the current study was to assess the extent of diversity of *Ilyonectria*-like fungi occurring in a single young vineyard. To this end, we studied a collection of isolates obtained from one vineyard showing symptoms of early decline that was established with propagating material from three different nurseries. Isolates were characterised using morphological, cultural and molecular methods (Inter-Simple Sequence Repeat (ISSR) and Random Amplified Polymorphic DNA (RAPD) markers and histone H3 (HIS) nucleotide sequence).

Material and methods

Isolates

This study included 33 isolates of *Ilyonectria*-like fungi obtained from a 2-year-old vineyard located in the southern region of Portugal (Vidigueira, Alentejo), that was showing severe symptoms of early decline (Table 1). The total vineyard area was 60 ha and the planting material was obtained from three independent commercial nurseries, two located in Portugal (nurseries A and B), and the third located abroad (nursery C).

Sample vines were washed with abundant water and small pieces of blackened rootstock woody tissues were collected from 2–4 cm above the rootstock base. Tissue pieces were disinfected by immer-

Table 1. *Ilyonectria*-like isolates obtained from a young vineyard showing symptoms of early decline: species identified, isolate, grapevine cultivar and rootstock, nursery origin (A and B, Portugal; C, abroad) and GenBank accession number.

Species and isolate	Cultivar / Rootstock	Nursery origin	GenBank accession H3
"*Cylindrocarpon*" *pauciseptatum*			
Cy238	Petit Verdot / 110R	B	JF735591
Cy239	Cabernet Sauvignon / 110R	B	…
Ilyonectria estremocensis			
Cy243	Touriga Nacional / 110R	B	JF735626
I. europaea			
Cy241	Petit Verdot / 110R	C	JF735567

(*Continued*)

Table 1. *Continues.*

Species and isolate	Cultivar / Rootstock	Nursery origin	GenBank accession H3
I. liriodendri			
Cy252	Petit Verdot / 110R	C	…
Cy253	Petit Verdot / 110R	B	KC119445
Cy254	Touriga Nacional / 110R	B	…
Cy255	Touriga Nacional / 110R	B	…
Cy256	Petit Verdot / 110R	A	…
Cy257	Petit Verdot / 110R	A	KC119446
I. macrodidyma			
Cy244	Petit Verdot / 110R	A	JF735655
Cy250	Chardonnay / 110R	B	KC119443
Cy258	Cabernet Sauvignon / 110R	C	JF735656
Cy264	Petit Verdot / 110R	B	KC119449
I. torresensis			
Cy234	Chardonnay / 110R	B	KC119438
Cy235	Cabernet Sauvignon / 110R	C	JF735685
Cy236	Cabernet Sauvignon / 110R	C	…
Cy237	Chardonnay / 110R	C	JF735686
Cy240	Touriga Nacional / 140RU	A	JF735687
Cy242	Touriga Nacional / 110R	A	KC119439
Cy245	Petit Verdot / 110R	A	…
Cy246	Antão Vaz / 110R	B	JF735688
Cy247	Cabernet Sauvignon / 110R	B	KC119440
Cy248	Touriga Nacional / 110R	B	KC119441
Cy249	Touriga Nacional / 110R	B	KC119441
Cy251	Chardonnay / 110R	A	KC119444
Cy259	Touriga Nacional / 110R	B	KC119447
Cy260	Cabernet Sauvignon / 110R	B	JF735689
Cy261	Touriga Nacional / 110R	B	…
Cy262	Cabernet Sauvignon / 110R	A	JF735690
Cy263	Touriga Nacional / 110R	B	KC119448
Cy265	Touriga Nacional / 110R	A	KC119450
I. vitis			
Cy233	Touriga Nacional / 110R	A	JF735580

sion for 1 min in a solution of sodium hypochlorite (0.35% w/w active chlorine), rinsed with sterile distilled water (sdw), dried on filter paper and placed in Petri dishes (90 mm diam.) containing potato dextrose agar (PDA, Difco, BD, Sparks, MD, USA) amended with 250 mg L^{-1} chloramphenicol (BioChemica, AppliChem, Darmstadt, Germany). Inoculated dishes were incubated in darkness at 20°C for 12 to 15 days and all *Ilyonectria*-like cultures were single-spored and stored in the collection of the Laboratório de Patologia Vegetal "Veríssimo de Almeida" (LPVVA), ISA, Lisbon, Portugal. Representative isolates were obtained from either the LPVVA collection or Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS) (Table 2).

Cultural and morphological variability

Cultural characteristics (density, growing margin, zonation, texture, transparency) of all isolates were evaluated on PDA and oatmeal agar (OA, Difco, BD, Sparks, MD, USA) after incubation at 20°C, in the dark for 12 days (Samuels and Brayford, 1990). Colony colour (surface and reverse) was determined using Rayner's colour chart (1970).

In order to study the morphological characters, isolates were grown for 10 days on synthetic nutrient agar (SNA) (Nirenberg, 1976) under 12h light (Philips TL 15W/33), at 20°C. Measurements were made by removing 1 cm^2 squares of agar and placing them on microscope slides, to which a drop of water was added and a cover slip laid (Brayford, 1992). For each isolate, 20 measurements were obtained for each type of conidia at a 1000 × magnification, using a Leica DM2500 microscope. Minimum, average, and maximum conidial measurements were determined.

Molecular characterisation

DNA was extracted according to a protocol of Cenis (1992), modified by Nascimento *et al.* (2001). DNA analysis was based on ISSR and RAPD markers, as well as HIS nucleotide sequences. Four ISSR primers were tested: $HVH(TG)_7$ (Gilbert *et al.*, 1999), $(AG)_8YT$ (Fang and Rose, 1997), $(TCC)_5$ and MR (Bridge *et al.*, 1997). PCR amplifications were performed using 1 × PCR buffer, 3 mM $MgCl_2$ (Fermentas, Vilnius, Lithuania), 200 μM of each dNTP, 0.5 μM of each primer, 0.5 units of *Taq* DNA Polymerase (recombinant) (Fermentas) and 2.5 μL of diluted gDNA, in a final volume of 12.5 μL. The cycle conditions in a Biometra T-Gradient termocycler were 94°C for 1 min, followed by 40 cycles of 94°C for 30s, 50°C for 45s, 72°C for 2 min, and a final elongation of 10 min at 72°C. In the case of the $HVH(TG)_7$ and $(AG)_8YT$ primers, there was a slight change in the hybridization temperature from 50°C to 52°C (Talhinhas *et al.*, 2003). Four RAPD markers were also used: OPA-09, OPA-10, OPB-01 and OPD-13 (previously validated for black foot pathogens by Rego, 2004). Each PCR amplification was performed using 1 × PCR buffer, 3.5 mM $MgCl_2$ (Fermentas), 200 μM of each dNTP, 0.6 μM of each primer, 1 unit of *Taq* DNA Polymerase (recombinant) (Fermentas), and 2 μL of diluted gDNA, in a final volume of 20 μL. The cycle conditions, also in a Biometra T-Gradient termocycler were 94°C for 5 min, followed by 35 cycles of 94°C for 2 min, 37°C for 1 min, 72°C for 2 min, and a final elongation at 72°C for 15 min.

PCR products were separated by electrophoresis on 2% agarose gels in 0.5 × TBE buffer for 18 h at 40V. The gels were stained with ethidium bromide, visualized under ultraviolet light and photographed.

Gel images were analyzed using the software GelCompar II version 5.10 (Applied Maths NV, Sint-Martens-Latem, Belgium). Molecular weights were assigned to each band using a 1 Kb Plus ladder marker (Invitrogen, Gaithersburg, MD, USA) and DNA polymorphic fragments were scored automatically and rectified manually as present (1) or absent (0), generating a binary matrix. Clustering was performed using Dice's similarity coefficients and the unweighted pair group method (UPGMA). Internal branch support was evaluated by bootstrap analysis with 2,000 replicates.

Sequencing of part of the HIS gene was performed by STAB Vida, Lda. (Monte de Caparica, Portugal), after PCR amplification as described by Cabral *et al.* (2012a). 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). The sequences obtained were then blasted in GenBank against the corresponding sequences from the epitype strains for *Ilyonectria* spp. to confirm the identity of the isolates.

Table 2. Details of *Ilyonectria* spp. and "*Cylindrocarpon*" spp. isolates used in phylogenetic studies.

Species and isolate[a]	Collected (year)	Location	Host (cultivar/rootstock)
"*Cylindrocarpon*" sp.			
Cy228	2003	Lisbon, Portugal	*Ficus* sp.
"*C.*" *pauciseptatum*			
Cy217	2007	Torres Vedras, Portugal	*V. vinifera* (Gouveio/-)
Ilyonectria alcacerensis			
Cy134; IAFM Cy20-1	…	Ciudad Real, Villarubia de los Ojos, Spain	*V. vinifera*
CBS 129087; Cy159	2004	Alcácer do Sal, Torrão, Portugal	*V. vinifera* (Sangiovese/1103P)
I. anthuriicola			
CBS 564.95	1995	Bleiswijk , The Netherlands	*Anthurium* sp.
I. estremocensis			
Cy135	2003	Estremoz, Portugal	*V. vinifera* (Aragonez/3309C)
CBS 129085; Cy145	2003	Estremoz, Portugal	*V. vinifera* (Aragonez/3309C)
I. europaea			
Cy155	2004	Alter do Chão, Portugal	*V. vinifera* (Alfrocheiro/SO4)
CBS 537.92	1992	Liège, Belgium	*Aesculus hippocastanum*
I. liriodendri			
CBS 117640; IMI 357400; Cy1	1992	Dois Portos, Torres Vedras, Portugal	*V. vinifera* (Seara Nova/99R)
CBS117526; Cy68	1999	Ribatejo e Oeste, Portugal	*Vitis* sp. (99R)
macrodidyma			
CBS 112603	1999	Darling, Western Cape, South Africa	*V. vinifera*
I. novozelandica			
Cy117	…	Califórnia, EUA	*Vitis* sp.
CBS 113552	2003	Candy P, New Ground, New Zealand	*Vitis* sp.
I. pseudodestructans			
CBS 129081; Cy20	1996	São Paio, Gouveia, Portugal	*V. vinifera* (Malvasia Fina/1103P)
Cy22	1996	Silgueiros, Viseu, Portugal	*Vitis* (Aragonez/99R)
I.robusta			
Cy23	1997	Ribatejo e Oeste, Portugal	*Vitis* sp. (99R)
CBS 773.83	…	Utrecht, The Netherlands	water
I. torresensis			
Cy214	2007	Torres Vedras, Portugal	*V. vinifera* (Grenache/-)

[a] CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; Cy, *Cylindrocarpon* collection housed at Laboratório de Patologia Vegetal Veríssimo de Almeida, ISA, Lisbon, Portugal; IAFM, Instituto Agroforestal Mediterraneo, Universidad Politecnica de Valencia, Spain; IMI, International Mycological Institute, CABI-Bioscience, Egham, UK.

Results

Morpho-cultural characteristics

Only 21 out 33 isolates produced abundant conidia on SNA, predominantly (1−)3(−4)-septate macroconidia, straight or minutely curved, cylindrical with round extremities, sometimes widening towards the tip. The values obtained from the analysis of conidia size were within those described for species of *Ilyonectria* and "*C.*" *pauciseptatum* associated with black foot disease. Seven groups were created according to macro- and microconidia measurements, as well as conidial features (Table 3).

Isolates Cy252, Cy253, Cy254 and Cy256 were within the range described for *I. liriodendri*. They formed 1−3-septate macroconidia, straight or slightly curved, cylindrical, base mostly with a visible, centrally located or laterally displaced hilum. The microconidia were formed in heads or on the agar surface; 0−1-septate, ellipsoidal, cylindrical or ovoid, more or less straight, with a laterally displaced hilum.

Conidia morphology and size for the isolate Cy241 were within those described for *I. europaea*. Macroconidia were 1−3-septate, straight or minutely curved, cylindrical with both ends more or less broadly rounded, occasionally narrow towards the tip and mostly without a visible hilum. Microconidia were 0−1-septate, ellipsoid to ovoid, more or less straight and without a visible hilum.

Isolates Cy244 and Cy258 were within the range described for *I. macrodydima*. Macroconidia usually 1−3(4−)-septate, mostly straight, sometimes slightly curved and apically rounded. The typical apical cell was slightly bent to one side with a laterally displaced hilum. Microconidia were usually 0−1-septate, ellipsoid or ovoid, more or less straight and with a laterally displaced hilum.

For isolates Cy238 and Cy239 the values recorded were within those described for "*C.*" *pauciseptatum*. Macroconidia were predominately 3-septate, straight or slightly curved, more or less cylindrical, with both ends rounded. Usually no hilum was visible. Conidia morphology and size of isolates Cy237, Cy240, Cy242, Cy246, Cy247, Cy249, Cy259, Cy261, Cy262 and Cy263 were within those described for *I. torresensis*. Macroconidia were predominately, (1−)3(−4)-septate, straight or minutely curved, cylindrical, or with minute widening towards the tip and appearing rather clavate, particularly when still attached to the phialide. The apex or apical cell was typically slightly bent to one side and minutely beaked. The base typically had a visible, centrally located or laterally displaced hilum. The microconidia were 0−1-septate, ellipsoidal to ovoid, more or less straight, with a minutely or clearly laterally displaced hilum and a constriction at the septum.

For isolate Cy243, the values recorded were within those described for *I. estremocensis*. Macroconidia predominated, and were formed on simple conidiophores. On SNA macroconidia formed in flat domes or slimy masses, 1(3)-septate, straight or slightly curved, cylindrical, but typically with a slight widening towards the apex and appearing somewhat clavate. The apex was obtuse and the base mostly with a visible, centrally located or laterally displaced hilum. Microconidia were 0−1-septate, cylindrical, more or less straight and with a minutely or clearly laterally displaced hilum.

Finally, isolate Cy233 was slightly different from all others and fitted the characteristics described for *I. vitis*. Macroconidia were predominantly 3-septate, commonly 4−5-septate, but rarely 1−2-septate, straight or minutely curved, cylindrical with both ends more or less broadly rounded, mostly without a visible hilum. Microconidia formed in heads and were aseptate, subglobose to ovoid, mostly with a centrally located or slightly laterally displaced hilum.

Morpho-cultural differences among isolates are reported in Table 4, revealing nine different groups of isolates, mainly based on mycelium coloration (surface and reverse) and growing margin. Although seven different types of mycelium coloration have been observed on PDA, differences on OA medium and/or differences between the growing margins resulted in the identification of the nine groups referred to above (Table 4). Only isolates of *I. torresensis* and *I. macrodidyma* could not be clearly distinguished from each other by morphological features in culture and were scattered over four groups rather than two as would be expected.

Molecular characterisation

The combined analysis of RAPD and ISSR fingerprints enabled the clustering of the *Ilyonectria*-like isolates into three major groups (Figure 1). Group A, which includes species from *I. radicicola* complex and contains isolates belonging to *I. europaea*, *I. liriodendri*, *I. pseudodestructans* and *I. robusta.* Group B includes isolates belonging to *I. alcacerensis*, *I. macrodidyma*, *I.*

Table 3. Average conidia size for 21 isolates of *Ilyonectria* spp. and "*Cylindrocarpon*" *pauciseptatum* in SNA medium, after 20 days incubation (20°C, 12 h light).

Species and isolate	Microconidia (µm)		Macroconidia (µm)	
	0-1-septate	1-septate	2-septate	3-septate
"*Cylindrocarpon*" *pauciseptatum*				
Cy238, Cy239	…	(21.2-)31.2(-43.4) ×	(25.6-)41.7(-55.4) ×	(38.9-)45.0(-53.9) ×
	…	(3.1-)7.0(-9.3)	(6.4-)7.9(-9.3)	(6.7-)8.2(-9.3)
Ilyonectria estremocensis				
Cy243	(12.0-)16.6(-20.0) ×	(27.3-)30.9(-34.1) ×	(26.4-)34.4(-42.6) ×	(34.0-)39.0(-43.1) ×
	(4.0-)4.4(-5.0)	(4.1-)4.9(-5.4)	(4.7-)5.5(-6.3)	(4.8-)5.6(-6.5)
I. europaea				
Cy241	(3.0-)9.1(-17.0) ×	(16.4-)22.6(-34.0) ×	(22.0-)27.2-(34.0) ×	(22.0-)30.6(-40.0) ×
	(1.7-)3.4(-5.0)	(4.0-)5.4(-7.8)	(4.4-)6.1(-8.0)	(5.0-)6.7(-8.6)
I. liriodendri				
Cy252, Cy253, Cy254, Cy256	(5.7-)9.2(-14.3) ×	(12.9-)16.7(-20.0) ×	(11.4-)21.1(-28.6) ×	(20.0-)23.6(-30.0) ×
	(1.4-)3.5(-5.7)	(2.9-)4.15(-5.7)	(2.9-)4.1(-7.1)	(2.9-)5.4(-7,1)
I. macrodidyma				
Cy244, Cy258	(7.1-)9.4(-11.4) ×	(17.2-)20.1(-28.6) ×	(18.6-)26.6(-34.3) ×	(20.0-)29.1(-38.6) ×
	(2.9-)3.6(-5.7)	(4.0-)5.0(-8.9)	(4.3-)5.8(-7.1)	(5.6-)6.6(-8.6)
I. torresensis				
Cy237, Cy240, Cy242, Cy246, Cy247, Cy249, Cy259, Cy261, Cy262, Cy263	(5.7-)10.7(-14.3) ×	(12.8-)22.3(-34.1) ×	(15.7-)27.9(-42.6) ×	(20.0-)35.2(- 48.6) ×
	(1.4-)3.7(-4.3)	(2.9-)4.7(-6.4)	(4.3-)5.4(-8.6)	(5.6-)6.1(-8.6)
I. vitis				
Cy233	(3.7-)5.1(-6.7) ×	(23.-)34.3(-46.0) ×	(37.0-)42.5(-47.7) ×	(43.7-)45.0(-53.7) ×
	(3.2-)3.8(- 4.6)	(5.4-)7.5(-9.1)	(7.5-)8.1(-9.0)	(7.6-)8.6(-9.1)

novozelandica and *I. torresensis* that were formerly included in the *I. macrodidyma* complex. Finally, group C that included isolates belonging to *I. anthuriicola*, "*Cylindrocarpon*" sp., *I. vitis* and "*C.*" *pauciseptatum*. This combined analysis of ISSR and RAPD fingerprints enabled the clustering of *Ilyonectria*-like isolates belonging to the same species together (bootstrap value of 100%) at a similarity greater than 65% (Figure 1). These results were also corroborated by DNA sequence analysis of part of the HIS gene, with the isolates in the study assigned to seven species (Table 1).

The analysis of the frequency of the species within the collection revealed the predominance of *I. torresensis* (55%), followed by *I. liriodendri* (18%) and *I. macrodidyma* (12%). The isolates of these species were recovered from plants obtained from all three nursery suppliers. The two isolates of "*C.*" *pauciseptatum* were isolated from plants supplied by nursery B. The remaining species, *I. estremocensis*, *I. europaea* and *I. vitis*, were each represented by only one isolate; each one originating from a vine from a different nursery supplier (Table 1).

Discussion

Until recently, the *Ilyonectria* species most widely associated with black foot disease of grapevine

Table 4. Cultural characteristics of 33 *Ilyonectria* spp. and "*Cylindrocarpon*" *pauciseptatum* isolates grown on PDA and OA, after 10 days incubation (20°C in the dark).

Isolate	Texture	Density	Colour	Growing margin	Transparency	Zonation	Reverse
Cy235, Cy242, Cy247, Cy249	Felty	Average	PDA: chestnut with aerial mycelium buff to cinnamon; OA: cinnamon to dark sienna, mycelium buff to luteous	Uneven	Homogeneous on PDA	Concentric on PDA, sometimes absent on OA	Same as surface, except for the colour (PDA: sienna to chestnut; OA: light cinnamon to dark fulvous)
Cy237, Cy240, Cy244, Cy250, Cy259, Cy262, Cy263, Cy245, Cy260, Cy261, Cy264, Cy265	Felty	Average	PDA: chestnut with aerial mycelium buff to cinnamon; OA: cinnamon to dark sienna, mycelium buff to luteous	Even	Homogeneous on PDA	Concentric on PDA, sometimes absent on OA	Same as surface, except for the colour (PDA: sienna to chestnut; OA: light cinnamon to dark fulvous)
Cy251	Felty	Average	PDA: chestnut with aerial mycelium buff to cinnamon; OA: chestnut, mycelium buff	Even	Homogeneous on PDA	Concentric on PDA, sometimes absent on OA	Same as surface, except for the colour (PDA: sienna to chestnut; OA: light chestnut)
Cy234, Cy236, Cy246, Cy248, Cy258	Cottony on PDA, felty on OA	Average to strong	PDA: dark sienna to chestnut with aerial mycelium buff to luteous; OA: light sienna to chestnut with aerial mycelium buff to luteous and growing margin saffron to luteous	Uneven sometimes even on OA	Homogeneous on PDA	Concentric or absent	Same as surface, except for the colour (PDA: chestnut to dark chestnut; OA: light cinnamon to light chestnut)
Cy238, Cy239	Felty to cottony	Average on PDA, average to weak on OA	PDA: orange to sienna with aerial mycelium saffron, growing margin buff to pale luteous; OA: pale luteous to luteous with growing margin luteous	Even	Homogeneous on PDA	Concentric on PDA, absent on OA	Same as surface, except for the colour (PDA: dark orange to sienna; OA: luteous to orange)
Cy243	Cottony	Average to strong	PDA: sienna with dark luteous to sienna mycelium and growing margin buff to luteous; OA: buff to saffron to cinnamon and growing margin amber to pure yellow	Even	Homogeneous on PDA	Absent	Same as surface, except for the colour (PDA: chestnut; OA: light sienna)

(*Continued*)

Table 4. *Continues.*

Isolate	Texture	Density	Colour	Growing margin	Transparency	Zonation	Reverse
Cy233	Felty	Weak to average	PDA: chestnut with aerial mycelium sienna and growing margin luteous; OA: sienna with aerial mycelium saffron and growing margin luteous	Even	Homogeneous on PDA	Concentric on PDA, absent on OA	Same as surface, except for the colour (PDA: chestnut to umber, OA: sienna to saffron
Cy241	Felty	Average	PDA: sienna to saffron with aerial mycelium luteous; OA: chestnut with aerial mycelium saffron	Even	Homogeneous on PDA	Concentric on PDA	Same as surface, except for the colour (PDA: chestnut to umber; OA: sepia)
Cy252, Cy253, Cy254, Cy255, Cy256, Cy257	Felty to cottony	Average to strong on pda, weak to average on OA	PDA: dark saffron to cinnamon with aerial mycelium buff to light luteous; OA: sienna with aerial mycelium buff	Even	Homogeneous on PDA	Absent	Same as surface, except for the colour (PDA: ochreous to fulvous; OA: ochreous to light fulvous)

were *I. liriodendri*, *I. macrodydima* (Halleen *et al*., 2004, 2006a) and, to a lesser extent, "C." *pauciseptatum* (Schroers *et al*., 2008). Latterly, Cabral *et al.* (2012c) demonstrated that *I. macrodidyma* was a species complex encompassing six different species, as well as the *I. radicicola* complex (Cabral *et al*., 2012a). From this complex, at least *I. europaea*, *I. lusitanica*, *I. pseudodestructans* and *I. robusta* were recognized as causal agents of black foot disease of grapevine, as well as *I. vitis* (Cabral *et al*., 2012b).

In the present study, a young vineyard showing symptoms of early decline was surveyed and *Ilyonectria*-like fungi were consistently isolated from declining vines. The variation in morpho-cultural characteristics of the collected isolates clearly indicated that several species were present in the collection. Conidial morphology, especially septation, shape and size, was the most informative phenotypic characteristic and enabled the clustering of isolates into seven distinct groups. Results of conidia measurements were in accordance with those described for *I. liriondendri* (Halleen *et al*., 2006b), *I. macrodydima* (Halleen *et al*., 2004), *I. europaea* and *I. vitis* (Cabral *et al*., 2012a), *I. estremocensis* and *I. torresensis* (Cabral *et al*., 2012c), and "C". *pauciseptatum* (Schroers *et al*., 2008). However, this grouping did not match that determined by cultural characteristics, thus revealing that morpho-cultural characteristics alone are not a reliable means of identifying cryptic species.

RAPD and ISSR molecular markers were used to first disentangle species within the *Ilyonectria*-like fungi collection, followed by sequencing part of the HIS gene. Although the establishment of species boundaries from DNA polymorphisms should be done carefully (Menzies *et al*., 2003), the combined use of RAPD and ISSR analyses revealed its usefulness for disentangling species. Cluster analysis based on RAPD and ISSR marker data were further supported by results of HIS gene sequencing and were mostly in agreement with results of morpho-cultural characters. Several clusters were obtained and a predominance of one particular species, *I. torresensis*, was revealed, followed by *I. liriodendri*, *I. macrodidyma* and "C." *pauciseptatum*. The remaining species namely *I. estremocensis*, *I. europaea* and *I. vitis,* were only represented by one isolate each. There was no clear relationship between most isolates and the nursery of origin. However, the three species represented by one isolate each came from vines from three different nurseries.

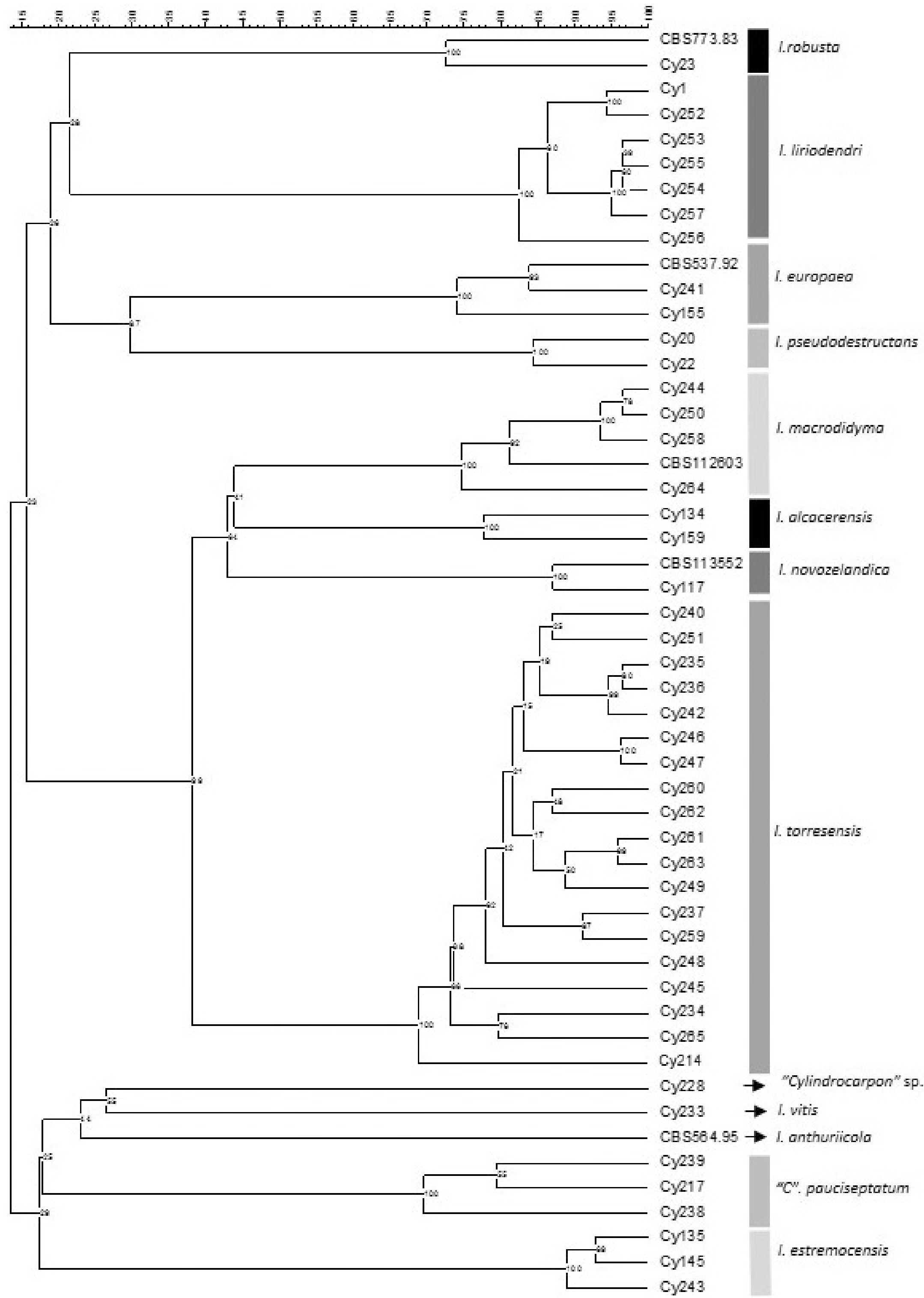

Figure 1. Dendrogram showing the diversity and relationships among *Ilyonectria* spp. and "*Cylindrocarpon*" spp. isolates based on cluster analysis (UPGMA) of a similarity matrix (Dice) generated from ISSR-PCR and RAPD-PCR profile data with four primers each. A total of 2,000 bootstrap replicates were used.

All *Ilyonectria* spp. identified during the present study, as well as "*C.*" *pauciseptatum* are pathogenic to grapevine and potentially to other hosts. Representatives of each species found during this survey were previously tested for pathogenicity on the grapevine rootstock 1103P (Cabral *et al*., 2012b). The results showed high virulence of *I. estremocensis* and *I. europaea* to grapevine followed by *I. liriodendri* and *I. macrodidyma* or *I. torresensis*, ranging over the positions of intermediate virulence.

The present study revealed the predominance of *I. torresensis* within the collection, and available data indicate that this pathogen is well established in at least some Mediterranean countries. In fact, in addition to Portugal, *I. torresensis* has been recently reported as one of the most frequent pathogens associated with black foot disease of grapevine in Spain (Agustí-Brisach *et al*., 2013a, 2013b) and with a root rot disease of kiwifruit in the Black Sea Region of Turkey (Erper *et al*., 2013). It is also very probable that previous records of *I. macrodidyma* from grapevine and other host plants may correspond in part to *I. torresensis*.

During the course of this study six *Ilyonectria* species and "*C.*" *pauciseptatum* were recovered. A maximum of five *Ilyonectria*-like species were collected from material coming from one nursery, thus revealing the primary role of propagation material movement in spreading black foot pathogens. The presence of these pathogens in grapevine propagation material is well documented, as a result of infections that occur in grapevine mother-fields, at different stages of the grapevine propagation process or during the rooting phase in nursery fields (Agustí-Brisach *et al*., 2013; Cardoso *et al*., 2013).

Ilyonectria species found from this survey are not grapevine specific and the potential for cross-infection from other host species was recently demonstrated (Tewoldemedhin *et al*., 2011; Agustí-Brisach *et al*., 2011; Cabral *et al*., 2012b). Furthermore, "*C.*" *pauciseptatum* is also not grapevine-specific and this pathogen has been recovered from apple orchards affected by apple replant disease (Tewoldemedhin *et al*., 2011) and from kiwifruit trees (Erper *et al*., 2013).

Overall, our results reinforce the potential of grapevine propagating material to carry a multitude of *Ilyonectria*-like fungi to new growing areas, thus contributing to the establishment of foreign pathogens in the soil, and potentially compromising not only grapevine but also other plant hosts.

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RESEARCH PAPER

Hot water treatment to reduce incidence of black foot pathogens in young grapevines grown in cool climates

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Summary. Black foot disease causes death of infected grapevines but management of this soil-borne disease by preventative measures such as pre-planting fungicide dips has not been totally effective. Hot water treatment (HWT; 50°C for 30 min) of young dormant grapevine plants has been shown to significantly reduce infection. However, it has been reported to cause unacceptable damage to young vines in cooler climate countries like New Zealand, so this study examined the effects of different HWT protocols on the New Zealand black foot isolates. *In vitro* testing of different HWT protocols was conducted on conidia, mycelium and detached, inoculated grapevine canes using three isolates each of the species *I. liriodendri* (*"C". liriodendri*) and the complexes, *I. radicicola ("C". destructans)* and *I. macrodidyma* (*"C". macrodidymum)*. Heat treatments greater than 40°C for 5 min killed all conidia ($P<0.001$), and treatments greater than 47°C for 30 min inhibited ($P\leq0.003$) further growth of treated mycelium plugs for all but one isolate. Within cane pieces, infection by *Ilyonectria* (*"Cylindrocarpon"*) isolates was significantly reduced ($P<0.001$) by 30 min at 48.5 and 50°C. Additionally, these studies showed different responses to the different treatments for the three isolates of each species complex and differences between species. In field trials, HWT of 48.5 and 50°C for 30 min significantly reduced disease incidence in dormant plants to 0% ($P\leq0.001$). This study confirmed that HWT of 48.5°C for 30 min could be used to eliminate black foot disease in dormant nursery grapevines grown in New Zealand prior to their use for establishing new vineyards.

Key words: "Cylindrocarpon", New Zealand, *in vitro*, field experiments, *Ilyonectria.*

Introduction

Black foot is a serious disease of grapevines in nurseries and vineyards world-wide which causes stunting, chlorosis, late bud break and often death of vines. Infected vines often show small necrotic lesions on roots and the butts of affected vines show dark purplish or reddish brown necrotic streaks which spread across the whole trunk eventually causing death of the vine. The disease has been identified in all major viticulture regions throughout the world, including Italy (Grasso and Magnano Di San Lio, 1975; Grasso, 1984), California (Scheck *et al.*, 1998; Petit and Gubler, 2005), Portugal (Rego *et al.*, 2000), South Africa (Fourie *et al.*, 2000), New Zealand (Halleen *et al.*, 2004c), Australia (Whitelaw-Weckert *et al.*, 2007), Chile (Auger *et al.*, 2007) and Spain (Alaniz *et al.*, 2009) and in less well known viticultural regions such as Lebanon (Choueiri *et al.*, 2009), Iran (Mohammadi *et al.*, 2009), Uruguay (Abreo *et al.*, 2010) and Canada (Petit *et al.*, 2011).

The pathogens reported to cause black foot include *"Cylindrocarpon" liriodendri, "C". macrodidymum* and *"C". destructans* and in New Zealand were isolated from 121 of 141 symptomatic vines, (86%) contributed by 49 growers (Bleach *et al.*, 2006). These were later identified in a study by Mostert *et al*. (2006) by molecular analyses of the internal transcribed spacers 1 and 2 (ITS1 and ITS2), the 5.8S ribosomal RNA gene and the partial β-tubulin genes. However, recent publications used new molecular techniques that reclassified these species into mul-

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tiple new species. Chaverri *et al*. (2011) divided the *Neonectria* into four groups: 1) *Neonectria/Cylindrocarpon* sensu stricto, 2) *Rugonectria,* 3) *Thelonectria* and 4) *Ilyonectria.* Cabral *et al.* (2012a) introduced 12 new taxa within the *Ilyonectria radicicola* (*"C". destructans*) species complex and more recently Cabral *et al*. (2012b) demonstrated that there are six monophyletic species within the *Ilyonectria macrodidyma* complex. They used a combination of morphology, culture characteristics and multi gene analysis which included the β-tubulin, nuclear ribosomal RNA internal transcribed spacer (ITS), translation elongation factor 1-α genes and the histone H3 sequences, which were most useful. However the purpose of this research was to develop control strategies which were effective against a broad range of *"Cylindrocarpon"* species, and so the precise taxonomy of the isolates within the three originally designated species was considered of relatively low importance for the overall outcome.

Ilyonectria macrodidyma species complex and *Ilyonectria radicicola* species complex have been most commonly reported (Rego *et al*., 2000; Armengol *et al*., 2001; Halleen *et al*., 2003; Gubler *et al*., 2004; Oliveira *et al*., 2004) and in South Africa, *Campylocarpon fasciculare* and *Campyl. pseudofasciculare* were also frequently associated with black foot of grapevines (Halleen *et al*., 2004b). In New Zealand vineyards, *Ilyonectria liriodendri*, *Ilyonectria macrodidyma* complex and *Ilyonectria radicicola* complex, have been found associated with black foot, were widespread and of similar importance (Bleach *et al*., 2007). More recently *Cylindrocladiella parva* (Jones *et al*., 2012) has been reported as a pathogen responsible for black foot of grapevines in New Zealand.

Black foot is a soil-borne disease, thus the pathogen propagules and infected host debris that remain in the soil after infected host plants have been removed can infect subsequent plantings. Severe levels of disease were reported in replanted vineyards in Portugal (Oliveira *et al*., 2004) and in New Zealand vineyards which replaced apple orchards (Bonfiglioli, 2005). Nursery field sites were also reported to harbour the pathogens, with more than 50% of the grafted vines becoming infected after 7 months growth in previously used nursery soils in South Africa (Halleen *et al*., 2003). Although the disease cycle of *"Cylindrocarpon"* spp. in vineyards has not been well studied, the behaviours of these pathogens on other hosts (Booth, 1966; Brayford, 1993) has indicated that conidia and chlamydospores are likely to be produced on the diseased roots and stem bases of infected vines. Agustí-Brisach *et al*. (2011) also reported isolation of *"C" macrodidymum* from 26 of 52 weed species growing in propagation nurseries and vineyards, with these being pathogenic to vineyard weeds and therefore may provide sources of spores. Previous research reports (Rego *et al*., 2001; Halleen *et al*., 2003; Probst, 2011) have shown that contact between these spores and the grapevine roots or callused stem bases results in high rates of infection.

Few specific recommendations are currently available for the control of back foot disease. Research reports on the use of pre-planting fungicide treatments have provided varying results. In South Africa, Halleen *et al*. (2007) tested the efficacy of fungicides in reducing mycelium growth of four black foot pathogens, *"C". liriodendri*, *"C". macrodidymum*, *Campyl. fasciculare* and *Campyl. pseudofasciculare.* They found that prochloraz manganese chloride was the most effective at reducing mycelium growth of all four pathogens, while benomyl, flusilazole and imazalil were only effective in reducing mycelium growth of *"C". liriodendri* and *"C". macrodidymum.* In contrast, their nursery field trials showed that these four fungicides as pre-planting soak treatments were not able to prevent infection by *"C". liriodendri* and *"C". macrodidymum*. In a field experiment in 2002–2003, Halleen *et al*. (2007) showed that these fungicides were not totally effective, with incidence of black foot pathogens in bases of uprooted plants being 53.3, 40.7, 54 and 31.3%, respectively, compared to the untreated controls (45.3%). However, other more recent trials have demonstrated that these fungicides can reduce incidence of infection when applied before planting into inoculated pots or naturally infested nursery fields. Nascimento *et al*. (2007) reported significantly reduced incidence of *"C". liriodendri* for potted grapevines treated with cyprodinil + fludioxonil (21.7%) and chitosan (32.0%) than for the control (80.8%), but the carbendazim + flusilazole treatment caused similar incidence to the control (40.8%). Rego *et al*. (2009) reported that in a field site with a previous grapevine history, plants treated with cyprodinil + fludioxonil and fludioxonil alone had significantly less disease incidence (40 and 25%, respectively) and severity (4.2 and 2.9%, respectively) than the water treated control plants (75 and 10.4%, respectively). Alaniz *et al*. (2011) found cuttings dipped into the fungicides captan, carbendazim, copper oxychloride, prochlo-

raz, didecyldimethyl-ammonium chloride and hydroxyquinoline sulphate, significantly decreased the severity of root disease for both "*Cylindrocarpon*" species compared with the control treatment. However, disease incidence for cuttings inoculated with "*C*". *liriodendri* was significantly reduced by only captan, carbendazim and didecyldimethyl-ammonium chloride, while for cuttings inoculated with "*C*". *macrodidymum* only prochloraz reduced disease incidence (Alaniz *et al*., 2011).

Hot water treatment (HWT) at 50°C for 30 min has been reported to effectively eliminate the black foot pathogens and some other grapevine pests and pathogens from grapevine materials in Spain (Gramaje *et al*., 2009), South Africa (Halleen *et al*., 2004a), California (Rooney and Gubler, 2001) and Australia (Waite and Morton, 2007). The industry standard HWT (50°C for 30 min) has been advocated for Petri disease (Ferreira, 1999a; Pascoe *et al*., 2000), however, there are contradictory reports about the efficacy of the treatment. In experiments with dormant cutting material, both Laukart *et al*. (2001) and Rooney and Gubler (2001) did not observe a significant reduction in infection and concluded that HWT was ineffective. However, Edwards *et al*. (2003b) and Fourie and Halleen (2003) observed an average reduction of 76% in the incidence of infected vines and concluded that while the treatment did not eliminate the pathogen, this level of reduction suggested it may be of use in disease management. However, HWT has sometimes been reported to cause damage to propagation material (Crocker and Waite, 2004; Waite and May, 2005) and "negative side effects on vine development" (Habib *et al*., 2009) especially to grapevines grown in cooler climates like New Zealand (Graham, 2007b; Waite and Morton, 2007). The damage seems to be most common in cool climates and is thought to be related to the lower heat tolerance within these grapevines (Crocker *et al*., 2002; Crocker and Waite, 2004). In grapevine cuttings grown in New Zealand, HWT at 47°C for 30 min reduced "known pathogens and endophytes" in grafted rootstock to 3% compared to the untreated controls (15%) and reduced vine mortality to less than 10% in vines that were treated at lower temperatures (45–47°C for 30 min) compared to vines that were HWT using the industry standard (60%) (Graham, 2007b). A field experiment which hot water treated 2-year-old rootstock plants at 47°C for 30 min and then grew them on for 8 months in a greenhouse found no adverse effects on shoot development (Bleach *et al*., 2009). The aim of this experiment was to examine the effects of different HWT temperature and time combinations on nine New Zealand *Ilyonectria* isolates in laboratory and field experiments, as well as to evaluate the differential sensitivity of the isolates to heat treatments.

Materials and methods

Isolates

Nine *Ilyonectria* isolates, three each of *I. liriodendri*, *I. macrodidyma* complex and *I. radicicola* complex that had been isolated from the trunks and roots of symptomatic grapevines, collected throughout New Zealand (Bleach *et al*., 2006) were used for this study (Table 1). These isolates were maintained on Spezieller Nährstoffarmer Agar (SNA) slants (Brayford, 1993) at 4°C until required, when they were subcultured to potato dextrose agar (PDA; Oxoid Ltd, Basingstoke, Hampshire, England,) plates and incubated at 20°C for 2–4 weeks.

Effect of different HWT treatments on viability of mycelium and conidia

Treatments at 40, 45, 47, 50 and 55°C for 5, 15 and 30 min or 40, 45, 47 and 50°C for 5, 15 and 30 min were applied to mycelia and conidia, respectively, of the nine *Ilyonectria* isolates (Table 1), using a PCR temperature cycler (Eppendorf Mastercycler®, Biorad iCyclerTM, California, USA). For each temperature/time treatment there were three replicate Eppendorf plastic tubes (0.6 mL) per isolate, comprising 27 tubes of agar plugs of mycelium or of conidium suspensions in total. The 27 tubes were randomly arranged in the PCR temperature cycler and hot water treated together. After HWT, the Eppendorf Mastercycler® automatically dropped the temperature to 4°C for a holding period of 3–5 min, after which the tubes were removed and their viability assessed. The controls comprised untreated tubes that were randomly positioned on a laboratory bench and held at ambient room temperature of 20°C ± 2°C for 30 min.

Mycelium

For each of the nine isolates, three replicate 3 mm plugs of agar with mycelium and conidia, were cut from the growing edges of 2–4 week old agar colonies. Each agar plug was placed into a separate tube

Table 1. Source of *Ilyonectria* isolates obtained from characteristic necrotic lesions on grapevine roots and trunks in New Zealand.

Strain Code	Species (isolate)	LUPP [a] No.	Geographic origin
1R	*I. radicicola* complex	1071	Marlborough
2R	*I. radicicola* complex	1022	Central Otago
3R	*I. radicicola* complex	989	Waipara
1L	*I. liriodendri* (1L)	1000	Central Otago
2L	*I. liriodendri* (2L)	1102	Marlborough
3L	*I. liriodendri* (3L)	953	Hawkes Bay
1M	*I. macrodidyma* complex (1M)	974	Hawkes Bay
2M	*I. macrodidyma* complex (2M)	1039	Gisborne
3M	*I. macrodidyma* complex (3M)	1120	Marlborough

[a] LUPP, Lincoln University Plant Pathology.

containing 100 μL of sterile distilled water (SDW) and the lid closed. Immediately after the designated treatments, each mycelium plug was removed from its tube and placed onto malt extract agar (MEA; 2% agar, 2% Maltexo). The plates were sealed with cling film and randomly allocated to positions in a 20°C incubator for 12 days under a diurnal light schedule (12 h light, 12 h dark). Growth was assessed on each plate after 7 and 12 days by measuring the perpendicular diameters with a digital calliper (Mitutoyo, Mitutoyo Corp, Kanogawa, Japan), and the mean percent mycelium growth was determined in relation to the growth on untreated control plates.

Conidium suspension

For each of the nine isolates, a 2–4 week-old PDA colony was flooded with 5 mL of SDW and the colony surface was rubbed with a sterile hockey stick to release the conidia. Each resulting suspension was poured into a sterile Universal bottle, the conidium concentration assessed using a haemocytometer and then the final concentration was adjusted to 1×10^4 conidia mL^{-1} with SDW. Three replicate 120 μL aliquots of each conidium suspension (for each isolate) were placed into separate tubes and the lids closed. After the designated HWT, three 40 μL droplets were pipetted from each tube onto a glass slide, 20 mm apart. The three slides for each isolate were then placed into separate 85 mm diameter Petri dishes; each Petri dish contained three slides that contained conidium suspensions of different isolates. Each Petri dish was placed inside a larger square Petri dish (100 × 100 mm) that contained 5 mL of water and covered with the lid to act as a humidity chamber. The dishes were randomly allocated to positions in a 25°C incubator and incubated for 5 h in the dark. To maintain the 5 h germination rate and prevent further development during the assessment period, individual glass cover slips were placed onto each conidium droplet on removal from the incubator and the slides were then held in a 7–8°C temperature controlled room until counting was completed (up to 4 h). Germination of 100 randomly selected conidia in each droplet was assessed. A conidium was considered germinated if the length of the germ tube exceeded half the length of the conidium. The mean percent germinated conidia was determined for each slide relative to the untreated controls. To confirm that lack of germination reflected non-viability of the conidia, the coverslips were removed from the slides which were replaced in their Petri dishes and humid chambers. These were randomly replaced in the 25°C incubator for a further 24 h and conidium germination assessed again.

In vitro trunk inoculation

One year old dormant cuttings of rootstock 101-14 were cut into 40 cm pieces, surface sterilised (Halleen *et al.*, 2003) and left to air dry in a laminar flow cabinet on paper towels for 1 h. After drying, the

canes were clamped into a bench vice ('P&B, England') and an electric drill with a 2 mm bit (Makita™ New Zealand Limited), which had been surface sterilised with 70% ethanol and flamed, was used to drill three 5 mm deep holes into the pith of each cane approximately 80 mm apart. These holes provided inoculation ports for the 3 mm diameter mycelium plugs cut from the colony edges of the nine *Ilyonectria* isolates as described. Each hole in a cane was inoculated with a different *Ilyonectria* sp. and isolate using aseptic techniques and the remaining cavity was filled with sterilised sawdust, which was previously collected from drill holes in surplus canes and autoclaved at 121°C for 15 min. The mycelium plug and sterilised sawdust were held in place by wrapping the inoculation area with a single layer of waterproof grafting tape (Aglis & Co. Ltd, Fukuoka, Japan). There were three replicate canes for each isolate and HWT protocol. The inoculated canes were placed into separate, new plastic bags and randomly allocated to positions in a 25°C incubator for 7 d in the dark to allow colonisation of the wood by the pathogen. The canes were then removed from their bags and HWT, by placing them into HWT baths set at 47, 48.5 or 50°C for 30 min, followed by immediately plunging them into cold water for 30 min. The control canes were inoculated in a similar manner but they were not HWT. The canes were air dried in a laminar flow cabinet and when dry the grafting tape was removed. Isolations were made from the inoculation point (0 cm) and ~1 cm above and below that point; a 1–2 mm section was sliced across the cane and divided into four pieces which were placed equidistantly around the perimeter of a PDA plate amended with 250 mg L^{-1} chloramphenicol (PDAC) (Sigma-Aldrich® Inc., St. Louis, MO, USA). The data available for analysis were the presence or absence of the inoculating *Ilyonectria* spp. (incidence) in each plate.

HWT of dormant grapevine rootstock plants from a field nursery

The experiment was conducted in a commercial field nursery located in Auckland, New Zealand from September 2007 to May 2008. Conidium suspensions were prepared by first dislodging conidia from ten replicate plates for each isolate as described for the *in vitro* experiment. The contents of the plates (agar and conidial suspensions) for each isolate were then placed in a new plastic bag and emulsified with 100 mL of SDW using a Colworth Stomacher 400, (A.J. Seward & Co., Blackfriars Rd, London, UK) set at 50 Hz for 10 min. A further 500 mL of SDW was mixed into the resulting mash and it was strained through a series of sterilised sieves (pore sizes of 710, 500 and 150 μm), with the filtrate from the 150 μm sieve being collected into a sterile 2 L bottle. The mash that remained on the first sieve (710 μm) was replaced into the plastic bag with a further 100 mL of SDW and the emulsifying process repeated. The resulting mash was again mixed with 500 mL of SDW and strained through two sieves (500 μm and 150 μm), combining the final filtrates from the 150 μm sieves. The conidial concentration for each isolate was adjusted to 1×10^8 conidia mL^{-1} using a haemocytometer to ensure equivalent numbers of conidia for each isolate before mixing them. The bottle containing the mixed conidial suspension was placed into an insulated container filled with crushed ice for overnight transport to the field site. On site, the suspension was diluted with enough water to give 1×10^4 conidia mL^{-1} immediately prior to soil inoculation.

Preparation of the field site was carried out in line with standard nursery practices; the soil was cultivated, then mounded and the mounds covered in black polythene. Planting holes were made through the polythene to a depth of 15 cm, in double rows 100 mm apart. They were inoculated immediately prior to planting using a drench pack and gun suitable for dosing farm animals (N J Phillips Pty Limited, NSW, Somersby, Australia). The 20 mL of mixed conidium suspension (1×10^4 conidia mL^{-1} of the nine isolates, three each of *I. liriodendri*, *I. macrodidyma* and *I. radicicola* groups used in the *in vitro* experiment) was injected at the base of each planting hole. Apparently healthy, two node cuttings of callused rootstocks varieties: 101-14 MGT (101-14; *Vitis riparia* × *V. rupestris*) and Teleki 5C (5C; *V. berlandieri* × *V. riparia*) supplied by the commercial nursery, were inserted into the planting holes. The experiment was laid out in a completely randomised split plot (rootstock varieties, 101-14 and 5C) design with six blocks, each containing 14 plots (seven treatments by two rootstock varieties) that were 600 mm long, and separated by 200 mm buffer zones. The experimental site was managed by the on-site nursery staff according to standard nursery practices, which included a calendar spray program every 14 days of sulphur (3 kg

ha^{-1} Kumulus®; BASF Canada Inc. Ontario, Canada), Dithane® (2 kg ha^{-1}; Dow AgroSciences, Indianapolis, USA), and Tracel Plus (5 kg ha^{-1}; Fruitfed Supplies, PGG Wrightson Limited, New Zealand). The vines were trimmed after 4 months growth to reduce canopy density and thus plant susceptibility to mildew diseases. After 8 months, the dormant plants were harvested, then washed under running tap water. Control plants were set aside and those allocated to HWT of 47, 48.5 and 50°C for 15 and 30 min were treated according to standard practice in the nursery HWT plant (7,500 L with accuracy of ± 0.5°C) followed by 30 min immersion in cold water.

Assessment

The roots and shoots were removed from the plants and the bare trunks that remained were washed in cold water, air dried and then surface-sterilised (Halleen *et al*., 2003) one plot at a time. From each trunk, the ~1 cm root crown was removed and discarded. A 1–2 mm section was sliced from across the basal end of the trunk (0 cm) and divided into four pieces which were placed equidistantly around the perimeter of a PDAC plate and another 1–2 mm slice cut from further up the trunk (5 cm) to assess the progression of the pathogen was transferred to the centre of the same PDAC plate. Plates were incubated as described above.

Statistical analysis

The *in vitro* experimental design was a randomised factorial design with nine isolates (three isolates from the *I. liriodendri*, *I. macrodidyma* and *I. radicicola* groups), and three replications for each of the experimental combinations. The percent growth and germination data and incidence data (trunk inoculation) were analysed by general analysis of variance using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) to determine HWT temperature/time and isolate/species effects. The field experiment data of disease incidence (from 0 cm and 5 cm isolations) and severity (the mean proportion of tissues infected at 0 cm) were analysed by general linear model (GenStat Release 14.1, VSN International Ltd, Hemel Hempstead, UK) with terms appropriate to the design and the two-way interactions amongst the factors of interest. Where significant, main effects or two-way interactions were identified and the significance of differences between individual treatments was further explored using Fisher's protected LSD tests at $P \leq 0.05$.

Results

Mycelium

There was a significant effect of species ($P=0.034$) on mycelium growth, with temperature treatments causing significantly less inhibition for isolates of *I. liriodendri* than for *I. macrodidyma* complex but not for *I. radicicola* complex (46.3, 40.4 and 42.0%, respectively, of the untreated control growths, LSD = 4.60). The HWT temperatures significantly affected mycelium growth of isolates overall ($P<0.001$), means as proportions of the control growth being 99.7, 56.6, 35.7, 20.5 and 1.8%, respectively for 40, 45, 47, 50 and 55°C (LSD = 5.08). Similarly, HWT time had a significant effect on mycelium growth ($P<0.001$), with means as proportions of the control growth being 69.1, 37.8 and 21.8% for 5, 15 and 30 min, respectively, (LSD = 3.94; Figure 1). There was also a significant effect for isolates within individual species which sometimes conflicted with the apparent species effects ($P<0.003$; Figure 1).

The significant interaction of HWT temperature × time × isolates ($P<0.001$, LSD = 26.41) was particularly evident with the 45°C treatment for 30 min and 47°C treatment for 15 and 30 min. The 30 min treatment at 45 and 47°C treatment for 30 min totally inhibited growth for all isolates except 3L (30.8%) and 1L (16.1%), respectively (Figure 1) and the 15 min treatment at 47°C totally inhibited growth of all isolates except 1R, 2M and 3M.

However, these different isolate effects for the 30 min HWT were due to the different results from the three replicates, with mycelium growth on only one of the three replicate plates, being 48% (1L) and 93% (3L) growth, respectively, which accounted for the 16.1 and 30.8% mean growths, respectively. Treatment at 50°C for 15 and 30 min provided complete inhibition of all isolates except 3L and treatment at 55°C for 15 and 30 min completely inhibited growth of all isolates (Figure 1).

Conidia

The HWT temperature and time combinations were significant for all isolates ($P<0.001$), however all treatments completely inhibited germination of

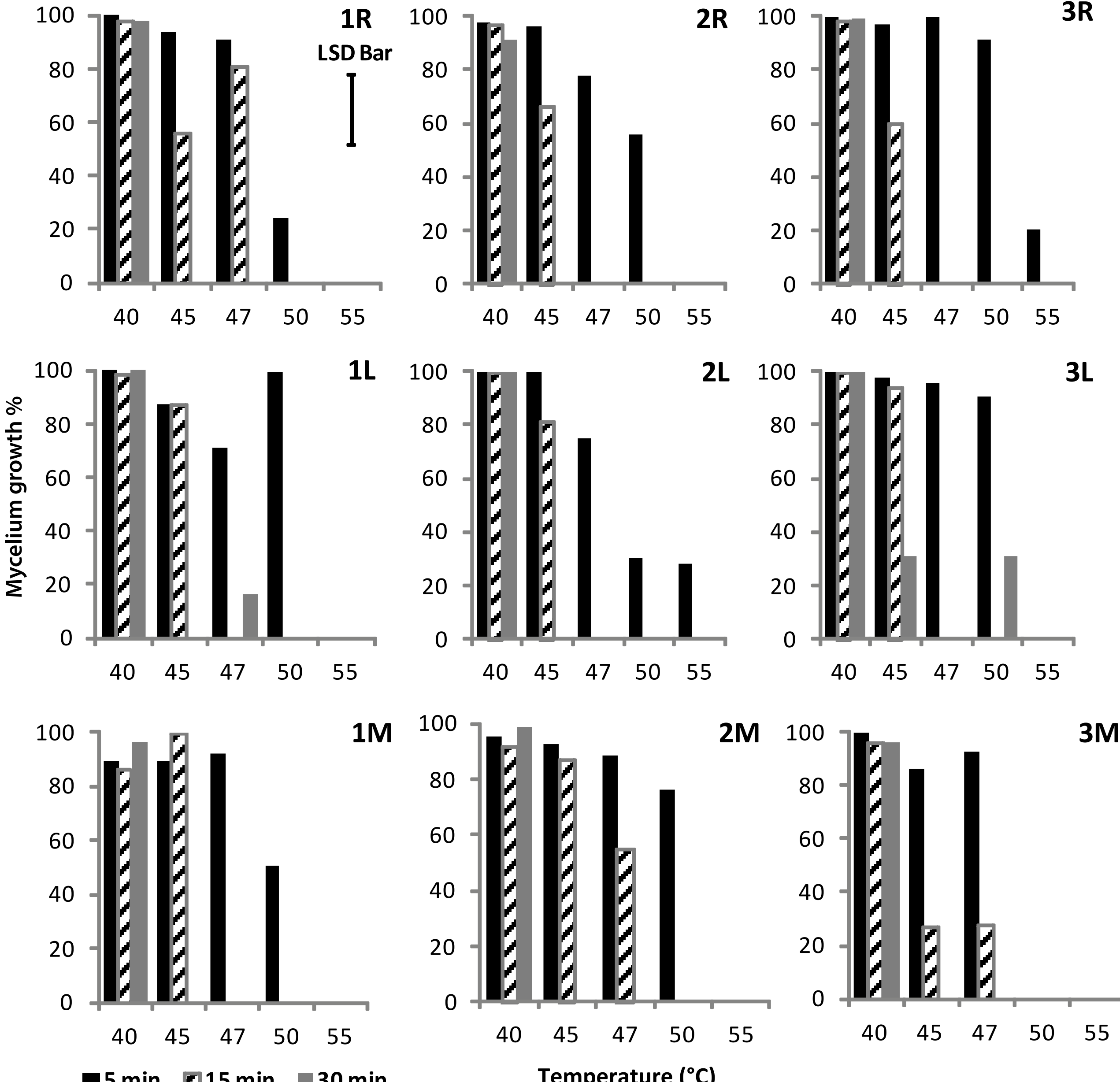

Figure 1. Percent mycelium growth of *Ilyonectria* isolates for *I. radicicola* (1R, 2R and 3R), *I. liriodendri* (1L, 2L and 3L) and *I. macrodidyma* (1M, 2M and 3M) compared to the untreated controls after hot water treatment at 40, 45, 47, 50 or 55°C for 5, 15 or 30 min. Results are the means of three replicates for each isolate for each temperature and time combination ($P<0.001$, LSD = 26.41).

conidia of the three *Ilyonectria* species except 40°C for 5 min. Germination was also assessed after a further 24 h incubation to allow more time for inhibitory effects to be overcome but there was no further germ tube development for any of the nine *Ilyonectria* isolates. After HWT at 40°C for 5 min, germination of *I. radicicola* conidia was more than for *I. liriodendri* and *I. macrodidyma* (20.2, 11.2 and 10.3%, respec-

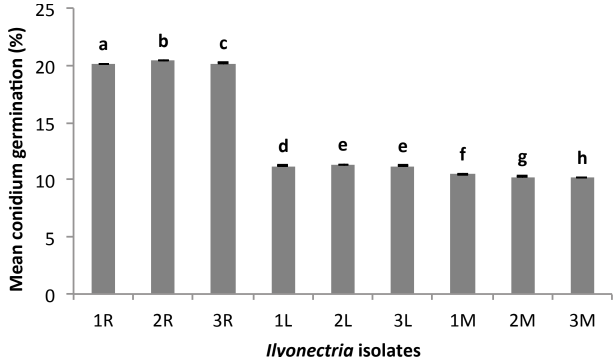

Figure 2. Mean conidium germination compared to the untreated control for nine *Ilyonectria* isolates, after HWT at 40°C for 5 min. Bars with the same letter are not significantly different according to Fisher's protected LSD ($P \leq 0.001$, LSD = 0.06).

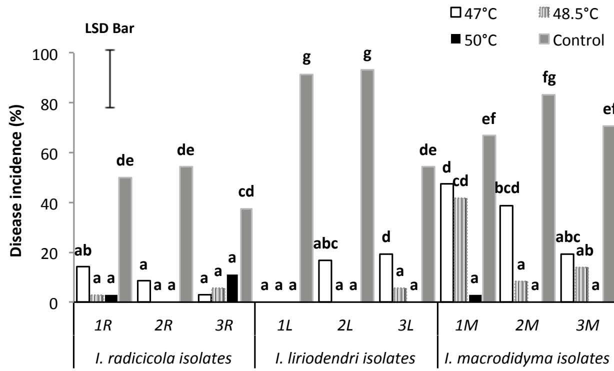

Figure 3. Mean pathogen incidence of three isolates for each of the *Ilyonectria* groups after the nine isolates were allowed to grow for 7 d inside grapevine canes and then HWT at 47, 48.5 and 50°C for 30 min. Bars with the same letter are not significantly different according to Fisher's protected LSD ($P \leq 0.001$, LSD = 22.68).

tively, LSD = 0.06), with some differences between isolates ($P<0.001$; Figure 2). However, these minor differences were not considered to have biological significance.

In vitro trunk inoculation

Incidence of infection within trunks was significantly affected by HWT temperature, *Ilyonectria* species, isolates and interactions for species × temperature (all $P<0.001$) and species × isolate × temperature ($P=0.029$). Treated canes inoculated with *I. macrodidyma* isolates had greater disease incidence than those inoculated with *I. radicicola* and *I. liriodendri* isolates (19.1, 5.3 and 4.6 %, respectively, LSD = 8.62). The species × HWT temperature interaction was associated with the significantly greater disease incidence for *I. macrodidyma* than *I. radicicola* and *I. liriodendri* after treatment at 47°C (35.2, 8.3 and 12.0%, respectively) and 48.5°C (21.3, 2.8 and 1.9%, respectively) but not 50°C when incidences were similar (0.9, 4.6 and 0%, respectively, LSD = 15.12). The significant isolate effects and their interactions (Figure 3) show that some isolates were generally more resistant than others and these differences diminished as the HWT temperature increased. Incidence of infection in wood pieces from the treated canes differed between isolation positions ($P<0.001$), being significantly greater for those from the inoculation site (0 cm) than 1 cm above or 1 cm below that site (47.7, 27.9 and 20.5%, respectively, LSD = 9.13).

Dormant grapevine rootstock plants from a field nursery

There were significant effects of the HWT treatments on disease severity and incidence (both $P<0.001$), which reduced with increasing temperature and time (Figure 4). There was zero disease incidence in plants HWT at 48.5 and 50°C for 30 min. However, these zero incidences did not differ significantly from the disease incidences after HWT at 48.5 and 50°C for 15 min and 47°C for 30 min (3.1, 1.0 and 2.1%, respectively LSD = 7.26). The least effective treatment (47°C for 15 min) caused significantly less disease incidence than in control plants (44.8%).

Disease severity was similarly low for all HWT plants and significantly less than in the inoculated control plants ($P \leq 0.05$), reflecting a similar trend as observed for disease incidences (Figure 4). Zero disease severity occurred in plants that were HWT at 48.5 and 50°C for 30 min, and the remaining treatments had significantly reduced severities (2.9–0.3%) compared to the control plants (20.8%, LSD = 4.74). There was no significant difference in disease incidence and severity between rootstock varieties ($P=0.225$) nor any interaction between HWT treatment and rootstock varieties ($P=0.540$) although mean disease incidence and severity were less in rootstock variety 5C (7.1 and 3.4%, respectively) than rootstock variety 101-14 (9.5 and 4.1%, respectively).

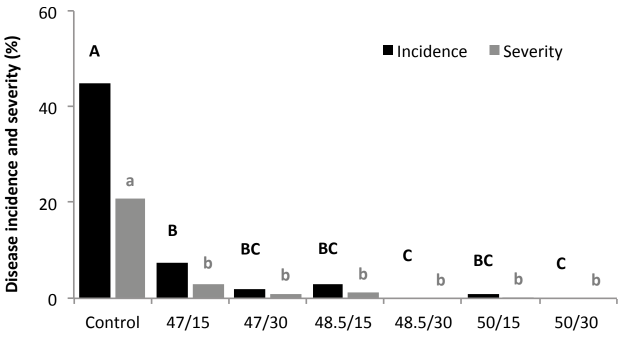

Figure 4. Mean percent incidence and severity of isolates of *I. radicicola*, *I. macrodidyma* and *I. liriodendri* spp. in grapevine plants after hot water treatment. Columns which are the same colour, black for disease incidence LSD = 7.26 and grey for disease severity LSD = 4.74, with the same letters above are not significantly different (Fishers protected LSD $P<0.05$).

Discussion

This study showed that some of the different HWT temperature and time combinations tested inhibited mycelium growth and conidium germination but that effects differed between isolates of *I. radicicola* and *I. macrodidyma* complexes and *I. liriodendri,* the pathogens commonly associated with black foot disease in grapevines. The increasing HWT temperatures and times used for treating the mycelium plugs, caused the subsequent mycelium growth to be progressively more inhibited. Comparable HWT effects were reported by Gramaje *et al.* (2010) who investigated the effects of 41–55°C for 30-45 min for single isolates of three black foot pathogens and eight *Phaeoacremonium* species. They reported complete inhibition after 30 min at different temperatures for *I. liriodendri* (48°C) and *I. macrodidyma* (49°C). However in this study, growth of all *I. macrodidyma* isolates was completely inhibited if the mycelium plugs were treated for 30 min at 45°C while the effects on *I. liriodendri* isolates were generally similar, although one replicate each of 1L and 3L had greater resistance to the treatments. These uneven trends may have been caused by presence of occasional chlamydospores on the treated plugs. Chlamydospores are more heat resistant than conidia and mycelium (Smith *et al.*, 2009), and Halleen *et al.* (2004b) reported that *I. liriodendri* and *I. radicicola* readily produced chlamydospores when growing on agar, unlike *I. macrodidyma* which rarely produced them. The Petri disease pathogen, *Cadophora luteo-olivacea* (Gramaje *et al.*, 2010) was reported as more resistant to HWT requiring 55°C for 30 min to completely inhibit growth however, this pathogen has been reported once only in New Zealand (Manning and Mundy, 2009) and was not found in the New Zealand survey conducted by these authors (Bleach *et al.*, 2006). Comparisons could not be made regarding *I. radicicola* isolates as they were not investigated in the study by Gramaje *et al.* (2010).

Conidial germination in this study was completely inhibited after very little heat treatment. Germination occurred only after 5 min at 40°C when mean germination rates differed slightly between species, being greater for *I. radicicola* than *I. liriodendri* and *I. macrodidyma* (20.2, 11.3 and 10.3%, respectively). In contrast, Gramaje *et al.* (2010) found that much greater heat treatments were required to provide 100% inhibition of germination. They found that 41°C for 30 min reduced conidium germination of *"C". liriodendri* and *"C". macrodidymum* isolates to 38.8% and 65.7% respectively, and complete inhibition occurred after 30 min at 45°C for *"C". liriodendri,* 46°C for *"C". macrodidymum* and 51°C for *Ca. luteo-olivacea.* This study clearly showed that conidia of the New Zealand *"Cylindrocarpon"* isolates were more sensitive to HWT than those of the Spanish isolates. The significant differences between isolates from a species, with respect to mycelium growth after different HWT treatments in this study, clearly indicates that more isolates should be tested from a range of climatic regions before a final conclusion can be reached about the effects of climate on plant tolerances to heat treatment, as suggested by Crocker *et al*. (2002).

When the inoculum of the isolates was inserted into canes, and mycelium allowed to grow in each cane sample prior to HWT, 48.5 and 50°C were similarly effective, with 50°C treatment reducing incidence to 4.6, 0.0 and 0.9% for *I. radicicola, I. liriodendri* and *I. macrodidyma,* respectively. In this experiment *I. macrodidyma* appeared to be more resistant to HWT than *I. liriodendri* and *I. radicicola*, as mean incidences at 47°C were 35.2, 12.1 and 8.3%, respectively, whereas in the *in vitro* experiments, HWT of 45°C for 30 min completely inhibited mycelium growth of the three *I. macrodidyma* isolates. Two isolates, (mycelium of isolate 3L of *I. liriodendri* in the *in vitro* HWT experiment and mycelium of isolate 1M of *I. macrodidyma*,

in the cane inoculation experiment) appeared to be more tolerant to HWT than the other seven isolates since temperatures which had inhibited their growth did not completely inhibit growth of isolates 3L and 1M. Interestingly, both isolates 3L and 1M originated from grapevines grown in the warmer Hawke's Bay region of New Zealand.

Although Gramaje *et al.* (2010) suggested that the industry standard HWT (50°C for 30 min) was the minimum required to eliminate *Ilyonectria* spp. of Spanish origin from grapevines, this *in vitro* study indicated that a reduced HWT may be effective for New Zealand *Ilyonectria* isolates and the cane inoculation experiment showed that HWT temperatures at 48.5 and 50°C, which were similarly and significantly more effective than 47°C for inhibiting mycelium growth, could be used in New Zealand. However, other HWT protocols may be more suitable for grapevines and isolates from other countries, which should be determined by similar investigations.

The field experiment demonstrated the efficacy of a less stringent HWT protocol (48.5°C for 30 min) than the industry standard treatment of 50°C for 30 min to completely eliminate *Ilyonectria* spp. from grapevine rootstocks plants, which had become naturally infected while growing in soil infested with the black foot pathogens. In addition, the 15 min HWT at 48.5 and 50°C significantly reduced the levels of disease incidence (3.1 and 1%) and could be considered by grapevine propagators who feared that HWT could reduce viability in their plants. Although plant viability was not tested in the current experiment, it was tested in a later and similar field experiment undertaken by the authors (data not provided); none of the grafted grapevines that had been HWT at 48.5°C for 30 min died or displayed retarded growth. In other HWT experiments carried out in New Zealand, Graham (2007a) reported that vines which had been HWT at 45 and 47°C for 30 min and grown in a field nursery for three weeks had reduced mortality (<10%) compared to vines treated at 50°C (60%), and reduced 'pathogen' incidence (11 and 3%, respectively) compared to untreated controls (15%). In earlier experiments (Graham, 2007b), the mortality of rootstock cuttings 5C and 101-14 six weeks after HWT at 50°C was 60 and 95%, respectively. These reports concurred with the current study in showing that in a cooler climate, lower HWT temperatures were effective against grapevine pathogens and reduced the physiological damage to the grapevine tissues. In this study, the rates of mortality were considerably less than those of Graham (2007a; 2007b). Clearly, further investigations should be conducted with more grapevine varieties and in conjunction with cold storage, which commonly occurs before HWT of dormant plants and after HWT of grapevine cuttings.

HWT injury has been reported as less common for grapevines grown in warmer regions than in cool regions (Crocker *et al.*, 2002), which was concluded to be due to the higher levels of thermotolerance reported for grapevines grown in warmer regions than those from cooler regions (Crocker and Waite, 2004). This was attributed to the heat shock proteins synthesised by plants during hot weather, which persist into dormancy and provide protection to the plant during HWT (Crocker and Waite, 2004). Because of the increased tolerance of grapevines to HWT in regions warmer than New Zealand, those research programs have mainly used HWT protocols with temperatures equal to or above 50°C, for example, 50°C in South Africa (Crous *et al.*, 2001; Fourie and Halleen, 2004) and Australia (Edwards *et al.*, 2004; Waite and May, 2005), 51°C in California (Rooney and Gubler, 2001; Whiting *et al.*, 2001) and 50 to 53°C in Spain (Gramaje *et al.*, 2008; Gramaje *et al.*, 2009; Gramaje *et al.*, 2010) and Italy (Mannini, 2007). In the latter study, which was conducted over three years, HWT at 52°C for 45 min was found to reduce vine losses after planting to an acceptable level (zero to 20%) compared to the untreated controls (Mannini, 2007).

The HWT protocol used by grapevine propagators in any region must be effective for many of the prevalent trunk diseases, such as Petri disease, caused by *Phaeomoniella chlamydospora* and *Phaeoacremonium* species. The HWT protocols published for these pathogens have indicated that they are also affected by climatic factors. In Spain, *Pa. chlamydospora* and *P. aleophilum* in grapevine cuttings were reported to require HWT of 51–53°C for 30 min to eliminate them (Gramaje *et al.*, 2009). In contrast, in South African field trials conducted over two growing seasons, Halleen *et al.* (2007) reported that HWT at 50°C for 30 min effectively eliminated the Petri disease pathogens, *Pa. chlamydospora and Phaeoacremonium* spp. and the black foot pathogens "*C*". *liriodendri* and "*C*". *macrodidymum* as well as the *Campylocarpon* species, *Campyl. fasciculare* and *Campyl. pseudofasciculare*, from dormant grapevine plants grown in naturally infested soil. These results showed pathogen, isolate and country variability to HWT and concur with the current study.

Since HWT temperatures equal to or above 50°C have not reduced viability of grapevine cuttings grown in warmer regions and were required to control the Petri disease pathogens (Rooney and Gubler, 2001; Gramaje *et al*., 2008; Gramaje *et al*., 2009), the warm climate grapevine industries have not needed to modify the standard HWT. Hence little *in vivo* research has been conducted on the effectiveness of HWT temperatures below 50°C for control of grapevine pathogens until this study, which has indicated the efficacy of reduced temperatures against New Zealand *Ilyonectria* isolates, and the study into *Pa. chlamydospora* infection of grapevines by (Graham, 2007a).

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RESEARCH PAPER

Soaking grapevine cuttings in water: a potential source of cross contamination by micro-organisms

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Summary. Grapevine nurseries soak cuttings in water during propagation to compensate for dehydration and promote root initiation. However, trunk disease pathogens have been isolated from soaking water, indicating cross contamination. Cuttings of *Vitis vinifera* cv. Sunmuscat and *V. berlandieri x V. rupestris* rootstock cv. 140 Ruggeri were immersed in sterilized, deionised water for 1, 2, 4, 8 and 16 h. The soaking water was cultured (25°C for 3 days) on non-specific and specific media for fungi and bacteria. The base of each cutting was debarked and trimmed and three 3 mm thick, contiguous, transverse slices of wood cultured at 25°C for 3 days. The soaking water for both cultivars became contaminated with microorganisms within the first hour. Numbers of fungi isolated from the wood slices soaked for one hour were significantly greater than those from non-soaked cuttings. The number of bacterial colonies growing from the wood slices increased after soaking for 2–4 h in Sunmuscat. In a second experiment Shiraz cuttings were soaked for 1, 2, 4, 8 and 24 h. The soaking water became contaminated within the first hour but only the bacterial count increased significantly over time. Microorganisms also established on the container surfaces within the first hour although there were no significant increases over 24 h. These results confirm that soaking cuttings is a potential cause of cross contamination and demonstrate contamination of cuttings occurs after relatively short periods of soaking. Avoiding exposing cuttings to water will reduce the transmission of trunk diseases in propagation.

Key words: trunk diseases, soaking, cross contamination, grapevine propagation.

Introduction

In modern times the grapevine propagation process has been mechanized and streamlined, enabling individual nurseries to reduce costs and produce large numbers of vines over an extended season. Bench mounted grafting machines that automatically cut and join a dormant rootstock cutting and one bud scion prior to rooting, along with cool rooms to hold cuttings in the dormant state until required for grafting, are now common in the grapevine nursery industry. Bench grafting, as this technique is known, has enabled individual nurseries to produce many hundreds of thousands of vines in a single season. However, the efficiency gains resulting from mechanization and the use of cool rooms have been somewhat offset by losses in quality. Incompletely healed graft unions and biotic contamination of the rootstocks, scions and graft unions are common problems in bench grafted vines (Stamp, 2001) and spasmodic, large scale failures of grafted vines in the nursery and vineyards are a serious problem for nurseries and grape growers (Morton, 2000; Halleen *et al.*, 2003; Gramaje and Armengol, 2011; Whitelaw-Weckert *et al.*, 2012).

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The large scale vine failures that occur soon after planting have been attributed to infection by trunk disease pathogens (Rego *et al*., 2000; Sidoti *et al*., 2000; Gramaje and Armengol, 2011), pointing to propagation as a potential source of infection in young vines (Whitelaw-Weckert *et al.,* 2012). There is indirect evidence that contaminating microorganisms enter the propagation chain in (Halleen *et al*., 2003; Whiteman *et al*., 2007), or on (Whiteman *et al*., 2004a), asymptomatic cuttings from infected mother vines and contaminate other cuttings via water used to soak cuttings during propagation (Rego *et al*., 2001; Edwards *et al*., 2007, 2011; Aroca *et al*., 2010).

In addition, *Rahnella aquatilis*, *Enterobacter intermedius* and *Trichosporon pullulans,* microorganisms that are not recognised grapevine pathogens but are associated with dust and faecal contamination of water, have been isolated from compromised vine tissues and have also been shown to interfere with graft healing and to inhibit shoot growth (Waite and Cole, 2006). Botryosphaeriacea spp. (Phillips, 2002) and *Phaeomoniella chlamydospora* (Wallace *et al*., 2004) are also reported to interfere with graft healing.

Grapevine nurseries routinely soak many hundreds of cuttings together in water as a means of compensating for dehydration that occurs during cutting harvest and transportation to nurseries (Fourie and Halleen, 2006; Waite and Morton, 2007; Gramaje and Armengol, 2011). Soaking occurs for periods typically between 1 and 12 hours, though sometimes for longer, and is often performed more than once during the propagation cycle.

Soaking is thought to facilitate the entry of epiphytes into cutting tissue through the wounds made at each end of the cuttings, disbudding wounds and graft unions (Whiteman *et al*., 2004b; Fourie and Halleen, 2006; Retief *et al*., 2006; West *et al*., 2010) and has been implicated in cross contamination by the trunk disease pathogens *Ca. luteo-olivacea*, *Pa. chlamydospora* and *Phaeoacremonium aleophilum* (Gramaje *et al*., 2009, 2011). Some of these organisms have been detected in nursery environments. *Pa. chlamydospora* DNA has been detected in untreated rainwater collected from the roofs of houses and sheds that is used in Australian nurseries in hydration tanks, hot water treatment tanks and post HWT cool down tanks (Edwards *et al*., 2007) and in pre storage fungicide / rehydration tanks and pre grafting hydration tanks in New Zealand nurseries (Whiteman *et al*., 2004b). Retief *et al*. (2006) also detected *Pa. chlamydospora* in a high percentage of water samples collected after pre-storage hydration (40%) and during grafting (67%) in South African commercial nurseries. *Phaeoacremonium* spp. were also detected using molecular techniques in post-storage hydration tanks in Spanish nurseries (Aroca *et al*., 2010) and species of Botryosphaeriaceae and Petri disease pathogens (*Pa. chlamydospora* and *Phaeoacremonium* spp.) were detected by PCR on the surface of grafted varieties and rootstocks and in hydration and callusing baths in French nurseries (Vigues *et al*., 2009). In Italy, Pollastro *et al*. (2009) detected *Pa. chlamydospora* DNA in 28% of pre-grafting and 23% of pre-callusing hydration tanks. Viable propagules of *Ca. luteo-olivacea* were also obtained from hydration tanks in Spanish nurseries by filtering the water samples and culturing the filtrate on appropriate media (Gramaje *et al*., 2011).

Some nurseries use fungicides in the soaking water, but their efficacy cannot always be relied upon (Fourie and Halleen, 2004), particularly if multiple batches of cuttings are soaked in the same fungicidal dip. Apart from *Pa. chlamydospora* (Pascoe and Cottral, 2000) and Botryosphaeriaceae spp. (Billones-Baaijens *et al*., 2013) many more micro-organisms including bacteria are also found on the surfaces of grapevines (Munkvold and Marois, 1993). The potential for endophytic microorganisms to suppress pathogens in grapevine wood tissue has been raised by several authors (Martini *et al*., 2009; Gonzáles and Tello, 2010), but knowledge of the influence of endophytes on the development of diseases is incomplete (Crous *et al*., 2001; Gonzáles and Tello, 2010) and previously unidentified microorganisms might be opportunistic pathogens when accidentally introduced into the tissues of vines when cuttings are soaked. Although it is probable that the cuttings themselves are a major source of microbial contaminants identified in soaking water, particularly if potable water is used, this has not been tested.

To determine the capacity of epiphytic microorganisms to contaminate soaking water and thus the wood of cuttings, we investigated the rate of movement of microorganisms into soaking water and the woody tissue of cuttings at different soaking intervals. We also investigated the ability of microorganisms to form bio-films on surfaces of soaking vessels and thus present a potential contamination threat to subsequent batches of cuttings; an issue raised by the nursery supplying the cuttings for the first experiment.

Materials and Methods

Microbial contaminants in soaking water and woody tissue

Healthy, dormant cuttings (approximately 8 mm diameter) of *V. vinifera* cv. Sunmuscat and rootstock 140 Ruggeri from the Sunraysia district of the Murray Valley were obtained from a New South Wales vine nursery in September 2010. The cuttings were held in cold storage at 2–3°C at the nursery between harvesting in early winter (June) and shipping in September. Cuttings were trimmed to size (300 mm, approximately 19 g) with fresh cuts 10 mm above the top bud and 5 mm below the bottom bud. The freshly trimmed cuttings were randomly divided between 3 replicates of 6 soaking treatments (0, 1, 2, 4, 8 and 16 h) per variety with 3 cuttings per replicate. Cuttings were completely submerged in sterile (autoclaved at 131°C for 30 min at 2770 mbar) deionised water to a depth of 30 mm (0.5 L/cutting as per industry standard) at ambient room temperature (18°C) in sterile plastic containers (260 × 180 × 90 mm) with loosely fitting lids. Sterile deionised water was used in these experiments to allow the assays to be standardized as domestic supply water varies widely in pH and the ions present over time and between regions (Batarseh, 2006).

A 50 mL sample of water was taken from each treatment replicate using sterile plastic centrifuge tubes (30 mm diameter) after agitation with a sterile spatula to ensure the water was well mixed and all levels of water were sampled. Immediately prior to sub-sampling the 50 mL aliquots were vigorously inverted several times and vortexed for 20 seconds to ensure even mixing. After a preliminary trial to determine appropriate dilutions, three replicate 30 μL aliquots of undiluted sample water and sample water diluted to 10^{-1} in phosphate-buffered saline (PBS) were spread plated onto selective media for fungi [Dichloran Rose Bengal Chloramphenicol (DRBC) agar, Oxoid], bacteria [Nutrient agar (Oxoid) with 30 ppm benomyl (NA-B)] and Pseudomonas agar (Oxoid) for *Pseudomonas* spp., and the non selective fungal medium potato dextrose agar (PDA, Oxoid) and incubated in darkness at 25°C for 3 days and the number of colony forming units (cfu) counted.

Following soaking treatments, the cuttings were debarked and surface sterilized by flaming briefly with ethanol (70%). The base of each cutting was trimmed by 3 mm and three contiguous 3 mm thick cross sections taken from the base of each cutting and incubated in darkness on PDA at 25°C. After 3 days incubation, the numbers of fungal and bacterial colonies emerging from the wood were counted. The cfu g^{-1} (Cg) was calculated using equation 1:

$$Cg = \frac{Cc}{S} \quad (1)$$

where Cc is the number of cfus per slice and S (g) is the weight of the slice.

Formation of biofilms

Arising from the comments of the nursery that supplied the cuttings for the first experiment, a second experiment was performed the following season (2011) to determine the capacity of fungal and bacterial epiphytes to form bio-films on the surfaces of soaking vessels and thus become an additional source of contamination for subsequent batches of cuttings soaked in the same container. Australian nurseries use a variety of large vessels for soaking cuttings, usually plastic, stainless steel or galvanised steel all of which can be colonised by microorganisms (Percival *et al*., 1998; Webb *et al*., 2000; Ilhan-Sungur and Çotuk, 2010) that can be very difficult to remove from container surfaces (Batté *et al*., 2003).

For this experiment, cuttings of *V. vinifera* cv. Shiraz were obtained in late June from the Charles Sturt University vineyard at Wagga Wagga, NSW and held for 3 months in cold storage at 2–3°C in ventilated plastic bags with damp paper to prevent dehydration. Cuttings were removed from cold storage, allowed to come to ambient temperature, trimmed to size (300 mm) with fresh cuts 10 mm above the top bud and 5 mm below the bottom bud and randomly divided between 5 soaking treatments (1, 2, 4, 8 and 24 h) per variety with 3 replicates of 3 cuttings per replicate. The cuttings were soaked in sterile deionised water (0.5 L/cutting as per industry standard) in sterile plastic containers with loosely fitting lids.

Following the method used in experiment 1, a 50 mL sample of water was taken from each treatment replicate after stirring and diluted to 10^{-1} in phosphate-buffered saline (PBS). Three replicate 30 μL aliquots of each diluted sample were spread plated onto selective media for fungi (DRBC) and bacteria (NA-B) and incubated as above before colonies were counted.

Numbers of microorganisms on the inside surfaces of the containers were sampled by draining the

water from the containers and swabbing a 25 cm^2 area with a sterile cotton swab wetted in 10 mL sterile PBS. The swabs were then aseptically returned to the PBS, agitated vigorously and three replicate 30 µL aliquots spread onto selective media for fungi (DRBC) and bacteria (NA-B) and incubated as above and colonies counted.

Statistical analyses

Culturable microbial concentrations from water samples, container surface swabs and colonies emerging from wood samples were subjected to analysis of variance (ANOVA) test and least significant differences test by Genstat for Windows, 15th Edition.

Results

Microorganisms in soaking water and woody tissue

In this experiment the sterile soaking water of both varieties (Sunmuscat and 140 Ruggeri) became contaminated with *Pseudomonas* bacteria, NA-B bacteria, DRBC fungi and PDA fungi and bacteria within the first hour of soaking (Table 1). Microbial populations in the soaking water of Sunmuscat increased significantly (P<0.05) over the second hour except in populations culturable on NA-B where the increase was not significant. Thereafter populations generally decreased until climbing again in the time between 8 h and 16 h, although the later increases were significant (P<0.05) only for NA-B (bacteria) and DRBC (fungi). In the case of 140 Ruggeri the pattern was somewhat different. The number of microorganisms in soaking water increased in the second hour, but only significantly (P<0.05) in populations culturable on DRBC (fungi) and PDA (mixed populations). Numbers reached a maximum after 4 h of soaking in populations culturable on DRBC (fungi) and PDA (mixed populations) and after 8 h of soaking in populations culturable on PS (Pseudomonads) and NA-B (bacteria). After peaking, there were no significant differences over the time of the experiment in all populations except fungi cultured on DRBC where there was a significant (P<0.05) drop in numbers before beginning to increase again between 8 and 16 h (Table 1).

The culturable fungal populations in wood samples of both varieties also increased significantly (P<0.05) during the first hour of soaking, but then remained

Table 1. Colony forming units (cfu) of bacteria and fungi isolated from soaking water of 140 Ruggeri and Sunmuscat cuttings.

Culture media	Soaking time (hours)[a]				
	1 10^4 cfu mL^{-1}	2 10^4 cfu mL^{-1}	4 10^4 cfu mL^{-1}	8 10^4 cfu mL^{-1}	16 10^4 cfu mL^{-1}
140 Ruggeri					
PS	0.57 ± 0.49	3.34 ± 1.80	6.73 ± 3.80	7.79 ± 4.58	4.11 ± 1.38
NA-B	0.95 ± 0.78	3.66 ± 1.56	6.50 ± 4.17	10.78 ± 8.56	6.21 ± 2.98
DRBC	0.04 ± 0.01 b	5.84 ±3.19 a	6.02 ± 0.53 a	1.12 ± 0.59 b	2.01 ± 1.18 b
PDA	0.50 ± 0.40 c	3.90 ± 2.68 b	7.51 ± 1.34 a	4.49 ± 0.27 ab	6.13 ± 2.14 ab
Sunmuscat					
PS	1.20 ± 0.24 c	10.59 ± 1.55 a	3.11 ± 0.62 b	1.76 ±0.28 bc	2.90 ± 0.45 b
NA-B	1.18 ± 0.20 b	17.90 ± 8.75 ab	5.11 ± 1.06 b	4.09 ± 0.55 b	13.69 ± 2.27 a
DRBC	0.74 ± 0.25 b	5.46 ± 1.45 a	1.83 ± 0.78 b	2.30 ± 1.67 b	7.14 ± 2.71 a
PDA	2.26 ± 0.34 c	14.02 ± 6.52 a	5.77 ± 0.47 bc	7.74 ± 1.14 b	10.15 ± 3.55 ab

[a] For data in a single row, values followed by the same letter are not significantly different (P<0.05).

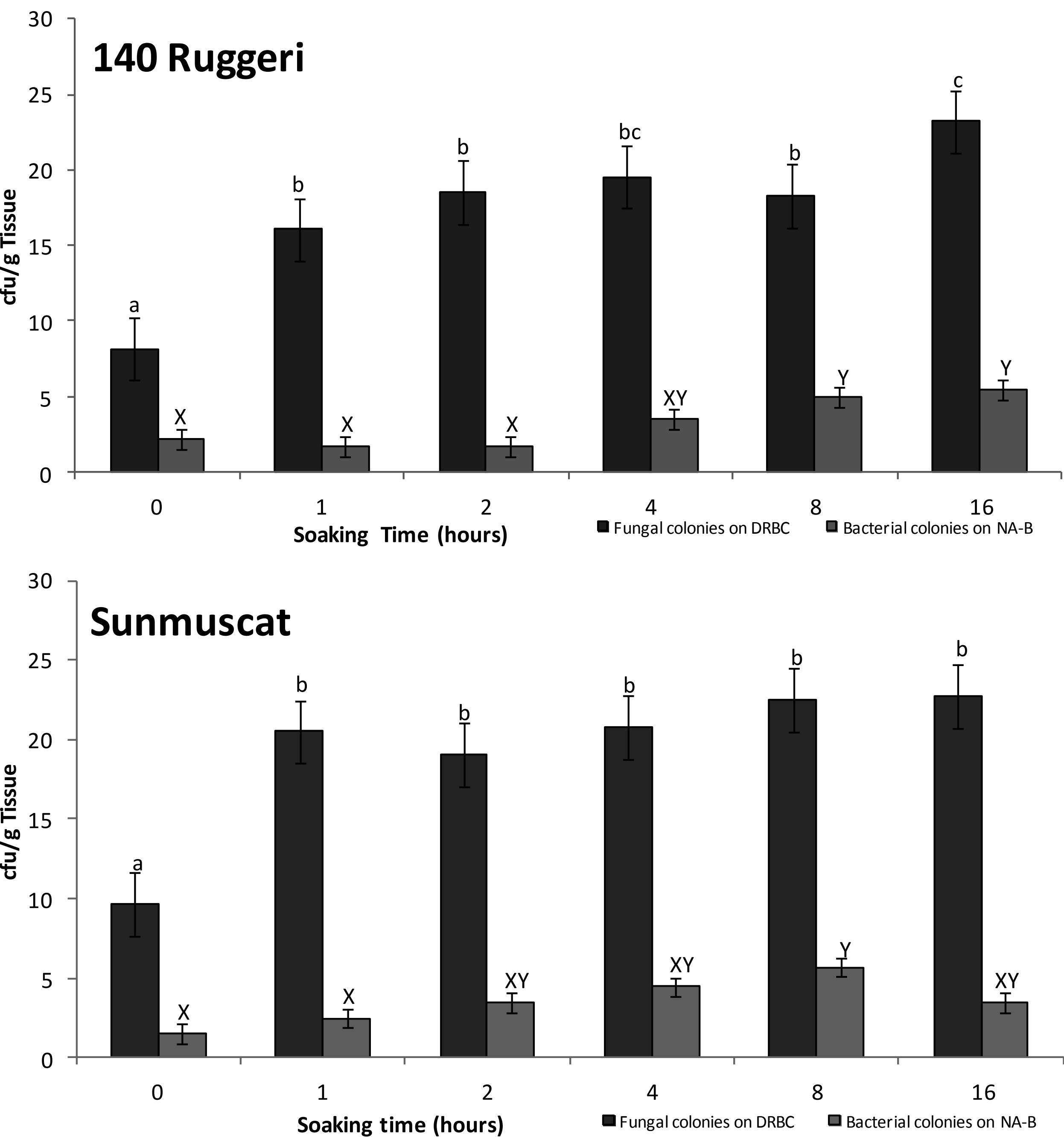

Figure 1. Fungi and bacteria isolated from rootstock 140 Ruggeri and *V. vinifera* cv. Sunmuscat after different periods of soaking. Vertical bars are the standard error of the means. Letters are for comparison of means of fungal colonies (a, b) and bacterial colonies (X, Y). Bars with a different letter are significantly different according to the Student's Least Significant Difference (P<0.05).

largely static for the duration of the experiment, although there was a significant (*P*<0.05) increase in fungal colonies in the 140 Ruggeri after 16 h (Figure 1).

There were also increases in the number of bacterial colonies isolated from the wood of both varieties, but the increases were not significant until the cuttings had been soaked for 8 h (Figure 1).

Although the titre of all microorganisms in the woody tissue of both 140 Ruggeri and Sunmuscat increased over the time of the experiment, the rate at which the epiphytic microorganisms on the bark of cuttings moved into the soaking water and thus the woody tissue of soaked cuttings varied with variety and type of microorganism. It is interesting to note that despite the high titre of culturable bacteria (NA-B) in the soaking water of both varieties compared to the number of culturable fungi (DRBC) (Table 1), the numbers of fungi isolated from the wood of Sunmuscat and 140 Ruggeri were significantly (*P*<0.05) greater than the numbers of bacterial isolated from the wood of both varieties (Figure 1). The rate of increase in the titre of bacteria isolated from the wood of both varieties was also slower than the rate of increase in the titre of fungi in the wood.

Microorganisms on container surfaces

In this experiment, only the NA-B bacterial count in the Shiraz soaking water increased significantly (*P*<0.05) beyond the initial contamination that occurred in the first hour of soaking (Table 2). Bacterial and fungal populations became established on the surface of the soaking container within the first hour of the commencement of soaking, followed by non-significant increases for fungi (*P*=0.173) and NA-B bacteria (*P*=0.053) over the time of the experiment (Table 3). These upward trends indicate that the increases in the populations, particularly of bacteria, are likely to have become significant if the experiment had continued for longer.

Discussion

All healthy plants, including grapevines, are colonised by a diverse range of bacteria, fungi and yeasts that inhabit both the phylloplane and the internal tissues (Bell *et al*., 1995; Halleen *et al*., 2003; West *et al*., 2010). These epiphyte and endophyte communities vary between plant species and with environmen-

Table 2. Colony forming units (cfu) of bacteria and fungi isolated from soaking water of Shiraz cuttings.

Culture medium	Soaking time (hours)				
	1 10^4 cfu mL^{-1}	2 10^4 cfu mL^{-1}	4 10^4 cfu mL^{-1}	8 10^4 cfu mL^{-1}	24 10^4 cfu mL^{-1}
NA-B	3.66 ± 2.40 c	10.51 ± 4.96 b	7.82 ± 2.20 bc	9.52 ±3.46 bc	19.65 ± 4.65 a
DRBC	0.26 ± 0.12	0.40 ± 0.12	0.36 ± 0.16	0.50 ± 0.25	0.32 ± 0.13

[a,b] For data in a single row, values followed by the same letter are not significantly different (*P*<0.05).

Table 3. Colony forming units (cfu) of bacteria and fungi recovered from the surfaces of containers used for soaking Shiraz cuttings.

Culture medium	Soaking time (hours)				
	1 10^2 cfu cm^{-2}	2 10^2 cfu cm^{-2}	4 10^2 cfu cm^{-2}	8 10^2 cfu cm^{-2}	24 10^2 cfu cm^{-2}
NA-B	15.64 ± 10.56	8.57 ± 4.94	9.07 ± 5.16	19.06 ± 13.31	68.70 ± 50.36
DRBC	1.60 ± 0.35	1.68 ± 1.20	2.27 ± 1.01	2.00 ± 1.62	5.91 ± 4.45

tal conditions (Quadt-Hallmann *et. al*., 1997; Yang *et al*., 2001; Compant *et al*., 2005b). Endophytes normally gain entry through roots, and the aerial parts of plants including stomata and flowers (Sette *et al*., 2006) and although some are pathogenic, many form complex symbiotic relationships with the host plants (Compant *et al*., 2005a; Rodriguez *et al*., 2009). In the case of grapevine propagation, the DNA and propagules of known grapevine pathogens have been isolated from water used to soak cuttings at various stages of the propagation process (Whiteman *et al*., 2004a; Retief *et al*., 2006; Edwards *et al*., 2007; Aroca *et al*., 2010), potentially causing cross contamination of entire batches of cuttings by exposing the wounds necessarily made during propagation to the pathogens in the water (Waite and Morton, 2007).

We have now established a direct link between soaking grapevine cuttings in water at the beginning of the propagation chain and immediate increases in the titre of microorganisms in the bases of cuttings and demonstrated that contamination of cuttings occurs after relatively short periods of soaking, thus confirming that soaking cuttings is a cause of cross contamination during propagation.

A number of other interesting observations arising from this work highlight the complex and dynamic nature of microbial epiphyte and endophyte populations (Ramey *et al*., 2004; Whipps *et al*., 2008). Although numbers of culturable fungi isolated from the wood of Sunmuscat and 140 Ruggeri were significantly ($P<0.05$) greater than the numbers of culturable bacterial isolated from the wood of both varieties the bacterial numbers in the soaking water were generally greater than the fungal numbers ($P<0.05$). This was also true for the second experiment where numbers of bacteria in the soaking water were higher than the numbers of fungi (Table 2). The higher levels of wood colonization by fungi than bacteria may have been a reflection of lower suitability of the woody tissue as a substrate for bacteria, greater capacity of fungi to adhere to and colonize the wood of the cuttings, or antagonistic interactions between the two groups of microorganisms (Ramey *et al*., 2004). Suppressive interactions within and between fungal and bacterial endophyte populations are well known (Compant *et al*., 2005a; Sette *et al*., 2006; Berg, 2009; Rodriguez *et al*., 2009).

The factors causing the fluctuations in the size of soaking water microbial populations within and between sampling times are likely to have arisen from a number of sources including: differences in microbial populations between the cuttings used in each replicate; microbial settling due to gravity between the end of agitation and sample collection (Hornby, 1969); variations in solute concentrations due to substrates leaching from the cuttings into the soaking water; and changes in population dynamics resulting from interactions between the different groups within the microbial populations (Mian *et al*., 1997; Jordan *et al*., 1999; Yang *et al*., 2001; Whipps *et al*., 2008).

Culturable fungal concentrations in the Sunmuscat soaking water were generally greater than those of 140 Ruggeri. This may be a reflection of different inoculum loads in the source vineyards or the general variability in epiphytes that occurs between plant species (Whipps *et al*., 2008; Yang *et al*., 2001). Differences in spray regimes and variations in the suitability of the bark as a habitat may have caused these differences (Yang *et al*., 2001; Lindow and Brandl, 2003). Rootstock mother vines are not normally trained on trellises in the manner of *V. vinifera* scions, but are instead allowed to sprawl on the ground (Stamp, 2003; Hunter *et al*., 2004) and it is likely that the differences reflect different fungal populations.

It is possible that much of the epiphyte population could have consisted of fastidious and unculturable microflora that did not grow on the media used in this experiment (Yang *et al*., 2001). Large populations of unculturable epiphytic and endophytic microorganisms associated with grapevines have been revealed by molecular techniques (West *et al*., 2010) and the possibility of these organisms impacting on the results of this work cannot be excluded.

The significant decline in culturable fungal and bacterial populations in Sunmuscat soaking water after the early initial maxima might be explained by both adsorption to the container surfaces and the death of microbial populations due to the osmotic effects of the sterile water used as the soaking medium and/or the lack of suitable substrate to enable the population of microorganisms associated with this variety to persist (Straka and Stokes, 1957; Mian *et al*., 1997; Jordan *et al*., 1999). With the exception of DRBC culturable fungi, the microbial populations associated with 140 Ruggeri generally increased over time. Possible causes may be the increase in microbial substrate and reduced osmotic stress caused by leaching of soluble carbohydrates from the ends of the cuttings that is often observed by the author in rootstock varieties in nurseries following soaking. Lower levels

of suitable microbial substrates may explain the slow increase in the microorganisms in the water of the Shiraz cuttings in the second experiment (Straka and Stokes, 1957; Mian *et al*., 1997; Jordan *et al*., 1999).

There is a trend in nurseries to shorten soaking times to less than 8 h to avoid cross contamination, but the fungal concentrations in the water after soaking for 1 hour were sufficient to result in significant increases in endophytic fungi, demonstrating that enough fungi move into the water from soaked cuttings for cross contamination to occur earlier in the soaking cycle than is generally thought.

Most nurseries change the soaking water between batches, but microorganisms established on the container surfaces are a potential source of cross contamination for subsequent batches of cuttings. The upward, but not significant trend in bacterial and fungal populations on container surfaces over the 24 h of this experiment is typical of the colonization patterns that are reported by authors investigating the establishment and growth of biofilms in other situations. Hood and Zottola (1997) reported that after initial colonization of stainless steel surfaces in the first hour of exposure, there was little increase in colonization over time (0–80 h). The authors noted that the adherence of microorganisms to stainless steel surfaces over time varies between species and is influenced by factors such as the growth stage of the organism, substrate availability and other substances, including proteins in the liquid environment. The upward trend in biofilm populations in this experiment is in contrast to the fluctuating populations in the water (Tables 1 and 2). However, biofilm species represent only a subset of the species in the liquid matrix (Costerton *et al*., 1994; Tait and Sutherland, 2002) and the characteristics and behaviour of microorganisms in an established biofilm are independent of the same species in the liquid matrix. Biofilm populations interact differently and are more stable than the population of origin (Costerton *et al*., 1994; Hansen *et al*., 2007).

The slow colonization of the container surfaces reported here may also indicate that plastic is a relatively poor surface for colonization by microorganisms compared to the bark of the cuttings. Factors such as the competition for space between the colonizing microorganisms, the lack of suitable substrate in the water and the osmotic stress imposed by using distilled deionised water may have also affected the rate of colonization (Webb *et al*., 2000; Chmielewski and Frank, 2003). However, microorganisms are particularly difficult to dislodge from container surfaces with common biocides (Costerton *et al*., 1994; Batté *et al*., 2003; Harding *et al*., 2009) and are therefore potential source of cross contamination in nurseries where successive batches of cuttings or vines are soaked in the same container. This is particularly likely to be the case if soaking time is prolonged (Waite and Morton, 2007; Aroca *et al*., 2010) and the level of substrates increases (Hood and Zottola, 1997; Chmielewski and Frank, 2003), containers are not thoroughly cleaned between batches and the soaking water is not treated with an active biocide. Commonly used fungicides tested in the vine nursery context can reduce inoculum of known pathogens, particularly *Pa. chlamydospora* and *P. aleophilum* in soaking water (Fourie and Halleen, 2006) and cuttings (Retief *et al*., 2006; Gramaje *et al*., 2009; Rego *et al*., 2009) but, these and other general purpose biocides have not been evaluated for the specific purpose of cleaning soaking containers.

The results of this study support the growing body of evidence that soaking water is a source of cross contamination and trunk disease transmission during propagation (Whiteman *et al*., 2004b; Retief *et al*., 2006; Edwards *et al*., 2007; Aroca *et al*., 2010). If untreated water is used there is also potential for other microorganisms that are not known grapevine pathogens, to enter the cutting tissue during soaking and impede shoot growth (Waite and Cole, 2006).

Although fungicides and general biocides used in nurseries have been evaluated against a number of known trunk disease pathogens (Groenewald *et al*., 2000; Jaspers, 2001; Fourie and Halleen, 2006; Gramaje *et al*., 2009; Rego *et al*., 2009), the research on biocides in the nursery context has been outpaced by the identification of pathogens implicated in trunk diseases. Therefore, development of nursery management strategies that avoid exposing cuttings to water during propagation is an essential first step in reducing the transmission of trunk diseases in propagation.

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CURRENT TOPICS

Variations in phytosanitary and other management practices in Australian grapevine nurseries

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Summary. Sporadic and costly failure of newly planted vines is an ongoing problem in the Australian wine industry. Failed vines are frequently infected with wood pathogens, including the fungi associated with Young Vine Decline. Hot water treatment (HWT) and other nursery practices have also been implicated in vine failure. We undertook a survey of Australian grapevine nurseries to develop an understanding of current propagation practices and to facilitate the development of reliable propagation procedures that consistently produce high quality vines. A survey covering all aspects of grapevine propagation including sources of cuttings, HWT, sanitation and cold storage was mailed to all 60 trading Australian vine nurseries. In all, 25 nurseries responded, a response rate of 41.7%. Practices were found to vary widely both within and between nurseries. The vast majority of respondents (20) reported that they currently used, or had used, HWT, but the reliability of HWT was questioned by most nursery operators. A majority (18) felt that some *Vitis vinifera* varieties were more sensitive to HWT than others. Hydration also emerged as an important factor that had the potential to affect vine quality. All respondents used hydration and although the majority used treated water, cuttings were not generally seen as a source of cross contamination. Our study identified a clear need for further research into the effects of HWT on cutting physiology and the role of hydration in the epidemiology of grapevine pathogens, and the importance of incorporating the results of such research into practical and comprehensive propagation guidelines for vine nurseries.

Key words: hot water treatment, black-foot disease, Petri disease, young vine decline, *Vitis vinifera*.

Introduction

Since the Australian wine industry planting boom in the 1990s, the quality of grapevine planting material available in Australia has been variable. Sporadic and costly failures of large batches of vines have been an ongoing problem for both grape growers and nurseries in Australia and overseas (Whiting *et al.*, 2001; Waite and Morton, 2007). Many of the failed vines that were planted during the boom between 1995 and 2005 were found to be infected with endogenous pathogens (Morton, 2000; Edwards *et al*., 2001; Gatica *et al*., 2001; Halleen *et al*., 2003; Fourie and Halleen, 2004b). *Cylindrocarpon* spp. and Botryosphaeriaceae spp., with and without *Phaaeomoniella chlamydospora* and *Phaeoacremonium* spp., are associated with Young Vine Decline (Pascoe and Cottral, 2000; Alaniz *et al*., 2007; Petit *et al*., 2011; Whitelaw-Weckert *et al*., 2012). *P. chlamydospora* and *Phaeoacremonium* spp. alone also cause Petri disease in younger vines (Crous and Gams 2000; Edwards and Pascoe, 2001) and later, in complexes with various basidiomycete pathogens, cause esca in mature vines (Mugnai *et al*., 1999)

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A range of other factors including hot water treatment (HWT), which is commonly used to control endogenous pathogens in dormant cuttings and rooted vines, poor nursery sanitation and cold storage (Caudwell *et al*. 1997) are also implicated in vine failure (Fourie and Halleen, 2006; Waite and Morton, 2007; Gramaje and Armengol, 2011). HWT, the immersion of dormant cuttings in hot water at 54°C for 5 min for short duration HWT, or 50°C for 30 min for long duration HWT in Australia (Waite and Morton, 2007), 50°C for 30 min in South Africa (Crous *et al.*, 2001), 50°C for 45 min in France (Caudwell *et al*., 1997), 53°C for 30 min in Spain (Gramaje *et al*., 2009a) and 47°C in New Zealand (Graham, 2007), is widely accepted to be important for the production of high quality planting material (Fourie and Halleen, 2004b; Gramaje *et al*., 2009a) and was adopted in Australia to prevent the transmission of endogenous diseases in planting material following the emergence and identification of the phytoplasma disease, Australian Grapevine Yellows (AGY) in the early 1990's (Smart *et al.*, 1995; Wilson & Hayes, 1996), and the identification of *P. chlamydospora* in mother vines used for propagation (Edwards and Pascoe, 2004). HWT is also an effective control for phylloxera (Stonerod and Strik, 1996) and as such satisfies the requirements of the National Phylloxera Management Protocols (National vine Health Steering Committee, 2002) and thus the quarantine regulations for the movement of cuttings and rooted vines between Australian states. However, the transfer of HWT from small batch research laboratory treatments to commercial practice has met with mixed success and significant losses have been attributed to HWT (Waite and Morton, 2007). Concerns expressed by nurseries and growers resulted in a significant body of research into the effects of HWT on cuttings and rootlings (Waite, 1998; Crocker *et al*., 1999; Crocker *et al*., 2002; Waite and May, 2005; Gramaje *et al*., 2009a). Considerable progress was made and protocols were improved and refined (Waite and Morton, 2007), but the effects of HWT and surrounding nursery practices on cuttings and rooted vines cannot yet be predicted with certainty. Further research is needed to develop propagation procedures that are reliable and result in the production of high quality vines. To facilitate the planning of relevant and targeted research (Kelley *et al*., 2003), a systematic survey of Australian grapevine nurseries was undertaken to develop an understanding of current practices and identify those likely to have the most impact on the quality of planting material. The survey was also a means of acknowledging the value and importance of industry engagement with the research and its outcomes, a critical factor in the development and adoption of best practice (Black, 2000; Piderit, 2000; Pannell *et al*., 2006).

Materials and methods

Survey type

A mailed paper and pencil survey (Hoyle, 2002) was sent to all 65 identifiable Australian vine nurseries. Telephone interviews, in the first instance, were ruled out as a first approach because of the difficulty of contacting busy nursery operators who work mainly out of doors and are often out of mobile telephone range during the day, and evening calls are perceived negatively. Face to face interviews and nursery visits were impractical because of remote locations and high cost of travel (McHorney *et al*., 1994; Hoyle, 2002). Electronic surveys would have also been difficult to administer as there were no public email addresses for more than half of the nurseries and many of the nursery managers are known to be in the age group (over 50) where familiarity with the internet cannot be automatically assumed.

The relative anonymity of pencil and paper surveys also places some physical and psychological distance between author and participant and elicits a less biased response, as does the identification of a university as the sponsor of the survey (Hoyle, 2002; Pennings *et al*., 2002). A disadvantage shared with other survey types where participation is not compulsory, is the empirical bias resulting from the self selecting nature of the respondents (Kelley *et al*., 2003). Primary producers often complain of a constant flood of bureaucratic and regulatory correspondence that is time consuming, of little perceived benefit and takes them away from essential farming activities. Consequently non essential paperwork is often ignored and surveys frequently go unanswered (Heberlein and Baumgartner, 1978; Pennings *et al.*, 2002; Conelly *et al.*, 2003).

Survey design

The survey consisted of 26 questions divided into two sections; 1) hot water treatment and 2) other

nursery practices. Section 1 questioned nursery operators about their use of and opinions about HWT. Section 2 covered the use of hydration (immersion of dormant cuttings and or rooted vines in water), water treatment and general sanitation and cold storage and fungicides, factors that are known to interact with HWT and affect the quality of the vines produced (Waite and Morton, 2007). A draft of the survey was sent to the executive of the Vine Industry Nursery Association for review before final formatting and distribution.

Each section included both closed and open questions and covered all aspects of grapevine propagation. The closed format was used for both factual and subjective questions. Closed questions have a number of benefits including reduction of time taken to complete the survey and standardization of responses, but may not offer all alternative responses (Kalton and Schuman, 1982; Kelley *et al*., 2003). To ensure that vital information was not inadvertently excluded, open questions were linked with some closed questions to provide the opportunity for respondents to elaborate on their replies.

As an incentive to complete the survey, respondents were offered feedback in the form of a summary of the survey results and the opportunity to comment further by participating in more extended telephone interviews following the initial survey (Powers and Alderman, 1982). The surveys were mailed out in autumn 2009, a relatively quiet time in the nursery calendar when operators are more likely to have time to attend to non essential matters.

Results

Initially 20 replies were received and a further five responded after a follow up letter. It became apparent from received phone calls, emails and returned mail, that at least five nurseries had ceased trading, or were no longer growing grapevines, reducing the cohort of extant nurseries from 65 to 60, thus giving a response rate of 42%, a relatively high response rate for primary producers (Hayman and Alston, 1999; Kelley *et al*., 2003). Furthermore, ten respondents agreed to a telephone interview to discuss the survey results and 17 respondents (68% of all respondents) requested a copy of the results indicating a high level of interest and engagement among respondents (Powers and Alderman, 1982).

Statistical analysis

Although the response rate was relatively high (Hayman and Alston, 1999), the low actual numbers of responses (25) and the self selecting nature of the respondents, mean that the capacity to apply statistical tests to the data is very limited and results may be biased, particularly as the capacity to collect demographic information was not available (Kanuk and Berenson, 1975; McHorney *et al*., 1994; Groves, 2006). Therefore, we report proportions and summary statistics only.

Survey Part I - Hot water treatment

The majority of respondents (80%) reported that they currently used, or had used, short and/or long duration HWT (SdHWT and LdHWT). Both on-site and off-site HWT plants were used (56% of respondents each), with 24% of nurseries reporting using both on and off site plants; a consequence of moving material between quarantine jurisdictions, rather than one of insufficient on-site capacity. Eight percent of respondents reported that they did not use HWT at all, but acknowledged using HWT plants in a later question (one on-site and one off-site), thus 88% rather than 80% of respondents had either used, or were currently using, HWT. More respondents (72%) reported using LdHWT than SdHWT (64%), most likely because the long duration treatment satisfies quarantine requirements in all Australian jurisdictions and controls endogenous pathogens, in addition to the external pathogens controlled by SdHWT. Four respondents gave reasons for not currently using HWT, two because of safety concerns and two because they did not have on-site HWT plants.

Grower perceptions of the efficacy of HWT in controlling grapevine pests and diseases are summarized in Table 1. A majority of growers agreed that LdHWT provided some level of control for internal and external pests and diseases and a majority also were of the opinion that SdHWT provided some level of control for external pests and diseases. The number of respondents who did not believe in the efficacy of either HWT was very small. However, a significant level of doubt about the efficacy of HWT among nursery operators is indicated by the substantial number of respondents who ticked either the" *frequently*" or "*sometimes*" response options. This uncertainty might have arisen as a result of variable results from poorly applied hot water treatments, or

Table 1. Grower perception of the efficacy of hot-water treatment for pathogen control expressed as a percentage of whole respondent cohort.

Activity	Long duration HWT (%)			Short duration HWT (%)		
	Always	Frequently/ sometimes	Never	Always	Frequently/ sometimes	Never
Controls internal pests and pathogens	36	52	8	NAa	NA	NA
Controls external pests and pathogens	52	44	0	40	44	4

[a] NA short duration HWT is not recommended for control of internal pests and pathogens.
Note: not all respondents answered these questions.

from a lack of clear, concise and unambiguous information regarding the efficacy of HWT (Vanclay and Lawrence, 1994).

Scientific research that investigates the efficacy of HWT against endogenous pathogens including phytoplasmas (Caudwell *et al*., 1997) trunk disease pathogens (Crous *et al*., 2001; Rooney and Gubler, 2001; Whiting *et al.*, 2001; Fourie and Halleen, 2004b; Gramaje *et al*., 2009a) is generally published in peer reviewed journals which are not normally read by industry, or is reported in technical magazines aimed at the wine industry more broadly (Waite, 1998; Waite *et al*., 2001; Crocker *et al*., 2002), rather than directly at the vine nursery industry. The terminology used in the scientific literature and conference presentations may also be a source of confusion, particularly the term "*control*". In scientific literature control often means that the target pest or pathogen cannot be detected in the treated material, but stops short of claiming that the treatment eliminates the target pest or pathogen. This may suggest to some readers that the treatment is not fully effective. In the 1990s and early 2000s both the Australian Vine Improvement Association and the Vine industry Nursery Association conducted more than ten HWT workshops across all states, specifically for the vine nursery industry, aimed at addressing these issues, but not all nursery operators were represented at these workshops and, as yet, no manual or reference book has been published specifically aimed at assisting nursery operators in relation to this issue.

Most nursery operators were less sanguine about the safety of HWT. A substantial number felt that LdHWT was never a safe treatment for cuttings (24%) or rooted vines (32%). Fewer thought that LdHWT was always or frequently a safe and reliable treatment for cuttings (28% and 20% respectively). Confidence in LdHWT for rooted vines was even lower. Only 8% of respondents thought that it was always a safe and reliable treatment and only three felt it was frequently safe and reliable. SdHWT was viewed slightly more favourably. Only 16% and 12% of respondents respectively took the view that SdHWT was never a safe and reliable treatment for cuttings and rooted vines. More respondents (24% and 32% respectively) thought SdHWT was always or frequently a safe and reliable treatment for cuttings and rooted vines.

Respondents also had the opportunity to make any general comments at the end of the survey. Of the 64% of respondents who exercised this option, 36% commented about HWT. Negative comments were made by 20% of the respondents and included remarks such as: 1) "*HWT for interstate vines has cost us a fortune in failed vines. HWT cuttings is (sic) avoided like the plague for losses of up to 100% at times. Never again*"; 2) "*I heard a colleague say once when investigating HWT and finding no real hard evidence to provide benefit, that HWT is like treating everyone in the community with chemotherapy in case they have cancer*"; 3) "*My belief is that HWT exacerbates any other problems the vines may have to a high degree. E.g. Healthy vines may experience 5% loss in take. Problem vines may experience 50% loss in take*". However, four respondents indicated that they thought other factors either directly affected propagation success or the success of hot water treated material. Comments included: 1) "*Look beyond the HWT treatment for most of the issues with grafting, e.g. quality of cuttings*"; 2) "*stress of plant material needs to be considered, e.g. frost, dryness, heavy crop*"; 3) "*We have found out that cool room temperature is most important and planting as soon as possible after coming out of cold room*" (sic).

A majority of respondents (72%) thought that some *V. vinifera* varieties were more sensitive to HWT than others and only 12% respondents thought that there were no differences between varieties. By contrast, those who believed that some rootstock varieties were more sensitive than others (32%) were in the minority. Eleven respondents felt that there was no difference in the sensitivity of rootstocks to HWT and the remaining 12% of respondents either did not reply to the question or stated that they did not know. The *V. vinifera varieties* Pinot Noir, Chardonnay, Merlot, Riesling, Petit Verdot and the rootstock varieties Ramsey and Ruggeri 140 were identified as being sensitive to HWT. These results agree broadly with anecdotal reports and research results (Crocker *et al.*, 1997).

Negative effects of HWT that have sometimes been observed by growers, or reported previously, include delayed callusing and rooting of cuttings (Orffer and Goussard, 1980; Waite and May, 2005), delayed development or death of buds in cuttings and rooted vines (Wample, 1993; Caudwell *et al*., 1997; Laukart *et al*., 2001; Gramaje *et al.*, 2009a), failed or incomplete healing of graft unions and fermentation in cold storage. However, the response of cuttings and vines is variable and other authors reported no negative effects of HWT on treated material (Ophel *et al.*, 1990; Fourie and Halleen, 2004). Sixteen respondents reported negative effects of HWT on cuttings (Table 2); 52% reported problems with grafted cuttings and 11 reported negative effects on rooted vines. The most commonly reported effects of HWT were delayed development of callus (44%) and roots (48%), delayed bud development (44%), death of buds (44%) and death of whole cuttings in the nursery (40%) There were fewer reports of deaths of one-year-old grafted or ungrafted vines in the vineyard (24% and 36% respectively). This might be a reflection of lower rates of treatment in rooted vines than in cuttings rather than of a difference in sensitivity between cuttings and rooted vines. Anecdotal reports indicate that many nurseries avoid treating one-year-old rooted vines for fear of litigation if the vines die or perform poorly in the vineyard. However, two of the respondents who agreed to a telephone interview commented that they thought rooted vines were generally less susceptible to injury from HWT than were cuttings and that this was a general perception in the nursery industry. It is notable that reports of HWT cuttings and rooted vines fermenting in cold storage were less frequent than other forms of injury (20% and 12% reports respectively). Anecdotally this is seen as being a common problem, but respondents to telephone interviews commented that fermentation in cold storage had declined in recent years as a result of better cool room management practices and improved sanitation.

Survey Part II – Other nursery practices

The second part of the survey examined if other potentially harmful practices were widespread among Australian nurseries. The quality and disease status of source material, cool room management, packaging and general nursery sanitation all affect the viability of hot water treated cuttings or vines (Waite and May, 2005). The water used in HWT and other process such as hydration (the practice of soaking cuttings in water for variable periods) is a potential source of contamination (Fourie and Halleen, 2006; Retief *et al*., 2006; Whiteman *et al*., 2007; West *et al*., 2010) and physiological stress.

Origins of cuttings

Vine improvement associations have been formed in all wine producing states to select and disseminate high quality propagating material for the wine industry. The Australian Vine Improvement Association (AVIA) is the peak national body. These associations provide high health, true to type certified cuttings to the vine nursery industry. These cuttings are generally regarded as being the best available material compared to cuttings from unregistered vineyards where disease status and type are not able to be verified. However, there are reports of nurseries obtaining cuttings from unregistered sources, usually as a result of a shortage of registered material. There are also reports that some nurseries on-sell surplus cuttings to other nurseries. Cuttings sourced from outside the vine improvement associations, or cuttings that have passed through a number of hands are more likely to be affected by diseases and environmental stress and potentially less able to tolerate HWT and other stresses imposed during propagation (Hartmann *et al*., 1990b). A narrow majority (56%) of nurseries that responded to the survey stated that they "*frequently*" use registered cuttings from vine improvement associations, only five nurseries reported that they "*always*" use registered

Table2. Respondents reporting of negative effects of hot-water treatment on cuttings and rooted vines.

Symptoms of HWT Sensitivity	%
Slow or delayed callusing of V. vinifera cuttings	44
Slow or delayed rooting of V. vinifera cuttings	48
Unusually high deaths of buds in V. vinifera cuttings	44
Slow or delayed bud burst in V. vinifera cuttings	44
Unusually high deaths of V. vinifera cuttings in cold storage	24
Unusually high deaths of HWT V. vinifera cuttings in the nursery	40
Slow or delayed healing of graft unions	32
Incomplete healing of graft unions	24
Slow or delayed rooting of bench grafted vines	24
Slow or delayed scion bud burst in newly grafted vines	32
Unusually high deaths of scion buds in newly grafted vines	28
Slow or delayed bud burst in one-year-old vines	32
Unusually high deaths of roots in one-year-old vines	28
Unusually high deaths of one-year-old vines in cold storage	24
Unusually high deaths of HWT one-year-old own rooted vines in the vineyard	24
Unusually high deaths of HWT one-year-old grafted vines in the vineyard	36

material. More than half the respondents (56%) also reported obtaining registered cuttings from third parties rather than directly from the original supplier. It is evident from these responses that the quality of cuttings entering the propagation chain is highly variable and this may have consequences in terms of predictability of response to HWT and other nursery processes.

Hydration of cuttings and rooted vines

Repeatedly soaking cuttings, pre-cut buds and also rooted vines in water in the belief that it reverses the effects of dehydration and promotes root initiation is a widespread practice in grapevine nurseries. The origin of soaking as a routine practice in grapevine nurseries appears to be a paper by Spiegel (1954) who reported that prolonged hydration (up to 96 h) of rootstock and *V. vinifera* cuttings improved rooting by leaching auxin inhibitors. However, Spiegel (1954) also reported that these inhibitors disappeared naturally as the cuttings emerged from dormancy in spring after exposure to natural or artificial chilling, thus obviating the need for soaking.

Despite this, the practice remains entrenched in grapevine nurseries around the world.

In nurseries that propagate other species, every effort is made to prevent dehydration of cuttings, untreated water is not used and cuttings are not soaked for fear of cross contamination and creating conditions favourable to pathogens such as *Pythium* spp. *Rhizoctonia* spp. and *Botrytis cinerea* that kill cuttings during the callusing and rooting phase (Baker, 1957; Hartmann *et al*., 1990a; Preece, 2003). Trunk disease pathogens including *P. chlamydospora* (Fourie and Halleen, 2004a; Retief *et al*., 2006; Whiteman *et al.*, 2007), Botryosphaeriaceous specie and *black foot fungal agents* have been detected in cuttings (Giménez-Jaime *et al*., 2006) and DNA of both *P. chlamydospora* (Edwards *et al*., 2007) and *Phaeoacremonium* spp. (Aroca *et al.*, 2010) has been detected in soaking water sampled from hydration tanks in commercial grapevine nurseries, evidence that hydrating tanks are a potential source of cross contamination.

The value of soaking has been questioned by researchers for more than a decade (Crocker *et al*., 2002; Fourie and Halleen, 2006; Retief *et al*., 2006), but the survey results indicate that the practice is still widespread. All respondents reported soaking material at least once during the propagation cycle and the majority (76%) reported soaking material on three or more occasions. Soaking times varied from less than 1 hour to more than 12 hours (most commonly less than eight hours). One respondent reported soaking one-year-old rooted vines for more than 12 hours on four occasions (following lifting from the field nursery, before cold storage, after cold storage and before despatch to customers). Although clean water was used by all respondents, there are ample opportunities for cross contamination from bark inhabiting organisms that enter the water during soaking if the active chemical has dissipated, or been inactivated by soil and organic material on the surfaces of the treated cuttings and vines. Thus hydration continues to threaten the phytosanitary status of grapevine planting material in Australia.

Cold storage conditions

Cold storage at 1–3°C is a convenient way of storing dormant cuttings (Hartmann *et al*, 1990b). Cold storage delays root initiation and bud burst in cuttings and enables nurseries to extend the propagating season by several weeks, and make better use of labour, and has largely replaced the practice of storing cuttings in sand or sawdust callusing pits. However, poorly managed cool rooms can be the source of microbial and chemical contamination and of stress from temperature fluctuations. A majority of nurseries reported always using on site cool rooms for storing cuttings and one-year-old vines (56% and 40% respectively). Off site cool rooms operated by third parties were also utilized; 16% of respondents always used off site cool rooms for storing cuttings and 24% of respondents always used off site cool rooms for storing one-year-old vines. A majority of respondents (72%) reported always monitoring cool room temperature to ensure temperatures remained within the range of 1–3°C, but only four respondents reported always monitoring the temperature inside bins or crates. However, another 36% of respondents reported that they either "*frequently*" or "*sometimes*" monitored the temperature inside bins or crates indicating that there is a level of awareness that storage temperature affects the quality of the stored material and that the temperature in a bin of cuttings may differ from the cool room atmosphere.

There is also an awareness of the importance of cool room sanitation. The majority of respondents (72%) reported cleaning on site cool rooms at the beginning of the propagating season, but off site cool rooms operated by third parties were rarely cleaned by survey respondents (20% of those using off-site cool rooms). Cleaning of on-site cool rooms was usually relatively thorough. Floors were swept and walls and floors were washed, usually with chlorine based disinfectants, but the cleaning of storage bins and crates was less common because they were not seen as potential sources of contamination. Eight respondents stated that there was no need to clean bins and crates because they lined them with new plastic bags, or packaged cuttings and one-year-old vines in new plastic bags. Responses such as; *"Have crates that are only used for cuttings storage"* and "*No. Because we use new plastic liners in packs or bins. Our hydration tanks are regularly disinfected with appropriate chemicals"* demonstrated a relatively poor understanding of microbial ecology and epidemiology. However, others demonstrated a much better grasp of these issues with replies such as; *"Steam sterilization 75°C for 30 min"* and *"Before use, bins are pressure washed and soaked in chlorinated water".*

Materials other than cuttings or vines can also be a source of contaminants in cool rooms, particularly if atmospheres are shared with vegetables such as potatoes and onions treated with sprouting inhibitors, or climacteric fruits such as apples that produce ethylene, a hormone associated with senescence in plants (Saltviet, 1999; Pierik *et al*., 2006) that may also prompt emergence from dormancy (Ophir *et al*., 2009); undesirable in long term storage where the purpose is to delay sprouting. Only 28% of respondents reported storing other materials (beer, vegetables and deciduous fruits) with cuttings and vines indicating that the risks from these sources of contamination while high, particularly if fruits and vegetables are stored with vines, the problem is not common and therefore not a major cause of cutting and vine failure across the nursery industry.

The move from callusing pits to cold storage entailed significant changes to packaging. The risk of dehydration in cold storage resulted in the use of sealed plastic bags for cutting storage. The unintended consequences of this were the development of an-

aerobic conditions and excessive growth of surface moulds. A moderate exposure to anoxia can result in enhanced bud burst (Halaly *et al*., 2008; Ophir *et al*., 2009), but decreasing oxygen levels, accumulation of toxic fermentative metabolites, and the growth of anaerobic microorganisms (Phillips, 1996) can result in fatal tissue damage when material is stored for 3–6 months in bags with limited head space (Chen *et al*., 2011). Storage in bags with a small number of perforations (6–8) made with a ball point pen or instrument of similar diameter is recommended to allow some air flow without causing excessive dehydration if cool rooms are not humidified, but anecdotal reports indicate that sealed bags are common. Five respondents reported using sealed bags and seven used perforated or unsealed bags, but a number of others did not state whether the bags they used were sealed or unsealed, perhaps indicating that they did not see this as an important detail. More precise data may have been elicited by designing the question with fixed choices (Miles and Huberman, 1994). However, the survey results do show that the approach to packaging and cold storage is inconsistent and likely contributes to the variable quality of planting material.

Use of chemical control

Chemical sprays, dips and drenches that are widely used in the nursery industry to control the fungal pathogens including *Botrytis cinerea* that are favoured by the warm, moist and sheltered nursery environments (Daughtry and Benson, 2005) are also employed by the vine nursery industry. and commonly used fungicidal dips including captan, carbendazim and didecyldimethylammonium chloride can also assist in reducing infection by trunk disease pathogens including "*Cylindrocarpon*" spp. (Alaniz *et al*., 2011), *P. chlamydospora* and *Phaeoacremonium* spp. (Fourie and Halleen, 2004b; Gramaje *et al*., 2009b). In this survey, only 12% of nurseries reported not using fungicides at any of the stages in the propagation process. Of the remaining 88% of nurseries, 64% used fungicides more than twice, but only 8% used fungicides at all stages of the cycle; in hydrating tanks, in callusing boxes, as a dip for stored cuttings and one-year-old vines and as a dip before despatch to customers. The use of more than one type of fungicide was reported. Captan was the most commonly used fungicide, but iprodione, mancozeb, carbendazim, 8-hydroxyquinoline sulphate (Chinosol), the biocontrol agent *Trichoderma* and the multipurpose biocides didecyldimethylammonium chloride (Sporekill) and sodium chlorite (Vibrex) were also used. Two nurseries also reported using benomyl, a fungicide that is no longer registered for use in Australia.

Discussion

While the small actual numbers and the self selecting nature of respondents prevent a comprehensive statistical analysis, the survey has provided a useful insight into the vine nursery industry in Australia and the factors that influence the quality of planting material. Our results have revealed wide variations in practices both within and between nurseries and confirmed the previous anecdotal evidence that reliability, and efficacy of HWT continues to be questioned by a majority of nursery operators. The literature examining the effects of heat treatment on plant physiology is not large and there is a need for further research on the effects of HWT on the growth and development of cuttings, particularly *V. vinifera*, before the concerns of the nursery industry can be fully answered. By contrast, the consistent use of treated water and fungicides and other sanitary practices demonstrate a better than expected, although imperfect, understanding of factors that can lead to microbial contamination and consequent loss of quality. However, the common thread running through all the responses is the inappropriate use of soaking (often coupled with poor storage practices) that is likely to be a major source of contamination regardless of when it is applied during the propagation process. Respondents seemed to be generally unaware that cuttings and vines themselves, as well as untreated water, can be a source of contamination from bark inhabiting pathogens that can gain entry through propagation wounds, affecting the short and long term health and vigour of the vine. A small experiment to quantify and illustrate this point would be a useful precursor to the development of improved propagation protocols, as would an investigation into the appropriate use and choice of fungicides. However, there is no need for comprehensive research in nursery sanitation. The value of good sanitation in plant nurseries has been known since the 1950s when the first editions of the seminal works on modern nursery practice "The UC System for Producing Healthy Container Grown Plants" (Baker, 1957) and "Plant

Propagation Principles and Practices," (Hartmann and Kester, 1959) were published.

Vine nurseries do not usually belong to the Nursery and Garden Industry Australia association and therefore do not utilize the best practice management guides attached to the accreditation scheme of that organization. They rather belong to the Vine Industry Nursery Association that has not yet completed development of similarly comprehensive guidelines. If the quality and consistency of grapevine planting material is to improve to the standard required for the establishment of healthy, productive, long lived vineyards it is in the interest of all participants in the grape and wine industries to support the development of comprehensive propagation procedures for incorporation into accreditation schemes as a matter of urgency. Until such time, the current *ad hoc* approaches to nursery practice are likely to persist and the quality of planting material will continue to be erratic. However, it will not be sufficient to simply codify a set of standard operating procedures and expect them to be adopted. The benefits of standard operating procedures to the nurseries themselves must also be clearly demonstrated (Vanclay and Lawrence, 1994) and the principles of good nursery practice be explicitly stated to ensure that nursery operators understand the reasons for, and the importance of, each sequential step in the propagation process (Baker, 1957; Hartmann *et al*., 1990a). It is also essential that codified standard operating procedures be practical and flexible enough to accommodate the needs and resources of individual businesses without compromising the quality of the end product. It is only by a comprehensive approach that engages, and is supported by, all interested parties that the quality of grapevine planting material will meet the standard required for the production of high quality grapes and wine.

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Short Note

Fungal trunk pathogens associated with table grape decline in North-eastern Brazil

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Summary. During the last five years a decline of table grape plants has been noticed in nurseries, young plantations and vineyards of the Northeastern region of Brazil, where the management systems for grapevine production are adapted to the specific environmental conditions of a tropical viticulture. Samples of table grape plants showing decline symptoms were obtained from grapevine nurseries, young plantations and vineyards located in the São Francisco, Assú and Siriji Valleys in 2010, and were subjected to fungal isolation. Grapevine trunk pathogens were identified using morphological and molecular methods. Species recovered included *Botryosphaeria mamane*, *Campylocarpon fasciculare*, *C. pseudofasciculare*, *Lasiodiplodia crassispora*, *L. parva*, *L. pseudotheobromae*, *L. theobromae*, *Neofusicoccum parvum*, *Phaeoacremonium aleophilum*, *Pm. parasiticum* and *Phaeomoniella chlamydospora*. They are all reported for the first time on grapevine in Brazil, with the exception of *L. theobromae*. Moreover, *Botryosphaeria mamane*, *Lasiodiplodia parva* and *L. pseudotheobromae* are reported for the first time on grapevine, and *C. fasciculare* is reported for the first time on the American continent.

Key words: Botryosphaeriaceae, *Vitis vinifera*.

Introduction

In 2011, 58,236 t of table grapes (*Vitis* spp.) were exported from Brazil, being the main fresh fruit export from this country and accounting for US$ 141 million (Agrianual, 2012). Most of these table grapes are produced in the Northeastern region, where 9,600 ha are cultivated in three different areas: the São Francisco Valley, located in the semi-arid region of Bahia and Pernambuco States; the Assú Valley, located in the semi-arid region of Rio Grande do Norte State; and the Siriji Valley, located in the humid region of Pernambuco State. The São Francisco Valley is the main table grape growing area in the region, accounting for 98% of the production. The Assú Valley is a new area of production of fine table grapes for export, which was implemented five years ago, while in the Siriji Valley table grapes have been grown for over 40 years with a production intended only for the local market (Araújo and Ramalho, 2009). North-eastern Brazil is a tropical region, thus the management systems for grapevine production are adapted to the specific environmental conditions of a tropical viticulture. In both the dry and wet tropics, the growth and cropping cycle of the vine can be manipulated to extend from 5 to 12 months by

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a combination of pruning, modifying vine water status and the use of chemical regulators. Thus, it is possible to achieve two and a half to three vegetative cycles per year (Camargo *et al*., 2008; Possingham, 2008).

During the last five years a decline of table grape plants has been noticed in nurseries, young plantations and vineyards of the Northeastern regions of Brazil. Symptoms included poor early growth, reduced vigour showing leaf yellowing, wilting and dieback, and different symptoms in wood such as V-shaped necroses when affected arms and trunks were cut in cross-section, and longitudinal brown and black streaking that appeared as necrotic black spots in cross-sections. These symptoms are similar to those described in other viticultural regions worldwide (Luque *et al*., 2009; Gramaje and Armengol, 2011), which have been associated with several decline diseases such as black dead arm, black foot, eutypiose, esca or Petri disease (Moller and Kasimatis, 1981; Mugnai *et al*., 1999; Halleen *et al*., 2006; Mostert *et al*., 2006; Úrbez-Torres, 2011). Nevertheless, grapevine decline and its associated pathogens are yet to be studied in tropical viticulture. Thus, the aim of this work was to determine the occurrence of fungal trunk pathogens in declining table grapes in Northeastern Brazil.

Figure 1. Table grape growing areas surveyed in North-eastern Brazil (the São Francisco, Assú and Siriji Valleys) in 2010.

Materials and methods

Sampling and isolation of fungi

Samples of table grape plants showing decline symptoms were obtained from grapevine nurseries, young plantations and vineyards located in the São Francisco, Assú and Siriji Valleys (Northeastern Brazil) in 2010 (Figure 1), and were subjected to fungal isolation. At least 5–7 plants were analyzed per sample.

Rootstocks, graft unions and scions were examined. Symptomatic wood fragments taken from the margin of dead and healthy tissue, and from internal necroses and brown-black vascular streaking were washed under running tap water, surface-disinfected for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Small pieces of necrotic, discolored or decayed tissues were plated on malt extract agar (MEA) (Oxoid Ltd., Basingstoke, England) supplemented with 0.5 g L^{-1} of streptomycin sulphate (MEAS) (Sigma-Aldrich, St. Louis, MO, USA). Plates were incubated at 25ºC in the dark for 14 to 21 days, and all colonies were transferred to 2% potato dextrose agar (PDA; Biokar-Diagnostics, Zac de Ther, France). Thirty two representative isolates were selected for further analyses (Table 1). They were hyphal-tipped or single-spored with the serial dilution method, prior to morphological and molecular identification (Dhingra and Sinclair, 1995).

Fungal identification

Morphological identification

Species of the Botryosphaeriaceae were identified by colony and conidial morphology (Phillips, 2006). In order to enhance sporulation, cultures were placed with sterilized pine needles on 2% water agar (WA; Biokar-Diagnostics) at 25°C with a 12-h day (Philips TDL18W/33) (Slippers *et al*., 2004). Isolates were examined weekly for formation of pycnidia and conidia. Conidial morphology (cell wall, shape, color, and presence or absence of septa) from pycnidia was recorded. Species of *Campylocarpon* were identified by macroscopic characters such as colony texture, color, and the margin on PDA. Colonies grown on PDA were incubated for a further 20 days to determine the presence/absence of chlamydospores. Co-

Table 1. List of fungal trunk pathogens isolated from table grapes in Northeastern Brazil, with their geographical origin.

Species	Isolate	State/Town		Rootstock/scion[a]
Botryosphaeria mamane	BV1	Rio Grande do Norte	Assú	572/Red Globe
Campylocarpon fasciculare	BV2		Assú	572/Red Globe
	BV3		Assú	572/Red Globe
	BV4		Assú	572/Red Globe
	BV5		Assú	572/Red Globe
	BV6		Assú	572/Red Globe
Campylocarpon pseudofasciculare	BV7		Assú	572/Red Globe
Lasiodiplodia crassispora	BV8	Pernambuco	Petrolina	766/nd
Lasiodiplodia parva	BV9		Petrolina	572/nd
Lasiodiplodia pseudotheobromae	BV10		Machados	cv. Isabel
	BV11		Machados	cv. Isabel
	BV12		Machados	cv. Isabel
Lasiodiplodia theobromae	BV13		Machados	cv. Isabel
	BV14		Machados	cv. Isabel
	BV15		Petrolina	572/nd
	BV16		Petrolina	313/nd
	BV17		Petrolina	SO4/nd
	BV18		Machados	cv. Isabel
	BV19		Machados	cv. Isabel
	BV20		Petrolina	420 A/nd
	BV21	Rio Grande do Norte	Assú	572/Red Globe
	BV22		Assú	572/Red Globe
Neofusicoccum parvum	BV23	Pernambuco	Petrolina	313/nd
	BV24		Machados	cv. Isabel
Phaeoacremonium aleophilum	BV25		Petrolina	572/nd
	BV26		Petrolina	SO4/nd
	BV27		Petrolina	766/nd
Phaeoacremonium parasiticum	BV28		Machados	cv. Isabel
	BV29		Machados	cv. Isabel
Phaeomoniella chlamydospora	BV30		Petrolina	SO4/nd
	BV31		Machados	cv. Isabel
	BV32		Machados	cv. Isabel

[a] nd = not determined. Grapevines cv. Isabel are planted without rootstock.

nidia size was also measured on Spezieller Nährstoffarmer Agar (SNA) with the addition of a 1×1 cm piece of filter paper to the colony surface (Halleen *et al*., 2004). Morphological characters to distinguish species of *Phaeoacremonium* included conidiophore morphology, phialide type and shape, size of hyphal warts and colony characters and pigment production on MEA, PDA and oatmeal agar (OA, 60 g oatmeal; 12.5 g 16 agar; Difco, Osi, Maurepas, France) (Mostert *et al*., 2006). *Phaeomoniella chlamydospora* was identified by conidiophore morphology, conidial size and shape, and its cultural characteristics on PDA and MEA (Crous and Gams, 2000).

DNA isolation and sequencing

Fungal mycelium and conidia from pure cultures grown on PDA for 2 to 3 weeks at 25ºC in the dark were scraped and mechanically disrupted by grinding to a fine powder under liquid nitrogen using a mortar and pestle. Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Doraville, GA, USA) following manufacturer's instructions. DNA was visualized on 0.7% agarose gels stained with ethidium bromide and stored at -20ºC.

Identification of Botryosphaeriaceae species was confirmed by analysis of elongation factor 1-α gen (EF) amplified using EF1-728F and EF1-986R primers (Carbone and Kohn, 1999). For *Campylocarpon* species identification, the internal transcribed spacer (ITS) region of DNA was amplified using the fungal universal primers ITS1F and ITS4 (Gardes and Bruns, 1993) and partial sequence of the first part of the β-tubulin gene, BT, were amplified using primers BT1a and BT1b (Petit and Gubler, 2005). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and sequenced in both directions by Macrogen Inc., Sequencing Center (Seoul, South Korea). *Phaeoacremonium* species were confirmed by sequence analysis of the β-tubulin gene using primer sets T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995), and by comparison with the polyphasic, online identification system for *Phaeoacremonium* species recognition (http://www.cbs.knaw.nl/phaeoacremonium/biolomics.aspx) developed by Mostert *et al*. (2006). *Pa. chlamydospora* was identified by PCR using primers Pch1-Pch2 (Tegli *et al*., 2000), and confirmed by sequencing the ITS region of DNA using the primers ITS1F and ITS4 (Gardes and Bruns, 1993).

Results and discussion

Based on their appearance in culture, the isolates obtained from the margin of dead and healthy tissue, and from internal necroses and brown-black vascular streaking of symptomatic wood fragments of trunks and branches could be assigned to three main fungal groups.

The first group was characterized by dark green or gray to dark gray fast-growing mycelium on PDA. With age, most of these cultures developed single or grouped, black, globose fruiting bodies (pycnidia) on the surface of pine needles on WA releasing either pigmented or hyaline conidia characteristic of Botryosphaeriaceae spp. (Van Niekerk *et al*., 2004; Phillips, 2006). BLASTn searches in GenBank showed that EF sequences of Botryosphaeriaceae isolates from Northeastern Brazil had 99 to 100% identity with isolates of *Botryosphaeria mamane* CMW 13416 (GU134938), *Lasiodiplodia crassipora* CMW 22653 (FJ888452), *L. parva* CMW 28309 (GQ469904), *L. pseudotheobromae* CMW 26702 (GQ471796), *L. theobromae* CMW 28311 (GQ469898) and *Neofusicoccum parvum* CMW 26718 (FJ900658).

The second group of isolates was characterized by pale to medium brown flat slow-growing cultures on MEA. Different types of phialides that were variable in size and shape were observed in the aerial mycelium, and either discrete or integrated in conidiophores. Sporulation was abundant and conidia were hyaline and aseptate. All morphological characters corresponded to the genus *Phaeoacremonium* (Mostert *et al*. 2006). BLASTn searches showed that BT sequences of these isolates had 99 and 100% identity with isolates of *Pm. aleophilum* CBS 631.94 (JQ691663) and *Pm. parasiticum* P46 (HQ605022), respectively.

The last fungal group was characterized by grey-olivaceous to olivaceous-black, slow-growing colonies with sparse aerial mycelium. They showed abundant straight, pigmented conidia and dark green-brown conidiophores with light green to hyaline conidiogeneus cells. These morphological characters corresponded to the genus *Phaeomoniella* (Crous and Gams, 2000). BLASTn searches showed that the ITS sequences of these isolates had 100% identity with isolates previously identified as *Phaeomoniella chlamydospora* Pach-302 (JQ822210).

Additionally, isolates obtained from the crown area showed colonies with white to off-white or slightly brownish cottony to felty aerial mycelium. Abundant curved macroconidia, up to 6-septate with

obtuse apical and basal cells, were observed. Microconidia were absent. These morphological characters corresponded to the genus *Campylocarpon* (Halleen *et al.*, 2004). In this case, BLASTn searches showed that the BT sequences of these isolates had 98 and 100% identity with isolates of *C. fasciculare* CBS 112611 (AY677225) and *C. pseudofasciculare* CBS 112679 (AY677214), respectively, and the ITS sequences of these isolates had 99% identity with isolates of *C. fasciculare* CBS 113559 (AY677303) and *C. pseudofasciculare* FI2034 (GU198190). Sequences and Blast results of representative isolates of each species derived in this study were lodged in GenBank (Table 2).

These results show the high diversity of fungal trunk pathogens found in table grapes in Northeastern Brazil. These include species of Botryosphaeriaceae (*Botryosphaeria mamane*, *Lasiodiplodia crassispora*, *L. parva*, *L. pseudotheobromae*, *L. theobromae* and *Neofusicoccum parvum*), species belonging to the genera *Campylocarpon* (*C. fasciculare* and *C. pseudofasciculare*) and *Phaeoacremonium* (*Pm. aleophilum* and *Pm. parasiticum*), and *Phaeomoniella chlamydospora*. These are all reported for the first time on grapevine in Brazil, with the exception of *L. theobromae* (Gava *et al.*, 2010), which was the only species present in all production areas surveyed. Moreover, *Botryosphaeria mamane*, *Lasiodiplodia parva* and *L. pseudotheobromae* are reported for the first time on grapevine, and *C. fasciculare* is reported for the first time in the American continent. These species could be distinguished

Table 2. List of fungal trunk pathogens isolated from table grapes in Northeastern Brazil, with their corresponding GenBank accession numbers and data of Blast results obtained from GenBank.

Species	Isolate	GenBank accessions[a]	Blast accessions[b]	Query length	Gaps[c]	Identities[d]	Maximum identity (%)
Elongation factor 1-α gen							
Botryosphaeria mamane	BV1	JX521846	GU134938	440	1/425	424/425	99
Lasiodiplodia crassispora	BV8	JX521847	FJ888452	458	0/443	443/443	100
Lasiodiplodia parva	BV9	JX521848	GQ469904	482	0/464	459/464	99
Lasiodiplodia pseudotheobromae	BV10	JX521849	GQ471796	480	0/462	462/462	100
	BV11	JX521850	GQ471796	485	0/462	462/462	100
	BV12	JX521851	GQ471796	478	0/457	457/457	100
Lasiodiplodia theobromae	BV13	JX521852	GQ469898	481	1/470	467/470	99
	BV14	JX521853	GQ469898	477	1/468	465/468	99
	BV15	JX521854	GQ469898	476	0/467	464/467	99
	BV16	JX521855	GQ469898	488	0/475	473/475	99
	BV17	JX521856	GQ469898	468	0/454	453/454	99
	BV18	JX521857	GQ469898	488	1/476	473/476	99
	BV19	JX521858	GQ469898	472	1/459	456/459	99
	BV20	JX521859	GQ469898	478	1/467	465/467	99
	BV21	JX521860	GQ469898	481	1/470	467/470	99
	BV22	JX521861	GQ469898	483	0/470	468/470	99
Neofusicoccum parvum	BV23	JX521862	FJ900658	457	0/440	435/440	99
	BV24	JX521863	FJ900658	447	0/431	427/431	99

(*Continued*)

Table 2. *Continues.*

Species	Isolate	GenBank accessions[a]	Blast accessions[b]	Query length	Gaps[c]	Identities[d]	Maximum identity (%)
β-tubulin gene							
Campylocarpon fasciculare	BV2	JX521835	AY677225	244	0/242	238/242	98
	BV3	JX521836	AY677225	212	0/212	208/212	98
	BV4	JX521837	AY677225	250	0/247	243/247	98
	BV5	JX521838	AY677225	227	0/227	223/227	98
	BV6	JX521839	AY677225	249	0/249	244/249	98
Campyl. pseudofasciculare	BV7	JX521840	AY677214	215	0/215	215/215	100
Phaeoacremonium aleophilum	BV25	JX521841	JQ691663	651	0/651	650/651	99
	BV26	JX521842	JQ691663	657	0/655	655/655	100
	BV27	JX521843	JQ691663	649	0/649	649/649	100
Phaeoacremonium parasiticum	BV28	JX521844	HQ605022	673	0/673	673/673	100
	BV29	JX521845	HQ605022	694	1/695	694/695	99
Internal Transcribed Spacer region of DNA							
Campylocarpon fasciculare	BV2	JX521864	AY677303	563	0/495	493/495	99
	BV3	JX521865	AY677303	563	0/495	493/495	99
	BV4	JX521866	AY677303	562	0/495	493/495	99
	BV5	JX521867	AY677303	558	0/495	493/495	99
	BV6	JX521868	AY677303	552	0/493	491/493	99
Campyl. pseudofasciculare	BV7	JX521869	GU198190	564	2/510	508/510	99
Phaeomoniella chlamydospora	BV30	JX521870	JQ822210	404	0/404	404/404	100
	BV31	JX521871	JQ822210	383	0/383	383/383	100
	BV32	JX521872	JQ822210	399	0/399	399/399	100

[a] Corresponding GenBank accession numbers of fungal trunk pathogens isolated from table grapes in Northeastern Brazil.
[b] GenBank accession numbers blasted with the isolates obtained in this study.
[c] Number of spaces introduced into the alignment to compensate for insertions and deletions in our sequence relative to blasted sequences.
[d] Number of nucleotides of our sequences/Number of nucleotides of blasted sequences.

based on their DNA sequence data and unique morphological characters.

The most frequently isolated Botryosphaeriaceae sp. was *L. theobromae*, which is an important pathogen of mango (*Mangifera indica* L.) and papaya (*Carica papaya* L.) crops in Northeastern Brazil (Costa *et al*., 2010; Pereira *et al*., 2012). *Neofusicoccum parvum* is also prevalent in this region causing tip dieback and stem-end rot symptoms on mango (Costa *et al*., 2010). Although further research is needed to explain the role that inoculum of Botryosphaeriaceae spp. from non-grapevine hosts surrounding vineyards has on the disease cycle, the wide host range of this family could potentially provide an important source of primary inoculum in table grapes, due to the numerous fruiting bodies produced on non-grapevine hosts (Úrbez-Torres, 2011).

Regarding the genus *Campylocarpon*, it was established in 2004 with two species *C. fasciculare* and *C. pseudofasciculare* associated with black-foot disease

of grapevine in South Africa (Halleen *et al*., 2004). Subsequently *C. pseudofasciculare* and *C. fasciculare* were reported in Uruguay (Abreo *et al*., 2010) and Spain (Alaniz *et al*., 2011), respectively.

Phaeomoniella chlamydospora and *Pm. aleophilum* are species commonly isolated from young vines showing a general decline (Mugnai *et al*., 1999). Numerous other species of the genus *Phaeoacremonium* have also been associated with grapevine decline in grape-growing regions throughout the world, although their importance is thought to be minor (Mostert *et al*., 2006; Essakhi *et al*., 2008; Gramaje *et al*., 2009). One of these species is *Pm. parasiticum*, which in the American continent had already been reported in Argentina, Chile and Peru (Mostert *et al*., 2006; Romero-Rivas *et al*. 2009). It is interesting to note that *Pm. parasiticum*, under its original name *Phialophora parasitica*, was the first species of *Phaeoacremonium* reported to cause phaeohyphomycosis in humans (Ajello *et al*., 1974). This species has also been reported in Brazil causing subcutaneous infections in humans (Guarro *et al*., 2003; Marques *et al*., 2006) and is a good example of the wide substrate range of *Phaeoacremonium* spp. (Mostert *et al*., 2006).

Fungal trunk pathogens are becoming an important problem to the table grape industry in Brazil. Our study improves the knowledge of the fungal species involved in table grape decline in this country and contributes to a better understanding of this complex syndrome under the specific conditions of a developing tropical viticulture.

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NEW OR UNUSUAL DISEASE REPORTS

First report of *Neofusiccoccum vitifusiforme* and presence of other Botryosphaeriaceae species associated with Botryosphaeria dieback of grapevine in Sicily (Italy)

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Summary. Since 2007, when a grapevine decline caused by *Lasiodiplodia theobromae* was reported for the first time in Sicily, vines showing similar declining symptoms have been also found in other grape-growing areas of western and central Sicily. We report the result of a study on fungi associated with grapevine decline in Sicily, with particular regard to those belonging to the *Botryosphaeriaceae*. Four species were found to be associated with declining vines, namely *Diplodia seriata, Lasiodiplodia* sp., *Neofusicoccum parvum* and *Neofusicoccum vitifusiforme*, the latter species reported for the first time on *Vitis vinifera* in Italy.

Key words: *Diplodia seriata*, *Lasiodiplodia* sp., *Neofusicoccum parvum*, *Vitis vinifera*, grapevine trunk disease.

Introduction

Species in the Botryosphaeriaceae Theiss & P. Syd. are cosmopolitan and have been reported as endophytes, parasites, and saprophytes on a broad range of both annual and perennial hosts (Barr, 1972; Punithalingam, 1980; von Arx, 1987; Smith *et al*., 1996; Burgess *et al*., 2005; Slippers and Wingfield, 2007) including grapevines (Chamberlain *et al*., 1964; Lehoczky, 1974, 1988; Hewitt, 1988; Leavitt, 1990, Úrbez-Torres, 2011). Investigations conducted during the last decade in different countries have shown that actually several species of Botryosphaeriaceae have a pathogenic role in *Vitis vinifera* causing trunk diseases (Phillips, 2002; van Niekerk *et al*., 2004, 2006; Taylor *et al*., 2005; Úrbez-Torres *et al*., 2008; Úrbez-Torres and Gubler, 2009; Linaldeddu *et al*., 2010; Pitt *et al*., 2010), recently reported as Botryosphaeria dieback (Úrbez-Torres, 2011). Geographical distribution of some Botryosphaeriaceae species has been shown to be associated with climate. In particular, *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. is the prevalent species in warmer grape-growing areas of many countries such as Australia (Taylor *et al*., 2005; Pitt *et al*., 2010), California, Arizona, Mexico (Úrbez-Torres *et al*., 2006, 2008), Egypt (El-Goorani and El Meleigi, 1972), Spain (Aroca *et al*., 2008) and also in Italy (Burruano *et al*., 2008).

Among the 21 Botryosphaeriaceae reported to be associated with decline symptoms on grapevine (Úrbez-Torres, 2011), only five species have been to date recognized in Italy on *V. vinifera*: *Botryosphaeria dothidea* in Apulia (Carlucci *et al*., 2009), Marche (Romanazzi *et al*., 2009) and Central Italy (Spagnolo *et al*., 2011); *Diplodia seriata* in Molise (Cristinzio, 1978), Apulia (Pollastro *et al*., 2000) and Central Italy (Spagnolo *et al*., 2011); *L. theobromae* in Sicily (Burruano *et al*., 2008) and Apulia (Carlucci *et al*., 2009); *Neofusicoccum australe* in Sardinia (Linaldeddu *et al*., 2010) and *Neofusicoccum parvum* in Apulia (Carlucci *et al*., 2009) and Central Italy (Spagnolo *et al*., 2011). These

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species are associated with grapevine decline symptoms, as late sprout and or dead buds, sub-cortical brown streaking and wedge-shaped wood necrosis, and with "esca" symptoms. After the first report of Botryosphaeria dieback in a vineyard in West Sicily by Burruano *et al*. (2008), similar grapevine decline symptoms were also observed in other Sicilian grape-growing areas. Thus, the aim of the present study was to ascertain the occurrence and identity of the Botryosphaeriaceae species associated with Botryosphaeria dieback in Sicily.

Materials and methods

Over the four years (2008–2011) since the first report on grapevine decline in Marsala (Trapani), declining plants were gradually detected and some of these collected in different grape-growing areas in Sicily: 3 plants cultivar Insolia in Marsala, annually from 2008–2010, and in 2010–2011, 2 plants cv. Merlot in Salemi (Trapani), 2 cv. Grillo in Alcamo (Trapani), 2 cv. Alicante Bouchet in Montevago (Agrigento) and 2 cv. Insolia in Milena (Caltanisetta).

Sample collection and fungal isolation

In the summer of each year (July–September), declining grapevines were collected and sectioned in several portions both to detect and describe wood symptoms and for isolations. Symptomatic wood portions, after bark removal, were further cut into disks, about 2–3 cm in diameter, which were flame sterilized. Fragments at the margin between the healthy and affected tissue were excised aseptically and plated on 2% malt-extract agar (MEA, Oxoid, Milan, Italy). Petri dishes were kept at 25±1°C in the dark and examined daily for fungal development during two weeks. The isolation frequency (IF) of each fungal genus was calculated using the formula: IF = (Nif/Ntf)×100, where Nif is the number of colonies of a given fungus, and Ntf the total number of isolations attempted ×100 (Ragazzi *et al*., 2001).

The Botryosphaeriaceae isolates, which were selected on the basis of gross colony morphology, were transferred to PDA (Oxoid) and incubated until fungal spore production. Identification of fungal species was based on the morphology of monosporic colonies and conidial characters. Conidia (100) were observed and measured at 40× magnification with a light microscope equipped with an HRc Axiocam digital camera and accompanying software (Carl Zeiss Ltd, Germany).

The optimum growth temperature of three isolates of each morphologically characterized species was determined by incubating the cultures in the dark at temperatures ranging from 5 to 40°C at 5°C intervals, with three replicates per temperature. For each colony two orthogonal diameters were measured after 2 and 3 days, and the colony diameter was expressed as mean radial growth in millimeters (Úrbez-Torres *et al*., 2008).

Molecular identification

Fungal genomic DNA of all isolates was extracted from monosporic cultures following a standard CTAB-based protocol (O'Donnell *et al*., 1998). Internal Transcribed Spacer (ITS) regions, ITS1 and ITS2, including the 5.8S gene, of the ribosomal DNA (rDNA) operon, were amplified with the primers ITS1-F (Gardes and Bruns, 1993) and ITS4 (White *et al*., 1990). Part of the translation elongation factor 1-alpha (EF1-α) gene was amplified with the primers EF1-728F and EF1-986R (Carbone and Kohn, 1999). The primers Bt2a and Bt2b (Glass and Donaldson, 1995) were used to amplify a portion of the β-tubulin (BT) gene. The PCR reactions were performed following the PCR protocol described by Slippers *et al*. (2004). The ITS-RFLP technique was applied to identify groups among the collected isolates and to select representative isolates for sequencing. The PCR amplicons of the ITS regions were digested separately with *Cfo*I and *Hae*III restriction endonucleases (Slippers *et al*., 2007) following the manufacturer's instructions (Fermentas, Milan, Italy). The digestion reaction was incubated at 37°C overnight. The resulting restriction fragments were separated by electrophoresis on 2% (w/v) agarose gel and then molecular weights determined. Isolates for which identical RFLP patterns were obtained with both endonucleases were considered to belong to the same RFLP group. ITS, EF1-α and BT regions of a representative isolate of each RFLP group were sequenced in both directions using the same primers as for PCR reactions. Nucleotide sequences were compared to GenBank sequences through BLASTn searches.

Pathogenicity

Six-month-old shoots, 8–10 mm in diameter and 30 cm long, were collected from healthy, mature In-

solia grapevines, the leaves and tendrils removed, and the shoots surface sterilized with 70% ethanol. Four isolates of each species were used and inoculated onto three shoots. The shoots were first wounded, 10–15 cm from the apex, by removing the bark with a sterile scalpel. A 6-mm-diam. plug from of a 7-day-old colony on PDA was then placed on each wound and immediately covered with Parafilm. Control shoots were inoculated with non-colonised plugs of PDA. All inoculated shoots, were placed into a 3-cm-diameter tubes containing 200 mL of tap water, and covered with plastic bags to maintain humidity. After 21 days at 25°C with natural light, each shoot was evaluated for the length of vascular discoloration around the point of inoculation. Data were submitted to ANOVA and to the Student's *t*-test. Re-isolations from symptomatic tissues were carried out on PDA to fullfill Koch's postulates.

Results and discussion

Declining grapevines observed in the five vineyards of West and Central Sicily showed in field late sprouting and/or mortality bud, delayed growth, cankers and dieback. In particular, bud and canopy symptoms were more evident in spring, since as the season progressed the grapevines seemed to recover their vegetative growth. Longitudinal sections of symptomatic samples always showed, along the whole length or nearly so of the trunk, brown wood necrosis (Figure 1a) that was often wedge-shaped

Figure 1. Symptoms of *Botryosphaeria* dieback observed in the trunk of a grapevine: a) sub-cortical, dark longitudinal bands observed when bark has been removed and b) in cross section, often arc-shaped.

Table 1. Number of isolates and isolation frequency (IF) of the Botryosphaeriaceae species and other fungal taxa obtained from symptomatic wood of diseased grapevines in Sicily.

Fungal *taxa*	No. of isolates (IF) per vineyard					Total
	Alcamo	Marsala	Milena	Montevago	Salemi	
Diplodia seriata	47 (23.5)	7 (5.6)	153 (76.5)	4 (2.0)	89 (44.5)	300 (24.0)
Lasiodiplodia sp.	--	197 (43.8)	--	--	38 (18.9)	235 (18.8)
Neofusicoccum parvum	--	25 (1.6)	--	--	--	25 (2.0)
Neofusicoccum vitifusiforme	--	--	--	114 (57.0)	--	114 (9.1)
Total No. of Botryosphaeriaceae	47 (23.5)	229 (50.9)	153 (76.5)	118 (59.0)	127 (63.5)	670 (53.6)
Penicillium	18 (9.0)	69 (15.3)	59 (15.3)	32 (16.0)	15 (7.5)	193 (15.4)
Aspergillus	23 (11.5)	30 (6.7)	6 (3.5)	17 (8.5)	5 (2.5)	81 (6.4)
Alternaria	25 (12.5)	10 (2.2)	7 (3.5)	6 (3.0)	15 (7.5)	63 (5.0)
Cladosporium	5 (2.5)	7 (1.6)	8 (4.0)	8 (4.0)	4 (2.5)	32 (2.6)
Fomitiporia	--	--	23 (11.5)	--	--	23 (1.8)
Fusarium	6 (3.0)	--	--	--	--	6 (0.4)
Acremonium	4 (2.0)	--	--	--	--	4 (0.3)
Phoma	4 (2.0)	--	--	--	--	4 (0.3)
Rhizopus	--	3 (0.7)	--	--	--	3 (0.2)
Total No. of fungal isolates	132	348	256	181	166	1079
No. of wood fragments	200	450	200	200	200	1250

in cross section (Figure 1b). In addition, sub-cortical longitudinal brownish discolored bands were often detected (Figure 1c). Only in Milena and Salemi samples, white rot starting from pruning wounds spreading along the trunk was also observed.

From the symptomatic wood of all sampled grapevines, a total of 1079 fungal colonies were isolated (Table 1). Among these, Botryosphaeriaceae species predominated (670 isolates) with IF ranging between 23.5% (Alcamo samples) and 76.5% (Milena samples). Saprophytic and wood contaminant fungi belonging to *Penicillium*, *Aspergillus* and *Alternaria* were also present, while colonies of *Acremonium*, *Cladosporium*, *Fusarium*, *Phoma*, *Rhizopus* and *Fomitiporia mediterranea* were sporadically observed. Contrary to what is often reported by other authors (Pollastro *et al*., 2000; van Niekerk *et al*., 2006; Úrbez-Torres *et al*., 2006; Pitt *et al*., 2010), other causal agents of grapevine decline such as *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp. and *Eutypa lata* were never isolated.

On the basis of colony and conidial morphology, and optimum growth temperature, Botryosphaeriaceae isolates were classified into four groups (Table 2), which were supported by the ITS-RFLP profiles (Figure 2). BLASTn searches of the ITS sequences of the four selected isolates showed a high homology with *D. seriata* (99%), *L. theobromae* (98%), *N. parvum* (99%) and *Neofusicoccum vitifusiforme* (99%) (Table 3). Sequences from EF1-α and β-tubulin gene regions were used to confirm identification that could not be clearly resolved with ITS sequence. The comparison of sequence data of both EF1-α and β-tubulin with those in GenBank confirmed the identification based on ITS sequences, with the exception of *L. theobromae*. Since a phylogenetic analysis is in progress in order to distinguish the two potential cryptic species in *L. theobromae* (Alves *et al*., 2008), we report this species as *Lasiodiplodia* sp.

Among these four species, *D. seriata* was associated with all samples from various grapevine growing

Table 2. Morphological features of the four groups of Botryosphaeriaceae isolated in this study.

No. group	No. isolates	Colony features	Mature conidia: Colour and shape	Mature conidia: Septa	Dimension (µm)[a]: Lenght	Dimension (µm)[a]: Width	Opt. growth T (°C)	Identity
1	296	Dense grey-brown aerial mycelium	Brown, cylindrical to ellipsoid	no	(17–)20–22(-26.5)	(8.5–)10–11(–13)	30	*Diplodia seriata*
2	235	Abundant aerial mycelium that became dark green with age	Hyaline becoming dark brown, oblong with irregular longitudinal striations	1	(15–)19–22(–27)	(8.5–) 9–12-(16.5)	35	*Lasiodiplodia* sp.
3	25	Mycelium dull gray	Thin-walled, fusiform or ellipsoidal olivaceous or light brown	1–2	(12.5–)13–18(–23)	(5.0)6–7(–10)	30	*Neofusicoccum parvum*
4	114	Aerial mycelium that became grey whit age	Hyaline, fusoid to ellipsoid, widest in upper third, apex obtuse, base flattened and sub-truncate	no	(18–)20–21.4(–22)	(4.5–)5.0–5.6(–7)	30	*Neofusicoccum vitifusiforme*

[a] Minimum and maximum and dimensions in parentheses.

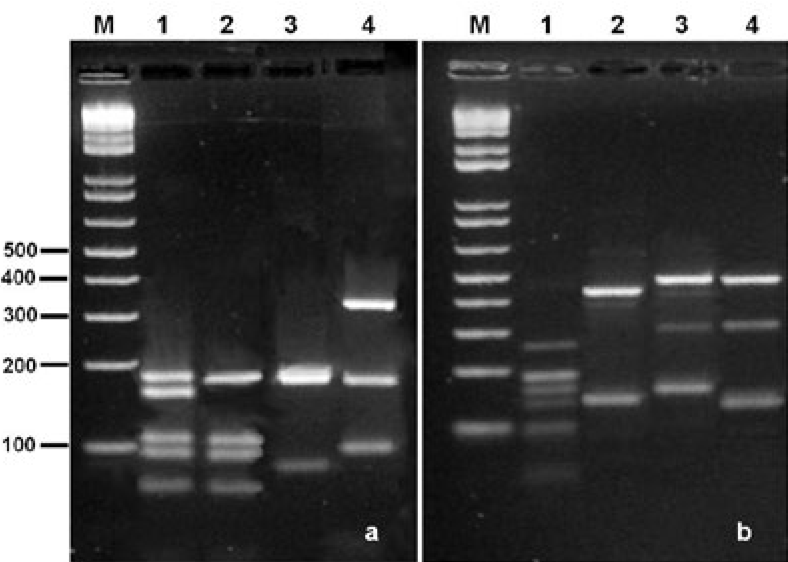

Figure 2. Restriction profiles with *Cfo*I (a) and *Hae*III (b) of the ITS PCR products of *N. parvum* (lane 1), *N. vitifusiforme* (lane 2), *D. seriata* (lane 3) and *Lasiodiplodia* sp. (lane 4). M, 1 Kb plus DNA ladder (Invitrogen).

Table 3. Molecular identification and GenBank accession numbers of the representative isolates of the *Botryosphaeriaceae* species isolated in this study.

Isolate	Molecular identification	DNA target[a]	GenBank accession No.	Blast match sequence		
				Reference accession No.[b]	Coverage (%)	Identity (%)
B4	*Lasiodiplodia* sp.	ITS	JN251118	*L. theobromae* AY640255	100	98
		EF1-α	--	--	--	--
		β -tubulin	--	--	--	--
B8	*Neofusicoccum vitifusiforme*	ITS	KC469638	*N. vitifusiforme* **AY343383**	87	99
		EF1-α	KC884948	*N. vitifusiforme* **AY343343**	99	99
		β -tubulin	KC884951	*N. vitifusiforme* HM176500	97	99
B19	*Neofusicoccum parvum*	ITS	JN251119	*N. parvum* **AY236943**	95	99
		EF1-α	KC884949	*N. parvum* **AY236888**	100	99
		β -tubulin	KC884952	*N. parvum* **AY236917**	96	99
B25	*Diplodia seriata*	ITS	JN251120	*D. seriata* **AY259094**	100	99
		EF1-α	KC884950	*D. seriata* **AY573220**	99	99
		β -tubulin	KC884953	*D. seriata* **DQ458856**	98	99

[a] ITS, internal transcribed spacer; EF1-α, elongation factor.
[b] Accession numbers of the ex-type or ex-epitype strains are in bold.

Figure 3. Discolouration on excised shoots of *V. vinifera* cv. Insolia 21 days after artificial inoculation with a) *D. seriata,* b) *Lasiodiplodia* sp., c) *N. parvum* and d) *N. vitifusiforme*. Arrows indicate the point of inoculation. The shoot in e) was wounded but was not inoculated with a fungus.

Table 4. Mean lesion length on excised grapevine green shoots cv. Insolia 21 days after artificial inoculation with Botryosphaeriaceae species.

Inoculum	Mean lesion length (cm) ±SE [a]	Re-isolation[b]
Diplodia seriata	4.9 ±2.0 b	100
Lasiodiplodia sp.	14.8 ±1.8 b	100
Neofusicoccum parvum	5.6 ±3.6 b	100
Neofusicoccum vitifusiforme	4.5 ±2.8 b	100
Control	1.1 ±0.1 a	0

[a] Values are means of three replicates per treatment. SE= standard error of the mean. Equal letters refers to not significantly different values (Student *t*-test; $P>0.05$).
[b] Reisolation percentage.

areas (Table 1). In particular, it was the only species isolated from the sub-cortical longitudinal brown bands and, exclusively in those of Alcamo (23.5%) and Milena (76.5%), also from the wood necrosis. This species was also obtained from wood necrosis of grapevines in Marsala, Montevago and Salemi, but not singularly and in different percentages (5.6, 2.5 and 44.5, respectively). *Lasiodiplodia* sp., on the contrary, was obtained both from Marsala and Salemi samples, with IF of 43.8% and 18.9%, respectively. *N. parvum* was only and sporadically isolated from Marsala grapevines (IF 1.6%), while *N. vitifusiforme,* from Montevago samples, with a high isolation percentage (57%).

With regard to the preliminary tests of pathogenicity, the inoculated fungi caused vascular discoloration, extending differently both upward and downward from the inoculation point, in the sampled shoots (Figure 3). However, the length of vascular discolorations was very variable both between the different species and within each species. The control shoots developed only a slight discolouration only around the wound site. ANOVA analysis showed significant differences in the extent of vascular discoloration between control and inoculated shoots, but no significant differences between Botryosphaeriaceae species (Table 4). The inoculated fungi were always reisolated from the inoculated canes and no botryosphaeriaceous fungi were reisolated from the control. These assays, even if with low number of replicates, allowed to ascertain the pathogenicity of the assayed Botryosphaeriaceae species on *V. vinifera.*

Our results show several Botryosphaeriaceae species associated with Botryosphaeria dieback in Sicily, as well as already observed in other countries (Úrbez-Torres *et al*., 2006; Candolfi-Arballo, *et al*., 2010; Pitt *et al*., 2010; Úrbez-Torres, 2011); this could be due to environmental conditions of every grape-growing area.

Regarding *N. vitifusiforme*, reported in *V. vinifera* for first the time in South Africa (van Niekerk *et al*., 2004), is now frequently associated with Botryosphaeria dieback in several grape-growing areas worldwide: Spain (Luque *et al*., 2009), New Mexico (Candolfi-Arballo, *et al*., 2010) and USA (Úrbez-Torres, 2011). The occurrence of *N. vitifusiforme* on *Olea europaea* in South Italy (Lazzizzera *et al*., 2008) shows the capability of this species to colonize different hosts, and may confirm the hypothesis of the role of secondary host as a inoculum source for grapevine trunk disease pathogens (Cloete *et al*., 2011). At present, *N. vitifusiforme* has been also recently reported as pathogen in fruit trees: *Prunus* spp. (Damm *et al*., 2007), *Malus* and *Pyrus* spp. (Cloete *et al*., 2011), *O. europaea* (Lazzizzera *et al*., 2008; Úrbez-Torres *et al*., 2012) and in blueberry (*Vaccinium corymbosum,* Kong *et al*., 2010), mostly associated with host wood necrosis. To date, this is the first report of *N. vitifusiforme* in *V. vinifera* in Italy.

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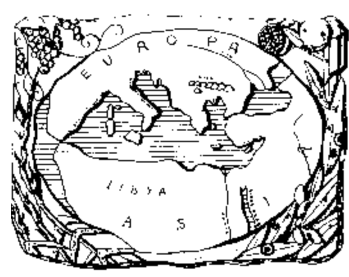

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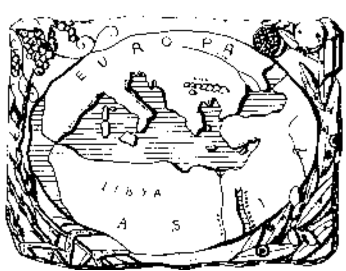

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