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Studies on the epidemiology of black-foot disease of grapevine in Spain

Carlos Agustí Brisach

Director:

Dr. Josep Armengol Fortí

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Summary

Since the early 1990s, an important decrease in the survival rate of grafted grapevines in nurseries and young vineyards has been noted worldwide. Fungi involved in wood decay are among the most destructive pathogens either infecting grapevine propagation material, newly planted vines and mature established vineyards. Among the grapevine trunk diseases, a serious disease in most wine and grape-producing regions of the world, particularly in nurseries and young vineyards, is black-foot disease. The causal agents of this disease are included into the genera *Campylocarpon*, "*Cylindrocarpon*", *Cylindrocladiella* and *Ilyonectria*. It is well known that these pathogens are common in the soil and it has been demonstrated that nursery field sites can harbor them, causing infection of grafted vines after some months of growth in nursery soils. Nevertheless, the presence of black-foot disease pathogens in grapevine nurseries, as well as the potential inoculum sources of these pathogens in soils from nurseries and commercial vineyards, has not been explored in Spain. Thus, the main objective of this Thesis has been to study the epidemiology of black-foot disease in Spain.

Firstly, the different stages in the propagation of grapevine in Spanish nurseries were evaluated as potential sources of black-foot disease pathogens. To this end, samples were taken from four sources of the propagation process: pre-grafting hydration tanks, scissors used for cutting buds, omega-cut grafting machines and peat used for callusing. DNA from these samples was extracted and a multiplex nested-PCR with primers specific for "*Cylindrocarpon*" *pauciseptatum*, *Ilyonectria liriodendri* and *I. macrodidyma*-complex was used to identify the species present. *Ilyonectria liriodendri* and *I. macrodidyma*-complex were detected at different stages of the grapevine propagation process. Additionally, the detection of *Ilyonectria* spp. was also studied by multiplex, nested PCR and by isolation and genotyping in the grapevine planting material before and after the rooting phase in nursery fields. We confirmed that during the rooting phase in nursery fields the number of plants infected with black-foot pathogens increases markedly. By isolation on culture media, only one *I. torresensis* isolate was obtained from one of the cuttings sampled immediately after callusing. However, after one growing season in nursery fields, *I. liriodendri*, *I. novozelandica* and *I. torresensis* were more frequently isolated from rooted plants. Regarding the molecular detection of *Ilyonectria* spp. on grafted cuttings and plants, a greater number of positive samples were found before and after the rooting phase in nursery fields.

The soil of grapevine rootstock mother fields was evaluated as a potential inoculum source of black-foot disease pathogens by using four different techniques: fungal isolation from roots of grapevine seedlings used as bait plants; fungal isolation from roots of weeds; multiplex, nested PCR and qPCR. Four *Ilyonectria* spp., named *I. alcacerensis, I. macrodidyma*, *I. novozelandica* and *I. torresensis*, were isolated from the roots of bait plants grown in a rootstock mother field. "*Cylindrocarpon" macrodidymum* was also commonly isolated from weeds collected in rootstock mother fields showing a high rate of isolation. The analysis of soils collected from rootstock mother fields with the multiplex, nested PCR as well as with qPCR showed a high rate of detection of *I. macrodidyma*-complex from soil DNA samples, while the rate of detection of *I. liriodendri* was markedly lower in the same DNA samples.

Then, the contribution of soils of nursery fields as well as commercial vineyards in increasing the infections caused by black-foot pathogens on grapevine cuttings during the rooting phase in nurseries or in new plantations was also investigated. To this aim we used the same techniques described before. *Ilyonectria alcacerensis*, *I. macrodidyma*, *I. novozelandica* and *I. torresensis*, were isolated from the roots of bait plants grown in nursery fields. In addition, "*Cylindrocarpon" macrodidymum* was also commonly isolated from weeds collected in nursery fields showing a high rate of isolation. The results obtained with the multiplex, nested PCR as well as with the qPCR showed a high rate of detection of *I. macrodidyma*-complex from soil DNA samples collected in nursery fields, being the rate of detection of *I. liriodendri* markedly lower in the same DNA samples. Regarding the soil of commercial vineyards, three *Ilyonectria* spp., named *I. alcacerensis, I. novozelandica* and *I. torresensis*, were isolated from the roots of bait plants grown in pots filled with soils sampled from ten different commercial vineyards. "*Cylindrocarpon*" *macrodidymum* was also frequently isolated from weeds collected in several commercial vineyards.

It is interesting to note that in all soil types: roostock mother fields, nursery fields and commercial vineyards, species belonging to *I. macrodidyma*-complex were the most frequently detected.

Finally, the effects of temperature, pH and water potential (Ψ_s) on mycelial growth, sporulation and chlamydospore production of "*C.*" *liriodendri*, "*C.*" *macrodidymum* and "*C.*" *pauciseptatum* were evaluated in order to provide further information on factors affecting growth, reproductive and survival of these pathogens. 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 over a range of pHs, from 4 to 8. Regarding the effect of Ψ_s, in general, mycelial growth was greater on amended media at -0.5, -1.0 or/and -2.0 MPa compared with that obtained on nonamended PDA (-0.3 MPa), and was reduced at Ψ_s values lower than -2.0 MPa. Most of the "*Cylindrocarpon*" spp. isolates sporulated at all temperatures, pHs and water potentials tested. In all studied conditions, "*C.*" *liriodendri* had the greatest sporulation capacity compared with "*C.*" *macrodidymum* and "*C.*" *pauciseptatum.* In general, chlamydospore production was not much affected by temperature, pH and Ψ_s. Chlamydospores were observed in PDA cultures of all isolates at all pH values studied, while some isolates did not produce chlamydospores at 5 and 10ºC or -4.0 and/or -5.0 MPa.

Additionally, in this Thesis, pathogens associated with Petri disease of grapevines were also detected on bait plants and weeds. *Cadophora luteo-olivacea*, *Phaeoacremonium aleophilum*, *Pm. parasiticum* and/or *Phaeomoniella chlamydospora* were isolated from xylem vessels of bait plants grown both in a rootstock mother field and a nursery field as well as from weeds collected in rootstock mother fields, nursery fields and commercial vineyards, confirming soil and weeds as inoculum sources of these pathogens.

Resumen

Desde principios de los años 1990, se viene observando una elevada mortalidad de plantas jóvenes de vid, tanto en vivero como en plantaciones jóvenes, en todas las áreas vitivinícolas del mundo. Las enfermedades de la madera se encuentran entre las patologías más dañinas que afectan al cultivo de la vid. De entre ellas, el pie negro es una de las más destacadas, afectando principalmente a las plantas en vivero y en plantaciones jóvenes. Los agentes causales de esta enfermedad están incluidos dentro de los géneros *Campylocarpon*, "*Cylindrocarpon*", *Cylindrocladiella* e *Ilyonectria*. Los hongos incluidos en estos géneros se caracterizan por ser habitantes comunes del suelo y, en los últimos años, se ha demostrado que pueden permanecer en él, infectando al material de propagación cultivado en los campos de vivero durante la fase de enraizamiento. Sin embargo, la presencia de hongos asociados al pie negro de la vid en vivero, así como sus fuentes potenciales de inóculo tanto en suelos de vivero como en suelos de viñedos comerciales, no han sido nunca estudiados en España. En este sentido, el principal objetivo de esta Tesis ha sido estudiar la epidemiología de hongos que causan el pie negro de la vid en España.

En primer lugar, las distintas fases del proceso viverístico se evaluaron como fuentes potenciales de inóculo de hongos que causan el pie negro de la vid. Para ello, se tomaron muestras en cuatro fases del proceso de propagación: balsas de hidratación, tijeras, máquinas injertadoras y turba utilizada para la inducción del callo. Posteriormente, se extrajo el ADN de estas muestras, detectándose las especies causantes del pie negro de la vid mediante multiplex, nested PCR utilizando tres pares de cebadores específicos para *I. liriodendri*, el complejo *I. macrodidyma* y "*C.*" *pauciseptatum*. En las distintas fases estudiadas se detectaron *I. liriodendri* y el complejo *I. macrodidyma*. Además, también se estudió la detección de especies de *Ilyonectria* en material de propagación de vid, antes y después de la fase de enraizamiento en campos de vivero, mediante técnicas de aislamiento y multiplex, nested PCR. Este estudio confirmó que el número de plantas infectadas con especies asociadas al pie negro de la vid aumenta considerablemente durante el proceso de enraizamiento en los campos de vivero. *Ilyonectria torresensis* fue la única especie que se aisló de las plantas injertadas después de la fase de inducción del callo. Sin embargo, las especies *I. liriodendri*, *I. novozelandica* e *I. torresensis* se aislaron frecuentemente de las raíces de las plantas tras el período de cultivo en los campos de vivero. Respecto

a la detección molecular, se detectaron un número elevado de muestras positivas tanto en planta injertada tras la inducción del callo como después del proceso de enraizamiento en campo de vivero.

Mediante el uso de cuatro técnicas diferentes, aislamiento fúngico a partir de raíces de plántulas de vid obtenidas de semilla que se utilizaron como plantas trampa; aislamiento a partir de raíces de malas hierbas; multiplex, nested PCR y qPCR, se estudió el suelo de campos de plantas madre de viña como posible fuente de inóculo de hongos asociados al pie negro de la vid. A partir de raíces de plantas trampa cultivadas en campos de plantas madre se aislaron cuatro especies de *Ilyonectria*: *I. alcacerensis*, *I. macrodidyma*, *I. novozelandica* e *I. torresensis*. "*Cylindrocarpon*" *macrodidymum* fue la única especie que se aisló de las raíces de malas hierbas recogidas en campos de plantas madre, mostrando un elevado porcentaje de aislamiento. En los análisis de suelos de campos de plantas madre realizados mediante multiplex, nested PCR así como mediante qPCR se observó un elevado porcentaje de detección del complejo *I. macrodidyma* en muestras de ADN de suelo, mientras que el porcentaje de detección de *I. liriodendri* fue mucho menor.

Las mismas técnicas descritas para campos de plantas madre se utilizaron para estudiar los suelos de campos de vivero y de viñedos comerciales. En campos de vivero, los resultados obtenidos mediante el uso de plantas trampa fueron similares a los obtenidos en campos de plantas madre. En este caso, de las raíces de las plantas trampa se aislaron las especies *I. alcacerensis*, *I. macrodidyma*, *I. novozelandica* e *I. torresensis*. Además, "*Cylindrocarpon*" *macrodidymum* también se aisló con elevada frecuencia de las raíces de malas hierbas recogidas en campos de vivero. En los análisis de suelos de campos de vivero realizados mediante multiplex, nested PCR, así como mediante qPCR, se observaron resultados muy parecidos a los obtenidos en campos de plantas madre, detectándose frecuentemente el complejo *I. macrodidyma* en muestras de ADN de suelo, mientras que la frecuencia de detección de *I. liriodendri* fue mucho menor. Respecto al suelo de campos comerciales, las especies *I. alcacerensis*, *I. novozelandica* e *I. torresensis* se aislaron de raíces de plantas trampa cultivadas en macetas conteniendo suelos procedentes de diez viñedos comerciales. "*Cylindrocarpon*" *macrodidymum* también se aisló con elevada frecuencia a partir de raíces de malas hierbas recogidas en viñedos comerciales, mostrando un elevado porcentaje de aislamiento

Es importante destacar que las especies comprendidas dentro del complejo *I. macrodidyma* fueron las que se aislaron con mayor frecuencia en todos los tipos de suelos estudiados: suelos de campos de plantas madre, de campos de enraizamiento y de campos comerciales.

Finalmente, se estudió el efecto de la temperatura, pH y potencial osmótico (Ψ_s) sobre el crecimiento miceliar, la esporulación y la producción de clamidosporas de "*C.*" *liriodendri*, "*C.*" *macrodidymum* y "*C.*" *pauciseptatum*, con el objetivo de mejorar el conocimiento de los factores que afectan al crecimiento, reproducción y supervivencia de estos patógenos. Todos los aislados estudiados fueron capaces de crecer en un rango de temperaturas comprendido entre 5 y 30ºC, con un óptimo de temperatura entre 20 y 25ºC. También se observó crecimiento miceliar en un rango de pH comprendido entre 4 y 8. Respecto al efecto del Ψ_s, en general, el crecimiento miceliar fue mejor en medio de cultivo PDA ajustado a -0,5, -1,0 y/o -2,0 MPa en comparación con el crecimiento miceliar observado en PDA sin ajustar a ningún Ψ_s (-0,3 MPA), reduciéndose a valores de Ψ_s por debajo de -2,0 MPa. La mayoría de los aislados de "*Cylindrocarpon*" esporularon a todas las temperaturas, pHs y valores de Ψ_s estudiados. "*Cylindrocarpon*" *liriodendri* mostró una mayor capacidad de esporulación en comparación con "*C.*" *macrodidymum* y "*C.*" *pauciseptatum* en todas las condiciones estudiadas. En general, la producción de clamidosporas no se vio afectada por la temperatura, el pH y el Ψ_s. A todos los valores de pH estudiados se observaron clamidosporas en todos los aislados cultivados en PDA, mientras que a 5 y 10ºC o a -4,0 y/o -5,0 MPa algunos de ellos no produjeron clamidosporas.

Además, en esta Tesis, también se detectaron algunas de las especies que causan la enfermedad de Petri en vid a partir de los aislamientos realizados en plantas trampa y en malas hierbas. Las especies *Cadophora luteo-olivacea*, *Phaeoacremonium aleophilum*, *Pm. parasiticum* y/o *Phaeomoniella chlamydospora* se aislaron a partir de los tejidos xilemáticos de las plantas trampa cultivadas tanto en los campos de plantas madre como en los campos de vivero, así como a partir de los tejidos xilemáticos de malas hierbas recogidas en campos de plantas madre, campos de vivero y viñedos comerciales.

Resum

Des de principis dels anys 1990, s'ha constatat una alta mortalitat de plantes joves de vinya, tant en vivers com en plantacions joves, en totes les àrees vitivinícoles del món. Les malalties de la fusta es troben entre les patologies més greus que afecten al cultiu de la vinya. De entre elles, el peu negre és una de les més destacades, afectant principalment a les plantes en el viver i en plantacions joves. Els agents causals d'aquesta malaltia estan inclosos dins dels gèneres *Campylocarpon*, "*Cylindrocarpon*", *Cylindrocladiella* i *Ilyonectria*. Els fongs inclosos en aquestos gèneres es caracteritzen per ser habitants del sol i, durant els últims anys, s'ha demostrat que poden romandre en ell, infectant el material de propagació cultivat en els camps de viver durant la fase de enrailament. No obstant això, la presència de fongs associats al peu negre de la vinya en viver, així com les seves fonts potencials d'inòcul, tant en sols de viver com en sols de vinya comercials, no han estat mai estudiats a Espanya. En este sentit, el principal objectiu d'aquesta Tesi ha estat estudiar l'epidemiologia de fongs que causen el peu negre de la vinya a Espanya.

En primer lloc, les diferents fases del procés viverístic van ser avaluades com a fonts potencials d'inòcul de fongs que causen el peu negre de la vinya. Per a això, es van prendre mostres en quatre fases del procés de propagació: basses d'hidratació, tisores, màquines empeltadores i torba utilitzada per a la inducció del call. Posteriorment, es va extraure el ADN d'aquestes mostres, detectant les espècies causants del peu negre de la vinya mitjançant multiplex, nested PCR, utilitzant tres parells de encebadors específics per a *I. liriodendri*, el complex *I. macrodidyma* i "*C.*" *pauciseptatum*. En les diferents fases estudiades es detectaren *I. liriodendri* i el complex *I. macrodidyma.* A més a més, també es va estudiar la detecció d'espècies d'*Ilyonectria* en material de propagació de vinya, avanç i després de la fase d'enrailament en camps de viver, mitjançant tècniques d'aïllament i multiplex, nested PCR. Aquest estudi confirma que el nombre de plantes infectades amb espècies associades al peu negre de la vinya augmenta considerablement durant el procés d'enrailament en els camps de viver. No obstant això, les espècies *I. liriodendri*, *I. novozelandica* i *I. torresensis* s'aïllaren freqüentment de les arrels de les plantes després del període de cultiu en els camps de viver. Respecte a la detecció molecular, es detectaren un nombre elevat de mostres positives tant en planta empeltada després de la inducció del call com després del procés d'enrailament en camp de viver.

Mitjançant l'ús de quatre tècniques diferents, aïllament fúngic a partir d'arrels de plàntules de vinya obtingudes de llavor que es van utilitzar com a plantes parany; aïllament a partir d'arrels de males herbes; multiplex, nested PCR i qPCR, es va estudiar el sol de camps de plantes mare de vinya com a possible font d'inòcul de fongs associats al peu negre de la vinya. A partir d'arrels de plantes parany cultivades en camps de plantes mare s'aïllaren quatre espècies d'*Ilyonectria*: *I. alcacerensis, I. macrodidyma, I. novozelandica* i *I. torresensis*. "*Cylindrocarpon*" *macrodidymum* fou la única espècie que es va aïllar de les arrels de males herbes recollides en camps de plantes mare, mostrant un elevat percentatge d'aïllament. En els anàlisis de sols de camps de plantes mare realitzats mitjançant multiplex, nested PCR, així com mitjançant qPCR s'observà un elevat percentatge de detecció del complex *I. macrodidyma* en mostres de ADN de sol, mentre que el percentatge de detecció de *I. liriodendri* fou molt menor.

Aquestes mateixes tècniques descrites per a camps de plantes mare es van utilitzar per a estudiar els sols de camps de viver i camps de vinya comercials. En camps de viver, els resultats obtinguts mitjançant l'ús de plantes parany foren similars als obtinguts en camps de plantes mare. En aquest cas, de les arrels de les plantes parany s'aïllaren les espècies *I. alcacerensis*, *I. macrodidyma, I. novozelandica* i *I. torresensis*. A més a més, "*Cylindrocarpon*" *macrodidymum* també s'aïllà amb elevada freqüència de les arrels de males herbes recollides en camps de viver, mostrant un elevat percentatge d'aïllament. En el anàlisi de sols de camps de viver realitzats mitjançant multiplex, nested PCR, així com mitjançant qPCR, s'observaren resultats molt pareguts als obtinguts en camps de plantes mare, detectant freqüentment el complex *I. macrodidyma* en mostres de ADN de sol, mentre que la freqüència de detecció d'*I. liridodendri* fou molt menor. Respecte al sol de camps comercials, les espècies *I. alcacerensis, I. novozelandica* i *I. torresensis* s'aïllaren d'arrels de plantes parany cultivades en test contenint sols procedents de deu camps de vinya comercials. "*Cylindrocarpon*" *macrodidymum* també s'aïllà amb elevada freqüència de les arrels de males herbes recollides en vinyers comercials, mostrant un elevat percentatge d'aïllament.

És important destacar que les espècies compreses dins del complex *I. macrodidyma* foren les que s'aïllaren amb major freqüència en tots els tipus de sol estudiats: sols de camp de plantes mare, de camps d'enrailament i de camps comercials.

Finalment, es va estudiar l'efecte de la temperatura, pH i el potencial osmòtic (Ψ_s) sobre el creixement micelià, la esporulació i la producció de clamidòspores de "*C*." *liriodendri*, "*C*." *macrodidymum* i "*C*." *pauciseptatum*, amb l'objectiu de millorar el coneixement dels factors que afecten al creixement, reproducció i supervivència d'aquestos patògens. Tots els aïllats estudiats foren capaços de créixer en un rang de temperatures comprés entre 5 i 30ºC, amb un òptim de temperatura entre 20 i 25ºC. També s'observà creixement micelià en un rang de pH comprés entre 4 i 8. Respecte a l'efecte del Ψ_s, en general, el creixement micelià fou millor en medi de cultiu PDA ajustat a -0,5, -1,0 i/o -2,0 MPa en comparació amb el creixement micelià observat en PDA sense ajustar a cap Ψ_s (-0,3 MPA), reduint-se a valors de Ψ_s per davall de -2,0 MPa. La majoria dels aïllats de "*Cylindrocarpon*" *liriodendri* mostraren una major capacitat d'esporulació en comparació amb "*C*." *macrodidymum* i "*C*." *pauciseptatum* a totes les condicions estudiades. En general, la producció de clamidòspores no es va veure afectada per la temperatura, el pH i el Ψ_s. A tots els valors de pH estudiats s'observaren clamidòspores en tots els aïllats cultivats en PDA, mentre que a 5 i 10ºC o a -4,0 i/o -5,0 MPa alguns d'ells no produïren clamidòspores.

A més a més, en aquesta Tesi, també es detectaren algunes espècies que causen la malaltia de Petri de la vinya a partir dels aïllaments realitzats en plantes parany i en males herbes. Les espècies *Cadophora luteo-olivacea*, *Phaeoacremonium aleophilum*, *Pm. parasiticum* i/o *Phaeomoniella chlamydospora* s'aillaren a partir dels teixits xilemàtics de les plantes parany cultivades tant en els camps de plantes mare com en els camps de viver, així com a partir dels teixits xilemàtics de males herbes recollides en camps de plantes mare, camps de viver i camps de vinya comercials.

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Chapter 1

1.- GENERAL INTRODUCTION

1.1.- Young grapevine decline

Since the early 1990s, decline symptoms in young vineyards have dramatically increased causing a drastic reduction in the survival rate of grafted grapevines (*Vitis vinifera* L.) all over the world, coinciding with the extensive establishment of new vineyards (Graniti *et al*., 2000; Gramaje and Armengol, 2011). Decline symptoms have also been identified during the grapevine nursery propagation process. Evaluation of declining young vineyards has revealed that many factors are involved in the poor performance of vines. Grapevine wood fungi have been extensively investigated and are now considered among the most destructive pathogens either infecting grapevine propagation material or newly planted vines (Oliveira *et al*., 2004; Gramaje and Armengol, 2011).

The intensification of fungal trunk pathogens in young vineyards has been evaluated by several authors, who revealed that many factors such as changes in vineyard management and cultural practices, reduced sanitary care of rootstocks and vine propagation material, poor protection of pruning wounds, or the replacement of arsenite treatments with less efficient fungicides, are associated with young grapevine decline (Scheck *et al.,* 1998b; Chiarappa, 2000; Graniti *et al*., 2000; Morton, 2000; Stamp, 2001; Waite and May, 2005). These factors have been divided into three major groups: (i) nursery induced stress (structural vine defects, extended cold storage, limited vine carbohydrates and pot-bound root systems), (ii) vineyard establishment and management stresses (inadequate ground preparation, incorrect planting, inappropriate irrigation and nutritional deficiencies or excesses), and (iii) biological stresses (nematodes, root-colonizing fungal pathogens, viruses, rootstock/scion incompatibilities, or fungal trunk pathogens) (Stamp, 2001; Gramaje and Armengol, 2011). Nevertheless, as highlighted before, although the stress factors could be associated with this syndrome in young vineyards, the extensive research carried out on fungal trunk pathogens supports that they can be considered one of the major causes of this syndrome.

Among the grapevine trunk diseases, those associated with young grapevine decline are Petri disease, black dead arm and black-foot disease. They have been reported in most grapevine producing regions of the world and are responsible for the decline of young vines, loss of productivity and young vine death (Oliveira *et al*., 2004; Halleen *et al*., 2006a; Mostert *et al*., 2006; Gramaje and Armengol, 2011). Afterward, each of these diseases will be listed and described separately, with the exception of black-foot disease, which will be explained in more detail in a different section.

1.1.1.- Petri disease

Petri disease is a serious disease of grapevines which causes significant losses of young vines in newly planted vineyards (Mostert *el al*., 2006). External symptoms include stunted growth, reduced vigor, retarded or absent sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins, wilting, and dieback (Scheck *et al*., 1998b; Oliveira *et al*., 2004; Mostert *et al*., 2006; Luque *et al*., 2009). Internal symptoms can normally be seen in the trunk and cordons. Dissected vines show black spot when vines are cut transversally, and dark brown to black streaking when trunks or shoots are cut longitudinally (Mostert *et al*., 2006), which is a result of tyloses, gums, and phenolic compounds formed inside these vessels by the host in response to the fungus growing in and around the xylem vessels (Mugnai *et al*., 1999). Root system of declining vines also has less total root biomass and dry brown lesions on feeder roots (Whiting *et al*., 2001).

The causal agents of this disease are *Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams, as well as several species of *Phaeoacremonium* W. Gams, Crous & M. J. Wingf. (Mugnai *et al*., 1999; Mostert *et al*., 2006), *Pm. aleophilum* W. Gams, Crous, M. J. Wingf. & L. Mugnai, being the most commonly isolated and studied species of this genus (Mostert *et al*., 2006). Several *Phialophora*-like and *Acremonium* species have also been involved in the decline in young vines, mainly species of the genus *Cadophora* Lagerberg & Melin (Overton *et al*., 2005; Halleen *et al*., 2007b). Of those, *Cadophora luteo-olivace*a (F.H. Beyma) Harrington & McNew has been recently shown to be quite common on grapevines affected by fungal trunk pathogens in several viticulture areas worldwide (Navarrete *et al*., 2011; Gramaje *et al*., 2011b). In addition, *Pleurostomophora richardsiae* (Nannf.) L. Mostert, W. Gams & Crous (basionym: *Ca. richardsiae* Nannf.) has also been

associated with Petri disease in California (Rolshausen *et al*., 2010), causing vascular discoloration after field and glasshouse inoculations similar to that seen in Petri diseased grapevines in South Africa (Halleen *et al*., 2007b).

In Spain, the main fungal agents associated to this disease are *Pa. chlamydospora*, *Pm. aleophilum* and *Pm. parasiticum* (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf. (Armengol *et al*., 2001; Aroca *et al*., 2006; Giménez-Jaime *et al*., 2006), which have been reported affecting young grapevines. These species have also been recovered from rootstock mother-vines and from different stages of the grapevine nursery process. Other *Phaeoacremonium* species have also been reported in Spain associated with Petri disease: *Pm .cinereum* D. Gramaje, H. Mohammadi, Z. Banihashemi, J. Armengol & L. Mostert, *Pm. hispanicum* D. Gramaje, J. Armengol & L. Mostert, *Pm. inflatipes* W. Gams, Crous & M.J. Wingf., *Pm. iranianum* L. Mostert, Gräfenhan, W. Gams & Crous, *Pm. krajdenii* L. Mostert, Summerb. & Crous, *Pm. mortoniae* Crous & W. Gams, *Pm. scolyti* L. Mostert, Summerb. & Crous, *Pm. sicilianum* Essakhi, Mugnai, Surico & Crous and *Pm. viticola* J. Dupont (Gramaje *et al*., 2007; Aroca *et al*., 2008a; Gramaje *et al*., 2008, 2009a, b, 2011a). In addition, *Ca. luteo-olivacea* and *Ca. melinii* Nannf. have already been recovered from grapevines showing symptoms characteristic of Petri disease, and also from different stages of the grapevine nursery process over the last few years in Spain (Gramaje *et al.*, 2011b).

The main sources of inoculum of these fungi in vineyards include: (i) infected propagation material, which is already infected at different stages of the grafted plant production process (Retief *et al*., 2006; Aroca *et al*., 2010; Gramaje and Armengol, 2011; Gramaje *et al*., 2011b; Serra *et al*., 2011), (ii) aerial inoculum, which infects grapevines through pruning wounds (Eskalen *et al*., 2007; Quaglia *et al*., 2009; Rolshausen *et al*., 2010; Gramaje and Armengol, 2011), and (iii) infested soils, in which soilborne pathogens such as *Pa. chlamydospora* and *Ca. luteo-olivacea* could persist over time and also could develop the capacity to infect grapevine roots and reach the xylem vessels (Bertelli *et al*., 1998; Mugnai *et al*., 1999; Sidoti *et al*., 2000; Eskalen *et al*. 2001; Whiteman *et al*., 2002; Damm and Fourie, 2005; Retief *et al*., 2006; Spadaro *et al*., 2011).

1.1.2.- Black dead arm

The most severe losses due to black dead arm, caused by species of the family Botryosphaeriaceae, occur in grapevines that are 8 years and older. However, Botryosphaeriaceae species have been frequently isolated from declining young vineyards in different grapevine-growing areas worldwide (Crous *et al.*, 2006; Ridgway *et al*., 2011; Úrbez-Torres, 2011). Moreover, they have also been detected on grapevine propagation material in Greece (Rumbos and Rumbou, 2001), Italy (Spagnolo *et al*. 2011), New Zealand (Billones-Baaijens *et al*., 2012a, b), Portugal (Oliveira *et al*. 2004) and Spain (Aroca *et al.* 2006).

The distinctive characteristic of black dead arm is the wood necrosis of the trunk and arms of infected vines. Typical disease symptoms include cankers and other dieback symptoms such as bud mortality, leaf chlorosis, fruit rot, sectorial wood necrosis, shoot dieback, cane bleaching, and graft union failure (Luque *et al*., 2009; Úrbez-Torres and Gubler, 2011; Bertsch *et al.*, 2012). Buds from affected vines sometimes did not burst and eventually die. The bark tissue of the diseased parts (cordons and/or trunks) collapse at the sites of infection, becoming discolored to dark-brown, a characteristic symptom which gave black dead arm its name. The internal wood symptoms that result from the wood infection can usually be seen in cross-sectioned arms or trunks as wedge- or irregular-shaped necrotic sectors. After colonization of the xylem by the fungus the wood became black and this discoloration spread downwards and upwards in a narrow straight stripe. Foliar symptoms are characterized by reddening and scorching of the leaves which showed a dramatic loss of turgor with death of the plant within 2 years (Luque *et al*., 2009; Ridgway *et al*., 2011; Úrbez-Torres, 2011). Yellowish-orange or wine-red spots develop on leaf margins and the blade well in advance of what is generally observed for young affected plants, usually from May to June instead of late June or early July in the northern hemisphere. As the disease progresses, these spots merge to finally form large interveinal necroses (Bertsch *et al.*, 2012). Although the development and spread of these symptoms is slow, Botryosphaeriaceae species are considered among the major contributors to the grapevine trunk disease syndrome, which have caused decline in vigor and yield of whole vineyards, with severe financial losses to growers (Ridgway *et al*., 2011).

The Botryosphaeriaceae comprise cosmopolitan Ascomycetes occurring worldwide as saprophytes, endophytes or pathogens on a wide range of annual and

perennial hosts, including grapevines (Spagnolo *et al.*, 2011; Úrbez-Torres, 2011). To date, 17 potentially phytopathogenic species belonging to the genera *Diplodia* Fr., *Dothiorella* Sacc., *Fusicoccum* Corda, *Lasiodiplodia* Ellis & Everh., and *Neofusicoccum* Crous, Slippers & A.J.L. Phillips have been associated with cases of dieback of grapevines (Spagnolo *et al.*, 2011; Úrbez-Torres, 2011).

In Spain, a total of 12 Botryosphaeriaceae species have been reported associated with black dead arm disease: *Botryosphaeria dothidea* (Moug.) Ces. & De Not. (Armengol *et al*., 2001), *Diplodia mutila* (Fr.) Mont. (Martin and Cobos, 2007), *D. seriata* De Not. (Armengol *et al*., 2001), *Dothiorella iberica* A.J.L. Phillips, J. Luque & A. Alves and *Do. sarmentorum* (Fr.) A.J.L. Phillips, A. Alves & J. Luque (Martin and Cobos, 2007), *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Aroca *et al*., 2008b; Martin *et al*., 2009), *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips (Aroca *et al*., 2010), *Neof. luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (Luque *et al*., 2009), *Neof. mediterraneum* Crous, M.J. Wingf. & A.J.L. Phillips (Aroca *et al*., 2010), *Neof. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (Aroca *et al*., 2006), *Neof. vitifusiforme* (Van Niekerk & Crous) Crous, Slippers & A.J.L. Phillips (Aroca *et al*., 2010) and *Spencermartinsia viticola* (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous (Phillips *et al*., 2008).

Infection of grapevines by these fungal pathogens primarily occurs through pruning wounds. Pycnidiospores are primarily dispersed by rain splash and spread the disease by infecting the exposed xylem of pruning wounds, where spore germination and colonize exposed wood vessels (Rolshausen *et al.*, 2010; Úrbez-Torres and Gubler, 2011). In nurseries, the sources of the infection are likely to be the infected canes, from infected mothervines or contamination of propagation tools or materials (Billones-Baaijens *et al*., 2012a, b).

1.2.- Black-foot disease

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 (Rumbos and Rumbou, 2001; Halleen *et al*. 2003; Fourie and Halleen, 2004; Oliveira *et al*. 2004; Aroca *et al*., 2006; Dubrovsky and Fabritius, 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, 2007a; Chaverri *et al*. 2012). Thus, several authors indicated that black-foot pathogens are the most common pathogenic fungi associated with young nursery vines (Halleen *et al*. 2003, 2007a; Dubrovsky and Fabritius, 2007).

1.2.1.- Symptoms

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). Moreover, roots show abnormal development characterized by shallow growth parallel to the soil surface and become necrotic. 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 (Fig. 1.1A, B). A cross-section through these lesions reveals the development typical symptoms of vascular streaking which include dark-brown to black discoloration and gum inclusions in xylem vessels. The pith is also compacted and discolored (Scheck *et al*., 1998b; Larignon, 1999; Fourie and Halleen, 2001; Halleen *et al*., 2006a).

External symptoms show reduced vigor with small-sized trunks, shortened internodes, uneven wood maturity, sparse foliage, and small leaves with interveinal

chlorosis and necrosis (Fig. 1.1C, D). 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).

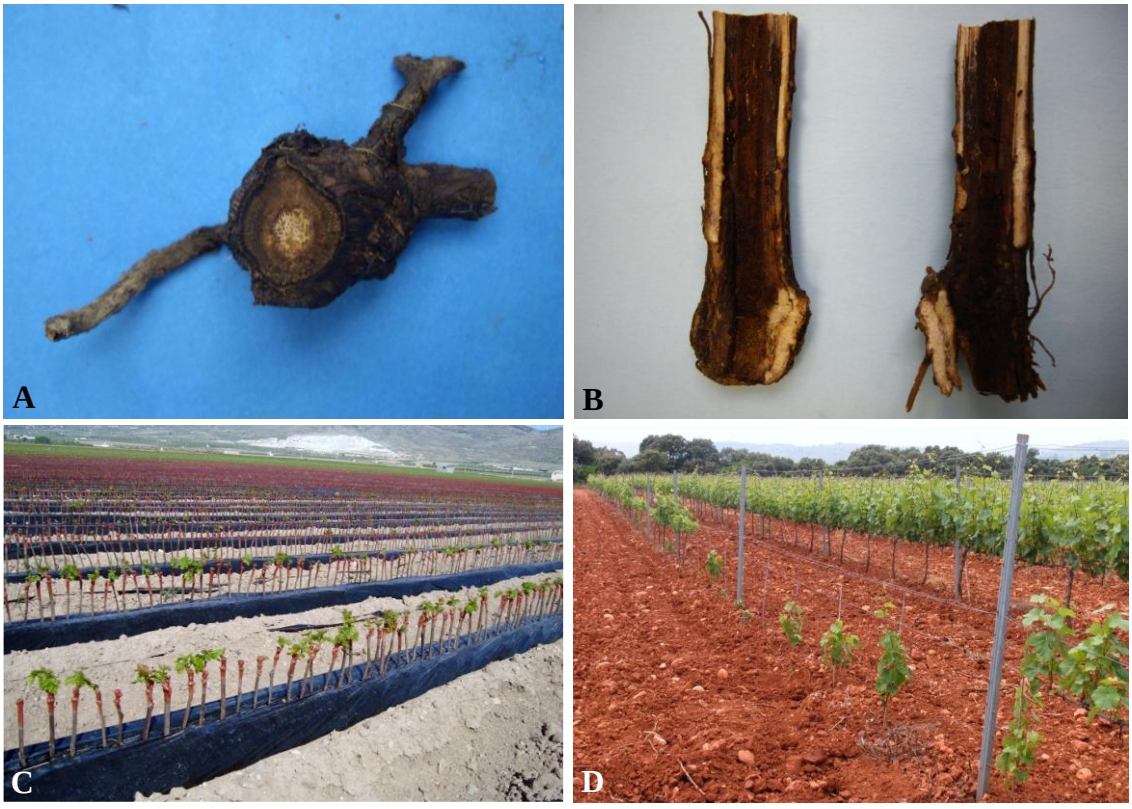

Figure 1.1. A, black discoloration and necrosis of wood tissue which develops from the base of the rootstock, characteristic of black-foot disease; B, longitudinal section of a rootstock showing dark-brown to black discoloration; C, Un-sprouted grapevine propagation material in a grapevine nursery; D, grapevine plants showing stunded growth, reduced vigour and retarded sprouting in a young plantation.

1.2.2.- 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 *et al*., 2000), Argentine (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), California (Petit and Gubler, 2005) 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 is the anamorph of the type species of *Neonectria*, (ii) "*C.*" *cylindroides* Wollenw., which is the type species of the genus "*Cylindrocarpon*", (iii) "*C.*" *destructans*, which is 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 have been 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, 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), 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), Portugal (Cabral *et al*., 2012c) 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 has 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 internal transcribed spacer (ITS) and partial β-tubulin genes (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) and Spain (Martin *et al*., 2011). 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*.

In addition, other "*Cylindrocarpon*" species have been associated 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).

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 2011, Chaverri *et al*., performed a phylogenetic study of *Neonectria*, "*Cylindrocarpon*" and related genera with "*Cylindrocarpon*"-like anamorphs (Chaverri *et al*., 2011). 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* are included into *Ilyonectria* genus 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*. (2012c), have just demonstrated the existence of polymorphism into *I. macrodidyma*-complex. This hypothesis is 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* (*Ilyonectria* sp. 1, *I.* sp. 2, *I. estremocensis* A. Cabral, Nascimento & Crous, *I. alcacerensis* A. Cabral, Oliveira & Crous, *I. novozelandica* A. Cabral & Crous, and *I. torresensis* A. Cabral, Rego & Crous) 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 and New Zealand (Cabral *et al*., 2012a, c).

Other *Ilyonectria* species have been 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, *I. robusta* (A.A. Hildebr.*)* A. Cabral, Rego & Crous and *I. vitis* A. Cabral, Rego & Crous which have been reported in

Portugal (Cabral *et al*., 2012a, c); and *I. crassa* (Wollenw.) A. Cabral & Crous which has been reported in Uruguay (Cabral *et al*., 2012a). Isolates belonging to *Neonectria mammoidea* group have also been associated with this disease in Canada (Petit *et al*., 2011).

Regarding, "*C*." *pauciseptatum* is not clear in which genera it has to included, although 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 (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). Recently, 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 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).

The taxonomy and distribution of "*Cylindrocarpon*"/*Ilyonectria*, *Campylocarpon* and *Cylindrocladiella* species associated with black-foot disease of grapevine are summarized in Table 1.1.

These genera have characteristic distinctive morphological and cultural patterns (Fig. 1.2 and 1.3; Table 1.2). The anamorphs of "*Cylindrocarpon*"/*Ilyonectria* produce abundant microconidia and chlamydospores. Macro- and microconidia apparently are

Table 1.1. Fungal species associated with black-foot disease of grapevines and their distribution.

Species	Distribution
Campylocarpon fasciculare Schroers, Halleen & Crous	South Africa (Halleen *et al*., 2004), Brazil (Correia *et al*., 2012), and Spain (Alaniz *et al*., 2011b)
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), Portugal (Rego *et al*., 2000), Argentine (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), California (Petit and Gubler, 2005) 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) and Spain (Martin *et al.*, 2011)
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)
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	Spain, and Portugal (Cabral *et al*., 2012c)
Ilyonectria crassa (Wollenw.) A. Cabral & Crous	Uruguay (Abreo *et al*., 2010; Cabral *et al*., 2012a)
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*., 2006), Australia (Whitelaw-Weckert *et al*., 2007), California (Petit and Gubler, 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), 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), Spain (Alaniz *et al*., 2007), Uruguay (Abreo *et al*., 2010), northeastern United States and southeastern Canada (Petit *et al.*, 2011), Portugal (Cabral *et al.*, 2012c) and Turkey (Özben *et al*., 2012)
Ilyonectria novozelandica A. Cabral, Nascimento & Crous	South Africa, USA and New Zealand (Cabral *et al*., 2012c)
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	Portugal (Cabral *et al*., 2012c)
Ilyonectria vitis A. Cabral, Rego & Crous	Portugal (Cabral *et al*., 2012)
Ilyonectria sp. 1 (Cabral *et al*. 2012c)	Portugal (Cabral *et al*., 2012c)
Ilyonectria sp. 2 (Cabral *et al*. 2012c)	Portugal (Cabral *et al*., 2012c)
Isolates belonging to *Neonectria mammoidea* group	Canada (Petit *et al.*, 2011)

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] (Fig. 1.2A, B). Chlamydospores are abundant, generally intercalary, globose, single or in chains, becoming brownish. In addition, colony morphology on PDA is very heterogeneous (Fig. 1.2C). Aerial mycelium is floccose to felted, and the color varied from white to yellow or light to dark brown. Colony margins are entire, slightly lobulated, or lobulated (Fig. 1.3A-H) (Booth, 1966; Samuels and Brayford, 1990; Chaverri *et al*., 2011).

Campylocarpon is similar to "*Cylindrocarpon*"/*Ilyonectria*. They 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 (Fig. 1.2D). Conidial heads form pionnotes-like aggregates. Conidiophore show an 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- septate, [(24–)35-60(–62)×6.5-9 µm], apical cell obtuse, basal cell obtuse or with inconspicuous hilum (Fig. 1.2E). 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 (Fig. 1.3I, J) (Halleen *et al*., 2004; Chaverri *et al*., 2011).

Cylindrocladiella species produce hyaline, single, subverticillate, as well as penicilliate conidiophores, 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 (Fig. 1.2F, G). Conidia are cylindrical, rounded at both ends, straight, hyaline, (0)-1-septate, [(9–)11-13(–15)×2-4 µm], sometimes becoming swollen at one end with age (Fig. 1.2H). Chlamydospores are

abundant or moderate, more frequently arranged in chains than clusters (Fig. 1.2I). Aerial mycelium ranges from dark to light brown (Fig. 1.3K, L) (Crous and Wingfield, 1993; Lombard *et al*., 2012).

1.2.3.- Biology, epidemiology and host range

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 openings (Fourie and Halleen, 2006; Halleen *et al*., 2006a, 2007a; 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).

Table 1.2. 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 penicilliate, with primary and secondary branches
Conidia			Cylindrical, rounded at both ends, straight, hyaline, (0)-1-septate, sometimes becoming swollen at one end with age
Microconidia	Abundant, ellipsoidal to ovoid, hyaline, 0−1-septate, with a lateral or basal hilum	Absent	
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	
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

They are generally regarded as pathogens and/or saprobes of to a wide range of angiosperm and gymnosperm hosts and substrates in temperate, sub-tropical and tropical regions wordwide (Victor *et al*., 1998; Chaverri et *al*., 2011; Lombard *et al*., 2012). In general, black-foot pathogens contribute to young plant decline, reducing productivity and longevity, thereby causing considerable economic loss to the industry. There are few reports about virulence diversity of "*Cylindrocarpon*"/*Ilyonectria* spp. to grapevine. Alaniz *et al*. (2009) detected virulence diversity in "*C*." *macrodidymum* showing that the isolates belonging to ISSR groups G6 and G7 were significantly more virulent than other isolates of "*C*." *macrodidymum* (ISSR groups G3, G4 and G5) and "*C*." *liriodendri* (ISSR groups G1 and G2). Recently, research studies 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*.

Although black-foot disease pathogens are associated with young grapevine decline, they have also been associated with rot root diseases of other agricultural, forestry or horticultural crops (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*., 2011) or *Pinus sylvestris* L. (Menkis and Burokiene, 2012).

Black-foot pathogens are frequently isolated from rootstock mother-plants, rooted rootstock cuttings, bench-grafts and young grafted vines (Oliveira *et al*., 2004; Halleen *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*., 2001; 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). The results of these studies suggest that new plants were infected during the propagation process in nurseries and that even the planting material used in the propagation process might be infected with these pathogens (Halleen *et al*., 2003; Aroca *et al*. 2010).

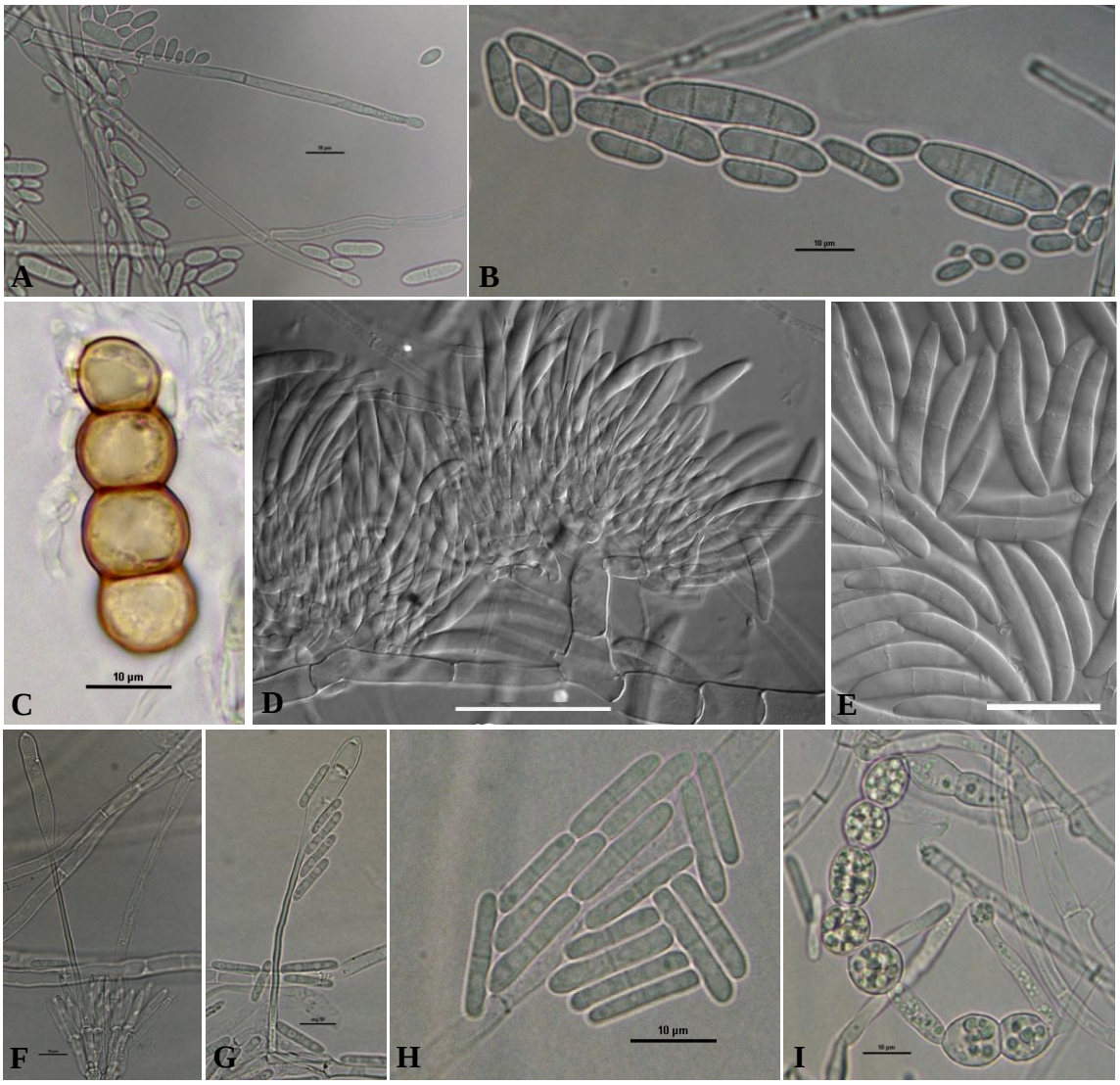

Figure 1.2. A, Conidiophores of *Ilyonectria liriodendri*; B, Macro- and microconidia of *I. liriodendri*; C, Chlamydospores in chains of "*Cylindrocarpon*" *pauciseptatum*; D, Conidiophores of *Campylocarpon fasciculare* (Halleen *et al*., 2004); E, Macroconidia of *Campyl. fasciculare* (Halleen *et al*., 2004); 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 = 50 µm; e = 30 µm.

In this sense, 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. Thus, 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.

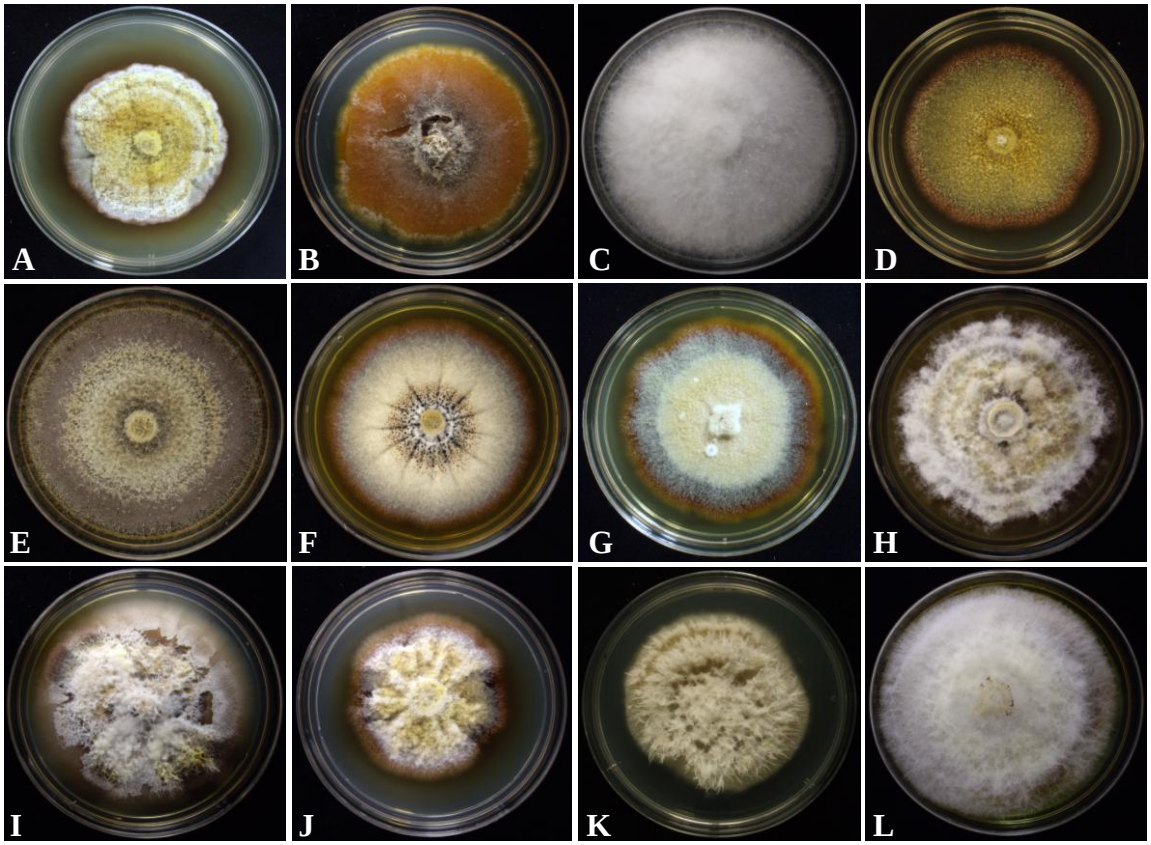

Figure 1.3. Colonies of black-foot pathogens grown on PDA. A, "*Cylindrocarpon*" *destructans*; B, "*C.*" *pauciseptatum*; C, "*C.*" *obtusisporum*; D, *Ilyonectria alcacerensis*; E, *I. liriodendri*; F, *I. novozelandica*; G, *I. macrodidyma*; H, *I. torresensis*; I, *Campylocarpon fasciculare*; J, *Campyl. pseudofasciculare*; K, *Cylindrocladiella parva*; L, *Cyl. peruviana*.

Moreover, 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). Recently, research studies carried out in New Zealand by Brown *et al*. (2012), confirm that stress factors such as defoliation can contribute to black-foot disease severity in young vines.

Finally, 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 pathogens such as Petri disease pathogens, Botryosphaeriaceae, *Phomopsis* spp., *Pythium* spp. or *Phytophthora* spp. (Halleen *et al*., 2007a).

1.2.4.- Control

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*., 2007a). 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*., 2007a). 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 mycelal 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, 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*. (2007a) evaluated various chemical pre-planting treatments for prevention of infection by black-foot and Petri disease pathogens. A total of 13 fungicides were evaluated *in vitro* against "*C.*" *liriodendri* and "*C." macrodidymum*. 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*. (2007a), 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-45 min, while treatments above 48ºC-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*. (2007a) 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*., 2007a).

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.) incorporated 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.

Recently, Sun *et al*. (2013) studied the ability of methyl jasmonate (MeJa) to induce plant defense against "*Cylindrocarpon*" *destructans*, the causal agent of "Cylindrocarpon" root rot on ginseng. In this study, the authors concluded that low concentrations of MeJa had not effect on "*C.*" *destructans*, but it stimulated the activities of defense enzymes in treated ginseng roots and alleviated the damage of roots from "*C.*" *destructans*.

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Chapter 2

OBJECTIVES

Black-foot is a severe disease of grapevines which affects propagation material, newly planted vines or established vineyards in most of the wine-producing countries worldwide, including Spain.

Black-foot disease pathogens have been frequently isolated from symptomatic or asymptomatic rootstock mother-plants, rooted rootstock cuttings, bench-graft and young grafted vines in different grapevine growing areas around the world. 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. Nevertheless, the presence of black-foot disease pathogens in grapevine nurseries as well as the potential inoculum sources of these pathogens in soils from nurseries and commercial vineyards has not been explored yet in Spain.

Thus, this Thesis will focus on the study of the epidemiology of black-foot disease pathogens in Spain, with the following objectives:

1. Detect *Ilyonectria* spp. at the different stages of the propagation process by multiplex nested-PCR and to determine the contribution of the rooting phase in open-root nursery fields to increase infections caused by *Ilyonectria* spp. in grapevine planting material, in order to evaluate the nursery grapevine propagation process as a potential infection source for *Ilyonectria* spp. (Chapter 3).

2. Determine the presence of black-foot pathogens in the soil of grapevine rootstock mother fields, open-root field nurseries and commercial vineyards by using seedlings of a grapevine rootstock and cultivars as bait plants, and to confirm the capacity of soilborne inoculum of these pathogens to naturally infect these plants (Chapter 4).

3. Detect the presence of *Ilyonectria* spp. in nursery soils with multiplex, nested PCR and to quantify the concentration of the *Ilyonectria* spp. in the same DNA samples by quantitative real-time PCR (Chapter 5).

4. Study the role that weeds could play as potential hosts of black-foot pathogens in the soil of grapevine fields (Chapter 6).

5. Study the effects of temperature, pH and Ψ_s on mycelial growth, sporulation and chlamydospore production of "*C.*"*liriodendri,* "*C.*" *macrodidymum* and "*C.*" *pauciseptatum* isolated from grapevines (Chapter 7).

Chapter 3

Detection of black-foot disease pathogens in the grapevine nursery propagation process in Spain

Carlos Agustí-Brisach[1], David Gramaje[2], José García-Jiménez[1] and Josep Armengol[1]

[1]Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain.

[2]Department of Crop Protection, Institute for Sustainable Agriculture (IAS), Spanish National Research Council (CSIC), Alameda del Obispo s/n, APDO. 4084, 14004 Córdoba, Spain.

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Keywords

Black-foot, inoculum sources, planting material, *Vitis vinifera*

Abstract Two commercial nurseries located in Comunidad Valenciana region (central-eastern Spain) were sampled in 2010 to evaluate whether the grapevine nursery propagation process could be a source of black-foot disease pathogens. Samples were taken from four sources of the propagation process: pre-grafting hydration tanks, scissors used for cutting buds, omega-cut grafting machines and peat used for callusing. DNA from these samples was extracted and multiplex nested-PCR using primers specific for "*Cylindrocarpon*" *pauciseptatum*, *Ilyonectria liriodendri* and *I. macrodidyma*-complex (composed of *I. alcacerensis*, *I. estremocensis*, *I. macrodidyma*, *I. novozelandica*, *I. torresensis*, *I.* sp. 1 and *I.* sp. 2) was used to identify the species present. *Ilyonectria liriodendri* and *I. macrodidyma*-complex were detected in hydration tanks, scissors, grafting machines and peat, *I. macrodidyma*-complex being the most frequent. Additionally, ten grafted cuttings each from five grapevine scion/rootstock combinations were collected from each nursery immediately after callusing, and again after one growing season in a nursery field. Roots of these grafted cuttings and plants were sampled to isolate the fungal pathogens. Only *I. torresensis* was isolated after callusing, while *I. liriodendri*, *I. novozelandica* and *I. torresensis* were isolated after one growing season, showing the highest incidence at this latter sampling time. Moreover, DNA was extracted from roots and analyzed as described before. *Ilyonectria liriodendri* and *I. macrodidyma*-complex were also detected at both sampling times. The use of the multiplex nested-PCR technique improved the detection of *I. liriodendri* and *I. macrodidyma*-complex from grafted cuttings and plants in both nurseries. This work shows that the grapevine nursery propagation process should be considered as potential infection source for black-foot disease pathogens, and confirms that infections caused by *Ilyonectria* spp. in grapevine planting material increase markedly after one growing season in nursery fields.

Introduction

Over the last decade, the importance of infected grapevine propagation material as a major means of spread of fungal trunk pathogens has been emphasized (Gramaje and Armengol, 2011). Although these pathogens usually manifest in older, mature grapevines as trunk diseases, some of them are frequently associated with nursery

grapevines as symptomatic or asymptomatic infections (Halleen et al. 2003; Fourie and Halleen, 2004).

Traditional grapevine propagation techniques use rootstock or scion mother vines, from which dormant cuttings are taken for bench grafting, rooting, or field budding. Some practices, such as hydration, cold storage conditions, and general standards of nursery hygiene, can have a significant effect on the quality of the vines produced (Gramaje and Armengol, 2011). When apparently healthy grafted nursery plants are examined, a relevant number of them show black discolorations and brown to dark streaks in longitudinal or transversal sections of the wood on stems, rootstocks or roots, from which Petri or black-foot disease pathogens, or Botryosphaeriaceae spp., can be isolated (Halleen et al. 2003; Oliveira et al. 2004; Aroca et al. 2006; Halleen et al. 2006; Mostert et al. 2006).

Regarding Petri disease pathogens, *Phaeomoniella chlamydospora* and species of the genus *Phaeoacremonium* have been detected in grapevine propagation material from nurseries in California (Dubrovsky and Fabritius, 2007), Greece (Rumbos and Rumbou, 2001), Italy (Serra et al. 2011), New Zealand (Whiteman et al. 2003), Portugal (Oliveira et al. 2004), South Africa (Halleen et al. 2003; Retief et al. 2006) and Spain (Aroca et al. 2006; Giménez-Jaime et al. 2006). Different stages of the grafted plant production process have been identified as potential inoculum sources of Petri disease pathogens. In Spain, Aroca et al. (2010) detected *Pa. chlamydospora*, *Phaeoacremonium aleophilum* and *Pm. parasiticum* in hydration tanks, omega-cut grafting machines, scissors and peat for callusing during the grapevine propagation process by nested-PCR. These authors also isolated *Pm. aleophilum* and *Pm. parasiticum* from scissors washings and *Pa. chlamydospora* from peat samples using a semi-selective culture medium, demonstrating that viable propagules are present at different stages of the grafted plant production process (Aroca et al. 2010). Recently, Gramaje et al. (2011) isolated *Cadophora luteo-olivacea* and *Ca. melinii* from planting material showing black vascular streaking and decline symptoms characteristic of Petri disease, and also detected viable inoculum of *Ca. luteo-olivacea* from different stages of the grapevine nursery process. Species of Botryosphaeriaceae have also been detected on grapevine propagation material in Greece (Rumbos and Rumbou, 2001), Italy (Spagnolo et al. 2011), Portugal (Oliveira et al. 2004), Spain (Aroca et al. 2006) and New Zealand (Billones-Baaijens et al. 2012a, b).

Black-foot disease pathogens, included into the genera *Campylocarpon*, "*Cylindrocarpon*" and *Ilyonectria* (Halleen et al. 2004; Halleen et al. 2006b; Schroers et al. 2008; Chaverri et al. 2011; Cabral et al. 2012a, b), have also been frequently isolated from rootstock mother-plants, rooted rootstock cuttings, bench-graft and young grafted vines in California (Dubrovsky and Fabritius, 2007), Greece (Rumbos and Rumbou, 2001), Portugal (Oliveira et al. 2004) and South Africa (Halleen et al. 2003; Fourie and Halleen, 2004). It is well known that these pathogens are common in the soil and it has been demonstrated that nursery field sites can harbor them, causing infection of grafted vines after some months of growth in nursery soils (Halleen et al. 2003, 2007; Chaverri et al. 2011). In fact, isolation studies conducted in South African grapevine nurseries by Halleen et al (2003) demonstrated that soilborne inoculum of black-foot pathogens infected grafted grapevines once planted in nursery fields. Nevertheless, the presence of black-foot disease pathogens in grapevine nurseries in Spain has not been explored.

Molecular techniques have played an important role in the identification and detection of black-foot pathogens. In 2011, described "*Cylindrocarpon"* species associated with black-foot were renamed in the genus *Ilyonectria* or referred to as "*Cylindrocarpon"*, because true *Cylindrocarpon* species belong to the genus *Neonectria* (Chaverri et al. 2011). "*Cylindrocarpon"* has been divided into five informal groups (*Neonectria*/"*Cylindrocarpon*" *sensu stricto*, *Rugonectria*, *Thelonectria*, *Ilyonectria* and the anamorph genus *Campylocarpon*) that are recognized as genera, including "*Cylindrocarpon" liriodendri* and "*C". macrodidymum*, causal agents of black-foot, into *Ilyonectria* genus (Chaverri et al. 2011; Cabral et al. 2012a). Moreover, Cabral et al. (2012b) revealed the existence of polymorphism in a wide collection of isolates previously identified as *I. macrodidyma* by using internal transcribed spacer, β-tubulin gene, histone H3 gene and translation elongation factor 1-α sequence analysis. Consequently, six new species of *Ilyonectria* (*I. alcacerensis, I. estremocensis, I. novozelandica, I. torresensis, Ilyonectria* sp. 1 and *I*. sp. 2) and *I. macrodidyma,* which are morphologically rather similar, were recognised into the *I. macrodidyma*-complex. Dubrovski and Fabritius (2007) developed genus-specific PCR primers (Cyl-F and Cyl-R) for the simultaneous detection of "*Cylindrocarpon" liriodendri* and "*C." macrodidymum* in grapevine nurseries. Alaniz et al. (2009) designed species-specific primer pairs (Mac1/MaPa2, Lir1/Lir2 and Pau1/MaPa2) which allow the quick and easy detection of *I. liriodendri, I. macrodidyma*-complex and "*C." pauciseptatum* and have

been recently used to detect inoculum sources of these pathogens in a commercial grapevine nursery in Portugal (Cardoso et al. 2012).

The objective of this work was to evaluate which of the different stages in the propagation of grapevine in Spanish nurseries could be a source of black-foot disease pathogens. To this end, *Ilyonectria* spp. were detected at different stages of the propagation process by multiplex nested-PCR and by isolation and genotyping in the grapevine planting material before and after the rooting phase in nursery fields.

Material and Methods

Sampling of grapevine nurseries

Two commercial nurseries (A and B), located in Comunidad Valenciana region (central-eastern Spain) were surveyed in 2010.

Samples from nurseries were taken arbitrarily at four stages of the propagation process: pre-grafting hydration tanks, washings from scissors used for cutting buds, washings from omega-cut grafting machines and peat from the callusing stage. Samples of tank water (approximately 100 ml each) were taken from the middle of the tank stirring slightly. Sampling was done in each hydration period when cuttings had been immersed for two to three days. A sample of the tap water used to fill the tanks was also collected. Scissors and omega-cut grafting machines were washed with a sterile solution of 0.2% Tween-20 at the end of one working day, and 30 to 60 ml of every washing was recovered in a sterile tube. Samples of about 100 g of peat were taken after the callusing stage for a batch of finished cuttings. One sample of peat was also taken before use. In nursery A, the total number of samples taken was 28 from hydration tanks, 38 from scissors, 13 from omega-cut grafting machines and 5 from peat after the callusing process, and in nursery B, the total number of samples taken was 19 from hydration tanks, 23 from scissors, 16 from omega-cut grafting machines and 5 from peat after the callusing process.

In addition, ten grafted cuttings from five bundles (200 grafted cuttings each) of different grapevine scion/rootstock combinations were collected per nursery after

callusing (50 grafted cuttings per nursery). The remaining grafted cuttings were immediately planted in a nursery field in both nurseries. After one growing season, ten plants of the same five grapevine scion/rootstock combinations were also collected in each nursery (50 plants per nursery).

Detection of *Ilyonectria* spp. and "*C.*" *pauciseptatum* from liquid samples

Liquid samples from hydration tanks, scissors and omega-cut grafting machines were centrifuged for 15 min at 5000 rpm; supernatant was discarded and the resulting liquid sample pellets were mixed. This liquid was centrifuged again for 5 min at 10000 rpm in order to obtain a sample pellet which was stored at -20ºC. Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Doraville, USA). Before following the manufacturer's instruction of the E.Z.N.A. Plant Miniprep Kit, pellets samples were shaked using small steel balls in a vial filled with 600 µl of DNA extraction buffer (P1) for 10 min at 50 Hz using TissueLyser LT (QIAGEN, Hilden, Germany). Mix liquid (600 µl) were then transferred to new vial and the manufacturer's instructions were followed to complete the DNA extraction.

A nested-PCR combined with a multiplex PCR approach was used for the detection of *Ilyonectria* spp. and "*C.*" *pauciseptatum* (Alaniz et al. 2009). The first PCR reaction was carried out with primers ITS1F/ITS4 (Gardes and Bruns, 1993) containing 1× PCR buffer, 1.25 mM $MgCl_2$, 80 µM of each dNTP, 0.2 µM of each primer, 0.7 U of *Taq* polymerase, and 1 µl of template DNA. The PCR reaction mix was adjusted to a final volume of 25 µl with water. The program consisted of an initial step of 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 45 s. A final extension was performed at 72°C for 10 min. PCR product was diluted 1:100 and 1 µl was used as template DNA for the secondary PCR which was carried out with specific primers Lir1/Lir2, Mac1/MaPa2 and Pau1/MaPa2 (Alaniz et al. 2009) to amplify *I. liriodendri*, *I. macrodidyma*-complex and "*C". pauciseptatum*, respectively. Each reaction contained 1× PCR buffer, 2.0 mM $MgCl_2$, 80 µM of each dNTP, 0.2 µM of each primer, 0.7 U of *Taq* polymerase, and 1 µl of template DNA. The PCR reaction mix was adjusted to a final volume of 25 µl with water. The program consisted of an initial step of 3 min at 94°C, followed by 30 cycles

of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and elongation at 72°C for 45 s. A final extension was performed at 72°C for 10 min. Multiplex nested-PCR product was visualized on 1.5% agarose gels stained with ethidium bromide.

Detection of *Ilyonectria* spp. and "*C*". *pauciseptatum* from peat samples

For the direct extraction of DNA from peat samples, they were lyophilized overnight and then, were crushed using FRITSCH Variable Speed Rotor Mill-PULVERISETTE 14 (ROSH, Oberstein, Germany) until all the peat was ground to a fine powder. A total of 250 mg of each air-dried peat sample was placed in 1.5 ml vial with 700 µl warm (65ºC) extraction buffer (120 mM NaH_2PO_4, 2 % CTAB or SDS, 1.5 mM NaCl, pH 8.0, and 1 % w/v polyvinylpyrrolidone). Vials were then shaken and incubated at 65 ºC for 15 min, followed by centrifugation at 13000 rpm for 10 min. Supernatants (400 µl) were then transferred to a new vial and ZR Soil Microbe DNA MiniPrep™ (Zymo Research, Irvine, U.S.A.) was used for the further steps of DNA extraction. Extracted DNA was kept at −20°C until its use in PCR amplifications. Multiplex nested-PCR reactions to detect *Ilyonectria* spp. and "*C*". *pauciseptatum* were carried out as described before.

Isolation and molecular detection of *Ilyonectria* spp. and "*C*". *pauciseptatum* in grafted cuttings and plants

In order to isolate *Ilyonectria* spp. and "*C*". *pauciseptatum* from grafted cuttings and plants in each sampling time, root sections were cut from necrotic areas, washed under running tap water, surface-disinfested for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Small root pieces were plated on potato dextrose agar (PDA, Biokar-Diagnostics, Zac de Ther, France) supplemented with 0.5 g l^{-1} of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA). Plates were incubated for 10-15 days at 25ºC in the dark and all colonies were transferred to PDA. Isolates were single-spored prior to morphological and molecular identification with the serial dilution method (Dhingra and Sinclair, 1995).

Species of *Ilyonectria* were identified morphologically by macroscopic characters including colony texture, color, and the shape of the growing margin on PDA. Conidia were observed and measured on Spezieller Nährstoffarmer Agar (SNA) with the addition of a 1×1 cm piece of filter paper to the colony surface (Alaniz *et al*., 2007; Chaverri et al. 2011). For DNA extraction, 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 following manufacturer's instructions. DNA was visualized on 0.7% agarose gels stained with ethidium bromide and was stored at –20ºC. The identification of *Ilyonectria* spp. was confirmed by sequencing part of the HIS using CYLH3F and CYLH3R primers (Crous et al. 2004, Cabral et al. 2012a, b). The sequences obtained were then blasted in GenBank.

For the molecular detection of *Ilyonectria* spp. from grafted cuttings and plants, DNA extraction from 0.2 g of necrotic roots and multiplex nested-PCR reactions were carried out as described for liquid and peat samples.

Results

Detection of *Ilyonectria* spp. in liquid and peat samples

Ilyonectria liriodendri and *I. macrodidyma*-complex were detected in hydration tanks, scissors, omega-cut grafting machines, or in peat for callusing, *I. macrodidyma*-complex being the most frequent (Table 3.1 and Figure 3.1). However, *"C." pauciseptatum* was not detected. In nursery A, *I. liriodendri* was detected in 5 of 28 samples from hydration tanks and in 2 of 5 peat samples, while *I. macrodidyma*-complex was detected in 10 of 28 samples from hydration tanks, in 6 of 38 from scissors, and in all peat samples. *Ilyonectria* spp. were not detected from omega-cut grafting machines. In nursery B, *I. liriodendri* was only detected in 2 of 19 samples from hydration tanks, while *I. macrodidyma*-complex was detected in 5 of 19 samples from hydration tanks, in 6 of 23 samples from scissors, in 4 of 16 samples from omega-

cut grafting machines and in 4 of 5 peat samples. None of these pathogens were detected in tap water and peat taken before plant processing.

Table 3.1 Detection of *Ilyonectria liriodendri* and *I. macrodidyma*-complex in samples collected from nurseries at four different propagation stages.

Stage of grapevine propagation process	Nursery A		Nursery B	
	I. liriodendri[a]	*I. macrodidyma*-complex	*I. liriodendri*	*I. macrodidyma*-complex
Hydration tanks	5/28[b]	10/28	2/19	5/19
Scissors	0/38	6/38	0/23	6/23
Omega-cut grafting machines	0/13	0/13	0/16	4/16
Peat for callusing	2/5	5/5	0/5	4/5

[a]Detection was performed by multiplex nested-PCR

[b]Number of samples from which *I. liriodendri* and *I. macrodidyma*-complex were identified/number of total samples evaluated

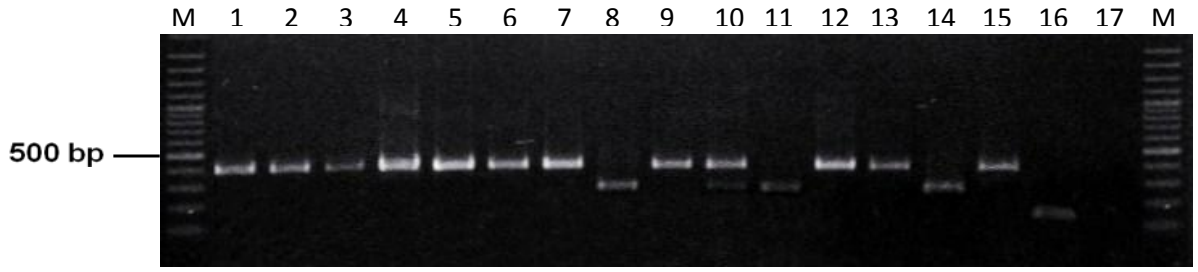

Figure 3.1 Identification of *Ilyonectria liriodendri* and/or *I. macrodidyma*-complex from liquid samples by nested multiplex polymerase chain reaction (PCR). PCR amplification products with three primer pairs: Lir1/Lir2, Mac1/MaPa2, and Pau1/MaPa2. Lanes 1 to 13: liquid samples from scissors sampled in nursery A (Lanes 1 and 2) and in nursery B (Lanes 3 to 5), liquid samples from hydration tanks sampled in nursery A (Lanes 6 to 8) and in nursery B (Lanes 9 to 11), and liquid samples from omega-cut grafting machines sampled in nursery B (Lanes 12 and 13); lanes 14 to 16, positive control of *I. liriodendri*, *I. macrodidyma*-complex and *"Cylindrocarpon" pauciseptatum,* respectively; lane 17, negative control; lanes M, 100-bp DNA ladder.

Isolation and molecular detection of *Ilyonectria* spp. in grafted cuttings and plants

Results of the isolation and detection of *Ilyonectria* spp. from grafted cuttings and plants sampled after callusing and after growth in nursery fields, respectively, are shown in Table 3.2.

After callusing, only *I. torresensis,* was isolated from one grafted cutting in nursery A, while no *Ilyonectria* spp. were isolated in nursery B. However, the use of the multiplex nested-PCR technique allowed the detection of *I. liriodendri* and *I.*

Table 3.2 Isolation and molecular detection of *Ilyonectria* spp. in grapevine nursery plants sampled after callusing stage and after one growing season in nursery fields.

		After callusing		After growth in nursery fields	
Nursery	**Scion/rootstock**	**Isolation[a]**	**Nested PCR**	**Isolation**	**Nested PCR**
A	Cabernet Sauvignon/110R	-	-	*I. torresensis* (2/10)	*I. macrodidyma*-complex (3/10)
	Cardenal/110R	-	*I. macrodidyma*-complex (1/10)[c] *I. liriodendri* (1/10)	*I. liriodendri* (1/10) *I. novozelandica* (3/10)	*I. macrodidyma*-complex (4/10)
	Pardina/110R	-	*I. macrodidyma*-complex (2/10) *I. liriodendri* (2/10)	*I. liriodendri* (3/10) *I. novozelandica* (1/10) *I. torresensis* (1/10)	*I. macrodidyma*-complex (1/10)
	Mazuelo/110R	*I. torresensis* (1/10)[b]	-	*I. novozelandica* (2/10)	*I. liriodendri* (1/10) *I. macrodidyma*-complex (4/10)
	Victoria/140Ru	-	*I. macrodidyma*-complex (7/10)	*I. liriodendri* (2/10) *I. novozelandica* (1/10) *I. torresensis* (1/10)	*I. liriodendri* (5/10) *I. macrodidyma*-complex (6/10)
B	Tintorera/SO4	-	*I. macrodidyma*-complex (1/10)	*I. liriodendri* (2/10) *I. novozelandica* (1/10)	*I. liriodendri* (1/10) *I. macrodidyma*-complex (4/10)
	Bobal/161.49	-	*I. macrodidyma*-complex (1/10)	*I. novozelandica* (3/10)	-
	Airén/420	-	-	*I. liriodendri* (2/10) *I. torresensis* (3/10)	*I. liriodendri* (7/10) *I. macrodidyma*-complex (9/10)
	Red Globe/140Ru	-	*I. macrodidyma*-complex (3/10)	*I. liriodendri* (1/10) *I. novozelandica* (2/10) *I. torresensis* (2/10)	*I. liriodendri* (3/10) *I. macrodidyma*-complex (7/10)
	Airén/41B	-	-	*I. liriodendri* (2/10) *I. novozelandica* (2/10) *I. torresensis* (1/10)	*I. liriodendri* (7/10) *I. macrodidyma*-complex (9/10)

[a]Identification was confirmed by sequencing part of the histone H3 gene (HIS) using CYLH3F and CYLH3R primers

[b]Number of plants from which *Ilyonectria* spp. were isolated/number of total plants evaluated

[c]Number of plants from which *Ilyonectria* spp. were detected/number of total plants evaluated

macrodidyma-complex in both nurseries with higher frequency. In nursery A, *I. liriodendri* was detected from 3 of 50 grafted cuttings, belonging to two different scion/rootstock combinations, and *I. macrodidyma*-complex was detected from 10 of 50 grafted cuttings belonging to three scion/rootstock combinations. In nursery B, *I. macrodidyma*-complex was detected from 5 of 50 grafted cuttings belonging to three scion/rootstock combinations.

After a growth season in nursery fields, *I. liriodendri*, *I. novozelandica* and *I. torresensis* were isolated in both nurseries, *I. novozelandica* being the most frequent species. In nursery A, *I. liriodendri* was isolated from 6 of 50 plants belonging to three scion/rootstock combinations, *I. novozelandica* was isolated from 7 of 50 plants belonging to four scion/rootstock combinations and *I. torresensis* was isolated from 4 of 50 plants belonging to three scion/rootstock combinations. In nursery B, *I. liriodendri* was isolated from 7 of 50 plants belonging to four scion/rootstock combinations, *I. novozelandica* was isolated from 8 of 50 plants analyzed belonging to four scion/rootstock combinations and *I. torresensis* was isolated from 6 of 50 plants analyzed belonging to three scion/rootstock combinations. Regarding the detection of *Ilyonectria* spp. by multiplex nested-PCR, *I. liriodendri* and *I. macrodidyma*-complex were detected in both nurseries more frequently than by isolation. In nursery A, *I. liriodendri* was detected from 6 of 50 plants, belonging to two different scion/rootstock combinations, and *I. macrodidyma*-complex was detected from 18 of 50 plants belonging to all scion/rootstock combinations analyzed. In nursery B, *I. liriodendri* was detected from 18 of 50 plants belonging to four different scion/rootstock combinations, and *I. macrodidyma*-complex was detected from 29 of 50 plants belonging to four different scion/rootstock combinations.

Co-infections caused by *I. liriodendri* and *I. macrodidyma*-complex were detected both immediately after callusing and after a growth season in nursery fields.

Discussion

Previous studies had detected Petri disease pathogens such as *Ca. luteo-olivacea*, *Pa. chlamydospora* and *Phaeoacremonium* spp., as well as Botryosphaeriaceae spp. at

different stages of the grapevine nursery process or in propagation material (Aroca et al. 2010; Gramaje et al. 2011; Spagnolo et al. 2011).

The present study demonstrates for the first time that inoculum of *Ilyonectria* spp. is also present at the different stages of the grapevine nursery propagation process. We detected *I. liriodendri* and *I. macrodidyma*-complex in hydration tanks, omega-cut grafting machines, scissors and/or callusing peat from two different nurseries by multiplex nested-PCR, *I. macrodidyma*-complex being the most frequent. These pathogens were not detected in tap water and peat before use at their respective stages, which indicates that they were present in water and peat after being in contact with infected planting material. This is in concordance with the results obtained by Aroca et al. (2010) and Gramaje et al. (2011), in commercial Spanish grapevine nurseries, who detected *Ca. luteo-olivacea*, *Pa. chlamydospora*, *Pm. aleophilum* and *Pm. parasiticum* in hydration tanks, omega-cut grafting machines, scissors and peat for callusing during the grapevine propagation process by multiplex nested-PCR or by isolating them on culture medium, but no from water and peat before use.

The high potential risk of mother vines as an inoculum source of fungal grapevine trunk pathogens in the vegetative propagation process has been demonstrated (Pascoe and Cottral, 2000; Feliciano and Gubler, 2001; Edwards et al. 2003). Thus, water used in hydration tanks to soak cuttings could be contaminated by field-acquired microorganisms on the bark of the bud sections dispersing into the tank being also a potential inoculum source (Waite and May, 2005, Aroca et al. 2010; Gramaje et al. 2011). Moreover, buds of hydrated canes are manually removed with scissors, and rootstock and scion cuttings are mechanically-grafted with an omega-cut grafting machine causing wounds which make the propagation material very susceptible to infection by fungal trunk pathogens (Gramaje and Armengol, 2011). According to this, our results suggest that infections caused by *Ilyonectria* spp. can also occur during these stages of the nursery propagation process.

Moreover, in this work we confirmed that during the rooting phase in nursery fields the number of plants infected with black-foot pathogens increases markedly. By isolation on culture media, only one *I. torresensis* isolate was obtained from one of the cuttings sampled immediately after callusing. However, after one growing season in nursery fields, *I. liriodendri, I. novozelandica* and *I. torresensis* were more frequently isolated from rooted plants in both nurseries. These results are in agreement with those

obtained by Halleen et al. (2003) who demonstrated that in South African grapevine nurseries less than 1% of the callused cuttings are infected with *Ilyonectria* spp. prior to planting in nursery soils for rooting, whereas 50% or more of the plants are infected by the end of the season. Similar results have been obtained in Spain with Petri disease pathogens such as *Pa. chlamydospora* and *Pm. aleophilum*, which are more frequently isolated from grafted plants after plantation in nursery fields (Giménez-Jaime et al. 2006).

Regarding the molecular detection of *Ilyonectria* spp. on grafted cuttings and plants, a greater number of positive samples were found in both nurseries, as well as in the two different sampling times. This could be due to the fact that these pathogens may require a higher level of host colonization to be detected by isolation on culture media (Aroca and Raposo, 2007; Aroca et al. 2010) and to the high sensitivity of the multiplex nested-PCR technique (minimum of 100 pg of genomic DNA for "*C.*" *pauciseptatum*, 10 pg for *I. liriodendri* and 100 fg for *I. macrodidyma*-complex) (Alaniz et al. 2009). Moreover, another advantage of the multiplex nested-PCR technique was its rapidity. The analysis was performed in 6 hours, while culturing and subsequent fungal isolation took between 10 and 20 days and misidentification was not ruled out. Thus, the multiplex nested-PCR technique will be useful for the detection of these pathogens in a high throughput manner from many nurseries and planting material.

All together, these results demonstrate that the soil in the nursery fields, where grafting plants are planted once the grafting propagation process is over, is an important source of inoculum for *Ilyonectria* spp. During this period, the susceptible basal ends of most of the nursery grafted cuttings are partly or even fully exposed. The young callus roots also break during the planting process, resulting in small wounds susceptible to infection by *Ilyonectria* spp., which are common and may be isolated as soil inhabitants, saprobes on dead plant material, root colonizers or pathogens, or weak pathogens of various herbaceous and woody plants (Brayford, 1993; Halleen et al. 2003, 2007). Recently, *Ilyonectria* spp. have been detected in natural soils from grapevine nurseries in Spain by using bait plants (Agustí-Brisach et al. 2012).

It is interesting to note that species belonging to *I. macrodidyma*-complex were the most frequently detected. These results agree with those obtained by Alaniz et al. (2007), who reported the prevalence of the *I. macrodidyma*-complex associated with black-foot disease in Spain. This fungal complex was also isolated frequently in Spain

from roots of weeds and bait plants grown in grapevine rootstock mother fields, nursery fields and commercial vineyards (Agustí-Brisach et al. 2011, 2012).

The use of propagation material infected with *Ilyonectria* spp. is contributing to the low percentage survival of vine plants observed in the last few years in Spain (Giménez-Jaime et al. 2006; Gramaje et al. 2009), as well as the dissemination of these pathogens. In this context, a sanitation program is required to improve the quality of grapevine planting material. Chemical control with fungicides (Fourie and Halleen, 2006; Rego et al. 2006; Halleen et al. 2007; Alaniz et al. 2011) and biological methods such as the use of *Trichoderma* (Fourie and Halleen, 2006; Halleen et al. 2007), arbuscular mycorrhizae (Petit and Gubler, 2006; Bleach et al. 2008) or soil biofumigation (Bleach et al. 2009) can be used as a strategy to decrease the incidence and severity of *Ilyonectria* spp. during the nursery propagation process. Finally, standard hot water treatment protocols at 50ºC for 30 min may be sufficient to control *Ilyonectria* spp. in grapevine propagation material (Gramaje et al. 2010). Thus, an integrated management program that includes biological, chemical, hot water treatment and other control measures would ensure high phytosanitary quality plant production with low levels of infection.

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Chapter 4

Detection of black-foot and Petri disease pathogens in soils of grapevine nurseries and vineyards using bait plants

Carlos Agustí-Brisach[1], David Gramaje[2], José García-Jiménez[1] and Josep Armengol[1]

[1]Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain.

[2]Department of Crop Protection, Institute for Sustainable Agriculture (IAS), Spanish National Research Council (CSIC), Alameda del Obispo s/n, APDO. 4084, 14004 Córdoba, Spain.

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Keywords

Cadophora luteo-olivacea, *Ilyonectria* spp., *Phaeoacremonium* spp., *Phaeomoniella chlamydospora*, soilborne inoculum

Abstract

Background and Aims Little information is currently available regarding the number of species of black-foot and Petri disease pathogens present in soil and their capacity to infect grapevine roots and reach the xylem vessels.

Methods Seedlings of grapevine rootstock 41-B, and cvs. Bobal and Palomino were planted both in pots containing soil samples collected from commercial vineyards and in nursery fields. Roots and xylem vessels were later analyzed for fungal isolation.

Results Black-foot pathogens: *Ilyonectria alcacerensis, I. macrodidyma*, *I. novozelandica* and *I. torresensis* were frequently isolated from roots of seedlings grown in all soils evaluated, whereas Petri disease pathogens: *Cadophora luteo-olivacea*, *Phaeoacremonium aleophilum*, *Pm. parasiticum* and *Phaeomoniella chlamydospora* were only isolated from xylem vessels of seedlings grown in nursery soils, with a low incidence. *Ilyonectria alcacerensis, I. novozelandica* and *I. torresensis* were isolated for the first time from grapevines in Spain, and *Pm. parasiticum* and *Ca. luteo-olivacea* were detected for the first time in nursery soils.

Conclusions Our results confirm nursery and vineyard soils as an important inoculum source for black-foot pathogens and demonstrate the presence of several Petri disease pathogens in nursery soils.

Introduction

Grapevine fungal trunk diseases are among the most destructive either affecting grapevine propagation material, newly planted vines or established vineyards. They have been reported in most grapevine production regions over the world and are responsible for loss of productivity and vine death. Among them, special emphasis has been placed on black-foot and Petri diseases, which cause the decline of young vines (Oliveira et al., 2004; Gramaje and Armengol, 2011).

Based on current data, black-foot is caused by several species belonging to the genera *Campylocarpon* Halleen, Schroers & Crous, *Cylindrocarpon* Wollenw. and *Ilyonectria* P. Chaverri & C. Salgado (Halleen et al., 2004; Halleen et al., 2006a and 2006b; Schroers et al., 2008; Chaverri et al., 2011; Cabral et al., 2012a and 2012b).

External symptoms of this disease include stunted growth, reduced vigor, retarded or absent sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins, wilting, and dieback. Characteristic symptoms of vines affected by black-foot disease are sunken necrotic root lesions with a reduction in root biomass and root hairs (Halleen et al., 2006a).

Petri disease is caused by *Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams, and numerous species of the genus *Phaeoacremonium* W. Gams, Crous & M. J. Wingf. (Mostert et al., 2006). Moreover, *Cadophora luteo-olivacea* (F.H. Beyma) Harrington & McNew has recently been shown to be quite common on grapevines affected by Petri disease in several viticultural areas worldwide (Rooney-Latham, 2005; Halleen et al., 2007; Gramaje et al., 2011; Navarrete et al., 2011). Field symptoms of Petri disease affected vines are frequently indistinguishable from those of black-foot. Nevertheless, dissected vines affected by Petri disease show a typical black discoloration of the xylem vessels, which is result of tyloses, gums, and phenolic compounds formed inside these vessels by the host in response to the fungus growing in and around the xylem vessels (Mugnai et al., 1999).

Control of these diseases in grapevines is problematic. Management strategies recommend the use of healthy planting material as well as the prevention of pruning wound infections (Gramaje and Armengol, 2011). Over the last decade, several studies have demonstrated the role of infected propagation material (Retief et al., 2006; Halleen et al., 2007; Aroca et al., 2010; Gramaje and Armengol, 2011; Gramaje et al., 2011; Serra et al., 2011) as a primary source of inoculum of black-foot or Petri disease pathogens in vineyards. Grapevines can also be infected by aerial inoculum of Petri disease pathogens through pruning wounds (Eskalen et al., 2007; Quaglia et al., 2009; Rolshausen et al., 2010).

Additionally, considerable effort has been placed by researchers on studying the soil as a potential source of inoculum for these pathogens. *Pa*. *chlamydospora* is considered a soilborne pathogen due to its ability to produce chlamydospores in culture (Bertelli et al., 1998; Mugnai et al., 1999; Sidoti et al., 2000). Moreover, this species was detected in vineyard soils in New Zealand by nested-Polymerase Chain Reaction (PCR) (Whiteman et al., 2002) and in South Africa by species-specific PCR (Damm and Fourie, 2005) and nested-PCR (Retief et al., 2006). Ridgway et al. (2005) used a nested-PCR/Restriction Fragment Length Polymorphism procedure to determine the

persistence of viable and nonviable spores of *Pa. chlamydospora* in soil and observed that nonviable spores were not detected after 8 weeks of being inoculated into dried soil, whereas viable spores could still be detected at 17 weeks after inoculation, indicating that spores could persist in soil over time. Eskalen et al. (2001) confirmed the presence of *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingfield & L. Mugnai in soil by nested-PCR. This species was also recovered from soil and standing water under grapevine drip systems (Rooney et al., 2001). *Campylocarpon*, "*Cylindrocarpon*" 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 openings (Fourie and Halleen, 2006; Halleen et al., 2006a and 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).

Given these findings, the soil could be considered as a source of inoculum for black-foot and Petri disease pathogens. However, very little information is currently available regarding their quantity and incidence in soil and their capacity to infect grapevine roots and reach the xylem vessels. Thus, in this work we used seedlings of a grapevine rootstock and cultivars as bait plants with the following objectives: (i) to determine the presence of black-foot and Petri disease pathogens in the soil of grapevine rootstock mother fields, open-root field nurseries and commercial vineyards, (ii) and to confirm the capacity of soilborne inoculum of these pathogens to naturally infect these plants.

Materials and methods

Planting material

Seeds obtained from grapevine rootstock 41-B Millardet-Grasset and from cvs. Bobal and Palomino were planted individually in 220 cm^3 plastic pots containing sterilized peat moss and incubated in a growth chamber at 25ºC. Two-month old seedlings were used for the greenhouse or field experiments when three to four leaves had emerged.

Greenhouse experiments

Soil sampling and experimental design

Soil samples were collected in June 2010 in ten commercial vineyards from Albacete, Castellón, Murcia and Valencia provinces in eastern Spain (Table 4.1). In each field, the soil samples consisted of five sub-samples, which were taken at different locations in a "W" shaped pattern at a depth of 5–30 cm, at least 10 cm from the trunk of grapevines. These samples were thoroughly mixed into one compound sample that was split into four replicates which were used to fill four plastic pots (18 cm diameter). Eight seedlings of grapevine cv. Palomino (two seedlings per pot) were planted in July 2010. Additionally, eight seedlings were planted as control plants in four pots which were filled with sterilized peat moss. All plants were grown in a temperature-controlled greenhouse (25-30ºC), and irrigated twice a week. Nine months later, after one growing season, sprouted plants were carefully dug out from the soil to keep the root system intact and taken back to the laboratory for immediate processing.

Field experiments

Experimental design

Seedlings were planted in June 2009 in a grapevine rootstock mother field and in an open-root field nursery in Aielo de Malferit and Rotglà i Corberà, respectively, both in Valencia province (Spain). In each field, plant groups (10 seedlings) were spaced 100 cm from other groups, with seedlings 50 cm apart from center to center and an interrow spacing of 100 cm. Each field plot was 7 m long and included 4 rows, each with 3 seedling groups (120 seedlings per field). In both sites, the experimental design consisted of four rows with three randomized blocks per row, each containing 10 seedlings of 41-B rootstock, and cvs. Bobal and Palomino. Standard cultural practices were used in both sites, but plants were never pruned.

Additionally, ten seedlings of each 41-B rootstock, and cvs. Bobal and Palomino were planted as control plants in pots (18 cm diameter), which were filled with sterilized peat moss. These plants were grown in a temperature-controlled greenhouse (25-30ºC), and irrigated twice a week.

Plant sampling

Plants were collected and analyzed at two different sampling moments. Nine months after planting (March 2010), sprouted plants from two rows in both fields, and from five control pots per type of planting material were carefully dug out from the soil to keep the root system intact and taken back to the laboratory for immediate processing. Twenty-one months after planting (March 2011), sprouted plants from the two remaining rows in both fields and from the five remaining control pots were sampled as described before.

Fungal isolation and identification

Black-foot disease pathogens

In order to isolate black-foot pathogens, root sections were cut from necrotic areas, washed under running tap water, surface-disinfested for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Small root pieces were plated on potato dextrose agar (PDA) (Biokar-Diagnostics, Zac de Ther, France) supplemented with 0.5 g l^{-1} of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA) (PDAS). Plates were incubated for 10-15 days at 25ºC in the dark and all colonies were transferred to PDA. Isolates were single-spored prior to morphological and molecular identification with the serial dilution method (Dhingra and Sinclair, 1995).

Species of *Ilyonectria* were identified morphologically by macroscopic characters including colony texture, color, and the shape of the growing margin on PDA. Conidia were observed and measured on Spezieller Nährstoffarmer Agar (SNA) with the addition of a 1×1 cm piece of filter paper to the colony surface (Alaniz *et al*., 2007; Chaverri et al. 2011).

For DNA extraction, 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, USA) following manufacturer's instructions. DNA was visualized on 0.7% agarose gels stained with ethidium bromide and was stored at –20ºC.

The identification of *Ilyonectria* spp. was confirmed by sequencing part of the histone H3 gene (HIS) using CYLH3F and CYLH3R primers (Crous et al., 2004, Cabral et al., 2012a and 2012b). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany), sequenced in both directions by Macrogen Inc. (Seoul, Republic of Korea). The sequences obtained were then blasted in GenBank.

Petri disease pathogens

In order to recover Petri disease pathogens, isolations were made from sections (10 cm long) that were cut from the basal stem and disinfected as previously described. Small pieces of internal xylem tissues were plated on malt extract agar (MEA) (Oxoid Ltd., Basingstoke, Hants, England) supplemented with 0.5 g l^{-1} of streptomycin sulphate (MEAS). Plates were incubated for 10-15 days at 25ºC in the dark and all colonies were transferred to PDA. Isolates were single-spored prior to morphological and molecular identification as described before.

Phaeomoniella chlamydospora and *Phaeoacreomonium* spp. were identified by conidiophore morphology, conidial size and shape, and its cultural characteristics (radial growth after 8 days at 25ºC, cardinal temperatures for growth and colony colors) on PDA and MEA (Crous and Gams, 2000). *Cadophora luteo-olivacea* was identified by conidiophore morphology, size of phialides and conidia, and cultural characteristics (radial growth after 8 days at 25ºC, cardinal temperatures for growth, colony colors and pigment production) on MEA, PDA and oatmeal agar (Gams, 2000; Harrington and McNew, 2003; Gramaje et al., 2011).

DNA extractions were carried out as previously described. Identification of *Ca. luteo-olivacea* isolates was confirmed by sequencing the Internal Transcribed Spacer (ITS) region of rDNA amplified using the fungal universal primers ITS1F and ITS4 (Gardes and Bruns, 1993), and *Phaeomoniella chlamydospora* was identified by using specific primers Pch1-Pch2 (Tegli et al., 2000). PCR products were purified and sequenced as described before. The sequences obtained were then blasted in GenBank. Identification of *Phaeoacremonium* spp. isolates was confirmed by sequencing of the β-tubulin (TUB) gene fragment using primers T1 and Bt2b (Dupont et al., 2000) 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).

Results

Greenhouse experiments

No xylem necroses were observed in all plants sampled but roots showed slight discolorations or necrotic lesions. Controls appeared healthy with no discolorations.

Results of the incidence and isolation of black-foot pathogens are shown in Table 4.1. *Ilyonectria* spp. were isolated from roots of plants in all soils evaluated. These isolates were identified as *Ilyonectria alcacerensis* A. Cabral, Oliveira & Crous, *I. novozelandica* A. Cabral & Crous and *I. torresensis* A. Cabral, Rego & Crous. *Ilyonectria alcacerensis* and *I. torresensis* were isolated from plants grown in most of the soils evaluated, with the exception of plants grown in soil from vineyard 1 in which *I. novozelandica* and *I. torresensis* were found, and vineyard 5, in which only *I. novozelandica* was isolated. Mean incidence of *Ilyonectria* spp. on infected plants ranged from 12.5 to 100%, while mean percentage of isolation ranged from 0.9 to 38.4%. In general, soils from vineyards located in Valencia province showed the highest mean incidences and percentages of isolation. Black-foot disease pathogens were not isolated from control plants.

Petri disease pathogens were not isolated from any plant including the controls.

Field experiments

In general, at both sampling times, roots of the plants showed slight discolorations or necrotic areas. These lesions were more abundant twenty-one months after planting. Xylem necroses were only observed in few of them. No symptoms were observed in control plants and black-foot and Petri disease pathogens were not isolated from them.

Table 4.1. Incidence and isolation of *Ilyonectria* spp. from grapevine seedlings of cv. Palomino nine months after plantation in pots filled with soil from ten different vineyards.

	Geographical origin				
Vineyard	**Location**	**Province**	**Species[x]**	**Incidence[y]**	**Isolation[z]**
1	Albacete	Albacete	*I. novozelandica*	12.5	0.9
			I. torresensis	12.5	0.9
2	Les Useres	Castellón	*I. alcacerensis*	37.5	3.6
			I. torresensis	25.0	2.7
3	Les Useres	Castellón	*I. alcacerensis*	37.5	2.7
			I. torresensis	25.0	4.5
4	Vilanova d'Alcolea	Castellón	*I. alcacerensis*	50.0	5.4
			I. torresensis	25.0	2.7
5	Jumilla	Murcia	*I. novozelandica*	25.0	2.7
6	Yecla	Murcia	*I. alcacerensis*	62.5	11.6
			I. torresensis	12.5	0.9
7	Requena	Valencia	*I. alcacerensis*	62.5	19.6
			I. torresensis	37.5	2.7
8	Requena	Valencia	*I. alcacerensis*	100	31.3
			I. torresensis	62.5	9.8
9	Requena	Valencia	*I. alcacerensis*	100	38.4
			I. torresensis	50.0	6.3
10	Aielo de Malferit	Valencia	*I. alcacerensis*	87.5	31.3
			I. torresensis	20.0	1.8

[x] Species were identified using morphological and molecular methods.

[y] Mean incidence (%) of infected seedlings (8 seedlings for each commercial vineyard soil)

[z] Mean percentage of root segments which *Ilyonectria* spp. were isolated (112 root fragments for each commercial vineyard soil; 14 segments per seedling)

Rootstock mother field

Results of the incidence and isolation of black-foot pathogens in the rootstock mother field are shown in Table 4.2. Nine months after planting four *Ilyonectria* spp., named *I. alcacerensis, I. macrodidyma* (Halleen, Schroers & Crous) P. Chaverri & C. Salgado, *I. novozelandica* and *I. torresensis*, were isolated from the roots of cv. Bobal. In roostock 41-B, three *Ilyonectria* spp., *I. alcacerensis, I. novozelandica* and *I. torresensis* were isolated from the roots, while only *I. alcacerensis* was detected on cv. Palomino. Mean incidence of these pathogens ranged from 5.0 to 55.0% of infected plants and mean percentage of isolation ranged from 0.7 to 7.5%. Twenty-one months after planting, only the rootstock 41-B was evaluated in this field because plants of cvs. Bobal and Palomino died due to the very low and unusual temperatures in winter. *Ilyonectria alcacerensis, I. novozelandica* and *I. torresensis* were isolated from roots of rootstock 41-B, with a higher mean incidence in comparison with the first sampling moment, which ranged from 10.0 to 90.0%. The mean percentage of isolation was also higher, ranging from 1.1 to 22.5%.

Petri disease pathogens were isolated at very low frequency. Three isolates of *Ca. luteo-olivacea* and one isolate of *Pm. aleophilum* were obtained nine months after planting, all from rootstock 41-B. Twenty-one months after planting, three isolates of *Pm. aleophilum* and one isolate of *Pa. chlamydospora* were obtained also from rootstock 41-B.

Table 4.2. Incidence and isolation of *Ilyonectria* spp. from seedlings of grapevine rootstock 41-B, and cvs. Bobal and Palomino, nine or twenty-one months after plantation in a rootstock mother field and in an open root field nursery.

Rootstock mother field					
Grapevine Seedlings	**Species[w]**	**Nine months**		**Twenty-one months**	
		Incidence[x]	**Isolation[y]**	**Incidence**	**Isolation**
41-B	*I. alcacerensis*	55.0	7.5	90.0	22.5
	I. macrodidyma	0.0	0.0	15.0	1.4
	I. novozelandica	10.0	0.7	0.0	0.0
	I. torresensis	5.0	0.7	10.0	1.1
Bobal	*I. alcacerensis*	40.0	7.5	n/d[z]	n/d
	I. macrodidyma	10.0	1.1	n/d	n/d
	I. novozelandica	5.0	1.1	n/d	n/d
	I. torresensis	5.0	1.1	n/d	n/d
Palomino	*I. alcacerensis*	50.0	7.5	n/d	n/d
Open-root field nursery					
Grapevine Seedlings	**Species**	**Nine months**		**Twenty-one months**	
		Incidence	**Isolation**	**Incidence**	**Isolation**
41-B	*I. alcacerensis*	0.0	0.0	37.5	4.5
	I. macrodidyma	0.0	0.0	37.5	3.6
	I. novozelandica	10.0	1.4	50.0	3.6
Bobal	*I. alcacerensis*	0.0	0.0	13.3	1.4
	I. macrodidyma	0.0	0.0	6.7	1.0
	I. novozelandica	13.3	1.0	6.7	1.0
Palomino	*I. alcacerensis*	8.0	2.0	0.0	0.0
	I. novozelandica	28.0	3.1	41.2	2.9
	I. torresensis	12.0	4.4	0.0	0.0

[w] Species were identified using morphological and molecular methods.

[x] Mean incidence (%) of infected seedlings (20 seedlings for each rootstock or cultivar per growing season)

[y] Mean percentage of root segments from which *Ilyonectria* spp. were isolated (280 root fragments per rootstock or cultivar in each growing season; 14 segments per seedling)

[z] Not determined

Open-root field nursery

Results of the isolation of black-foot pathogens in the open-root field nursery are shown in Table 4.2. Nine months after planting, three *Ilyonectria* spp., named *I. alcacerensis, I. novozelandica* and *I. torresensis*, were isolated from roots of cv. Palomino, while in rootstock 41-B and cv. Bobal only *I. novozelandica* was isolated. Mean incidence ranged from 8.0 and 28.0% of infected plants and mean percentage of

isolation ranged from 1.0 to 4.4%. Twenty-one months after planting, *I. alcacerensis, I. macrodidyma* and *I. novozelandica* were isolated from both rootstock 41-B and cv. Bobal. Nevertheless, in cv. Palomino only *I. novozelandica* was isolated. In general, twenty-one months after planting the mean incidence of *Ilyonectria* spp. increased, ranging from 6.7 and 50.0%, and the mean percentage of isolation ranged from 1.0 to 4.5%.

Regarding Petri disease pathogens, nine months after planting only four isolates of *Ca. luteo-olivacea* (two from cv. Bobal and two from cv. Palomino) and three isolates of *Pm. parasiticum* (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf. (from cv. Palomino) were obtained. Twenty-one months after planting, two isolates of *Ca. luteo-olivacea* (from 41-B rootstock and cv. Palomino) and one isolate of *Pm. aleophilum* (from cv. Bobal) were obtained.

Discussion

To our knowledge, this is the first study to evaluate the incidence of black-foot and Petri disease pathogens in natural nursery and vineyard soils using bait plants. *Ilyonectria* spp. were frequently isolated from plants grown in all soils evaluated, whereas the causal agents of Petri disease were only isolated from nursery soils, with a low incidence.

In our work, four *Ilyonectria* spp. were found. *Ilyonectria macrodidyma* was already reported in Spain as the predominant species associated with black-foot disease, being present in all grapevine growing regions studied (Alaniz et al., 2007). Recently, this species was also isolated frequently from roots of weeds collected in Spanish vineyards (Agustí-Brisach et al., 2011). This study represents the first report of *I. alcacerensis, I. novozelandica* and *I. torresensis* isolated from grapevines in Spain. To date, *Ilyonectria* spp. had been reported in most grapevine-producing areas world-wide (Halleen et al., 2006a; Cabral et al., 2012b). Recently, Cabral et al. (2012b), revealed the existence of polymorphism in a wide collection of isolates previously identified as *I. macrodidyma* by using ITS, TUB, HIS and translation elongation factor 1-α sequence analysis. Consequently, six new species of *Ilyonectria*, which are morphologically rather similar, were recognised in the *I. macrodidyma* complex. Four of this species

were named as *I. estremocensis*, *I. alcacerensis*, *I. novozelandica* and *I. torresensis*, while two *Ilyonectria* species have not been named yet (Cabral et al., 2012b). According to our results and to the high levels of diversity detected by Alaniz et al. (2009) in Spanish isolates of *I. macrodidyma* using inter-simple sequence repeat (ISSR) markers, all previous reports of *I. macrodidyma* in Spain (Alaniz et al., 2007; Agustí-Brisach et al., 2011) could be probably different *Ilyonectria* species included into the *I. macrodydima* complex, as stated by Cabral et al. (2012b).

Regarding Petri disease pathogens, isolates of *Ca. luteo-olivacea*, *Pm. aleophilum*, *Pm. parasiticum* and *Pa. chlamydospora* were obtained from xylem vessels of plants grown both in a rootstock mother field and an open-root field nursery. *Ca. luteo-olivacea* had been recently shown to be quite common on grapevines affected by esca and Petri disease in California, South Africa, Uruguay, New Zealand, Northeastern America and Spain (Gramaje and Armengol, 2011). Of the *Phaeoacremonium* spp. occurring in grapevines, *Pm. aleophilum* is the most common and widely distributed species and *Pm. parasiticum* is also encountered frequently (Mostert et al., 2006; Gramaje et al., 2009). *Pa. chlamydospora* is regarded as the most important fungal organism associated with Petri disease because of its predominance in affected grapevines (Ridgway et al., 2005; Mostert et al., 2006; Serra et al., 2011). Among them, only *Pm. aleophilum* (Eskalen et al., 2001; Rooney et al., 2001) and *Pa. chlamydospora* (Whiteman et al., 2002; Retief et al., 2006) were previously detected in vineyard soils by using molecular methods. Consequently, this is the first report of the presence of *Pm. parasiticum* and *Ca. luteo-olivacea* in grapevine nursery soils.

To date, pruning wounds (Eskalen et al., 2007; Rolshausen et al., 2010) and wounds produced during the grapevine propagation process (Gramaje and Armengol, 2011) had been indicated as the main ways for grapevine infections caused by Petri disease pathogens. *Phaeoacremonium* spp. and *Pa. chlamydospora* were re-isolated from plants grown in artificially inoculated soils which were infested by drenching them with spore suspensions (Adalat et al., 2000; Gramaje et al., 2007 and 2008; Aroca et al., 2008; Aroca and Raposo; 2009). However, this is the first study to demonstrate that soilborne inoculum of Petri disease pathogens can also infect grapevines through the root system in naturally infested soils. Although previous studies confirmed the presence of some *Phaeoacremonium* spp. and *Pa. chlamydospora* in soil by molecular techniques (Eskalen et al., 2001; Rooney et al., 2001; Whiteman et al., 2002; Retief et

al., 2006), the positive result by PCR does not necessarily imply that propagules of these fungi were viable for infection.

The mean incidence and isolation of Petri disease pathogens were lower than those obtained for *Ilyonectria* spp., and they were not isolated from plants grown in pots filled with vineyard soils. This could indicate low inoculum densities of Petri disease patoghens in soil or not suitable environmental conditions or a slow infection process. There is no information about how these pathogens penetrate grapevine roots and invade the xylem vessels. It is well known that Petri disease pathogens are slow-growing fungi which usually take up to 20 days to grow on enriched medium, and their ability to compete with other fungi and bacteria arising from plant material is limited (Aroca and Raposo, 2007). Thus, in contrast with *Ilyonectria* spp., probably more time would be needed for the soilborne inoculum of Petri disease pathogens to infect grapevine planting material. In addition, several authors already demonstrated the presence of *Phaeoacremonium* spp. and *Pa. chlamydospora* in asymptomatic vines, which indicate that these pathogens can behave as endophytes or latent pathogens until the moment in which grapevines are stressed (Edwards and Pascoe, 2004; Ridgway et al., 2005; Gramaje et al., 2010).

The use of bait plants allowed us to increase the knowledge about the different species of black-foot and Petri disease pathogens present in Spanish soils. Our ability to isolate them from plants grown in nursery and vineyard soils confirms both soil types as important inoculum sources for black-foot pathogens and demonstrates the presence of several Petri disease pathogens in nursery soils. In addition, among them, *Ilyonectria* spp. and *Pa. chlamydospora*, have the ability to produce chlamydospores, possibly as an adaptation for survival in soil for extended periods (Bertelli et al., 1998; Mugnai et al., 1999; Halleen et al., 2004; Chaverri et al 2012). All together, these results emphasize the importance of suitable control measures to prevent or eradicate infections caused by soilborne inoculum of black-foot and Petri disease pathogens, mainly in grapevine nurseries in order to prevent the spread of the diseases. Further research on the epidemiology of these pathogens as well as developing molecular techniques such as qPCR methods for their early and accurate detection, and quantification is therefore important and should aid in finding an effective way to control.

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Chapter 5

Detection and quantification of *Ilyonectria* spp. associated with black-foot disease of grapevine in nursery soils using multiplex, nested PCR and real-time PCR

Carlos Agustí-Brisach[1], Lizel Mostert[2] and Josep Armengol[1]

[1]Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022-Valencia, Spain.

[2]Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Stellenbosch 7602, South Africa.

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Abstract Three nursery fields and three rootstock mother fields from commercial nurseries located in Comunidad Valenciana region (central-eastern Spain) were surveyed in July 2011 to detect the presence and quantify *Ilyonectria* spp. in the soil. In each field, ten soil samples were taken randomly at a depth 10 to 30 cm and 10 to 20 cm from the trunk of grapevines. Three replicate subsamples (10 g each) were taken from each soil sample. DNA was extracted and a multiplex, nested PCR with species-specific primer pairs (Mac1/MaPa2, Lir1/Lir2 and Pau1/MaPa2) was used to identify the species present. Among the 180 soil DNA samples analyzed, *Ilyonectria* spp. were detected in 172 of them. *Ilyonectria macrodidyma* complex was the most frequently detected, being identified in 141 samples from all the fields evaluated. However, *I. liriodendri* was detected in only 16 samples, but was present in all open-root field nurseries and in two rootstock mother fields. In addition, quantitative real-time PCR (qPCR) assays were done to assess the levels of *I. liriodendri* and *I. macrodidyma*-complex DNA in the soil samples. The presence of *Ilyonectria* spp. DNA with qPCR correlated with the fields found positive with the nested, multiplex PCR. DNA concentrations of *Ilyonectria* spp. ranged from 0.004 to 1,904.8 pg μl^{-1}. In general, samples from rootstock mother fields showed the highest DNA concentrations. Our ability to detect and quantify *Ilyonectria* spp. genomic DNA in soil samples from nursery fields and rootstock mother fields confirms soils from both field types as important inoculum sources for black-foot pathogens.

Introduction

Black-foot is an important disease of grapevines which affect grapevine propagation material, newly planted vines or established vineyards in most of the wine-producing countries worldwide (Halleen *et al*., 2006a; Cabral *et al*., 2012b). Over the last decades, the disease has increased in incidence and severity and has been reported as responsible of the decline of young vines, loss of productivity and death of young vines (Oliveira *et al*., 2004; Alaniz *et al*., 2009a). External symptoms of this disease include stunted growth, reduced vigor, retarded or absent sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins, wilting, and dieback. Specific symptoms of the disease are sunken necrotic crown and root lesions, with a reduction in root biomass and root hairs (Halleen *et al*., 2006a; Gramaje & Armengol, 2011).

Based on current data, black-foot is caused by several species belonging to the genera *Campylocarpon* Halleen, Schroers & Crous, "*Cylindrocarpon*" Wollenw. and *Ilyonectria* P. Chaverri & C. Salgado (Halleen *et al*., 2004, 2006a, 2006b; Schroers *et al*., 2008; Chaverri *et al*., 2011; Cabral *et al*., 2012a, 2012b). Described "*Cylindrocarpon"* species associated with black-foot have been renamed in the genus *Ilyonectria* or referred to as "*Cylindrocarpon"*, because true *Cylindrocarpon* species belong to the genus *Neonectria* (Chaverri *et al*., 2011). These species have been frequently isolated from rootstock mother-plants, rooted rootstock cuttings, bench-grafts and young grafted vines (Rumbos & Rumbou, 2001; Halleen *et al*., 2003; Fourie & Halleen, 2004; Oliveira *et al*., 2004; Dubrovsky & Fabritius, 2007).

Nursery soils are the major source of black-foot inoculum, causing infection of grafted vines (Halleen *et al*., 2003, 2007; Chaverri *et al*., 2011). In fact, Halleen *et al*. (2006b) confirmed that new infections are frequent when cuttings come in contact with infected nursery soils during nursery practices such as covering grafted cuttings with soil and, more commonly, after planting of callused cuttings. These findings are in agreement with those obtained recently by Agustí-Brisach *et al*. (2012a), who detected black-foot pathogens at different stages of the grapevine nursery propagation process and confirmed that grapevine planting material is highly infected by *Ilyonectria* spp. after growing in nursery fields. In addition, Agustí-Brisach *et al*. (2011, 2012b) isolated black-foot pathogens from grapevine seedlings used as bait plants grown in a nursery field and in a rootstock mother field, as well as from weeds collected from nursery fields and vineyards.

Campylocarpon, "*Cylindrocarpon*" and *Ilyonectria* species are known to be saprobes in soil, 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 openings (Fourie & Halleen, 2006; Halleen *et al*., 2006a; 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 and remain in the soil when an infected crop is removed (Halleen *et al*., 2004; Probst *et al*., 2012).

Molecular techniques have played an important role in detection of black-foot pathogens in grapevine propagation material as well as young or adult vines. Dubrovski & Fabritius (2007) developed genus-specific PCR primers (Cyl-F and Cyl-R) for the simultaneous detection of "*Cylindrocarpon" liriodendri* and "*C." macrodidymum* in

grapevine nurseries. Soon thereafter, Alaniz *et al*. (2009b) designed species-specific primer pairs (Mac1/MaPa2, Lir1/Lir2 and Pau1/MaPa2) which allow the quick and easy detection of "*C." liriodendri*, "*C." macrodidymum* and "*C." pauciseptatum*. To date, these species-specific primer pairs have been used for the identification of black-foot pathogens as well as for their detection at the different stages of the grapevine propagation process (Agustí-Brisach *et al*., 2011, 2012a, 2012b; Cardoso *et al*., 2012). However, this technique has never been used to detect black-foot pathogens from soil samples.

Soilborne plant pathogens are difficult to identify, detect and quantify from soil and plant roots using conventional isolation techniques (Tewoldemedhin *et al*., 2011). PCR based methods that use pathogen specific primers can detect the presence of specific pathogens in soil and are also less time consuming and labour intensive (Lievens *et al*., 2006). However, conventional PCR based methods do not allow accurate quantification of DNA due to variability in the efficiency of amplification between PCR tubes (Filion *et al*., 2003). Quantitative real-time PCR (qPCR) has the advantage of being more sensitive as well as quantitative in comparison to conventional PCR. Kernaghan *et al*. (2007) developed a qPCR assay to quantify the levels of "*Cylindrocarpon" destructans* f. sp. *panacis* in soils previously cropped with ginseng. Probst *et al*. (2009) developed a qPCR method for detecting "*C". macrodidymum* and "*C*". *liriodendri* in soil and their results showed that the species-specific primers used in this qPCR system could detect as little as 3 pg of pure DNA. Recently, a qPCR method has been developed for the simultaneous detection of "*C." destructans, "C". liriodendri, "C." macrodidymum* and "*C." pauciseptatum* associated with apple tree roots in South Africa (Tewoldemedhin *et al*., 2011).

Soil is an important source of inoculum for black-foot pathogens causing infections in grapevine nursery plants. The occurrence of *Ilyonectria* spp. in the soil of Spanish nurseries and mother fields is unknown. Therefore, the objectives of this study were: (i) to detect the presence of *Ilyonectria* spp. in nursery soils with multiplex, nested PCR and (ii) to quantify the concentration of the *Ilyonectria* spp. in the same DNA samples.

Materials and methods

Field sites and sampling

Three nursery fields (A, B and C) and three rootstock mother fields (D, E and F) from commercial nurseries located in Comunidad Valenciana region (central-eastern Spain) were surveyed in July 2011. Nursery fields, which previously were cultivated with other crops, consisted of one-year-old grapevine cuttings, whereas rootstock mother fields consisted of grapevine rootstocks that were more than 15 years old.

In each field, ten soil samples were taken randomly at a depth 10 to30 cm and 10 to 20 cm from the trunk of grapevines. Three replicate samples (10 g each) were taken from each soil sample. A total of 30 soil samples per field were then evaluated.

Soil DNA extraction

For the direct extraction of DNA from soil samples, they were lyophilized overnight and then, were crushed using FRITSCH Variable Speed Rotor Mill-PULVERISETTE 14 (ROSH, Oberstein, Germany) until all the soil was ground to a fine powder. A total of 250 mg of each air-dried soil sample were placed in 1.5 ml vials with 700 µl warm (65 ºC) extraction buffer (120 mM NaH_2PO_4, 2 % CTAB or SDS, 1.5 mM NaCl, pH 8.0, and 1 % w/v polyvinylpyrrolidone). Vials were then shaken and incubated at 65 ºC for 15 min, followed by centrifugation at 13000 rpm for 10 min. Supernatants (400 µl) were then transferred to new vials and ZR Soil Microbe DNA MiniPrep™ was used for the further steps of DNA extraction. Extracted DNA was kept at −20 °C until its use in PCR amplifications.

Detection of *Ilyonectria* spp. from soil samples with multiplex, nested PCR

A nested PCR combined with a multiplex PCR approach was used for the detection of three pathogens from soil samples. The first PCR reaction was carried out with primers

ITS1F/ITS4 (Gardes & Bruns, 1993) containing 1× PCR buffer, 1.25 mM $MgCl_2$, 80 µM of each dNTP, 0.2 µM of each primer, 0.7 U of BIOTAQ DNA polymerase, and 1 µl of template DNA. The PCR reaction mix was adjusted to a final volume of 25 µl with water. The program consisted of an initial step of 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 45 s. A final extension was performed at 72 °C for 10 min. PCR product was diluted 1:100 and 1 µl was used as template DNA for the secondary PCR which was carried out with species-specific primers Mac1/MaPa2, Lir1/Lir2, and Pau1/MaPa2 (Alaniz *et al*., 2009b) to amplify *I. liriodendri*, *I. macrodidyma*-complex, and "*C.*" *pauciseptatum*, respectively. Each reaction contained 1× PCR buffer, 2.0 mM $MgCl_2$, 80 µM of each dNTP, 0.2 µM of each primer, 0.7 U of Netzyme DNA polymerase, and 1 µl of template DNA. The PCR reaction mix was adjusted to a final volume of 25 µl with water. The program consisted of an initial step of 3 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and elongation at 72 °C for 45 s. A final extension was performed at 72 °C for 10 min.

qPCR detection of *Ilyonectria* species

qPCR analyses for *I. liriodendri* and *I. macrodidyma*-complex were conducted using SensiMix™ SYBR No-Rox Kit (Bioline Ltd., United Kingdom) on a Rotor-Gene™ 6000 real-time rotary analyzer (Qiagen GmbH, Hilden, Germany). The total reaction volume of 20 µl contained 2 µl pure culture DNA extract (10 ng μl^{-1}), 10 µl SensiMix (includes SYBR Green I and $MgCl_2$ at a concentration of 3 mM) and 0.3 µM of each primer YT2F/Cyl-R for the genus *Cylindrocarpon* (Dubrovsky & Fabritius, 2007; Tewoldemedhin *et al*., 2011). Additional $MgCl_2$ was added to attain a final concentration of 4.5 mM. The qPCR reaction mix was adjusted to a final volume of 20 µl with clean water. Each set of qPCR reactions included three replications of each sample and a negative (water) control. All reactions were first denatured at 95 °C for 10 min, followed by 60 cycles of denaturing at 95 °C for 10 s, annealing at 60 °C for 10 s and extension 72 °C for 30 s. The generated qPCR data were analyzed using Rotor-Gene 6000 Series Software 1.7.

Sensitivity of the qPCR assay was assessed using standard curves that were constructed for *I. liriodendri* (DNA from isolate Cy89) and *I. macrodidyma*-complex (DNA from isolate Cy81) species using one isolate per species. Both isolates were obtained from the culture collection of the Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Spain. The standard curve was constructed from nine fold serial dilutions of pure culture DNA from target taxon, yielding concentrations from 20 ng μl^{-1} to 5.12 fg μl^{-1}, with three replicates per concentration. The standard curves were generated by plotting cycle threshold (C_q) values obtained for each specific DNA concentration, versus the log of the initial concentration of species DNA. The standard curves were also used to quantify the amount of pathogen DNA from soil material, based on C_q values obtained in the soil DNA qPCR assays.

To investigate the presence of PCR inhibitors in the extracted root DNA a constant amount (10 ng) of *I. liriodendri* DNA was added to serial dilutions of soil DNA from uninfected soil samples which did not show any amplification with the nested PCR. The degree to which the quantification cycles (C_q)-values differed between the serial dilutions was used to determine inhibition.

The same DNA samples that were used for the multiplex, nested PCR were again used for qPCR. Two µl DNA extracted from soil (10 ng μl^{-1}) was used in each reaction. The amount of *Cylindrocarpon* DNA in the soil was quantified using the standard curves for both *I. liriodendri* and *I. macrodidyma*-complex. The mean DNA concentration and the standard deviation were determined from three DNA repeats per soil sample.

Results

Detection of *Ilyonectria* spp. from soil samples with multiplex, nested PCR

Ilyonectria spp. were detected in 172 out of 180 soil DNA samples evaluated. *Ilyonectria liriodendri* and *I. macrodidyma*-complex were the only species found, with the *I. macrodidyma*-complex being the most frequent (Tables 5.1 and 5.2)

Table 5.1. Fungal identification (*Ilyonectria liriodendri* and *I. macrodidyma*-complex) and mean pathogen DNA concentrations detected through nested multiplex PCR and qPCR analyses, respectively, of DNA obtained from three nursery field soils.

	Field A		Field B		Field C	
Sample [a]	Fungal identification with nested PCR[b]	Mean pathogen DNA[c] (pg µl^{-1})[d] ± STD[e]	Fungal identification with nested PCR	Mean pathogen DNA (pg µl^{-1}) ± STD	Fungal identification with nested PCR	Mean pathogen DNA (pg µl^{-1}) ± STD
1.1	*I. macrodidyma*-complex	2.45 ± 2.11	Both species	*	*I. macrodidyma*-complex	9.89 ± 4.16
1.2	*I. macrodidyma*-complex	7.08 ± 4.08	*I. liriodendri*	0.02 ± 0.0021	*I. macrodidyma*-complex	24.27 ± 8.87
1.3	Both species[f]	11.64 ± 6.88	Both species	28.74 ± 9.28	*I. macrodidyma*-complex	20.71 ± 7.77
2.1	Both species	16.21 ± 9.76	*I. macrodidyma*-complex	305.46 ± 115.96	*I. macrodidyma*-complex	83.31 ± 22.91
2.2	*I. macrodidyma*-complex	*[h]	*I. macrodidyma*-complex	129.88 ± 19.51	*I. macrodidyma*-complex	34.01 ± 2.52
2.3	*I. macrodidyma*-complex	20.77 ± 12.66	*I. macrodidyma*-complex	12.66 ± 4.53	*I. macrodidyma*-complex	114.18 ± 30.06
3.1	*I. macrodidyma*-complex	*	*I. liriodendri*	0.17 ± 0.22	Both species	17.04 ± 5.35
3.2	*I. liriodendri*	25.33 ± 15.58	*I. macrodidyma*-complex	0.89 ± 0.98	*I. macrodidyma*-complex	9.57 ± 5.11
3.3	-[g]	29.89 ± 18.49	-	*	*I. macrodidyma*-complex	45.33 ± 21.99
4.1	*I. liriodendri*	34.46 ± 21.42	*I. macrodidyma*-complex	122.17 ± 49.14	*I. macrodidyma*-complex	3.57 ± 2.32
4.2	*I. macrodidyma*-complex	39.03 ± 24.35	*I. macrodidyma*-complex	144.55 ± 45.44	*I. macrodidyma*-complex	4.07 ± 1.89
4.3	-	*	*I. macrodidyma*-complex	59.09 ± 25.68	*I. macrodidyma*-complex	7.02 ± 2.92
5.1	*I. macrodidyma*-complex	43.59 ± 27.28	*I. macrodidyma*-complex	9.69 ± 13.27	*I. macrodidyma*-complex	185.69 ± 51.77
5.2	*I. macrodidyma*-complex	*	*I. liriodendri*	*	*I. macrodidyma*-complex	757.79 ± 157.72
5.3	*I. macrodidyma*-complex	48.15 ± 30.21	*I. macrodidyma*-complex	92.96 ± 42.85	*I. macrodidyma*-complex	98.59 ± 9.89
6.1	*I. liriodendri*	51.96 ± 34.39	Both species	*	*I. liriodendri*	0.04 ± 0.04
6.2	*I. liriodendri*	0.007 ± 0.003	Both species	0.05 ± 0.03	*I. macrodidyma*-complex	*
6.3	*I. macrodidyma*-complex	55.76 ± 35.09	*I. macrodidyma*-complex	0.03 ± 0.02	*I. macrodidyma*-complex	14.76 ± 9.28
7.1	*I. macrodidyma*-complex	60.32 ± 38.02	*I. macrodidyma*-complex	6.51 ± 7.15	-	*
7.2	*I. macrodidyma*-complex	64.89± 40.96	*I. macrodidyma*-complex	17.16 ± 3.23	*I. macrodidyma*-complex	0.81 ± 0.68
7.3	*I. macrodidyma*-complex	69.45 ± 43.89	*I. macrodidyma*-complex	34.83 ± 6.29	*I. macrodidyma*-complex	6.16 ± 3.79
8.1	*I. macrodidyma*-complex	74.01 ± 46.82	*I. macrodidyma*-complex	23.15 ± 6.44	*I. macrodidyma*-complex	27.24 ± 17.11
8.2	*I. macrodidyma*-complex	78.58 ± 48.75	*I. macrodidyma*-complex	376.33 ± 98.72	*I. macrodidyma*-complex	0.48 ± 0.14
8.3	*I. liriodendri*	83.14 ± 52.68	*I. macrodidyma*-complex	125.35 ± 34.72	*I. macrodidyma*-complex	0.004 ±0.0007
9.1	*I. macrodidyma*-complex	87.71 ± 55.62	*I. macrodidyma*-complex	44.31 ± 8.52	*I. macrodidyma*-complex	14.05 ± 5.64
9.2	-	*	*I. macrodidyma*-complex	52.76 ± 2.54	*I. macrodidyma*-complex	69.51 ± 10.99
9.3	-	*	*I. macrodidyma*-complex	80.89 ± 16.25	*I. liriodendri*	*
10.1	*I. liriodendri*	0.005 ± 0.0007	*I. macrodidyma*-complex	145.89 ± 13.12	*I. liriodendri*	*
10.2	-	*	*I. macrodidyma*-complex	*	*I. macrodidyma*-complex	0.07 ± 0.07
10.3	*I. liriodendri*	0.025 ± 0.0148	*I. macrodidyma*-complex	4.55 ± 2.93	*I. macrodidyma*-complex	4.79 ± 2.71

[a] Ten soil samples per field, with three replicate samples from each soil, were collected.
[b] Multiplex, nested-PCR was performed with primers ITS1F/ITS4 and with species-specific primers Mac1/MaPa2, Lir1/Lir2, and Pau1/MaPa2
[c] qPCR was performed with primers YT2F/Cyl-R
[d] *Ilyonectria* spp. DNA concentration detected in 10 ng of soil genomic DNA that was extracted from 250 mg of soil.
[e] The mean DNA concentration per soil sample was determined from three DNA repeat samples (two qPCR concentrations per DNA sample). The standard deviation (STD) (±) is shown for the six replicates. Samples with a Cq-value >30 were designated a DNA concentration of 0 pg µl^{-1}.
[f] *I. liriodendri* and *I. macrodidyma*-complex were detected simultaneously.
[g] Nested, multiplex PCR reaction was negative.
[h] DNA of *Ilyonectria* spp. was not detected.

Table 5.2. Fungal identification (*Ilyonectria liriodendri* and *I. macrodidyma*-complex) and mean pathogen DNA concentrations detected through nested multiplex PCR and qPCR analyses, respectively, of DNA obtained from three rootstock mother field soils.

	Field D		Field E		Field F	
Sample[a]	Fungal identification with nested PCR[b]	Mean pathogen DNA[c] (pg µl^{-1})[d] ± STD[e]	Fungal identification with nested PCR	Mean pathogen DNA (pg µl^{-1}) ± STD	Fungal identification with nested PCR	Mean pathogen DNA (pg µl^{-1}) ± STD
1.1	*I. macrodidyma*-complex	109.81 ± 11.51	*I. macrodidyma*-complex	134.71 ± 14.62	*I. macrodidyma*-complex	371.58 ± 47.97
1.2	*I. macrodidyma*-complex	576.12 ± 174.85	*I. macrodidyma*-complex	107.28 ± 15.44	*I. macrodidyma*-complex	251.56 ± 17.75
1.3	*I. macrodidyma*-complex	640.95 ± 122.79	*I. macrodidyma*-complex	72.86 ± 9.68	*I. macrodidyma*-complex	1569.68 ± 560.98
2.1	*I. macrodidyma*-complex	222.68 ± 19.71	*I. macrodidyma*-complex	10.68 ± 1.04	*I. macrodidyma*-complex	0.11 ± 0.05
2.2	*I. macrodidyma*-complex	80.15 ± 28.13	*I. macrodidyma*-complex	12.99 ± 0.49	*I. macrodidyma*-complex	1.99 ± 1.25
2.3	*I. liriodendri*	1.86 ± 1.29	*I. macrodidyma*-complex	21.08 ± 2.54	-[g]	*
3.1	*I. macrodidyma*-complex	19.61 ± 8.77	*I. macrodidyma*-complex	25.65 ± 1.91	*I. macrodidyma*-complex	51.88 ± 26.35
3.2	Both species[f]	0.017 ± 0.004	*I. macrodidyma*-complex	11.65 ± 4.81	*I. macrodidyma*-complex	46.59 ± 15.79
3.3	Both species	0.56 ± 0.19	*I. macrodidyma*-complex	16.84 ± 7.49	*I. macrodidyma*-complex	53.47 ± 7.83
4.1	*I. macrodidyma*-complex	286.32 ± 81.51	*I. macrodidyma*-complex	0.81 ± 0.12	*I. macrodidyma*-complex	3.66 ± 0.32
4.2	*I. macrodidyma*-complex	73.16 ± 14.93	*I. macrodidyma*-complex	1.07 ± 0.22	*I. macrodidyma*-complex	2.74 ± 0.91
4.3	*I. macrodidyma*-complex	78.78 ± 50.65	Both species	0.12 ± 0.04	*I. macrodidyma*-complex	2.19 ± 0.49
5.1	*I. macrodidyma*-complex	551.67 ± 101	*I. macrodidyma*-complex	685.62 ± 87.57	*I. macrodidyma*-complex	0.99 ± 0.19
5.2	*I. macrodidyma*-complex	196.91 ± 19.6	*I. macrodidyma*-complex	183.57 ± 24.39	*I. macrodidyma*-complex	0.01 ± 0.005
5.3	*I. macrodidyma*-complex	625.8 ± 111.89	*I. macrodidyma*-complex	61.01 ± 6.6	*I. macrodidyma*-complex	1.2 ± 0.21
6.1	*I. macrodidyma*-complex	87.75 ± 7.78	*I. macrodidyma*-complex	56.09 ± 18.67	*I. macrodidyma*-complex	2.05 ± 0.37
6.2	*I. macrodidyma*-complex	47.53 ± 21.02	*I. macrodidyma*-complex	102.42 ± 14.97	*I. macrodidyma*-complex	0.56 ± 0.22
6.3	*I. macrodidyma*-complex	79.98 ± 25.39	*I. macrodidyma*-complex	87.99 ± 14.94	*I. macrodidyma*-complex	0.09 ± 0.02
7.1	*I. macrodidyma*-complex	92.91 ± 7.72	*I. macrodidyma*-complex	328.69 ± 147.02	*I. macrodidyma*-complex	4.59 ± 0.73
7.2	*I. macrodidyma*-complex	189.94 ± 17.99	*I. macrodidyma*-complex	716.14 ± 120.64	*I. macrodidyma*-complex	2.54 ± 0.57
7.3	*I. macrodidyma*-complex	279.15 ± 88.18	*I. macrodidyma*-complex	1904.8 ± 455.15	*I. macrodidyma*-complex	2.96 ± 1.28
8.1	*I. macrodidyma*-complex	58.86 ± 5.07	*I. macrodidyma*-complex	0.29 ± 0.07	*I. macrodidyma*-complex	1.22 ± 0.28
8.2	*I. macrodidyma*-complex	19.32 ± 3.65	Both species	0.05 ± 0.01	*I. macrodidyma*-complex	8.96 ± 1.87
8.3	*I. macrodidyma*-complex	107.32 ± 12.37	Both species	0.05 ± 0.04	*I. macrodidyma*-complex	1.77 ± 0.62
9.1	*I. macrodidyma*-complex	21.79 ± 3.9	*I. macrodidyma*-complex	0.06 ± 0.02	*I. macrodidyma*-complex	0.32 ± 0.06
9.2	*I. macrodidyma*-complex	583.93 ± 124.49	Both species	0.27 ± 0.07	*I. macrodidyma*-complex	2.52 ± 0.75
9.3	*I. macrodidyma*-complex	82.52 ± 8.1	Both species	0.14 ± 0.19	*I. macrodidyma*-complex	1.06 ± 0.16
10.1	*I. macrodidyma*-complex	67.97 ± 25.03	Both species	0.61 ± 0.11	*I. macrodidyma*-complex	2.31 ± 0.59
10.2	*I. macrodidyma*-complex	67.92 ± 5.29	*I. liriodendri*	0.51 ± 0.43	*I. macrodidyma*-complex	4.81 ± 0.55
10.3	*I. macrodidyma*-complex	340.11 ± 118.39	*I. liriodendri*	*[h]	*I. macrodidyma*-complex	4.58 ± 1.69

[a] Ten soil samples per field, with three replicate samples from each soil, were collected.
[b] Multiplex, nested-PCR was performed with primers ITS1F/ITS4 and with species-specific primers Mac1/MaPa2, Lir1/Lir2, and Pau1/MaPa2
[c] qPCR was performed with primers YT2F/Cyl-R
[d] *Ilyonectria* spp. DNA concentration detected in 10 ng of soil genomic DNA that was extracted from 250 mg of soil.
[e] The mean DNA concentration per soil sample was determined from three DNA repeat samples (two qPCR concentrations per DNA sample). The standard deviation (STD) (±) is shown for the six replicates. Samples with a Cq-value >30 were designated a DNA concentration of 0 pg µl^{-1}.
[f] *I. liriodendri* and *I. macrodidyma*-complex were detected simultaneously.
[g] Nested, multiplex PCR reaction was negative.
[h] DNA of *Ilyonectria* spp. was not detected.

A total of 141 soil DNA samples generated a product size of 387 bp characteristic of *I. macrodidyma*-complex. However, *I. liriodendri* was only found in 16 out of 180 soil DNA samples, generating a product size of 253 bp. This species was more frequent in samples collected from nursery fields (13 soil DNA samples), whereas it was only detected in three soil DNA samples collected from rootstock mother fields. *Ilyonectria liriodendri* and *I. macrodidyma*-complex were detected simultaneously in only 15 soil DNA samples, occurring in both types of fields evaluated.

Some variation in results was found in the three 10g soil samples taken at a vine. Field A had the most variation with only detecting *I. macrodidyma* or *I. liriodendri* or no DNA. This indicates the variation that can occur in 30g of soil. When comparing nursery fields with mother rootstock fields, more variation was found in the nursery soil samples.

qPCR detection of *Ilyonectria* species

Under optimized conditions the qPCR assay showed standard fluorescence amplification representing exponential growth of PCR products and standard curves were constructed with serial dilutions of the target DNA. The standard curves revealed that the primer set used in the present study was quite accurate over a linear range and high correlations between C_q and DNA quantities were obtained with a R^2 values of at least 0.98 and reaction efficiencies of 0.72 or higher.

Analyses for PCR inhibitors in the extracted soil DNA samples indicated the presence of little or no PCR inhibitors in the samples. When low levels of inhibitors were present, very little variation in overall inhibition was observed among the different samples.

The qPCR results confirmed the presence of *Ilyonectria* spp. in both nursery fields and rootstock mother fields. In the nursery fields the concentration ranged from 0.004 to 757.79 pg μl^{-1} (average of 57.68 pg μl^{-1}) (Table 1) and in the rootstock mother fields it ranged from 0.01 to 1,904.8 pg μl^{-1} (average of 141.92 pg μl^{-1}) (Table 2). In eleven of the samples that tested positive with the nested PCR, no DNA was detected with qPCR. A possible reason for this could be the presence of inhibitors that influenced the qPCR reaction.

In general, qPCR analyses showed that *Ilyonectria* DNA concentrations are higher in soil samples from rootstock mother fields than that obtained in soil samples from nursery fields.

Discussion

This is the first study evaluating the occurrence of *Ilyonectria* spp. in the soil of grapevine nursery fields and rootstock mother fields by using multiplex, nested PCR, and also quantifying the concentration of *Ilyonectria* spp. in the same nursery soils by qPCR.

The multiplex, nested PCR technique allowed the detection of *I. liriodendri* and *I. macrodidyma*-complex, in DNA samples from both nursery fields and rootstock mother fields. This methodology was carried out by using the species-specific primer pairs (Mac1/MaPa2, Lir1/Lir2 and Pau1/MaPa2) designed by Alaniz *et al*. (2009b) for the detection of "*C." liriodendri*, "*C." macrodidymum* and "*C." pauciseptatum*. Recently, "*C." liriodendri* and "*C". macrodidymum* species have been renamed in the genus *Ilyonectria* as *Ilyonectria liriodendri* and *I. macrodidyma*, respectively (Chaverri *et al*., 2011; Cabral *et al*., 2012a). Moreover, Cabral *et al*. (2012b) revealed the existence of polymorphism in a wide collection of isolates previously identified as *I. macrodidyma* by using internal transcribed spacer, β-tubulin gene, histone H3 gene and translation elongation factor 1-α sequence analysis. Consequently, six new species of *Ilyonectria*, which are morphologically rather similar, were recognised into the *I. macrodidyma*-complex. Thus, the species-specific primer pairs designed by Alaniz *et al*. (2009b), which have been used in this study, identify *I. liriodendri*, *I. macrodidyma*-complex and "*C." pauciseptatum* (Agustí-Brisach *et al*., 2012a)

The results obtained with the multiplex, nested-PCR showed a high rate of detection of *I. macrodidyma*-complex from soil DNA samples collected in nursery fields as well as in rootstock mother fields, while the rate of detection of *I. liriodendri* was markedly lower in the same DNA samples. This is in concordance with the results obtained by Alaniz *et al*. (2007), who reported the prevalence of *I. macrodidyma*-complex in all grapevine growing regions in Spain. Moreover, species belonging to this fungal complex such as *I. alcacerensis, I. macrodidyma, I. novozelandica* and *I. torresensis* have also been isolated frequently in Spain from roots of weeds grown in

grapevine rootstock mother fields, nursery fields and commercial vineyards (Agustí-Brisach *et al.*, 2011) and from roots of grapevines grown in nursery soils (Agustí-Brisach *et al*., 2012a, 2012b).

The qPCR assay was performed by using the genus primer pair YT2F/Cyl-R (Dubrovsky & Fabritius, 2007; Tewoldemedhin *et al*. 2011). The primer pair amplified only DNA derived from *Ilyonectria* spp./"*Cylindrocarpon*" spp., but not DNA of the other tested fungal genera such as *Campylocarpon*, *Fusarium*, *Pythium*, *Phytophthora* or *Rhizoctonia* (Tewoldemedhin *et al*. 2011). These authors also indicated that this primer pair could detect 1 fg/µl of "*C.*" *liriodendri* and "*C.*" *macrodidymum.* Our results confirmed the detection of *Ilyonectria* spp. in soil DNA samples from both nursery and rootstock mother fields, demonstrating that they are largely infested with these black-foot pathogens. The minimum and maximum levels of *Ilyonectria* spp. DNA detected in soil were 0.004 pg μl^{-1} and 1,904.8 pg μl^{-1}, respectively. During the last few years, several authors have used the qPCR technique in order to detect and quantify inoculum of "*Cylindrocarpon*" spp. from soil samples. Kernaghan *et al*. (2007) quantified "*C.*" *destructans* f. sp. *panacis* in several naturally infested soils of ginseng crop in North America, and calculated average concentrations of target "*C.*" *destructans* f. sp. p*anacis* DNA that ranged from 0 to 4.8 pg μl^{-1} of soil extract.

In general, in our study *Ilyonectria* DNA concentrations were higher in soil samples from rootstock mother fields than that obtained in nursery fields. This could be due to rootstock mother fields consisting of grapevine rootstocks that were more than 15 years old, whereas, according to the current Spanish nursery legislation, nursery fields used for the rooting phase can not be planted for more than two consecutive growing seasons, and must have not been used for grapevine cultivation, at least for the previous twelve years. In this sense, Halleen *et al*. (2003), indicated that in South Africa, where the same nursery soil has been used for decades, the standard nursery practice of a 2-year rotation system, whereby cuttings are planted every second year, alternated with a cover crop, might have led to a build-up of black-foot pathogens in these soils, and suggested that the duration of this rotation period should, therefore, be investigated to establish its effect on pathogen populations.

Our ability to detect and quantify *Ilyonectria* spp. genomic DNA in soil samples from nursery fields and rootstock mother fields confirms soils from both field types as important inoculum sources for black-foot pathogens. The multiplex, nested-PCR and

the qPCR techniques have much value for the rapid detection of black-foot pathogens in soil, to reduce the challenges with the difficult isolation of them from soil using culture media. These tools would also be valuable in future studies on the epidemiology and control of black-foot disease of grapevines. Moreover, with the qPCR assay, it is possible to detect soilborne pathogens in soil samples in 4-5 h, avoiding laborious post-PCR gel electrophoresis and reducing contamination which could be associated with nested PCRs (Huang *et al*., 2010; Huang & Kang, 2010). Quantitative real-time PCR is a tool with great value for rapid, specific, and sensitive detection and diagnosis of black-foot pathogens in soil. Testing of soils before planting and monitoring the levels of the pathogen over time will assist management decisions that could prevent or minimize infections caused by black-foot pathogens in grapevine nurseries as well as in commercial vineyards.

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Chapter 6

Evaluation of weeds as potential hosts of black-foot and Petri disease pathogens on grapevine fields

Carlos Agustí-Brisach, David Gramaje, Maela León, José García-Jiménez and Josep Armengol

Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain.

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Keywords

Cadophora luteo-olivacea, *Cylindrocarpon macrodidymum*, *Phaeomoniella chlamydospora*, sources of inoculum, *Vitis vinifera*

Abstract

Weeds were sampled in grapevine rootstock mother fields, open-root field nurseries and commercial vineyards of Albacete, Alicante, Castellón, Murcia and Valencia provinces in Spain between June 2009 and June 2010 and evaluated as potential hosts of black-foot and Petri disease pathogens. Isolations were conducted in the root system and internal xylem tissues for black foot and Petri disease pathogens, respectively. *Cylindrocarpon macrodidymum* was successfully isolated from the roots of 15 out of 19 weed families evaluated and 26 out of 52 weed species. Regarding Petri disease pathogens, one isolate of *Phaeomoniella chlamydospora* was obtained from *Convolvulus arvensis*, and three isolates of *Cadophora luteo-olivacea* were obtained from *Bidens subalternans, Plantago coronopus* and *Sonchus oleraceus.* Pathogenicity tests showed that *C. macrodidymum* isolates obtained from weeds were able to induce typical black foot disease symptoms. When inoculated in grapevines, isolates of *Ca. luteo-olivacea* and *Pa. chlamydospora* were also shown to be pathogenic on grapevine cuttings. Our ability to recover grapevine pathogens from vineyard weeds and to demonstrate pathogenicity of recovered strains on grape suggests that these weeds may serve as a source of inoculum for infection of grapevine.

INTRODUCTION

Over the last years, an important decrease in the survival rate of grafted grapevines in nurseries and young vineyards worldwide has been noted (24). Fungi involved in wood decay are among the most destructive pathogens either infecting grapevine propagation material, newly planted vines and mature established vineyards. They have been reported in most grapevine producing regions of the world and are responsible for the decline of young vines, loss of productivity and young vine death (25,35,37).

Young grapevine decline is caused by black-foot disease and Petri disease pathogens. Fungi associated with black-foot disease are *Cylindrocarpon liriodendri* MacDon. & Butler, *C. macrodidymum* Schroers, Halleen & Crous (25,39,40), *C. pauciseptatum* Schroers & Crous, *Campylocarpon fasciculare* Schroers, Halleen & Crous, and *Campyl. pseudofasciculare* Halleen, Schroers & Crous (27,47). Petri disease

is caused by *Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams, as well as several species of *Phaeoacremonium* W. Gams, Crous & M. J. Wingf. (35,36), with *Pm. aleophilum* W. Gams, Crous, M. J. Wingf. & L. Mugnai, being the most commonly isolated and studied species of this genus (35). Several *Phialophora*-like and *Acremonium* species have also been involved in the decline of young vines, mainly species of the genus *Cadophora* Lagerberg & Melin (26,38). Among those, *Cadophora luteo-olivace*a (F.H. Beyma) Harrington & McNew has been recently shown to be quite common on grapevines affected by esca and Petri disease in several viticultural areas worldwide (2,23,26,33,44,46).

Field symptoms of black-foot and Petri disease affected vines are frequently indistinguible. External symptoms show stunted growth, reduced vigor, delayed or absence of spring growth, shortened internodes, sparse and chlorotic foliage with necrotic margins, wilting, and dieback (17,41,48).

Specific symptoms of vines affected by *Cylindrocarpon* spp. and *Campylocarpon* spp. often are sunken necrotic root lesions with a reduction in root biomass and root hairs (5,25). Removal of rootstock bark reveals black discoloration and necrosis of wood tissues which develops from the base of the rootstock, giving the disease the name of black foot (25). Species of *Cylindrocarpon* are known to be saprobes in soil, 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 openings (25,47). The production of chlamydospores may allow *Cylindrocarpon* spp. to survive for extended periods in soil (27). However, very little information is currently available regarding the survival of these pathogens and the role that chlamydospores play during the infection processes (25).

Dissected vines affected by Petri disease show a typical black discoloration of the xylem vessels, which is a result of tyloses, gums, and phenolic compounds formed inside these vessels by the host in response to the fungus growing in and around the xylem vessels (36). *Phaeomoniella chlamydospora* is a soilborne pathogen due to its ability to produce chlamydospores in culture (8,36,49). *Pa. chlamydospora* was detected in vineyard soil in New Zealand by nested-Polymerase Chain Reaction (PCR) (52) and in South Africa by species-specific PCR (13) and nested-PCR (42). Rigdway *et al.* (43) used a nested-PCR/RFLP procedure to determine the persistence of viable and nonviable spores of *Pa. chlamydospora* in soil and observed that nonviable spores were

undetected after 8 weeks of being inoculated into dried soil, whereas viable spores still could be detected at 17 weeks after inoculation, indicating that spores could persist in soil and the inoculum could persist over time. Eskalen *et al*. (16) confirmed the presence of *Pm. aleophilum* in soil by nested-PCR. This species was also recovered from soil and standing water under grapevine drip systems (45).

Given these findings soil is a source of inoculum for black-foot and Petri disease. Likewise, weeds could serve as potential hosts and thus be a source of inoculum for young vine decline. Weeds have been shown to be an alternative host for several plant pathogens (54). Black *et al.* (9,10) found a wide range of weed species as potential hosts for *Diaporthe phaseolorum* var. *caulivora* Athow & Caldwell and *Rhizoctonia solani* J.G. Kühn AG-1 in soybean fields in Louisiana. Dissanayake *et al*. (15) isolated *Pythium arrhenomanes* Drechs. from roots of grass species native to the north central plains of North America. *Cyperus rotundus* L. and other weed species associated with peanut crop have also been identified as a host of *Sclerotinia minor* Jagger (31). Several weed plants in the genera *Datura, Geranium, Ipomoea* and *Solanum* were found as weed hosts for *Phytophthora infestans*, while *Portulaca oleracea* L. was found as an alternative host for *P. capsici* (18).

The present work was conducted to study the role of weeds as potential hosts of black-foot and Petri disease pathogens in vineyards.

MATERIALS AND METHODS

Field sites

A total of 32 fields (3 grapevine rootstock mother fields, 6 open-root field nurseries and 23 commercial vineyards) were sampled between June 2009 and June 2010 in Albacete, Alicante, Castellón, Murcia and Valencia provinces in eastern-central Spain (Figure 6.1).

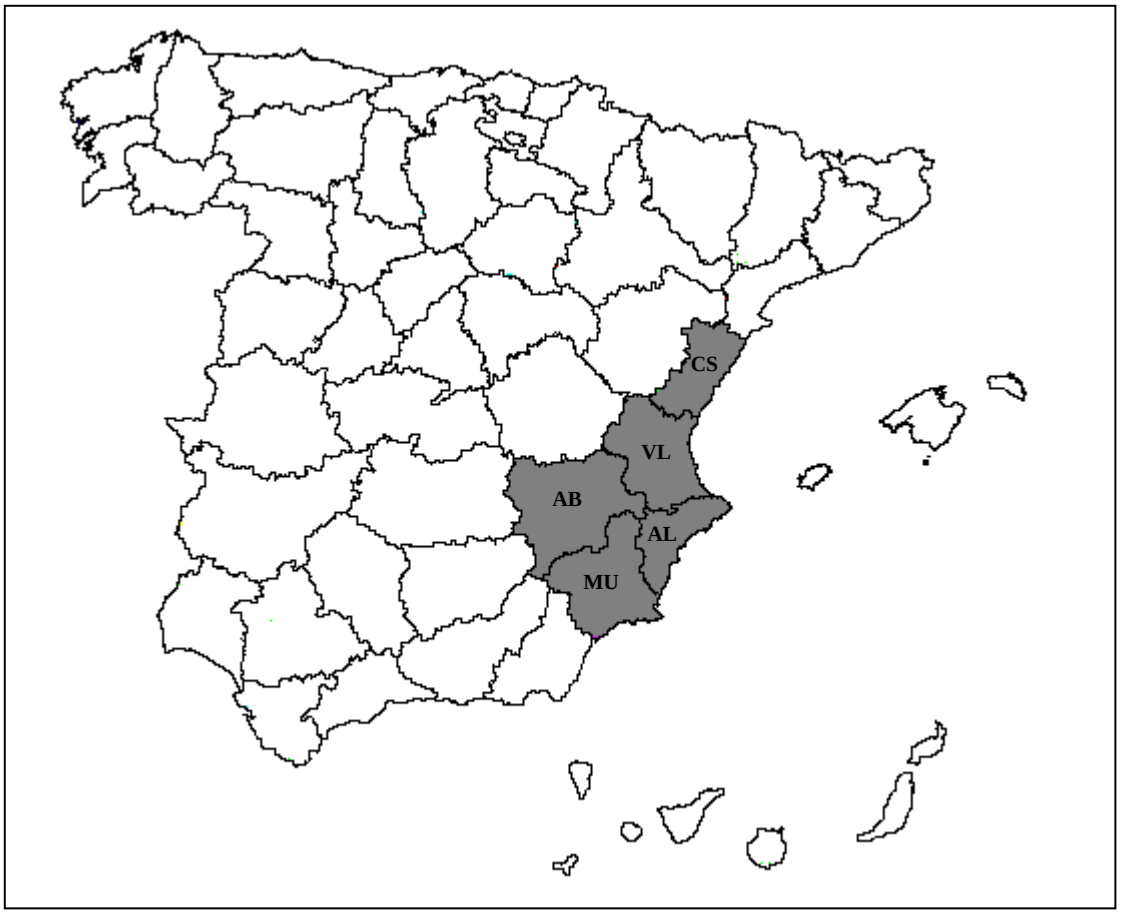

Figure 6.1. Geographic location of the Spanish provinces from which grapevine rootstock mother fields, open-root field nurseries, and commercial vineyards were sampled. Two-letter code indicates the name of the province: AB (Albacete), AL (Alicante), CS (Castellón), MU (Murcia) and VL (Valencia).

Sampling and fungal isolation

In each field, selected weed plants (three plants per species) were carefully dug out from the soil to keep the root system intact and taken back to the laboratory for immediate processing (Figure 6.2). Roots of each plant were exposed by carefully washing the soil away. Roots were visually inspected for evidence of root necrosis, then cut open and examined for signs of discolored xylem vessels.

In order to isolate *Cylindrocarpon* spp., root sections were cut from necrotic areas, washed under running tap water, surface-disinfested for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Small root pieces were plated on potato dextrose agar (PDA) (Biokar-Diagnostics, Zac de Ther, France) supplemented with 0.5 g l^{-1} of streptomycin sulphate (PDAS) (Sigma-Aldrich, St. Louis,

Figure 6.2. Weed species in grapevine rootstock mother fields, open-root field nurseries and commercial vineyards. A, Several weed species growing in a commercial vineyard; B, *Amaranthus blitoides* and *Diplotaxis erucoides* in a young vineyard and weeds growing close to grapevine plants; C, *Echinocloa crus-galli;* D, *Sonchus oleraceus;* E, *Bassia scoparia;* and F, *Amaranthus blitoides.*

MO, USA). In order to recover Petri disease pathogens, isolations were made from sections (10 cm long) that were cut from the basal stem and disinfected as previously described. Small pieces of internal healthy xylem tissues were plated on malt extract agar (MEA) (Oxoid Ltd., Basingstoke, Hants, England) supplemented with 0.5 g l^{-1} of streptomycin sulphate (MEAS).

Plates were incubated for 10-15 days at 25ºC in the dark and all colonies were transferred to PDA. Isolates were single-spored prior to morphological and molecular identification with the serial dilution method (14).

Fungal identification

Morphological identification

Species of *Cylindrocarpon* were identified by macroscopic characters including colony texture, color, and the shape of the growing margin on PDA. Conidial 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 (5).

Phaeomoniella chlamydospora was identified by conidiophore morphology, conidial size and shape, and its cultural characteristics (radial growth after 8 days at 25ºC, cardinal temperatures for growth and colony colors) on PDA and MEA (12). *Cadophora luteo-olivacea* was identified by conidiophore morphology, size of phialides and conidia, and cultural characteristics (radial growth after 8 days at 25ºC, cardinal temperatures for growth, colony colors and pigment production) on MEA, PDA and OA (19,29).

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, USA) following manufacturer's instructions. DNA was visualized on 0.7% agarose gels stained with ethidium bromide and was stored at –20ºC.

Identification of *Cylindrocarpon* species was confirmed by a multiplex PCR system using a set of three pairs of specific primers (3). *Phaeomoniella chlamydospora* was detected by PCR using specific primers Pch1-Pch2 (50). Identification of *Ca. luteo-olivacea* isolates was confirmed by analysis of the ITS region of rDNA amplified using the fungal universal primers ITS1F and ITS4 (20). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and sequenced in both directions by Macrogen Inc. (Seoul, Republic of Korea).

Pathogenicity tests

Ten *C. macrodidymum* isolates obtained from different weed species were selected to complete Koch's postulates on grapevines (Tables 6.1 and 6.2). Inoculum was produced on wheat (*Triticum aestivum* L.) seeds (11). Seeds were soaked for twelve hours in distilled water, air dried, and transferred to 300 ml flasks. Each flask was autoclaved three times on 3 successive days at 120ºC during 1 hour. Two fungal disks of a two-week old culture of each *C. macrodidymum* isolate grown on PDA at 25ºC were placed aseptically in separate flasks. The flasks were incubated at 25ºC for four weeks, and shaken once a week to avoid clustering of inoculum.

Plastic pots (220 cc) were filled with a mixture of sterilized peat moss and 10 g of inoculum per pot. Seedlings obtained from grapevines cv. Palomino were planted individually in each pot at the two-true-leaf stage. Controls were inoculated with sterile uninoculated seeds. Six replicates (each one in individual pots) for each isolate were used, with an equal number of control plants. After inoculation, plants were placed in a greenhouse at 25-30ºC in a completely randomized design and watered every 3 days or as needed.

Forty-five days after inoculation plants were observed for the development of foliar symptoms, and evaluated using a 0 to 5 rating scale: 0 = no symptoms, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, 4 = 76 to 100% chlorotic and necrotic leaves, and 5 = dead plant. Plants were gently uprooted and washed free of soil. Root symptoms of

individual plants were evaluated on the following scale: 0 = healthy with no lesions, 1 = slight discoloration with 0 to 25% of root mass reduction, 2 = discoloration with 26 to 50% of root mass reduction, 3 = moderate discoloration with 51 to 75% of root mass reduction, 4 = severe discoloration with >75% of root mass reduction and 5 = dead plant. In addition, dry weights of shoot and root were recorded for each plant. Symptomatic roots were aseptically plated on MEAS in an attempt to reisolate *C. macrodidymum* and complete Koch's postulates. The experiment was repeated.

Shoot and root disease severities were calculated using the McKinney's index (34), which expresses the percentage of the maximum disease severity according to the formula: MI= [Σ(*R* x *N*)] x 100 / *H* x *T*, where *R* = disease rating; *N*= number of plants with this rating; *H*= the highest rating; *T*= total number of plants counted. Statistical analysis of the results was done using one-way ANOVA with treatment as independent variable, and the following dependant variables: shoot disease severity (%), shoot dry weight (g), root disease severity (%), and root dry weight (g). The Student's Least Significant Difference (LSD) test was used to compare the overall means of each treatment at $P<0.05$. Statistical analyses were performed using Statistical Analysis System (SAS, version 9.0, SAS Institute Inc. Cary, NC, USA).

One isolate of *Pa. chlamydospora* (Pch-256) obtained from *Convolvulus arvensis* L. and two isolates of *Ca. luteo-olivacea* (Clo-65 and Clo-66) obtained from *Bidens subalternans* DC. and *Sonchus oleraceus* L. respectively, were used. Pathogenicity tests were conducted in 1-year-old grapevine cuttings of 110 Richter rootstock, as described by Gramaje *et al*. (23). Dormant cuttings were cut into uniform lengths containing four to five buds, and then hot-water treated at 53ºC for 30 min to eliminate the possible incidence of fungal trunk pathogens (21). In order to enhance callusing and rooting, dormant cuttings were buried into sterilized peat moss in plastic boxes, and placed in a callusing room at 25ºC and 100% humidity for 4 weeks. After callusing and rooting, cuttings were wounded between the two upper internodes with a 5 mm cork borer. A 5 mm mycelium agar plug from a 2-weeks-old culture was placed in the wound. Wounds were wrapped with parafilm. Eight cuttings per fungal isolate were used. Additionally, eight cuttings were inoculated with 5 mm noncolonized PDA agar plugs from two different plates for negative controls. Inoculated cuttings were planted immediately in individual pots, placed in a greenhouse at 25ºC and watered

Table 6.1. Weed families and species surveyed as potentials hosts of *Cylindrocarpon* spp., indicating those from which *Cylindrocarpon macrodidymum* was isolated.

Family	Species	Frequency of positive fields[y]	Frequency of positive plants[x]
Amaranthaceae	*Amaranthus blitoides*	3/7	6/9
	Amaranthus blitum	0/3	-
	Amaranthus retroflexus	1/4	1/3
	Salsola kali	1/6	1/3
Ambrosiaceae	*Xanthium stromarium*	1/1	3/3
Asteraceae	*Aster alpinus*	0/1	-
	Bidens subalternans	1/1	3/3
	Centaurea cyanus	0/1	-
	Cirsium arvense	1/3	3/3
	Conyza bonariensis	0/5	-
	Conyza canadiensis	0/1	-
	Helichrysum stoechas	1/1	3/3
	Picnomom acarna	0/2	-
	Senecio vulgaris	2/2	4/6
	Sonchus asper	0/1	-
	Sonchus oleraceus	4/10	9/12
	Sonchus terrenimus	3/5	6/9
Boraginaceae	*Heliotropium europaeum*	0/5	-
Brassicaceae	*Descurainia sophia*	0/1	-
	Diplotaxis erucoides	1/8	2/3
	Hirschfeldia incana	1/1	3/3
Chenopodiaceae	*Atriplex hortensis*	0/2	-
	Bassia scoparia	1/2	2/3
	Chenopodium glaucum	0/1	-
	Chenopodium murale	3/9	6/9
	Kochia scoparia	0/1	-
Convolvulaceae	*Convolvulus arvensis*	2/2	1/6
Cyperaceae	*Cyperus rotundus*	0/2	-
Euphorbiaceae	*Chamaesyce serpens*	0/1	-
	Chrozophora tinctoria	1/1	2/3
	Euphorbia segetalis	2/2	4/6
	Euphorbia serrata	0/2	-
Fumariaceae	*Fumaria officinalis*	0/1	-
Geraniaceae	*Erodium malacoides*	1/2	2/3
Leguminosae	*Medicago lupulina*	1/1	3/3
Liliaceae	*Allium oleraceum*	1/1	1/3
	Asparagus acutifolius	0/1	-
Malvaceae	*Lavatera cretica*	1/1	3/3
	Malva neglecta	0/1	-
Plantaginaceae	*Plantago coronopus*	1/1	3/3
	Plantago lanceolata	0/1	-
Poaceae	*Bromus madritensis*	1/1	1/3
	Bromus rubens	0/1	-
	Echinocloa crus-galli	2/3	2/6
	Hordeum leporinum	0/1	-
	Leptochloa uninervia	0/1	-
	Piptatherum miliaceum	0/1	-
Portulacaceae	*Portulaca oleracea*	1/3	1/3
Solanaceae	*Datura inoxia*	0/2	-
	Solanum nigrum	1/2	3/3
Umbelliferae	*Pimpinella anisum*	0/1	-
	Ridolfia segetum	0/1	-

[y] Number of fields from which *C. macrodidymum* was isolated/number of total fields in which the weed species was collected.

[x] Number of plants from which *C. macrodidymum* was isolated/number of total plants collected in positive fields (three plants per field).

every 3 days. Plants were arranged in a completely randomized design. The experiment was repeated.

Cuttings were collected after 14 weeks and inspected for lesion development. Extent of vascular discoloration was measured upward and downward from the inoculation point. Small pieces (0.5 to 1 cm) of necrotic tissue from the edge of each lesion were cut and placed on MEAS in an attempt to recover the inoculated fungi and complete Koch's postulates. Fungi were identified as previously described.

Lesion length data were subjected to analyses of variance using SAS and the LSD test was calculated at the 5% significance level to compare the treatment means for the different fungal species.

RESULTS

Isolation and identification of fungal species from weeds

No xylem necroses were observed in all weed plant samples, but roots showed slight discolorations or necrotic lesions.

Cylindrocarpon spp. were isolated from roots of weeds in 17 out of the 32 field sites surveyed including all type of fields (3 grapevine rootstock mother fields, 3 open-root field nurseries and 11 commercial vineyards) and in all provinces. All *Cylindrocarpon* isolates were identified by multiplex PCR as *C. macrodidymum*, because they generated a product size of 387 bp characteristic of this species (Figure 6.3). The families and species of weeds from which *C. macrodidymum* was isolated are shown in Table 6.1. This pathogen was recovered from 15 out of 19 weed families evaluated and 26 out of 52 species. In each family, the number of host weed species was variable. The family Asteraceae was the most prevalent with six weed species hosting *C. macrodidymum* followed by Amaranthaceae (three species) and Brassicaceae, Chenopodiaceae, Euphorbiaceae, and Poaceae (two species each). *C. macrodidymum* was isolated from only one weed species in the remaining families (Ambrosiaceae, Convolvulaceae, Geraniaceae, Leguminosae, Liliaceae, Malvaceae, Plantaginaceae, Portulacaceae and Solanaceae). The frequency of isolation of *C. macrodidymum* was

very variable depending on the number of field sites or plants evaluated for each weed species (Table 6.1).

Only one isolate of *Pa. chlamydospora* was obtained from *Convolvulus arvensis* in a grapevine commercial field. Three isolates of *Ca. luteo-olivacea* were obtained from *Bidens subalternans* (in a grapevine rootstock mother field), and from *Plantago coronopus* L. and *Sonchus oleraceus* (both in an open-root field nursery).

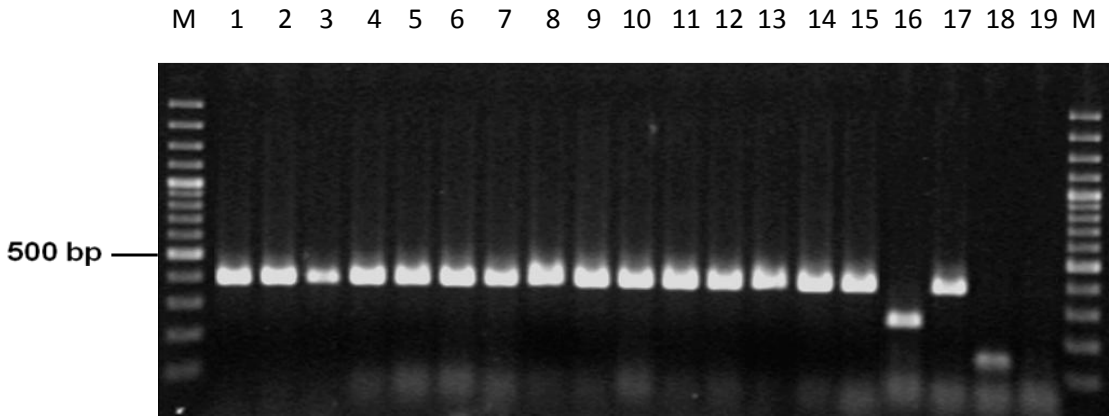

Figure 6.3. Identification of *Cylindrocarpon macrodidymum* isolates obtained from roots of weeds by polymerase chain reaction (PCR). PCR amplification products with three primer pairs: Mac1/MaPa2, Lir1/Lir2, and Pau1/MaPa2. Lanes 1 to 15, isolates from *Amaranthus blitoides* (Lanes 6 and13), *Amaranthus retroflexus* (Lane 12), *Bassia scoparia* (Lane 2), *Chrozophora tinctoria* (Lanes 3, 4 and 7), *Cirsium arvense* (Lane 10), *Convolvulus arvensis* (Lanes 11 and 14), *Diplotaxis erucoides* (Lane 9), *Hellycrysum stoechas* (Lane 15), *Solanum nigrum* (Lanes 5 and 8), and *Sonchus oleraceus* (Lane 1); lanes 16 to 18, positive control of *C. liriodendri, C. macrodidymum and C. pauciseptatum*, respectively; lane 19, negative control; lanes M, 100-bp DNA ladder.

Pathogenicity tests

Data of the two *C. macrodidymum* pathogenicity tests were combined because of the lack of significant differences between the two tests for all studied variables: shoot disease severity (F = 0.04; df = 1; P = 0.8390), shoot dry weigh (F = 0.27; df = 1; P = 0.6064), root disease severity (F = 0.20; df = 1; P = 0.6595) and root dry weight (F = 0.07; df = 1; P = 0.7941). All isolates used in this study were pathogenic to seedlings obtained from grapevines cv. Palomino. First symptoms were observed 10 days after inoculation and consisted in reduced vigor, leaves with internervial chlorosis and necrosis, necrotic root lesions with a reduction in root biomass, and death of some plants at the end of the forty-five days incubation period. Inoculation with *C.*

macrodidymum isolates had a significant effect on all variables (one-way ANOVA: shoot disease severity, $F = 14.39$; df = 12; $P < 0.001$; shoot dry weigh, $F = 33.59$; df = 12; $P < 0.001$; root disease severity, $F = 13.48$; df = 12; $P < 0.001$ and root dry weight, $F = 17.47$; df = 12; $P < 0.001$). All isolates caused a significant increase of shoot and root disease severity and a decrease of shoot and root dry weight when compared to the uninoculated controls. The isolate MH-46 was the most virulent, causing 100% mortality of grapevine seedlings at the end of the forty-five days incubation period (Table 6.2).

Table 6.2. Pathogenicity of *Cylindrocarpon macrodidymum* isolates obtained from twelve different weed species to seedlings of grapevine cv. Palomino fourty five days after inoculation[x].

		Shoot[y]		Root[z]	
Isolates	Weed species	Disease severity	Dry weight (g)	Disease severity	Dry weight (g)
MH-1	*Salsola kaly*	68.3a	0.18 b	68.8 b	0.20 b
MH-3	*Echinocloa crus-galli*	83.3 bcde	0.05 cdef	76.7 bc	0.07 cd
MH-9	*Sonchus oleraceus*	80.0 bcde	0.08 bcdef	68.8 b	0.16 bc
MH-11	*Euphorbia segetalis*	88.3 cde	0.03 ef	83.3 bc	0.01 d
MH-21	*Amaranthus blitoides*	66.7 b	0.12 bcde	70.0 b	0.10 bcd
MH-25	*Chenopodium murale*	86.7 bcde	0.06 cdef	79.6 bc	0.08 cd
MH-29	*Bassia scoparia*	73.3 bcd	0.08 cdef	81.3 bc	0.10 bcd
MH-34	*Chrozophora tinctoria*	75.0 bcd	0.14 bc	79.2 bc	0.17 bc
MH-42	*Convolvulus arvensis*	91.7 de	0.05 def	86.7 bc	0.06 cd
MH-44	*Hellychrisum stoechas*	76.7 bcd	0.08 cdef	83.3 bc	0.07 cd
MH-46	*Diplotaxis erucoides*	100.0 e	0.02 f	91.7 bc	0.01 d
MH-55	*Sonchus terrenimus*	66.7 b	0.13 bcd	70.0 b	0.14 bc
Control	-	0.00 a	0.74 a	16.7 a	0.68 a

[x]Values represent the means of 12 replications for each isolate; six per experiment. Analysis of variance: means in a column followed by the same letter are not significantly different according to Student's least significant difference test at $P < 0.05$.

[y]Shoot symptoms were evaluated on the following scale: 0 = no symptoms; 1 = 1 to 25, 2 = 26 to 50, 3 = 51 to 75, and 4 = 76 to 100% chlorotic and necrotic leaves; and 5 = dead plant. Shoot disease severity was calculated using the McKinney's index: MI = $[\Sigma(R \times N)] \times 100/H \times T$, where R = disease rating, N = number of plants with this rating, H = the highest rating, and T = total number of plants counted.

[z]Roots symptoms were evaluated on the following scale: 0 = healthy, with no lesions; 1 = slight discoloration, with 0 to 25% of root mass reduction; 2 = discoloration, with 26 to 50% of root mass reduction; 3 = moderate discoloration, with 51 to 75% of root mass reduction; 4 = severe discoloration, with >75% of root mass reduction; and 5 = dead plant. Root disease severity was calculated using the McKinney's index.

Similar effects were obtained in the two experiments of lesion length caused by Petri disease pathogens ($F = 2.99$; df = 1; $P = 0.0894$), thus the data were combined in a single analysis. Analysis of variance of the lesion length data on grapevine cuttings indicated a significant treatment effect ($F = 201.47$; df = 3; $P < 0.001$). According to the Least Significant Difference at $P < 0.05$ (LSD = 16.4), *Pa. chlamydospora* isolate Pch-

256 (mean 57.1 mm), *Ca. luteo-olivacea* isolate Clo-65 (66.3 mm), and Clo-66 (65.5 mm) caused lesions on the xylem of grapevine cuttings that were significantly longer than the controls (mean 3.9 mm).

DISCUSSION

This is the first study that examined weeds as potential hosts for black-foot and Petri disease pathogens on grapevine fields. *Cylindrocarpon macrodidymum* was commonly isolated from weeds collected in grapevine rootstock mother fields, open-root field nurseries and commercial vineyards, while only one isolate of *Pa. chlamydospora* and three isolates of *Ca. luteo-olivacea* were recovered.

This study shows a high rate of isolation of *C. macrodidymum* from roots of weeds collected in grapevine fields. Although several species of *Cylindrocarpon* have been identified worldwide as a common cause of vine death in nurseries and in young vineyards (25,47), only *C. macrodidymum* was found in the present study. This is in agreement with the results obtained by Alaniz *et al*. (5), who reported *C. macrodidymum* as the predominant species in all grapevine growing regions in Spain. To date, *C. macrodidymum* had been shown to be species specific to grapevines, being isolated only from young grapevines in Australia (51), California (39,40,48), Chile (7), New Zealand (27), South Africa (27), Spain (5) and Uruguay (1), but not from other hosts. There was variation among weed families and species in the percentage of plants colonized by *C. macrodidymum* and its field frequency. This variation could affect their importance as alternative hosts as described for weed hosts of *Pythium arrhenomanes* in sugarcane fields by Dissanayake *et al*. (15), but further research is needed to determine if *C. macrodidymum* has a preference for some weed families or species.

Cylindrocarpon spp. are soilborne pathogens which produce chlamydospores to survive for extended periods of time in the soil (25). However, very little information is currently available regarding the long-term survival of the species of this genus (11,25). The high frequency of isolation of *C. macrodidymum* obtained in this study demonstrates that this species may have the ability to alternatively survive in weeds.

The vascular pathogens *Ca. luteo-olivacea* and *Pa. chlamydospora* were also isolated from several weeds within grapevine fields but in a very small percentage

compared to *C. macrodidymum*. Recently, *Ca. luteo-olivacea* has been identified in grapevines showing decline symptoms in California (46), South Africa (26), Uruguay (2), New Zealand (33), Northeastern America (44), and Spain (23). This work represents the first report of *Pa. chlamydospora* on a host different from grapevine. *Phaeomoniella chlamydospora* is regarded as the most important fungal organism associated with Petri disease because of its predominance in affected grapevines (35,43); therefore, although only one isolate was recovered, this finding could indeed be a very important aspect to consider in further epidemiological studies of this species.

In contrast with *C. macrodidymum*, *Ca. luteo-olivacea* and *Pa. chlamydospora* were isolated from asymptomatic xylem tissues. In similar studies, weeds have been also found to be symptomless hosts for other plant pathogens, such as *Rhizoctonia solani* and *Diaporthe phaseolorum* var. *caulivora* in soybean (9,10). Our results could indicate that *Ca. luteo-olivacea* and *Pa. chlamydospora* may be surviving in weeds as endophytes. The potential role of these pathogens as grapevine endophytes and the erratic manifestation of leaf symptoms in infected grapevines have been previously reported by different authors. Whiting *et al*. (53) suggested that *Pa. chlamydospora* and *Phaeoacremonium* spp. may exist as endophytes or as latent infections in vine tissue and elicit plant response only when vines are stressed. Infected grapevine rootstock mother plants with no external foliar symptoms were reported by Fourie and Halleen (17) and Aroca *et al*. (6). Halleen *et al*. (28) observed that primary pathogens associated with young vine decline in South Africa, such as *Pa. chlamydospora, Pm. aleophilum* and *Ca. luteo-olivacea* were already present in the apparently healthy rootstock propagation material as endophytes or latent pathogens. Despite the isolation of *Phaeoacremonium* spp. and *Pa. chlamydospora* from wood discoloration of young grapevines, Zanzotto *et al.* (55) did not observe classic foliar symptoms of either Petri disease or esca in the vineyards during the 4 years after planting. Gramaje *et al*. (22), who inoculated five species of *Phaeoacremonium, Pa. chlamydospora* and *Ca. luteo-olivacea* on rootstock cuttings under field conditions, observed that these species were not able to cause foliar symptoms on inoculated plants during the grapevine growing season.

Pathogenicity tests showed that *C. macrodidymum* isolates obtained from weeds were able to induce typical black foot disease symptoms and caused shoot and root mass reduction on grapevine seedlings. Different levels of virulence were also observed

in the *C. macrodidymum* isolates inoculated. These results are in agreement with those obtained by Alaniz *et al*. (4), who detected a relevant genetic and virulence diversity in *C. macrodidymum* by Inter Simple Sequence Repeat (ISSR) analysis and pathogenicity tests. Isolates of *Ca. luteo-olivacea* and *Pa. chlamydospora* were also shown to be pathogenic on grapevine cuttings. The expression of symptoms caused by *Pa. chlamydospora* and *Ca. luteo-olivacea* on grapevines has been extensively investigated and successfully reproduced with artificial inoculations under field (22,26,36) or greenhouse conditions (23,26,32,55).

Our ability to recover grapevine pathogens from vineyard weeds and to demonstrate pathogenicity of recovered strains on grape suggests that these weeds may serve as a source of inoculum for infection of grapevine. These weed species are among the most common and widely distributed in Spanish vineyards (30). Sampling of weeds prevalent in other viticultural regions in different countries might reveal a larger host range for them and contribute to determine if they are likely to play an important role in maintaining or increasing the inoculum density of the pathogens in grapevine fields. In Spanish vineyards, weeds are often well-managed during the growing season. During the off-season, when land is fallow, weeds are not managed and their population increase size. Large populations of host weed species may increase the amount of infected plant debris in grapevine fields and thus contribute to elevate inoculum levels the following spring. These results emphasize the importance of weed control programs in possibly reducing the inoculum levels of soilborne pathogens.

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Chapter 7

Effects of temperature, pH and water potential on mycelial growth, sporulation and chlamydospore production in culture of *Cylindrocarpon* spp. associated with black-foot of grapevines

Carlos Agustí-Brisach and Josep Armengol

Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022-Valencia, Spain

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Summary The effects of temperature, pH and water potential (Ψ_s) on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon liriodendri*, *C. macrodidymum* and *C. pauciseptatum* isolated from grapevines was studied. 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 over a range of pHs, from 4 to 8. Regarding the effect of Ψ_s, in general, mycelial growth was greater on amended media at -0.5, -1.0 or/and -2.0 MPa compared with that obtained on nonamended PDA (-0.3 MPa), and was reduced at Ψ_s values lower than -2.0 MPa. Most of the *Cylindrocarpon* spp. isolates were sporulated at all temperatures, pHs and water potentials tested. In all studied conditions, *C. liriodendri* had the greatest sporulation capacity compared with *C. macrodidymum* and *C. pauciseptatum.* In general, chlamydospore production was not much affected by temperature, pH and Ψ_s. Chlamydospores were observed in PDA cultures of all isolates at all pH values studied, while some isolates did not produce chlamydospores at 5 and 10ºC or -4.0 and/or -5.0 MPa. These results improve understanding of the biology of these important grapevine pathogens.

Introduction

Black foot disease of grapevines, caused by *Cylindrocarpon* spp., is a serious disease in most wine and grape-producing regions of the world, particularly in nurseries and young vineyards (Halleen *et al.*, 2006a). It was first described in 1961 (Grasso and Magnano Di San Lio, 1975), and over the last decade, black foot disease has been reported in most grapevine production areas of the world, including Portugal (Rego *et al*., 2000), Argentina (Gatica *et al*., 2001), Germany (Fischer and Kassemeyer, 2003), New Zealand and South Africa (Halleen *et al*., 2004), Brazil (Garrido *et al*., 2004), California (Petit and Gubler, 2005), Chile (Auger *et al*., 2007), Australia (Whitelaw-Weckert *et al*., 2007), Spain (Alaniz *et al*., 2007), Iran (Mohammadi *et al*., 2009), Uruguay (Abreo *et al*., 2010), northeastern United States and southeastern Canada (Petit *et al*., 2011).

Vines affected by *Cylindrocarpon* spp. often show sunken necrotic root lesions with a reduction in root biomass and root hairs. Removal of rootstock bark reveals black

discoloration and necrosis of wood tissues which develops from the base of the rootstock. Moreover, affected vines show low vigour with small trunks and short internodes, a reduction in total foliage and leaf size, with leaves depicting interveinal chlorosis and necrosis, frequently leading to death of the plants (Halleen *et al*., 2006a; Alaniz *et al*., 2007, 2009; Abreo *et al.*, 2010).

Black foot disease of grapevine is caused by *Cylindrocarpon* spp. (*Cylindrocarpon destructans* [Zinnsm.] Scholten, *C. liriodendri* J.D. MacDonald & E.E. Butler, *C. macrodidymum* Schroers, Halleen & Crous and *C. pauciseptatum* Schroers & Crous) and *Campylocarpon* spp. (*Campylocarpon fasciculare* Schroers, Halleen & Crous and *Campyl. pseudofasciculare* Halleen, Schroers & Crous) (Halleen *et al*., 2004; Halleen *et al*., 2006a, 2006b; Schroers *et al*., 2008).

In Spain, surveys carried out in recent years in grapevine nurseries and young vineyards have confirmed the importance of *Cylindrocarpon* spp. affecting this crop. These pathogens were found in grapevine nurseries very early in the planting material production process, in grapevine plants ready to be planted and in young vineyards showing decline symptoms (Aroca *et al*., 2006; Giménez-Jaime *et al*., 2006; Alaniz *et al*., 2007; Gramaje *et al*., 2010). In all cases, *Cylindrocarpon* spp. were mostly isolated from rootstocks, especially from the basal ends. To date, *C. liriodendri*, *C. macrodidymum* and *C. pauciseptatum* are the species which have been identified associated with young vines showing symptoms of black foot disease in Spain (Alaniz *et al*., 2007; Martin *et al*., 2011).

Species of *Cylindrocarpon* are common and may be isolated as soil inhabitants, saprobes on dead plant material, root colonizers or pathogens, or weak pathogens of various herbaceous and woody plants (Brayford, 1993). The production of chlamydospores may allow *Cylindrocarpon* spp. to survive for extended periods in soil (Halleen *et al*., 2004). Given these findings, it could be assumed that black foot disease pathogens could survive in the soil to infest grapevine plants. However, very little information is currently available regarding the basic biology of these pathogens, such as mycelial growth, sporulation and chlamydospore production under various environmental conditions, and the role that chlamydospores might play during the infection processes (Halleen *et al*., 2006a).

In common with all microorganisms, fungi are profoundly affected by physical and physicochemical factors, such as temperature, aeration, pH, water potential ($\Psi_{s)}$ and

light. These factors not only affect the growth rate of fungi but can also act as triggers in developmental pathways (Deacon, 2006). These factors are known to influence host-pathogen interactions in *C. destructans* on Ginseng (Rahman and Punja, 2005) and in a number of other soilborne pathogens such as *Monosporascus cannonballus* Pollack & Uecker on muskmelon and watermelon (Ferrin and Stanghellini, 2006; Armengol *et al*., 2011), *Rhizoctonia solani* J.G. Kühn on lupin and potato (Kumar *et al*., 1999; Ritchie *et al*., 2006; Ritchie *et al*., 2009), and diseases caused by *Pythium* and *Phytophthora* spp. (Sommers *et al*., 1970; Abdelzaher *et al*., 1997).

In *Cylindrocarpon* spp. associated with black foot disease of grapevines, only growth temperature experiments have been conducted so far. These studies were performed when these pathogens were recently described and/or characterized (Halleen *et al*., 2004; Petit and Gubler, 2005; Halleen *et al*., 2006b; Alaniz *et al*., 2007; Schroers *et al*., 2008). The aim of the present study was to expand knowledge of the effects of temperature, pH and Ψ_s on mycelial growth, sporulation and chlamydospore production of *C. liriodendri, C. macrodidymum* and *C. pauciseptatum* isolated from grapevines.

Materials and methods

Fungal isolates

Three isolates of *C. liriodendri* (Cy59, Cy89 and Cy100), three isolates of *C. macrodidymum* (Cy47, Cy14 and Cy81) and one isolate of *C. pauciseptatum* (Cy593), obtained from roots or the basal ends of rootstocks from grapevines exhibiting symptoms of black foot in Spain were used in this study. Additionally, two isolates of *C. pauciseptatum* from roots of grapevines in Slovenia (CBS120171 and CBS120173) were obtained from the collection of the Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands) (Table 7.1). Single spore isolates were stored in 15% glycerol solution at –80°C in cryovials (1.5 mL capacity). Prior to use, a small plug of the colonized agar from each cryovial was transferred to potato dextrose agar (PDA) (Biokar-Diagnostics, Zac de Ther, France) plates and allowed to grow at 25ºC in darkness for 14 d.

Table 7.1. Sources of isolates of *Cylindrocarpon* spp. associated with black-foot disease and their optimum growth temperatures.

Species/Isolate	Year	Geographical origin		Scion/rootstock	Optimum growth
		Town	Province		
C. liriodendri					
Cy59	2003	Tarazona de la Mancha	Albacete	Cencibel/1103 P	21.9
Cy89	2004	Aielo de Malferit	Valencia	Garnacha/110R	23.7
Cy100	2004	Alesanco	La Rioja	Garnacha/110R	24.3
C. macrodidymum					
Cy14	2002	n/d[z]	Burgos	Tempranillo/110R	24.5
Cy47	2003	Mollina	Málaga	Pedro Ximénez/1103 P	23.3
Cy81	2003	Beneixama	Alicante	Tempranillo/161-49C	24.1
C. pauciseptatum					
Cy593	2009	Tobarra	Albacete	Syrah/S04	24.4
CBS120171[x]	2005	Krsko (Eslovenia)	n/d	n/d	24.0
CBS120173[x]	2005	Doljenska (Eslovenia)	n/d	n/d	22.9

[x]Reference isolates of *C. pauciseptatum* (CBS 120171 and CBS 120173) were obtained from the collection of Centralbureau voor Schimmelcultures inUtrecht, the Netherlands (CBS).

[y]For each *Cylindrocarpon* spp. isolate, temperature average growth rates were adjusted to a regression curve to estimate the optimum growth temperature.

[z]n/d: not determined.

Effects of temperature on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon* spp.

To determine the effect of temperature on mycelial growth, all isolates were maintained and grown on PDA plates at 25°C. Agar plugs (8 mm diam.) were cut from the leading edges of 14-d-old colonies and placed in the center of PDA plates (one plug per plate) that were then incubated in the dark at 5, 10, 15, 20, 25, 30 or 35°C. There were four pseudoreplicates for each isolate and temperature combination. After 10 d, colony diameter was measured along two perpendicular axes, and data were converted to radial growth (mm d^{-1}).

Colonies grown on PDA were further incubated over 20 days to evaluate sporulation and determine the presence/absence of chlamydospores. The number of conidia produced on mycelia from agar plugs was measured following the method described by Whiting *et al*. (2001) and Alaniz *et al*. (2007). After 30 d of incubation, two plugs of agar (4 mm diam.) with mycelia and spores, were cut from the growing

edge of each colony, and each was placed in an Eppendorf vial (1.5 mL capacity) containing 1 mL of sterile water. Vials with plugs were vortexed for 5 s, and the number of conidia per mL was counted using a haemocytometer. To observe chlamydospores, a small quantity of fungal material was removed from each colony surface with a sterile needle taking care to minimize disturbance of the fungal structures, and placed in a drop of distilled water in the centre of a clean slide, and a cover slide was carefully lowered on to the drop. Two preparations per Petri dish were observed microscopically at ×100 and ×400 magnification. The experiment was repeated.

Effects of pH on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon* spp.

The effects of pH on mycelial growth and sporulation of *Cylindrocarpon* spp. in culture was determined on PDA. Mycelial plugs (8 mm diam.) obtained from the growing edges of colonies were transferred to the center of PDA plates (one plug per plate) which were adjusted to pH 4, 5, 6, 7 and 8 with the addition of 50 mM citrate phosphate buffer (pH 4–7) or 50 mM Tris-HCl buffer (pH 8) (Gomori, 1955). Plates were incubated in the dark at 25ºC. There were two replicates for each isolate and pH combination. Mean mycelial growth rates, the number of conidia produced on mycelia and the presence/absence of chlamydospores were evaluated as described above. The experiment was repeated.

Effects of water potential (Ψ_s) on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon* spp.

The effect of Ψ_s on mycelial growth and sporulation of *Cylindrocarpon* spp. in culture was determined on PDA. Mycelial plugs (8 mm in diam.) obtained from the growing edges of colonies were transferred to the center of PDA plates (one plug per plate) amended with KCl or NaCl prior to sterilization to obtain six Ψ_s values: -0.5, -1.0, -2.0, -3.0, -4.0 and -5.0 MPa, according to Robinson and Stokes (1959). Non-amended PDA (-0.3 MPa) was used as and experimental control. Plates were incubated in the dark at 25ºC. There were two replicates for each isolate, type of solute and Ψ_s combination. Mean mycelia growth rates, the number of conidia produced on mycelia and the

presence/absence of chlamydospores were evaluated as described above. The experiment was repeated.

Statistical analyses

Data from each *Cylindrocarpon* spp. were analyzed separately. Two way analyses of variance (ANOVA) were conducted with radial growth and sporulation data obtained from temperature and pH experiments, and a three way ANOVA was conducted for Ψ_s experiments using Statgraphics Plus 5.1 software (Manugistics Inc., Rockville, MD, USA). For all *Cylindrocarpon* spp., ANOVA analyses indicated that the radial growth and sporulation data between the two repetitions in temperature, pH and Ψ_s experiments were similar (P>0.05). Thus, in all cases, data from both experiments were combined.

For each *Cylindrocarpon* spp. isolate, temperature, pH or Ψ_s average mycelial growth rates were adjusted to a regression curve using Statgraphics Plus 5.1 software, and the best polynomial model was chosen based on parameter significance (P<0.05) and coefficient of determination (R^2). Previous to this analysis, data of mycelial growth from the Ψ_s experiment were converted to relative growth rate as a percentage of the experimental controls. Additionally, the polynomial models in the temperature experiments were used to estimate the optimum growth temperature for each isolate.

Results

Effects of temperature on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon* spp.

Statistically significant effects of the isolate on radial growth were observed for *C. macrodidymum* (P=0.0019) and *C. pauciseptatum* (P=0.0073), but not for *C. liriodendri* (P=0.4999). The effect of isolate × experiment was also not significant in all of the species studied (P>0.05) (Table 7.2).

The effects of temperature on mycelial radial growth and sporulation of the nine *Cylindrocarpon* spp. isolates are shown in Figure 7.1. All isolates were able to grow on PDA over a range of temperatures from 5 to 30ºC, and no growth was obtained at 35ºC. At 5ºC, *C. liriodendri* isolates showed growth rates between 0.038 cm day^{-1} for isolate

Table 7.2. Analysis of variance for the effects of temperature, pH and Ψ_s on radial growth and sporulation of *Cylindrocarpon liriodendri*, *C. macrodidymum* and *C. pauciseptatum* isolates.

	C. liriodendri			*C. macrodidymum*			*C. pauciseptatum*		
	d.f.[a]	MS[b]	*P* < *F*[c]	d.f.	MS	*P* < *F*	d.f.	MS	*P* < *F*
Temperature									
Radial growth									
Experiment (A)	1	0.00012	0.8911	1	<0.0001	0.9894	1	0.00007	0.9199
Isolate (B)	2	0.00427	0.4999	2	0.04492	0.0019	2	0.03480	0.0073
A x B	2	0.00019	0.9688	2	0.00011	0.9839	2	0.00001	0.9980
Residual	326	0.00615		329	0.00703		330	0.00697	
Sporulation									
Experiment (A)	1	17.5208	0.1418	1	17.2798	0.0629	1	5.17642	0.3420
Isolate (B)	2	0.56193	0.9328	2	379.699	<0.001	2	195.172	<0.001
A x B	2	4.69997	0.5596	2	2.11654	0.6532	2	4.39406	0.4645
Residual	330	8.08063		330	4.96344		330	5.71707	
pH									
Radial growth									
Experiment (A)	1	<0.0001	0.9790	1	0.00005	0.8122	1	<0.0001	0.9793
Isolate (B)	2	0.00318	0.0895	2	0.04015	<0.001	2	0.00557	0.0006
A x B	2	0.00017	0.8787	2	0.00005	0.9459	2	<0.0001	0.9987
Residual	226	0.00131		221	0.00089		225	0.00073	
Sporulation									
Experiment (A)	1	0.02486	0.8548	1	<0.0001	0.9132	1	<0.0001	0.2186
Isolate (B)	2	2.26406	0.0492	2	<0.0001	<0.001	2	<0.0001	<0.001
A x B	2	0.09818	0.8759	2	<0.0001	0.9882	2	<0.0001	0.2346
Residual	212	0.74085		210	<0.0001		208	<0.0001	
Ψ_s									
Radial growth									
Experiment (A)	1	0.00004	0.8891	1	0.00002	0.9411	1	0.00002	0.9329
Isolate (B)	2	0.02469	<0.001	2	0.05107	<0.001	2	0.05301	<0.001
Salt type (C)	1	0.05410	<0.001	1	0.05925	<0.001	1	0.16224	<0.001
A x B	2	0.00020	0.8982	2	0.00016	0.9547	2	<0.0001	0.9992
A x C	2	0.00247	0.2742	2	0.02014	0.0026	2	0.00127	0.6735
B x C	1	0.00182	0.3290	1	0.00021	0.8009	1	0.00005	0.9011
A x B x C	2	0.00042	0.8029	2	0.00019	0.9449	2	0.00006	0.9817
Residual	639	0.00191		658	0.00335				
Sporulation									
Experiment (A)	1	4.06421	0.1092	1	2.37550	0.5960	1	0.52946	0.8002
Isolate (B)	2	0.88494	0.5723	2	68.3923	0.0003	2	91.4964	<0.001
Salt type (C)	1	0.67427	0.0391	1	1.26907	0.6983	1	118.754	0.0002
A x B	2	1.70771	0.3409	2	1.70329	0.8175	2	3.05828	0.6908
A x C	2	10.0494	0.0019	2	297.890	<0.001	2	52.2602	0.0019
B x C	1	3.36179	0.1452	1	0.62988	0.7848	1	15.4059	0.1721
Residual	2	0.06835	0.9578	2	7.15412	0.4293	2	8.13652	0.3742
A x B x C	660	1.58416		660	8.44962		660	8.26433	

[a] Degrees of freedom

[b] Mean square

[c] Probabilities associated with individual *F*-tests

Cy89 and 0.043 cm day^{-1} for isolate Cy59, while the growth rates of the *C. macrodidymum* and *C. pauciseptatum* isolates were almost negligible at this temperature. Optimum growth temperatures for all isolates ranged between 21.9ºC for

isolate Cy59 (*C. liriodendri*) and 24.5ºC for isolate Cy14 (*C. macrodidymum*) (Table 7.1).

Regarding sporulation, significant effects of the isolate on sporulation were observed for *C. macrodidymum* (P<0.001) and *C. pauciseptatum* (P<0.001), but not for *C. liriodendri* (P=0.9328). The effect of isolate × experiment was also not significant in all species studied (P>0.05) (Table 7.2).

Most of the *Cylindrocarpon* spp. isolates produced conidia at all temperatures, showing broad variation. In general, the three *C. liriodendri* isolates sporulated more abundantly than *C. macrodidymum* and *C. pauciseptatum* isolates in all studied temperatures, with values greater than 10^4 conidia mm^{-2}. In *C. macrodidymum*, there was more variability among the isolates, isolate Cy14 being the only one for which values greater than 10^4 conidia mm^{-2} were obtained at 15, 20, 25 and 30ºC. Isolate Cy47 only sporulated at 15, 20 and 25ºC. Sporulation of *C. pauciseptatum* isolates was also variable among isolates, isolate Cy593 being the only one in which values greater than 10^4 conidia mm^{-2} were obtained at 5, 20, 25 and 30ºC. Isolate CBS120173 did not sporulate at 5ºC.

Chlamydospores were observed in PDA cultures of all isolates from 15 to 30ºC. No chlamydospores were observed at 5 or 10ºC.

Effects of pH on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon* spp.

Statistically significant effects of the isolates on radial growth were observed for *C. macrodidymum* (P<0.001) and *C. pauciseptatum* (P=0.0006), but not for *C. liriodendri* (P=0.0895). The effect of isolate × experiment was also not significant in the three species studied (P>0.05) (Table 7.2).

The effects of pH on mycelial radial growth and sporulation of the nine *Cylindrocarpon* spp. isolates are shown in Figure 7.2. All isolates were able to grow on pH-adjusted PDA at all pH values studied. In general, for each *Cylindrocarpon* spp. all isolates showed similar growth rates from pH 4 to pH 8, although in *C. liriodendri* and *C. macrodidymum* radial growth increased slightly as pH increased. In *C. liriodendri*, mycelial radial growth was greatest at pH 8 for all isolates, and in *C. macrodidymum*,

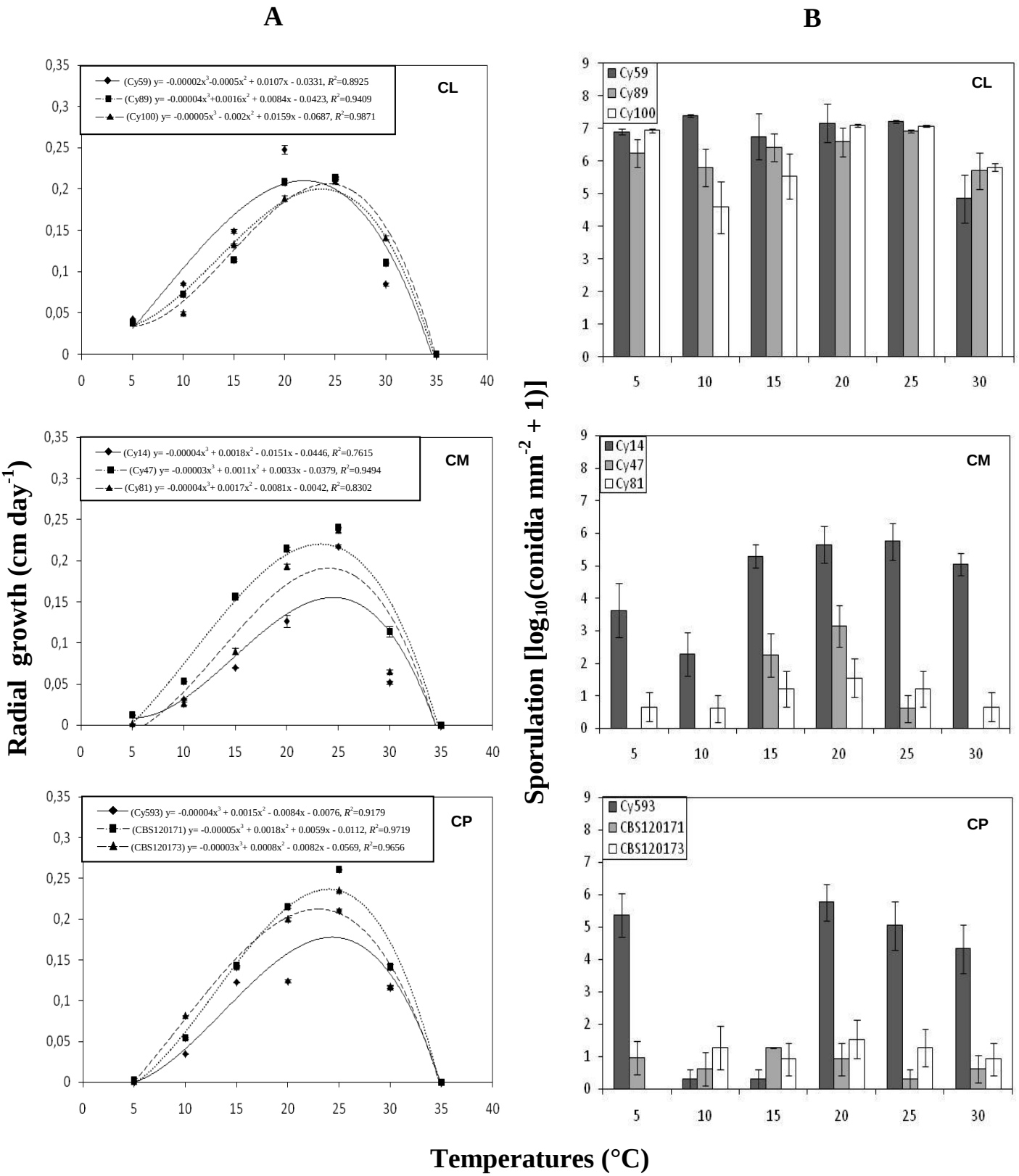

Figure 7.1. A, mean colony radial growth rates (cm day^{-1}) of three isolates of *C. liriodendi* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 10 days of incubation in darkness on PDA at 5, 10, 15, 20, 25, 30 or 35ºC; **B,** mean amounts of sporulation [$\log_{10}$(conidia mm^{-2} + 1)] of three isolates of *C. liriodendri* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 30 days of incubation in darkness on PDA at 5, 10, 15, 20, 25, 30 or 35ºC. Results are the mean of two independent sets of four pseudoreplicates for each temperature. Vertical bars are the standard error of the means.

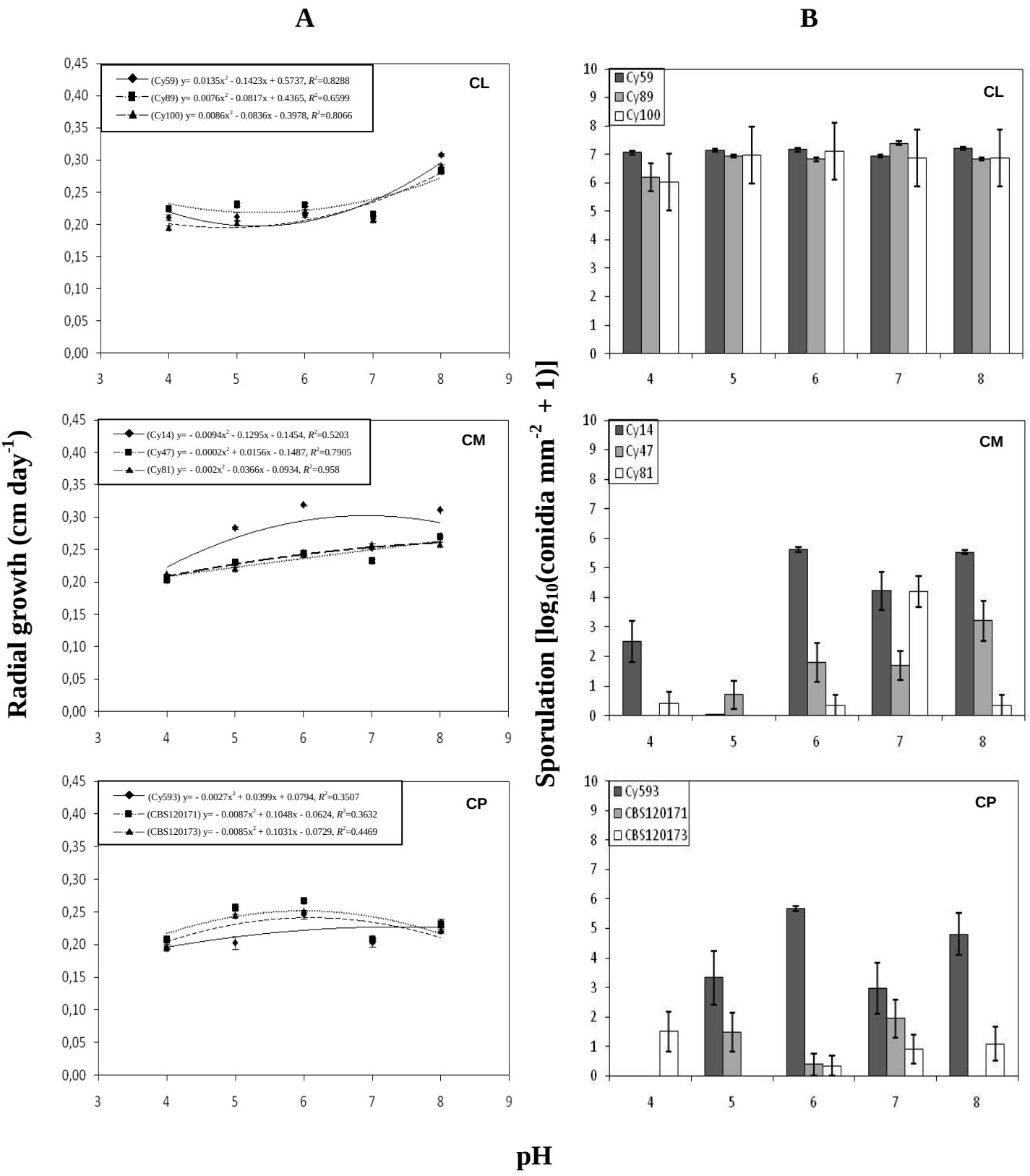

Figure 7.2. A, mean colony radial growth rates (cm day^{-1}) of three isolates of *C. liriodendi* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 10 days of incubation in darkness on PDA at pHs 4, 5, 6, 7 or 8; **B,** mean amounts of sporulation [$\log_{10}$(conidia mm^{-2} + 1)] of three isolates of *C. liriodendri* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 30 days of incubation in darkness on PDA at pHs 4, 5, 6, 7 or 8. Results are the mean of two independent sets of four pseudoreplicates in each pH.Vertical bars are the standard error of the means.

mycelial growth of Cy47 and Cy81 isolates was also greatest at pH 8, while for Cy14 this was greatest at pH 6. In *C. pauciseptatum*, mycelial growth of isolates CBS120171 and CBS120173 was greatest at pH 6, while for isolate Cy593 this was greatest at pH 8.

Regarding sporulation, significant effects of the isolate on sporulation were observed for *C. liriodendri* (P=0.0492), *C. macrodidymum* (P<0.001) and *C. pauciseptatum* (P<0.001). The effect of isolate × experiment was not significant for all *Cylindrocarpon* spp. (P>0.05) (Table 7.2).

Most of the isolates produced conidia at all pH values, showing a broad range of variation among *Cylindrocarpon* spp. In general, the three *C. liriodendri* isolates sporulated more abundantly than *C. macrodidymum* and *C. pauciseptatum* at all studied pH values, producing more than 10^5 conidia mm^{-2}. In *C. macrodidymum*, there was variability among the isolates, isolate Cy14 being the only one for which values greater than 10^5 conidia mm^{-2} were obtained at pH 6 and 8. Isolates Cy47 did not sporulate at pH 5 and Cy81 did not sporulate at pH 6. Sporulation of *C. pauciseptatum* isolates was also variable among isolates, isolate Cy593 being the only one for which values greater than 10^5 conidia mm^{-2} were recorded at pH 6, but this isolate did not sporulate at pH 4. Isolate CBS120171 only sporulated at pH 5, 6 and 7, and isolate CBS120173 did not sporulate at pH 5.

Chlamydospores were observed in PDA cultures of all isolates at all pH values studied.

Effects of water potential (Ψ_s) on mycelial growth, sporulation and chlamydospore production of *Cylindrocarpon* spp.

Statistically significant effects of the isolate and salt type on mycelia growth were observed for all three species (P<0.001). All the interactions were not significant with the exception of experiment × salt type for *C. macrodidymum* (P<0.0026) (Table 7.2).

The effect of Ψ_s on mycelial growth of the nine *Cylindrocarpon* spp. isolates is shown in Figure 7.3, and on sporulation is shown in Figure 7.4. The patterns of the mycelial radial growth responses of the isolates to decreasing Ψ_s were similar for the two osmotica tested, but, in general, *Cylindrocarpon* spp. were more tolerant to NaCl than KCl. Mycelial growth generally increased compared with mycelial growth on

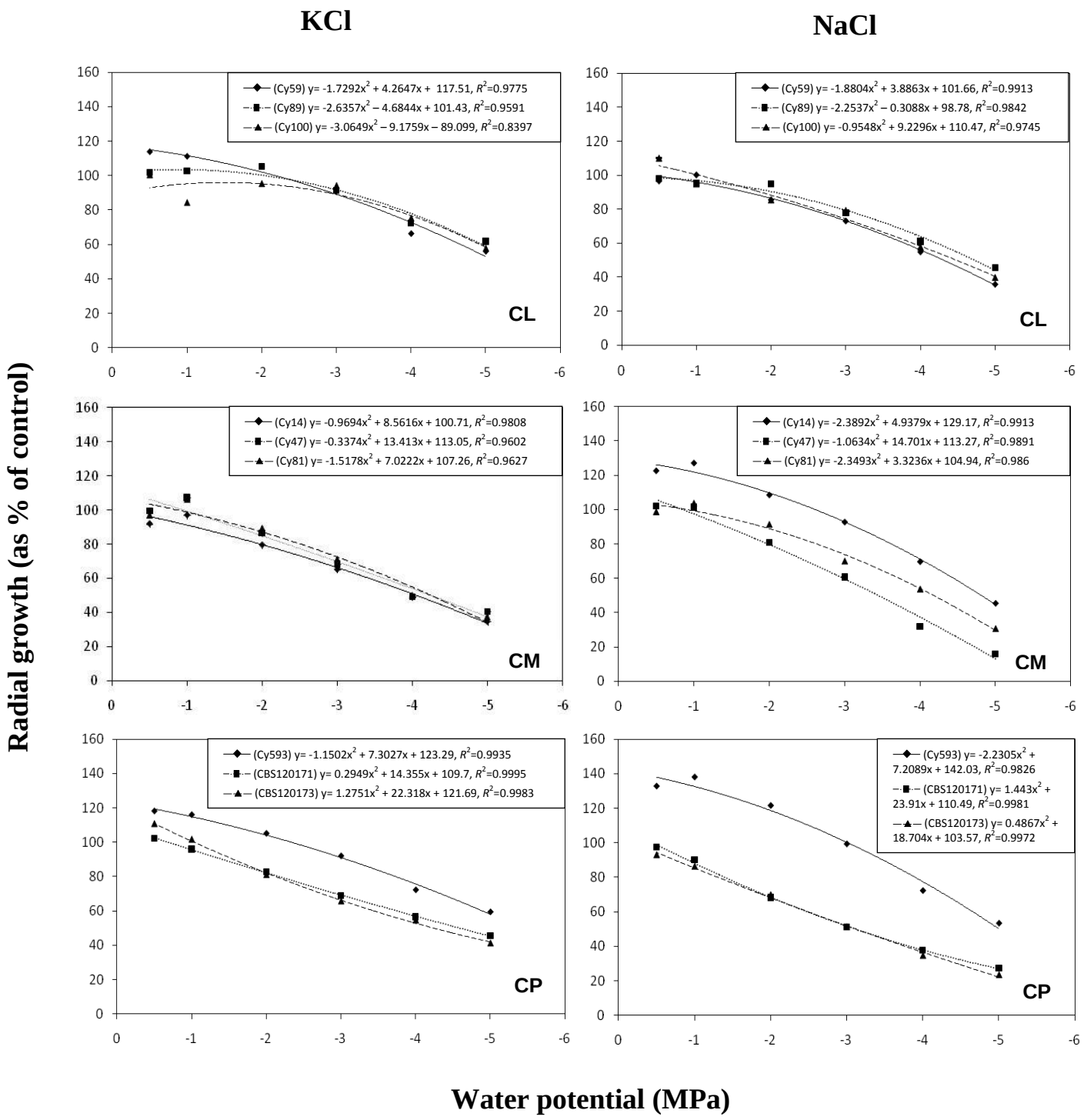

Figure 7.3. Mean relative colony radial growth (as a percentage of that on nonamended media) of three isolates of *C. liriodendri* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 10 days of incubation in darkness at 25ºC on PDA amended with KCl or NaCl to give the following water potentials -0.5, -1, -2, -3, -4 or -5 MPa. The water potential of nonamended PDA was -0.3MPa. Data points are the means of two independent sets of four pseudoreplicates in each experiment. Vertical bars are the standard error of the means.

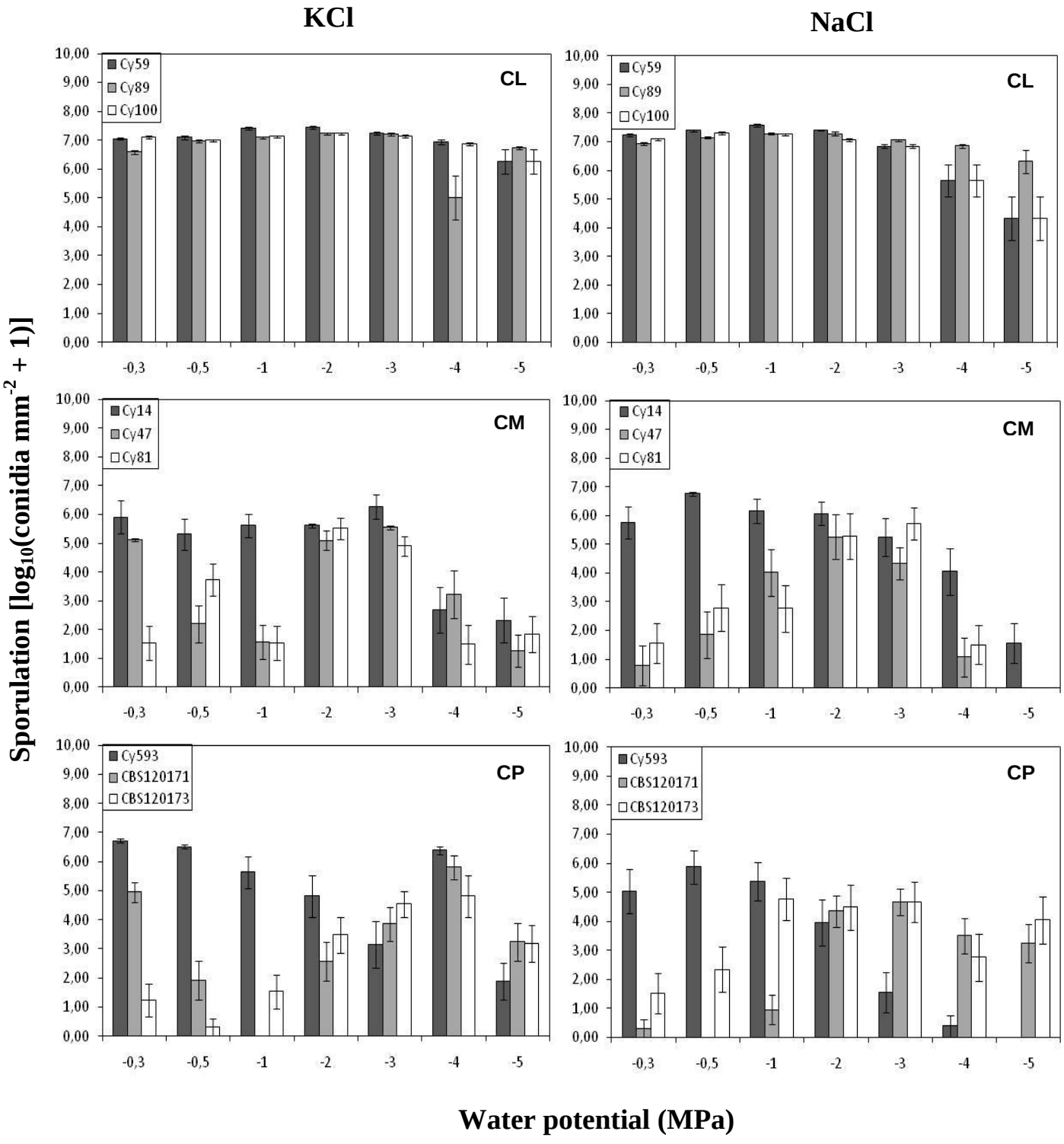

Figure 7.4. Mean amounts of sporulation [$\log_{10}$(conidia mm^{-2} + 1)] of three isolates of *C. liriodendri* (CL), *C. macrodidymum* (CM) and *C. pauciseptatum* (CP), after 30 days in darkness at 25ºC on PDA amended with KCl or NaCl to give the following water potentials -0.5, -1, -2, -3, -4 or -5 MPa. The water potential of nonamended PDA was -0.3MPa. Data points are the means of two independent sets of four pseudoreplicates in each experiment. Vertical bars are the standard error of the means.

nonamended PDA (-0.3 MPa) at -0.5 MPa and -1.0 Mpa by the addition of KCl and NaCl. The exception was isolate Cy89 which showed the greatest mycelia growth at -2.0 MPa. At lower water potentials, mycelial growth decreased as Ψ_s reduced, showing the lowest percentages at -5.0 MPa.

Regarding sporulation, a significant effect of the isolate on sporulation was observed for *C. macrodidymum* (P=0.0003) and *C. pauciseptatum* (P<0.001), but not for *C. liriodendri* (P=0.5723). The effect of salt type was significant for *C. liriodendri* (P=0.0391) and *C. pauciseptatum* (P=0.0002), but not for *C. macrodidymum* (P=0.6983). All the interactions were not significant with the exception of experiment × salt type for all *Cylindrocarpon* spp. (Table 7. 2).

Most of the isolates were able to produce conidia at all Ψ_s values, showing a broad range of variation. In general, the three *C. liriodendri* isolates sporulated more abundantly than *C. macrodidymum* and *C. pauciseptatum* isolates in all studied Ψ_s values for each salt tested, with values greater than 10^5 conidia mm^{-2} in KCl and 10^{-4} conidia mm^{-2} in NaCl. In *C. macrodidymum*, there was more variability among the isolates, and isolates Cy47 and Cy81 did not sporulate at -5.0 MPa in NaCl. In *C. pauciseptatum*, sporulation was also variable among isolates. Isolate CBS120171 did not sporulate at -1.0 MPa in KCl and at -0.5 in NaCl, and isolate Cy593 did not sporulate at -5.0 MPa in NaCl.

Chlamydospores were observed in PDA cultures of all isolates at almost all Ψ_s values in both salts. No chlamydospores were observed in isolate Cy593 (*C. pauciseptatum*) at -4.0 MPa in NaCl and at -5.0 MPa both in NaCl and KCl, and in Cy59 (*C. liriodendri*) at -4.0 and -5.0 MPa in KCl.

Discussion

This study has identified differences in the effects of temperature, pH and Ψ_s on mycelial growth, sporulation and chlamydospore production of *C. liriodendri, C. macrodidymum* and *C. pauciseptatum*.

In general, these *Cylindrocarpon* spp. were able to grow over a range of temperatures from 5 to 30ºC, with optimum temperatures for growth between 20 to 25ºC, but they did not grow at 35ºC. These results are in agreement with those obtained in previous studies (Halleen *et al*., 2004; Petit and Gubler, 2005; Halleen *et al*., 2006b;

Alaniz *et al*., 2007; Schroers *et al*., 2008), and indicate that *Cylindrocarpon* spp. associated to black foot disease of grapevine are mesophilic, as most fungi, which commonly grow within the range 10–40ºC (Deacon, 2006). In addition, our study showed differences among *Cylindrocarpon* spp. in the effect of low temperatures on mycelial growth. Growth of *C. macrodidymum* and *C. pauciseptatum* at 5 and 10ºC was almost negligible compared with that of *C. liriodendri*. This is in agreement with the results obtained by Alaniz *et al*. (2007), who demonstrated that *C. macrodidymum* can be differentiated from *C. liriodendri* by growing more slowly at 5 and 10ºC.

Previous research has suggested that species belonging to the genus *Cylindrocarpon* are calcicolous, with optimal pH around 7.0 and being poorly represented in acid soils (Matturi and Stenton, 1964a). Nevertheless, in our study *Cylindrocarpon* spp. associated with black foot of grapevine showed broad pH tolerance for mycelial growth, although with slight differences among isolates. All isolates were able to grow between pH 4.0 to 8.0. This corresponds with the optimum pH range indicated by Deacon (2006) for mycelial growth of most fungi.

Regarding the effect of Ψ_s, the response of *Cylindrocarpon* spp. isolates was reduced mycelial growth as Ψ_s decreased. Our results indicate that the isolates may have benefited from small to modest additions of solutes. In general, mycelial growth was greater on amended media at -0.5, -1.0 and/or -2.0 MPa compared with that on nonamended PDA (-0.3 MPa), and was reduced at Ψ_s values less than -2.0 MPa. Moreover, the effect of Ψ_s on mycelial growth was similar whether KCl or NaCl was used as the osmoticum, indicating that the observed responses were caused by changes in Ψ_s rather than by toxicity of the osmotic. These results, together with those obtained in the pH experiments, suggest that *Cylindrocarpon* spp. pathogenic to grapevine are likely to proliferate in most vineyard soils.

In all studied conditions, *C. liriodendri* was the species with the greatest capacity for sporulation compared with *C. macrodidymum* and *C. pauciseptatum.* Specifically, regarding the effect of temperature, Alaniz *et al*. (2007) indicated that *C. macrodidymum* can be differentiated from *C. liriodendri* by producing fewer conidia at 5 and 10ºC, which agrees with our results. It is also interesting to note that the *C. macrodidymum* isolates Cy47 and Cy81, which showed reduced mycelial growth and sporulation in the temperature, pH and Ψ_s experiments when compared with *C.*

liriodendri isolates and *C. macrodidymum* isolate Cy14, belong to the genetic Groups G7 and G6, respectively, described by Alaniz *et al*. (2009) using inter-simple sequence repeats analysis. These authors demonstrated that isolates included in these groups were significantly more virulent to grapevine than other *C. macrodidymum* and *C. liriodendri* isolates. Thus, our results suggest a possible relationship between high virulence of the isolates and a low mycelial growth and sporulation.

Previous studies have shown that *C. liriodendri*, *C. macrodidymum* and *C. pauciseptatum* are able to produce chlamydospores in culture media such as carnation leaf agar (CLA), oatmeal agar (OA), PDA, Spezieller Nährstoffarmer Agar (SNA) or Diluted V8-juice Agar (V8) after 14–21 dys of incubation in darkness at 20–25ºC (Halleen *et al*., 2004; Halleen *et al.*, 2006b; Schroers *et al*., 2008). Moreover, chlamydospores of *C. destructans*, which causes root rot of *Panax ginseng*, formed over a range of temperatures from 5 to 30ºC (Yoo *et al*., 1996). In general, our results showed that chlamydospore production was not much affect by temperature, pH and Ψ_s. 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 in both osmotic media tested. Petit and Gubler (2005), indicated that *C. macrodidymum* isolates produced relatively more chlamydospores than *C. liriodendri*, although they considered this characteristic too inconsistent to be used for identification purposes. Chlamydospores allow *Cylindrocarpon* spp. to survive for extended periods in soil and remain dormant until they are stimulated to germinate by plants or are destroyed by other soil organisms (Matturi and Stenton, 1964b; Booth, 1966). However, further research is needed to determine the role of chlamydospores during subsequent infections on grapevines.

Our study provides further information on factors affecting growth, reproductive and survival potential of *C. liriodendri, C. macrodidymum* and *C. pauciseptatum*. From the results reported here, it can be concluded that these species have abilities to be active over a wide pH range, while temperature and Ψ_s changes can affect their growth and reproductive potential. In this sense, the results obtained here improve understanding of the biology of these important grapevine pathogens. This is likely to be important for developing *in vitro* assays for assessing pathogenicity of *Cylindrocarpon* spp. (Pathrose *et al*., 2010), or new methods for control of black foot

disease of grapevines, such as the use of arbuscular mycorrhizae (Petit and Gubler, 2006; Bleach *et al*., 2008) or soil biofumigation (Bleach *et al*., 2009).

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Chapter 8

GENERAL DISCUSSION

During the four years period of research work covered by this Thesis (2009-2013), the taxonomy of the genus "*Cylindrocarpon*" has been revised and the methodologies used to identify fungal isolates belonging to this genus have changed as well. This fact has affected the way in which the results are presented in the different Chapters.

In 2011, described "*Cylindrocarpon"* species associated with black-foot were renamed in the genus *Ilyonectria* or referred to as "*Cylindrocarpon"*, because true *Cylindrocarpon* species belong to the genus *Neonectria* (Chaverri *et al*., 2011). "*Cylindrocarpon*" has been divided into five informal groups (*Neonectria*/"*Cylindrocarpon*" *sensu stricto*, *Rugonectria*, *Thelonectria*, *Ilyonectria* and the anamorph genus *Campylocarpon*) that are recognized as genera, including "*Cylindrocarpon" liriodendri* and "*C". macrodidymum*, causal agents of black-foot, into *Ilyonectria* genus (Chaverri *et al*., 2011; Cabral *et al*., 2012a). Moreover, Cabral *et al*. (2012c) revealed the existence of polymorphism in a wide collection of isolates previously identified as *I. macrodidyma* by using internal transcribed spacer, β-tubulin gene, histone H3 gene (HIS) and translation elongation factor 1-α sequence analysis. Consequently, six new species of *Ilyonectria* (*I. alcacerensis*, *I. estremocensis*, *I. novozelandica*, *I. torresensis*, *Ilyonectria* sp. 1 and *I*. sp. 2) and *I. macrodidyma*, which are morphologically rather similar, were recognised into the *I. macrodidyma*-complex. For this reason, in the different chapters of this Thesis we have used different names for some of the species belonging to the genus "*Cylindrocarpon"*/*Ilyonectria*, depending on the year in which the experiments were conducted. In addition, the multiplex, nested PCR technique used in this Thesis, which was initially designed by Alaniz *et al*. (2009a) for the detection of "*C.*" *liriodendri*, "*C.*" *macrodidymum* and "*C.*" *pauciseptatum*, allowed the detection of *I. liriodendri*, *I. macrodidyma*-complex and "*C.*" *pauciseptatum* in the DNA samples evaluated. Recently, this technique has been used as a useful tool to detect inoculum sources of these pathogens in a commercial grapevine nursery in Portugal (Cardoso *et al*., 2012).

The main objective of this Thesis has been to study the epidemiology of black-foot disease pathogens in Spain. First, in Chapter 3, different stages of the propagation process of grapevine in Spanish nurseries such as hydration tanks, omega-cut grafting machines, scissors and callusing peat, were evaluated as potential sources of black-foot disease pathogens. In addition, the infection of grafted plants during the rooting phase in nursery fields was also determined. To this end, the detection of *Ilyonectria* spp. was studied by multiplex, nested PCR and by isolation and genotyping in the grapevine planting material before and after the rooting phase in nursery fields.

Ilyonectria liriodendri and *I. macrodidyma*-complex were detected at the different stages of the grapevine propagation process in two different nurseries. These pathogens were not detected in tap water and peat before use at their respective stages, which indicates that they were present in water and peat after being in contact with infected planting material. This is in concordance with the results obtained by Aroca *et al*. (2010) and Gramaje *et al*. (2011), in commercial Spanish grapevine nurseries, who detected *Cadophora luteo-olivacea*, *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *Pm. parasiticum* in hydration tanks, omega-cut grafting machines, scissors and peat for callusing during the grapevine propagation process by multiplex, nested PCR or by isolating them on culture medium, but no from water and peat before use. Thus, our results demonstrate for the first time 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, we confirmed that during the rooting phase in nursery fields the number of plants infected with black-foot pathogens increases markedly. By isolation on culture media, only one *I. torresensis* isolate was obtained from one of the cuttings sampled immediately after callusing. However, after one growing season in nursery fields, *I. liriodendri*, *I. novozelandica* and *I. torresensis* were more frequently isolated from rooted plants. These results are in agreement with those obtained by Halleen *et al*. (2003), who demonstrated that in South African grapevine nurseries less than 1% of the callused cuttings are infected with *Ilyonectria* spp. prior to planting in nursery soils for rooting, whereas 50% or more of the plants are infected by the end of the season. Similar results have been obtained in Spain with Petri disease pathogens such as *Pa. chlamydospora* and *Pm. aleophilum*, which are more frequently isolated from grafted plants after plantation in nursery fields (Giménez-Jaime *et al*., 2006). Regarding the

molecular detection of *Ilyonectria* spp. on grafted cuttings and plants, a greater number of positive samples were found before and after the rooting phase in nursery fields. This could be due to the high sensitivity of the multiplex, nested PCR technique and to the fact that these pathogens may require a higher level of host colonization to be detected by isolation on culture media (Aroca and Raposo, 2007; Aroca *et al*., 2010).

Once the presence of inoculum of black-foot pathogens in the grapevine propagation process has been demonstrated, further research is needed to determine the role of rootstock mother fields, from which dormant cuttings are taken for bench grafting, rooting, or field budding, in the epidemiology of these pathogens. In fact, mother vines have been reported as an inoculum source of other fungal grapevine trunk pathogens such as *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusiccocum parvum*, *Pa. chlamydospora*, *Pm. aleophilum* or *Pm. parasiticum* (Pascoe and Cottral, 2000; Feliciano and Gubler, 2001; Edwards *et al*., 2003; Aroca *et al*., 2010). Thus, another objective of this thesis was to evaluate the soil of grapevine rootstock mother fields as a potential inoculum source of black-foot disease pathogens by using four different techniques: fungal isolation from roots of grapevine seedlings used as bait plants grown in rootstock mother fields (Chapter 4), multiplex, nested PCR and qPCR (Chapter 5), and fungal isolation from roots of weeds collected from rootstock mother fields (Chapter 6).

Four *Ilyonectria* spp., named *I. alcacerensis*, *I. macrodidyma*, *I. novozelandica* and *I. torresensis*, were isolated from the roots of bait plants grown in a rootstock mother field after nine and/or twenty-one months after planting. In general, *Ilyonectria* spp. were isolated with a higher mean incidence in plants sampled after twenty-one months planting in comparison with the plants sampled after nine months planting. "*Cylindrocarpon" macrodidymum* was also commonly isolated from weeds collected in rootstock mother fields showing a high rate of isolation. The analysis of soils collected from rootstock mother fields with the multiplex, nested PCR showed a high rate of detection of *I. macrodidyma*-complex from soil DNA samples, while the rate of detection of *I. liriodendri* was markedly lower in the same DNA samples. Regarding qPCR results, the minimum and maximum levels of *Ilyonectria* spp. DNA detected in soil were 0.01 pg μl^{-1} and 1,904.8 pg μl^{-1}, respectively.

As hypothesized before, these results confirmed the presence of black-foot pathogens in soils from rootstock mother fields. Then, a new hypothesis that needed to

be investigated is the role of soils from nursery fields as well as from commercial vineyards as additional inoculum sources of black-foot pathogens. To this aim we used the same techniques described before: fungal isolation from roots of bait plants (Chapter 4), multiplex, nested PCR and qPCR (Chapter 5), and fungal isolation from roots of weeds (chapter 6).

In nursery fields, the results obtained by using bait plants were similar than those obtained in a rootstock mother field. *Ilyonectria alcacerensis*, *I. macrodidyma*, *I. novozelandica* and *I. torresensis*, were isolated from the roots of grapevine seedlings after nine and/or twenty-one months planting, being the mean incidence of *Ilyonectria* spp. higher twenty-one months after planting. In addition, "*Cylindrocarpon" macrodidymum* was also commonly isolated from weeds collected in nursery fields showing a high rate of isolation. The results obtained with the multiplex, nested PCR as well as with the qPCR showed a high rate of detection of *I. macrodidyma*-complex from soil DNA samples collected in nursery fields, being the rate of detection of *I. liriodendri* markedly lower in the same DNA samples. Regarding qPCR results, the minimum and maximum levels of *Ilyonectria* spp. DNA detected in soil were 0.004 pg μl^{-1} and 757.79 pg μl^{-1}, respectively. In general, *Ilyonectria* DNA concentrations were lower in soil samples from nursery fields than that obtained in rootstock mother fields. This could be due to rootstock mother fields studied in this Thesis consisted of grapevine rootstocks that were more than 15 years old, whereas, according to the current Spanish nursery legislation, nursery fields used for the rooting phase can not be planted for more than two consecutive growing seasons, and must have not been used for grapevine cultivation, at least for the previous twelve years. In this sense, Halleen *et al*. (2003), indicated that in South Africa, where the same nursery soil has been used for decades, the standard nursery practice of a 2-year rotation system, whereby cuttings are planted every second year, alternated with a cover crop, might have led to a build-up of black-foot pathogens in these soils, and suggested that the duration of this rotation period should, therefore, be investigated to establish its effect on pathogen populations.

Regarding the soil of commercial vineyards, three *Ilyonectria* spp., named *I. alcacerensis*, *I. novozelandica* and *I. torresensis*, were isolated from the roots of bait plants grown along nine months in pots filled with soils sampled from ten different commercial vineyards. "*Cylindrocarpon" macrodidymum* was also frequently isolated from weeds collected in several commercial vineyards.

It is interesting to note that in all soil types: roostock mother fields, nursery fields and commercial vineyards, species belonging to *I. macrodidyma*-complex were the most frequently detected. These results are in agreement with those obtained by Alaniz *et al*. (2007), who reported the prevalence of the *I. macrodidyma*-complex associated with black-foot disease in Spain.

When weeds were evaluated as potential host of black-foot pathogens in the three different soil types, variation among weed families and species in the percentage of plants colonized by "*C.*" *macrodidymum* and its field frequency were observed. This variation could affect their importance as alternative hosts as described for weed hosts of *Pythium arrhenomanes* in sugarcane fields by Dissanayake *et al*. (1997), but further research is needed to determine if "*C.*" *macrodidymum* or different *Ilyonectria* spp. have a preference for some weed families or species. Pathogenicity tests showed that "*C.*" *macrodidymum* isolates obtained from weeds were able to induce typical black-foot disease symptoms and caused shoot and root mass reduction on grapevine seedlings. Different levels of virulence were also observed in the "*C.*" *macrodidymum* isolates inoculated. These results are in agreement with those obtained by Alaniz *et al*. (2009b), who detected a relevant genetic and virulence diversity in "*C.*" *macrodidymum* by Inter Simple Sequence Repeat (ISSR) analysis and pathogenicity tests. Consequently, our results suggest that weeds may serve as a source of inoculum for infection of grapevine.

The qPCR assay used in this Thesis was performed by using the genus primer pair YT2F/Cyl-R (Dubrovsky and Fabritius, 2007; Tewoldemedhin *et al*., 2011). The primer pair amplified only DNA derived from *Ilyonectria* spp./"*Cylindrocarpon*" spp., but not DNA of the other tested fungal genera such as *Campylocarpon*, *Fusarium*, *Pythium*, *Phytophthora* or *Rhizoctonia* (Tewoldemedhin *et al*., 2011). These authors also indicated that this primer pair could detect 1 fg/µl of "*C.*" *liriodendri* and "*C.*" *macrodidymum.* During the last few years, several authors have used the qPCR technique in order to detect and quantify inoculum of "*Cylindrocarpon*" spp. from soil samples. Kernaghan *et al*. (2007), quantified "*C.*" *destructans* f. sp. *panacis* in several naturally infested soils of ginseng crop in North America, and calculated average concentrations of target "*C.*" *destructans* f. sp. *panacis* DNA that ranged from 0 to 4.8 pg μl^{-1} of soil extract.

The multiplex, nested PCR and the qPCR techniques have much value for the rapid detection of black-foot pathogens in soil, to reduce the challenges with the

difficult isolation of them from soil using culture media, and would be a valuable tool in any future studies on the epidemiology and control of black-foot disease of grapevines. Moreover, with the qPCR assay, it is possible to detect soilborne pathogens in soil samples in 4-5 h, avoiding laborious post- PCR gel electrophoresis and greatly reducing opportunity for contamination of reaction mixtures with target DNA, further increasing the suitability of this technique for routine diagnostic testing, and also reducing sensitivity to PCR inhibition (Huang *et al*., 2010; Huang and Kang, 2010).

Although this Thesis has focused on the detection of black-foot disease pathogens, Petri disease pathogens, which are also important fungi involved in young grapevine decline (Gramaje and Armengol, 2011), were detected in the experiments conducted in Chapters 4 and 6 . Isolates of *Ca. luteo-olivacea*, *Pm. aleophilum*, *Pm. parasiticum* and *Pa. chlamydospora* were obtained from xylem vessels of bait plants grown both in a rootstock mother field and an open-root field nursery.

Petri disease pathogens were also isolated from several weeds within grapevine fields. Only one isolate of *Pa. chlamydospora* was obtained from *Convolvulus arvensis* L. in a grapevine commercial field and three isolates of *Ca. luteo-olivacea* were obtained from *Bidens subalternans* DC. (in a grapevine rootstock mother field), and from *Plantago coronopus* L. and *Sonchus oleraceus* L. (both in an open-root field nursery). In addition, isolates of *Ca. luteo-olivacea* and *Pa. chlamydospora* were also shown to be pathogenic on grapevine cuttings. In contrast with *Ilyonectria* spp., *Ca. luteo-olivacea* and *Pa. chlamydospora* were isolated from asymptomatic xylem tissues. In similar studies, weeds have been also found to be symptomless hosts for other plant pathogens, such as *Rhizoctonia solani* J.G. Kühn and *Diaporthe phaseolorum* var. *caulivora* Athow & Caldwell in soybean (Black *et al*., 1996a and b). These results could indicate that *Ca. luteo-olivacea* and *Pa. chlamydospora* may be surviving in weeds as endophytes or latent pathogens until the moment in which grapevines are stressed (Edwards and Pascoe, 2004; Ridgway *et al*., 2005; Gramaje *et al*., 2010b). The potential role of these pathogens as grapevine endophytes and the erratic manifestation of leaf symptoms in infected grapevines have been previously reported by different authors (Whiting *et al*., 2001; Fourie and Halleen, 2004; Aroca *et al*., 2010; Halleen *et al*.; 2005; Zanzotto *et al*., 2007; Gramaje *et al*., 2010b).

The mean incidence and isolation of Petri disease pathogens were lower than those obtained for *Ilyonectria* spp. in bait plants grown in a rootstock mother field and

nursery field as well as in weeds, while they were not isolated from plants grown in pots filled with vineyard soils. This could indicate low inoculum densities of Petri disease patoghens in soil or not suitable environmental conditions or a slow infection process. There is no information about how these pathogens penetrate grapevine roots and invade the xylem vessels. It is well known that Petri disease pathogens are slow-growing fungi which usually take up to 20 days to grow on enriched medium, and their ability to compete with other fungi and bacteria arising from plant material is limited (Aroca and Raposo, 2007). Thus, in contrast with *Ilyonectria* spp., probably more time would be needed for the soilborne inoculum of Petri disease pathogens to infect grapevine planting material.

It is well known that *Ilyonectria* spp. are soilborne pathogens which produce chlamydospores to survive for extended periods of time in the soil and to remain dormant until they are stimulated to germinate by plants or are destroyed by other soil organisms (Matturi and Stenton, 1964b; Booth, 1966), but there is few information concerning the biology of *Ilyonectria* spp. In this sense, in Chapter 7, we studied the effects of temperature, pH and water potential (Ψ_s) on mycelial growth, sporulation and chlamydospore production of "*C.*" *liriodendri*, "*C.*" *macrodidymum* and "*C.*" *pauciseptatum*, in order to provide further information on factors affecting growth, reproductive and survival of these pathogens (Chapter 7).

In general, "*C.*" *liriodendri*, "*C.*" *macrodidymum* and "*C.*" *pauciseptatum* were able to grow over a range of temperatures from 5 to 30ºC, with optimum temperatures for growth between 20 to 25ºC, but they did not grow at 35ºC. These results are in agreement with those obtained in previous studies (Halleen *et al*., 2004; Petit and Gubler, 2005; Halleen *et al*., 2006; Alaniz *et al*., 2007; Schroers *et al*., 2008), and indicate that "*Cylindrocarpon*" spp. associated to black foot-disease of grapevine are mesophilic, as most fungi, which commonly grow within the range 10−40ºC (Deacon, 2006).

Previous research has suggested that species belonging to the genus "*Cylindrocarpon*" are calcicolous, with optimal pH around 7.0 and being poorly represented in acid soils (Matturi and Stenton, 1964a). Nevertheless, in our study "*Cylindrocarpon*" spp. associated with black-foot of grapevine showed broad pH tolerance for mycelial growth, although with slight differences among isolates. All

isolates were able to grow between pH 4.0 to 8.0. This corresponds with the optimum pH range indicated by Deacon (2006) for mycelial growth of most fungi.

Regarding the effect of Ψ_s, the response of "*Cylindrocarpon*" spp. isolates was reduced mycelial growth as Ψ_s decreased. Our results indicated that the isolates may have benefited from small to modest additions of solutes. In general, mycelial growth was greater on amended media at -0.5, -1.0 and/or -2.0 MPa compared with that on nonamended PDA (-0.3 MPa), and was reduced at Ψ_s values less than -2.0 MPa. Moreover, the effect of Ψ_s on mycelial growth was similar whether KCl or NaCl was used as the osmoticum, indicating that the observed responses were caused by changes in Ψ_s rather than by toxicity of the osmotic. These results, together with those obtained in the pH experiments, suggest that "*Cylindrocarpon*" spp. pathogenic to grapevine are likely to proliferate in most vineyard soils.

In all studied conditions, "*C.*" *liriodendri* was the species with the greatest capacity for sporulation compared with "*C.*" *macrodidymum* and "*C.*" *pauciseptatum.* Specifically, regarding the effect of temperature, Alaniz *et al*. (2007) indicated that "*C.*" *macrodidymum* can be differentiated from "*C.*" *liriodendri* by producing fewer conidia at 5 and 10ºC, which agrees with our results. It is also interesting to note that the "*C.*" *macrodidymum* isolates Cy47 and Cy81, which showed reduced mycelial growth and sporulation in the temperature, pH and Ψ_s experiments when compared with "*C.*" *liriodendri* isolates and "*C.*" *macrodidymum* isolate Cy14, belong to the genetic Groups G7 and G6, respectively, described by Alaniz *et al*. (2009b) using ISSR analysis. These authors demonstrated that isolates included in these groups were significantly more virulent to grapevine than other "*C.*" *macrodidymum* and "*C.*" *liriodendri* isolates.

Due to the recent taxonomic changes in the genus *Cylindrocarpon* based on the multigene analysis strategy (Cabral *et al*., 2012c), we decided to revise the identification of the isolates Cy14, Cy 47 and Cy81. HIS sequence of these isolates revealed that isolate Cy14 belongs to the species *I. macrodidyma*, while isolate Cy47 and Cy81 belong to the species *I. alcacerensis*. Thus, this could explain while isolate Cy14 showed a different pattern in the temperature, pH and Ψ_s experiments when compared with the isolates Cy47 and Cy 81. Nevertheless, according to this, the results obtained by Alaniz *et al*. (2009b) about virulence of "*Cylindrocarpon*" isolates to grapevine seem not to be in concordance with those obtained by Cabral *et al*. (2012b), who revealed that only *I. lusitanica*, *I. estremocensis* and *I. europaea* are more virulent to grapevine than

species previously accepted to represent the main causal agents of black-foot, such as *I. liriodendri* and *I. macrodidyma*.

Chlamydospore production of "*C.*" *liriodendri*, "*C.*" *macrodidymum* and "*C.*" *pauciseptatum* was not much affect by temperature, pH and Ψ_s. 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 in both osmotic media tested. Petit and Gubler (2005), indicated that "*C.*" *macrodidymum* isolates produced relatively more chlamydospores than "*C.*" *liriodendri*, although they considered this characteristic too inconsistent to be used for identification purposes.

The results obtained in this Thesis confirm that soils from rootstock mother fields, nursery fields as well as commercial vineyards are largely infested with black-foot pathogens and can be considered as important inoculum sources for black-foot pathogens. The presence of several Petri disease pathogens in the three soil types has also been demonstrated. In addition, we have improved our understanding of the biology of some "*Cylindrocarpon*"/*Ilyonectria* species, which showed the ability to be active over a wide pH range, while temperature and Ψ_s changes can affect their growth and reproductive potential.

Finally, it is important to note that the results obtained in this Thesis represent the first report of *I. alcacerensis, I. novozelandica* and *I. torresensis* isolated from grapevines in Spain, and also the first report of *Pa. chlamydospora* on a host different from grapevine. Moreover, *Pm. parasiticum* and *Ca. luteo-olivacea* have been reported for the first time in grapevine nursery soils.

All together, these results emphasize the importance of suitable control measures to prevent or eradicate infections caused by soilborne inoculum of black-foot and Petri disease pathogens, mainly in grapevine nurseries in order to improve the quality of grapevine planting material and to prevent the spread of the diseases. Chemical control with fungicides (Fourie and Halleen, 2006; Rego *et al*., 2006; Halleen *et al*., 2007; Alaniz *et al*., 2011) and biological methods such as the use of *Trichoderma* (Fourie and Halleen, 2006; Halleen *et al*., 2007), arbuscular mycorrhizae (Petit and Gubler, 2006; Bleach *et al*., 2008) or soil biofumigation (Bleach *et al*., 2010) can be used as a strategy to decrease the incidence and severity of *Ilyonectria* spp. during the nursery propagation process. Finally, standard hot water treatment protocols at 50ºC for 30 min may be sufficient to control *Ilyonectria* spp. in grapevine propagation material (Gramaje *et al*.,

2010a). Thus, an integrated management program that includes biological, chemical, hot water treatment and other control measures would ensure high phytosanitary quality plant production with low levels of infection.

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Chapter 9

CONCLUSIONS

1.- Inoculum of *Ilyonectria* spp. is present at different stages of the grapevine nursery propagation process: hydration tanks, scissors, omega-cut grafting machines and peat used for callusing.

2.- During the rooting phase in nursery fields the number of grapevine plants infected with *Ilyonectria* spp. increases markedly.

3.- *Ilyonectria alcacerensis*, *I. macrodidyma*, *I. novozelandica* and *I. torresensis* were frequently isolated from bait plants grown in soils from rootstock mother fields, nursery fields and commercial vineyards.

4.- The multiplex, nested-PCR and the qPCR techniques allowed a high rate of detection of *Ilyonectria macrodidyma*-complex from soil DNA samples collected in nursery fields as well as in rootstock mother fields, while the rate of detection of *I. liriodendri* was lower in the same DNA samples.

5.- *Ilyonectria* DNA concentrations determined with qPCR were higher in soil samples from rootstock mother fields than that obtained in nursery fields.

6.- The detection of *Ilyonectria* spp. in soils from nurseries and commercial vineyards using bait plants; nested, multiplex PCR and qPCR, demonstrates that they are largely infested with these pathogens, confirming them as important sources of inoculum.

7.- "*Cylindrocarpon*" *macrodydimum* was commonly isolated from weeds collected in grapevine rootstock mother fields, nursery fields and commercial vineyards, being recovered from 15 out of 19 weed families evaluated and 26 out of 52 species. The family Asteraceae was the most prevalent with six weed species hosting this pathogen followed by Amaranthaceae, Brassicaceae, Chenopodiaceae, Euphorbiaceae and Poaceae.

8.- Our ability to recover grapevine pathogens from vineyard weeds and to demonstrate pathogenicity of recovered strains on grape, suggests that these weeds may also serve as a source of inoculum for infection of grapevine.

9.- In all studies performed in this Thesis, species belonging to *I. macrodidyma*-complex were the most frequently detected.

10.- The study of the effects of temperature, pH and water potential (Ψ_s) on mycelial growth, sporulation and chlamydospore production of "*Cylindrocarpon*" *liriodendri*, "*C.*" *macrodidymum* and "*C.*" *pauciseptatum* concluded that these species have abilities to be active over a wide pH range, while temperature and Ψ_s changes can affect their growth and reproductive potential.

11.- Petri disease pathogens were also detected on bait plants and weeds. *Cadophora luteo-olivacea, Phaeoacremonium aleophilum, Pm. parasiticum* and/or *Phaeomoniella chlamydospora* were isolated from xylem vessels of bait plants grown both in a rootstock mother field and a nursery field as well as from weeds collected in rootstock mother fields, nursery fields and commercial vineyards, confirming soil and weeds as inoculum sources of these pathogens.

12.- The results obtained in this Thesis represent the first report of *I. alcacerensis*, *I. novozelandica* and *I. torresensis* isolated from grapevines in Spain, and also the first report of *Pa. chlamydospora* on a host different from grapevine. Moreover, *Pm. parasiticum* and *Ca. luteo-olivacea* have been reported for the first time in grapevine nursery soils.