UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Escola Tècnica Superior
d'Enginyeria Agronòmica
i del Medi Natural

# Detection and Identification of *Phytophthora* spp. in WoodyPlant Nurseries and Holm Oak Forests

**Doctoral Thesis**

**Beatriz Mora Sala**

Supervisors, Dra. Paloma Abad-Campos & Dra. Mónica Berbegal Martínez

Valencia, June 2020

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# Summary

*Phytophthora* is one of the most relevant and aggressive plant pathogenic genus in agriculture and forestry. Due to the increasing environmental threat of invasive plant pathogens, monitoring new areas in the last decade has revealed a large number of new *Phytophthora* species-plant host interactions. The introduction of soilborne pathogens, such as *Phytophthora* in *Fagaceae* forests modifies the microbial community present in the rhizosphere with relevant environmental and economic consequences. The genus *Quercus* is one of the most extended *Fagaceae* genera in Europe, and *Q. ilex* is the dominant tree in Spain.

The link between *Phytophthora* dispersion in natural ecosystems and human derived activities has been previously studied. Numerous sampling in nurseries and public spaces revealed a great diversity of *Phytophthora* species that compromised production and threated natural ecosystems. In this context, sampling ornamental and forest nurseries in four Spanish regions was carried out focusing on possible symptoms associated to *Phytophthora* on different hosts and including water samples from the nurseries. The results showed 17 *Phytophthora* phylotypes affecting 22 plant species included in 19 plant genera and some of them reported for the first time in Spain.

Among the soilborne pathogens isolated in the nurseries, a large number of Cylindrocarpon-like asexual morphs were identified from the roots of woody hosts. A collection of Cylindrocarpon-like isolates recovered from Spanish nurseries was characterised by morphological and molecular studies. Twelve species belonging to the genera *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria* were identified from damaged roots of 15 different host genera and other four species were newly described. The study demonstrated the prevalence of this fungal group associated with seedlings of diverse hosts showing decline symptoms in forest nurseries in Spain.

The susceptibility of *Quercus ilex* to the inoculation with eight *Phytophthora* species obtained from nurseries was evaluated. The most aggressive species were *Phytophthora cinnamomi*, *Phytophthora cryptogea*, *Phytophthora gonapodyides*, *Phytophthora plurivora* and *Phytophthora*

*psychrophila* followed by *Phytophthora megasperma*, while *Phytophthora quercina* and *Phytophthora nicotianae* were the least aggressive species. Results obtained in the pathogenicity test confirmed that all *Phytophthora* species tested could represent a threat to holm oak ecosystems. In this context, a study to verify the presence and/or detection of *Phytophthora* species was conducted in two holm oak areas of Spain (southwestern dehesas and northeastern woodland) using different isolation and detection methods.

Direct isolation and baiting methods in declining and non-declining holm oak trees revealed *Phytophthora cambivora*, *P. cinnamomi*, *P. gonapodyides*, *P. megasperma*, and *Phytophthora pseudocryptogea* in the dehesas, while in the northeastern woodland, no *Phytophthora* spp. were recovered. Statistical analyses indicated that there was not a significant relationship between the *Phytophthora* spp. isolation frequency and the disease expression of the holm oak stands in the dehesas. *Phytophthora quercina* and *P. cinnamomi* TaqMan real-time PCR probes showed that *P. quercina* was detected in a higher frequency than *P. cinnamomi* in both studied areas.

A better understanding of the *Phytophthora* spp. diversity in holm oak forests was assessed using Next Generation Sequencing (NGS) in six *Q. ilex* stands located in three regions in Spain. Thirty-seven *Phytophthora* phylotypes belonging to clades 1 to 12, except for clades 4, 5 and 11, were detected in this study, demonstrating a high diversity of *Phytophthora* species in holm oak Spanish forests. The most abundant phylotypes were *P. quercina*, *P. psychrophila*, *P. cinnamomi* and *P. plurivora*.

In summary, this Thesis demonstrated a high diversity of *Phytophthora* and Cylindrocarpon-like species in Spanish nurseries, reporting new pathogen-plant host interactions and new species. Furthermore, pathogenicity test showed that holm oak, which is the dominant tree species in Spain, was susceptible to eight *Phytophthora* spp. detected in the nurseries and in Spanish *Q. ilex* stands. Coupling direct isolation methods with DNA-based techniques, provided a better understanding of *Phytophthora* diversity in holm oak ecosystems.

# Resumen

*Phytophthora* es uno de los géneros fitopatógenos más relevantes y agresivos en la agricultura y silvicultura. Muestreos realizados en la última década han revelado una gran cantidad de interacciones entre especies de *Phytophthora* y plantas, desconocidas con anterioridad. La introducción de nuevos patógenos de suelo, como *Phytophthora* en los bosques de Fagaceae, modifica la comunidad microbiana presente en la rizosfera, con importantes consecuencias ambientales y económicas. El género *Quercus* es uno de los géneros de Fagaceae más extendidos en Europa, y *Quercus ilex* es la especie dominante en España.

El vínculo entre la dispersión de *Phytophthora* en los ecosistemas naturales y las actividades del ser humano, ha sido previamente estudiado. Numerosos muestreos en viveros y espacios públicos, mostraron la presencia de gran diversidad de especies de *Phytophthora* que podían suponer una amenaza para la producción y los ecosistemas naturales. En este contexto, se realizó un muestreo de viveros ornamentales y forestales en cuatro comunidades autónomas españolas, centrándose en los posibles síntomas asociados a *Phytophthora* en diferentes hospedantes e incluyendo muestras de agua de los viveros. Los resultados mostraron 17 filotipos de *Phytophthora* que afectan a 22 especies vegetales incluidas en 19 géneros. Algunas de estas interacciones se citaron por primera vez en España.

Entre los patógenos de suelo aislados en los viveros, se identificó una gran cantidad de formas asexuales tipo Cylindrocarpon en las raíces de hospedantes leñosos. Se caracterizó una colección de aislados mediante estudios morfológicos y moleculares. Se identificaron 12 especies pertenecientes a los géneros *Cylindrodendrum*, *Dactylonectria* e *Ilyonectria* en hospedantes pertenecientes a 15 géneros y otras cuatro nuevas especies se describieron. El estudio demostró la prevalencia de este grupo fúngico asociado con plántulas de diversos hospedantes que muestran síntomas de decaimiento en viveros forestales.

Se evaluó la susceptibilidad de *Q. ilex* a la inoculación con ocho especies de *Phytophthora* obtenidas de muestreos en viveros. Las especies más agresivas

fueron *Phytophthora cinnamomi*, *Phytophthora cryptogea*, *Phytophthora gonapodyides*, *Phytophthora plurivora* y *Phytophthora psychrophila*, seguidas de *Phytophthora megasperma*, mientras que *Phytophthora quercina* y *Phytophthora nicotianae* fueron las especies menos agresivas. Los resultados obtenidos en el ensayo de patogenicidad confirmaron que todas las especies de *Phytophthora* evaluadas podrían representar una amenaza para los encinares. En este contexto, se realizó un estudio para verificar la presencia y / o detección de especies de *Phytophthora* en dos áreas de España (dehesas del sudoeste y bosque del noreste) utilizando diferentes métodos de aislamiento y detección.

El aislamiento directo y el método de trampeo vegetal en muestras obtenidas a partir de encinas con y sin decaimiento, identificaron *Phytophthora cambivora*, *P. cinnamomi*, *P. gonapodyides*, *P. megasperma* y *Phytophthora pseudocryptogea* en las dehesas, mientras que, en el bosque del noreste, no se aisló *Phytophthora* spp. Los análisis estadísticos indicaron que no había una relación significativa entre la frecuencia de aislamiento de las especies de *Phytophthora* y la expresión de los síntomas de la enfermedad en las encinas de las dehesas. Además, *P. quercina* se detectó con mayor frecuencia que *P. cinnamomi* en las dos áreas estudiadas utilizando sondas TaqMan de PCR a tiempo real.

Se evaluaron seis masas de *Q. ilex* ubicadas en tres comunidades autónomas de España mediante “Next Generation Sequencing” (NGS) para tener un mayor conocimiento sobre la diversidad de *Phytophthora* spp. en los bosques de encinas. Se detectaron 37 filotipos de *Phytophthora* pertenecientes a los clados 1 al 12, excepto los clados 4, 5 y 11, lo que demuestra una gran diversidad de *Phytophthora* en los encinares estudiados. Los filotipos más abundantes fueron *P. quercina*, *P. psychrophila*, *P. cinnamomi* y *P. plurivora*.

# Resum

*Phytophthora* és un dels gèneres fitopatògens més rellevants i agressius en l'agricultura i la silvicultura. Mostrejos realitzats en l'última dècada han revelat una gran quantitat d'interaccions desconegudes fins ara entre espècies de *Phytophthora* i plantes. La introducció de nous patògens del sòl, com *Phytophthora* en els boscos de Fagaceae, modifica la comunitat microbiana present en la rizosfera, amb importants conseqüències ambientals i econòmiques. El gènere *Quercus* és un dels gèneres de Fagaceae més estesos a Europa, i *Quercus ilex* és l'espècie dominant a Espanya.

El vincle entre la dispersió de *Phytophthora* en els ecosistemes naturals i les activitats de l'ésser humà, ja ha sigut estudiat prèviament. Nombrosos mostrejos en vivers i espais públics, van mostrar la presència d'una gran diversitat d'espècies de *Phytophthora* que podien suposar una amenaça per a la producció i els ecosistemes naturals. En aquest context, es va realitzar un mostreig de vivers ornamentals i forestals en quatre comunitats autònomes espanyoles, centrant-se en els possibles símptomes associats a *Phytophthora* en diferents hostes i incloent mostres d'aigua dels vivers. Els resultats van mostrar 17 filotipus de *Phytophthora* que afecten 22 espècies vegetals incloses en 19 gèneres. Algunes d'aquestes interaccions es van citar per primera vegada a Espanya.

Entre els patògens del sòl aïllats en els vivers, es va identificar una gran quantitat de formes asexuals tipus Cylindrocarpon en les arrels de plantes llenyoses. Es va caracteritzar una col·lecció d'aïllats mitjançant estudis morfològics i moleculars. Es van identificar 12 espècies pertanyents als gèneres *Cylindrodendrum*, *Dactylonectria* i *Ilyonectria* en hostes pertanyents a 15 gèneres i altres quatre noves espècies es van descriure. L'estudi va demostrar la prevalença d'aquest grup fúngic associat amb plàntules de diversos hostes que mostren símptomes de decaïment en vivers forestals espanyols.

Es va avaluar la susceptibilitat de *Q. ilex* a la inoculació amb huit espècies de *Phytophthora* obtingudes de mostrejos en vivers. Les espècies més agressives van ser *Phytophthora cinnamomi*, *Phytophthora cryptogea*, *Phytophthora gonapodyides*, *Phytophthora plurivora* i *Phytophthora psychrophila*, seguides de *Phytophthora megasperma*, mentre que *Phytophthora quercina* i *Phytophthora nicotianae* van ser les espècies menys agressives. Els resultats obtinguts en l'assaig

de patogenicitat van confirmar que totes les espècies de *Phytophthora* avaluades podrien representar una amenaça per als ecosistemes d'alzines. En aquest context, es va realitzar un estudi per a verificar la presència i / o detecció d'espècies de *Phytophthora* en dues àrees d'Espanya (deveses del sud-oest i bosc del nord-est) utilitzant diferents mètodes d'aïllament i detecció.

L'aïllament directe i el mètode de parany vegetal en mostres obtingudes a partir d'alzines, amb i sense decaïment, van identificar *Phytophthora cambivora*, *P. cinnamomi*, *P. gonapodyides*, *P. megasperma* i *Phytophthora pseudocryptogea* en les deveses, mentre que, en el bosc del nord-est, no es va aïllar *Phytophthora* spp. Les anàlisis estadístiques van indicar que no hi havia una relació significativa entre la freqüència d'aïllament de les espècies de *Phytophthora* i l'expressió dels símptomes de la malaltia en les alzines en les deveses. A més, *P. quercina* es va detectar amb major freqüència que *P. cinnamomi* en les dues àrees estudiades utilitzant sondes TaqMan de PCR a temps real.

Es van avaluar sis masses de *Q. ilex* situades en tres comunitats autònomes d'Espanya mitjançant "Next Generation Sequencing" (NGS) per a tindre un major coneixement sobre la diversitat de *Phytophthora* spp. en els boscos d'alzines. Es van detectar 37 filotipus de *Phytophthora* pertanyents als clades 1 al 12, excepte els clades 4, 5 i 11, la qual cosa demostra una gran diversitat d'espècies de *Phytophthora* en els alzinars estudiats. Els filotipus més abundants van ser *P. quercina*, *P. psychrophila*, *P. cinnamomi* i *P. plurivora*.

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# Chapter 1

## GENERAL INTRODUCTION

### 1. *Quercus ilex* L.

### *1.1.* Characteristics and distribution

The genus *Quercus* (Fagaceae) comprises more than 600 species that extends through the northern hemisphere and reaches the south to Central America and Ecuador (Ruiz de la Torre & Ceballos y Fernández de Córdoba, 1979). The number of species increases from west to east, from Europe and Africa to North America´s Pacific coast, being Mexico the country with the highest *Quercus* diversity (Ruiz de la Torre & Ceballos y Fernández de Córdoba, 1979).

In the Mediterranean basin, *Quercus* species are the most spread forests trees. The Iberian Peninsula is one of the main centers of diversity of *Quercus* in Eurasia, with more than nine native species forming extensive forests (Ruiz de la Torre, 2002). *Quercus canariensis* Willd., *Quercus cerris* L., *Quercus coccifera*, *Quercus faginea* (Cout) Camus, *Quercus ilex* L., *Quercus petraea* (Matts.) Liebl., *Quercus pubescens* Willd., *Quercus pyrenaica*, *Quercus robur* L., Willd. and *Quercus suber* L. are some of the most important species present in Spanish forests (Ruiz de la Torre & Ceballos y Fernández de Córdoba, 1979).

*Quercus ilex*, also known as holm oak, is an evergreen tree native to Southern Europe and Northwest Africa having its ecological optimum in the Western and Central Mediterranean (Romane & Terradas, 1992). It is the most characteristic tree species in Spanish forests covering an area of 2.8 M ha (15.3 % of the forested area) and also in the dehesas (2.4 M ha), that are oak rangelands which consist mainly of holm oaks mixed with cork oaks (*Q. suber*) and even a deciduous oak (*Q. faginea*) (MAGRAMA 2014). In fact, Spain is the country with the largest *Q. ilex* surface (Ruiz de la Torre & Ceballos y Fernández de Córdoba, 1979), and this species is very common in almost all provinces but being scarce in the Canary Islands and Galicia regions the Canary Islands and Galicia regions, where it is scarce (Romane & Terradas, 1992). *Quercus ilex* includes two subspecies *Quercus ilex* subsp. *ballota* (Desf.) Samp. and *Quercus*

*ilex* subsp. *ilex* L. In Spain, *Q. ilex* subsp. *ilex* is located in Northeast while *Q. ilex* subsp. *ballota* is elsewhere distributed.

*Quercus ilex* is a broadleaved tree or shrub that can reach 25 m high with a variable appearance, as it has been modified by the direct or indirect action of man. This species is characterised by coriaceous dark green leaves with a woolly lower side, and produces acorns from November to January with high production every 4-6 years (de Rigo & Caudullo, 2016). It does not require specific soil conditions, growing either on calcareous or in siliceous soils, improving especially the quality of the first ones (Ruiz de la Torre & Ceballos y Fernández de Córdoba, 1979; Sainz Ollero *et al.*, 2005). *Quercus ilex* extends from sea level to 2,000 – 2,800 m high depending on the latitude and the region, having great resistance to drought, continental features, heat and cold (Ruiz de la Torre & Ceballos y Fernández de Córdoba, 1979; Romane & Terradas, 1992). In Spain the most dense holm oak forests´ altitude ranges from 200 to 800 m (Moro, 2007). *Quercus ilex* has a high environmental, socio-economic and cultural value (Sainz Ollero *et al.*, 2005).

Mediterranean holm oak forests have been heavily disturbed by human activities for centuries, which have exploited (food source for acorns, fuel - charcoal and construction material – cork), modified the species mixture and in many cases replaced forests by crop production and urban areas (Romane & Terradas, 1992; Sainz Ollero *et al.*, 2005; de Rigo & Caudullo, 2016). Forests are advanced successional stages that use most of their production in their own maintenance, which prevents intensive continued exploitation without altering their characteristics (Sainz Ollero *et al.*, 2005). Man has simplified holm oak natural ecosystems that would show a high heterogeneity in their distribution and floristic composition by reversing the succession process to obtain more productive resources (Romane & Terradas, 1992; Sainz Ollero *et al.*, 2005; Trumbore *et al.*, 2015; Cernadas *et al.*, 2018). Due to this process, it is difficult to find mature holm oak forests in Spain, in which diverse *Quercus* species would grow together favouring mixed *Quercus* formations (Sainz Ollero *et al.*, 2005). In the Western and Southwestern Iberian Peninsula, the holm oak forests are mainly managed as agro-silvo-pastoral systems known as dehesas in Spain and "montados" in Portugal. These ecosystems include large isolated trees (*Q. ilex* and/or *Q. suber* with densities ranging 30 – 60 trees/ha) where livestock feed on

the acorns and grass (Romane &Terradas, 1992; Sainz Ollero *et al.*, 2005; Cernadas *et al.*, 2018). This management promotes the production of acorn, cork extraction and alternates cultivation or grazing favouring soils fertility as the *Quercus* trees extract water and nutrients from deep in the soils and increase organic matter under its canopy protecting soils from erosion (Romane & Terradas, 1992; Sainz Ollero *et al.*, 2005). In the dehesas, the wood is also a valuable asset. In addition, these ecosystems host migrant birds from Central and Northern Europe during the winter season.

### *1.2.* Nursery industry and reforestations

There is a failure in the tree population dynamic of the dehesas due to exceeded holm oak regeneration threshold (Carmona *et al.*, 2013; Corcobado *et al.*, 2013). Most stands are overaged and seedlings and saplings are sparse (Plieninger *et al.*, 2003). Regeneration has been inhibited since stands were opened for agriculture and grazing (Plieninger *et al.*, 2003). The increasing livestock pressure due to the shift from transhumance practices, to permanent grazed soils, has reduce the juvenile oak saplings growth rate (increasing the age to reach the height threshold to avoid being eaten by livestock) and promoted soil degradation (increase of soil compaction and nitrogen content) (Carmona *et al.*, 2013; Corcobado *et al.*, 2013). Juvenile holm oaks in grazed dehesas use to grow via asexual reproduction because if grazing is present, even at low pressure, acorns cannot germinate (Carmona *et al.*, 2013).

Holm oak acorn dispersal is very limited, so it has a low replacement rate by seed germination (Romane & Terradas, 1992). Moreover, acorn production is highly variable (Cernadas *et al.*, 2018). Plieninger *et al.*, (2003) reported a two-years-old lack of seedlings and saplings from seed origin in the dehesas. Holm oak seedlings survival requires the presence of shrubby areas to avoid a high animal feeding pressure (Plieninger *et al.*, 2003). According to Plieninger *et al.* (2003), there are four options to face holm oak regeneration: (i) reduction of livestock densities and the intensity of farming, (ii) afforestation (directive CEE 2080/92 by which the European Union provides for the conversion of agricultural lands into forests), (iii) abandonment and conversion of the dehesas into game hunting estates, (iv) spatially and temporally limited set-aside of grazing and cultivation (20-30 years).

*Quercus ilex* is used in artificial reforestation programmes in Spain, Portugal and Morocco (Corcobado *et al.*, 2017). According to FAO (2015), in Spain 8.9 M ha/year were artificially reforested during the period 1990-2010. Reforestation can be conducted by direct seeding of acorns and by planting nursery stock. Drawbacks of direct sowing of acorns is that are more vulnerable to predation, survive less than one-year-old holm oak planted seedlings and that are susceptible to oomycetes infection (damping-off associated with *Phytophthora* spp. and *Pythium* spp.) if they are present in the soils (Corcobado *et al.*, 2017). A previously published study about early survival of holm oak concluded that in soils infested with *Phytophthora* species, the bigger size of the acorn, the previous co-infection by other *Phytophthora* species less virulent than *P. cinnamomi* and the subspecies of *Q. ilex* present were determinant for the successful germination and development of the seedling (Corcobado *et al.*, 2017). Planting nursery stock has the inconvenience of the high probability of being infected by *Phytophthora* species, as this genus is present in almost all nurseries worldwide, with the consequent risk of *Phytophthora* dispersion to natural and semi-natural ecosystems (Forsberg, 1985; Ham & Hansen, 1986; Ferguson & Jeffers, 1999; Rizzo *et al.*, 2002, 2003; Schwingle *et al.*, 2007; Donahoo & Lamour, 2008; Parke *et al.*, 2014; Bienapfl & Balci, 2014; Corcobado *et al.*, 2017; Jung *et al.*, 2016; Jung *et al.*, 2018).

Jung *et al.* (2018) estimated that in Europe between 1990 and 2010, approximately 680,000 new afforestations were conducted with *Phytophthora*-infested nursery stock dispersed through an area of almost 5 M ha. These authors also suggested that during the same period, the area of potentially *Phytophthora*-infested reforestations might have exceeded 17 M ha (Jung *et al.*, 2016; Jung *et al.*, 2018). Most of these newly introduced *Phytophthora* species are considered invasive pathogens in Europe (Jung *et al.*, 2018). In the two last decades, 50% approximately of the European reforestations were made in the Iberian Peninsula, mainly by planting pure or mixed stands of *Q. ilex* and *Q. suber*, and this material was probably infested by *Phytophthora cinnamomi* Rands. at a rate of 86% (Jung *et al.*, 2016).

International plant trade is responsible for the introduction of invasive forests pathogen species that in many cases cause relevant ecological disturbances and reduce the biodiversity of the ecosystem (Weste & Marks,

1987; Anagnostakis, 1988; Brasier *et al.*, 1993; Rizzo *et al.*, 2002, 2003; Brasier, 2008, Wingfield *et al.*, 2015). The application of fungicides or fungistatic chemicals in nurseries masks *Phytophthora* symptoms and the asymptomatic plants are introduced avoiding being intercepted in the phytosanitary controls (Brasier, 2008). These infected nursery stock act as inoculum reservoir and continues infecting other plants of the nursery and/or escapes the nursery boundaries infecting the vegetation from natural ecosystems. Heavy losses are often the result of delays in recognition of *Phytophthora* as the causal agent of the disease (Erwin & Ribeiro, 1996). Moreover, as *Phytophthora* alien species have not co-evolved with the vegetation and the native *Phytophthora* species, if the new environment is suitable, highly aggressive diseases occur and hybridisation could take place infecting a wide range of hosts (Schwingle *et al.*, 2007; Moralejo *et al.*, 2009a; Kroon *et al*., 2012).

### *1.3.* Decline associated to *Phytophthora* infection

In addition to the reduction of holm oak regeneration, the problem is exacerbated by the oak decline caused by the genus *Phytophthora* that is happening in Spain from the early twentieth century and that probably has been enhanced by the more frequent and extreme weather events (Brasier, 1992a, b, 1996; Sánchez *et al.*, 2002; Plieninger *et al.*, 2003; Cernadas *et al.*, 2018). *Phytophthora cinnamomi* was already present in Europe in 1940 affecting *Quercus* species and it was dispersed from chestnut decayed forests to neighbouring oak forests in Spain around 1920-1940, when chestnut disease intensity increased (Brasier, 1992b). The dispersion would probably occur via infested soil (human and animal movement), watercourses and/or nursery stock (Brasier, 1992b).

The flow of *Phytophthora* species associated with plant trade may start with an accidental dispersion of infected plant material or soil from its natural habitat. Once in the nursery, the favourable environmental conditions (mild temperatures and free water), may enhance the inoculum production and dispersion improving pathogen establishment. Nurseries unintentionally encourage fungicide resistance, as it is a common practice to spray plants preventively or curatively. Therefore, plants may remain asymptomatic when they are actually infected by *Phytophthora* spp. (Bienapfl & Balci, 2014;

Migliorini *et al.*, 2015). Finally, the infected asymptomatic plant is sold, thus the pathogen escapes the nursery (Moralejo *et al.*, 2009a; Migliorini *et al.*, 2015).

Probably, the most relevant disease affecting *Quercus* spp. in nurseries is damping off caused by *Phytophthora* spp., *Pythium* spp. and Cylindrocarpon-like asexual morphs (Andicoberry *et al.*, 2001; Sánchez *et al.*, 2002, 2005ref). After the outbreak of *Phytophthora ramorum* (Werres, De Cock, and Man in't Veld), phytosanitary inspections to detect other *Phytophthora* spp. such as *Phytophthora kernoviae* Brasier, Beales & Kirk, *Phytophthora pinifolia* Durán, Gryzenh. & Wingf. or *Phytophthora lateralis* Tacker & Milbrath, increased in nurseries, gardens and forestry stands, revealing new *Phytophthora* hosts and unknown *Phytophthora* species (Donahoo *et al.*, 2006; Schwingle *et al.*, 2006; Balci *et al.*, 2008; Hüberli *et al.*, 2008; Hulvey *et al.*, 2010; Pérez-Sierra *et al.*, 2012; Abad *et al.*. 2014; Bienapfl & Balci, 2014).

Invasive species are one of the major threats for forest sustainability. *Quercus ilex* mortality associated to *Phytophthora* root rot is a serious risk for the sustainability of the Mediterranean ecosystem, including the dehesas (Ruiz-Gómez *et al.*, 2019). An integrated management strategy should be implemented for *Phytophthora* diseases based on the prevention principle. The objective should be to reduce the dispersion risk and/or decrease the density and infective capacity of the inoculum in the soils.

## *2.* The genus *Phytophthora*

### *2.1.* Taxonomy

*Phytophthora* ("plant destroyer") is an oomycete that was first described in the nineteenth century by De Bary (1876) as the causal agent of potato late blight. The plant pathogenic oomycetes group has many taxa and exhibit remarkably diverse lifestyles ranging from obligate biotroph to necrotroph (Erwin & Ribeiro, 1996; Crone *et al*., 2013a, b), including some genera such as *Phytophthora*, *Pythium*, *Peronospora*, *Albugo* or *Aphanomyces*. Oomycetes group was initially included in the kingdom Fungi but due to its differences with true fungi it was positioned in the kingdom Chromista (Cavalier-Smith, 1986, 1987; Barr, 1992; Dick, 1995a, Erwin & Ribeiro, 1996), which afterwards it was known as Straminipila (Dick, 1995b). Some of the characteristics that set it apart

from true fungi are the composition of the cell wall, composed of β-1,3 and β-1,6 glucans and cellulose (β-1,4 glucans), instead of chitin; storage reserves, are β-1,3 glucans instead of glycogen; somatic cells morphology, the somatic nucleus and mitochondrial cristae (Erwin & Ribeiro, 1996).

Oomycetes are characterized by the formation on oospores as a result of sexual reproduction. Oospores develop in the oogonium after the union of two gametangia, in which, meiosis takes place before fertilization (Sansome, 1965). In addition, they are characterized by having a coenocytic mycelium and by the production, in the presence of water, of uninucleate motile zoospores with two cilia (flagella) of different size (Erwin and Ribeiro, 1996). These infective units are able to synthesize a cyst cell wall within minutes (Erwin & Ribeiro, 1996).

The genus *Phytophthora* is closely related to the genus *Pythium* and both genera were classified in the family Pythiaceae, so named because the genus *Pythium* was described first (Erwin & Ribeiro, 1996). Recent phylogenetic studies indicate a closer affiliation of *Phytophthora* with the downy mildews and white rusts in the Peronosporales (Thines *et al.*, 2009, Beakes and Sekimoto, 2009) but the relationship between Peronosporales and Pythiales (*Pythium*) needs clarification (Martin *et al.*, 2012).

Nowadays, the genus *Phytophthora* with more than 150 known species, includes well-known plant pathogens with a wide host range that are devastating natural ecosystems and it is among the top world pathogens but also include saprophytic taxa (Erwin& Ribeiro, 1996; Mideros *et al.*, 2018; Tremblay *et al.*, 2018). When mycelium is grown in suitable media, or when it grows outside of infected tissue under wet conditions, it is hyaline (unpigmented). Hyphal growth occurs at the hyphal tips and the hyphae may grow smooth, swollen, nodose or tuber-shaped (Bartnicki-García, 1990). Variations in branching patterns are associated with environmental effects (Ho, 1978).

Firstly, the genus *Phytophthora* was only classified morphologically into six groups (Waterhouse, 1963) based primarily on the papillation of sporangium (papillate, semi-papillate and non-papillate), sexual type (homothallic and heterothallic) and the attachment of the antheridium to the oogonium (amphigynous or paragynous). Other morphological features are: the ornamentation of the oospore wall, whether the oospores are plerotic or aplerotic, if the sporangium pedicel is deciduous or non-deciduous, the branching patterns

of the sporangiophores, the internal proliferation of the sporangium, the morphology of hyphae and hyphall swellings. Afterwards, in 2000 Cooke *et al.* stablished the first comprehensive phylogeny classification based on the rDNA internal transcribed spacer (ITS) sequences and supported by subsequent analyses of many additional DNA regions (Martin & Tooley 2003; Kroon *et al.*, 2004; Villa *et al.*, 2006; Blair *et al.*, 2008; Robideau *et al.*, 2011; Runge *et al.*, 2011). This phylogeny resulted in ten clades and the majority of the studied *Phytophthora* species can be grouped in clades 1 to 8. As new species were discovered, new phylogenies were constructed trying to fit the new species in the ten clades (Martin *et al*, 2014). Currently, with the description of *Phytophthora lilii* Rahman, Uematsu, Kimishima & Kageyama and the basal position of *Phytophthora quercina* Jung, a twelve clade phylogeny was proposed by Rahman *et al.* (2015) and seconded by Jung *et al* (2017a; 2019).

## *2.2.* Reproduction and dissemination

Asexual reproduction

*Phytophthora* reproduces asexually through motile zoospores produced in sporangia, which are released to an aqueous medium at mild temperatures. These are attracted to roots and encyst, producing a germ tube. Chlamydospores are asexual globose, dormant-resistance spores, which germinate depending on exogenous sources of stimulant compounds. Initially are hyaline but they become yellowish-brownish over time. They can be distinguished from hyphal swellings because they are delimitated from the mycelium by septa (Blackwell, 1949; Hemmes, 1983).

Sexual reproduction

Oospores are thick-walled sexual spores that form after an antheridium (male) and oogonium (female) fuse their nuclei. The oospore can fill completely the oogonium cavity (plerotic) or not (aplerotic). The spores of homothallic (self-fertile) species form in agar or liquid media or in infected plant tissue. Heterothallic species (self-sterile) form oospores after A1 and A2 mating types have grown together, either in a suitable medium or in an infected plant.

*Phytophthora* life cycle is presented in Figure 1.1.

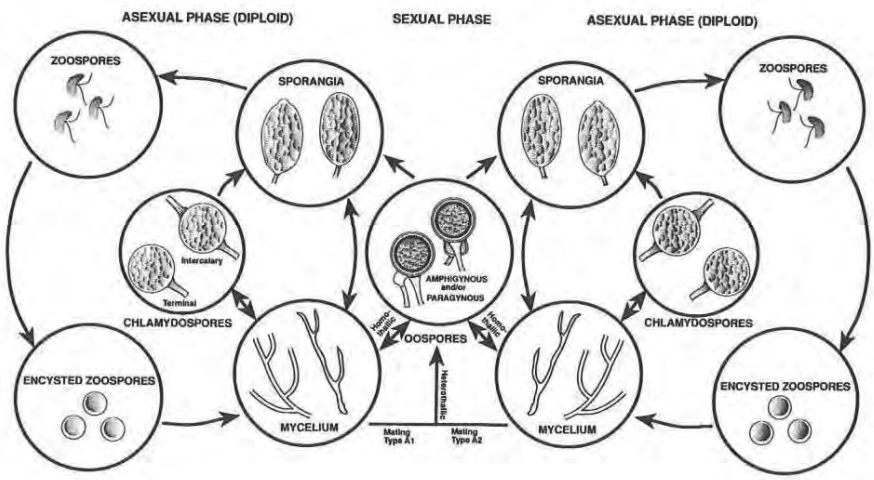


**Figure 1.1.** *Phytophthora* life cycle (Erwin & Ribeiro, 1996).

Sporangia can be long-distance dispersed, under favourable conditions, by water or wind. It has been demonstrated that free water is a remarkable vector for *Phytophthora* dispersion and irrigation sources become perfect reservoirs for many species such as *Phytophthora*. *cactorum* Schroeter, *Phytophthora cambivora* (Petri) Buisman, *Phytophthora capensis* Bezuidenhout, Denman, McLeod & Kirk, *P. cinnamomi*, *Phytophthora citricola* Sawada, *Phytophthora citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, *P. cryptogea*, *Phytophthora frigida* Maseko, Coutinho & Wingf., *Phytophthora intercalaris* Yang, Balci, Brazee, Loyd & Hong, *P. megasperma*, *Phytophthora multivora* Scott & Jung, *Phytophthora nicotianae* Breda de Haan, *Phytophthora plurivora* Jung & Burgess, *Phytophthora syringae* (Kleb.) Kleb., *Phytophthora tropicalis* Aragaki & Uchida, *Phytophthora* sp. *canthium*, *Phytophthora* sp. *emzansi*, *Phytophthora* sp. *kununurra*, *Phytophthora chlamydospora* Brasier & E.M. Hansen, *Phytophthora* sp. *stellaris*, *Phytophthora* sp. *umtamvuna*, *Phytophthora sp. xHennops*, *Phytophthora sp. xWS* (MacDonald *et al.*, 1994; Yamack *et al.*, 2002; Hong *et al.*, 2006; Oh *et al.*, 2013; Zappia *et al.*, 2014; Yang *et al.*, 2016). Zoospores can only be short-distance transported due to its short-term survival ability and because they are only adapted to localize host tissue. Zoospores not only can swim short distances in soils with high humidity but they can be

dispersed by irrigation or precipitation water. *Phytophthora* can also be transmitted by invertebrates like flies from the genus *Drosophila* (Hunter & Buddenhagen, 1969), ants (Turner, 1972) and snails (Álvarez *et al.*, 2008).

### *2.3.* Disease associated to *Quercus* caused by *Phytophthora*

There is a long history of diseases caused by *Phytophthora* in the genus *Quercus* worldwide. Regarding Europe, dieback of branches and parts of the crown, formation of epicormics shoots, high transparency of the crown, yellowing and wilting of leaves and tarry exudates from the bark are some of the most frequent symptoms present in oak forests (Jung *et al.*, 1999). *Phytophthora quercina* stands as the main *Phytophthora* species associated to *Quercus* decline (*Quercus robur* L., *Q. petraea* (Mattuscka) Liebl., *Q. cerris* L., *Q. pubescens* Willd. and *Q. ilex*) in Europe (Jung *et al.*, 1999, 2000). Nevertheless, it is not the only species associated to oak decline. *Phytophthora bilorbang* Aghighi & Burgess, *P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. citricola*, *P. cryptogea*, *Phytophthora drechsleri* Tucker, *Phytophthora europaea* Hansen & Jung, *Phytophthora gonapodyides* (Petersen) Buisman, *P. megasperma*, *P. plurivora*, *Phytophthora pseudosyringae* Jung & Delatour, *Phytophthora psychrophila* Jung & Hansen, *P. syringae* and *Phytophthora uliginosa* Jung & Hansen have been associated to declined oak forests (Hansen & Delatour, 1999; Vettraino *et al.*, 2002, Jung *et al.*, 2002, 2003; Balci & Halmschlager, 2003a, b; Sánchez *et al.*, 2002, 2005; Jankowiak *et al.*, 2014).

In the Iberian Peninsula, the invasive pathogen *P. cinnamomi* is from far known to contribute to the oak decline since 1985 (Brasier 1992a, b, 1993, 1996; Tuset *et al.*, 1996; Gallego *et al.*, 1999; Sánchez *et al.*, 2002; Camilo-Alves, 2013). The disease is characterized by a severe decline and occasional collapse of the oak trees (*Q. ilex*, *Q. suber*) caused by root-invading *Phytophthora* species (Moralejo *et al.*, 2009b). Other *Phytophthora* species such as *P. gonapodyides* or *P. psychrophila* have been identified as active pathogens that contribute to the Iberian oak decline (Corcobado *et al*, 2010, Pérez-Sierra *et al.*, 2013). This disease has in fact a relevant interaction of abiotic and biotic factors (Sánchez *et al.*, 2002; de Rigo & Caudullo, 2016). The first ones weakens the tree, such as the characteristics Mediterranean summer droughts followed by episodes of flooding. Then the pathogens present in the oak rhizosphere, such as

*Phytophthora* spp., attack the tree roots more easily and invades the healthy tissues, colonising the tree. As *Phytophthora* progresses and colonizes oak tissues, symptoms become visible. Sometimes the tree is infected with *Phytophthora* spp. and remains symptomless due to the tree vigour and/or to the colonisation rate of the pathogen (Tsao, 1990). These drives to a progressive decline, or to a quick collapse and death of the oak. Monitoring and surveying Iberian oak areas have revealed a higher diversity of *Phytophthora* species than expected that probably are involved in the disease.

In America, since 1995 several *Quercus* species are threatened by the aerial *P. ramorum*, the causal agent of the Sudden Oak Death (SOD) (Rizzo *et al.*, 2002, 2005). *Phytophthora ramorum* causes the disease at the plant community level, killing millions of oak trees and infecting more than 26 understory species (Moralejo *et al.*, 2009a). The symptomatology of *P. ramorum* includes stem cankers, twig dieback, and/or foliar lesions, depending on the host (Balci & Bienapfl, 2013). *Quercus agrifolia* Née, *Quercus kelloggii* Newb. and *Lithocarpus densiflorus* Blume are suffering a severe decline showing above ground infections in northern and central coastal California forests and restricted areas of Southwestern Oregon (Moralejo *et al.*, 2009b; Balci & Bienapfl, 2013). Even there are differences in oak susceptibility, Moralejo *et al*. (2009b) reported that Iberian oaks (*Q. canariensis*, *Q. pyrenaica*, *Q. faginea*, *Q. pubescens*, *Q. suber*, *Q. ilex*) were highly to moderate susceptible to *P. ramorum* if the pathogen was established in the canopy or surrounding vegetation.

### *2.4.* Management (nurseries/crops/natural ecosystems)

Chemical control

Fungicides are a useful tool for the control of foliar and root diseases (Chase, 1993. Bledsoe *et al.*, 2004). Nevertheless, in recent years, registration of active ingredients has declined leading farmers to look for alternatives to the control of these diseases (Gullino & Garibaldi, 2005).

However, when pesticides are available, their effectiveness is only partial, because plant pathogens have developed resistances. Fungicide resistance is quite widespread, especially in greenhouse conditions, due to the high number of chemicals needed for the control of some pathogens. Resistant populations are

a big problem in pathogens like *Phytophthora*. Resistance of *Phytophthora* spp. to mefenoxam has been demonstrated in gerbera, aster, maritime cinery, african thought and violet in the United States (Hwang & Benson, 2005).

*Phytophthora* management include the use of systemic fungicides. Metalaxyl is not allowed in forests, as it has to be applied to soils and there is a high risk of contamination of the soils and the underground watercourses (González *et al.*, 2017a). Phosphonate resistance inducers trunk injections (endotherapy) are used in natural ecosystems to control *Phytophthora* outbreaks (Shearer and Fairman, 2007; Scott *et al*., 2013; Scanu *et al*., 2015) or aerial spraying (Hardy *et al.*, 2001; Pilbeam *et al.*, 2011). Phosphonates applied to healthy trees have preventive effect, while applied to infected asymptomatic trees have a curative effect. Potassium phosphite is a plant defense stimulant worldwide used against *Phytophthora*. Potassium phosphite trunk injections were the most used product for *Phytophthora* diseases but this chemical was prohibited in Spain when registered as fertilizer (González *et al.*, 2017a). González *et al.* (2017a) reported that fosetyl-Aluminium is an alternative to potassium phosphite to manage oak decline as it is effective at 75%, compared to potassium phosphite in *in vitro* tests and showing better results preventing root loss in 1-year-old *Q. ilex* and *Q. suber* seedlings pot experiments with *P. cinnamomi*. Moreover, potassium phosphite reduced *Phytophthora cryptogea* Pethybr. & Laff., *Phytophthora oleae* Schena, Ruano-Rosa, Agosteo & Cacciola, *Phytophthora megasperma* Drechsler and *Pythium spiculum* B. Paul growth in the *in vitro* test., while fosetyl-Aluminium was only effective against *P. cryptogea* and *P. oleae*. Both chemicals were successful against all the *Phytophthora* species tested *in planta*. In a next step, González *et al.* (2017b) conducted a field experiment (in a dehesa) testing fosetyl-Aluminium against *P. cinnamomi* root infection in mature *Q. suber* declined trees and the results validated the use of fosetyl-Aluminium as a preventive treatment against *P. cinnamomi* in the management of oak decline.

As regards soil disinfection, following the ban on the use of methyl bromide in 2005, growers had to look for alternatives; other fumigants (sometimes less effective), use of steam or solarisation in the soil, biofumigation buried in green or application of plant/seed preserved to the soil (Gullino *et al.*, 2007; Rosskopf *et al*., 2005; Ríos *et al*., 2016; Widmer *et al*., 2018). In Florida,

soil solarisation was effective against *P. nicotianae* in *Catharanthus roseus* (McGovern *et al.*, 2000).

The use of natural products such as salts, extracts and oils, are a booming alternative for the control of fungal diseases, being more effective for the control of foliar problems (Gullino & Garibaldi, 2007; Neves *et al*., 2014).

Cultural practices

Beside the use of chemicals, cultural practices should be implemented. Serrano *et al.* (2012a, 2013) reported that limestone supplements are recommended as a measure against root rot caused by *P. cinnamomi* in dehesas in southern Spain, as a good option for control of oak root disease. This option confers tolerance to disease inhibiting the production and germination of sporangia (Serrano *et al.*, 2012a). In the field, some practices in order to reduce infections would be: to maintain plants at a certain distance from other crops, avoid growing potential host plants (Serrano *et al.*, 2012b), limit the movement of soil and. avoid soil tillage, leave an herbaceous residue on the ground and ensure good drainage of the soil. Regarding livestock density, avoid high livestock loads in high soil moisture conditions and animal hooves disinfestation if they come from an infected dehesa. Moreover, limit traffic to vehicles, machines and people; reduce displacement in periods when the soil is wet and cleaning of shoes, tools, vehicles, contribute to limit the spread of the disease (Scanu *et al*., 2015).

Genetic resistance is an effective strategy for disease management (Serrano *et al.*, 2012c). The production of resistant cultivars offers the possibility of reducing the use of pesticides and possibly working costs without sacrificing crop quality (Gullino & Garibaldi, 2007). On certain occasions, as is the case with ornamental plants, the use of disease-resistant plant material also reduces maintenance costs (Hagan, 2001). A negative aspect of breeding programs is that improved cultivars can show greater susceptibility to certain diseases. Even sometimes, the resistance to the disease is not complete and under conditions very favourable for the development of the disease, can easily be overcome by the pathogen (Gullino & Garibaldi, 2007).

Over the past two decades, numerous composted organic waste has partially replaced peat in containers for ornamental production. The recycling of these wastes means an economic saving, both in the cost of compost and production, as well as in the reduction of the number of dead plants, by suppression, through compost, of soil pathogens (Hoitink & Bohem, 1999). The use of organic amendments has the potential to reduce the need for the use of fungicides (Gullino & Garibaldi, 2007). In addition, the use of thermosterilized substrates helps to prevent the growth of soil pathogens (Widmer *et al*., 2018).

In closed systems, such as greenhouses, climate factors can be controlled to reduce infection risk, for example, reducing heat in greenhouses helps to avoid the typical root rot caused by *Phytophthora*. Avoiding optimal temperatures for *P. infestans* and reducing moisture levels, can reduce the incidence of "late blight" in petunia (Becktell *et al.*, 2005a) and can be used, effectively, combined with lower use of fungicides (Becktell *et al.*, 2005b). Aeration between seedling banks is a simple method to avoid seedling rot. The disease can also be partially controlled by taking into account parameters such as pH; a low pH (3.5 to 4.5) suppresses the release of spores, thus reducing the disease. Excessive nitrogen fertilization increases the susceptibility to *Phytophthora*. Changes in salt levels in crop containers after a high nitrogen fertiliser, can also promote the activity of *Phytophthora*, aggravating the disease (Bledsoe *et al.*, 2004).

Water management is a key factor in ornamental plant nurseries. Trying to avoid overwatering, ensuring good drainage and trying to cover the nursery surface of systems that avoid splashing and the direct contact of containers with the ground (mesh, gravel layer or height containers) are practices that have to be implemented. It is also necessary to avoid the recirculation of water, but failing that, incorporate sand filters into the irrigation system, as well as perform treatments with chlorine dioxide (Brasier, 2008).

Routine disinfection of tools and work materials, by immersion of them in a solution of 10-20% bleach with water after use, prevents the mechanical transmission of diseases from one plant to another, although it leads to some corrosion. All entrances-exits of the nursery's production area should have hand health dispensers, tools and footwear tools; which usually carry alcohol or other disinfectant agents. Washing, followed by steam sterilization, is a very effective method for disinfecting trays, pots and other production tools (always taking into

account the manufacturer's temperature indications to avoid heat damage) (Bledsoe *et al.*, 2004).

## Biological control

The ornamental sector may be more favoured in terms of the effectiveness of biological agents for disease control, since it is possible to control the growing environment of plants. Currently, the environmental control in greenhouses, the high value of crops and the limited number of registered fungicides, offers a unique scenario for the biological control of plant diseases (Paulitz & Bélanger, 2001). Nevertheless, the importance of aesthetics in this sector complicates the practical application of biological control agents, which in many cases provide only partial control of the disease (Gullino & Wardlow, 1999). There is the problem in practice of moving the antagonists observed in the laboratory to production systems. In nature, biological control agents have their own antagonists, besides that it is difficult to commit to choosing between the optimal conditions for biocontrol activity and those optimal and economical for production. It is known that there are suppressive soils for certain soil diseases or where the disease is less severe; as well as conductive soils, favour the development of the disease (Tousson, 1975). Some substrates used in floriculture have suppressive effects. In some countries, biocontrol agents (among others) commercially applied to ornamental crops to prevent soil diseases caused by *Phytophthora* are *Streptomyces griseoviriridis*, *Streptomyces plicatus*, *Gliocladium catenulatum*, *Bacillus subtilis*, *Trichoderma harzianum* and other *Trichoderma* species (Jones & Samac, 1996; Gullino & Garibaldi, 2007; Bae *et al*., 2016; Chen *et al*., 2016; Widmer *et al*., 2018). In addition, the use of bioagents together with chemical control agents increases the effectiveness of the latter, such as *Pseudomonas* sp., *Aspergillus* sp., *Trichoderma* sp. and *Bacillus* sp. (Bledsoe *et al.*, 2004). Trials conducted with *Streptomyces* for the control of the rot produced by *Phytophthora medicaginis* E.M. Hansen & D.P. Maxwell in alfalfa, showed that it increased the frequency of healthy plants in the susceptible alfalfa variety, as well as decreasing the rate of medium severity for both varieties, susceptible and resistant (Jones & Samac, 1996). It was also found in the study that the combined use of metalaxil fungicide together with *Streptomyces*, controlled the disease better than both methods separately;

suggesting that fungicide helps in the establishment of the biological control agent. A slow sand filtering technique, along with the application of different strains of *Fusarium* spp antagonists and *Trichoderma* spp., proved to be effective against *P. cryptogea* in gerbera (Garibaldi *et al.*, 2003).

## *3.* Monitoring *Phytophthora*

### *3.1.* Traditional detection techniques

*Phytophthora* is a difficult genus to work with compared to fungi or other oomycetes like *Pythium* species. Baiting and culture-based detection have been for many years the only recommended techniques for *Phytophthora* detection (Tsao, 1990). These techniques provide valuable information but require a level of expertise that is not easy to achieve in order to do a correct identification. *Phytophthora* traditional identification is based on morphological, physiological and cultural traits that researchers have to be thoroughly trained in. Despite that they are time-consuming techniques, these require the pathogen to be alive and active for isolation. *Phytophthora* selective culturing media has been improved to increase the isolation frequency (Tsao, 1990). Nevertheless, if the quantity of inoculum is not abundant the isolation may fail, not identifying the pathogen when present (false negatives).

Traditional detection need specific tools, plant hosts and big sample sizes to obtain a correct *Phytophthora* diversity assessment (Jeffers & Martin, 1986; Jeffers & Aldwinckle, 1987, Tsao, 1990, Cooke *et al.*, 2007). Appropriate selective media is essential for a successful *Phytophthora* isolation, especially in environmental samples (Cooke *et al.*, 2007). Moreover, not all selective media act the same way for all *Phytophthora* species. *Phytophthora* species do not synthesize sterols but demand an external source of β-hydroxi sterols for sporulation. Some Pythiales (like *Phytophthora* and *Pythium*) are resistant to polyene antibiotics such as pimaricin, while fungi are sensitive (Ho, 1978; Bae *et al.*, 2016). The incorporation of pimaricin to selective media favoured *Phytophthora* isolation from plant tissue (Jeffers & Martin, 1986). Likewise, until the concentration of pimaricin was not reduced enough to allow germination of propagules, chlamydospores and oospores, *Phytophthora* isolation from soils failed (Jeffers & Martin, 1986). Hymexazol incorporation to selective media inhibited most *Pythium* and *Mortierella* species, which grow faster, allowing

development of most *Phytophthora* colonies (Jeffers & Martin, 1986). Some species reported some sensitivity to hymexazol especially when the fungicide PCNB was present (Jeffers & Martin, 1986). According to baitings, using different hosts, adjusting air drying of the soils and adjusting soil moisture have improved the detection of *Phytophthora* spp. (Jeffers & Aldwinckle, 1987; Erwin & Ribeiro, 1996; Cooke *et al.*, 2007; O´Brien *et al.*, 2009).

Traditional methods are not sensitive enough to assess the *Phytophthora* diversity in a sample since many species can be excluded during the detection process. Moreover, as the number of species has been increasing it has been more difficult to characterise the new species due to intra-specific variation and the overlapping of some of the characters. These limitations have led to the development of molecular procedures for detecting and identifying plant pathogens such as *Phytophthora* species. The advantage of traditional techniques is the successful isolation proof of the presence of the pathogen and having the *Phytophthora* culture for further characterisation (Cooke *et al.*, 2007).

### *3.2.* Molecular detection techniques

The increasing number of invasive species and the development of plant pathogens in nurseries requires an early detection and diagnosis for the implementation of efficient control strategies (Grote *et al.*, 2002). Nucleic acid-based methods can reduce diagnosis time and can allow detection in samples from which the pathogen is not culturable (Duncan & Cooke, 2002; Hughes *et al.*, 2011). Target-specific measurements may improve the efficiency of control strategies in the future (Duncan & Cooke, 2002; Grote *et al.*, 2002; Schena & Cooke, 2006). Speed, sensitivity and specificity are characteristics provided by molecular methods. In addition, these methods can be automated for high throughput testing or adapted for use in the field (Hughes *et al.*, 2011).

Firstly, hybridisation probes were used but they were replaced by polymerase chain reaction (PCR)-based approaches for the identification of plant pathogens (Cooke *et al.*, 2007). These methods include techniques like conventional PCR (simple or nested), quantitative PCR, digital PCR, cloning or massive sequencing, which allow fast and accurate pathogen detection and identification even when the inoculum amount is low in a complex mixture.

From these techniques, the most used and more informative are the Real-Time PCR and those methods based on High-Throughput DNA sequencing (HTS) (Schena *et al.*, 2006, 2008; Cooke *et al.*, 2007).

There is a high number of *Phytophthora* species endangering forest and natural ecosystems and in many cases are found coinfecting the same tree (Schena *et al.*, 2006; Cooke *et al.*, 2007). Faced with the threat of invasive species, monitoring increased and thanks to improved detection tools, the number of *Phytophthora* species found increased as well (Schena *et al.*, 2006, 2008). Despite the fact that molecular methods have improved *Phytophthora* identification and detection, they are not enough to assess *Phytophthora* diversity in forest and natural ecosystems. The internal transcribed spacer (ITS) has been used as barcode for oomycetes due to its high stability, easy amplification and sequencing with universal primers and high both conserved and variable sequences to identify individual species (White *et al.*, 1990, Scibetta *et al.*, 2012). However, since new *Phytophthora* spp, have been reported, for most cases the ITS sequences may not be informative enough to identify and detect closely related species (Martin & Tooley, 2003; Brasier *et al.*, 2004; Schena & Cooke, 2006; Donahoo *et al.*, 2006; Martin *et al.*, 2012; Yang *et al.*, 2017; Khaliq *et al.*, 2018). Nevertheless, ITS region is still the main target for HTS of environmental samples, which enables the assessment of the *Phytophthora* diversity in forest and natural ecosystems (Vettraino *et al.*, 2012; Vannini *et al.*, 2013; Català *et al.*, 2015; Prigigallo *et al.*, 2016; Català *et al.*, 2017; Riddell *et al.*, 2019).

### *3.3.* Metabarcoding analysis

Environmental samples are complex to analyse since many organisms are present. The analysis of environmental DNA for the identification of microbial taxa present was coined “metagenomics” by microbiologists, even it is not its original definition (Taberlet *et al.*, 2012a). Metagenomic analysis require the capacity of performing simultaneous readings in parallel, which massive sequencing technologies perform effectively and at a lower price compared to Sanger technologies (Taberlet *et al.*, 2012a). Massive DNA Sequencing (also known as high-throughput sequencing or NGS-Next Generation Sequencing) were developed in 2005 allowing simultaneous detection (sequencing) of thousands to millions of DNA molecules in a single sample (Margulies *et al.*,

2005). DNA metabarcoding is the targeted sequencing of taxonomically informative genetic markers, which allows to measure biodiversity quickly, cheaply, comprehensively, repeatedly and verifiably (Hajibabaei *et al.*, 2011; Taberlet *et al.*, 2012a, b, c). Metabarcoding allows biodiversity analysis combining two technologies: a selection of the genetic material of interest (barcode) through PCR followed by a massive sequencing to identify the range of organisms present in the environmental sample. The selection and combination of target regions (delimited by primers) in the PCR amplification process determines which group of organisms to analyse (Taberlet *et al.*, 2012a, b, c).

Metabarcoding has facilitated biomonitoring programs in natural ecosystems allowing large-scale analysis of microbial communities (Buée *et al.*, 2009; Hajibabaei *et al.*, 2011; Orgiazzi *et al.*, 2012; Taberlet *et al.*, 2012b, Thomsen *et al.*, 2012; Oulas *et al.*, 2015; Tremblay *et al.*, 2018; Ruiz-Gómez *et al.*, 2019). Molecular methods for detection, identification and monitoring of *Phytophthora* species have proved important tools to predict the threats posed by native pathogens and minimize the risk of further invasive *Phytophthora* diseases in terrestrial and water environments. A better understanding of this group of pathogens and their impact on natural and managed vegetation systems can be provided by metabarcoding analysis. The combination of traditional and molecular methods can encourage focussing on isolating new molecular *Phytophthora* phylotypes and to clarify ambiguous results, confirming the presence of the pathogen in new hosts or at new outbreak sites (Hughes *et al.*, 2011; Khaliq *et al.*, 2018).

## *4.* Diversity

The Mediterranean basin is one of the 25 most important biodiversity hotspots on Earth (Orgiazzi *et al.*, 2012; Scanu *et al*., 2015). The Mediterranean biome covers 2% of the Earth´s land surface but houses 20% of the world´s floristic richness and represent one of the most vulnerable of the Earth´s 13 terrestrial biomes (Orgiazzi *et al.*, 2012).

Natural ecosystems including the Mediterranean basin are threatened by the movement of plants associated to trade and globalisation. In this context, migration is a powerful evolutionary force that has showed a visible effect on the

genetic structure of *Phytophthora* species populations (Goodwin, 1997). Migration is the movement of individuals from one population to another and may result in gene flow when the migrants contribute their genes to the gene pool of the new population (Milgroom, 2015).

Many *Phytophthora* species surely evolved in limited geographic areas, especially species associated with a single host (Goodwin, 1997). *Phytophthora* migrations in the past would probably be limited in presence and in the number of introduced individuals as most *Phytophthora* species are hemibiotrophs (Goodwin, 1997). Man has increased the geographical ranges of most species through the dispersal of the crops to which they were associated (Prospero *et al*., 2013; Franić *et al.*, 2019). New stablished populations outside the center of origin of the pathogen would have lower genetic variability compared to the source population. Moreover, mode of reproduction would dramatically influence diversity. New populations of heterothallic species would reproduce asexually, if only one of the mating types had been introduced, with the consequent vulnerability to extinction. In contrast, homothallism would be advantageous for colonizing populations. Recurrent migrations would increase genetic variability between populations counteracting the effect of other evolutionary forces such as drift or selection.

For example, the migration pathway of *Phytophthora infestans* (Mont.) de Bary has been traced by Ristaino *et al.* (2001) who identified the center of diversity in the Andes. The uniformity of genotypes among isolates from different countries revealed by several genetic markers provided evidence that a single clonal lineage had dispersed worldwide (first migration from Mexico to USA 1842 or 1843, second from USA to Europe in 1844 or 1845 and from Europe to the rest of the world after 1846) via trade of infected plant material. Since only one mating type escaped originally, all *P. infestans* populations were asexual until recently (Guha Roy & Grünwald, 2014). Beginning in the late 1970s, new clones escaped from Mexico, including the opposite mating type and now genotypic diversity of *P. infestans* populations in increasing worldwide (Shakya *et al.*, 2018).

Similar types of studies have shown the long-distance dispersal of genotypes of *Phytophthora ramorum*, the causal agent of the "Sudden Oak Death". The center of origin is not clear but it was apparently introduced in

Europe and North America by the trade of infected nursery plants around the 90s, surely rhododendrons (Brasier *et al.*, 2004a; Rizzo *et al.*, 2005; Ivors *et al.*, 2006; Grünwald *et al.*, 2019). From the nurseries, it expanded through the natural of the new geographical areas, causing serious ecological damage, especially in California and Oregon forests (Grünwald *et al.*, 2012) Another example is *Phytophthora kernoviae*, apparently introduced in Europe from the center of origin located in Asia likely with infected ornamental material imported via New Zealand (Brasier *et al.*, 2005).

The spread of invasive plant-pathogenic organisms such as *Phytophthora* species is a constant global worry for nature conservation (Brasier *et al.*, 2008; Hulbert *et al.*, 2017). The European Union has contradictory measures and an open door phytosanitary system, under which any plant that is not specifically regulated can be imported (Brasier *et al.*, 2008; Migliorini *et al.*, 2015). On the one hand, it is forbidden importing soil from non-European countries, but on the other hand, potted plants if not present in alert lists can be imported with its substrate (Migliorini *et al.*, 2015). Currently, only five *Phytophthora* species are proscribed in European regulations: *Phytophthora fragariae* Hickman, *P. kernoviae*, *Phytophthora lateralis* Tucker & Milbrath, *P. ramorum*, *Phytophthora rubi* (Wilcox & Duncan) Man in´t Velt (subject to official quarantine regulations and/or present in the A2 European and Mediterranean Plant Protection Organization list). Controlling invasive species is essential to slow the loss of biodiversity and to avoid the ecosystem degradation. In this context, prevention is the best management strategy but for already introduced pathogens, detection followed by monitoring is the usual process to determine which strategy should be implemented afterwards depending the abundance and distribution of the pathogen (Wingfield *et al.*, 2015; Hulbert *et al.*, 2017).

Hulbert *et al.* (2017) reported seventy-two publications that described 98 *Phytophthora* species; from 91 species with data on geographic location isolation, 22% were isolated from urban environments (including nurseries), 33% from agricultural environments (including nursery plantations for afforestation) and 45% from natural environments. Irrigation reservoirs and natural waterways are also hotspots for *Phytophthora* species that favour its dissemination (Zappia *et al.*, 2014; Català *et al.*, 2015). Recent studies in undisturbed ecosystems have

revealed new *Phytophthora* species (Jung & Burgess, 2009; Scanu *et al*., 2015 Jung *et al* 2017b, c; Burgess *et al.*, 2018).

There is a need to improve the knowledge about *Phytophthora* diversity in the different ecosystems in order to have a better idea of which species are present and to understand the origin and distribution of these species, which will help to prevent ecological disasters (Keriö *et al.*, 2019). Monitoring and detecting *Phytophthora* in different sites is essential to anticipate potential threats. Implementation of all available techniques, especially new molecular technologies based on environmental DNA can help to assess the real *Phytophthora* diversity and to overcome failures in the plant health sanitary system.

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# Chapter 2

# Chapter 2

## SCOPE OF THE THESIS

*Phytophthora* is commonly found in nursery industry worldwide (horticultural, ornamental as well as forest tree nurseries) and the species diversity seems to have increased in recent years. European Fagaceae forests made up primarily by genus *Castanea*, *Fagus* and *Quercus* are suffering a general decline in which *Phytophthora* species are involved. In particular, the decline of *Quercus* stands in Spain, as highlighted in the general introduction, is traditionally associated to *P. cinnamomi*. Nevertheless, the *Phytophthora* species involved in the holm oak Spanish decline is increasing as new studies and new techniques develop.

Since the first detection of *P. ramorum* in Spain (2002), surveys have been done in ornamental nurseries, garden centers, public gardens and forest masses to eradicate. In these surveys, other *Phytophthora* species have been found to affect many ornamental plants and *Q. ilex* stands, representing a risk to nurseries and natural ecosystems. Moreover, in these surveys, Cylindrocarpon-like asexual morphs were isolated very frequently. As *Phytophthora* identification is not an easy task, an appropriate and quick *Phytophthora* diagnosis is the cornerstone to implement the preventing principle in the Spanish phytosanitary system.

In this context, the objectives of the Thesis were to:

- Gather information on the occurrence of *Phytophthora* spp. and Cylindrocarpon-like asexual morphs in Spanish nurseries and irrigation water (**Chapter 3 and 4**).
- Evaluate the susceptibility of *Q. ilex* (as it is the most frequent tree in Spain) to *Phytophthora* species recovered from the nurseries (**Chapter 5**).
- Study the potential implication of new *Phytophthora* species in the *Q. ilex* decline in Spain by applying different traditional isolation and molecular detection approaches to natural and seminatural *Q. ilex* stands (**Chapter 6 and 7**).

- Unravel the *Phytophthora* diversity by applying a Next Generation Sequencing approach to the previously monitored holm oak stands (**Chapter 7**).

# Chapter 3

## Survey and identification of *Phytophthora* spp. in Spanish nurseries

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# Chapter 3


## Abstract

Nursery industry has become an ideal reservoir for the development and recombination of *Phytophthora* species worldwide. Considering the detection of *Phytophthora ramorum* in 2002, numerous samplings in nurseries and public spaces were carried out, revealing a great diversity of *Phytophthora* species that compromised production, posing a risk to natural ecosystems. In this context, sampling ornamental and forest nurseries in four Spanish regions (Cataluña, Comunidad Valenciana, Extremadura and País Vasco) was carried out focusing on possible symptoms associated to *Phytophthora* on different hosts and on water sources used by the nurseries. The results of these surveys conducted from 2012 to 2014, showed a high diversity of *Phytophthora* species in Spanish nurseries and water sources, which confirm previous studies. Thirteen *Phytophthora* species and one informally designated taxon were isolated from 547 plant samples belonging to 22 species included in nineteen plant genera. The isolated species were *P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. citrophthora*, *P. crassamura*, *P. gonapodyides*, *P. hedraiandra*, *P. nicotianae*, *P. niederhauserii*, *P. palmivora*, *P. plurivora*, *P. pseudocryptogea*, *P. sansomeana* and *Phytophthora* sp. tropicalis-like 2. Nine species were detected on water sources, seven of those coincided with species detected on plants and two species were only detected on water samples: *P. bilorbang* and *P. lacustris*. This is the first report of *P. crassamura* in Spain where it was detected on *Pinus pinea* and this species-host combination is the first record worldwide. Similarly, this is the first detection of *P. pseudocryptogea* in *Chamaecyparis lawsoniana* and *Yucca rostrata* in Spain.


## Introduction

Spain stands in the fifth position in the European nursery production with a value of 1,989 million of euros that represents approximately 11% of the European total production. It has a land area over 29.000 hectares with an increasement of 6.4% in 2016 over the previous year (EUROSTAT 2017).

Different pests and diseases can affect nursery production, which can turn plants into pathogen vectors (Parke *et al.*, 2014). Pathogens affect all plant industries across agriculture, horticulture, forestry and amenity and can have a significant impact on yield, market access, sustainability of production, food

security and product integrity (Hyam, 2008). The kingdom Fungi is considered the kingdom with the largest number of phytopathogenic species (Tremblay *et al.*, 2018). In addition to this, the kingdom Straminipila, embraces other important plant pathogens such as *Phytophthora* and *Pythium* (Tremblay *et al.*, 2018). *Phytophthora* species are responsible for large losses of nursery stock throughout the world (Benson & Jones, 1980; Erwin & Ribeiro, 1996; Ferguson & Jeffers, 1999; Werres *et al.*, 2001; Osterbauer *et al.*, 2004; Pérez-Sierra *et al.*, 2012; Panabières *et al.*, 2012, Jung *et al.*, 2016).

*Phytophthora*, one of the most destructive pathogens, includes currently over 150 known species and about 100 more that are in the process of being described (Lévesque, 2011; Kroon *et al.*, 2012; Martin *et al.*, 2012; Jung *et al.*, 2016), which duplicates the species known in 1996 (Erwin & Ribeiro, 1996). Almost all *Phytophthora* species are ecological and economical important plant pathogens worldwide, some of them with a broad host range. *Phytophthora* species possess wide environmental adaptation that ranges from terrestrial to aquatic habitat. Some species are responsible for the most important epidemics in the past (*Phytophthora infestans* de Bary), for disrupting and diminishing biodiversity in natural ecosystems nowadays (*Phytophthora cinnamomi* Rands, *Phytophthora ramorum* Werres, de Cock & Man in't Veld) or for producing major losses in the nursery industry worldwide (*P. ramorum*, *Phytophthora citrophthora* [R.E. Sm. & E.H. Sm.] Leonian, *Phytophthora nicotianae* Breda de Haan, *Phytophthora hedraiandra* de Cock & Man in't Veld, *Phytophthora niederhauserii* Z.G. Abad *et* J.A. Abad, sp. nov., *Phytophthora 'kelmania'* and *Phytophthora chlamydospora* Brasier & Hansen) (Brasier *et al.*, 1993; Hüberli *et al*, 2001; Werres *et al.*, 2001; Rizzo *et al.*, 2002; Brasier *et al.*, 2004a; Tooley *et al.*, 2004; Ivors *et al.*, 2006; Cooke *et al.*, 2007; Moralejo *et al.*, 2009; Martin *et al.*, 2012; Leonberger *et al.*, 2013; Camilo-Alves *et al.*, 2013; Abad *et al.*, 2014; Jung *et al.*, 2016).

The movement of plants and plant products between biogeographical zones due to human activity constitutes the leading pathway of/for pathogens and exotic pests introduction (Brasier, 2008; Tremblay *et al.*, 2018). The inocula of *Phytophthora* spp., which cause foliar as well as root diseases, increase from low to high levels within a few days or weeks. Multicyclic diseases can turn into serious epidemics when environmental conditions favour a rapid production of *Phytophthora* propagules (Erwin & Ribeiro, 1996).

*Phytophthora* in its centre of origin do not necessarily constitutes an ecological problem or even noticeable because the binomial pathogen-host has co-evolutionated (Brasier, 2008). When a pathogen is associated to certain hosts,

these can offer some tolerance or resistance among them; moreover, populations are regulated by the presence of natural enemies. Nevertheless, when the pathogen is transferred to a new habitat with favourable conditions, it can likely extend to a wide range of new hosts causing serious ecological and economical losses (Prigigallo *et al.*, 2015; Hulbert *et al.*, 2017). The arrival of new genotypes, lineages or exempt mating types into a non-native habitat, can lead to an additional risk for the ecosystem and a possible host range expansion for that species (Brasier *et al.*, 2006; Ivors *et al.*, 2006; Prigigallo *et al.*, 2015; Jung *et al.*, 2016; Hulbert *et al.*, 2017).

Invasive pathogens have been causing damage to native plant communities, woodlands and landscapes on a global scale for over a century (Brasier, 2008; Peterson *et al.*, 2014; Migliorini *et al.*, 2015). Nursery trade encourages the dispersal and establishment of invasive and exotic *Phytophthora* spp. (Reichard & White, 2001; Jones & Baker, 2007; Jung *et al.*, 2016; Tremblay *et al.*, 2018). Even more, the high specialisation and intensification of nursery production favours the reproduction and hybridisation of invasive species enhancing the dispersion and settlement of these on natural ecosystems. The diversity of the genus has increased rapidly in the last decade due to the appearance of new alien species, which requires routine samplings for their early detection, and due to the numerous surveys on unexplored habitats, such as water reservoirs (Hulbert *et al.*, 2017).

In Spain since 2002 that *P. ramorum* was firstly detected (Moralejo & Werres, 2002; Pintos *et al.*, 2003), surveys have been done in ornamental nurseries, garden centres, public gardens and forest masses to detect and eradicate this pathogen. In these surveys, it has been found that other species of *Phytophthora* affect many ornamental plants, posing a risk also to nurseries and natural ecosystems (Perez-Sierra *et al.*, 2012).

Due to the increasing threat of invasive *Phytophthora* species, specially quarantine pathogens, and the high risk of hybridisation between them, a survey was carried out in producing woody and/or ornamental plant nurseries to assess the *Phytophthora* diversity present and to exclude the presence of quarantine *Phytophthora* species.

## Materials and methods

### Study sites

Surveys were conducted in 25 Spanish nurseries located in four geographically different regions during the period 2012-2013: Cataluña (provinces of Barcelona, Girona, Tarragona and Lleida), Comunidad Valenciana (provinces of Alicante, Castellón and Valencia), Extremadura (province of Cáceres) and País Vasco (province of Guipúzcoa) (Figure 3.1).

In ornamental nurseries, only symptomatic plants were collected, plants showing signs of ill-health. Non-symptomatic plants were also randomly collected in forest nurseries for habitat restoration, giving a final number of 547 samples. Foliar symptoms (leaf blotch, blight, chlorosis, defoliation), wilting–decline, dieback, growth reduction, cankers with or without gummosis, rot and presence of dead plants were considered symptoms associated to possible *Phytophthora* infection (Figure 3.2). These plants presented in most cases root rot and/or loss of the feeder roots with the presence of necrotic lesions (Figure 3.3). Plant samples were collected together with its pot media or soil, individually stored in labelled plastic bags and kept in cold conditions until they were processed in the laboratory at the Instituto Agroforestal Mediterráneo, Universitat Politècnica de València (IAM-UPV).

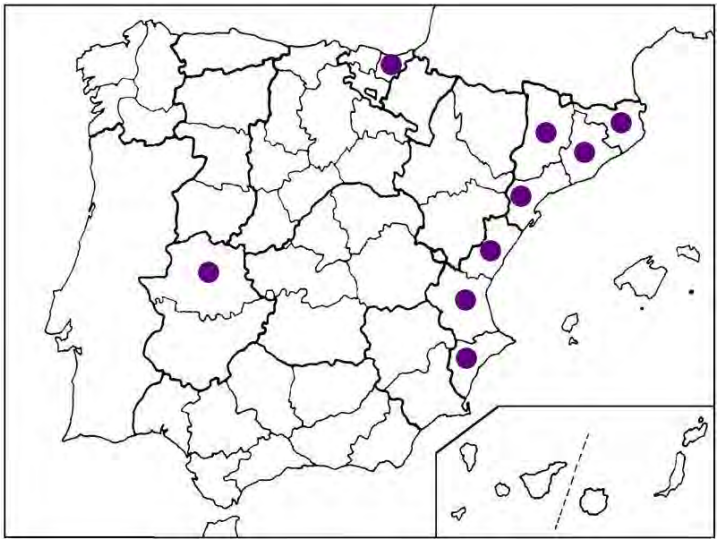

**Figure 3.1.** Spanish provinces in which nursery surveys were conducted.

Thirteen water samples from recirculating irrigation ponds and from streams located close to the nurseries were also collected during the survey in Cataluña region and from one nursery located in Comunidad Valenciana. Ten litres of water were filtered using three cellulose membranes (5.0 µm pore diameter, Millipore Corporation), which were placed in Petri dishes, sealed with parafilm, labelled and stored in a cooler during its transport to the laboratory. Furthermore, leaves floating in two streams located in Cataluña region were collected, labelled and transported for its processing as vegetal samples, which raises the total number of plant samples to 549.

**Isolation**

Once in the laboratory, samples were processed for fungal and oomycetes isolation. Plant samples (leaves and/or roots) were separated from its substrate media, and the roots were washed and kept 24 hours immersed in tap water repeatedly renewing for its oxygenation. These were superficially disinfested spraying alcohol at 70% for oomycete isolation and disinfested for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water for fungi isolation. Small fragments from the lesion edge were plated on semi-selective media for oomycetes [CMA-PARPB supplemented or not with hymexazol, (Jeffers & Aldwinckle, 1987)] and on potato dextrose agar (PDA) (Biokar-Diagnostics, Zac de Ther, France) amended with 0.5 g litre$^{-1}$ of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA) (PDAS) for fungi isolation. Plates were incubated at 20ºC in the dark for 3 to 5 days for fungi and up to 7 days for oomycetes. All the colonies grown on the isolation media were transferred to PDA plates, incubated at 20ºC in darkness for 7 days for its further identification. Pure cultures of all putative *Phytophthora* isolates were obtained by transferring single hyphal tips to PDA plates.

Substrate media or rhizosphere soil removed from each plant sample was baited using Granny Smith apples targeting oomycetes species isolation (Erwin & Ribeiro, 1996). Four 10 mm diameter and 1-1.5 cm depth holes were performed on the apple with a cork borer, each one was filled with the soil sample, saturated with distilled water, sealed with adhesive tape and incubated at room temperature until lesions appeared (4 to 7 days). Small tissue fragments from the edge of the lesion were plated on CMA-PARPB with and without hymexazol and incubated at 20ºC in darkness. Each emerging fragment colony was transferred to PDA and incubated as described before for plant samples.

Oomycetes isolation from filtered water samples was undertaken also by apple baiting; three longitudinal flap-like cuts (one per each membrane filter) were performed on a Granny Smith apple. In each flap cut, half of the subsample membrane was placed, sealed with parafilm and incubated at room temperature until symptoms develop (4 to 7 days). The re-isolation from the apple lesions was conducted as explained above for soil samples.

## Identification

### *Phytophthora* isolates

*Phytophthora* colony patterns were compared to those described in the literature for initial confirmation of *Phytophthora* species (Stamps *et al.*, 1990; Erwin & Ribeiro, 1996). *Phytophthora* isolates were grown on four different culturing media and incubated at 25ºC in darkness for 7 days to determine the colony morphology and pattern. The axenic media used were PDA, MEA [20 g malt extract (Difco laboratories), 15 g agar, 1 litre distilled water], V8 [200 ml vegetable juice, 2 g $CaCO_3$, 15 g agar (Difco laboratories), 800 ml distilled water] and OA (20 g oatmeal, 13 g agar, 1 litre distilled water).

### Fungal and other oomycete isolates

Once cultures were obtained in PDA, most fungal isolates were classified up to the genus level attending to the colony morphology.

Oomycetes that were not *Phytophthora* isolates were classified in different types attending to the colony morphology and one isolate from each morphology type was selected for its subsequent molecular identification.

### DNA extraction, sequencing and molecular identification

DNA from *Phytophthora* and *Pythium* isolates was extracted from pure cultures grown on PDA by scraping the mycelium and mechanically disrupting it by grinding to a fine powder under liquid nitrogen, using the EZNA Plant Miniprep Kit (Omega Bio-tek, Doraville, USA) following the manufacturer´s instructions.

Nuclear ribosomal DNA ITS amplifications were carried out using the universal primers ITS4 and ITS6 that target conserved regions in the 18S and 28S rDNA genes (White *et al.*, 1990; Cooke *et al.*, 2000). All PCR reactions

were performed using HotBegan™ Taq DNA Polymerase (Canvax Biotech SL, Córdoba, Spain), according to the manufacturer's instructions, in a PTC 200 thermo-cycler (MJ Research, Waltham, MA) with the following parameters: 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and 72°C for 10 min. Amplified products were purified and sequenced in an extern sequencing service (Macrogen, Amsterdam, Netherlands).

The isolates were identified to the species level by conducting Basic Local Alignment Search Tool (BLAST) searches with the sequence data on international collection databases (*Phytophthora* Database, PhyID and GenBank) and customized database. Assignation of a species name to an isolate occurred when the identity was above the 99% cut-off in respect to the ex-type isolates. There were ten isolates, in which the ITS did not resolve their identity. Therefore, for these isolates it was also amplified the mitochondrial cytochrome c oxidase I (COI) region using the primers OomCoxI-Levup and Fm85mod (Robideau *et al.*, 2011).

**Conservation of *Phytophthora* and *Pythium* isolates**

Pure cultures obtained by hyphal tipping were conserved in the oomycete culture collection maintained at the IAM-UPV. Each isolate was grown on V8 and incubated at 20ºC for 7 days in darkness. Among 15 mycelium plugs (6 mm diameter) from the border of the colony were extracted and placed into a 12 $cm^3$ glass flask which contained 1.5% sterile soil extract solution for its long term conservation at 14ºC. The sterile soil extract solution was prepared mixing 100 g of soil with 900 ml distilled water. The mixture was stirred and allowed to stand for 24 hours. Subsequently, 50 ml of the supernatant was taken and added to 950 ml of distilled water to be autoclaved.

*Phytophthora* isolates were also conserved in tubes filled with OA medium (72.5 g $litre^{-1}$ oatmeal agar, Sigma Aldrich) for its long-term storage. A single 6 mm diameter agar disk was placed in each OA tube, incubated at 25ºC until mycelium growth was observed, and then it was sealed with parafilm for its conservation at 14ºC.

## Results

### Symptomatology

In all nurseries, a broad range of symptoms was observed: cankers (with or without gummosis exudates), collar rot, dead plants, dieback (partial dieback or the whole plant), foliar symptoms (chlorosis, defoliation, leaf spots, irregular shaped blotches in the leaf margins or starting at the leaf apex or petiole, necrotic spots), growth reduction, wilting and/or decline (Figures 3.2 and 3.3). Figure 3.4 shows the percentage distribution of symptoms observed in the sampled plants collected in the nurseries. The most frequent symptoms were dieback (37.1%), followed by foliar symptoms (29.4%) and growth reduction (11.3%).

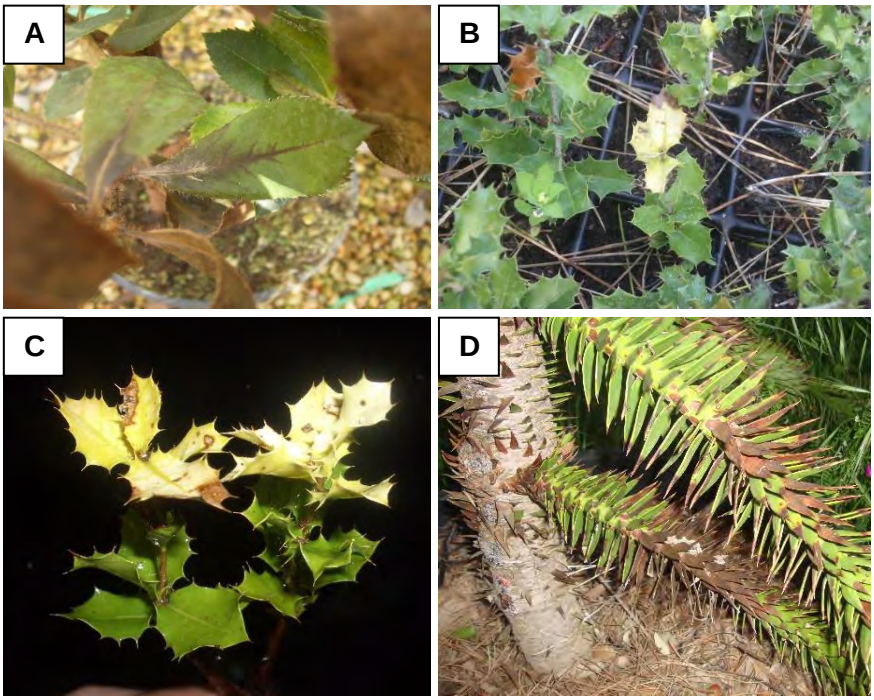


**Figure 3.2.** Foliar symptoms. **A:** *Arbutus unedo* with black leaf necrosis that advances along the middle vein from the petiole to the apex. **B** and **C:** *Quercus ilex* showing chlorotic symptoms and leaf spots. **D:** *Araucaria araucana* with leaf necrosis symptoms..

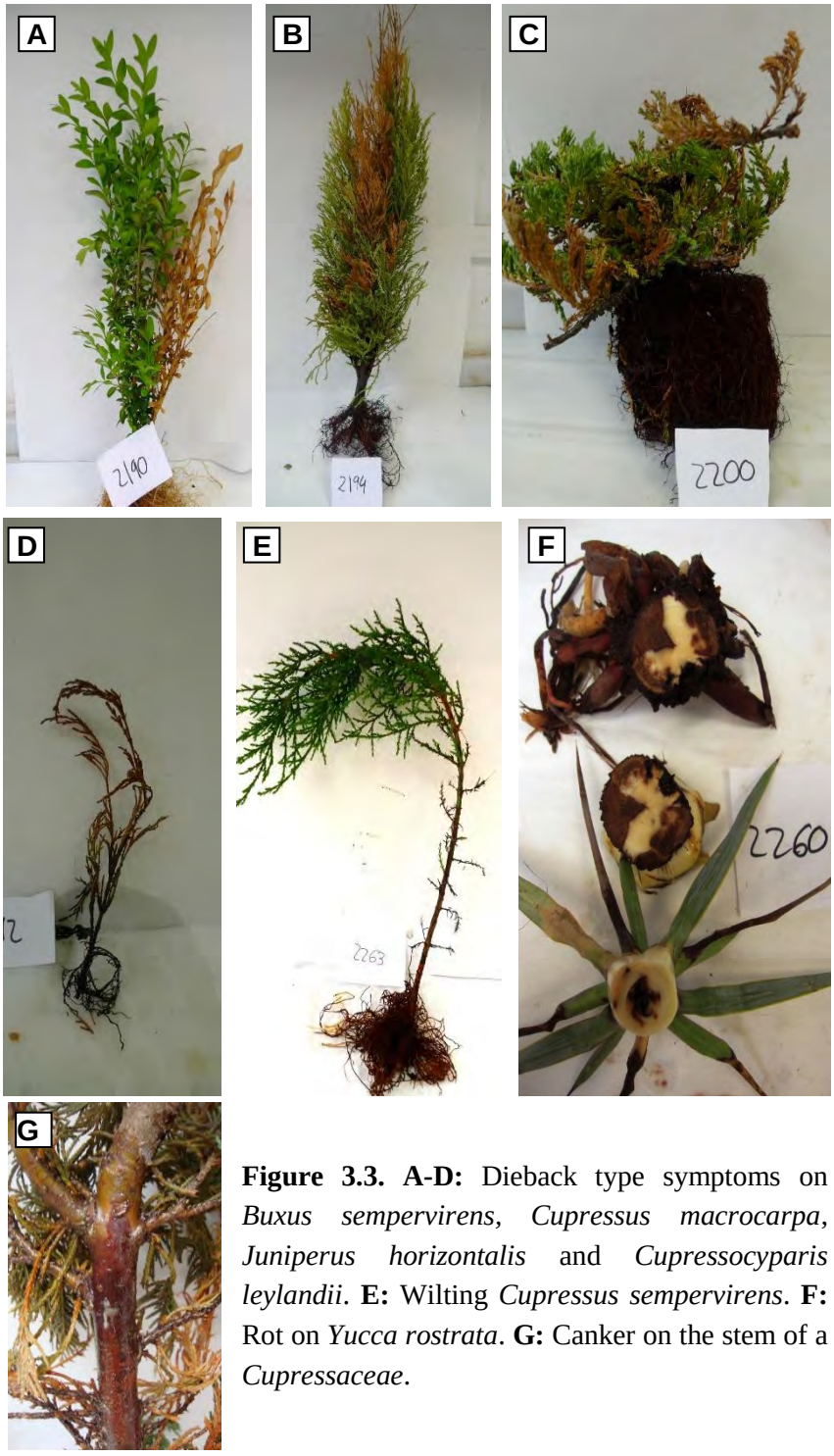


**Figure 3.3. A-D:** Dieback type symptoms on *Buxus sempervirens*, *Cupressus macrocarpa*, *Juniperus horizontalis* and *Cupressocyparis leylandii*. **E:** Wilting *Cupressus sempervirens*. **F:** Rot on *Yucca rostrata*. **G:** Canker on the stem of a *Cupressaceae*.

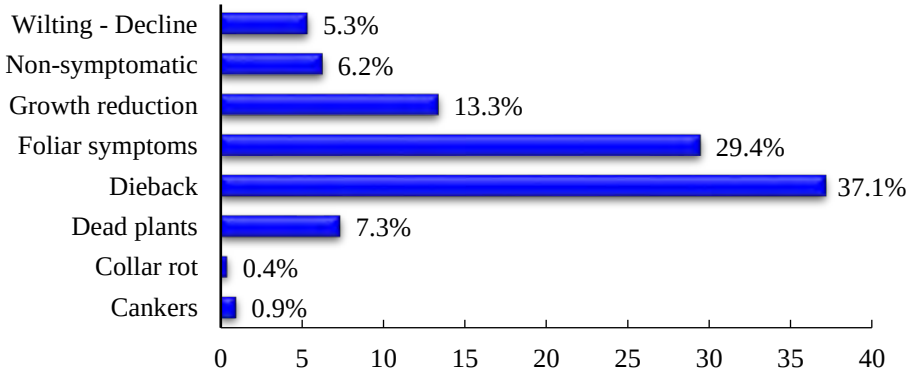


**Figure 3.4.** Symptomatology observed in the sampled nurseries expressed in percentage.

A total number of 547 samples were collected and oomycetes were identified in 30.7% of the plant samples, and the most frequent symptoms were dieback (43.5%), foliar symptoms (28.6%) and growth reduction (11.3%). *Phytophthora* was isolated on 59 plants, which means 10.8% of total plant samples collected in this survey. On plants affected by *Phytophthora*, dieback was the most frequent symptom observed (59.3%), followed by foliar symptoms (23.7%), wilting-decline and growth reduction (both 6.8%) (Figure 3.5).

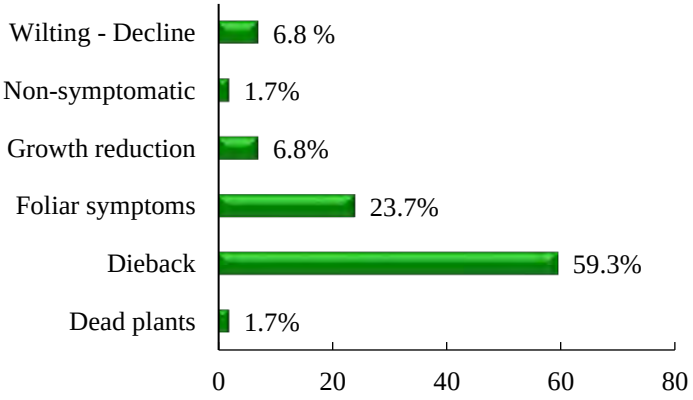


**Figure 3.5.** Symptomatology associated to *Phytophthora*: percentage of plants infected by *Phytophthora*.

### *Phytophthora* species isolated in the sampled nurseries

Seventy-one isolates of *Phytophthora* were obtained from infected tissues and/or the rhizosphere of the plants (Table 3.1) and 36 were isolated from water samples collected from the irrigation ponds or streams close to the nurseries (Table 3.2).

Molecular identification of the isolates revealed the presence of 17 *Phytophthora* phylotypes in the surveyed nurseries. The species isolated were *Phytophthora bilorbang* Aghighi & Burgess, *Phytophthora cactorum* (Lebert & Cohn) J. Schr., *Phytophthora cambivora* (Petri) Buisman, *Phytophthora cinnamomi* Rands, *Phytophthora citrophthora* (R.E. Smith & E.H. Smith) Leonian, *Phytophthora crassamura* Scanu, Deidda & Jung, *Phytophthora hedraiandra* de Cock & Man in 't Veld, *Phytophthora gonapodyides* (H.E. Petersen) Buisman, *Phytophthora lacustris* Brasier, Cacciola, Nechwatal, Jung & Bakonyi, *Phytophthora nicotianae* Breda de Haan, *Phytophthora niederhauserii* Z.G. Abad & J.A. Abad, *Phytophthora palmivora* E.J. Butler, *Phytophthora plurivora* T. Jung & T.I. Burgess, *Phytophthora pseudocryptogea* Safaiefarahani, Mostowfizadeh, Hardy & Burgess and *Phytophthora sansomeana* Hansen & Reeser. Two *Phytophthora* isolates recovered from the roots of *Arbutus unedo* and *Juniperus communis* were identified as the informally designated taxon *Phytophthora* sp. tropicalis-like 2 (Jung *et al.*, 2020). There were four *Phytophthora* isolates that could not be identified to the species level, so they were tentatively named as *Phytophthora* sp. 1 clade 2.

According to the ITS, *Phytophthora* sp. 1 clade 2 was closely related to *Phytophthora meadii* McRae showing differences in two positions with the ex-type, but the COI results put these isolates close to *P. citrophthora*. Therefore, we decided to designate these isolates as *Phytophthora* sp. 1 clade 2.

Among plant samples, which included rhizosphere substrate or soil, *P. pseudocryptogea* was the species with the highest incidence (21.1%), followed by *P. plurivora* (15.5%), *P. hedraiandra* (9.9%), *P. citrophthora* and *P. nicotianae* (8.5% each species), *P. cactorum*, *P. cinnamomi* and *Phytophthora* sp. 1 clade 2 (5.6% each species), *P. crassamura* and *P. sansomeana* (4.2% each species), *P. gonapodyides*, *P. palmivora* and *Phytophthora* sp. tropicalis-like 2 (2.8% each species). Two other species, *P. cambivora* and *P. niederhauserii*, had the lowest incidence values (1.4% each species). Some plants were co-infected with more than one *Phytophthora* species.

In the aquatic habitats, *P. cactorum*, *P. lacustris* and *P. gonapodyides* were the most abundant species (26.3%, 21.1% and 18.4% respectively),

followed by *P. cambivora*, *P. citrophthora* and *P. plurivora* (7.9% each species), *P. pseudocryptogea* (5.3%). The lowest value of presence in water was 2.6%, shared by *P. bilorbang* and *P. palmivora*.

The growing pattern on PDA of some of the *Phytophthora* spp. found in this study is shown Figure 3.6.

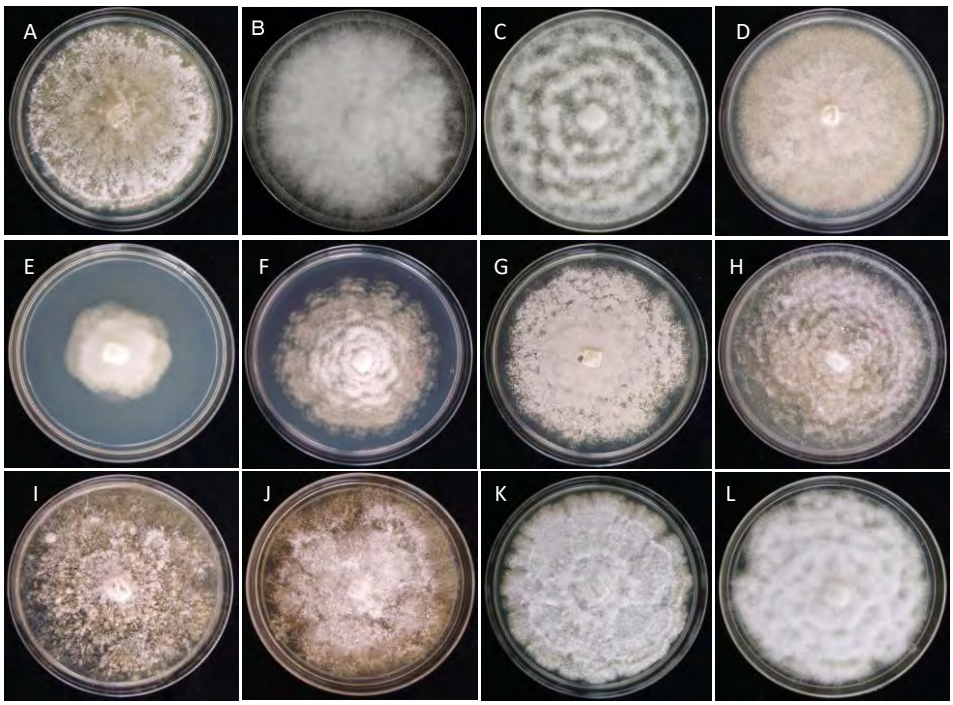


**Figure 3.6**. Colony morphology of *Phytophthora* spp. on PDA medium. **A:** *P. cactorum*. **B:** *P. cambivora.* **C:** *P. cinnamomi*. **D:** *P. citrophthora.* **E:** *P. crassamura*. **F:** *P. gonapodyides*. **G:** *P. hedraiandra*. **H:** *P. lacustris*. **I:** *P. nicotianae*. **J:** *P. palmivora.* **K:** *P. plurivora*. **L:** *P. pseudocryptogea*.

**Table 3.1.** *Phytophthora* species isolated from vegetal material.

| ***Phytophthora* spp.** | **Host** | **Nursery** | **Source** | **N. samples** | **Region** |
|---|---|---|---|---|---|
| CAC | *Fagus sylvatica*, *Photinia* "Red Robin", *Pinus pinea* | 6, 7, 10, 19 | R, S | 4 | Cat., Com.Val., P.Vas. |
| CAM | *Quercus ilex* | 7 | R | 1 | Com.Val. |
| CIN | *Arbutus unedo*, *Pinus radiata*, *Pseudotsuga menziesii* | 9, 18 | R, S | 4 | Cat., P.Vas. |
| CIP | *Citrus sinensis*, *Escallonia* sp., *Picea pungens* "Glauca Globosa", *Quercus faginea*, *Rosmarinus officinalis* | 4, 10, 11, 15, 16 | R, S | 6 | Cat., Com.Val. |
| CRA | *Pinus pinea* | 17 | S | 3 | Ext. |
| HED | *Juniperus phoenicea*, *Quercus ilex*, *Viburnum tinus* | 5, 7, 8, 10, 17 | R, S | 7 | Cat., Com.Val., Ext. |
| GON | *Juniperus communis* "Hibernica", *Quercus ilex* | 2, 13 | R, S | 2 | Cat., Com.Val. |
| NIC | *Buxus sempervirens*, *Citrus sinensis*, *Escallonia* sp., *Myrtus communis* "Tarentina", *Pistacia lentiscus*, *Rosmarinus* sp. | 7, 10, 15, 16 | R | 6 | Cat. |
| NIE | *Arbutus unedo* | 24 | S | 1 | Ext. |
| PAL | *Cupressus sempervirens*, *Pistacia lentiscus* | 7, 16 | R | 2 | Cat. |
| PLU | *Chamaecyparis lawsoniana* "Elwoodii", *Cupressus sempervirens*, *Juniperus chinensis* "Expansa", *Quercus faginea*, *Quercus ilex* | 2, 4, 12, 16 | R, S | 11 | Cat., Com.Val. |
| PSC | *Chamaecyparis lawsoniana* "Elwoodii", *Quercus ilex*, *Yucca rostrata* | 2, 8, 12,15, 23 | R, S | 15 | Cat., Com.Val., Ext. |
| SAN | *Quercus ilex* | 2 | R, S | 3 | Com.Val. |
| TRO | *Arbutus unedo*, *Juniperus communis* "Hibernica" | 13, 23 | R | 2 | Cat., Ext. |
| SP. 1 | *Citrus sinensis* | 16 | R | 3 | Cat. |

CAC: *P. cactorum*. CAM: *P. cambivora*. CIN: *P. cinnamomi*. CIP: *P. citrophthora.* CRA: *P. crassamura.* HED: *P. hedraiandra*. GON: *P. gonapodyides*. NIC: *P. nicotianae*. NIE: *P. niederhauserii*. PAL: *P. palmivora.* PLU: *P. plurivora*. PSC: *P. pseudocryptogea*. SAN: *P. sansomeana*. TRO: *Phytophthora* sp. tropicalis-like 2. SP. 1: *Phytophthora* sp. 1 clade 2. R: Roots. S: Soil. Cat.: Cataluña. Com.Val.: Comunidad Valenciana. Ext: Extremadura. P.Vas.: País Vasco. Each nursery was coded with a number.

**Table 3.2.** *Phytophthora* species isolated from water samples in surveyed nurseries and from floating leaves from two streams that were next to the nurseries.

| Collection code | Nursery | Water fluxes | *Phytophthora* species | Clade |
|---|---|---|---|---|
| Ps-961 | 2 | Nursery irrigation | *P. lacustris* | 6 |
| PS-1389 | 9 | Nursery irrigation | *P. cambivora* | 7 |
| PS-1390 | 9 | Nursery irrigation | *P. plurivora* | 2 |
| PS-1391 | 9 | Nursery irrigation | *P. plurivora* | 2 |
| PS-1392 | 9 | Stream | *P. cambivora* | 7 |
| PS-1393 | 9 | Stream | *P. cactorum* | 1 |
| PS-1394 | 7 | Nursery irrigation | *P. cambivora* | 7 |
| PS-1395 | 7 | Nursery irrigation | *P. cactorum* | 1 |
| PS-1396 | 10 | NurseryPond 1 | *P. cactorum* | 1 |
| PS-1397 | 10 | Nursery Pond 2 | *P. cactorum* | 1 |
| PS-1398 | 10 | Nursery Pond 3 | *P. pseudocryptogea* | 8 |
| PS-1399 | 10 | Nursery Pond 3 | *P. cactorum* | 1 |
| PS-1400 | 10 | Nursery Pond 3 | *P. cactorum* | 1 |
| PS-1401 | 10 | Nursery Pond 3 | *P. lacustris* | 6 |
| PS-1402 | 10 | Nursery Pond 3 | *P. gonapodyides* | 6 |
| PS-1403 | 10 | Nursery Pond 3 | *P. lacustris* | 6 |
| PS-1404 | 10 | Nursery Pond 3 | *P. lacustris* | 6 |
| PS-1405 | 10 | Stream | *P. plurivora* | 2 |
| PS-1406 | 10 | Stream | *P. pseudocryptogea* | 8 |
| PS-1407 | 10 | Stream | *P. citrophthora* | 2 |
| PS-1408 | 10 | Stream | *P. lacustris* | 6 |
| PS-1409 | 12 | Nursery irrigation | *P. gonapodyides* | 6 |
| PS-1410 | 12 | Nursery irrigation | *P. gonapodyides* | 6 |
| PS-1411 | 12 | Nursery pond | *P. cactorum* | 1 |
| PS-1412 | 8 | Nursery irrigation | *P. lacustris* | 6 |
| PS-1413 | 8 | Nursery Pond | *P. gonapodyides* | 6 |
| PS-1414 | 8 | Nursery Pond | *P. gonapodyides* | 6 |
| PS-1415 | 8 | Nursery Pond | *P. gonapodyides* | 6 |
| PS-1416 | 13 | Nursery Pond | *P. palmivora* | 4 |
| PS-1417 | 13 | Nursery Pond | *P. cactorum* | 1 |
| PS-1418 | 13 | Nursery Pond | *P. citrophthora* | 2 |
| PS-1419 | 13 | Nursery Pond | *P. citrophthora* | 2 |
| PS-1420 | 13 | Nursery Pond | *P. bilorbang* | 6 |

| Collection code | Nursery | Water fluxes | *Phytophthora* species | Clade |
|---|---|---|---|---|
| PS-1444 | 10 | Nursery Pond 2 | *P. cactorum* | 1 |
| PS-1445 | 10 | Nursery Pond 2 | *P. cactorum* | 1 |
| PS-1446 | 10 | Nursery Pond | *P. lacustris* | 6 |
| PS-1423 | 9 | Leaves from stream | *P.gonapodyides* | 6 |
| PS-1424 | 10 | Leaves from stream | *P. lacustris* | 6 |

### *Pythium* and *Phytopythium* species isolated in the sampled nurseries

The isolates obtained in PARPB media were grown on PDA plates to differentiate *Phytopthhora* isolates from other oomycetes according to the morphology of their colonies, DNA extraction was done in these young cultures not considered *Phytophthora*, and the sequence results of the amplified ITS region revealed six *Phytopythium* species (*Pp. chamaehyphon*, *Pp. helicoides*, *Pp. litorale*, *Pp. mercuriale*, *Pp. montanum* and *Pp. vexans*) and eleven *Pythium* species (*Py. sterilum*, *Py. intermedium*, *Py. attrantheridium*, *Py. rostratifingens*, *Py. opapillum*, *Py. irregulare*, *Py. ultimum*, *Py. undulatum*, *Py. sylvaticum*, *Py. pleroticum* and *Py. diclinum*) (Figures 3.7 and 3.8).

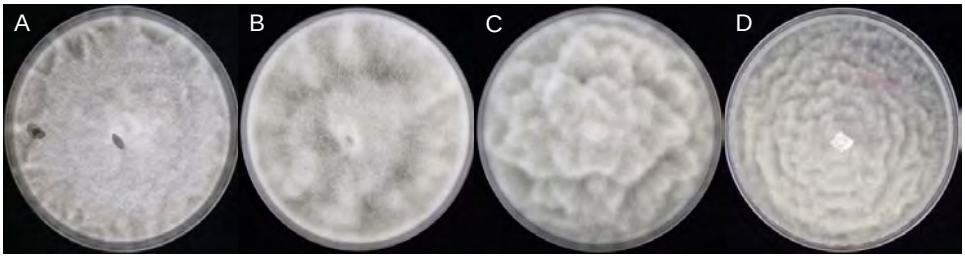


**Figure 3.7**. Colony morphology of *Phytopythium* types on PDA. **A:** *Pp. chamaehyphon*. **B:** *Pp. helicoides*. **C:** *Pp. vexans*. **D:** *Pp. litorale*.

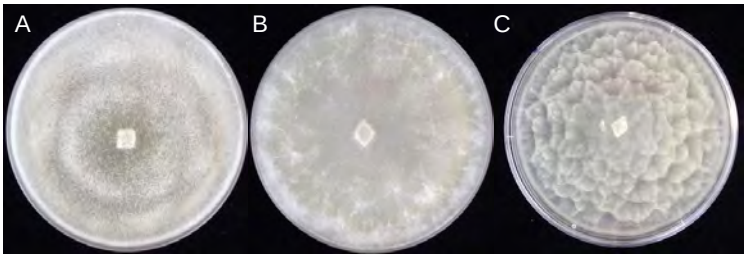


**Figure 3.8**. Colony morphology of some *Pythium* types. **A:** *Py. undulatum*. **B:** *Py. intermedium*. **C:** *Py. rostratifingens*.

**Fungal isolates obtained from sampled nurseries**

Cultures on PDA were grouped by colony morphology and were identified as: *Alternaria* sp. (7.1%), *Aspergillus* sp. (3.8%), *Botrytis* sp. (1.3%), *Chaetomium* sp. (1.1%), *Cladosporium* sp. (1.1%), *Colletotrichum* sp. (1.6%), *Cylindrocarpon*-like sp. [(39.3%) (nowadays reclassified into "*Dactylonectria*", *Ilyonectria* and *Campylocarpon*), *Fusarium* sp. (26.8%), *Gliocladium* sp. (1.8%), *Mortierella* sp. (4.7%), *Penicillium* sp. (14.8%), *Pestalotiopsis* sp. (10%), *Phoma* sp. (2.4%), *Phomopsis* sp. (0.4%), *Rhizoctonia* sp. (14%), *Stemphylium* sp. (0.5%), *Thielaviopsis* sp. (0.9%) and *Trichoderma* sp. (24.8%).

**Discussion**

The results of this study provide evidence of *Phytophthora*'s ubiquity in ornamental and forest nurseries, as it has been isolated from 18 surveyed nurseries, from both plant material and water samples. It was also noteworthy the presence of Cylindrocarpon-like asexual morphs in most of the nurseries.

In the surveyed nurseries, the plants that were collected showed an aerial symptomatology that could be associated with *Phytophthora* infections, such as dieback, shoot blight, chlorosis, defoliation, irregular leaf blotches, wilting, decline, cankers with gummosis, and presence of dead plants. This aerial symptomatology was generally associated with root damage such as change of colour in the roots, lesions, absence and/or rot of the feeder roots. Nevertheless, in some plants, the damage was limited to the aerial part, with no visible root symptoms. In plants affected by *Phytophthora* spp., dieback was the most frequent symptom, followed by diverse foliar symptomatology (leaf blotch, defoliation and chlorosis), wilting and/or decline. These set of observed

symptoms agree with the symptomatology described in the literature (Cacciola *et al.*, 1997; Schwingle *et al.*, 2007; Grünwald *et al.*, 2008; Moralejo *et al.*, 2009; Perez-Sierra *et al.*, 2012; Abad *et al.*, 2014; Bienapfl & Balci, 2014; Jung *et al.*, 2016). It should be noted that disease symptoms may be suppressed due to prophylactic fungicide treatments or the natural lag period between root and crown rots and the development of foliar symptoms (Leonberger *et al.*, 2013).

Seventy-one *Phytophthora* isolates from clades 1, 2, 4, 6, 7 and 8 were recovered from a total of 547 plant samples belonging to 22 species included in 19 plant genera. In some of the analysed plants more than one species of *Phytophthora* was isolated, revealing the existence of mixed infections, which should be common as it has also been detected in other nursery surveys (Cacciola & Polizzi, 1996; Perez-Sierra *et al.*, 2012; Schwingle *et al.*, 2007; Prigigallo *et al.*, 2015; Panabières *et al.*, 2016). Thirteen *Phytophthora* species, one informally designated taxon and one *Phytophthora* phylotype that could not be identified to the species level were isolated from plant samples, being the most frequent species isolated *P. pseudocryptogea*, *P. plurivora*, *P. hedraiandra*, *P. citrophthora* and *P. nicotianae*. In lower frequencies, *P. cactorum*, *P. cinnamomi*, *Phytophthora* sp. 1 clade 2, *P. crassamura*, *P. sansomeana*, *P. gonapodyides* and *P. palmivora* were also present. The species with the lowest frequencies were *P. cambivora* and *P. niederhauserii*, as well as the informally designated taxon, *Phytophthora* sp. tropicalis-like 2.

*Phytophthora crassamura* sp. nov. was described by Scanu *et al*. (2015) based on the morphological and genetic variation of few isolates belonging to the *Phytophthora megasperma* species complex. These isolates were obtained from infected tissue of *Picea abies* grown in a nursery and rhizosphere soil of *Juniperus phoenicea*, a component of the Mediterranean maquis vegetation in Sardinia (Italy). Since then, it has been isolated from the rhizosphere of *Fraxinus oxycarpa* in natural stands of Sicily, Italy (Jung *et al.*, 2019), and over twenty different hosts collected in both nurseries and restoration sites in California (Sims *et al*., 2019). This study is the first report of *P. crassamura* in Spain and the first worldwide record on *Pinus pinea* worldwide.

Safaiefarahani *et al.* (2015) identified *Phytophthora pseudocryptogea*, after re-evaluating isolates from Iran and Australia from the *P. cryptogea* species complex. This species has already been reported in 2018 in different regions in Spain affecting *Q. ilex* and the present study confirms its presence in the nurseries from those regions (Mora-Sala *et al.*, 2018a, 2018b). This study reports for the first time in Spain, *P. pseudocryptogea* in *Chamaecyparis lawsoniana* and *Yucca rostrata*. Sims *et al.* (2019) have also reported *P. pseudocryptogea* in

nurseries and Bourret (2018) in wildlands, both in California. Jung *et al.* (2019) confirmed the presence of *P. pseudocryptogea* in Sicily forests, considering this species as one of the most widespread *Phytophthora* species although it is considered an introduced pathogen. In spite *Phytophthora cryptogea* Pethybr. & Laff. was a frequently reported pathogen in European nurseries and forests (Vettraino *et al.*, 2002, 2005; Sánchez *et al.*, 2005; Prigigallo *et al.*, 2015; Jung *et al.*, 2016) it was not detected in this study.

*Phytophthora sansomeana* was segregated from the *P. megasperma* complex in 2009 and as far as we are concerned, until now it has only been reported in North America (United States) and in Asia (China). It was isolated from diverse forest and agricultural hosts, such as Douglas-fir nursery seedlings, weeds and soybean. Therefore, it is the first report of *P. sansomeana* in Europe and in *Q. ilex* seedlings worldwide. As it is the second time it is identified in nursery stock, it seems probable that the presence of *P. sansomeana* in nursery industry was not casual. In this context, pathogenicity trials should be performed to understand how this *Phytophthora* species affects holm oak seedlings.

The known distribution of *Phytophthora tropicalis* Aragaki & J.Y. Uchida currently comprises Pacific Islands (United States: HI), North America (United States: VA) and Europe (the Netherlands). In 2020, Jung *et al*. reported from *Alnus nepalensis* in Vietnam a species close to *P. tropicalis* that was designated as *Phytophthora* sp. tropicalis-like 2. Two isolates from our study were identified as *Phytophthora* sp. tropicalis-like 2. The ITS blast assigned a 100% identity with the isolate VN830 designated by Jung *et al*. (2020) as *Phytophthora* sp. tropicalis-like 2. Our study reports for the first time *Phytophthora* sp. tropicalis-like 2 on *Arbutus unedo* and *Juniperus communis*.

In previously Spanish surveyed nurseries, *P. cactorum*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. hibernalis*, *P. multivora*, *P. nicotianae*, *P. niederhauserii*, *P. palmivora*, *P. plurivora*, *P. syringae*, *P. tentaculata* and *P. tropicalis* have been reported (Sánchez *et al.*, 2005; Moralejo *et al.*, 2009; Pérez-Sierra *et al.*, 2012). According to Moralejo *et al.* (2009), *P. cinnamomi* and *P. cryptogea* (probably *P. pseudocryptogea*) have escaped from nurseries and are currently spreading in *Q. ilex* forests, and also infect associated shrubs such as *Arbutus unedo* and *Cistus monspeliensis* in the lowlands of northern Mallorca.

Other studies carried out in nurseries worldwide recovered almost the same species which demonstrates the fact that global nursery trade constitutes the main pathway for *Phytophthora* dispersion (Cacciola & Polizzi, 1996; Cacciola

*et al.*, 1997; Ferguson & Jeffers, 1999; Schwingle *et al.*, 2007; Brasier, 2008; Donahoo & Lamour, 2008; Moralejo *et al.*, 2009; Olson & Benson, 2011; Perez-Sierra *et al.*, 2012; Leonberger *et al.*, 2013; Prospero *et al.*, 2013; Abad *et al.*, 2014; Bienapfl & Balci, 2014; Parke *et al.*, 2014; Prigigallo *et al.*, 2015, 2016; Jung *et al.*, 2016; Sims *et al.*, 2019; Rooney-Latham *et al.*, 2019).

In Europe, a very extensive analysis of incidence of *Phytophthora* spp. was conducted, based on data from 23 countries between 1972 and 2013, in order to study the pathway of *Phytophthora* from nurseries into natural, semi-natural and horticultural ecosystems (Jung *et al.*, 2016). From nursery plant material, 49 *Phytophthora* taxa were identified, being *P. plurivora, P. cinnamomi, P. cactorum, P. nicotianae, P. ramorum* and *P. citrophthora* the most commonly sampled species, considered all alien pathogens in Europe. From forest and landscape plantings, 56 *Phytophthora* taxa were recovered, and invasive species with wide host range, such as *P. plurivora, P. cinnamomi, P. nicotianae, P. cryptogea* and *P. cactorum*, were most common. This large-scale study demonstrated that *Phytophthora* compromises nursery stock across Europe and, what is much more serious, the spread of these pathogens with infested nursery stock into natural ecosystems.

In California Sims *et al.* (2019) reported *P. cactorum* as the most frequent species in restoration nurseries but it was also isolated *P. hedraiandra, P. multivora, P. occultans, P. crassamura, P. thermophila* and *P. pseudocryptogea*. Rooney-Latham *et al.* (2019) reported *P. tentaculata, P. cactorum, P. cryptogea* complex, *P. cambivora, P. cinnamomi, P. citricola, P. hedraiandra, P. megasperma, P. multivora, P. nicotianae, P. niederhauserii, P. parvispora, P. pini, P. plurivora*, and *P. riparia* in their survey in Californian nurseries.

Regarding water surveys, the nine species reported in this study, once again agree with *Phytophthora* spp. recovered from irrigation water, waterways or riparian ecosystems published in other studies (Hwang *et al.*, 2008; Reeser *et al.*, 2011; Huai *et al.*, 2013; Hüberli *et al.*, 2013; Nagel *et al.*, 2013; Zappia *et al.*, 2014; Català *et al.*, 2015; Sims *et al.*, 2015; Redondo *et al.*, 2018). It is not surprising that as *Phytophthora* is adapted for aquatic dispersal, multiple *Phytophthora* spp. have been recovered from waterways or irrigation waters. Indeed several novel species have been detected in the last decade from water fluxes or riparian ecosystems such as *Phytophthora lateralis* (clade 8) causing *Chamaecyparis lawsoniana* decline (Hansen *et al.*, 2000), *Phytophthora alni* (clade 7) causing *Alnus* spp. decline (Brasier *et al.*, 2004b), and *Phytophthora ramorum* (clade 8) causing sudden oak death on *Quercus* spp. and

*Notholithocarpus densiflorus* (Rizzo *et al.*, 2002). Detection of *Phytophthora* taxa belonging to clade 6 has increased in the last years as riparian systems have grown in attention (Crous *et al.*, 2012; Kroon *et al.*, 2012; Nagel *et al.*, 2013; Yang *et al.*, 2017). *Phytophthora* spp. from clade 6 are thought to be adapted to survive in rivers due to its rapid colonisation of leaves and plant debris (Brasier *et al.*, 2003; Jung *et al.*, 2011). Jung *et al.* (2011) consider the possibility that species from clade 6 are probable saprotrophs, as these *Phytophthora* spp. depend on their ability to rapidly colonise fresh plant material (such as fallen leaves) in order to outcompete other saprotrophic organisms. There is a significant gap in understanding waterborne plant pathogens, particularly in open irrigation systems (Guha Roy & Grünwald, 2014; Zappia *et al.*, 2014; Redondo *et al.*, 2018)

Among other plant pathogens that were also isolated, the most important genera were *Pythium*, *Phytopythium*, Cylindrocarpon-like or *Fusarium*. The percentage of recovered *Pythium* and *Phytopythium* species, highlight again the importance of sanitary measures in nursery industry. *Pythium* and *Phytopythium* are also among the most frequent plant pathogens in nurseries (seed rot and damping-off), *Pythium* species require free water to complete their cycle but compared with *Phytophthora*, they have a quicker development and growth, so their colonisation ability is bigger than the one of *Phytophthora*. In some cases, recovering *Phytophthora* in a sample in which *Pythium* is present is not possible due to its rapid growth. Most *Pythium* and *Phytopythium* species are used to be considered saprotrophs but nowadays the pathogenicity of some species has been demonstrated such as *Pythium anandrum*, *Pythium dissotocum*, *Pythium irregulare*, *Pythium macrosporum*, *Pythium mamillatum*, *Pythium oopapillum*, *Pythium rostratifingens*, *Pythium spiculum*, *Pythium sylvaticum*, *Pythium ultimum* and *Phytopythium chamaehyphon* (Jung *et al.*, 1996; Romero *et al.*, 2007; Ivors *et al.*, 2008; Weiland *et al.*, 2013).

Cylindrocarpon-like asexual morphs were isolated in 84% of the surveyed nurseries and the most frequent symptomatology associated to these pathogens were foliar symptoms, dieback and growth reduction. Cylindrocarpon-like asexual morphs were present in all regions affecting 27 plant genera: *Acer*, *Aesculus*, *Alnus*, *Araucaria*, *Arbutus*, *Buxus*, *Castanea*, *Chamaecyparis*, *Cupressus*, *Ephedra*, *Fagus*, *Fraxinus*, *Hebe*, *Juniperus*, *Myrtus*, *Photinia*, *Picea*, *Pinus*, *Pistacia*, *Polygala*, *Quercus*, *Rosmarinus*, *Sequoiadendron*, *Sorbus*, *Thuja* and *Viburnum*. These pathogens are usually associated to *Vitis vinifera* in Spain and the high diversity of hosts suggested that it has a broader scope. In this

context, a study was performed to analyse the Cylindrocarpon-like asexual morphs present in the Spanish woody plant nurseries (Chapter 4).

The impact that plant pathogens can have on plant industry can extend into billions of dollars but the worst is the environmental risk, which biodiversity, forestry and agriculture are currently undergoing (Zappia *et al.*, 2014; Prigigallo *et al.*, 2015; Yang *et al.*, 2016; Jung *et al.*, 2016; Li *et al.*, 2019). Biosecurity needs to be cornerstone of global nursery trade to avoid the possibility of *Phytophthora* spp. spreading to new habitats where they may be exposed to compatible species and potentially form new hybrids (Brasier, 2000, 2008; O´Brien *et al.*, 2009).

The exclusion of nursery pathogens from forested areas is a critical issue for forest health (Gonthier & Nicolotti, 2013; Sims *et al.*, 2019). Monitoring pathogen zone; restricting vehicle movement from infested to uninfested areas; cleaning vehicles before entering in uninfested areas; preventing infested and uninfested soil mixing; preventing water draining from infested to uninfested areas; education of public and forestry workers are some of the exclusion measures should come into full force and effect (Gonthier & Nicolotti, 2013; Sims *et al.*, 2019).

A high priority should be placed on the production of pathogen-free propagating material by appropriate sanitary practices (Gullino & Garibaldi, 2007). The microbial community plays an important role in the protective effect against oomycetes. Organic soils in the form of compost have long been found to supress a number of *Phytophthora* and *Pythium* spp. (Gullino & Garibaldi, 2007). Nursery sanitation measures such as the following ought be implemented in all nurseries and garden centres: use of new seedling containers, container media pasteurised; irrigation water *Phytophthora*-free (sand filters or chlorine interventions); water splash kept off leaves and wetness time minimised; containers kept off the ground; suppressive composts or fungicides avoided; sustained heat treatment to kill resting structures in plant or soil material via composting, solarisation, oven treatment or autoclaving, heating installation in greenhouses, correct aeration between seedling benches and plantations, pH control (a low pH [3,5-4,5] avoid spore liberation), moderate nitrogen fertilisation, routinely tool disinfection (Gonthier & Nicolotti, 2013).

Nursery stock has become the most common way for the introduction of new *Phytophthora* species into natural habitats worldwide (Brasier, 2008). The spread of pathogens is an unintended consequence of nursery activity that has to be avoided at all costs. Supplying healthy plants should be the fundamental

principle of nursery production. Following these aims, implementing the most recent available molecular techniques to nurseries monitoring may facilitate the diagnosis by allowing an accurate and rapid diagnosis in plant trade.

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# Chapter 4

## Survey, Identification and characterization of Cylindrocarpon-like asexual morphs in Spanish forest nurseries

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# Chapter 4


## Abstract

Cylindrocarpon-like asexual morphs infect herbaceous and woody plants, mainly in agricultural scenarios, but also in forestry systems. The aim of the present study was to characterize a collection of Cylindrocarpon-like isolates recovered from the roots of a broad range of forest hosts from nurseries showing decline by morphological and molecular studies. Between 2009 and 2012, 17 forest nurseries in Spain were surveyed and a total of 103 Cylindrocarpon-like isolates were obtained. Isolates were identified based on DNA sequences of the partial gene regions histone H3 (*his3*). For the new species, the internal transcribed spacer and intervening 5.8S nrRNA gene (ITS) region, β-tubulin (*tub2*), and translation elongation factor 1-α (*tef1*) were also used to determine their phylogenetic position. Twelve species belonging to the genera *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria* were identified from damaged roots of 15 different host genera. The species *C. alicantinum*, *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *D. pinicola*, *D. torresensis*, *I. capensis*, *I. cyclaminicola*, *I. liriodendri*, *I. pseudodestructans*, *I. robusta* and *I. rufa* were identified. In addition, two *Dactylonectria* species (*D. hispanica* sp. nov., and *D. valentina* sp. nov.), one *Ilyonectria* species (*I. ilicicola* sp. nov.) and one *Neonectria* species (*N. quercicola* sp. nov.) are newly described. The present study demonstrates the prevalence of this fungal group associated with seedlings of diverse hosts showing decline symptoms in forest nurseries in Spain.


## Introduction

Spain has the second largest forest area in the European Union (EU), 27.7 M ha, which accounts for the 15.4% of the total European forest. The most extensive forest systems in Spain include holm oak forests (*Quercus ilex*) (2.8 M ha, 15.3% of the forested area), oak range-lands consisting mainly of holm oaks (2.4 M ha), and Aleppo pines (*Pinus halepensis*) (2 M ha) (MAGRAMA 2014).

Forest systems not only offer market valuable services such as timber, hunting, fishing and tourism, but also important environmental and social contributions such as carbon capture, hydric and soil regulation, hosting

biodiversity and regulating climate. Climate change has substantially built public awareness about the need of developing forest management strategies to counterbalance the deforestation rate (Trumbore *et al.* 2015).According to FAO (2015), in 2010, the deforestation rate in Spain was 57,000 ha per year, while the reforestation rate shed a positive balance with 89,000 ha per year.

Forest nurseries provide woody plants for the afforestation process, but they are also a key point to prevent and control early infections by fungal pathogens, thus guaranteeing the phytosanitary quality of planting materials. An increasing incidence of invasive pathogens related to global tree-planting projects has been reported worldwide (Wingfield *et al.* 2015). Numerous examples of alien invasions have been reported in the forestry sector, such as: *i*) *Phytophthora cinnamomi* root disease or Jarrah dieback; *ii*) Sudden Oak Death caused by *P. ramorum*; *iii*) Dutch elm disease caused by *Ophiostoma ulmi*; *iv*) Chestnut blight caused by *Cryphonectria parasitica*; *v*) Cedar root rot caused by *P. lateralis* (Brasier 2008). Thus, nursery regulations should be implemented at a global scale and updated phytosanitary measures must be considered as the cornerstone of the all nursery systems (Wingfield *et al.* 2015).

Cylindrocarpon-like asexual morphs infect herbaceous and woody plants, mainly in agricultural scenarios, but also in forest systems (Agustí-Brisach and Armengol 2013). This group of fungi has been commonly associated with damping-off, root rot or bark necrosis in forest nurseries and also with cankers on forest stands (Jankowiak *et al.* 2016). In Swedish conifer nurseries, *Cylindrocarpon* (*Cy.*) *destructans*, was the main pathogen isolated from damaged root tissues (Beyer-Ericson*et al.* 1991). Lilja *et al.* (1992) reported the presence of *Cy. cylindroides*, *Cy. destructans*, *Cy. didymum*, *Cy. magnusiarum*, *Cy. obtusisporum* and *Cy. pineum* in seedlings of *Pinus* (*P.*) *sylvestris* and *Picea* (*Pc.*) *abies* in Finnish nurseries. None of these asexual morphs seemed to be pathogenic but they predisposed *P. sylvestris* to be colonised by the most common saprophytic *Cy. destructans*. Dumroese and James (2005) stated that in forest and conservation nurseries in the Pacific Northwest of USA, Cylindrocarpon-like asexual morphs were among the most ubiquous root pathogens, with *Cy. destructans* being the most frequently isolated. Menkis *et al.* (2006) confirmed the presence of *Nectria* species (*N. gliocladioides*, *N. inventa*, *N. lucida*, *N. macrodydima* and *N. radicicola*) in decayed roots of *P. sylvestris*

and *Pc. abies* nursery seedlings in Lithuania. In 2009, damping off of *P. radiata* seedlings was observed in a pine nursery in Spain. *Dactylonectria* (*D.*) *pauciseptata* was described as the causal agent of damping-off, extensive root necrosis, and root death, exhibited by the pine seedlings (Agustí-Brisach *et al.* 2011).

In forests, Cylindrocarpon-like asexual morphs have also been reported as the dominant fungi on roots of *Fraxinus excelsior*, *Fagus* (*F.*) *sylvatica* and *Quercus* spp. (Kubíková 1963; Krzan 1987; Halmschlager and Kowalski 2004).Cylindrocarpon-like asexual morphs can hinder the natural regeneration of different tree species. *Cylindrocarpon destructans* arose as the main root pathogen implicated in the absence of regeneration of *Taxus baccata* and *Abies* (*A.*) *alba* in Poland (Manka *et al.* 1968; Kowalski 1982). Damping off caused by *Cy. destructans* was observed in the natural regeneration of *Eucalyptus* trees in Australia (Mwanza and Kellas 1987;Iles *et al.* 2010). In Canada, Axelrood *et al.*(1998)isolated Cylindrocarpon–like asexual morphs from the roots of naturally regenerating seedlings of *Pseudotsuga* (*Ps.*) *menziesii*. Szewczyk and Szwagrzyk, (2010) reported that this complex of asexual morphs affected the regeneration of old stands of *F. sylvatica* and *A. alba* in Western Carpathians, Poland. In 2012, *Neonectria candida* (syn. *N. ramulariae*) was described as a pathogen of *F. crenata* seeds in Japan (Hirooka *et al.* 2012), also affecting its natural regeneration. Jankowiak *et al.*(2016) characterised a collection of Cylindrocarpon-like fungi associated with beech litter in Austria and Poland and identified five species from *F. sylvatica* and *P. sylvestris: Ilyonectria crassa, I. pseudodestructans, I. rufa, N. candida* and *N. obtusispora*, and seven species were identified to genus level (*Ilyonectria* or *Neonectria* species).

The taxonomy of Cylindrocarpon-like asexual morphs has been revised several times since the genus *Cylindrocarpon* was first introduced in 1913 by Wollenweber to describe the asexual morphs of the *Nectria* section *Willkomiotes* Wollenw., which included species without chlamydospores (Brayford 1993;Halleen *et al.* 2006). Afterwards, in 1917, the term *Cylindrocarpon* also embraced species with mycelial chlamydospores in culture, with *Cy. destructans* becoming the most important species of this group. Booth (1966) split the genus into four groups based on the presence or absence of microconidia and chlamydospores (Brayford 1993; Halleen *et al.* 2006).Further studies transferred

species of the *Nectria* group with Cylindrocarpon-like asexual morphs into *Neonectria* (Rossman *et al.* 1999; Martiri *et al.* 2001; Brayford *et al.* 2004). In 2004, the new asexual morph genus, *Campylocarpon*, was described by Halleen *et al.* (2004). Later, Chaverri *et al.* (2011) recognized five novel genera within *Neonectria* based on characters associated with perithecial anatomy and conidial septation: *Campylocarpon, Ilyonectria, Neonectria* (*Cylindrocarpon s. s.*), *Rugonectria*, and *Thelonectria*. Finally, in 2014, Lombard *et al.* stated that the genus *Ilyonectria* was paraphyletic, and therefore designating a new genus *Dactylonectria*, to resolve this. At the same time, the genus *Cylindrodendrum* was shown to form a well-supported monophyletic sister clade to the *Ilyonectria* clade.

Within *I. destructans* complex, twelve new taxa were delineated mainly based on isolates previously describe as *C.destructans s.l.* from a diverse host range (Cabral *et al.* 2012a). This study comprised, isolates of *I. liriodendri* from *Quercus suber* (*Q. suber*); isolates of *I. robusta* from *Quercus* sp. and *Quercus robur* (*Q. robur*); *I. rufa* from *A. alba, Ps. menziesii* and *Pc. glauca*; *I. pseudodestructans* from *Quercus* sp., and *I. europaea* from *Aesculus hippocastanum*.

*Dactylonectria* species have also been isolated from forest species; *D. estremocensis* was isolated from *Quercus* sp. and *Pc. glauca*, *D. torresensis from A. nordmanniana* and *Quercus sp.* (Cabral *et al.* 2012b), and *D. pinicola* from *P. laricio* (Lombard *et al.* 2014).

Several *Neonectria* species have been associated with forest species, which include *N. coccinea, N. ditissima, N. faginata, N. fuckeliana, N. lugdunensis, N. major, N. neomacrospora, N. obtusispora* and *N. tsugae* (Castlebury *et al.* 2006; Lombard *et al.* 2014).

In 2002, Sánchez *et al.* reported high mortality levels of *Quercus* seedlings (*Q. ilex, Q. suber* and *Q. faginea*) caused by *C. destructans* in a nursery in southeastern Spain, but no further studies have explored the occurrence of species with Cylindrocarpon-like asexual morphs associated with root rot and dieback in Spanish forest nurseries. There is a lack of knowledge about the relevance of this group of fungi in forest nurseries. Thus, the aim of the present study was to characterize a large collection of Cylindrocarpon-like isolates

recovered from forest nurseries and a broad range of hosts displaying decline symptoms, by means of phenotypical characterization and DNA analysis.

## Materials and methods

### Fungal isolation

Between 2009 and 2012, extensive surveys were conducted in 17 Spanish forest nurseries located in the provinces of Alicante, Castellón, Tarragona and Valencia (Eastern Spain), and León, Logroño, Soria, and Madrid (Central-northern Spain). The survey was focused on plants showing symptoms such as wilting, dieback, chlorosis, foliage discoloration, defoliation, growth reduction and general decline (Fig. 4.1 A-E).

Affected plants showed root rot and loss of the feeder roots with the presence of necrotic lesions. These symptoms led to a reduction of the root biomass and root hairs, diminishing the volume of the root system and its feeder abilities (Fig. 4.1 F, G), which resulted in plant collapse (Fig. 4.1 A-E). At least three plants per symptomatic species were collected in each nursery and transported to the laboratory for fungal isolation. Affected roots were 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 pieces of discolored tissues were plated on potato dextrose agar (PDA) (Biokar-Diagnostics, Zac de Ther, France) amended with 0.5 g liter$^{-1}$ of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA) (PDAS). Plates were incubated for 5 to 10 days at 25°C in darkness.

According to morphological characters (mycelium aspect and colony colour), 103 isolates of Cylindrocarpon-like asexual morphs representative of different hosts and geographical origins were selected for further analysis (Table 4.1). These isolates were single-spored with the serial dilution method prior to morphological and molecular characterization (Dhingra and Sinclair 1995). For long-term storage, agar plugs with mycelium and conidia from cultures were stored in 15% glycerol solution at -80ºC in1.5 mL cryovials.


**Figure 4.1.** Symptomatology of nursery plants from which Cylindrocarpon-like anamorphs were isolated. **A**, **D** and **E**: shoot dieback on *Myrtus* sp. (A), *Juniperus* sp. (D) and *Rosmarinus officinalis* (E). **B** and **C**: *Juniperus* sp. and *Pinus* sp. dieback; apical dieback (B) and internal basal dieback (C). **F**: *Pinus* sp. seedlings showing reduced root system, including loss and rot of the feeder roots. **G**: *Quercus ilex* seedlings showing decline aerial symptoms, uneven growth and a reduction of the root system.

**Table 4.1.** *Campylocarpon*, *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria* isolates used in this study.

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *Campylocarpon fasciculare* | **CBS 112613**; STE-U 3970; C 76 | *Vitis vinifera* | F. Halleen | South Africa, Western Cape, Riebeeck Kasteel | AY677301 | AY677221 | JF735502 | JF735691 |
| *C. pseudofasciculare* | **CBS 112679**; CPC5472; HJS-1227 | *V. vinifera* | F. Halleen | South Africa, Western Cape, Wellington | AY677306 | AY677214 | JF735503 | JF735692 |
| *Cylindrodendrum album* | CBS 110655; VC-51 | Pine forest soil | F.X. Prenafeta-Boldú | The Netherlands, De Veluwe | KM231765 | KM232022 | KM231485 | KM231890 |
| | **CBS 301.83**; ATCC 46842; IMI 255534; TRTC 49165; UBC 8265 | *Fucus distichus* | R.C. Summerbell | Canada, British Columbia, Vancouver, Wreck Beach | KM231764 | KM532021 | KM231484 | KM231889 |
| *C. alicantinum* | **CBS 139518**; Cyl-3 | *Eriobotrya japonica* | J. Armengol | Spain, Alicante, Callosa d'En Sarrià | KP456014 | KP400578 | KP639555 | KP452501 |
| | Cyl-11 | *E. japonica* | J. Armengol | Spain, Alicante, Callosa d'En Sarrià | KP456017 | KP400581 | KP639558 | KP452504 |
| | Cy-FO-25 | *Quercus ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709593 | - |
| *C. hubeiense* | **CBS 124071**; HMAS 98331, 5620 | *Rhododendron* | W.P. Wu, W.Y. Zhuang & Y. Nong | China, Hubei | FJ560439 | FJ860056 | KR909093 | HM054090 |
| | CBS 129.97 | *Viscum album* | W. Gams | France, Dép. Jura, Châtelneuf near St. Laurent | KM231766 | KM232023 | KM231486 | KM231891 |
| *Dactylonectria alcacerensis* | **CBS 129087**; Cy159 | *V. vinifera* | A. Cabral & H. Oliveira | Portugal, Alcácer de Sol Torrão | JF735333 | AM419111 | JF735630 | JF735819 |
| | Cy134; IAFM Cy20-1 | *V. vinifera* | J. Armengol | Spain, Ciudad Real, Villarrubia de los Ojos | JF735332 | AM419104 | JF735629 | JF735818 |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *D. anthuriicola* | **CBS 564.95**; PD 95/1577 | *Anthurium* sp. | R. Pieters, 1995 | Netherlands, Bleiswijk | JF735302 | JF735430 | JF735579 | JF735768 |
| *D. estremocensis* | **CBS 129085**; Cy145 | *V. vinifera* | C. Rego & T. Nascimento | Portugal, Estremoz | JF735320 | JF735448 | JF735617 | JF735806 |
| | CPC 13539; 94–1685; CCFC226730 | *Picea glauca* | R. C. Hamelin, 1994 | Canada, Quebec | JF735330 | JF735458 | JF735627 | JF735816 |
| *D. hispanica* | **CBS 142827**;Cy-FO-45 | *Pinus halepensis* | B. Mora-Sala. 2011 | Spain, Valencia, Ayora | KY676882 | KY676876 | KY676864 | KY676870 |
| | Cy228 | *Ficus* sp. | F. Caetano, 2003 | Portugal, Lisbon | JF735301 | JF735429 | JF735578 | JF735767 |
| *D. hordeicola* | **CBS 162.89** | *Hordeum vulgare* | M. Barth | Netherlands, Noordoostpolder, Marknesse, Lovinhhoeve | AM419060 | AM419084 | JF735610 | JF735799 |
| *D. macrodidyma* | **CBS 112615**; STE-U 3976; C98; CPC 20709 | *V. vinifera* | F. Halleen | South Africa, Western Cape, Malmesbury, Jakkalsfontein | AY677290 | AY677233 | JF735647 | JF735836 |
| | CBS 112601; STE-U 3983; C 82 | *V. vinifera* | F. Halleen, 1999 | South Africa, Western Cape, Tulbagh | AY677284 | AY677229 | JF735644 | JF735833 |
| | Cy-FO-1 | *Q. faginea* | P. Abad-Campos. 2011 | Spain, Alicante, Alcoi | - | - | KX709497 | - |
| | Cy-FO-9 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Alicante, Alcoi | - | - | KX709498 | - |
| | Cy-FO-10 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Alicante, Alcoi | - | - | KX709499 | - |
| | Cy-FO-13 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Quart de Poblet | - | - | KX709500 | - |
| | Cy-FO-18 | *Q. faginea* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709501 | - |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *D. macrodidyma* | Cy-FO-19 | *J. phoenicea* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709502 | - |
| | Cy-FO-20 | *J. phoenicea* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709503 | - |
| | Cy-FO-23 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709504 | - |
| | Cy-FO-24 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709505 | - |
| | Cy-FO-26 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709506 | - |
| | Cy-FO-29 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709507 | - |
| | Cy-FO-30 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709508 | - |
| | Cy-FO-31 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709509 | - |
| | Cy-FO-32 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709510 | - |
| | Cy-FO-34 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709511 | - |
| | Cy-FO-48 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709512 | - |
| | Cy-FO-51 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709513 | - |
| | Cy-FO-55 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709514 | - |
| | Cy-FO-61 | *P. halepensis* | B. Mora-Sala. 2011 | Spain, Valencia, Ayora | - | - | KX709515 | - |
| | Cy-FO-134 | *I. aquifolium* | B. Mora-Sala. 2009 | Spain, Valencia, El Puig | - | - | KX709516 | - |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *D. macrodidyma* | Cy-FO-147 | *Rosmarinus officinalis* | B. Mora-Sala. 2009 | Spain, Valencia, Cheste | - | - | KX709517 | - |
| | Cy-FO-154 | *Lonicera* sp. | B. Mora-Sala. 2009 | Spain, Valencia, Torrente | - | - | KX709518 | - |
| | Cy-FO-160 | *Pyracantha* sp. | B. Mora-Sala. 2009 | Spain, Castellon, Segorbe | - | - | KX709519 | - |
| | Cy-FO-195 | *Myrtus communis* | B. Mora-Sala. 2009 | Spain,Valencia, Chiva | - | - | KX709520 | - |
| | Cy-FO-223 | *P. halepensis* | B. Mora-Sala. 2010 | Spain, La Rioja, Projano | - | - | KX709521 | - |
| *D. novozelandica* | CBS 112608; STE-U 3987; C 62 | *V. vinifera* | F. Halleen | South Africa, Western Cape, Citrusdal | AY677288 | AY677235 | JF735632 | JF735821 |
| | **CBS 113552**; STE-U 5713; HJS-1306; NZ C 41 | *Vitis* sp. | R. Bonfiglioli | New Zealand, Candy P New Ground | JF735334 | AY677237 | JF735633 | JF735822 |
| | Cy-FO-5 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Quart de Poblet | - | - | KX709522 | - |
| | Cy-FO-6 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Quart de Poblet | - | - | KX709523 | - |
| | Cy-FO-11 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Alicante, Alcoi | - | - | KX709524 | - |
| | Cy-FO-14 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Quart de Poblet | - | - | KX709525 | - |
| | Cy-FO-15 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Quart de Poblet | - | - | KX709526 | - |
| | Cy-FO-16 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Quart de Poblet | - | - | KX709527 | - |
| | Cy-FO-21 | *J. phoenicea* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709528 | - |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *D. novozelandica* | Cy-FO-22 | *J. phoenicea* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709529 | - |
| | Cy-FO-27 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709530 | - |
| | Cy-FO-33 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709531 | - |
| | Cy-FO-35 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709532 | - |
| | Cy-FO-36 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709533 | - |
| | Cy-FO-40 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709534 | - |
| | Cy-FO-41 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709535 KX709536 | - |
| | Cy-FO-44 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709537 | - |
| | Cy-FO-46 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709538 | - |
| | Cy-FO-47 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709539 | - |
| | Cy-FO-56 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709540 | - |
| | Cy-FO-59 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709541 | - |
| | Cy-FO-60 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709542 | - |
| | Cy-FO-66 | *Quercus* sp. | P. Abad-Campos. 2011 | Spain, Castellón, Pobla de Benifassà | - | - | KX709543 | - |
| | Cy-FO-137 | *Q. suber* | B. Mora-Sala. 2009 | Spain, Valencia, El Puig | - | - | KX709544 | - |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *D. novozelandica* | Cy-FO-146 | *Santolina chamaecyparissus* | B. Mora-Sala. 2009 | Spain, Valencia, Cheste | - | - | KX709545 | - |
| | Cy-FO-153 | *R. officinalis* | B. Mora-Sala. 2009 | Spain, Valencia, Rugat | - | - | KX709546 | - |
| | Cy-FO-180 | *R. officinalis* | B. Mora-Sala. 2009 | Spain, Valencia, Picasent | - | - | KX709547 | - |
| | Cy-FO-188 | *Crataegus azarolus* | B. Mora-Sala. 2009 | Spain,Valencia, Chiva | - | - | KX709536 | - |
| | Cy-FO-191 | *Pinus* sp. | B. Mora-Sala. 2009 | Spain, Castellon, Segorbe | - | - | KX709548 | - |
| | Cy-FO-210 | *Pistacia lentiscus* | B. Mora-Sala. 2009 | Spain,Valencia, Chiva | - | - | KX709549 | - |
| | Cy-FO-211 | *Pi. lentiscus* | B. Mora-Sala. 2009 | Spain,Valencia, Chiva | - | - | KX709550 | - |
| | Cy-FO-222 | *P. halepensis* | B. Mora-Sala. 2010 | Spain, La Rioja, Projano | - | - | KX709551 | - |
| *D. pauciseptata* | CBS 100819; LYN 16202/2 | *Erica melanthera* | H.M. Dance, 1998 | New Zealand, Tauranga | EF607090 | EF607067 | JF735582 | JF735771 |
| | **CBS 120171**; KIS 10467 | *Vitis* sp. | M. Žerjav, 2005 | Slovenia, Krŝko | EF607089 | EF607066 | JF735587 | JF735776 |
| | Cy-FO-37 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709552 | - |
| | Cy-FO-38 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709553 | - |
| | Cy-FO-178 | *Abies nordmanniana* | B. Mora-Sala. 2009 | Spain, León | - | - | KX709554 | - |
| *D. pinicola* | **CBS 173.37**; IMI 090176 | *P. laricio* | T. R. Peace | UK, England, Devon, Haldon | JF735319 | JF735447 | JF735614 | JF735803 |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *D. pinicola* | CBS 159.34; IMI 113891; MUCL 4084; VKM F-2656 | - | H.W. Wollenweber, 1934 | Germany | JF735318 | JF735446 | JF735613 | JF735802 |
| | Cy-FO-177 | *A. concolor* | B. Mora-Sala. 2009 | Spain, León | - | - | KX709555 | - |
| *D. torresensis* | **CBS 129086**; Cy218 | *V. vinifera* | A. Cabral | Portugal, Torres Vedras | JF735362 | JF735492 | JF735681 | JF735870 |
| | CBS 119.41 | *Fragaria* sp. | H.C. Koning | Netherlands, Baarn | JF735349 | JF735478 | JF735657 | JF735846 |
| | Cy-FO-2 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Alicante, Alcoi | - | - | KX709556 | - |
| | Cy-FO-12 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Alicante, Alcoi | - | - | KX709557 | - |
| | Cy-FO-28 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709558 | - |
| | Cy-FO-43 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709559 | - |
| | Cy-FO-54 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709560 | - |
| | Cy-FO-64 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709561 | - |
| | Cy-FO-65 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709562 | - |
| | Cy-FO-127 | *Arbutus unedo* | B. Mora-Sala. 2009 | Spain,Valencia, Chiva | - | - | KX709563 | - |
| | Cy-FO-205 | *Cistus albidus* | B. Mora-Sala. 2009 | Spain, Castellón, Pobla de Benifassà | - | - | KX709564 | - |
| | Cy-FO-206 | *C. albidus* | B. Mora-Sala. 2009 | Spain, Castellón, Pobla de Benifassà | - | - | KX709565 | - |
| | Cy-FO-218 | *Juglans regia* | B. Mora-Sala. 2010 | Spain, Soria, Rejas de S. Este | - | - | KX709566 | - |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *D. torresensis* | Cy-FO-227 | *R. officinalis* | B. Mora-Sala. 2009 | Spain, Valencia, Llaurí | - | - | KX709567 | - |
| *D. vitis* | **CBS 129082**; Cy233 | *V. vinifera* | C. Rego, 2008 | Portugal, Vidigueira | JF735303 | JF735431 | JF735580 | JF735769 |
| *D. valentina* | **CBS 142826;** Cy-FO-133 | *Ilex aquifolium* | B. Mora-Sala. 2009 | Spain, Valencia, El Puig | KY676881 | KY676875 | KY676863 | KY676869 |
| *Ilyonectria capensis* | **CBS 132815**; CPC 20695 | *Protea* sp. | C. M. Bezuidenhout | South Africa, Western Cape, Stanford | JX231151 | JX231103 | JX231135 | JX231119 |
| | CBS 132816; CPC 20700 | *Protea* sp. | C. M. Bezuidenhout | South Africa, Western Cape, Stanford | JX231160 | JX231112 | JX231144 | JX231128 |
| | Cy-FO-63 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709568 | - |
| | Cy-FO-129 | *Ar. unedo* | B. Mora-Sala. 2009 | Spain, Valencia, El Puig | - | - | KX709569 | - |
| | Cy-FO-184 | *Juniperus* sp. | B. Mora-Sala. 2009 | Spain, La Rioja, Logroño | - | - | KX709570 | - |
| *I. coprosmae* | CBS 119606; GJS 85-39 | *Metrosideros* sp. | G. J. Samuels | Canada, Ontario | JF735260 | JF735373 | JF735505 | JF735694 |
| *I. crassa* | CBS 129083; NSAC-SH-1 | *Panax quinquefolium* | S. Hong, 1998 | Canada, Nova Scotia | AY295311 | JF735395 | JF735536 | JF735725 |
| | CBS 158.31; IMI 061536; NRRL 6149 | *Narcissus* sp. | W. F. van Hell | The Netherlands | JF735276 | JF735394 | JF735535 | JF735724 |
| *I. cyclaminicola* | **CBS 302.93** | *Cyclamen* sp. | M. Hooftman | The Netherlands, Roelofarendsveen | JF735304 | JF735432 | JF735581 | JF735770 |
| | Cy-FO-67 | *Quercus* sp. | P. Abad-Campos. 2011 | Spain, Castellón, Pobla de Benifassà | - | - | KX709571 | - |
| *I. destructans* | **CBS 264.65** | *Cyclamen persicum* | L. Nilsson | Sweden, Skåne, Bjärred | AY677273 | AY677256 | JF735506 | JF735695 |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *I. europaea* | CBS 102892 | *Phragmites australis* | W. Leibinger | Germany, Lake Constance | JF735295 | JF735422 | JF735569 | JF735758 |
| | **CBS 129078**; Cy241 | *V. vinifera* | C. Rego | Portugal, Vidigueira | JF735294 | JF735421 | JF735567 | JF735756 |
| *I. gamsii* | **CBS 940.97** | Soil | J. T. Poll | The Netherlands, Lelystad | AM419065 | AM419089 | JF735577 | JF735766 |
| *I. ilicicola* | Cy-FO-224 | *Ilex* sp. | B. Mora-Sala. 2012 | Spain,Tarragona | KY676883 | KY676877 | KY676865 | KY676871 |
| | **CBS 142828**; Cy-FO-225 | *Ilex* sp. | B. Mora-Sala. 2012 | Spain,Tarragona | KY676884 | KY676878 | KY676866 | KY676872 |
| | Cy-FO-226 | *Ilex* sp. | B. Mora-Sala. 2012 | Spain,Tarragona | KY676885 | KY676879 | KY676867 | KY676873 |
| *I. leucospermi* | **CBS 132809**; CPC 20701 | *Leucospermum* sp. | C. M. Bezuidenhout | South Africa, Western Cape, Stanford | JX231161 | JX231113 | JX231145 | JX231129 |
| | CBS 132810; CPC 20703 | *Protea* sp. | C. M. Bezuidenhout | South Africa, Western Cape, Stanford | JX231162 | JX231114 | JX231146 | JX231130 |
| *I. liliigena* | **CBS 189.49**; IMI 113882 | *Lilium regale* | M.A.A. Schippers | The Netherlands, Hoorn | JF735297 | JF735425 | JF735573 | JF735762 |
| | CBS 732.74 | *Lilium* sp. | G. J. Bollen | The Netherlands, Heemskerk | JF735298 | JF735426 | JF735574 | JF735763 |
| *I. liriodendri* | **CBS 110.81**; IMI 303645 | *Liriodendron tulipifera* | J.D. MacDonald & E.E. | USA, California | DQ178163 | DQ178170 | JF735507 | JF735696 |
| | CBS 117527; Cy76 | *V. vinifera* | C. Rego, 1999 | Portugal, Ribatejo e Oeste | DQ178165 | DQ178172 | JF735509 | JF735698 |
| | Cy-FO-50 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709572 | - |
| | Cy-FO-52 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709573 | - |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *I. liriodendri* | Cy-FO-57 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709574 | - |
| | Cy-FO-68 | *Quercus* sp. | P. Abad-Campos. 2011 | Spain, Castellón, Pobla de Benifassà | - | - | KX709575 | - |
| | Cy-FO-132 | *Ar. unedo* | B. Mora-Sala. 2009 | Spain, Valencia, El Puig | - | - | KX709576 | - |
| | Cy-FO-136 | *Q.suber* | B. Mora-Sala. 2009 | Spain, Valencia, El Puig | - | - | KX709577 | - |
| | Cy-FO-142 | *Ar. unedo* | B. Mora-Sala. 2009 | Spain, Valencia, Cheste | - | - | KX709578 | - |
| | Cy-FO-183 | *Juniperus* sp. | B. Mora-Sala. 2009 | Spain, La Rioja, Logroño | - | - | KX709579 | - |
| | Cy-FO-221 | *P. halepensis* | B. Mora-Sala. 2010 | Spain, La Rioja, Projano | - | - | KX709580 | - |
| *I. lusitanica* | **CBS 129080**; Cy197 | *V. vinifera* | N. Cruz | Portugal, Melgaço | JF735296 | JF735423 | JF735570 | JF735759 |
| *I. mors-panacis* | CBS 124662; NBRC 31881; SUF 811 | *Pa. ginseng* | Y. Myazawa | Japan, Nagano, Kitasaku-gun | JF735290 | JF735416 | JF735559 | JF735748 |
| | **CBS 306.35** | *Pa. quinquefolium* | A. A. Hildebrand | Canada, Ontario | JF735288 | JF735414 | JF735557 | JF735746 |
| *I. palmarum* | CBS 135753; CPC 22088; DiGeSA-HF7 | *Howea forsteriana* | G. Polizzi | Italy, Sicily, Catania province, Aci Castello | HF937432 | HF922609 | HF922621 | HF922615 |
| | **CBS 135754**; CPC 22087; DiGeSA-HF3 | *H. forsteriana* | G. Polizzi | Italy, Sicily, Catania province, Aci Castello | HF937431 | HF922608 | HF922620 | HF922614 |
| *I. panacis* | **CBS 129079**; CDC-N-9a | *Pa. quinquefolium* | K. F. Chang | Canada, Alberta | AY295316 | JF735424 | JF735572 | JF735761 |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *I. protearum* | **CBS 132811**; CPC 20707 | *Protea* sp. | C. M. Bezuidenhout | South Africa, Western Cape, Stanford | JX231157 | JX231109 | JX231141 | JX231125 |
| | CBS 132812; CPC 20711 | *Protea* sp. | C. M. Bezuidenhout | South Africa, Western Cape, Stanford | JX231165 | JX231117 | JX231149 | JX231133 |
| *I. pseudodestructans* | CBS 117824 | *Quercus* sp. | E. Halmschlager | Austria, Patzmannsdorf | JF735292 | JF735419 | JF735562 | JF735751 |
| | **CBS 129081**; Cy20 | *V.vinifera* | C. Rego | Portugal, Gouveia,São Paio | AJ875330 | AM419091 | JF735563 | JF735752 |
| | Cy-FO-71 | *Q. ilex* | B. Mora-Sala. 2015 | Spain, Valencia, Ayora | - | - | KX709581 | - |
| *I. robusta* | CBS 129084; Cy192 | *V. vinifera* | N. Cruz, 2005 | Portugal, Monção | JF735273 | JF735391 | JF735532 | JF735721 |
| | **CBS 308.35** | *Pa. quinquefolium* | A. A. Hildebrand | Canada, Ontario | JF735264 | JF735377 | JF735518 | JF735707 |
| | Cy-FO-217 | *Ju. regia* | B. Mora-Sala. 2010 | Spain, Soria, Rejas de S. Este | - | - | KX709582 | - |
| | Cy-FO-219 | *Ju. regia* | B. Mora-Sala. 2010 | Spain, Soria, Rejas de S. Este | - | - | KX709583 | - |
| *I. rufa* | **CBS 153.37** | Dune sand | F. Moreau | France | AY677271 | AY677251 | JF735540 | JF735729 |
| | CBS 640.77 | *A. alba* | F. Gourbière, 1977 | France, Villeurbanne | JF735277 | JF735399 | JF735542 | JF735731 |
| | Cy-FO-4 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Alicante, Alcoi | - | - | KX709584 | - |
| | Cy-FO-7 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Quart de Poblet | - | - | KX709585 | - |
| | Cy-FO-8 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Alicante, Alcoi | - | - | KX709586 | - |
| | Cy-FO-17 | *Q. faginea* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709587 | - |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *I. rufa* | Cy-FO-53 | *P. halepensis* | P. Abad-Campos. 2011 | Spain, Valencia, Ayora | - | - | KX709588 | - |
| | Cy-FO-128 | *Ar. unedo* | B. Mora-Sala. 2009 | Spain, Valencia, El Puig | - | - | KX709589 | - |
| | Cy-FO-130 | *Ar. unedo* | B. Mora-Sala. 2009 | Spain, Valencia, El Puig | - | - | KX709590 | - |
| | Cy-FO-161 | *Juniperus* sp. | B. Mora-Sala. 2009 | Spain, Castellon, Segorbe | - | - | KX709591 | - |
| | Cy-FO-179 | *A. nordmanniana* | B. Mora-Sala. 2009 | Spain, León | - | - | KX709592 | - |
| *I. venezuelensis* | **CBS 102032**; ATCC 208837; AR2553 | Bark | A. Rossman | Venezuela, Amazonas, Cerro de la Neblina | AM419059 | AY677255 | JF735571 | JF735760 |
| *I. vredehoekensis* | **CBS 132807**; CPC 20699 | *Protea* sp. | C. M. Bezuidenhout | South Africa, Western Cape, Stanford | JX231155 | JX231107 | JX231139 | JX231123 |
| | CBS 132814; CPC 20690[b] | *Protea* sp. | C.M. Bezuidenhout | South Africa | JX231158 | JX231110 | JX231142 | JX231126 |
| *Neonectria candida* | CBS 182.36; IMI 113893; UPSC 1903 | *Malus sylvestris* | H.W. Wollenweber | - | JF735314 | JF735439 | JF735603 | JF735792 |
| *N. candida*, authentic strain of *C. obtusiusculum* (=*C. magnusianum*) | **CBS 151.29**; IMI 113894; MUCL 28083; MUCL 28094 | *Ma. sylvestris* | H.W. Wollenweber | UK, England, Cambridge | JF735313 | JF735438 | JF735602 | JF735791 |
| *N. ditissima*, authentic strain of *C. willkommii* | **CBS 226.31**; IMI 113922 | *Fagus sylvatica* | H.W. Wollenweber | Germany, Tharandt | JF735309 | DQ789869 | JF735594 | JF735783 |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *N. ditissima*, representative strain of *N. galligena* | CBS 835.97 | *Salix cinerea* | W. Gams, 1997 | Belgium, Marais de Sampant | JF735310 | DQ789880 | JF735595 | JF735784 |
| *N. major*, type strain | **CBS 240.29**; IMI 113909 | *Alnus incana* | H.W. Wollenweber | Norway | JF735308 | DQ789872 | JF735593 | JF735782 |
| *N. neomacrospora* representative strain | **CBS 324.61**; DSM 62489; IMB 9628 | *A. concolor* | J.A. von Arx | Netherlands, Zwolle | JF735312 | DQ789875 | JF735599 | JF735788 |
| | CBS 503.67 | *A. alba*, wood | F. Roll-Hansen | Norway, Hordaland, Fana | AY677261 | JF735436 | JF735600 | JF735789 |
| | CBS 118984; GJS 03-28 | *Arceuthobium tsugense* | L. Reitman, 2005 | Canada, British Columbia, Vancouver Island, Spider Lake | JF735311 | DQ789882 | JF735598 | JF735787 |
| *N. obtusispora* | CBS 183.36; IMI 113895 | *Solanum tuberosum* | H.W. Wollenweber, 1936 | Germany | AM419061 | AM419085 | JF735607 | JF735796 |
| | CPC 13544; DAOM 182772; JAT 1366 | *Prunus armenica* | J.A. Traquair, 1982 | Canada, Ontario, Ruthven | AY295306 | JF735443 | JF735608 | JF735797 |
| *N.quercicola* | **CBS 143704**; Cy-FO-3 | *Q. ilex* | P. Abad-Campos. 2011 | Spain, Alicante, Alcoi | KY676880 | KY676874 | KY676862 | KY676868 |
| | CPC 13530; DAOM 185722; JAT 1591 | *Pyrus* sp. | J.A. Traquair, 1983 | Canada, Ontario, Harrow | AY295302 | JF735441 | JF735605 | JF735794 |

| Species | Strain number | Host | Collected/isolated by/year | Location | GenBank Accession Numbers. | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | *tub2* | *his3* | *tef1* |
| *N.quercicola* | CPC 13531; CCFC 226722; DAOM 226722; CR6 | *Pseudotsuga menziesii* | P. Axelrood | Canada, British Columbia | AY295301 | JF735442 | JF735606 | JF735795 |
| | CR21 | *Ps. menziesii* | P. Axelrood | Canada, British Columbia | JF735315 | JF735440 | JF735604 | JF735793 |
| *Neonectria* sp.1 | CPC 13545; DAOM 185212; # 5 | *Pyrus* sp. | J.A. Traquair & B. Harrison, 1982 | Canada, Ontario, Harrow | AY295303 | JF735437 | JF735601 | JF735790 |

**AR**: Amy Y. Rossman personal collection;**ATCC**: American Type Culture Collection, USA; **CBS**: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; **CCFC**: Canadian Collection of Fungal Cultures, Agriculture and Agri-Food Canada, Ottawa, Canada; **CDC**: Centers for Disease Control and Prevention, Atlanta, GA, USA; **CPC**: Culture collection of Pedro Crous, housed at CBS; **Cy**: Cylindrocarpon collection housed at Laboratório de Patologia Vegetal "Veríssimo de Almeida" - ISA, Lisbon, Portugal; **DAOMC**: Canadian Collection of Fungal Cultures, Canada; **DiGeSA**: Dipartimento di Gestione dei Sistemi Agroalimentari e Ambientali, Catania, Italy;**DSM**: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; **GJS**: Gary J. Samuels collection; **HJS**: Hans-Josef Schroers collection; **HMAS**: Mycological Herbarium, Institute of Microbiology, Chinese Academy of Sciences;**IAFM**: Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Spain; **ICMP**: International Collection of Microorganisms from Plants, Auckland, New Zealand; **IMI**: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.; **JAT**: J. A. Traquair collection; **KIS**: Agricultural Institute of Slovenia, Ljubljana, Slovenia; **LYN:** Lynchburg College, Biology Department, USA; **MUCL**: Mycothèque de l'Université Catholique de Louvain, Belgium; **NBRC**: NITE Biological Resource Center, Japan; **NRRL**: Agricultural Research Service Culture Collection, USA; **NZ**: Collection of L. Castlebury; **PD**: Collection of the Dutch National Plant Protection Organization (NPPO-NL), Wageningen, The Netherlands;**STE-U**: Stellenbosch University, South Africa; **TRTC**: Royal Ontario Museum Fungarium, Toronto, Ontario, Canada;**UPSC**: Fungal Culture Collection at the Botanical Museum, Uppsala University, Uppsala, Sweden; **VKM**: All-Russian Collection of Microorganisms, Russia.

Ex-type culture indicated in bold.

**Morphological characterization**

Single conidial cultures were grown for up to 5 weeks at 20ºC on synthetic nutrient-poor agar (SNA; Nirenberg 1976) with or without the addition of two 1cm$^2$ pieces of sterile filter paper on the medium surface, PDA, and oatmeal agar (OA; Crous *et al.* 2009) under continuous near-UV fluorescent light (NUV; 400-315 nm; Philips TL 8W BLB, The Netherlands). To induce perithecia of new species, homothallic and heterothallic crosses (for the cases that there is more than one isolate in the species) were performed as described by Cabral *et al.* (2012a).

Fungal structures were measured at a 1,000× magnification using a Leica DM2500 and images were captured using a Leica DFC295 digital camera with the Leica Application Suite (LAS) version 3.3.0. For this purpose, an agar square was removed and placed on a microscope slide, to which a drop of water was added and overlaid with a cover slip. For each isolate, 30 measurements were obtained for each informative structure. Measurements were obtained with LAS software and round to the nearest 0.5 µm. The 95% confidence intervals were determined and the extremes of the conidial measurements are shown in parenthesis. For the other structures, only the extremes are presented.

Culture characteristics (texture, density, color, growth front, transparency and zonation) were described on PDA and OA after incubation at 20ºC in the dark for 14 days. Color (surface and reverse) was described using the color charts of Rayner (1970).

Cardinal growth temperatures were assessed by inoculating 90 mm diameter PDA dishes with a 6 mm diameter plug cut from the edge of an actively growing colony. Growth was determined after 7 days in two orthogonal directions. Trials were conducted at 5 to 35ºC with 5ºC intervals, with three replicates per strain at each temperature.

**DNA isolation, sequencing and phylogenetic analysis**

For DNA extraction, fungal mycelium, from pure cultures grown on PDA for 2 to 3 weeks at 25ºC in darkness, were scraped and grinded to a fine powder with liquid nitrogen using a mortar and pestle. Total genomic DNA was

extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Doraville, USA) following manufacturer's instructions. DNA was visualized by electrophoresis on 1% agarose gels stained with REALSAFE (REALSAFE Nucleic Acid Staining Solution 20,000x, Durviz S. L., Valencia, Spain) and stored at –20ºC.

In order to identify the species involved, partial sequences of the histone H3 (*his3*) gene region was amplified according to Cabral *et al.* (2012a). Six isolates (Cy-FO-3, Cy-FO -45, Cy-FO-133, Cy-FO-224, Cy-FO-225 and Cy-FO-226) were additionally sequenced for the internal transcribed spacer and intervening 5.8S gene (ITS) region, partial regions of the β-tubulin (*tub2*) and translation elongation factor 1-α (*tef1*) genes to better resolve their phylogenetic position. PCR amplifications were carried out using 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4mM of each primer, 1 U of Taq polymerase (Canvax Biotech, S.L., Córdoba, Spain), and 1 µL of template DNA (20 ng/µL). The PCR reaction mix was adjusted to a final volume of 25 µL with ultrapure sterile water (Chromasolv Plus®, Sigma-Aldrich, Steinheim, Germany). The cycle conditions in a Peltier Thermal Cycler-200 (MJ Research) were: 94ºC for 3 min, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, elongation at 72ºC for 45 s, and a final extension at 72ºC for 10 min. Primers used were CYLH3F and CYLH3R (Crous *et al.* 2004b) for *his3*, ITS1F (Gardes and Bruns 1993) and ITS4 (White *et al.*1990) for ITS, T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) for *tub2*, and CylEF-1 (5'- ATG GGT AAG GAV GAV AAG AC-3'; J.Z. Groenewald, unpublished) and CylEF-R2 (Crous *et al.* 2004b) for *tef1*. After confirmation by agarose gel electrophoresis, PCR products were sequenced in both directions by Macrogen Inc., Sequencing Center (The Netherlands, Europe). Sequences were assembled and edited to resolve ambiguities and consensus sequences for all isolates were compiled into a single file (Fasta format) using Sequencher software v. 5.3 (Gene Codes Corporation, Ann Arbor, MI, USA).

Phylogenetic analysis was first conducted on the *his3* single-locus alignment for all isolates obtained in this study, as this locus were reported to be a very informative locus (Cabral *et al.* 2012a). For the cases, that was not possible to infer species level for a specific isolate with only the *his3* single-locus phylogeny a combined alignment of the four loci (*his3*, ITS, *tub2* and *tef1*) was

also analyzed. GenBank sequences (Table 4.1) from different species of *Cylindrodendrum*, *Dactylonectria*, *Ilyonectria* and *Neonectria* were selected based on their high similarity with our query sequences using MegaBLAST. These were added to the sequences obtained and aligned using MAFFT version 7.305 implemented on CIPRES Science Gateway V 3.3 (Miller *et al.* 2010) and edited manually, if necessary, using MEGA 7.0.26 (Kumar *et al.* 2016). The alignments for each locus were combined in a single file using the program SequenceMatrix 1.8 (Vaidya *et al.* 2011).The best nucleotide substitution model settings for each locus were determined by jModelTest 2.1.10 (Darriba *et al.* 2012), with the following likelihood settings: number of substitution schemes = 3 (24 models), base frequencies (+F), proportion of invariable sites (+I) and rate variation among sites (+G) (nCat = 4), using the Akaike information criterion (AIC). The Bayesian analyses of the combined four-loci dataset and individual locus data were performed with MrBayes v. 3.2.1 (Ronquist *et al.* 2012) based on the results of the jModelTest. The Markov Chain Monte Carlo sampling (MCMC) analysis of four chains started in parallel from a random tree topology. The number of generations was set at 10 M and the run was stopped automatically when the average standard deviation of split frequencies fell below 0.01. Trees were saved each 1,000 generations. Burn-in was set at 25% after which the likelihood values were stationary and the remaining trees were used to calculate posterior probabilities. Trees from different runs were then combined and summarized in a majority rule 50% consensus tree. Maximum likelihood (ML) was implemented in the CIPRES Science Gateway V 3.3 (Miller *et al.* 2010) using RAxML-HPC v.8 on XSEDE (8.2.9) using the GTRCAT model and 1,000 rapid bootstrap inferences were done.

Both analyses were performed, rooting the trees to *Campylocarpon* (*Ca.*) *fasciculare* (CBS 112613) and *Ca. pseudofasciculare* (CBS 112679) and tree topologies were compared on http://phylo.io (Robinson *et al.* 2016).

Sequences derived in this study were lodged in GenBank, the alignments and phylogenetic trees in TreeBASE under study number S22022 (http://purl.org/phylo/treebase/phylows/study/TB2:S22022), and taxonomic novelties in MycoBank (www.MycoBank.org) (Crous *et al.* 2004a). GenBank accession numbers of the isolates collected during this study are listed in Table 4.1.

## Results

### Phylogenetic analysis

One hundred and three isolates were amplified with the primers CYLH3F and CYLH3R and approximately 500 bp were obtained for all. The *his3*single-locus alignment contains 427 aligned characters (including gaps), from which 195 characters were parsimony-informative, 17 were variable but parsimony-uninformative and 212 were constant. The AIC best-fit nucleotide substitution model identify by jModelTest was general time reversible model with inverse gamma rates (GTR+I+G). The Bayesian consensus tree and Maximum Likelihood tree had similar topology, and therefore only the Bayesian consensus tree is presented with bootstrap support values (BS) and posterior probability values (PP). The phylogenetic analysis contained a total of 174 ingroup taxa and two outgroup taxa [*Ca. fasciculare*(CBS112613),and *Ca. pseudofasciculare* (CBS112679)].

The phylogeny obtained with *his3*alignmentresulted in four major clades: the first major clade, comprised the isolates from the genus *Ilyonectria*; the second, isolates from the genus *Neonectria*; the third, isolates from the genus *Cylindrodendrum* and the fourth, isolates from the genus *Dactylonectria* (Fig. 4.2). About 71% of the isolates obtained in this study belonged to the genus *Dactyonectria* and included: *D. hispanica* (0.97%), *D. macrodidyma* (24.27%), *D .novozelandica* (29.13%), *D. pauciseptata* (2.91%), *D. pinicola* (0.97%), *D. torresensis* (11.65%) and *D. valentina* (0.97%). The genus *Ilyonectria* included 27.18% of the isolates: *I. capensis* (2.91%), *I. cyclaminicola* (0.97%), *I. ilicicola* (2.91%), *I. liriodendri* (8.74%), *I. pseudodestructans* (0.97%), *I robusta* (1.94%) and *I. rufa* (8.74%). The genus *Neonectria* contained one species, *Neonectria quercicola* (0.97%). *Cylindrodendrum alicantinum* (*C. alicantinum*) (0.97%) was the only species of the genus *Cylindrodendrum*.

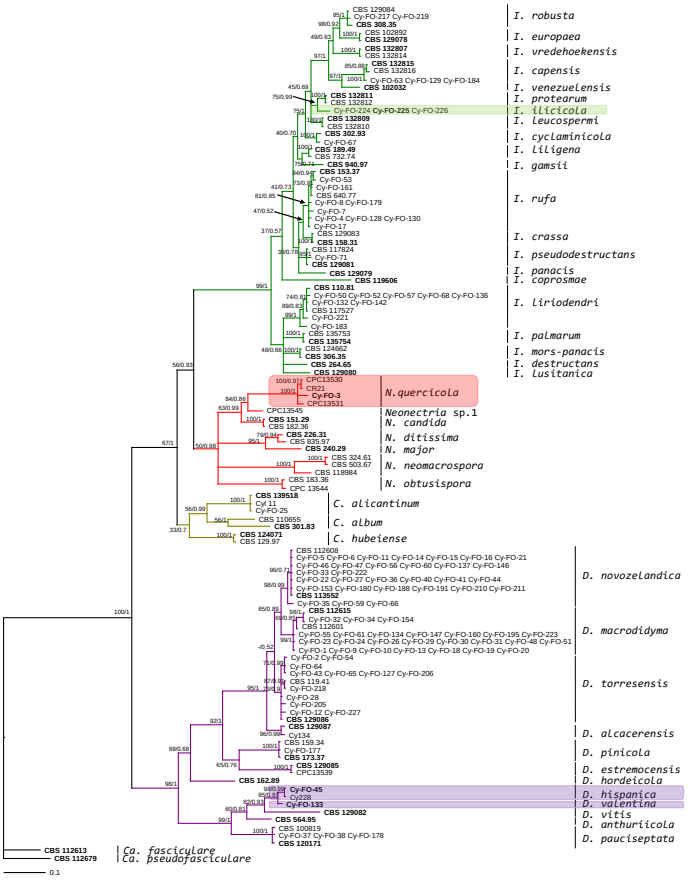


**Figure 4.** ı analysis based on the alignment of partial histone H3 gene sequences from the 103 Cylindrocarpon-like asexual morphs obtained from forest nurseries, and additional sequences of *Cylindrodendrum*, *Dactylonectria*, *Ilyonectria* and *Neonectria* species. The RAxML bootstrap support and Bayesian posterior probability values are indicated at the nodes (ML/PP). The tree was rooted to *Campylocarpon fasciculare* (CBS 112613) and *C. pseudofasciculare* (CBS 112679). The scale bar indicates 0.1 expected changes per site. Ex-type cultures are indicated in **bold**. Colors are used to indicate clades from the same genera *Ca.* – *Campylocarpon*, *C.* – *Cylindrodendrum*, *D.* – *Dactylonectria*, *I.* – *Ilyonectria*, *N.* – *Neonectria*. Tentative new species are indicated in colored boxes.

Six isolates (Cy-FO-3, Cy-FO-45, Cy-FO-133, Cy-FO-224, Cy-FO-225 and Cy-FO-226) could not be identified to the species level employing the *his3* sequences (Fig. 4.2). Therefore the ITS, *tub2*and *tef1*regions were analyzed additionally, and these sequences were concatenated with those obtained from the *his3*region for their identification (Fig. 4.3). The four loci alignment contained 79 taxa (including the two outgroups) and 1,935 aligned characters (including gaps), from which 718 characters were parsimony-informative, 83 were variable but parsimony-uninformative and 1,101 were constant. The AIC best-fit nucleotide substitution model identify by jModelTest was GTR+I+G model for ITS and *his3*and GTR+G for *tub2*and *tef1*.

The Bayesian and ML consensus trees obtained with the four-loci alignment confirmed the existence of four novel taxa within our set of isolates.

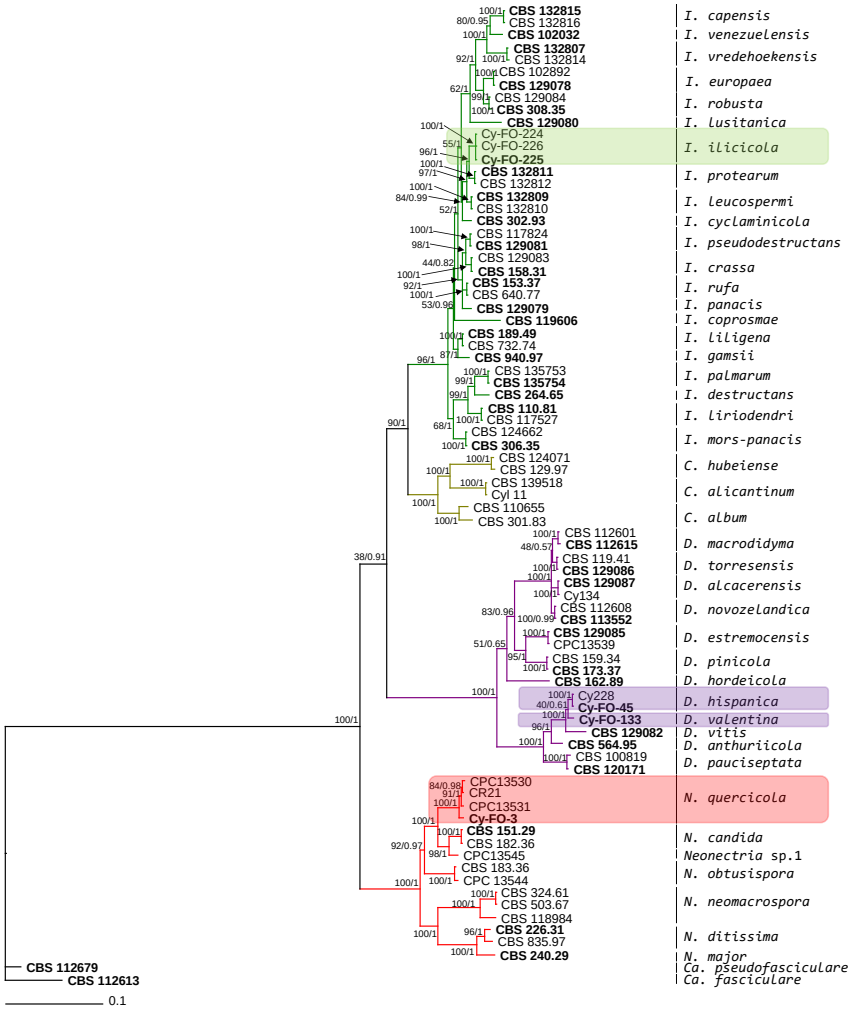


**Figure 4.3.** Fifty percent majority rule consensus tree from a Bayesian analysis based on the combined four gene dataset (ITS, *tub2*, *his3* and *tef1*). The RAxML bootstrap support and Bayesian posterior probability values are indicated at the nodes (ML/PP). The tree was rooted to *Campylocarpon fasciculare* (CBS 112613) and *C. pseudofasciculare* (CBS 112679). The scale bar indicates 0.1 expected changes per site. Ex-type cultures are indicated in **bold**. Colors are used to indicate clades from the same genera *Ca. –Campylocarpon*, *C. – Cylindrodendrum*, *D.– Dactylonectria*, *I. – lyonectria*, *N. –Neonectria*. New species are indicated in colored boxes.

## Taxonomy

Based on the phylogenetic analysis and morphological characters, two new species of *Dactylonectria*, one species of *Ilyonectria* and one species of *Neonectria* are described (Fig. 4.2, Fig. 4.3 and Fig. 4.4). No perithecia were observed in the homothallic or heterothallic crosses performed.

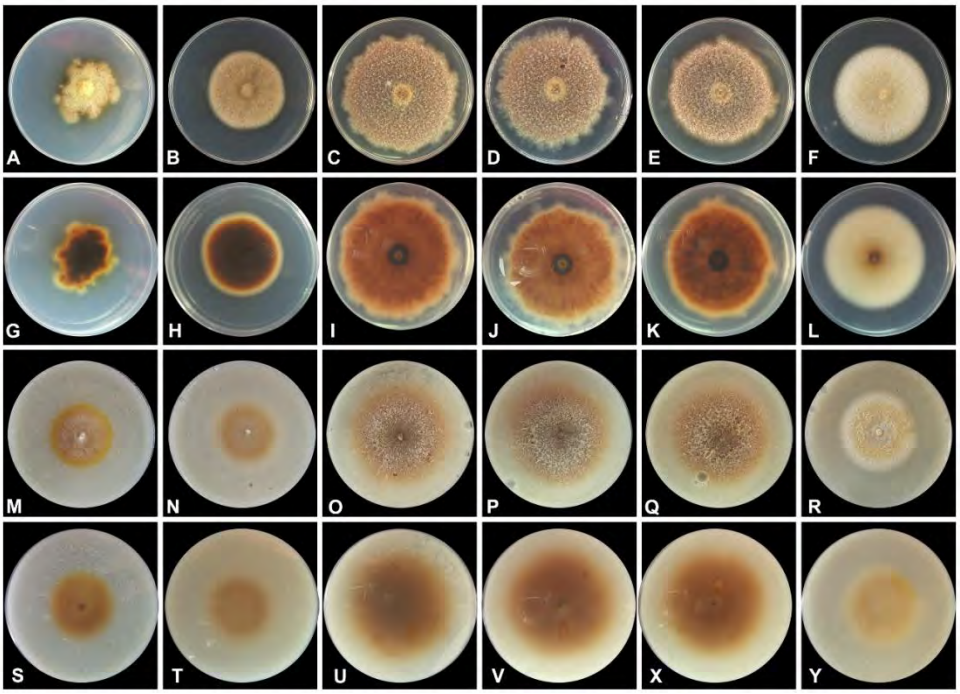


**Figure 4.4.**Ten-day-old colonies grown at 20ºC in darkness on PDA (A-F upper face; G-L bottom face) and Oat-meal agar (M-R upper face; S-Y bottom face) of: *Dactylonectria hispanica* isolate Cy-FO-45 (A, G, M and S); *D. valentina* isolate Cy-FO-133 (B, H, N and T); *Ilyonectria ilicicola* isolates Cy-FO-224 (C, I, O and U), Cy-FO-225 (D, J, P and V) and Cy-FO-226 (E, K, Q and X) and *Neonectria quercicola* Cy-FO-3 (F, L, R and Y).

***Dactylonectria hispanica*** B. Mora-Sala, A. Cabral, J. Armengol &P. Abad-Campos, **sp. nov**. MycoBank MB822023 (Fig. 4.5).

*Etymology*: Name refers to Spain, where the fungus was isolated.

*Diagnosis*: Morphologically *D. hispanica*, can be distinguished by its slightly larger 3-septate macroconidia when compared to *D. vitis* and *D. valentina*. Fourteen polymorphisms can distinguish *D. hispanica* from *D. valentina:* five in *tub2* locus at position 27 (A:T), 136 (G:A), 206 (A:G), 335 (T:C) and 434(C:A), five in *his3* locusatposition94(C:T), 104 (T:C); 214 (T:C); 291 (T:C) and 395 (C:T); and four in *tef1* locus at position 224(G:A), 266 (A:T), 289 (T:A) and 291 (A:C).

*Typus*: **Spain**: Valencia, Ayora, on *Pinus halepensis* (complete roots), 2011, *B. Mora-Sala* (CBS H-23154 – holotype; CBS 142827 = Cy-FO-45 – ex-type culture).

*Conidiophores* simple. Complex conidiophores not observed. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to aggregated, unbranched or sparsely branched with up to four phialides, 1 to 2-septate, 40 to 75 µm long; phialides monophialidic, cylindrical, tapering towards the apex, 16 to 28 µm long, 2 to 3.0 µm wide at the base, 3 to 4 µm at the widest point, 1.5 to 2.5 µm near the aperture.

*Macroconidia* (1 to) 3-septate, straight or minutely curved, cylindrical with both ends more or less broadly rounded, mostly with a visible centrally located to laterally displaced hilum; 1-septate (26–)31 to 37(–53) × (7.0–)7.5 to 8.5(–9.0) µm (av. 34 × 8 µm) L/W ratio (3–)3.9 to 4.7(–6.5) (av. 4.3), 2-septate (30.5–)37 to 43(–53) × (6–)7.5 to 8.5(–9) µm (av. 40 × 8 µm) L/W ratio (3.5–)4.5 to 5.5(–7) (av. 5.0), and 3-septate macroconidia (39–)45 to 47(–58) × (6.5–)7.8 to 8.2(–9.5) µm (av. 46 × 8 µm), L/W ratio (4.5–)5.5 to 6(–7.5) (av. 5.8). Macroconidia formed in heads or as flat domes of slimy masses.

*Microconidia* rarely formed, aseptate to 1-septate with a minutely or clearly laterally displaced hilum; aseptate microconidia ellipsoidal to fusiform (9.5–)10.5 to 14(–17)×(5.5–)6 to 7(–7.5) (av. 12.2×6.5 µm) L/W ratio (1.5–)1.7 to 2.1(–2.2) (av. 1.8); 1-septate, fusiform to subcylindrical (14–)19 to 21.5(–24.5)×(6–)7 to 7.5(–8.5) (av. 20.2×7.2 µm) L/W ratio (2–)2.6 to 3.0(–3.5) (av. 2.8). *Chlamydospores* observed on SNA; globose to subglobose to ellipsoidal, 7

to 10×6 to 9 µm diameter, smooth but often appearing rough due to deposits, thick-walled, in chains or in clumps, hyaline, becoming slightly brown.

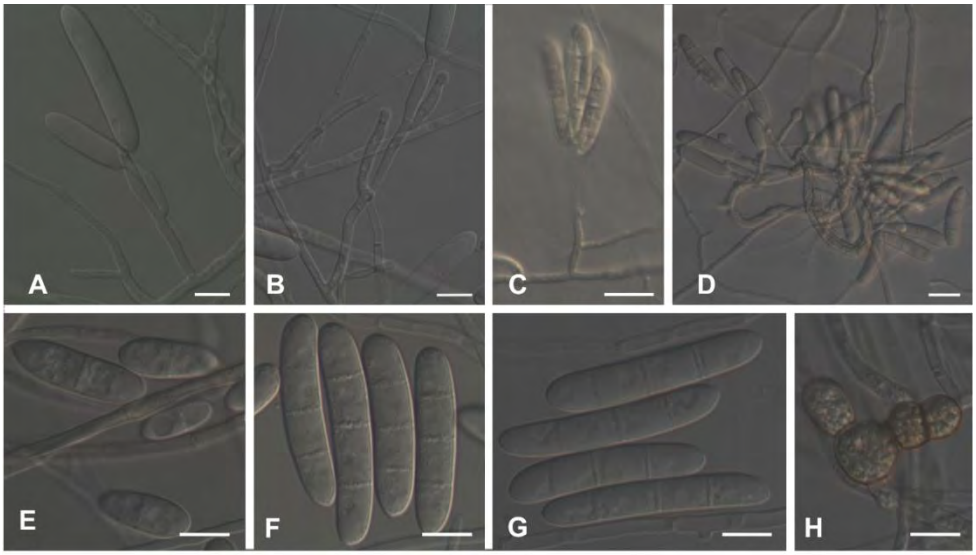


**Figure 4.5.** *Dactylonectria hispanica* (ex-type culture Cy-FO-45*).* **A–D** Simple, sparsely branched conidiophores of the aerial mycelium. **E–G** Micro- and macroconidia. **H** Chlamydospores in mycelium. Scale bars: C, D = 20 µm; A–B, E–H = 10 µm.

*Culture characteristics*: Mycelium felty with low to average density (OA) or average to strong density (PDA). Surface on OA buff to sepia with sparse cinnamon aerial mycelium; margin luteous. Surface on PDA honey to buff; margin buff. Zonation absent, transparency homogeneous and margins even (OA) and uneven (PDA). Reverse similar to surface, except in color, sepia to cinnamon (PDA). Colonies on PDA grow poorly, less than 1 mm diameter at 5°C after 7 days. Optimum temperature at 25°C, when colonies reach 28 mm diameter, after 7 days. Colony diameter was 9 mm at 30°C after 7days. No growth was observed at 35°C.

*Host and distribution*: *Pinus halepensis* (roots) (Spain, Valencia)

*Notes*: *Dactylonectria hispanica* is closely related to *D. valentina* and *D. vitis* based on phylogenetic inference. The morphology of these species is very

similar, but *D. hispanica* can be distinguished by its slightly larger 3-septate macroconidia when compared to *D. vitis* (34.9–)41.6 to 43.5(–51.6) × (6.2–)7.9 to 8.2(–9.5) µm (av. =42.5 × 8.0 µm; Cabral *et al.* 2012a) and *D. valentina* (30–)35.5 to 37(–44) × (6–)7.5 to 8(–9.0) µm (av. 36.3 × 7.6 µm); this study). No complex conidiophores or penicillate conidiophores with aseptate microconidia were observed in *D. hispanica*. The isolate Cy228 is classify as *D. hispanica*, as it form a clade very well supported (BS = 100% and PP =1.0) with the isolate Cy-FO-45.

***Dactylonectria valentina*** B. Mora-Sala, A. Cabral, J. Armengol &P. Abad-Campos, **sp. nov**.MycoBank MB822024(Fig. 4.6).

*Etymology*: Name refers to the province of Valencia, Spain, where the fungus was isolated.

*Diagnosis:* The morphologically *D. valentina* can be distinguished by its slightly smaller 3-septate macroconidia when compare to *D. vitis* and *D. valentina,* and for the absence of luteous margin in OA plates. Fourteen nucleotide differences can distinguish *D. valentina* from *D. hispanica* (as describe in diagnosis of *D. hispanica*).

*Typus*: **Spain**: Valencia, El Puig, 2009, on *Ilex aquifolium* (complete roots), *B. Mora-Sala* (CBS H-23155 – holotype; CBS 142826 = Cy-FO-133 – ex-type culture).

*Conidiophores* simple or complex. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to aggregated, unbranched or sparsely branched with up to four phialides, 1 to 4–septate, 55 to 130 µm long; phialides monophialidic, cylindrical, tapering towards the apex, 15 to 30.5 µm long, 2.1 to 3.2 µm wide at the base, 3.1 to 4.5 µm at the widest point, 1.5 to 3 µm near the aperture. Conidiophores forming aseptate microconidia arising from mycelium on agar surface, 1 to 4–septate, with a terminal arrangement of phialides, ranging from 2 to a dense cluster; sparsely branched or penicillate; monophialides narrowly flask-shaped, typically with widest point near the middle, 9 to 17 µm long, 1.5 to 3.0 µm wide at the base, 2 to 3.5 µm at widest point, 1 to 2 µm near the apex. *Sporodochial conidiophores* irregularly branched; phialides more or less cylindrical but slightly tapering towards the tip, or

narrowly flask-shaped, with widest point near the middle, 14 to 20 µm long, 2.5 to 3.5 µm wide at the base, 3.0 to 4.5 µm at widest point, 1.5 to 2.5 µm near the apex. *Macroconidia* (1 to)3-septate, straight or minutely curved, cylindrical with both ends more or less broadly rounded, mostly with a visible centrally located to laterally displaced hilum; 1-septate (18–)25 to 28(–33.5) × (5.5–)6.5 to 7(–8.5) µm (av. 26.3×6.9 µm) L/W ratio (3–)3.7 to 4.2(–5) (av. 3.9), 2-septate (26–)30 to 32(–35.5) × (6.5–)7.5 to 8(–9) µm (av. 31.1×7.6 µm) L/W ratio (3.0–)3.9 to 4.3(–5) (av. 4.1),and 3-septate macroconidia (30–)35.5 to 37(–44) × (6–)7.5 to 8(–9.0) µm (av. 36.3×7.6 µm), L/W ratio (3.5–)4.5 to 5(–6.5) (av. 4.8). Macroconidia formed in heads or as flat domes of slimy masses. *Microconidia* aseptate to 1-septate with a minutely or clearly laterally displaced hilum; aseptate microconidia subglobose to oval (3.5–)5 to 5.5(–7.5)×(3–)3.9 to 4.1(–4.5) (av. 5.2×4 µm) L/W ratio (1–)1.2 to 1.4(–2) (av. 1.3); 1-septate microconidia, rarely formed, fusiform to subcylindrical (14–)15 to 17(–18.5)×(4.5–)4.7 to 5.5(–6) (av. 16×5µm) L/W ratio (2.5–)2.8 to 3.3(–3.5) (av. 3.0).

*Chlamydospores* observed in the bottom of SNA plate; globose to subglobose to ellipsoidal, 9 to 19×7 to 12 µm diameter, smooth but often appearing rough due to deposits, thick-walled, mainly in chains or in clumps, hyaline, becoming slightly brown in the outer wall.

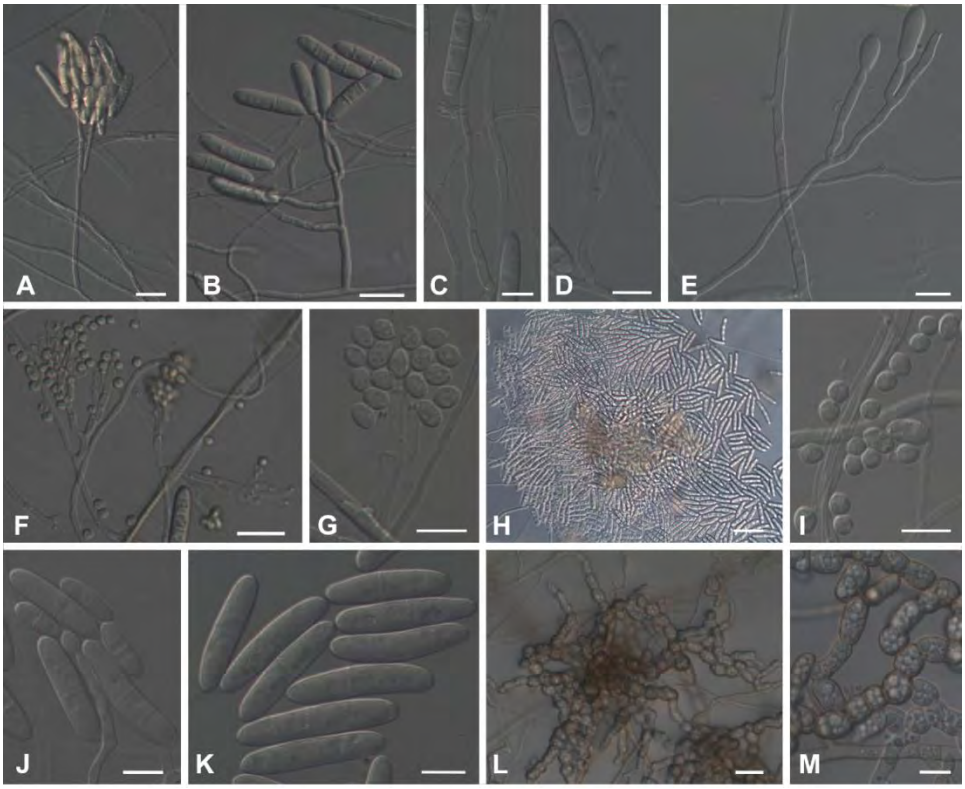


**Figure 4.6.** *Dactylonectria valentina.* **A–E** Simple, sparsely branched conidiophores of the aerial mycelium. **E–G** Conidiophores forming microconidia arising from mycelium at agar surface, with a terminal arrangement of phialides, ranging from 2 to a dense cluster; sparsely branched or penicillate. **H** Sporodochial conidiophores. **I–K** Micro- and macroconidia. **L–M** Chlamydospores in mycelium. Scale bars: H = 50 μm, A–B, F, L = 20 μm; C–E, G, I–K, M = 10 μm.

*Culture characteristics*: Mycelium felty with density low to average (OA) and average to strong (PDA). Surface on OA sienna, with sparse, saffron aerial mycelium, and buff growth at margin. Surface on PDA chestnut, with sienna aerial mycelium, with buff margin. Zonation was absent, transparency was homogeneous and growth margin even. Reverse similar to surface, except in color, chestnut to sienna on PDA. Colonies on PDA grow poorly, 1 mm diameter

at 5°C after 7 days. Optimum temperature at 25°C, when colonies reach 35 mm diameter after 7 days. Colony diameter was 8 mm at 30°C after 7days. No growth was observed at 35°C.

*Host and distribution: Ilex aquifolium* (roots) (Spain, Valencia).

***Ilyonectria ilicicola*** B. Mora-Sala, A. Cabral, J. Armengol&P. Abad-Campos, **sp. nov**. MycoBank MB822025(Fig. 4.7)

*Etymology*: Name refers to the plant host genus, *Ilex*, from which this fungus was isolated.

*Diagnosis*: *Ilyonectria ilicicola* can be distinguish morphologically from *I. cyclaminicola, I. leucospermi* and *I. protearum* by having slightly larger and narrower macroconidia. This taxa is best distinguish by *tub2* and *his3* genes.

*Typus*: **Spain**: Tarragona, 2012, on *Ilex* sp. roots, *B. Mora-Sala* (CBS H-23156 – holotype; CBS 142828=Cy-FO-225 – ex-type culture)

*Conidiophores* simple or complex. *Simple conidiophores* arising laterally or terminally from aerial mycelium, solitary to aggregated, unbranched or sparsely branched with up to three phialides, 1 to 3-septate, 49 to 178 µm long; phialides monophialidic, cylindrical, tapering towards the apex, 26 to 66 µm long, 2 to 4 µm wide at the base, 2.5 to 4.5 µm at the widest point, 1.5 to 3 µm near the aperture.

*Complex conidiophores* aggregated in sporodochia. Sporodochia consist of a pulvinate mass of short conidiophores, irregularly branched; phialides cylindrical, tapering towards the apex, 12 to 30 µm long, 1.5 to 2.5 µm wide at the base, 2.0 to 2.5 µm at the widest point, and 1 to 2 µm wide at the apex.

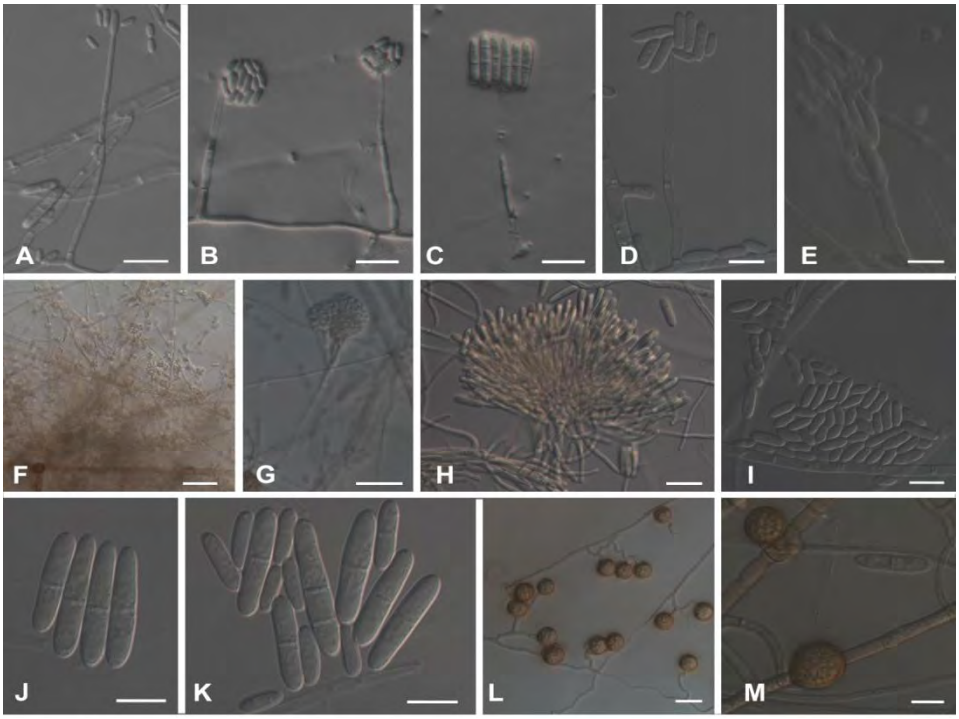


**Figure 4.7.** *Ilyonectria ilicicola* (ex-type culture Cy-FO-225). **A–E** Simple, sparsely branched conidiophores of the aerial mycelium. **F–H** Complex conidiophores. **I–K** Micro- and macroconidia. **L–M** Chlamydospores in mycelium. Scale bars: F, G = 50 µm, A–C, L = 20 µm; D–E, I–K, M = 10 µm; E, H, M from Cy-FO-224 and A–D, F–G, I–L from Cy-FO-225.

*Macroconidia* 1(to 3)-septate, straight, cylindrical, with both ends obtusely rounded, base sometimes with a visible, centrally located to laterally displaced hilum; 1-septate macroconidia (19.5–)25 to 26(–32.5)×(3.5–)5 to 5.5(-6.5) (av. 25.5×5.2µm), L/W ratio (3.5–)4.9 to 5.1(–7) (av. 5.0 µm); 2-septate macroconidia (26.5–)30 to 31.5(–35.5)×(5–)5.5 to 6(–7) (av. 30.7×5.7 µm), L/W ratio (4.5–)5.2 to 5.7(-7) (av. 5.4 µm); and 3-septate macroconidia (28–)31.5 to 34(–40.5)×(4.5–)5.7 to 6.2(–7) (av. 32.9×5.9 µm) L/W ratio (4.5–)5.3 to 5.9(–7.75) (av. 5.6 µm). Macroconidia predominant, formed by both types of conidiophores, forming flat domes of slimy masses.

*Microconidia* aseptate to 1-septate, with a minutely or clearly laterally displaced hilum; aseptate microconidia formed in simple conidiphores ellipsoidal to oval to fusiform (5–)9 to 9.5(–14.5)×(2.5–)3.4 to 3.6(–5) (av. 9.3×3.5 µm), L/W ratio (1.5–)2.6 to 2.8(–4.3) (av. 2.7µm); aseptate microconidia globose to subglobose formed in complex conidiophores 4.5 to 6×4 to 4.5 µm; 1-septate microconidia fusiform to ellipsoidal, (12–)15.5 to 16.5(–20)×(3.5–)4.3 to 4.6(–5.5) (av. 16×4.5µm), L/W ratio (2.5–)3.5 to 3.8(–4.5) (av. 3.7µm); microconidia formed in heads on simple conidiophores or as masses on complex conidiophores.

*Chlamydospores* globose to subglobose, 11 to 20×10 to 19 µm diam., smooth, but often appearing rough due to deposits, thick-walled, formed in lateral branches, rarely intercalary, mostly isolated, hyaline, becoming medium brown.

*Culture characteristics*: Mycelium felty with average density. Surface on OA fawn to cinnamon with aerial mycelium dark buff, with a buff margin. On PDA sepia with aerial mycelium, vinaceous buff, and margin buff. Zonation absent, with homogeneous transparency and margins even (OA) or lobate (PDA). Colonies similar in reverse, except in color, greyish sepia (OA) and dark brick to sepia (PDA). Colonies on PDA grow 6–7 mm diameter at 5°C after 7 days. Optimum temperature at 25°C, when colonies reach 62-64 mm diameter after 7 days. Colony diameter was 13-16 mm at 30°C after 7days. No growth was observed at 35°C.

*Additional cultures examined*: Cy-FO-224 and Cy-FO-226. Spain: Tarragona, isolated from *Ilex* sp. roots, 2012, B. Mora-Sala.

*Host and distribution*: *Ilex* sp. (roots) (Spain, Tarragona).

*Notes*: *Ilyonectria ilicicola* is phylogenetically closely related to *I. protearum*, *I. leucospermi* and *I. cyclaminicola* based on the phylogenetic inference in this study. The morphology of these four species overlap. *Ilyonectria ilicicola*, *I. protearum* and *I. cyclaminicola* formed sporodochia within 5 weeks, and have solitary chlamydospores and can be distinguished from *I. leucospermi* that failed to form sporodochia after 8 weeks incubation at 24°C under continuous UV light and have intercalary chlamydospores (Cabral *et al.* 2012a; Lombard et. al. 2013).

***Neonectria quercicola*** B. Mora-Sala, A. Cabral, J. Armengol & P. Abad-Campos, **sp. nov**. MycoBank MB 823852(Fig. 4.8).

*Etymology:* Name refers to the plant host genus, *Quercus*, from which this fungus was isolated.

*Diagnosis: Neonectria quercicola* can be distinguish morphologically by having long conidiophores that terminate in a whorl of phialides, and for production only 1-septate macroconidia. Phylogenetically it is better distinguished with the genes *his3* and *tef1*

*Typus*: **Spain**: Alicante, Alcoi, 2011, on *Quercus ilex* roots, *P. Abad-Campos* (CBS H-23353– holotype; CBS 143704=Cy-FO-3 – ex-type culture

*Conidiophores* simple or complex. Simple conidiophores short and sparsely branched 1 to 2-septate and 30 to 60 µm long, or long with 4 to 7-septate, 150 to 390 µm long and terminating in a whorl of phialides; phialides monophialidic, cylindrical, tapering towards the apex, 15 to 25 µm long, 1.5 to 4 µm wide at the base, 2 to 4.0 µm at the widest point, and 1.0 to 2.5 µm near the aperture. *Sporodochial conidiophores* irregularly branched.

*Macroconidia* 1-septate, straight, cylindrical with both ends more or less broadly rounded, mostly with a visible centrally located to laterally displaced hilum; 1-septate (17.5–)21.5 to 22.5(–26.5) × (4–)4.5 to 5(–6) (av. 22 × 4.7 µm) L/W ratio (3.5–)4.6–4.8(–6) (av. 4.7µm). Macroconidia formed in heads or as flat domes of slimy masses.

*Microconidia* rarely formed (0 to) 1-septate; 1-septate microconidia, mostly without a visible hilum ellipsoidal to oblong (10–)12 to 13.5(–15) × (4–)4.5to 5(–5.5) (av. 13 × 4.8 µm) L/W ratio (2–)2.5 to 3(–3.5) (av. 2.7µm).

*Chlamydospores* not observed.

*Culture characteristics***:** Mycelium felty with low density (OA) or strong density (PDA). Surface on OA buff with aerial mycelium cinnamon. Surface on PDA pale buff; with a pale luteous concentric ring. Zonation absent (OA) to concentric (PDA), transparency homogeneous and margins even. Reverse similar to surface, except in color, light cinnamon (OA) and buff with center sepia (PDA).

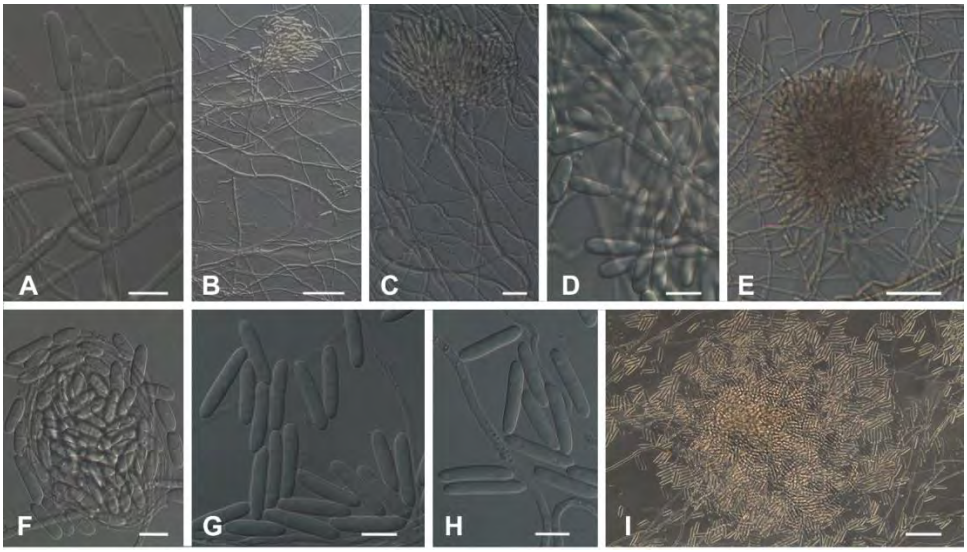


**Figure 4.8.** *Neonectria quercicola* **A** Simple, sparsely branched conidiophores of the aerial mycelium. **B–D** Long conidiophores of the aerial mycelium. **E** Sporodochial conidiophores. **F–I** Micro- and macroconidia. Scale bars: B, E, I = 50 μm; C = 20 μm; A, D, F–H = 10 μm.All from Cy-FO-3.

Colonies on PDA grow 5.7 mm diameter at 5°C after 7 days. Optimum temperature at 25°C, when colonies reach 17.2 mm diameter, after 7 days. Colony diameter was 6.4 mm at 30°C after 7days. No growth was observed at 35°C.

*Host and distribution: Quercus ilex* (Spain, Alicante, Alcoi)

*Notes*: Based on the phylogenetic inference in this study, *Neonectria quercicola* is closely related to other three isolates with no description available (CPC 13530, CPC 13531 and CR21). These isolates should also be considered *N. quercicola*, as they form a clade very well supported with 100% bootstrap support and a Bayesian posterior probability of 1.0.

**Host distribution**

The fungal species identified in this study were found associated with 15 host genera: *Abies* (2.9%), *Arbutus* (5.8%), *Cistus* (1.9%), *Crataegus* (1%), *Ilex* (4.9%), *Juglans* (2.9%), *Juniperus* (6.8%), *Lonicera* (1%), *Myrtus* (1%), *Pinus*(37.9%), *Pistacia* (1.9%), *Pyracantha* (1%), *Quercus* (26.2%), *Rosmarinus* (3.9%) and *Santolina* (1%) (Table 4.2). *Pinus* was the genus with the highest number of isolates recovered and from which eight species were identified: *D. hispanica, D. macrodidyma, D. novozelandica, D. pauciseptata, D. torresensis, I. capensis, I. liriodendri* and *I. rufa*. *Quercus* was the only host from which the four fungal genera were isolated, and the host with the highest number of fungal species isolated: *D. macrodidyma, D. novozelandica, D. torresensis, I. cyclaminicola, I. liriodendri, I. pseudodestructans, I. rufa, C. alicantinum* and *N. quercicola*. The third host regarding the number of species isolated was *Juniperus*: *D. macrodidyma, D. novozelandica, D. capensis, I. liriodendri* and *I. rufa*, followed by *Arbutus*, from which four fungal species were recovered, and *Abies* and *Rosmarinus*, from which three fungal species were recovered. In *Ilex* and *Juglans* only two fungal species were recovered and only one from *Cistus, Crataegus, Lonicera, Myrtus, Pistacia, Pyracantha* and *Santolina*.

Regarding the species, *D. novozelandica* was recovered from 66.67% of the hosts, followed by *D. macrodidyma* (60%), *D. torresensis* and *I. rufa* (40%), *I. liriodendri* (33.33%), *I. capensis* (20%) and *D. pauciseptata* (13.33%). The remaining fungal species were only recovered from one host, which represented 6.67% of the total number of hosts surveyed in this study (Table 4.2).

**Table 4.2.** Distribution of the Cylindrocarpon-like isolates according to the fungal species identified in the study. Number and percentages of isolates, hosts and forest nurseries.

| Fungal Species | Isolates [a] | | Host [b] | | | Nursery [c] | | |
|---|---|---|---|---|---|---|---|---|
| | No. | % | No | % | Genera | No | % | Location (Province) |
| *Cylindrodendrum alicantinum* | 1 | 0.9 | 1 | 6.67 | *Quercus* | 1 | 5.88 | Valencia |
| *Dactylonectria hispanica* | 1 | 0.9 | 1 | 6.67 | *Pinus* | 1 | 5.88 | Valencia |
| *Dactylonectria macrodidyma* | 25 | 24. | 9 | 60.00 | *Juniperus, Ilex, Lonicera, Myrtus,* | 9 | 52.94 | Alicante, Castellón, Logroño, Valencia |
| *Dactylonectria novozelandica* | 30 | 29. | 10 | 66.67 | *Crataegus, Juniperus, Pinus, Pistacia,* | 11 | 64.71 | Alicante, Castellón, Logroño, Valencia |
| *Dactylonectria pauciseptata* | 3 | 2.9 | 2 | 13.33 | *Abies,Pinus* | 2 | 11.76 | Valencia, León |
| *Dactylonectria pinicola* | 1 | 0.9 | 1 | 6.67 | *Abies* | 1 | 5.88 | León |
| *Dactylonectria torresensis* | 12 | 11. | 6 | 40.00 | *Arbutus, Cistus, Juglans, Pinus,* | 6 | 35.29 | Alicante, Castellón, Soria, Valencia |
| *Dactylonectria valentina* | 1 | 0.9 | 1 | 6.67 | *Ilex* | 1 | 5.88 | Valencia |
| *Ilyonectria capensis* | 3 | 2.9 | 3 | 20.00 | *Arbutus, Juniperus, Pinus* | 3 | 17.65 | Logroño, Valencia |
| *Ilyonectria cyclaminicola* | 1 | 0.9 | 1 | 6.67 | *Quercus* | 1 | 5.88 | Castellón |
| *Ilyonectria ilicicola* | 3 | 2.9 | 1 | 6.67 | *Ilex* | 1 | 5.88 | Tarragona |
| *Ilyonectria liriodendri* | 9 | 8.7 | 5 | 33.33 | *Arbutus, Juniperus, Pinus, Quercus* | 6 | 35.29 | Castellón, Logroño, Valencia |
| *Ilyonectria pseudodestructans* | 1 | 0.9 | 1 | 6.67 | *Quercus* | 1 | 5.88 | Valencia |
| *Ilyonectria robusta* | 2 | 1.9 | 1 | 6.67 | *Juglans* | 1 | 5.88 | Soria |
| *Ilyonectria rufa* | 9 | 8.7 | 6 | 40.00 | *Abies, Arbutus, Juniperus,* | 6 | 35.29 | Alicante, Castellón, León, Valencia |
| *Neonectria quercicola* | 1 | 0.9 | 1 | 6.67 | *Quercus* | 1 | 5.88 | Alicante |

[a] Number of isolates and percentages were calculated on the basis of a total of 103 isolates.

[b] Number of hosts and percentages were calculated on the basis of a total of 15 plant genera.

[c] Number of nurseries and percentages were calculated on the basis of a total of 17 surveyed nurseries

## Discussion

The present study represents the first attempt to characterize a wide collection of Cylindrocarpon–like asexual morphs collected from forest nurseries in Spain. This clearly demonstrates the prevalence of this fungal group associated with seedlings of diverse number of hosts showing decline symptoms. Cylindrocarpon–like asexual morphs are ubiquitous and can be easily found in soil or associated with plant roots, some of them having also a potential role as latent pathogens or endophytic organisms (Halleen *et al.* 2006, Agustí-Brisach and Armengol 2013).

Sixteen species belonging to the genera *Cylindrodendrum*, *Dactylonectria*, *Ilyonectria*, and *Neonectria* were identified from damaged roots of 15 forest plant genera. Six isolates were not identified to the species level with the *his3* data. Although *his3* region has previously showed to be a very informative locus (Cabral *et al.* 2012a), a combined analysis with ITS, *tub2* and *tef1* regions better resolved and confirmed that these isolates represented novel phylogenetic species, newly described as: *D. hispanica*, *D. valentina*. *I. ilicicola* and *N. quercicola*.

This is the first report of *C. alicantinum* on *Q. ilex*; of *D. macrodydima* on: *Ilex aquifolium, Juniperus phoenicea, Lonicera* sp., *Myrtus communis, P. halepensis, Pyracantha* sp., *Q. faginea, Q. ilex* and *Rosmarinus officinalis*; of *D. novozelandica* on: *Crataegus azarolus, J. phoenicea, Pinus* sp., *P. halepensis, Pistacia lentiscus, Quercus* sp., *Q. ilex, Q. suber, R. officinalis* and *Santolina chamaecyparissus*; of *D. pauciseptata* on: *Abies nordmanniana* and *P. halepensis*; of *D.pinicola* on: *A. concolor*, of *D. torresensis* on: *Ar. unedo, Cistus albidus, Ju. regia, P. halepensis, Q. ilex* and *R. officinalis; of I. capensis* in *Arbutus unedo, Juniperus* sp. and *P. halepensis*; of *I. cyclaminicola* on *Quercus* sp.; of *I. liriodendra* on: *Ar. unedo, Juniperus* sp., *P. halepensis*; of *I. pseudodestructans* in *Q. ilex*; of *I. robusta* in *Juglans regia*; and of *I. rufa* on: *A. nordmanniana, Ar. unedo, Juniperus* sp., *P. halepensis, Q. faginea* and *Q. ilex.* Furthermore, this is the first report of *I. capensis* in Europe because, to our knowledge, this fungus had only been recorded affecting *Protea* in South Africa (Lombard *et al.* 2013).

To date *D. novozelandica, D. macrodidyma, D. torresensis, I. liriodendri, I. robusta* and *C. alicantinum* had been reported only in cultivated crops such as grapevine (*Vitis vinifera*) (Agustí-Brisach and Armengol 2013) or loquat (*Eriobotrya japonica*)(Agustí-Brisach *et al.*2016) in Spain, but never affecting forest plants.

*Dactylonectria pauciseptata* had been reported as a pathogen of grapevines in Slovenia and New Zealand (Schroers *et al.* 2008), Uruguay (Abreo *et al.* 2010), Spain (Martín *et al.* 2011), Portugal (Cabral *et al.* 2012a), Brazil (dos Santos *et al.* 2014), and British Columbia, Canada (Úrbez-Torres *et al.* 2014). It has also been reported as a pathogen of apple trees in South Africa and of peach trees in Italy (Tewoldemedhin *et al.* 2011;Yaseen *et al.* 2012).This fungus had also been recorded in forest hosts: *Viburnum tinus* in Italy (Aiello 2015) and *P. radiata* in Spain (Agustí-Brisach *et al.* 2011). Thus, this study increases the range of forest hosts in nursery for this species, representing the first report of *D. pauciseptata* on *A. nordmanniana* and *P. halepensis.*

In our study, no correlation was found between the fungal pathogens isolated and nurseries from which they were collected. The fungal species did not show any distribution pattern among the different locations surveyed probably because the small sample size, as 3 plants per host is probably not enough to look for these correlations. Furthermore, it should be taken into account that there were locations (provinces) in which a higher number of nurseries were surveyed that included Valencia, Castellón and Alicante. Likewise, some of the surveyed hosts, in particular *Pinus* and *Quercus*, were the target hosts of the survey undertaken due to the importance of these genera in Spanish forests (MAGRAMA 2014). Therefore, these hosts had a higher number of sampled plants, also corresponding with a higher number of Cylindrocarpon-like isolates compared to other hosts. These two genera prevail in the Mediterranean landscape, as they constitute the characteristic vegetation of the Mediterranean forests. In this regard, the presence of nine Cylindrocarpon-like species on *Quercus* and eight on *Pinus* trees highlight the need for better management of nursery diseases to avoid the dispersal of these fungi through planting materials used for reforestation purposes. Management of Cylindrocarpon-like asexual morphs associated with black-foot disease has been intensively studied on grapevine nurseries, where the incorporation of holistic and integrated control

measures such as cultural practices and sanitation, chemical and biological control, has been shown as the best approach to improve the phytosanitary quality of planting material (Gramaje and Armengol 2011; Gramaje *et al.* 2018). Implementing a similar strategy could be advisable in forest nurseries.

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# Chapter 5

## Response of *Quercus ilex* seedlings to *Phytophthora* spp. root infection in a soil infestation test

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**Keywords:** *Quercus* spp.; Pathogenicity; Inoculation; Holm oak.

# Chapter 5


## Abstract

*Phytophthora* species are the main agents associated with oak (*Quercus* spp.) decline, together with the changing environmental conditions and the intensive land use. The aim of this study was to evaluate the susceptibility of *Quercus ilex* to the inoculation with eight *Phytophthora* species. Seven to eight months old *Q. ilex* seedlings grown from acorns, obtained from two Spanish origins, were inoculated with *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. megasperma*, *P. nicotianae*, *P. plurivora*, *P. psychrophila* and *P. quercina*. All *Phytophthora* inoculated seedlings showed decline and symptoms including small dark necrotic root lesions, root cankers, and loss of fine roots and tap root. The most aggressive species were *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. plurivora* and *P. psychrophila* followed by *P. megasperma*., while *Phytophthora quercina* and *P. nicotianae* were the less aggressive species. Results obtained confirm that these *Phytophthora* species could constituted a threat to *Q. ilex* ecosystems and the implications are further discussed.


## Introduction

The genus *Quercus* comprises over 450 species distributed mainly throughout the Northern hemisphere (Xia et al. 2014). Over 70 species are known to be present in Spain and approximately 20 % are native (Sánchez de Lorenzo-Cáceres 2001). The evergreen holm oak (*Quercus ilex* L.) is the dominant tree in Spanish woodlands covering an area of 2.8 M ha (15.3 % of the forested area) (MAGRAMA 2014). It can also be found in 2.4 M ha of oak rangelands mixed with cork oak (*Quercus suber* L.) and "quejigo" oak [*Quercus faginea* (Cout) Camus)] (MAGRAMA 2014).

Since the last century, European oak forests are suffering a decline (Brasier 1996; Jung et al. 1996, 2000). The increase of pathogens threatening *Quercus*, along with the changing environmental conditions and the intensive land use, has resulted in a serious complex syndrome that is diminishing *Quercus* woodlands (Brasier 1992ab, 1996, 2008; Brasier et al. 1993b; Jung et al. 1996, 1999, 2000; Moreira and Martins 2005; Camilo-Alves et al. 2013). Amongst

others, pathogens contributing to oak decline include: *Biscogniauxia mediterranea* (de Not.) Kuntze, *Botryosphaeria stevensii* Shoem., *Lembosia quercina* (Ellis & G. Martin) Tracy & Earle, *Pesotum piceae* J.L. Crane & Schokn., *Phomopsis quercina* (Sacc.) Höhn. ex Died., *Phytophthora* spp., *Pythium sterilum* Belbahri & Lefort and *Pythium spiculum* B. Paul (Brasier 1996; Jung et al. 1996, 2000; Gallego et al. 1999; Luque et al. 2000; Rizzo et al. 2002; Romero et al. 2007, Jiménez et al. 2008). Climate change, leading to an increase in mean temperatures, together with more frequent droughts followed by flooding episodes, are some of the abiotic factors causing weakening of the trees (Brasier 1992b, 1996; Sánchez et al. 2002; Corcobado et al. 2013). Once the tree health balance is disturbed, biotic damaging agents such as *Phytophthora* species can lead to the decline (Brasier et al. 1993b; Brasier 1996; Hansen and Delatour 1999; Jung et al. 2000; Sánchez et al. 2006; Camilo-Alves et al. 2013; Corcobado et al. 2013). The vigour of the tree is also affected by changes in the microbial composition of the rhizosphere. Jönsson (2006) suggested that the presence of microorganisms in the soil and mycorrhizal colonization made oak less susceptible to *Phytophthora* spp. infection. Lower ectomycorrhizal root colonization and diversity have been observed in *Phytophthora*-infected oak stands (Corcobado et al., 2014). Hence, disturbances in *Quercus* forests cause shifts in mycorrhizal soil communities, which in the presence of pathogens such as *Phytophora* spp., contribute to the decline (Corcobado et al., 2014).

The genus *Phytophthora* includes some of the most devastating plant pathogens comprising more than 150 species with different host ranges (Hardham and Blackman 2010; Scibetta et al. 2012; Thines 2013; Jung et al. 2016; Panabières et al. 2016). It is present in natural and anthropogenic ecosystems causing large environmental and economic losses (Erwin and Ribeiro 1996; Kroon et al. 2012; Jung et al. 2016). Numerous surveys conducted in Europe reported *Phytophthora* as the main damaging agent associated with oak decline. In Spain and Portugal, the invasive pathogen *P. cinnamomi* Rands was established as the causal agent of decline of *Q. ilex* and *Q suber* in the Iberian Peninsula, although it is not the only *Phytophthora* species involved (Brasier 1992ab, 1996; Brasier et al. 1993b; Tuset et al. 1996; Gallego et al. 1999; Sánchez et al. 2002, 2003, 2006; Moreira and Martins 2005; Navarro et al. 2004; Corcobado et al. 2010; Pérez-Sierra et al. 2013). This situation is similar in other oak woodlands and maquis from Mediterranean regions in France, Italy and

Turkey, where oaks are also affected by other *Phytophthora* species such as *P. cactorum* (Lebert & Cohn) J. Schröt., *P. cambivora* (Petri) Buisman, *P. citricola* complex, *P. cryptogea* Pethybr. & Laff., *P. gonapodyides* H.E. Petersen, *P. megasperma* Drechsler, *P. psychrophila* T. Jung & E.M. Hansen, *P. quercina* T. Jung and *P. syringae* (Kleb.) Kleb. (Brasier 1996; Robin et al. 1998; Hansen and Delatour 1999; Vettraino et al. 2002; Balci and Halmschlager 2003a; Linaldeddu et al. 2014; Scanu et al. 2015).

Several studies have been carried out to determine the susceptibility of oak (e.g. *Q. robur* and *Q. suber*) to different *Phytophthora* spp. *Phytophthora cinnamomi*, *P. cryptogea*, *P. drechsleri* Tucker, *P. gonapodyides*, *P. megasperma*, *P. psychrophila*, *P. quercina* and *P. syringae* have been inoculated on *Q. ilex*, in which lesions on the roots of young seedlings were observed (Tuset et al. 1996; Robin et al. 1998, 2001; Gallego et al. 1999; Maurel et al. 2001; Rodríguez-Molina et al. 2002; Sánchez et al. 2002, 2005; Pérez-Sierra et al. 2013; Linaldeddu et al. 2014; Martín-García et al. 2015). Considering the importance of *Q. ilex* as the most representative tree in the Spanish forest ecosystems and the lack of information regarding the role of some *Phytophthora* spp. in its decline, the aim of this study was to investigate the response of *Q. ilex* seedlings to the inoculation with eight different *Phytophthora* species using a soil infestation method.

## Material and Methods

### Plant material

Seven to eight months old *Q. ilex* subsp. *ballota* seedlings grown from acorns were used. Acorns were selected from two different origins; an oak rangeland (a silvopasture farming system) located in Cáceres in Extremadura region in western Spain (39º58´N, 6º5´W; mean T = 16.5 ºC; annual P = 803 mm), and from La Yesa, a Mediterranean mixed forest stand, in which holm oaks are grown competing with other tree species in Comunidad Valenciana in eastern Spain. In both cases acorns were collected from vigorous trees. The acorns from La Yesa were provided by the Forest Research Centre CIEF (Centro para la Investigación y Experimentación Forestal, Valencia). Acorns from both origins were surface sterilized and pre-germinated in trays with thermo-sterilized sand

incubated at 20 ºC under 12 h photoperiod. Once the roots emerged, pre-germinated acorns were transplanted to Quick pot PE trays (52 × 29 upper surface and 19 cm high). Each cell contained approximately 1,700 ml in volume of vermiculite-sand-peat substrate mixture (1:1:1, v/v/v) previously autoclaved three times. To avoid root disturbance during inoculation, two cavities were made in the substrate before sowing by placing 2 sterile 15 ml tubes 6 cm apart. One pre-germinated acorn per cell was planted between the tubes and plants maintained in the greenhouse at 20-25 ºC and watered every two weeks.

### *Phytophthora* isolates

*Phytophthora* isolates used in the pathogenicity tests were selected from the *Phytophthora* collection maintained in soil solution extract and oatmeal agar tubes at the Instituto Agroforestal Mediterráneo (IAM- UPV, Valencia, Spain). All were isolated from *Q. ilex* during previous surveys of forest ecosystems and nurseries. Eight *Phytophthora* species were selected: *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. megasperma*, *P. nicotianae*, *P. plurivora*, *P. psychrophila* and *P. quercina* (Table 5.1).

**Table 5.1.** *Phytophthora* isolates used in the pathogenicity test.

| ***Phytophthora* spp.** | **Code** | **Host** |
|---|---|---|
| *P. cinnamomi* | Ps 1630 | *Q. ilex* (roots) |
| *P. cryptogea* | Ps 962 | *Q. ilex* (roots) |
| *P. gonapodyides* | Ps 789 | *Quercus* sp. |
| *P. megasperma* | Ps 1619 | *Q. ilex* (soil) |
| *P. nicotianae* | Ps 956 | *Q. ilex*(roots) |
| *P. plurivora* | Ps 932 | *Q. ilex* |
| *P. psychrophila* | Ps 1030 | *Quercus* sp. |
| *P. quercina* | Ps 982 | *Q. ilex* (soil) |

**Soil infestation pathogenicity test**

The potting mix consisted of vermiculite-sand-peat (1:1:1, v/v/v), oat grains (20 $cm^3$) and V8 broth (200 mL/L V8 juice, 800 mL/L demineralized water and 3 g/L $CaCo_3$). The mixture was autoclaved 3 times and then inoculated with the selected *Phytophthora* species isolates previously grown on V8 media (V8A). The inoculated media were incubated for 6 weeks in the dark at room temperature (Pérez-Sierra et al. 2013). After this time, the inoculum mixture was rinsed with demineralized water before inoculations.

Seedlings were selected for the test based on morphological homogeneity and healthy appearance. For inoculation, 20 ml of inoculum mixture per 1 L potting medium was added to the cavities previously made in each cell where the seedling was grown. Negative control plants (henceforth called uninoculated plants) were inoculated with non-infested mixture and the experiment was repeated twice. In total, 24 seedlings were inoculated per *Phytophthora* spp. and control. Each 12 seedlings repetition contained 9 seedlings from Cáceres and 3 from La Yesa. For inoculations with *P. megasperma*, only 15 seedlings from La Yesa were included (7 and 8 seedlings in each repetition). All seedlings were watered the day before the inoculation. Immediately after inoculation, the seedlings were flooded for 48 h and the flooding was repeated every two weeks to stimulate formation of zoosporangia, as previously described (Pérez-Sierra et al. 2013). The experiment was harvested 6 months after inoculation.

Seedlings were uprooted and the root system was washed carefully under running water to remove the substrate. Reisolations from all seedlings were performed by plating symptomatic fine root fragments in CMA-PARPBH (Jeffers and Aldwinckle 1987) and baiting the substrate with Granny Smith apples (Erwin and Ribeiro 1996) to confirm Koch´s postulates.

**Seedling analysis**

Seedlings were evaluated immediately after inoculation and every two weeks thereafter in order to determine aerial condition. Number of leaves on each seedling and above-ground symptoms were evaluated using a visual scale, where 0 = symptoms-free plant, 1 = limited foliar chlorosis and necrosis, 2 = wilting, dieback, defoliation,and 3 = dead plant (Jönsson et al. 2003).

To assess root condition of inoculated seedlings two different approaches were used at the end of the experiment. First, symptom severity was assessed, using a visual descriptive scale from 1 to 4 (1 = root loss from 0-25 %, 2 = root loss from 26-50 %, 3 = root loss from 51-75 %, 4 = root loss from 76-100 %) (Pérez-Sierra et al. 2013). Symptom severity was calculated using the McKinney Index (MI; McKinney 1923) based on the scale given above, and a Kruskal-Wallis test applied to the data to compare between *Phytophthora* species. For the second approach, the dry weight of the root biomass was measured. The aerial tissues were separated from the root system by cutting at the root collar, placed into paper bags, and dried for 5 days in an oven at 35 ºC. The dry weights of the aerial tissues and root system were recorded. An analysis of variance (ANOVA) was performed for the factors treatment, origin and the interaction treatment x origin. Mean values were compared using the Student's least significant difference test at the 95 % confidence level. Correlation between the different parameters were determined by calculating Pearson´s coefficients (r). All analyses were performed using the package SPSS 16.0 (SPSS Inc., Chicago IL).

An ANOVA was performed to determine differences in mean number of final leaves, length of the stem, weight of fine roots (Ø<2mm), weight of main roots (Ø>2mm), weight of the complete root system, weight of the aerial tissues and survival days obtained from the different *Phytophthora* treatments and the acorn origin. Pearson´s coefficients were calculated to determine correlations between the measured parameters. Finally, survival time of the seedlings was also assessed using the Kaplan-Meier estimate, a product-limit estimate:

$$S(t) = \prod_{j=1}^{k} \cdot \left(\frac{nj-dj}{nj}\right)$$

where $n_j$ = number of seedlings alive before the time $t_{(j)}$ and $d_{(j)}$ = number of dead seedlings at time $t_{(j)}$ for $t_{(k)} \leq t \leq t_{(k+1)}$. This non-parametric analysis was carried out using the same software and the log-Rank test (Collett 2003) was used to compare the survival curves of the seedlings inoculated with the different *Phytophthora* species.

## Results

All *Q. ilex* seedlings inoculated with the different *Phytophthora* isolates showed root symptoms (small dark necrotic lesions, root cankers, loss of fine roots, tap root rot), as well as aerial symptoms (decline, chlorosis, wilting, dieback, defoliation, slow growth rate, leaf spots). Reisolations from symptomatic roots confirmed Koch´s postulates. In contrast, control treatment seedlings showed non-specific symptoms in the root system and the aerial tissues, which were not associated with positive reisolations of *Phytophthora* and new healthy rootlets were growing in almost all control seedlings at the end of the experiments.

ANOVA showed significant differences among the treatments (*Phytophthora* species inoculated) and the uninoculated control plants in number of leaves, weight of fine roots, symptom severity, MI and survival time with a confidence level of 99 % (Table 5.2). These parameters were not significantly different, based on the origin of the acorns. The interaction treatment x origin showed non-significant effect for these parameters. The analysis of MI showed *P. cinnamomi, P. cryptogea, P. gonapodyides, P. plurivora* and *P. psychrophila* were aggressive species causing severe symptoms ranging from 95.8 % to 98.8 %. The lowest MI corresponded to *P. nicotianae* (83.3 %) which was significantly higher than the uninoculated plants (Table 5.2). Seedlings inoculated with *P. nicotianae*, *P. quercina* and the uninoculated plants had significantly higher fine root weights compared with the other treatments (*P*-value > 0.05) (Table 5.2). The *P. gonapodyides* treatment caused the lowest survival time of the seedlings, followed by *P. cinnamomi*. These seedlings showed non-significant differences among them in terms of survival but they showed differences compared with *P. psychrophila, P. megasperma, P. quercina, P. nicotianae* and uninoculated controls (*P*-value < 0.05) (Table 5.2).

ANOVA also showed significant differences among the treatments and the uninoculated plants in length of the stem, weight of the complete root system, weight of the main roots and weight of the aerial tissues. For these parameters, the factor acorn origin was also significant (*P*-value < 0.05). Due to this finding, these parameters were examined separately by origin (Table 5.3). The interaction treatment x origin showed non-significant effect for these parameters. For seedlings from Cáceres, plants inoculated with *P. psychrophila, P. cinnamomi, P.*

*gonapodyides* and *P. cryptogea* showed significant lower stem length (*P*-value < 0.05). For seedlings from La Yesa, plants inoculated with *P. megasperma* showed lower stem length compared with plants inoculated with *P. cryptogea*, *P. quercina* and uninoculated controls.

**Table 5.2.** Kruskal-Wallis and one-way ANOVA for non-significant parameters according to the origin of the inoculated material. Results of the parameters analysed in the pathogenicity test coming from Cáceres and La Yesa acorn origins.

| ***Phytophthora* spp.** | **No. leaves** | **W fine roots (mg)** | **MI** | **Survival time (days)** |
|---|---|---|---|---|
| *P. cinnamomi* | 6.5 ± 0.95 | 63 ± 19.59 | 95 ± 2.46 | 92.1 ± 6.10 |
| | **ab** | **a** | **d** | **ab** |
| *P. cryptogea* | 8.9 ± 0.99 | 41 ± 14.51 | 96 ± 1.72 | 98.5 ± 7.31 |
| | **abc** | **a** | **d** | **b** |
| *P. gonapodyides* | 6.8 ± 1.08 | 45 ± 25.22 | 97 ± 1.44 | 78.8 ± 6.82 |
| | **ab** | **a** | **d** | **a** |
| *P. megasperma* | 4.6 ± 1.33 | 32 ± 10.84 | 95 ± 2.67 | 136.5 ± 11.50 |
| | **a** | **a** | **cd** | **cd** |
| *P. nicotianae* | 15.5 ± 1.91 | 230 ± 49.13 | 83 ± 4.43 | 147.5 ± 5.67 |
| | **d** | **b** | **b** | **d** |
| *P. plurivora* | 10.2 ± 2.05 | 41 ± 19.47 | 96 ± 2.29 | 101.4 ± 8.23 |
| | **bc** | **a** | **d** | **b** |
| *P. psychrophila* | 6.8 ± 1.08 | 35 ± 9.79 | 98 ± 1.14 | 122.4 ± 9.03 |
| | **ab** | **a** | **d** | **c** |
| *P. quercina* | 13.5 ± 2.11 | 185 ± 33.24 | 84 ± 3.30 | 139.3 ± 7.85 |
| | **cd** | **b** | **bc** | **cd** |
| Negative control | 16.7 ± 2.70 | 245 ± 77.30 | 72 ± 6.72 | 156.3 ± 1.28 |
| | **d** | **b** | **a** | **d** |

All *P*-values are significant at $P < 0.01$.
Values with the same letter for each column do not differ significantly according Fisher´s LSD test ($P = 0.05$)
W = weight; MI = McKinney Index

**Table 5.3.** Kruskal-Wallis one-way ANOVA. Results of the parameters obtained in the pathogenicity test of the different *Phytophthora* species inoculated on *Quercus ilex* seedlings from Cáceres.

| Species | L stem (cm) | W aerial tissues (mg) | W complete root system (mg) | W main roots (mg) |
|---|---|---|---|---|
| *P. cinnamomi* | 9.6 ± 1.19 | 393 ± 79.59 | 711 ± 172.58 | 640 ± 156.68 |
| | **a** | **a** | **a** | **a** |
| *P. cryptogea* | 11.14 ± 0.88 | 561 ± 69.96 | 421 ± 76.80 | 401 ± 61.30 |
| | **a** | **ab** | **a** | **a** |
| *P. gonapodyides* | 9.61 ± 0.60 | 486 ± 93.75 | 642 ± 183.31 | 602 ± 158.34 |
| | **a** | **ab** | **a** | **a** |
| *P. nicotianae* | 18.06 ± 1.80 | 1827 ± 292.60 | 898 ± 257.27 | 1627 ± 214.94 |
| | **c** | **c** | **b** | **b** |
| *P. plurivora* | 11.39 ± 1.17 | 607 ± 106.44 | 618 ± 186.71 | 566 ± 161.82 |
| | **ab** | **ab** | **a** | **a** |
| *P. psychrophila* | 8.25 ± 0.66 | 425 ± 83.75 | 590 ± 90.93 | 553 ± 87.04 |
| | **a** | **ab** | **a** | **a** |
| *P. quercina* | 11.08 ± 1.03 | 876 ± 151.35 | 1520 ± 221.89 | 1339 ± 192.15 |
| | **b** | **b** | **b** | **b** |
| Negative control | 14.6 ± 1.46 | 1372 ± 262.39 | 1925 ± 449.74 | 1642 ± 356.46 |
| | **b** | **c** | **b** | **b** |

All *P*-values are significant at $P < 0.01$.
Values with the same letter for each column do not differ significantly according Fisher´s LSD test ($P = 0.05$)
L = length; W = weight

Regarding the weight of the complete root system and the main roots, *P. cryptogea*, *P. psychrophila*, *P. plurivora*, *P. gonapodyides* and *P. cinnamomi* were the most aggressive species for both acorn origins. In seedlings grown from La Yesa acorns, the most aggressive species were also *P. megasperma* and *P. nicotianae*. Regardless of the origin of the acorns, seedlings inoculated with *P. quercina* did not differ significantly in weight of the complete root system from the uninoculated controls.

The survival curves (Fig. 5.1) agreed with the results obtained from the Kruskal-Wallis analysis. *Phytophthora megasperma*, *P. nicotianae*, *P. quercina*, and the uninoculated plants showed highest survival at the end of the experiment, ranging from 62.5 to 91.7 %. *Phytophthora cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. plurivora* and *P. psychrophila* were more aggressive causing lower survival of plants at the end of the experiment: 4.2 %, 4.2 %, 8.3 %, 20.8 % and 45.5 %, respectively.

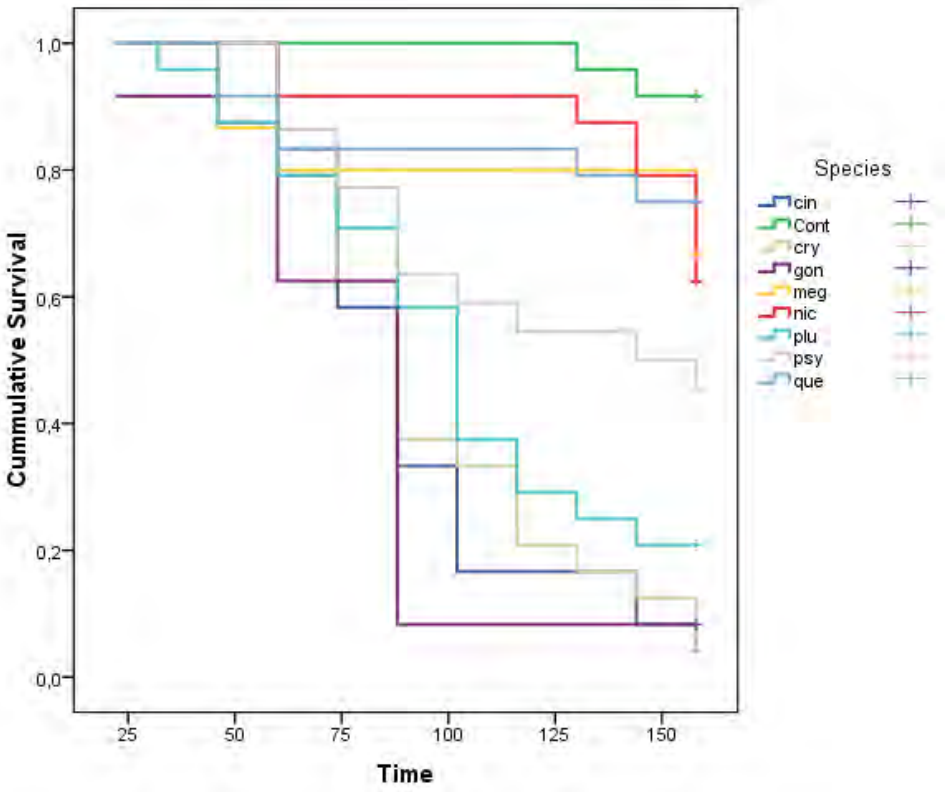


**Figure 5.1.** Survival probabilities plot using the Kaplan-Meier estimate (P-value < 0.001) of the survival function for *Quercus ilex* seedlings inoculated with *Phytophthora* spp. cry = *P. cryptogea*. gon = *P. gonapodyides*. meg = *P. megasperma*. nic = *P. nicotianae*. plu = *P. plurivora*. psy = *P. psychrophila*. que = *P. quercina*. Cont = control.

Pearson´s analysis of the global data set, showed that the correlation between the different parameters studied in the experiment (Table 5.4) was significant (*P*-value < 0.001). The coefficients between MI and the other parameters examined were negative and particularly strong between MI and the different weights (W) of the root system (W main roots r = -0.7591; W fine roots r = -0.7994) and the aerial tissues (r = -0.7066).

**Table 5.4.** Pearson´s correlation coefficient (r) between the different parameters studied in the *Phytophthora* spp. pathogenicity test on *Quercus ilex* seedlings.

| | **L** | **No. leaves** | **Waerial tissues** | **Wmain roots** | **Wfine roots** | **MI** | **Survival time** |
|---|---|---|---|---|---|---|---|
| **L** | | | | | | | |
| **No. leaves** | 0.5886 | | | | | | |
| **Waerial tissues** | 0.8310 | 0.6356 | | | | | |
| **Wmain roots** | 0.6519 | 0.5122 | 0.8048 | | | | |
| **Wfine roots** | 0.5847 | 0.5376 | 0.7407 | 0.8137 | | | |
| **MI** | -0.6200 | -0.5084 | -0.7066 | -0.7591 | -0.7994 | | |
| **Survival time** | 0.3301 | 0.4698 | 0.4265 | 0.4051 | 0.3391 | -0.3547 | |

All correlations are significant. *P*-values < 0.001
L = length; W = weight; MI = McKinney Index

The correlation between the weights of the aerial tissues and the root system of the seedlings was positive, also showing a strong relationship among these parameters (W main roots r = 0.8048; W fine roots r = 0.7407). Finally, there was a positive correlation between the total weight of the seedling (aerial tissues and root systems) and survival time (W aerial tissues r = 0.4265; W main roots r = 0.4051; W fine roots r = 0.3391) (Table 5.4).

## Discussion

All *Phytophthora* isolates inoculated on *Q. ilex* were pathogenic. The most aggressive species were *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. plurivora* and *P. psychrophila*, followed by *P. megasperma*, while *P. quercina* and *P. nicotianae* were the least aggressive species, with plants inoculated with *P. quercina* having the longest survival rates. For seedlings grown from Cáceres acorns, *P. nicotianae* was the least aggressive species, while seedlings grown from La Yesa acorns the least aggressive was *P. quercina*.

Results observed in seedlings inoculated with *P. cinnamomi* were in agreement with several studies and field observations, which demonstrated the devastating action of this wide-host range pathogen in the Iberian Peninsula (Brasier 1992a, 1992b, 1996; Brasier et al. 1993b; Robin et al. 1998; Tuset et al. 1996; Gallego et al. 1999; Luque et al. 2000, 2002; Sánchez et al. 2002, 2003, 2005, 2006; Moreira and Martins 2005; Navarro et al. 2004; Camilo-Alves et al. 2013, Hernández-Lambraño et al. 2018; Sena et al. 2018). The *P. cinnamomi* pathogenicity test showed high mortality rates that could be associated with a rapid root rot affecting not only the feeder roots but also the tap root. Loss of fine roots, cankers and dieback of the tap root with necrotic lesions were observed in inoculated seedlings as previously described for *P. cinnamomi* infection (Brasier et al. 1993b; Robin et al. 2001; Sánchez et al. 2005; Redondo et al. 2015). *Phytophthora cinnamomi* is well adapted to the Spanish environmental and edaphic conditions causing oak decline with the exception of the eastern limestone Mediterranean area, which constrains its development due to the high calcium content soils (Schmitthenner and Canaday 1983; Ríos et al. 2016).

*Phytophthora gonapodyides* was considered a ubiquitous and opportunistic or weak pathogen (Brasier 1993a, Hansen and Delatour 1999). However, Jung et al. (1996) reported that *P. gonapodyides* produced a wilting toxin able to cause root rot and stem lesions on *Q. robur* seedlings. In 2010, *P. gonapodyides* was reported as *Q. ilex* pathogen (Corcobado et al. 2010). Subsequent studies showed the aggressiveness of this species in holm oak (Pérez-Sierra et al. 2013; Corcobado et al. 2017). Corcobado et al. (2017) observed the highest necrosis lengths in the roots and high mortality rates in seedlings infected with *P. gonapodyides* compared with seedlings infected with *P. quercina*. Our results agree with these findings since seedlings inoculated with *P. gonapodyides*

caused the most rapid mortality, high MI, limited aerial tissue development and a significant reduction in the root system. *Phytophthora gonapodyides* could be considered along with *P. cinnamomi* in the category of main biotic threats to holm oak seedlings in Spanish forests as from our results it has similar behaviour as *P. cinnamomi*.

Regarding seedlings inoculated with *P. cryptogea*, results obtained agree with pathogenicity studies carried out with Spanish *Q. ilex* material by Sánchez et al. (2005). *Phytophthora cryptogea* zoospores attack the feeder roots, and the pathogen progresses through the root system reaching the main root and causing dieback with necrotic lesions and small cankers. As the root system diminishes rapidly, the aerial tissues do not develop correctly, leading to high mortality rates. *Phytophthora cryptogea* has been reported in several Mediterranean ecosystems associated with oak decline (Vettraino et al. 2002; Balci and Halmschlager 2003a; Sánchez et al. 2005; Pérez-Sierra et al. 2013; Linaldeddu et al. 2014; Scanu et al. 2015, Mora-Sala et al. unpublished data). The versatility of *P. cryptogea* and the ability to persist in water bodies, soil or plant tissue until favourable conditions appear, might allow it to establish and to develop throughout the oak forest ecosystems in Spain, then becoming then a dangerous pathogen.

*Phytophthora nicotianae* is a polyphagous, broad-range pathogen responsible for major economic losses in agricultural and ornamental sectors worldwide (Erwin and Ribeiro 1996; Álvarez et al. 2007; Panabières et al. 2016). It has been reported among the main *Phytophthora* species present in the nursery industry especially in Mediterranean regions threatening afforestation of *Quercus* stands (Moralejo et al. 2009; Pérez-Sierra et al. 2012; Pérez-Sierra and Jung 2013; Jung et al. 2016; Panabières et al. 2016). Climate change and global trade are driving *P. nicotianae* to an advantageous position over other *Phytophthora* species as its high optimum temperature, longevity, dispersal capacity and hybridisation capacity enable it to adapt to the changing worldwide climate scenarios (Panabières et al. 2016). This report is the first time that *P. nicotianae* was tested on *Q. ilex* and the results demonstrated that this host is susceptible to the pathogen, despite *P. nicotianae* being less aggressive than the other *Phytophthora* species tested. La Yesa seedlings were more susceptible to *P. nicotianae* than Cáceres seedlings, possibly due to the quality of the acorns, as

the management of the oaks in the two origins differ. While in Cáceres, the holm oak is the only tree species in this agricultural scenario, in La Yesa, oaks are part of a Mediterranean mixed forest. In Cáceres, oaks are maintained for the production of acorns to feed the livestock, which generally produces bigger acorns.

*Phytophthora plurivora* is a well-known aggressive oak pathogen (Jung et al. 1996, 2000; Hansen and Delatour 1999; Vettraino et al. 2002; Balci and Halmschlager 2003a, 2003b; Mrázková et al. 2013; Jung and Burgess 2009; Jankowiak et al. 2014), but our study represents the first soil infestation test conducted on holm oak with this species. *Q. ilex* seedlings inoculated with *P. plurivora* showed absence of fine roots, necrotic lesions, open cankers, dieback of the whole root system and collar rot. In some cases, no tap root was present. These symptoms agree with those reported in other woody hosts leading to a high mortality rate, high MI and low root and aerial tissues weight (Jung and Burgess 2009). As homothallic species, *P. plurivora* is a broad host range pathogen having high environmental versatility (Jung and Burgess 2009). It could be considered as a potentially easy spreading species in Spanish natural ecosystems. Indeed, it has already been detected in different areas of Spain (Català et al. 2017; Mora-Sala et al. unpublished data).

*Phytophthora psychrophila* was firstly recovered from soil from *Q. robur*, *Q. petraea* and *Q. ilex* in Bavaria and Southern France (Jung et al. 2002). In 2013, *P. psychrophila* was reported in Comunidad Valenciana (eastern Spain) causing *Q. ilex* and *Q. faginea* dieback in a Mediterranean oak forest (Pérez-Sierra et al. 2013) and it has also been detected in Spanish oak stands (Català et al. 2017; Mora-Sala et al. unpublished data). In the present study, *P. psychrophila* behaved as an aggressive pathogen, which caused dieback of the root system, mainly the fine roots, showed necrotic lesions and open cankers. The results observed agreed with a previous pathogenicity test performed on *Q. ilex* (Pérez-Sierra et al. 2013) and the symptoms obtained were similar to those observed by Jung et al. (2002) in a soil infestation test conducted on *Q. robur* seedlings.

*Phytophthora quercina* is a proven pathogen of oak, widespread in oak-dominated ecosystems (Hansen and Delatour 1999; Jung et al. 1999; Vettraino et al. 2002; Balci and Hamschlager 2003a, 2003b; Pérez-Sierra et al. 2013; Català

et al. 2017; Mora-Sala et al. unpublished data). *Phytophthora quercina* is considered a fine root nibbler, which causes major loses of fine roots weakening the tree progressively but effectively (Jung et al. 1999; Jönsson et al. 2003, Corcobado et al., 2017). Tsao (1990) stated that a tree can have substantial loss of fine roots without showing above-ground symptoms. Our results concur with this description, showing a pathogenic behaviour rotting feeder roots and causing small necrotic lesions and cankers. In this context, it can be hypothesized that a decrease in survival rate of inoculated seedlings would have occurred if the test lasted longer. As observed with *P. nicotianae*, La Yesa seedlings resulted to be more susceptible to the pathogen than Cáceres seedlings.

*Phytophthora megasperma* is considered an opportunistic pathogen and has been isolated and detected in declining oak forests (Hansen and Delatour 1999; Jung et al. 2000; Vettraino et al. 2002; Pérez-Sierra et al. 2013; Mora-Sala et al. unpublished data). The study shows a reduction of the root system and a limited development of the aerial tissues and survival rates were lower than when the other *Phytophthora* species were inoculated. *Phytophthora megasperma* behaves in a similar way to *P. quercina* and this result is similar to the one obtained previously by Pérez-Sierra et al. (2013).

In the present study, different parameters were evaluated to assess the pathogenicity of *Phytophthora* species on *Q. ilex*, and it is remarkable that most of these parameters agree in the results. Both Spanish acorn origins tested behaved the same way in terms of MI, fine root rot, defoliation and survival rates. Nevertheless, the seedlings from both acorn origins diverge in terms of weight of the aerial tissues, stem length and weight of the main roots. The results obtained showed that the McKinney index or the survival function were suitable to assess *Phytophthora* pathogenicity tests.

This pathogenicity test demonstrates that *Q. ilex* was susceptible to a range of *Phytophthora* species, apart from *P. cinnamomi*. The *Phytophthora* species tested are well known nursery pathogens affecting a broad range of host plants including woody hosts, such as *Quercus* species (Jung et al. 2016). The present and previous studies demonstrated that several *Phytophthora* species constituted a threat to *Quercus* ecosystems. The relevance of this group of plant pathogens and the increasing number of hosts that are emerging in different scenarios highlights the need for improving the control of plant material. In this

context, the nursery industry and international plant trade should implement effective phytosanitary measures to avoid *Phytophthora* dispersal to naïve natural ecosystems and geographical areas where the pathogen is not present.

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# Chapter 6

## The use of qPCR reveals a high frequency of *Phytophthora quercina* in two Spanish holm oak areas

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# Chapter 6


## Abstract

The struggling Spanish holm oak woodland situation associated with *Phytophthora* root rot has been studied for a long time. *Phytophthora cinnamomi* is considered the main, but not the only species responsible for the decline scenario. This study verifies the presence and/or detection of *Phytophthora* species in two holm oak areas of Spain (southwestern "dehesas" and northeastern woodland) using different isolation and detection approaches. Direct isolation and baiting methods in declining and non-declining holm oak trees revealed *Phytophthora cambivora*, *Phytophthora cinnamomi*, *Phytophthora gonapodyides*, *Phytophthora megasperma*, and *Phytophthora pseudocryptogea* in the dehesas, while in the northeastern woodland, no *Phytophthora* spp. were recovered. Statistical analyses indicated that there was not a significant relationship between the *Phytophthora* spp. isolation frequency and the disease expression of the holm oak stands in the dehesas. *Phytophthora quercina* and *P. cinnamomi* TaqMan real-time PCR probes showed that both *P. cinnamomi* and *P. quercina* are involved in the holm oak decline in Spain, but *P. quercina* was detected in a higher frequency than *P. cinnamomi* in both studied areas. Thus, this study demonstrates that molecular approaches complement direct isolation techniques in natural and seminatural ecosystem surveys to determine the presence and distribution of *Phytophthora* spp. This is the first report of *P. pseudocryptogea* in Europe and its role in the holm oak decline should be further studied.


## Introduction

Holm oak (*Quercus ilex* L.) grows spontaneously throughout the Mediterranean basin, from the Iberian Peninsula to Turkey in the North and from Morocco to Tunisia in the South, having its optimum growing conditions in the Western Mediterranean regions [1]. Holm oak is a low nutrient demanding species, which prefers dry soils situated in Spain from sea level up to 2000 m high, although the most dense holm oak forests' altitude ranges from 200 to 800 m. This species is well adapted to Mediterranean xeric conditions, with an early active taproot development and little branching at the expense of shoot development [2].

In Spain, holm oak is the most abundant evergreen *Fagaceae* tree species, covering almost all Spanish provinces except the Canary Islands and Galicia regions, where it is scarce [1]. About 2.8 M ha of the Spanish forestry surface are holm oak woodlands and 2.4 M ha are oak rangelands (henceforth called dehesas) (which consist mainly of holm oaks mixed with cork oaks (*Quercus suber* L.), and even a deciduous oak (*Quercus faginea* Lam.)) [3].

Holm oak constitutes a fundamental pillar of the Spanish dehesa, an agro–silvo–pastoral system, benefiting from the use of its fruit mainly for livestock during the autumn season and the grass growing underneath the canopy for grazing. Its wood it is also a valuable asset. In addition, it hosts migrant birds from Central and Northern Europe during the winter season. This complex system is suffering a significant decline due to biotic and abiotic factors [4,5]. The Spanish dehesas' decline associated with *Phytophthora* root rot has been studied since the end of the 20th century [6–11]. *Phytophthora cinnamomi* Rands. is considered the main pathogen responsible for the decline of this ecosystem [4,7–10,12,13], but it is not the only *Phytophthora* species infecting holm oaks [14–16].

On the other hand, Spanish natural oak woodlands are also undergoing this decline caused by *Phytophthora* spp. [17,18]. Several studies across Europe demonstrate the association of declining oak woodlands with *Phytophthora quercina* T. Jung, among other species, causing root infections [19–24]. In addition, abiotic factors, such as increasing temperatures and water stress, are being enhanced by climate changing conditions, which have a negative impact on the tree health status, weakening the stands and making holm oaks more susceptible to *Phytophthora* and *Pythium* infection [9,24–27]. Moreover, in view of the lack of regeneration of the stands, reforestations and afforestations are conducted with nursery material, with the consequent risk of introducing alien *Phytophthora* species to natural ecosystems [14,28–31].

Some *Phytophthora* species infect plants without causing external symptoms and this plant material is transported worldwide, allowing pathogens to be disseminated without generating any alert at the inspection points [30,32]. Denman *et al.* [33] reported that leaves from holm oak and rhododendron saplings remained asymptomatic when they were infected with *Phytophthora ramorum* Werres, De Cock, and Man in't Veld and *Phytophthora kernoviae*

Brasier, Beales, and S.A. Kirk, two invasive species affecting ornamental and natural ecosystems. In 2006, imported ornamental *Grevillea* plants, which were asymptomatic, were found to be infected with *Phytophthora niederhauserii* Z.G. Abad and J.A. Abad [30]. Thus, visual screening for monitoring *Phytophthora* without complementary tests is not an appropriate management tool. The direct isolation of *Phytophthora* species on semiselective media from affected tissue or baiting techniques do not always generate quick and sensitive results, making it difficult to accurately monitor forest areas [5,20]. Economic and environmental losses caused by *Phytophthora* worldwide [31,34,35] require the use of all available techniques to detect and identify invasive species as quickly as possible. Combining direct isolation and baiting techniques with molecular tools, such as quantitative real-time PCR, increases the specificity, reproducibility, and sensitivity of the assessments, adding efficiency and accuracy to the diagnosis, an essential part of forest management strategies.

The aim of this study was to verify the presence of *Phytophthora* species in the holm oak rhizosphere in southwestern Spanish dehesas and in a northeastern Spanish holm oak woodland. In addition, the association between the *Phytophthora* species and the symptomatology of the holm oaks was studied in the dehesas by taking samples from declining and non-declining stands. For this purpose, different *Phytophthora* spp. isolation and detection approaches were performed: Direct isolation on semiselective media and apple and soil baiting using leaf material. Moreover, as *P. cinnamomi* and *P. quercina* are considered among the main pathogens associated with holm oak decline, their presence and relative abundance were studied in the samples using specific TaqMan real-time PCR probes.

## Materials and Methods

### Study Sites and Sampling

Studies were conducted in autumn 2012 and 2013 at 10 and 15 mature dehesas, respectively, located in the Extremadura region (southwestern Spain) (Table 6.1). This region has siliceous soils with *Pyro bourgaeanae-Querceto rotundifoliae sigmetum* vegetation series, and calcareous soils with *Paeonio coriaceae-Querceto rotundifoliae sigmetum* vegetation series, within an altitude ranging from 300 to 600 m [36]. At each site, two different areas were studied: A declining area where three symptomatic trees were randomly selected and a non-declining area with three randomly selected asymptomatic trees. Trees severely affected by aerial pathogens or insect pests were discarded. In the 2012 survey, one soil sample including fine roots from the rhizosphere around the base of each tree was collected (60 samples in total) by making three 20–30 cm deep holes at approximately 1 m distance from the trunk and bulked, obtaining a representative 0.5 kg sample, as described by Pérez-Sierra *et al.* [18] (Table 6.1). In the 2013 survey, sites 1 to 5 were sampled as described above, but in the remaining ten sites, two pooled samples per site were collected (Table 6.1). In sites 6 to 15 from 2013, one pooled sample from 3 declining trees and one pooled sample from 3 non-declining trees were collected at approximately 1 m distance from the trunk of each tree at 20–30 cm depth. Fifty samples in total were collected in 2013.

**Table 6.1.** Description of the survey conducted in 2012 and 2013 in the dehesas of the Extremadura region and in 2013 in the oak woodland of Montseny Biosphere Reserve.

| 2012 Dehesas | | | |
|---|---|---|---|
| **Site** | **Number of Samples** | **X Coordinate** | **Y Coordinate** |
| 1 | 6 | 748324.99 | 4428259.51 |
| 2 | 6 | 248632.54 | 4460613.6 |
| 3 | 6 | 752500 | 4418487 |
| 4 | 6 | 694464.02 | 4431470.91 |
| 5 | 6 | 752500 | 4418487 |
| 6 | 6 | 750948.57 | 4437972.39 |
| 7 | 6 | 742685 | 4456109 |
| 8 | 6 | 753940.25 | 4450439.88 |
| 9 | 6 | 248428 | 4459568 |
| 10 | 6 | 749280 | 4457282 |

| 2013 Dehesas | | | |
|---|---|---|---|
| **Site** | **Number of Samples** | **X Coordinate** | **Y Coordinate** |
| 1 | 6 | 748324.99 | 4428259.51 |
| 2 | 6 | 248632.54 | 4460613.6 |
| 3 | 6 | 752500 | 4418487 |
| 4 | 6 | 694464.02 | 4431470.91 |
| 5 | 6 | 761398,91 | 4425067.28 |
| 6 | 2 | 750948.57 | 4437972.39 |
| 7 | 2 | 742685 | 4456109 |
| 8 | 2 | 753940.25 | 4450439.88 |
| 9 | 2 | 248428 | 4459568 |
| 10 | 2 | 749580 | 4457274 |
| 11 | 2 | 279799 | 4430500 |
| 12 | 2 | 285614.18 | 4435261.32 |
| 13 | 2 | 281973 | 4432507 |
| 14 | 2 | 724766.4 | 4438845.56 |
| 15 | 2 | 246007.77 | 4396525.56 |
| **2013 Montseny Biosphere Reserve (Oak Woodland)** | | | |
| **Site** | **Number of Samples** | **X Coordinate** | **Y Coordinate** |
| MS 2 | 1 | 450134 | 4625428 |
| MS 6 | 1 | 458610 | 4621206 |
| MS 12 | 1 | 457172 | 4620252 |
| MS 13 | 1 | 457197 | 4620078 |
| MS 14 | 1 | 455346 | 4619895 |
| MS 16 | 1 | 454763 | 4621083 |
| MS 18 | 1 | 455161 | 4621632 |
| MS 22 | 1 | 455266 | 4618911 |
| MS 23 | 1 | 454086 | 4619117 |
| MS 24 | 1 | 453979 | 4619403 |
| MS 25 | 1 | 452961 | 4620152 |
| MS 26 | 1 | 452734 | 4619947 |
| MS 27 | 1 | 451398 | 4622040 |
| MS 28 | 1 | 450537 | 4622715 |
| MS 29 | 1 | 449829 | 4622703 |

As differences in the *Phytophthora* spp. have been identified in eastern Spanish holm oak surveys [18], a study area located in northeastern Spain was included in the study. Montseny mountains is a 31,063 ha area located in Catalonia that since 1978 has been a biosphere reserve. It is made up of primarily siliceous rocks, with limestone rocks located on the western slopes of the

mountains [37,38]. *Quercus ilex* is located in the lower altitudes among a *Quercetum-ilicis-galloprovinciale* vegetation series [38,39]. Fifteen holm oak declining stands showing defoliation, dead branches, and dieback symptoms and whose altitude ranged from 293 to 868 m were sampled as described above for 2012 just after a precipitation period in autumn 2013 (Table 6.1).

All the samples from the different surveys were transported to the laboratory, where roots were separated from soil for processing and soil was conserved at 5 °C until processing.

### *Phytophthora* spp. Isolation

Roots from each sample were carefully washed under tap water and blotted on filter paper and direct isolation was performed on CMA-PARPB, as described by Jeffers and Martin [40], with and without the addition of hymexazol. Green apple baits were used for soil isolation. Granny Smith apples were surface disinfested with 95% ethanol. Four perpendicular 1 $cm^2$ holes were cut, filled with soil and remains of fine roots, and moistened with sterile water. These filled holes were sealed with tape and incubated in covered trays at 20 °C. The apples were examined daily until lesions developed. Small tissue fragments from the edge of the lesions were plated on CMA-PARPB with and without hymexazol and incubated at 20 °C in the dark. Oomycete-like colonies grown both from root and soil samples were transferred to potato dextrose agar (PDA) (Biokar-Diagnostics, Beauvais, France) and incubated at 20 °C in the dark for 7 days for further identification. Pure cultures of all putative *Phytophthora* isolates were obtained by transferring single hyphal tips to PDA plates.

Additionally, in the 2013 surveys, soils were also baited using leaflets of *Camellia* sp., *Rhododendron* sp., and *Viburnum* sp., following the methods described by Jung *et al.* [41,42]. Isolations were made using CMA-PARBPH as the selective agar medium [40] and processed as described above.

**Culture DNA Extraction, Sequencing, and Statistical Analyses**

DNA was extracted from pure cultures of putative *Phytophthora* grown on PDA by scraping the mycelium and grinding to a fine powder under liquid nitrogen, using the commercial kit EZNA Plant Miniprep Kit (Omega Bio-Tek, Doraville, GA, USA) following the manufacturer's instructions. Ribosomal DNA ITS amplifications were carried out using the universal primers ITS6 and ITS4 [43,44]. The PCR reaction final volume was 25 µL: PCR buffer 1×, 2.5 mM $MgCl_2$, 200 µM each dNTP, 0.4 µM of each primer, 1 U of DNA Taq polymerase (Dominion MBL, Córdoba, Spain), and 1 µL of template DNA. All PCR reactions were performed in a PTC 200 thermocycler (MJ Research Inc., Waltham, MA, USA) with the following parameters: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; and 72 °C for 10 min. Amplified products were sequenced at Macrogen Europe (Amsterdam, The Netherlands). The isolates were identified to the species level by conducting Basic Local Alignment Search Tool (BLAST) and comparing with the sequence data on international collection databases (*Phytophthora* Database, https://www.phytophthoradb.org and GenBank, https://www.ncbi.nlm.nih.gov/genbank/).

The total number of *Phytophthora* spp. isolates (*Phytophthora* pool) obtained and the number of isolates from each *Phytophthora* species were converted into frequencies relative to the total number of *Phytophthora* isolates recovered in the dehesas surveys. An analysis of variance (ANOVA) was performed with the 2012 and 2013 dehesas' data using a general linear model (GLM) in SAS version 9.0 (SAS Institute, Cary, NC, USA), in order to study the relationship between the frequency and diversity of *Phytophthora* spp. and the symptomatology shown by the trees in the dehesas. Mean values were compared using the Fischer´s least significant difference (LSD) procedure at $p$-value = 0.05.

## Environmental Samples: DNA Extraction and *P. cinnamomi* and *P. quercina* qPCRs

Roots and soil from both types of holm oak stands were tested with specific TaqMan probes for the main two oak *Phytophthora* pathogens, *P. cinnamomi* and *P. quercina* [16,45]. Each soil sample was passed through a 2 mm sieve to remove the organic matter and gravel. Once it was homogenized, 50–80 g per sample was lyophilized overnight and pulverized using FRITSCH Variable Speed Rotor Mill-PULVERISETTE 14 (ROSH, Oberstein, Germany). DNA was extracted in duplicate with the Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. The root samples were first ground using a mortar and pestle under liquid nitrogen and then extraction was performed from 60 to 80 mg using the Power Plant Pro DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA).

Real-time PCR was performed on a Rotor-Gene Q 5plex HRM (QIAGEN, Hilden, Germany) and data were analyzed with the Software Version 2.0.2. (QIAGEN) following the MIQE guidelines [46]. The primers quercina_F (GGTCTTGTCTGGCGTATGG), quercina_R (AGCTACTTGTTCAGACCGA-AG), and the hydrolysis probe (6-FAM/GCTGTAAAA/ZEN/GGCGGCGG-CTGTTGC/IaBlk-FQ/) designed by Català *et al.* [16] were used to detect *P. quercina* in DNA from all the soil and root samples collected in the study. In addition, *P. cinnamomi* was also tested with the primers P cin FF (CAATTAGTTGGGGGCCTGCT), P cin RF (GCAGCAGCAGCCGTCG), and the P cin hydrolysis probe (TTGACATCGACAGCCGCCGC) [45]. The qPCRs were performed in a total volume of 25 µL using Premix Ex TAQ (Probe qPCR; Takara Biotechnology (Dalian), Co., Ltd., China). Reactions consisted of 12.5 µL Premix Ex Taq (2×), 2.5 µL of primers–probe mix (500 nM of each primer and 250 nM probe), 1 µL of BSA (5 mg/mL) and 2 µL of template DNA. Two-step PCR was performed with the following cycling conditions: 95 °C for 1 min; 45 cycles of 95 °C for 5 s and 60 °C for 45 s for *P. quercina*, while for *P. cinnamomi*, 45 cycles of 95 °C for 5 s and 60 °C for 60 s. Two replicates were performed alongside standard dilution curves of *P. quercina* (isolate Ps-982 from Mediterranean Agroforestry Institute–UPV collection) and *P. cinnamomi* (isolate Ps-727 from Mediterranean Agroforestry Institute–UPV collection). Probe sensitivity was tested with serial dilution of each DNA ranging from 0.2 ng/µL to

2 fg/µL for *P. quercina* DNA; 2 ng/µL *P. cinnamomi* DNA (2 ng/µL) was serially diluted (1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $2:10^6$). Negative samples were diluted and tested again to avoid false negatives.

## Results

### *Phytophthora* spp. Isolation

In the 2012 survey, *Phytophthora* spp. were detected in three dehesas, which represented 30% of the sampled sites. Three isolates of *Phytophthora* were recovered through the apple baiting method, one of each of: *Phytophthora cambivora* (Petri) Buisman (from a non-declining site), *P. cinnamomi*, and *Phytophthora gonapodyides* (H.E. Petersen) Buisman (from declining sites) (Table 6.2).

**Table 6.2.** Number of isolates of *Phytophthora* spp. obtained from *Quercus ilex* roots and soil in the 2012 survey in the dehesas of the Extremadura region according to the symptomatology of the sampled trees and results of the TaqMan real-time PCR assays. Results obtained from samples in each site are grouped according to whether samples were from declining or non-declining trees.

| Site | Symptomatology | Isolates | | | qPCR | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | CIN | | QUE | |
| | | CAM | CIN | GON | Roots | Soil * | Roots | Soil * |
| 1 | d | 0 | 0 | 1 [a] | ndt | 0/3 | ndt | 3/3 |
| 1 | nd | 0 | 0 | 0 | ndt | 2/3 | ndt | 3/3 |
| 2 | d | 0 | 0 | 0 | ndt | 0/3 | ndt | 1/3 |
| 2 | nd | 0 | 0 | 0 | ndt | 0/3 | ndt | 1/3 |
| 3 | d | 0 | 0 | 0 | ndt | 0/3 | ndt | 2/3 |
| 3 | nd | 0 | 0 | 0 | ndt | 1/3 | ndt | 0/3 |
| 4 | d | 0 | 0 | 0 | ndt | 0/3 | ndt | 1/3 |
| 4 | nd | 0 | 0 | 0 | ndt | 0/3 | ndt | 3/3 |
| 5 | d | 0 | 0 | 0 | ndt | 0/3 | ndt | 2/3 |
| 5 | nd | 0 | 0 | 0 | ndt | 1/3 | ndt | 1/3 |
| 6 | d | 0 | 0 | 0 | ndt | 1/3 | ndt | 3/3 |
| 6 | nd | 0 | 0 | 0 | ndt | 0/3 | ndt | 2/3 |

| Site | Symptomatology | Isolates | | | qPCR | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | CIN | | QUE | |
| | | CAM | CIN | GON | Roots | Soil * | Roots | Soil * |
| 7 | d | 0 | 0 | 0 | ndt | 0/3 | ndt | 2/3 |
| 7 | nd | 0 | 0 | 0 | ndt | 0/3 | ndt | 3/3 |
| 8 | d | 0 | 0 | 0 | ndt | 2/3 | ndt | 2/3 |
| 8 | nd | 0 | 0 | 0 | ndt | 0/3 | ndt | 3/3 |
| 9 | d | 0 | 0 | 0 | ndt | 2/3 | ndt | 0/3 |
| 9 | nd | 1 [a] | 0 | 0 | ndt | 1/3 | ndt | 2/3 |
| 10 | d | 0 | 1 [a] | 0 | ndt | 1/3 | ndt | 3/3 |
| 10 | nd | 0 | 0 | 0 | ndt | 0/3 | ndt | 3/3 |

d = declining; nd = non-declining; CAM = *Phytophthora cambivora*; CIN = *Phytophthora cinnamomi*; GON = *Phytophthora gonapodyides*; QUE = *Phytophthora quercina*; [a] = isolated from soil with apple baiting; ndt = not determined; ***** = number of positive samples detected out of the total number of samples.

In 2013, the dehesas were surveyed and the soils baited in addition to the other methods already described for 2012. *Phytophthora* spp. were recovered in the 2013 dehesas survey from 21 holm oak samples, which represented 42% of the total samples, with 20% from declining samples and the remaining 22% from non-declining samples. A total of 165 Oomycetes isolates were obtained in 2013: 59 *Phytophthora* spp. isolates (clustered into four species) and 107 *Pythium* spp. isolates. In 2013, 39% of the *Phytophthora* spp. isolates were recovered from declining sites, and 61% were recovered from non-declining sites (Table 6.3). Regarding the isolation method, 13.4% of the *Phytophthora* spp. isolates were isolated directly from roots, 5.1% from apple baits, and 81.3% from leaf baits. As for the diversity of species obtained in 2013, *P. cinnamomi*, *P. gonapodyides*, *Phytophthora megasperma* Drechsler and *Phytophthora pseudocryptogea* Safaiefarahani, Mostowfizadeh, G.E. Hardy, and T.I. Burgess were isolated (Table 6.3). The range of abundance according to isolation was 39% *P. cinnamomi*, 35.6% *P. gonapodyides*, 20.3% *P. megasperma*, and 5.1% *P. pseudocryptogea*.

**Table 6.3.** Number of isolates of *Phytophthora* spp. obtained from *Q. ilex* roots and soil in the 2013 survey in the dehesas of the Extremadura region according to whether samples were from declining or non-declining trees and results of the TaqMan real-time PCR assays. PCR results obtained from samples in sites 1 to 5 are grouped according to whether samples were from declining or non-declining trees.

| Site | Sympt. | Isolates | | | | qPCR | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | CIN | | QUE | |
| | | CIN | GON | PSC | MEG | Roots * | Soil * | Roots * | Soil * |
| 1 | d | 0 | 1 [b] | 0 | 0 | 0/3 | 2/3 | 2/3 | 3/3 |
| 1 | nd | 4 [r,b] | 8 [r,b] | 0 | 4 [b] | 2/3 | 2/3 | 3/3 | 3/3 |
| 2 | d | 0 | 0 | 0 | 0 | 0/3 | 1/3 | 2/3 | 1/3 |
| 2 | nd | 0 | 3 [b] | 0 | 3 [b] | 1/3 | 2/3 | 1/3 | 3/3 |
| 3 | d | 4 [b] | 0 | 0 | 0 | 3/3 | 2/3 | 0/3 | 0/3 |
| 3 | nd | 0 | 0 | 0 | 0 | 2/3 | 1/3 | 1/3 | 1/3 |
| 4 | d | 0 | 0 | 0 | 2 [b] | 0/3 | 2/3 | 3/3 | 3/3 |
| 4 | nd | 0 | 3 [b] | 0 | 0 | 0/3 | 1/3 | 2/3 | 3/3 |
| 5 | d | 3 [b] | 0 | 0 | 0 | 2/3 | 0/3 | 1/3 | 2/3 |
| 5 | nd | 2 [b] | 6 [b] | 0 | 0 | 1/3 | 1/3 | 3/3 | 3/3 |
| 6 | d | 0 | 0 | 0 | 0 | − | − | + | − |
| 6 | nd | 0 | 0 | 0 | 0 | − | − | − | + |
| 7 | d | 0 | 0 | 0 | 0 | + | + | + | + |
| 7 | nd | 0 | 0 | 0 | 0 | − | − | + | + |
| 8 | d | 0 | 0 | 0 | 0 | + | − | − | + |
| 8 | nd | 0 | 0 | 0 | 0 | + | − | + | + |
| 9 | d | 0 | 0 | 0 | 0 | − | − | − | + |
| 9 | nd | 0 | 0 | 0 | 1 [b] | + | + | + | − |
| 10 | d | 3 [r,b] | 0 | 0 | 0 | + | + | + | − |
| 10 | nd | 0 | 0 | 0 | 0 | − | + | − | − |

| Site | Sympt. | Isolates | | | | qPCR | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | CIN | | QUE | |
| | | CIN | GON | PSC | MEG | Roots * | Soil * | Roots * | Soil * |
| 11 | d | 1 [b] | 0 | 0 | 0 | − | + | + | − |
| 11 | nd | 0 | 0 | 0 | 0 | − | − | + | + |
| 12 | d | 0 | 0 | 0 | 0 | + | − | − | + |
| 12 | nd | 0 | 0 | 0 | 0 | + | − | − | + |
| 13 | d | 0 | 0 | 0 | 0 | + | + | − | + |
| 13 | nd | 0 | 0 | 0 | 0 | − | − | + | − |
| 14 | d | 0 | 0 | 0 | 1 [b] | − | − | − | − |
| 14 | nd | 0 | 0 | 0 | 0 | − | − | + | + |
| 15 | d | 5 [a,b] | 0 | 2 [b] | 0 | + | + | + | + |
| 15 | nd | 1 [r] | 0 | 1 [a] | 0 | + | − | − | + |

Sympt. = Symptomatology; d = declining; nd = non-declining; CIN = *P. cinnamomi*; GON = *P. gonapodyides*; MEG = *Phytophthora megasperma*; PSC = *Phytophthora pseudocryptogea*; QUE = *P. quercina*; [r] = isolated from roots; [a] = isolated from soil with apple baiting; [b] = isolated from baiting soil with leaves; ***** = number of positive detected samples out of the total number of samples; + = positive; − = negative.

Twenty-three isolates of *P. cinnamomi* were isolated from eight samples in the dehesas in 2013 (Table 6.3); percentages from declining and non-declining samples are shown in Figure 6.1. Twenty-one cultures of *P. gonapodyides* were isolated from five samples, with most of the samples from non-declining sites (Table 6.3, Figure 6.1). Twelve *P. megasperma* isolates were isolated from five samples (Table 6.3), and most of these samples were from non-declining trees (Figure 6.1). Three *P. pseudocryptogea* cultures were isolated from two samples (Table 6.3, Figure 6.1).

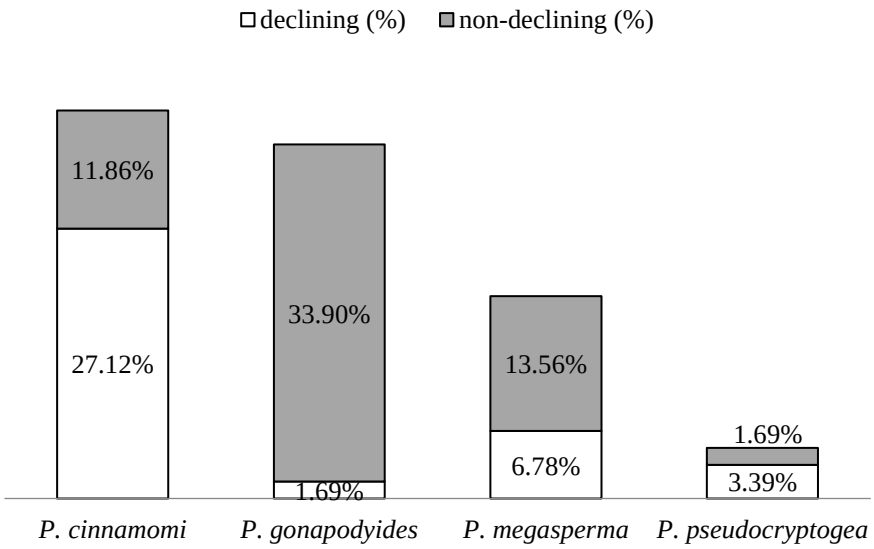


**Figure 6.1.** Percentage of each *Phytophthora* species cultures isolated in the dehesas 2013 survey according to whether the holm oaks were declining or non-declining.

The statistical analysis showed that the factors' symptomatology (*p*-value = 0.3626) and dehesa (*p*-value = 0.3087) were not significant for the frequency of the different *Phytophthora* species present in the dehesas in 2013. Considering the different species isolated separately, only the presence of *P. gonapodyides* was significantly higher in non-declining samples (*p*-value = 0.0366). The presence of either one species or another was not significantly associated with the dehesa factor (*P. cinnamomi p*-value = 0.2277, *P. gonapodyides p*-value = 0.9176 and *P. megasperma p*-value = 0.7029). *P. pseudocryptogea* was only isolated in one dehesa, but from both declining and non-declining sites.

No *Phytophthora* isolates were recovered from the 15 samples of the Montseny Biosphere Reserve by direct isolation on semiselective media from affected tissues and/or the baiting.

**Environmental Samples: Hydrolysis Probes—*P. cinnamomi* and *P. quercina* qPCRs**

The *Phytophthora quercina* standard curve plot showed that the correlation between the Cq-value and the DNA concentration was high ($r^2$ = 0.99966), with an efficiency of 0.90389. For *P. quercina*, the limit of detection (LOD) was established at a DNA concentration of 2 fg/µL.

*Phytophthora quercina* was detected in all the surveyed dehesas in 2012 (65.1% of the samples). Of these, 31.8% came from declining holm oak trees and 33.3% from non-declining trees (Table 6.2). In 2013, *P. quercina* was detected in all the surveyed dehesas (79.6% of the samples) (Table 6.3). A total of 66.7% of the soil samples were positive for *P. quercina*: 27.8% were from declining soil samples, while 38.9% were from non-declining soil samples. A total of 55.6% of the root samples were positive for *P. quercina*: 24.1% were from declining holm oak fine roots, while 31.5% were from non-declining holm oak roots. In the survey conducted in Montseny Biosphere Reserve in 2013, 66.7% of the samples were positive for *P. quercina*, of which 40% were from root samples and 53.3% from soil samples (Table 6.4).

The *Phytophthora cinnamomi* standard curve revealed a high correlation between the Cq-value and the DNA concentration ($r^2$ = 0.99731), with a reaction efficiency of 0.92014. The LOD was established at 4 fg/µL.

*Phytophthora cinnamomi* was detected in seven out of the ten surveyed dehesas in 2012 (19.7% of the samples) (Table 6.2). A total of 12.1% of the soil detections came from declining holm oak trees and 7.6% from non-declining. In 2013, *P. cinnamomi* was detected in 11 dehesas (57.4% of the samples) (Table 6.3). A total of 38.9% of the soil samples were positive for *P. cinnamomi*; 22.2% were from declining trees, while 16.7% were from non-declining trees. A total of 33.9% of the root samples were positive for *P. cinnamomi*, with 22.2% from declining oak fine roots and 14.8% from non-declining oak roots. In Montseny Biosphere Reserve, 53.3% of the samples were positive for *P. cinnamomi*, of which 46.7% were from root samples and 33.3% from soil samples (Table 6.4).

**Table 6.4.** Results of the TaqMan real-time PCR assays obtained from *Q. ilex* roots and soil in the 2013 survey in the oak woodland of Montseny Biosphere Reserve.

| Site | qPCR | | | |
|---|---|---|---|---|
| | CIN | | QUE | |
| | Roots | Soil | Roots | Soil |
| MS 2 | − | + | + | + |
| MS 6 | + | + | − | − |
| MS 12 | + | + | − | − |
| MS 13 | − | − | + | + |
| MS 14 | − | − | − | + |
| MS 16 | − | − | − | + |
| MS 18 | − | − | − | − |
| MS 22 | − | − | + | + |
| MS 23 | + | − | + | + |
| MS 24 | + | + | + | − |
| MS 25 | − | − | − | − |
| MS 26 | + | + | + | − |
| MS 27 | − | − | − | + |
| MS 28 | + | − | − | − |
| MS 29 | + | − | − | + |

CIN = *P. cinnamomi*. QUE = *P. quercina*. + = positive detection; − = negative detection.

## Discussion

This study provides evidence that molecular approaches complement direct isolation methods of *Phytophthora* species from fine roots from holm oak in natural (Montseny Biosphere Reserve) and seminatural (dehesas) ecosystems, confirming that it is not only *P. cinnamomi* that is involved in the holm oak decline in Spain, but *P. quercina* is also present. Moreover, this is the first report of *P. pseudocryptogea* in Europe.

Regarding traditional isolation methods, an increase in *Phytophthora* isolation was observed in the dehesas in 2013 compared with the sampling

conducted in the dehesas in 2012. This was probably not only due to the implementation of the leaf baiting technique, but also because in 2013, isolation of *Phytophthora* spp. from fine roots was more successful. This could be explained by the fact that under favorable environmental conditions, *Phytophthora* spp. infected the tree root systems and rotted fine roots containing the pathogens detached from the plant, so the pathogens can establish again in the soil [47]. *Phytophthora* spp. dispersion requires warm temperatures and free water to produce infective zoospores; if not, they remain as resistant structures in the soil [34]. Furthermore, the efficiency of *Phytophthora* isolation techniques can be compromised by the climatic conditions suffered during the period previous to the survey and by the presence of other microorganisms [8,34]. In fact, the dehesa regions where the surveys were conducted received less precipitation in 2012 than in 2013 [48]. Thus, according to this, the environmental conditions for *Phytophthora* spp. isolation were more favorable in 2013 than in 2012 in the southwestern Spanish dehesas, as they were recovered in 2013 from fresh lesions [49]. Another possible explanation for the low efficiency of *Phytophthora* recovery in Montseny Biosphere Reserve is the presence of other fast-growing species in the samples, such as *Pythium* spp., making the isolation difficult. *Pythium* spp. were recorded in very low numbers in dehesas in 2012 (explained by the absence of favorable environmental conditions), but their presence was very relevant in the 2013 dehesas and in the Montseny Biosphere Reserve surveys. The genus *Pythium* is present in almost all soils and, as the isolation medium used for *Phytophthora* isolation is semiselective [34,40], *Pythium* spp. were also isolated with a high frequency in our study and were able to mask *Phytophthora* spp. presence.

Oak decline, associated with abiotic and biotic factors, has been occurring across Europe during the past decades [4,11,22,28,42,50]. Among the several *Phytophthora* species that have been associated with this decline, *P. cinnamomi* has been considered the main biotic factor responsible for oak mortality in Spain since the 1990s [8,9,51]. In 2013, *P. cinnamomi* was the most frequent species isolated in the infested dehesas, as was expected according to previous studies [7,52]. In addition to *P. cinnamomi*, Corcobado *et al.* [15] reported that *P. gonapodyides* was also involved in oak decline in this region. In our surveys, *P. gonapodyides*, *P. megasperma*, and *P. pseudocryptogea* were recovered at low frequencies, and these species may play an important role as

causal agents of the disease, as reported in other studies, where they were also recovered at low frequencies [14,25,28,52]. There is no statistical evidence to support a differential distribution of *Phytophthora* species among the dehesas in 2013. Moreover, statistical results indicated that there was not a significant relationship between the *Phytophthora* spp. isolation frequency and the symptomatology of the holm oak stands. Our results showed a higher percentage of *Phytophthora* spp. recovery in 2013 from non-declining sites than from declining sites. *Phytophthora cinnamomi*, which was found in six dehesas, either from declining or from non-declining trees, is a primary root pathogen of woody species, considered a hemibiotrophic organism with life strategies which can change from biotrophic to necrotrophic, according to the environmental conditions [53–55]. This species is also present in plant reservoirs and, depending on its behavior, will determine if the plant remains asymptomatic or not [54–57]. Furthermore, *P. cinnamomi* is highly aggressive to holm oaks, as demonstrated previously [11,12,25,58–60]. Tsao [61] stated that a certain percentage of lost roots is required for symptoms to emerge and our results in the 2013 survey provide evidence that a tree symptomatology is not always an indication about the conditions of its root system. Statistical analyses showed that *P. gonapodyides* is more frequent in non-declining stands, in agreement with the results of Vettraino *et al.* [28], while the other species found did not show any statistical pattern. *Phytophthora gonapodyides* is known to attack the small or fine feeder roots [62] and to produce a wilting toxin [41]. Nevertheless, Brasier *et al.* [62] stated that *P. gonapodyides* is often in balance with the unstressed oak root system, but this can change under stress conditions, contributing to a rapid decline. Hansen *et al.* [63] suggested that some *Phytophthora* spp. from clade 6 ecologically related to *Phytophthora chlamydospora* could cause limited root damage with no above ground disease symptoms contributing to the oak decline. *Phytophthora megasperma* isolated in the present study had been previously associated with oak decline [28,42]. Although it is considered a pathogen of herbaceous plants and agricultural trees, it can become a serious problem when the oak balance is broken due to other factors, such as droughts or waterlogging [34]. A similar behavior has been indicated for other *Phytophthora* spp. such as *P. gonapodyides* [10]. *Phytophthora megasperma*, *P. quercina*, *P. psychrophila*, *Phytophthora drechsleri*, and *Phytophthora syringae* have also been associated with oak decline [18,19,25], although these species were not found in our

samples. Nevertheless, *P. pseudocryptogea* in the present study was isolated for the first time in Europe and from a holm oak-rangeland in Spain. This species was described by Safaiefarahani *et al.*, who re-evaluated the *P. cryptogea* complex [64]. Although it was isolated from three soil samples in the present study, from three soil samples, the role of *P. pseudocryptogea* in holm oak decline remains unknown. The pathogenicity of this species in holm oak should be further studied.

The results obtained with the *P. quercina* probe are relevant, since it has always been thought that the holm oak decline in acidic soils in Spain is caused primarily by *P. cinnamomi*. *Phytophthora cinnamomi* was present in a high number of samples in both study locations, as was expected, but surprisingly it was not the most frequent species detected. *Phytophthora quercina* was shown as the most frequent species in this study, and the number of positive samples was higher in both studied areas. Molecular diagnoses provide faster and more sensitive detection of *Phytophthora* spp. [16,45,65–72].

## Conclusions

Different *Phytophthora* species were detected and identified in the study areas, regardless of whether they cause symptoms of decline or not. Further research is needed to clarify the effect of these pathogens in combination and abiotic factors in the oak stands. The implementation of the different direct and baiting isolation techniques for the isolation of *Phytophthora* spp., along with the available molecular detection techniques, allows a better diagnosis and understanding of the role of *Phytophthora* spp. in the holm oak forest areas.

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# Chapter 7

## Diversity of *Phytophthora* species associated with *Quercus ilex* L. in three Spanish regions evaluated by NGS.

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# Chapter 7


## Abstract

The diversity of *Phytophthora* species in declining *Fagaceae* forests in Europe is increasing in the last years. The genus *Quercus* is one of the most extended *Fagaceae* genera in Europe, and *Q. ilex* is the dominant tree in Spain. The introduction of soil-borne pathogens, such as *Phytophthora* in *Fagaceae* forests modifies the microbial community present in the rhizosphere, and has relevant environmental and economic consequences. A better understanding of the diversity of *Phytophthora* spp. associated with *Q. ilex* is proposed in this study by using Next Generation Sequencing (NGS) in six *Q. ilex* stands located in three regions in Spain. Thirty-seven *Phytophthora* phylotypes belonging to clades 1 to 12, except for clades 4, 5 and 11, are detected in this study, which represents a high diversity of *Phytophthora* species in holm oak Spanish forests. *Phytophthora chlamydospora*, *P. citrophthora*, *P. gonapodyides*, *P. lacustris*, *P. meadii*, *P. plurivora*, *P. pseudocryptogea*, *P. psychrophila* and *P. quercina* were present in the three regions. Seven phylotypes could not be associated with known *Phytophthora* species, so they were putatively named as *Phytophthora* sp. Most of the detected phylotypes corresponded to terrestrial *Phytophthora* species but aquatic species from clades 6 and 9 were also present in all regions.


## Introduction

The genus *Phytophthora* comprises nowadays more than 150 species with a broad host range, and includes well-known plant pathogens that are devastating natural ecosystems [1–3]. *Fagaceae* forests, with *Phytophthora* hosts such as *Quercus*, *Castanea*, *Lithocarpus* or *Fagus* species, are an example of declining forests affected by *Phytophthora* worldwide [4–26]. In Europe, *Castanea*, *Fagus* and *Quercus* are the most extended *Fagaceae* genera [13]. The diversity of *Phytophthora* species found in the last thirty years associated with the rhizosphere of these genera, reveals a complex syndrome in the decline of these forests [10,13,27–31]. On one hand, there is the difficulty of diagnosing which *Phytophthora* species present is the primary pathogen. On the other hand, it is also known that depending on which *Phytophthora* species is established first, the damage on the host varies [32]. Furthermore, all *Phytophthora* species

present in the rhizosphere compromise forests regeneration, as they cause seedling damping off [13]. Within the genus *Phytophthora* we can find typical species of riparian ecosystems and/or water bodies that belong to clades 6 and 9. These species behave as saprotrophs or opportunistic organisms, causing in some cases tree decline, nevertheless its role is not well understood [32,33,34,35]. The remaining *Phytophthora* species included in the other clades (1 to 12, except 6 and 9) have a terrestrial life cycle, although they often end up in watercourses by runoff [13,35–37]. Either aquatic or terrestrial *Phytophthora* species are related to *Fagaceae* forests' decline [13].

Global trade increases the risk of unnoticed introductions of alien species into natural ecosystems [38,39]. The introduction of soil-borne pathogens, such as *Phytophthora* in *Fagaceae* forests modifies the microbial community present in the rhizosphere, and has relevant environmental and economic consequences [3]. Soil properties, land use, environmental conditions, the host plants and/or the microbial background determine the microbiota composition from a site [40]. Introduced pathogens have to compete with other microorganisms for available resources, which can lead to a decrease of the native microbiota. Hosts that co-evolved with soil microorganisms can adapt more easily to biotic and abiotic stresses, but a shift in the microbiota structure can trigger the host decline [3,40].

Next generation sequencing (NGS) technologies have stood out as an essential tool for environmental and ecological studies [41,42]. Pyrosequencing the Internal Transcribed Spacer (ITS1) is an efficient and accurate NGS technique for the detection and identification of *Phytophthora* spp. in environmental samples [29,35,43–45]. A better understanding of the diversity of *Phytophthora* spp. associated with a host can be provided by metagenomics, even if these *Phytophthora* spp. are not the most prevalent pathogens [3,45–47]. Nevertheless, a holistic study, combining biological and molecular identification tools, can substantially improve the diagnosis, because in many cases establishing species boundaries via molecular methods it is not an easy task [2,39,43]. In this context, it is interesting to note that samples from the current study were previously subjected to traditional isolation, baiting and real-time polymerase chain reaction (PCR) methods to detect *Phytophthora* spp. [48]. The objective of the present study was to unravel the *Phytophthora* community present in these previously studied areas using NGS technology. Thus, the

diversity and abundance of *Phytophthora* spp. in six holm oak forests located in southwestern and eastern Spain were investigated using an amplicon pyrosequencing approach and the implications are further discussed.

## Materials and Methods

### Study Site and Sampling

The six study areas are holm oak forests located in three different Spanish regions: Holm oak rangelands ("dehesas") in Extremadura in southwestern Spain (province of Cáceres), four holm oak stands in the Comunidad Valenciana region in eastern Spain (two in Valencia province, one in Castellón province and one in Alicante province) and one holm oak stand in Cataluña in northeastern Spain (Barcelona province). Samplings were conducted in the autumn (fall) and winter season in different years (2012–2015), and in some areas, it was repeated for two consecutive years.

Soil samples (0.5–1 kg approx.) were collected in all the surveys, consisting in a mixture of four subsamples taken from four different points 1 m around the selected holm oak. In the surveys conducted from 2013 to 2015, along with the soil samples, roots from the rhizosphere were also taken to be analyzed. Soil and roots samples were conserved at 5 °C until DNA extraction was performed. Baiting with leaflets of *Rhododendron* sp., *Viburnum tinus* L, *Quercus ilex* L, *Quercus suber* L, *Ceratonia siliqua* L. and/or *Dianthus caryophyllus* L. petals was performed as described in a previous study [48], and the vegetal material from the baitings was conserved at -80 °C until DNA extraction.

In the Extremadura region in 2012, 60 soil samples from the declining and non-declining *Q. ilex* rhizosphere were collected from 10 "dehesas" during the autumn. In 2013, 54 soil and root samples and 216 baiting samples from 15 "dehesas" were analyzed. In the Comunidad Valenciana region, during 2014 and 2015, holm oak stands were surveyed, generating 26 soil and roots samples and 104 baiting samples from declining trees. In the Cataluña region in 2013, 15 soil and root samples and 45 baiting samples were processed from declining holm oaks.

**DNA Extraction, PCR, and Preparation of the Amplicon Libraries**

Each soil sample was passed through a 2 mm sieve to remove the organic matter and gravel. Once it was homogenized, 50–80 g per subsample was lyophilized overnight and pulverized using a FRITSCH Variable Speed Rotor Mill-PULVERISETTE 14 (ROSH, Oberstein, Germany). DNA from each sample was extracted by duplicate with the Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer´s protocol. Roots and baiting vegetal material samples were firstly ground using a mortar and pestle under liquid nitrogen.

Healthy leaflets of the different vegetal species used as baitings were included as negative controls. Once homogenized, DNA extraction was performed from 60–80 mg of material per sample using the Power Plant Pro DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA).

Amplicon libraries were generated with a nested polymerase chain reaction (PCR) according to the methodology described by Català *et al.* [35]. The expected size of the amplicons ranged from 280 up to 450 bp. The duplicate amplicons obtained from each sample were pooled if they were positive. Negative samples were retried, firstly diluting 10 times the first round PCR product for the second round PCR. If the retried samples kept on being negative, DNA was diluted ten times, and nested PCR was performed without diluting the first round product. Positive amplicons were double purified using the Agencourt AMPureXP Bead PCR Purification protocol (Beckman Coulter Genomics, MA, USA). Samples were sequenced on the GS Junior 454 system (Roche 454 Life Science, Brandford, CT, USA) at the genotyping service facility from the Universitat de València (Burjassot, Spain).

### Bioinformatics Processing and Statistical Analysis

The sequence reads were processed as described in Català *et al.* [35]. MOTU (Molecular Operational Taxonomic Unit) clustering of the reads was done with the 90% length coverage threshold and a 99% score coverage threshold using BLASTCLUST software [49]. The consensus sequences of the MOTUs were identified using BLAST tool in the *Phytophthora* Database [50], the MegaBLAST tool from the GenBank database [49] and a customized database. Afterwards, our query sequences from each survey and type of material (soil/vegetal) were aligned separately using MUSCLE [51]. Phylogenetic analyses consisting of maximum likelihood were performed in MEGA 6.06 [52] with the suggested suitable model, where gaps and missing data were treated as complete deletions. The robustness of the topology was evaluated by 1000 bootstrap replications [53].

A *Phytophthora* operational taxonomic unit (OTU) table was created excluding other genus reads, unaligned sequences and singletons. OTUs represented globally by less than five reads were removed [54]. The resulting quality-filtered dataset was used for the assessment of diversity and richness. The relationship in OTU composition among samples were investigated by calculating Bray Curtis metrics, and visualized by means of PCoA plots. Samples without *Phytophthora* reads were excluded for the diversity analyses. Biodiversity indices and principle statistics analyses on taxonomic profiles were analyzed in the tool MicrobiomeAnalyst [55].

## Results

### Sequencing Results

In the Extremadura region, 66,732 total ITS1 sequences were generated from the pyrosequenced soil samples collected in 2012. These had an average length of 308 bp, and only 61,576 high quality sequences were considered for the analysis after trimming, and excluding singletons; 48.7% of the sequences were identified from declining trees, and 51.3% from non-declining trees.

From samples collected in 2013 in the Extremadura region, 377,799 ITS1 sequences reads were obtained with an average length of 302 bp. After trimming

and excluding singletons, 317,961 high quality sequences were used for the analysis; 103,333 from the root samples (45.05% from declining trees and 54.95% from non-declining trees), 56,112 sequences from the soil samples (45.3% from declining trees and 54.7% from non-declining trees) and 158,516 sequences from the baiting samples (51.68% from declining trees and 48.32% from non-declining trees). No *Phytophhtora* phylotypes are detected from the negative baiting controls.

In the Cataluña region, 63,105 total ITS1 sequences were generated from the pyrosequenced samples. These had an average length of 283 bp, and only 55,230 high quality sequences were considered for the analysis after trimming and excluding singletons (28,381 from roots, 9887 from soils and 16,962 from baitings).

In the Comunidad Valenciana region, 177,398 ITS1 raw sequences with an average length of 309 bp were obtained. After trimming and excluding singletons, 78,962 high quality sequences were considered for the analysis (3,977 from sampled roots, 18,861 from soils and 56,124 from baitings).

## Identification of *Phytophthora* Phylotypes

Extremadura Region

A total of 33 different *Phytophthora* phylotypes are identified in the Extremadura region during the surveys conducted in 2012 and 2013 (Table 7.1). Detected phylotypes belong to all clades except for clades 4, 5 and 11, the clade 6 having the highest number of *Phytophthora* phylotypes detected (Table 7.1). In 2012, 20 *Phytophthora* phylotypes were obtained, and 15 of them identified to the species level: *Phytophthora bilorbang* Aghighi, G.E. Hardy, J.K. Scott & T.I. Burgess, *Phytophthora botryosa* Chee, *Phytophthora cambivora* (Petri) Buisman, *Phytophthora chlamydospora* Brasier and Hansen, *Phytophthora cinnamomi* Rands., *Phytophthora cryptogea* Pethybr. and Laff., *Phytophthora gemini* Man in't Veld, Rosendahl, Brouwer and de Cock, *Phytophthora gonapodyides* (H.E. Petersen) Buisman, *Phytophthora hydropathica* Hong and Gallegly, *Phytophthora lacustris* Brasier, Cacciola, Nechwatal, Jung and Bakonyi, *Phytophthora lagoariana*, *Phytophthora multivora* Scott and Jung, *Phytophthora plurivora* Jung and Burgess, *Phytophthora psychrophila* Jung and Hansen,

*Phytophthora quercina* Jung and *Phytophthora riparia* Reeser, Sutton and Hansen. One phylotype belong to complex *Phytophthora uliginosa-europaea* as the ITS1 region used in the assay could not resolve its identity. Three phylotypes do not correspond to any *Phytophthora* sequence included in databases; therefore, these putative new species are named *Phytophthora* sp.1, *Phytophthora* sp.2 and *Phytophthora* sp.3.

In 2013, 25 *Phytophthora* phylotypes were recovered in Extremadura and 22 of them were identified to the species level: *Phytophthora* taxon *ballota*, *P. bilorbang*, *P. chlamydospora*, *P. cinnamomi*, *Phytophthora citrophthora* (R.E. Sm. and E.H. Sm.) Leonian, *Phytophthora clandestina* Taylor, Pascoe and Greenhalgh, *P. cryptogea*, *Phytophthora gallica* Jung and Nechwatal, *P. gonapodyides*, *P. hydropathica*, *Phytophthora insolita* Ann and Ko, *Phytophthora* sp. *kelmania*, *P. lacustris*, *Phytophthora lactucae* Bertier, Brouwer and de Cock, *P. lagoariana*, *Phytophthora meadii* McRae, *Phytophthora megasperma* Drechsler, *Phytophthora* sp. *palustris*, *P. plurivora*, *Phytophthora pseudocryptogea* Safaiefarahani, Mostowfizadeh, G.E. Hardy, and T.I. Burgess, *P. psychrophila*, *P. quercina* and *Phytophthora rosacearum* Hansen and Wilcox. The ITS1 region used in the assay could not resolve the identity of one *Phytophthora* phylotype complex: *Phytophthora uliginosa-europaea*. One *Phytophthora* phylotype does not match with any *Phytophthora* sequence included in the databases, therefore this putative new species is named as *Phytophthora* sp.4.

**Table 7.1.** *Phytophthora* phylotypes detected by next generation sequencing (NGS), based on the Internal Transcribed Spacer (ITS1) region.

| Phylotypes | Clade | Spanish Regions | | | | | |
|---|---|---|---|---|---|---|---|
| | | Extremadura | Cataluña | Comunidad Valenciana | | | |
| | | | | Font | Hunde | Pina | Alcublas |
| BAL | 1 | ✓ | | ✓ | ✓ | | |
| BIL | 6 | ✓ | | | | | |
| BOT | 2 | ✓ | | | | | |
| CAM | 7 | ✓ | | | | | |
| CHL | 6 | ✓ | ✓ | | | ✓ | ✓ |
| CIN | 7 | ✓ | ✓ | | | | |
| CIP | 2 | ✓ | ✓ | | | ✓ | |
| CLA | 1 | ✓ | | | | | |
| CRY | 8 | ✓ | | | | | |
| GAL | 10 | ✓ | | | | | |
| GEM | 6 | ✓ | | | | | |
| GON | 6 | ✓ | ✓ | | | | ✓ |
| HYD | 9 | ✓ | ✓ | | | | |
| INS | 9 | ✓ | ✓ | | | | |
| KEL | 8 | ✓ | | | | ✓ | |
| LAC | 6 | ✓ | ✓ | | | ✓ | ✓ |
| LCT | 8 | ✓ | | | | | |
| LAG | 9 | ✓ | ✓ | | | | |
| MEA | 2 | ✓ | ✓ | | | ✓ | |
| MEG | 6 | ✓ | | | | | ✓ |
| MUL | 2 | ✓ | | | | | |
| PAS | 9 | ✓ | | | | | |
| PLU | 2 | ✓ | ✓ | | | ✓ | ✓ |
| PSC | 8 | ✓ | ✓ | | ✓ | ✓ | ✓ |
| PSY | 3 | ✓ | ✓ | ✓ | ✓ | | ✓ |
| QUE | 12 | ✓ | ✓ | ✓ | ✓ | ✓ | |
| RIP | 6 | ✓ | | | | | |
| ROS | 6 | ✓ | | | | | |
| TEN | 1 | | | | | | ✓ |
| ULIG-EUR | 7 | ✓ | | ✓ | | | |
| SP.1 | 6 | ✓ | | | | | |
| SP.2 | 7 | ✓ | | | | | |
| SP.3 | 8 | ✓ | | | | | |
| SP.4 | 1 | ✓ | | | | | |
| SP.5 | 7 | | ✓ | | | | |
| SP.6 | 3 | | | ✓ | | | |
| SP.7 | 1 | | | | | | ✓ |

BAL, *P.* taxon *ballota*; BIL, *P. bilorbang*; BOT, *P. botryosa*; CAM, *P. cambivora*; CHL, *P. chlamydospora*; CIN, *P. cinnamomi*; CIP, *P. citrophthora*; CLA, *P. clandestina*; CRY, *P. cryptogea*; GAL, *P. gallica*; GEM, *P. gemini*; GON, *P. gonapodyides*; HYD, *P. hydropathica*; INS, *P. insolita*; KEL, *P.* sp. *kelmania*; LAC, *P. lacustris*; LCT, *P. lactucae*; LAG, *P. lagoariana*; MEA, *P. meadii*; MEG, *P. megasperma*; MUL, *P. multivora*; PAS, *P.* sp. *palustris*; PLU, *P. plurivora*; PSC, *P. pseudocryptogea*; PSY, *P. psychrophila*; QUE, *P. quercina*; RIP, *P. riparia*; ROS, *P. rosacearum*; TEN, *P. tentaculata*; ULIG-EUR, *P. uliginosa-P. europaea*; SP.1-SP.7, new phylotypes found not identified to the species level.

Cataluña Region

Fourteen *Phytophthora* phylotypes are identified in the Cataluña region during the survey conducted in 2013, belonging to clades 2, 3, 6, 7, 8, 9 and 12 (Table 7.1). Thirteen phylotypes are identified to the species level: *P. chlamydospora*, *P. cinnamomi*, *P. citrophthora*, *P. gonapodyides*, *P. hydropathica*, *P. insolita*, *P. lacustris*, *P. lagoariana*, *P. meadii*, *P. plurivora P. pseudocryptogea*, *P. psychrophila* and *P. quercina*. One *Phytophthora* phylotype does not match with any *Phytophthora* sequence included in the databases, therefore this putative new species is named *Phytophthora* sp.5.

Comunidad Valenciana Region

The *Phytophthora* phylotypes identified in Comunidad Valenciana during the surveys conducted in the period 2014-2015 is shown in Table 7.1. Sixteen *Phytophthora* phylotypes are identified in Comunidad Valenciana belonging to clades 1, 2, 3, 6, 7, 8 and 12 (Table 7.1). Thirteen phylotypes are identified to the species level: *P.* taxon *ballota*, *P. chlamydospora*, *P. citrophthora*, *P. gonapodyides*, *P.* sp. *kelmania*, *P. lacustris*, *P. meadii*, *P. megasperma*, *P. plurivora P. pseudocryptogea*, *P. psychrophila*, *P. quercina* and *Phytophthora tentaculata* Kröber and Marwitz. The ITS1 region used in the assay could not resolve the identity of one *Phytophthora* phylotype complex: *P. uliginosa-europaea*. Two *Phytophthora* phylotypes do not match with any *Phytophthora* sequences included in the databases and thus, these putative new species are named as *Phytophthora* sp.6 and *Phytophthora* sp.7.

## *Phytophthora* Diversity

The relative abundance of *Phytophthora* species detected from 2013 to 2015 in the three studied regions is shown in Figure 7.1A. The Extremadura region presents the greatest diversity of *Phytophthora* species. According to the type of material used for *Phytophthora* isolation, baiting material has the highest relative abundance of *Phytophthora* species, followed by roots and soils (Figure 7.1B).

Regarding symptomatology, non-declining holm oak samples in the Extremadura present a higher diversity of *Phytophthora* species either in the 2013 survey or analyzing 2012 and 2013 together, than samples from declining trees (Figure 7.2A, 7.2C), but this diversity was slightly higher in declining samples in the 2012 survey (Figure 7.2B).

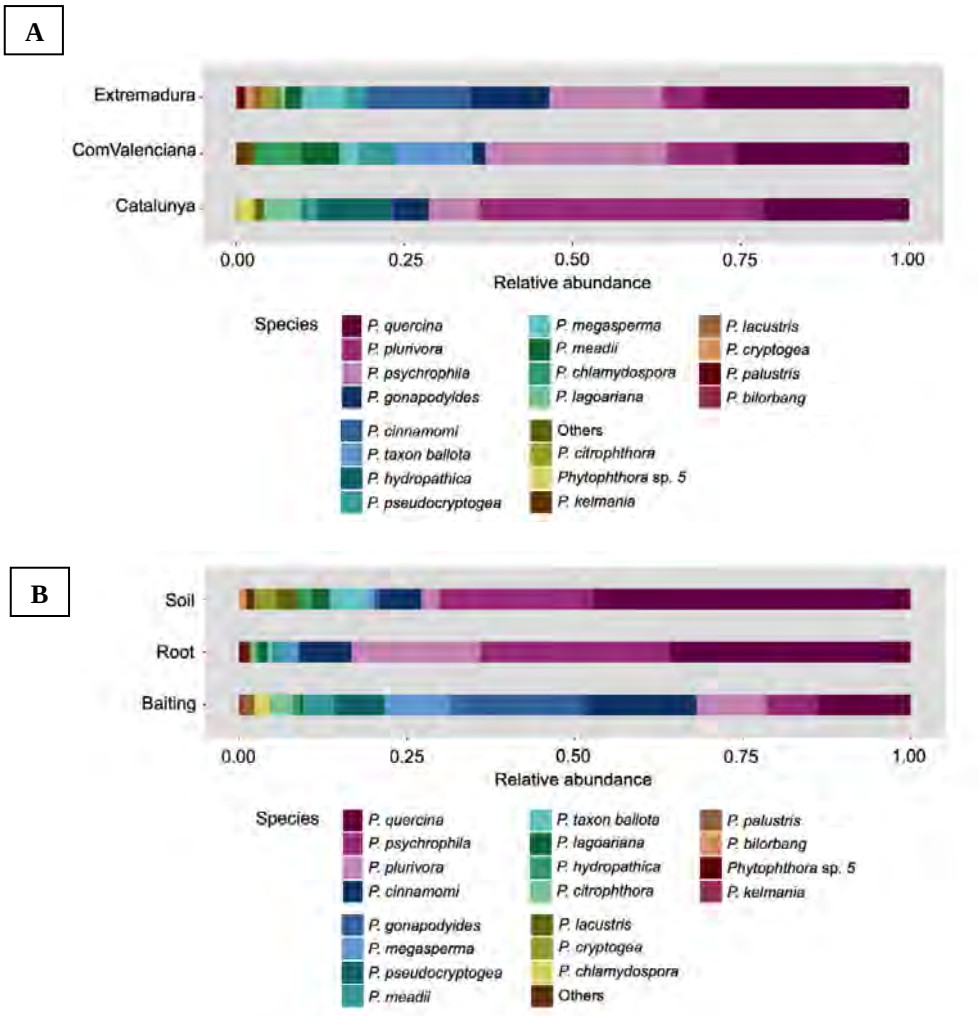


**Figure 7.1.** Relative abundance of *Phytophthora* species detected in the three studied regions from 2013 to 2015 showing more than 1% relative abundance of all reads. Species representing less than 1% of the total reads are grouped in "others". (**A):** According to the factor region; Extremadura, Comunidad Valenciana (ComValenciana) and Cataluña (Catalunya). (**B):** According to the factor type of material; soils, roots and baitings. Results of the survey performed in Extremadura in 2012 are excluded in this analysis, since only soil samples were included.

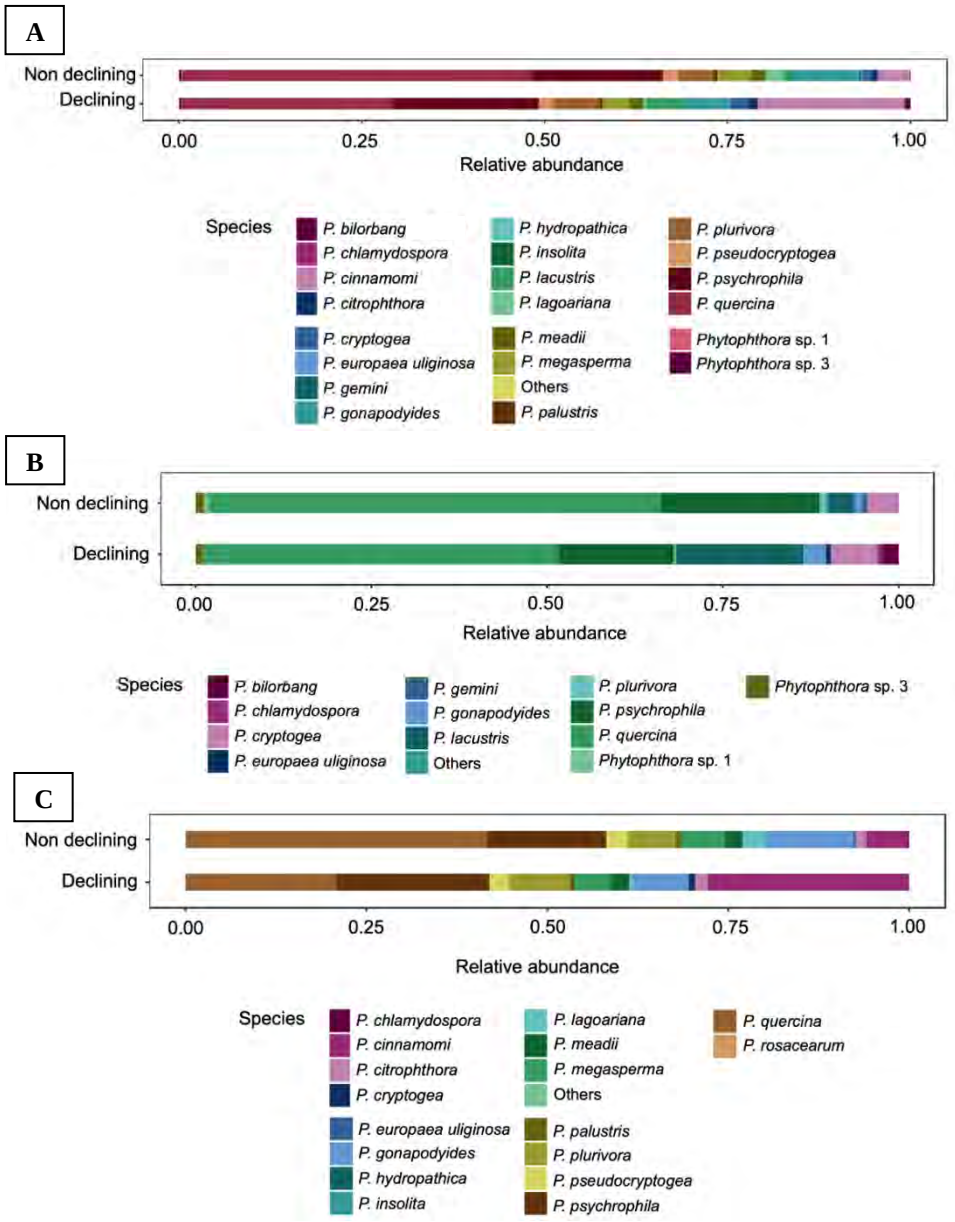


**Figure 7.2.** Relative abundance of *Phytophthora* species showing more than 1% relative abundance of all reads in Extremadura region according to the factor symptomatology. Species representing less than 1% of the total reads are grouped in others. (**A):** During the surveys conducted in 2012 and 2013. (**B):** Survey conducted in 2012. (**C):** Survey conducted in 2013.

The ANOVA shows that the alpha diversity was significantly higher in the Extremadura region, followed by Comunidad Valenciana and Cataluña (Figure 7.3). Both the Chao 1 estimator (*p value* < 0.05) and the Shannon index (*p value* < 0.01) were significant according to the factor region (Table 7.2). Regarding the factor material, the Shannon index was significant (*p value* = 0.033), showing greater diversity of *Phytophthora* species when using baiting material, while the Chao 1 estimator was non-significant (*p value* = 0.091) (Table 7.2).

**Table 7.2.** Analysis of variance (ANOVA) table for the alpha diversity of *Phytophthora* species detected in the study.

| **α Diversity** | | | |
|---|---|---|---|
| | **Region** | **Material** | **Region × Material** |
| **Chao 1 estimator** | $F_{2,245} = 10.8$<br>$p \leq 0.01$ | $F_{2,196} = 1.9$<br>$p = 0.091$ | $F_{4,188} = 5.5$<br>$p \leq 0.01$ |
| **Shannon index** | $F_{2,245} = 7.1$<br>$p \leq 0.01$ | $F_{2,196} = 2.3$<br>$p = 0.033$ | $F_{4,188} = 4.7$<br>$p \leq 0.01$ |

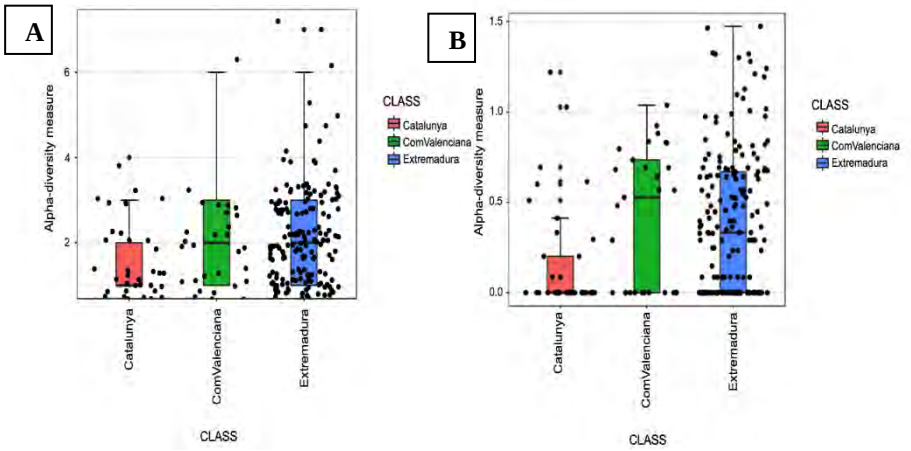


**Figure 7.3.** Boxplot showing alpha diversity measures of the *Phytophthora* diversity according to the factor region: Extremadura, Comunidad Valenciana (ComValenciana) and Cataluña (Catalonia). (**A)**: Chao 1 estimator (*p value* < 0.05). (**B)**: Shannon index (*p value* < 0.01).

In the Extremadura region where the factor symptomatology was studied, in 2012 alpha diversity measured by the Chao1 estimator results was significant for the factor symptomatology (*p value* = 0.0218), but the Shannon Index was non-significant (*p value* = 0.1303). Nevertheless, in 2013 the ANOVA shows that both estimators were significant for the factor material and non-significant for the factor symptomatology (Table 7.3).

**Table 7.3.** Analysis of variance (ANOVA) tables for the alpha diversity of *Phytophthora* species detected in Extremadura dehesas during the surveys conducted in 2012 and 2013.

| Survey | α Diversity | | | | | |
|---|---|---|---|---|---|---|
| | Chao 1 estimator | | | Shannon index | | |
| | Material | Symptoms | Material | Material | Symptoms | Material |
| 2012 | ndt | $F_{1,47}$= 2.39 | ndt | ndt | $F_{1,47}$= 1.54 | ndt |
| 2013 | $F_{2,126}$ = 6.03 | $F_{1,127}$ = 1.23 | $F_{2,123}$ = 2.7 | $F_{2,126}$ = 4.95 | $F_{1,127}$ = 0.83 | $F_{2,123}$ = 2.4 |

ndt = not determined.

The principal coordinates analysis (PCoAs) of Bray Curtis show that the *Phytophthora* population in Extremadura had more variation in the number of species present, although there were similarities with the populations of the other two regions (Figure 7.4A). There are no significant differences in the diversity of *Phytophthora* species detected in the type of material used or the symptomatology of the sampled trees (Figures 7.4B, 7.4C).

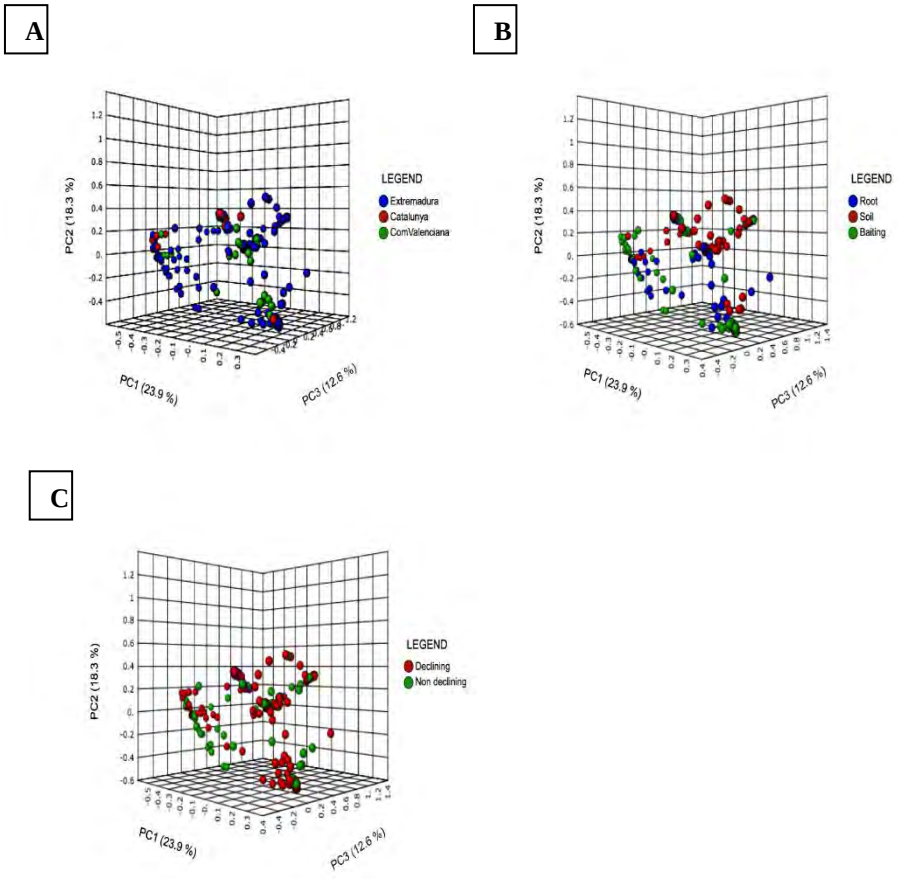


**Figure 7.4.** Principal Coordinate Analysis (PCoA) based on Bray Curtis dissimilarity metrics, showing the distance in the *Phytophthora* spp. composition according to the different factors studied. (**A)**: Regions surveyed; Extremadura, Comunidad Valenciana (ComValenciana) and Cataluña (Catalunya). (**B)**: Type of material used for the detection; roots, soils and baitings. (**C)**: Symptomatology of the holm oaks sampled: declining and non-declining.

Nine *Phytophthora* phylotypes were detected in the three regions (24.3%): *P. citrophthora*, *P. meadii*, *P. plurivora*, *P. psychrophila*, *P. quercina*, *P. chlamydospora*, *P. gonapodyides*, *P. lacustris* and *P. pseudocryptogea* (Figure 7.5A). Regarding the type of material, 11 *Phytophthora* phylotypes were common to root, soil and baiting material (28.9%): *P. plurivora*, *P. psychrophila*, *P. quercina*, *P. chlamydospora*, *P. gonapodyides*, *P. lacustris*, *P. megasperma*, *P. cinnamomi*, *P.* uliginosa - europaea, *P. insolita* and *P. lagoariana* (Figure 7.5B). Finally, 20 *Phytophthora* phylotypes were present in declining and non-declining holm oak trees, which represent 54.05% of the detected *Phytophthora* phylotypes (Figure 7.5C).

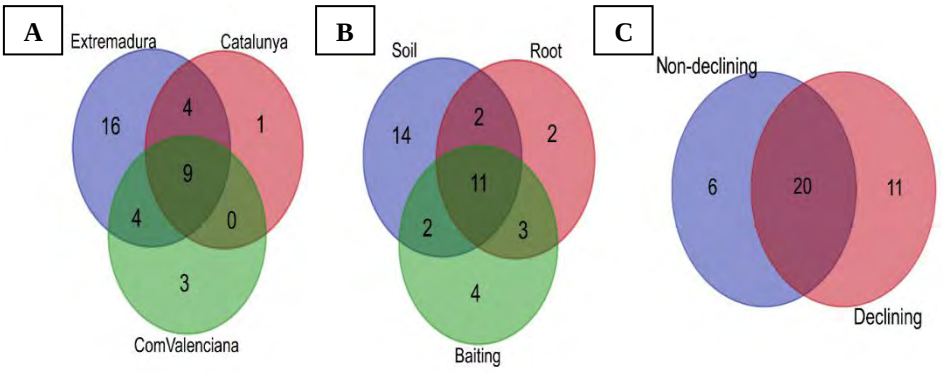


**Figure 7.5.** Venn diagram showing the overlap of operational taxonomic units (OTUs) identified in the *Phytophthora* population present in the holm oaks surveyed in the study according to the different factors studied. (**A)**: Regions surveyed; Extremadura, Comunidad Valenciana (ComValenciana) and Cataluña (Catalunya), (**B)**: Type of material used for the detection; roots, soils and baitings. (**C)**: Symptomatology of the holm oaks sampled: declining and non-declining.

## Discussion

Thirty-seven *Phytophthora* phylotypes belonging to clades 1 to 12, except for clades 4, 5 and 11, were detected in this study, which represents a high diversity of *Phytophthora* species in holm oak Spanish forests compared to previous Spanish NGS studies [44,56]. *Phytophthora chlamydospora*, *P. citrophthora*, *P. gonapodyides*, *P. lacustris*, *P. meadii*, *P. plurivora*, *P. pseudocryptogea*, *P. psychrophila* and *P. quercina* were present in the three regions. Seven of these phylotypes cannot be associated with known *Phytophthora* species, so they are putatively named as *Phytophthora* sp. Most of the detected phylotypes correspond to terrestrial *Phytophthora* species, but aquatic species from clades 6 and 9 were also present in all regions. In general, the most abundant phylotypes in the study are *P. quercina*, followed by *P. psychrophila*, *P. cinnamomi* and *P. plurivora*; all of them terrestrial species. Our results concur with Català *et al.* [44], which reported *P. quercina* and *P. psychrophila* as the most relevant species associated with *Q. ilex* in eastern Spain. Nevertheless, they differ from Ruiz Gómez *et al.* [56] describing *P. plurivora*, *P. quercina*, *P. cinnamomi* and *P. cactorum* as the most frequent detected species in Andalucía holm oak rangelands (dehesas) located in southern Spain.

In the Extremadura region, all *Phytophthora* clades are present with the exception of clades 4, 5 and 11. Terrestrial species dominate the *Phytophthora* community as *P. quercina* 30%, *P. psychrophila* 17% and *P. cinnamomi* 15%, are the most abundant species. Nevertheless, also aquatic species are identified, such as *P. gonapodyides* 12% and *P. megasperma* 7%, which follow in abundance. Hence, a mixture of *Q. ilex* pathogenic terrestrial and aquatic species is identified in the Extremadura region. In the Cataluña region, the *Phytophthora* community associated with the studied *Q. ilex* stand was dominated again by terrestrial species (*P. plurivora* 42%, *P. quercina* 22% and *P. psychrophila* 8%), followed by aquatic species (*P. hydropathica* 11%, *P. lagoariana* 6% and *P. gonapodyides* 5%). Clades 1, 4, 5, 8, 10 and 11 were not detected in the studied holm oaks. In the Comunidad Valenciana region, the *Phytophthora* community is made up primarily by terrestrial *Phytophthora* species from clades 1, 2, 3, 7, 8 and 12 (*P. psychrophila* 27%, *P. quercina* 26%, *P.* taxon *ballota* 11%, *P. plurivora* 10%); although there are also present in lower abundance aquatic

species from clade 6 such as *P. chlamydospora* 7%, *P. gonapodyides* 2% or *P. megasperma* 3%.

The weather conditions of the Extremadura region were more favorable for *Phytophthora* development in 2013 than in 2012, since in 2012 Extremadura received less precipitation than in 2013, as reported in 2018 by Mora-Sala *et al.* [48]. Redondo *et al.* [57] state that there is a decrease in the diversity of terrestrial *Phytophthora* communities when temperature and precipitation decreases, precipitation being the main driving factor, except for clades 2 and 8, in which temperature was more conditioning. According to Redondo *et al.* [57], aquatic *Phytophthora* species are inversely conditioned by temperature and precipitation. The *Phytophthora* diversity found in Extremadura in 2013 was higher than in 2012, which fits with the parameters stated in the study of Redondo *et al.* [57]. Moreover, more types of sampling materials (roots and baitings) were used for DNA extraction in 2013 than in 2012, which might potentially increase the phylotype diversity. Redondo *et al.* [57] conclude that the land use is only significant for aquatic species, having more diversity in urban or agricultural sites than in forests. In our study, we found more species belonging to the clade 6 in Extremadura oak rangelands, such as *P. gonapodyides* or *P. megasperma*, which are man-made forests, although they are also identified in lower abundance in Cataluña and Comunidad Valenciana. Clade 9 species (*P. hydropathica* and *P. lagoariana*) are also detected in Extremadura and in Cataluña.

*Phytophthora cinnamomi* is an important and devastating *Quercus* pathogen in Spain [27,58–61], although it is not the most abundant species detected in the *Quercus* forests studied. The pathogen is significantly more abundant in declining trees than in non-declining trees, as previously reported by Mora-Sala *et al.* using qPCR and traditional isolation methods [48], and that also corroborates the metabarcoding study of Ruiz Gómez *et al.* [56]. *Phytophthora cinnamomi* is not detected in Comunidad Valenciana.

This is in agreement with previously published reports, and may be due to the unsuitable conditions for the pathogen development in the area consisting on primarily calcareous soils with a high pH [26,44,48,62].

*Phytophthora quercina* is a specific oak pathogen, present in the three studied regions, that probably has co-evolved with *Q. ilex* in Spain, rotting the

trees slowly and progressively. As *P. quercina* was not described until 1999 [8], it could not be associated with the studies of the decline of Spanish holm oak conducted in the past. In addition, *P. quercina* is a difficult pathogen to isolate, due to its slow growth, thus its detection was not always possible in previous studies [8,15,26,30]. Moreover, as generally *P. cinnamomi* was present, and *P. quercina* is not a fast growing pathogen, the decline was associated with *P. cinnamomi*. Although recent studies report the presence of *P. quercina*, it is thought of as not being as frequent as *P. cinnamomi*. Mora-Sala *et al.* demonstrate that *P. quercina* is more frequent than *P. cinnamomi*, not only in Comunidad Valenciana, but also in the Extremadura and Cataluña regions by qPCR [48]. Ruiz Gómez *et al.* [56] report *P. quercina* as the fourth-most abundant oomycete in Andalucía holm oak rangelands, while *P. cinnamomi* stands in the ninth position. The present study based on amplicon pyrosequencing supports our previous results using qPCR and verifies that this pathogen is highly abundant in the studied *Quercus* Spanish regions.

*Phytophthora psychrophila* is detected in high abundance in the three regions sampled, especially in Comunidad Valenciana, either in declining or in non-declining trees. This species was previously related to *Quercus* spp. dieback in Spain where it is apparently well distributed and it is only able to be isolated during the winter and spring/autumn periods [10,26,56]. As Pérez-Sierra *et al.* report in 2013, it is perfectly adapted to xeric Mediterranean conditions due to the thick wall of its resting structures that enables it to overcome unsuitable environmental conditions. Pathogenicity tests with *Q. ilex* seedlings demonstrate that *P. psychrophila* is considered an aggressive pathogen, causing dieback of the root system, mainly the fine roots, necrotic lesions and open cankers [26,48].

*Phytophthora plurivora* is significantly detected in roots and it is even present in all regions, resulting more abundantly in the Cataluña region, where it is the most frequent detected species. *Phytophthora plurivora* is a widespread species in Europe, which had been already detected in *Q. ilex* in Spain [44,56], and its pathogenicity to *Q. ilex* is demonstrated in a previous study [63] causing the absence of fine roots, necrotic lesions, open cankers, dieback of the whole root system and collar rot. Ruiz Gómez *et al.* [56] report *P. plurivora* as the most abundant oomycete detected in the Spanish oak rangelands surveyed in their study. *Phytophthora plurivora* in our study seems to be more associated with

declining *Q. ilex* trees, as Ruiz Gómez *et al.* [56] report. The homothallic behavior of *Phytophthora* species, as *P. plurivora* or *P. psychrophila*, facilitates its reproduction and establishment in new areas, boosting the risk of forest decline [10,26,29,36,57,64].

From the aquatic species detected, *P. gonapodyides* was previously detected affecting *Q. ilex* in Spain [26,33,48]. *Phytophthora megasperma* is reported to reduce the root system [26,63] although this species is considered an opportunistic oak pathogen present in oak forests [9,15,16]. The role of the remaining aquatic *Phytophthora* species detected in the *Q. ilex* decline is still unknown.

The sequencing results support the presence of *P.* taxon *ballota* in two forests of the Comunidad Valenciana and in Extremadura regions. This uncultured phylotype was previously detected in oak forests in Comunidad Valenciana [44], but it is the first time that it has been detected outside the Comunidad Valenciana. The designation of new phylotypes from environmental DNA remains a committed issue, but it is reported in other studies [65–67]. Both in previous studies and the present study, no *Phytophthora* culture was isolated that coincided with this proposed phylotype using traditional methods. Further surveys targeting this organism should be performed to try the isolation.

This study detected the presence of *P. pseudocryptogea* in all three regions, and it has also been isolated from other *Fagaceae* such as *Castanea sativa* in the North of Spain (Mora-Sala and Català unpublished), suggesting that it is well established in Spain and probably in the past many isolates identified as *P. cryptogea*, were actually *P. pseudocryptogea*. In 2018, *P. pseudocryptogea* was firstly reported in Extremadura region from *Q. ilex* rhizosphere [48] and it has just been reported in Sicily affecting *Q. ilex* [36], so it seems to be well established in the Mediterranean basin.

The pathogenicity on *Q. ilex* has not been tested, but its pathogenicity on other *Fagaceae* (Mora-Sala and Català, unpublished), suggests that this species could contribute to the oak decline.

*Phytophthora* community diversity recovered from *Fagaceae* forests in Europe has increased in the last years [8–10, 13,16,20,21,23,24,26,29,30, 36,44,64,68]. The implementation of NGS technologies to forests surveys helps

to improve the knowledge about the *Phytophthora* spp. diversity associated with *Fagaceae* forests and to identify possible new introduced species [3,29,44,45,47]. *Phytophthora* spp. can adapt to a wide variety of environmental conditions [38]. In Spain, the oak decline due to *Phytophthora* spp. is related to the effect of the water stress; seasonal droughts followed by floods enhance the root damage induced by *Phytophthora* species. The versatility of these species to cope with the changing scenarios and the increase of extreme weather conditions that are occurring nowadays, focuses the attention on this destructive genus. The composition of *Phytophthora* species in these ecosystems is changing because of their adaptation to new environmental conditions and new species introduction. New technologies help us improve knowledge about species diversity in these new scenarios. In the present study, NGS reveals a higher *Phytophthora* species diversity than previously detected by traditional isolation, baiting and qPCR. However, the best approach should combine all available methodologies for a correct *Phytophthora* diagnosis, facilitating a quick answer facing the potential introduction of new and/or quarantine organisms.

## Conclusions

The use of amplicon pyrosequencing reveals a high diversity of *Phytophthora* species associated with *Q. ilex*. Baiting material has the highest relative abundance of *Phytophthora* species. The highest alpha diversity is obtained in the region of Extremadura, the diversity of Comunidad Valenciana and Cataluña being lower following this order. In general terms, the *Phytophthora* diversity is highest in non-declining *Q. ilex* than in declining trees. The implementation of molecular tools in *Phytophthora* forests monitoring, complement and help to overcome the limitations of traditional methods, being useful to improve the knowledge about the real composition of the species present in these ecosystems.

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# Chapter 8

# Chapter 8

## GENERAL DISCUSSION

The genus *Quercus* and especially holm oak (*Quercus ilex* L.) is a key element of Spanish Mediterranean forests. Oomycetes, and in particular *Phytophthora*, have been one of the factors associated with the decline of the stands of *Quercus* in Spain (Brasier 1992; Brasier *et al.*, 1993). The genus *Phytophthora* comprises nowadays more than 150 species with a broad host range, and includes well-known plant pathogens that are devastating natural ecosystems (Erwin & Ribeiro, 1996; Mideros *et al.*, 2018; Tremblay *et al.*, 2018).

Global trade increases the risk of unnoticed introductions of alien species into natural ecosystems (Brasier *et al.*, 2008; O'Brien *et al.*, 2009). Nursery industry has become an ideal reservoir for the development and recombination of *Phytophthora* species worldwide. High *Phytophthora* species diversity has been reported in both ornamental and forestry nurseries, so they are under continuous surveillance (Jung *et al.*, 2016; Sims *et al.*, 2019). In this context, sampling ornamental and forest nurseries in four Spanish regions (Cataluña, Comunidad Valenciana, Extremadura and País Vasco) was carried out focusing on possible symptoms associated to *Phytophthora* on different hosts but specially on *Quercus* and more specifically on *Q. ilex* if present. Moreover, water samples were also collected around the nurseries. The results of these surveys conducted from 2012 to 2014, showed a high diversity of *Phytophthora* species in Spanish nurseries and water sources, which confirmed results in previous studies (Pérez-Sierra *et al.*, 2012; Jung *et al.*, 2016). On plants affected by *Phytophthora*, dieback was the most frequent symptom observed, followed by foliar symptoms, wilting-decline and growth reduction. Thirteen *Phytophthora* species, one informally designated taxon and one *Phytophthora* phylotype that could not be identified to the species level were isolated from 547 plant samples belonging to 22 species included in 19 plant genera. The most frequent species isolated were *P. pseudocryptogea*, *P. plurivora*, *P. hedraiandra*, *P. citrophthora* and *P. nicotianae*. In lower frequencies, *P. cactorum*, *P. cinnamomi*, *Phytophthora* sp. 1 clade 2, *P. crassamura*, *P. sansomeana*, *P. citricola*, *P. gonapodyides* and *P. palmivora* were also present. The species with the lowest frequencies were *P.*

*cambivora* and *P. niederhauserii*, as well as the informally designated taxon, *Phytophthora* sp. tropicalis-like 2. In some of the analysed plants, more than one species of *Phytophthora* was isolated, revealing the existence of mixed infections. This is the first report of *P. crassamura* in Spain and in *Pinus pinea* worldwide and of *P. pseudocryptogea* in *Chamaecyparis lawsoniana* and *Yucca rostrata* in Spain. *Phytophthora cactorum, P. lacustris* and *P. gonapodyides* were the most abundant species from water samples, followed by *P. cambivora, P. citrophthora, P. plurivora and P. pseudocryptogea*. The species with the lowest isolation frequency in water samples were *P. bilorbang* and *P. palmivora*. Most of the *Phytophthora* species isolated from water have been also detected from the nursery vegetal samples. This confirms the hypothesis that the species of *Phytophthora* present in the vegetable samples are collected in the drainage and/or irrigation water and/or in the rivers close to the nurseries, dispersing the pathogens throughout the nursery and even sometimes outside the nursery boundaries. The sampling revealed a high number of Cylindrocarpon-like anamorphs present in the Spanish nursery industry, specially associated to *Q. ilex*. For this reason, a study was carried out to characterize a collection of Cylindrocarpon-like isolates recovered during the period 2009-2014, from the roots of a broad range of woody hosts showing decline. Sixteen species belonging to the genera *Cylindrodendrum*, *Dactylonectria*, *Ilyonectria*, and *Neonectria* were identified from damaged roots of 15 forest plant genera. The species *Cylindrodendrum alicantinum*, *Dactylonectria macrodidyma*, *Dactylonectria novozelandica*, *Dactylonectria pauciseptata*, *Dactylonectria pinicola*, *Dactylonectria torresensis*, *Ilyonectria capensis*, *Ilyonectria cyclaminicola, Ilyonectria liriodendri, Ilyonectria pseudodestructans*, *Ilyonectria robusta* and *Ilyonectria rufa* were identified. In addition, two *Dactylonectria* species (*D. hispanica* sp. nov. and *D. valentina* sp. nov.), one *Ilyonectria* species (*I. ilicicola* sp. nov.) and one *Neonectria* species (*N. quercicola* sp. nov.) were newly described.

In addition, the study reported for the first time *C. alicantinum* on *Q. ilex*; *D. macrodydima* on: *Ilex aquifolium, Juniperus phoenicea, Lonicera* sp., *Myrtus communis, Pinus halepensis, Pyracantha* sp., *Q. faginea, Q. ilex* and *Rosmarinus officinalis*; *D. novozelandica* on: *Crataegus azarolus, J. phoenicea, Pinus* sp., *P. halepensis, Pistacia lentiscus, Quercus* sp., *Q. ilex, Q. suber, R. officinalis* and *Santolina chamaecyparissus*; *D. pauciseptata* on: *Abies nordmanniana* and *P.*

*halepensis*; *D. pinicola* on: *Abies concolor*, *D. torresensis* on: *Arbutus unedo*, *Cistus albidus*, *Juglans regia*, *P. halepensis*, *Q. ilex* and *R. officinalis; I. capensis* in *Ar. unedo*, *Juniperus* sp. and *P. halepensis*; *I. cyclaminicola* on *Quercus* sp.; *I. liriodendra* on: *Ar. unedo*, *Juniperus* sp., *P. halepensis*; *I. pseudodestructans* in *Q. ilex*; *I. robusta* in *Ju. regia*; and *I. rufa* on: *Abies nordmanniana*, *Ar. unedo*, *Juniperus* sp., *P. halepensis*, *Q. faginea* and *Q. ilex.* Furthermore, *I. capensis* was first reported in Europe because, to our knowledge, this fungus had only been recorded affecting *Protea* in South Africa (Lombard *et al.*, 2013).

To date *D. novozelandica*, *D. macrodidyma*, *D. torresensis*, *I. liriodendri*, *I. robusta* and *C. alicantinum* had been reported only in cultivated crops such as grapevine (*Vitis vinifera*) (Agustí-Brisach & Armengol 2013) or loquat (*Eriobotrya japonica*) (Agustí-Brisach *et al.*2016) in Spain, but never affecting forest plants. Thus, this study increased the range of forest hosts in nursery for this species, representing the first report of *D. pauciseptata* on *A. nordmanniana* and *P. halepensis.*

*Quercus* and *Pinus* genera prevail in the Mediterranean landscape, as they are a major component of the Mediterranean forest vegetation. In this regard, the presence of nine Cylindrocarpon-like species on *Quercus* and eight on *Pinus* trees highlight the need for better management of nursery diseases to avoid the dispersal of these soilborne pathogens through planting materials used for reforestation purposes.

Considering the high *Phytophthora* diversity detected in Spanish nurseries, being a risk for planting material in natural ecosystems, one of the goals of the Thesis was to evaluate the susceptibility of *Q. ilex* to the inoculation with eight *Phytophthora* species present in Spanish nurseries. The results of the soil infestation test showed that all *Phytophthora* species tested were pathogenic to *Q. ilex* seedlings grown from acorns obtained from two Spanish origins. The most aggressive species were *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. plurivora* and *P. psychrophila*, followed by *P. megasperma*, while *P. quercina* and *P. nicotianae* were the least aggressive species, with plants inoculated with *P. quercina* having the longest survival rates.

Pathogenicity of *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. psychrophila*, *P. megasperma* and *P. quercina* on *Q. ilex* was already known but there was a lack of information of two of the most frequent *Phytophthora* species

in nurseries, *P. plurivora* and *P. nicotianae* (Tuset *et al.* 1996; Robin *et al.* 1998, 2001; Gallego *et al.* 1999; Maurel *et al.* 2001; Rodríguez-Molina *et al.* 2002; Sánchez *et al.* 2002, 2005; Pérez-Sierra *et al.*, 2013; Linaldeddu *et al.* 2014; Martín-García *et al.* 2015). Actually, our study was the first soil infestation test conducted on holm oak with these two species.

*Quercus ilex* seedlings inoculated with *P. plurivora* showed necrotic lesions, open cankers, dieback of the whole root system, collar rot, absence of fine roots and even sometimes, no tap root was present. These symptoms agreed with those reported in other woody hosts leading to a high mortality rate and low root and aerial tissues weight (Jung & Burgess 2009). Results obtained demonstrated that *Q. ilex* is susceptible to *P. nicotianae*, despite *P. nicotianae* being less aggressive than the other *Phytophthora* species tested. Climate change and global trade are driving *P. nicotianae* to an advantageous position over other *Phytophthora* species as its high optimum temperature, longevity, dispersal capacity and hybridisation capacity enable it to adapt to the changing worldwide climate scenarios (Panabières *et al.* 2016).

The pathogenicity test demonstrated that *Q. ilex* was susceptible to a range of *Phytophthora* species, apart from *P. cinnamomi* that unfortunately, are present in Spanish nurseries affecting a broad range of host plants including woody hosts, such as *Quercus* species (Jung *et al.* 2016). The present and previous studies demonstrated that several *Phytophthora* species constituted a threat to oak forests. The relevance of this group of plant pathogens and the increasing number of hosts that are emerging in different scenarios highlights the need for improving the control of plant material. In this context, the nursery industry and international plant trade should implement effective phytosanitary measures to avoid *Phytophthora* dispersal to naïve natural ecosystems and geographical areas where the pathogen is not present.

Another goal of the Thesis was to verify the presence of *Phytophthora* species in the holm oak rhizosphere in southwestern Spanish dehesas (seminatural ecosystem) and in a northeastern Spanish holm oak woodland (natural ecosystem). In addition, the association between the *Phytophthora* species and the symptomatology of the holm oaks was studied in the dehesas. As the efficiency of *Phytophthora* isolation techniques can be compromised by several factors, different isolation and baiting methods were implemented

(Brasier, 1992; Erwin & Ribeiro, 1996). Direct isolation and baiting methods in declining and non-declining holm oak trees revealed *P. cambivora*, *P. cinnamomi*, *P. gonapodyides*, *P. megasperma*, and *P. pseudocryptogea* in the dehesas, while in the northeastern woodland, no *Phytophthora* spp. were recovered. Statistical analyses indicated that there was not a significant relationship between the *Phytophthora* spp. isolation frequency and the disease expression of the holm oak stands in the dehesas.

Moreover, as *P. cinnamomi* and *P. quercina* are considered among the main pathogens associated with holm oak decline, their presence and relative abundance were studied in the samples using specific TaqMan real-time PCR probes. Results showed that both *P. cinnamomi* and *P. quercina* were involved in the holm oak decline in Spain, but *P. quercina* was detected in a higher frequency than *P. cinnamomi* in both studied areas.

This study provided evidence that molecular approaches complement direct isolation methods of *Phytophthora* species from fine roots from holm oak in natural (Montseny Biosphere Reserve) and seminatural (dehesas) ecosystems, confirming that not only *P. cinnamomi* is involved in the holm oak decline in Spain, but also *P. quercina*. Moreover, the study reported the detection of *P. pseudocryptogea* for the first time in Europe.

Economic and environmental losses caused by *Phytophthora* worldwide (Jung *et al.*, 2016; Erwin & Ribeiro, 1996; Hernández-Lambraño *et al.*, 2018) require the use of all available techniques to detect and identify invasive species as quickly as possible. Coupling direct isolation and baiting techniques with molecular tools increased the specificity, reproducibility, and sensitivity of the assessments, adding efficiency and accuracy to the diagnosis, which is an essential part of forest management strategies.

To improve knowledge about the diversity of *Phytophthora* spp. associated with *Q. ilex* in Spain, a study using Next Generation Sequencing (NGS) was performed in the two previous studied areas (Extremadura and Cataluña regions) and in other four *Q. ilex* stands located in a third region in Spain (Comunidad Valenciana). Thirty-seven *Phytophthora* phylotypes belonging to clades 1 to 12, except for clades 4, 5 and 11, were detected in this study, revealing a high diversity of *Phytophthora* species in holm oak Spanish forests. *Phytophthora chlamydospora*, *P. citrophthora*, *P. gonapodyides*, *P.*

*lacustris*, *P. meadii*, *P. plurivora*, *P. pseudocryptogea*, *P. psychrophila* and *P. quercina* were present in the three regions. Baiting material had the highest relative abundance of *Phytophthora* species. The highest alpha diversity was obtained in the region of Extremadura, followed by Comunidad Valenciana and Cataluña. In general terms, the *Phytophthora* diversity was higher in non-declining *Q. ilex* than in declining trees.

The sequencing results supported the presence of *P.* taxon *ballota* in two forests of the Comunidad Valenciana and in Extremadura regions. This uncultured phylotype was previously detected in oak forests in Comunidad Valenciana (Català *et al.*, 2017), but it was the first detection outside this region. Previous unsuccessful attempts had tried to isolate this phylotype, so further surveys targeting this organism should be performed.

Another important finding of this study was the detection of *P. pseudocryptogea* in all three regions. It was firstly isolated in 2018 in Extremadura region affecting *Q. ilex* and it has also been isolated from *Castanea sativa* in the North of Spain (Mora-Sala and Català unpublished). The isolations along with the amplicon pyrosequencing detection, suggests that it was present in Spain for long time ago and probably in the past many isolates identified as *P. cryptogea*, were actually *P. pseudocryptogea*. Moreover, this pathogen has just been reported in Sicily affecting *Q. ilex* and in restoration stock in Californian nurseries (Jung *et al.*, 2019; Sims *et al.*, 2019). The pathogenicity on *Q. ilex* has not been tested, but its pathogenicity on other *Fagaceae* (Mora-Sala and Català, unpublished), suggests that this species could contribute to the oak decline.

Overall, the results obtained in this Thesis represents an improvement in the knowledge of *Phytophthora* scenario in Spanish nurseries, and in how the main species of *Phytophthora* and Cylindrocarpon-like present in the nurseries may affect *Q. ilex* as potential reforestation elements. In addition, this Thesis has questioned the established idea that *P. cinnamomi* was the main cause of the oak decline in Spain, unraveling a high diversity of *Phytophthora* species in the Spanish *Q. ilex* stands, regardless of whether they cause symptoms of decline or not (Jung, 1999; Jung *et al.*, 2002; Corcobado *et al.*, 2010; Pérez-Sierra *et al.*, 2013). Moreover, the implementation of molecular tools has demonstrated a higher frequency of *P. quercina* than *P. cinnamomi* in two *Q. ilex* regions.

Further research is needed to clarify the effect of these pathogens in combination and abiotic factors in the oak stands. The implementation of molecular tools in *Phytophthora* forests monitoring complement and help to overcome the limitations of traditional methods, being useful to improve the knowledge about the species diversity in these ecosystems.

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# Chapter 9

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## CONCLUSIONS

1. The main soilborne pathogens isolated in the sampled nurseries were Cylindocarpon-like asexual morphs, *Phytophthora* spp., *Phytopythium* spp. and *Pythium* spp.

2. Seventeen *Phytophthora* phylotypes were isolated from 562 samples including 22 plant species belonging to 19 genera, water samples and baiting leaves.

3. Sixteen species belonging to the genera *Cylindrodendrum*, *Dactylonectria*, *Ilyonectria*, and *Neonectria* were identified from damaged roots of 15 forest plant genera and 4 new species were described: *D. hispanica*, *D. valentina*. *I. ilicicola* and *N. quercicola*.

4. *Phytophthora pseudocryptogea* and *Ilyonectria capensis* were reported for the first time in Europe. Forty-nine new host x pathogen combinations were reported in the genera *Phytophthora*, *Cylindrodendrum*, *Dactylonectria* and *Ilyonectria*. Among them, 12 were identified on *Quercus* species and, more specifically, seven on *Quercus ilex*.

5. The pathogenicity test demonstrates that *Q. ilex* was susceptible to a range of *Phytophthora* species. The most aggressive species were *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. plurivora* and *P. psychrophila*, followed by *P. megasperma*, while *P. quercina* and *P. nicotianae* were the least aggressive species.

6. Direct isolation and baiting methods in a seminatural holm oak ecosystem with declining and non-declining holm oak trees (Extremadura dehesas) revealed the presence of *P. cambivora*, *P. cinnamomi*, *P. gonapodyides*, *P. megasperma*, and *P. pseudocryptogea*.

Statistical analyses showed no significant relationship between the *Phytophthora* spp. isolation frequency and the disease expression of the holm oak stands in the studied dehesas.

7. *Phytophthora quercina* and *P. cinnamomi* TaqMan real-time PCR probes showed that both species are present in Extremadura dehesas and in Montseny Biosphere Reserve, but *P. quercina* was detected in a higher frequency than *P. cinnamomi* in both studied areas.

8. A Next Generation Sequencing (NGS) study conducted in six *Q. ilex* stands located in three regions in Spain revealed thirty-seven *Phytophthora* phylotypes belonging to clades 1 to 12, except for clades 4, 5 and 11. The most abundant phylotypes in the study were *P. quercina*, followed by *P. psychrophila*, *P. cinnamomi* and *P. plurivora*.

9. This study provides evidence that DNA-based techniques complement direct isolation methods for *Phytophthora* detection, confirming a high diversity present in Spanish holm oak forests. A broad range of *Phytophthora* species detected in natural ecosystems was previously identified in nurseries.