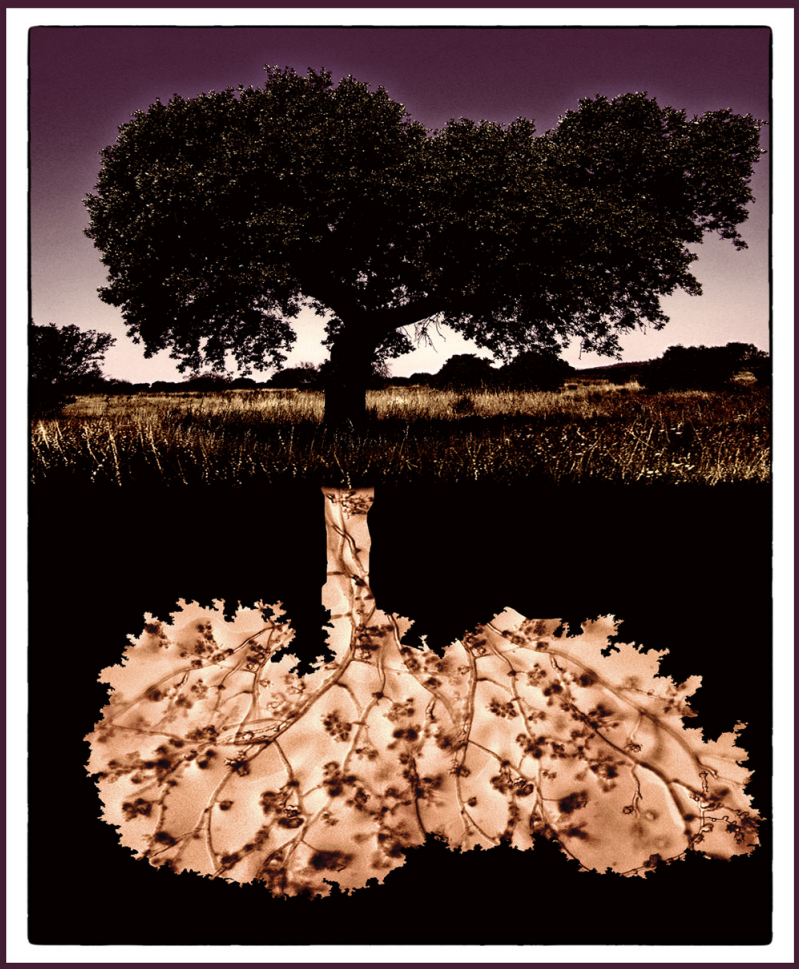
UNIVERSIDAD DE EXTREMADURA
Departamento de Ingeniería del Medio Agronómico y Forestal

TESIS DOCTORAL

Influencia de *Phytophthora cinnamomi* Rands en el decaimiento de *Quercus ilex* L. y su relación con las propiedades del suelo y las ectomicorrizas

Tamara Corcobado Sánchez



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CERTIFICAN:

Que la memoria descrita en este trabajo con el título «***Influencia de Phytophthora cinnamomi Rands en el decaimiento de Quercus ilex L. y su relación con las propiedades del suelo y las ectomicorrizas***», ha sido realizada bajo su dirección por Tamara Corcobado Sánchez y que a su juicio, salvo mejor criterio del tribunal que ha de juzgarlo, reúne todas las condiciones para poder optar al **Grado de Doctor**.
Y para que así conste a efectos legales, firman el presente documento en Plasencia a 11 de septiembre de 2013.

Fdo: Alejandro Solla Hach Fdo: Gerardo Moreno Marcos

2013

A mis padres

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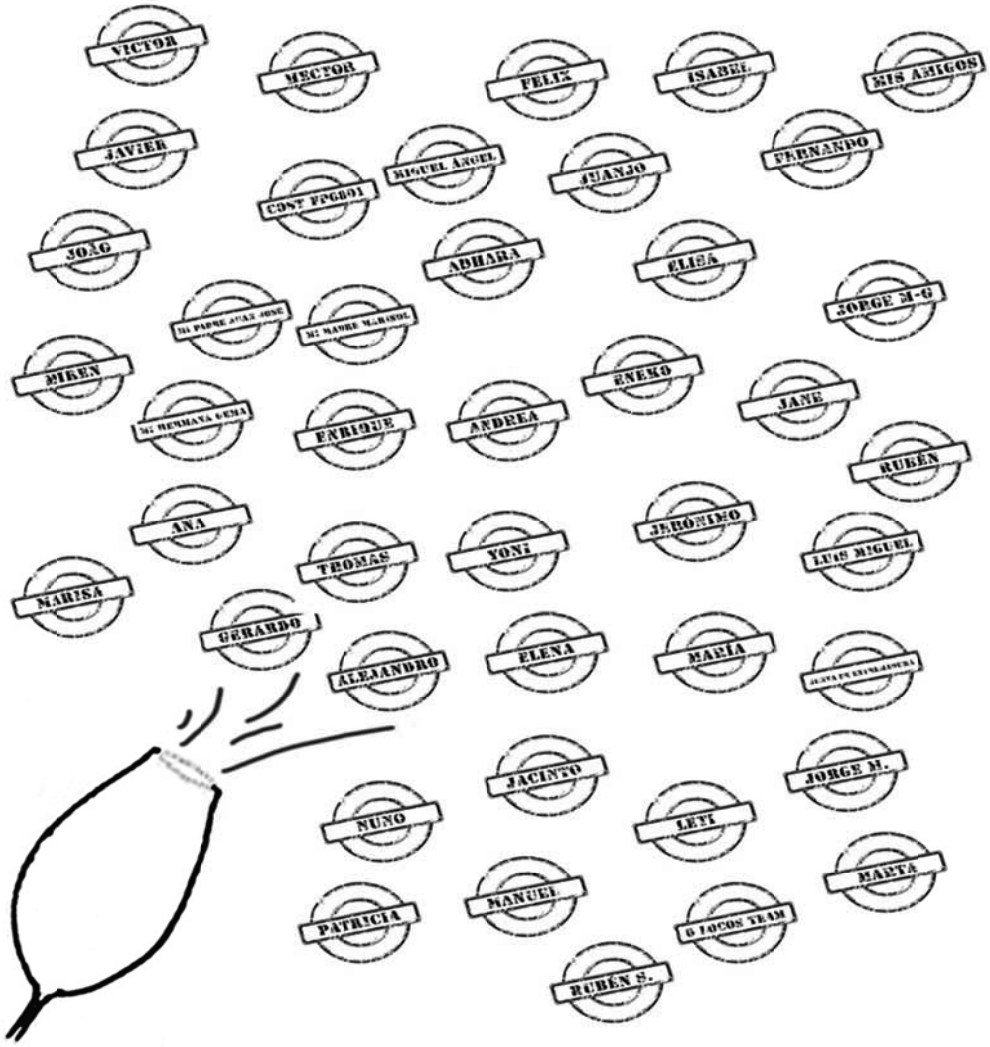
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SUMMARY

Since the 1980s it has been observed an acute *Quercus* decline in Europe and America, with special incidence and severity in one of the most dominant species in the Mediterranean, the holm oak (*Quercus ilex* L). Among the factors involved in holm oak decline, soil degradation associated to changes in woodland management, water stress, marked changes in soil water content and soil water table and shifts in mycorrhizal symbiosis have been described. The main biotic factor involved in holm oak decline is the oomycete *Phytophthora cinnamomi* Rands, with a worldwide distribution and a wide host range. This pathogen causes root necrosis and requires specific environmental conditions, especially of moisture and temperature for infecting. Additionally, physical and chemical soil properties, in some cases linked to moisture values, would affect *P. cinnamomi* infection. Its activity will also depend on the interactions with other organisms located on the rizosphere such as mycorrhizae and other oomycetes. The present work aims to assess the influence of *P. cinnamomi* on holm oak decline, and how physical and chemical soil properties, ectomycorrhizae, soil water content and soil water table affect *P. cinnamomi* infections of holm oak.

The work comprises three types of assessments: (i) extensive covering Extremadura region, (ii) following a temporal sampling and (iii) performed under greenhouse conditions. At extensive level, sampling was performed in 96 declining holm oak stands located on stream banks or on slopes, having both locations coarse or fine textured soils. In each stand, three declining and three non-declining holm oaks were selected and *P. cinnamomi* and *Pythium spiculum* presence, abundance of ectomycorrhizae, soil properties (soil depth, Ah horizon thickness, texture, pH, redox potential, soil bulk density and $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ concentrations) and fine root density were assessed. Within the temporal assessment, five holm oak declining stands were selected and soil water content and soil water table were measured

monthly, while abundance of ectomycorrhizae, *P. cinnamomi* presence and physiological parameters were seasonally assessed. Under greenhouse conditions, 140 holm oak seedlings were submitted to different watering regimes and subsequently inoculated with *P. cinnamomi*. Mortality, plant performance and some physiological parameters were measured.

Results showed that *P. cinnamomi* was the main biotic factor in holm oak decline. It was demonstrated that soil water content was higher under declining trees than under non-declining trees during the driest season, although they had lower soil water-holding capacity probably caused by their location on coarser texture soils. Water stress could be an important lethal factor as declining trees and those which were infected had a lower fine root biomass, and therefore a weaken capacity to uptake water. Soil water content plays an important role in holm oak decline when *P. cinnamomi* is present. This was demonstrated through examination of soil properties, such as soil bulk density, soil texture and Ah horizon thickness, which influence soil moisture values. When trees were infected, lower soil bulk densities, fine textured soils and a thicker Ah horizon favoured severity of symptoms and mortality. Concerning soil properties linked to disturbances caused by management, only $N\text{-}NO_3^-/N\text{-}NH_4^+$ ratio was lower under declining trees. Changes in soil water table seemed not to be involved in holm oak decline. The occurrence of long drought events, as climate change simulations predict, was also demonstrated to affect *Q. ilex* performance and to increase subsequent susceptibility to *P. cinnamomi*. Results also showed a low ectomycorrhizal abundance, which increased in spring and summer, and a scarce ectomycorrhizal diversity, highlighting the presence of *Cenococcum geophilum*, *Tomentella* spp. and *Russula* spp. Overall, a higher number of non-vital tips under declining than under non-declining trees, with independence of *P. cinnamomi* presence, was observed. A higher number of non-ectomycorrhizal tips was also detected under non-declining than under declining trees. The ectomycorrhizal

abundance was higher under non-declining than under declining trees in the 96 declining stands. The morphotypes *Cenococcum geophilum* and *Tomentella* spp. showed similar percentage values for all the trees, while the abundance of *Russula* spp. and other less abundant ectomycorrhizal fungi was altered with the presence of the pathogen between declining and non-declining trees. Some soil properties related to ectomycorrhizal abundance when trees were not infected, and the relation between physiological parameters and abundance of *C. geophilum* or *Russula* spp. changed depending on the tree status (declining or non-declining).

To sum up, root rot caused by *P. cinnamomi* infection in combination with factors which favour the pathogen activity (as properties associated with soil water content) play an important role in the vigour of holm oak. Disturbances in ectomycorrhizal abundance and diversity also related to holm oak decline.

RESUMEN

Desde los años 80 se viene observando un decaimiento generalizado de las masas de *Quercus* en Europa y América, con especial incidencia y severidad de daños en una de las especies dominantes del Mediterráneo, la encina (*Quercus ilex* L). Entre los factores involucrados en el decaimiento de la encina, se han descrito la degradación del suelo debido al manejo, el estrés hídrico, las alteraciones drásticas en la humedad edáfica y en el nivel freático y los cambios en la simbiosis micorrícica. El factor biótico más importante implicado en el debilitamiento de la encina es el oomiceto *Phytophthora cinnamomi* Rands, de distribución mundial y amplio rango de hospedantes. Este patógeno es causante de la necrosis del sistema radical y requiere de condiciones ambientales particulares, especialmente de humedad y temperatura, para llevar a cabo el proceso infectivo. Además, otros parámetros físico-químicos del suelo, que en algunos casos influyen en los valores de humedad, podrían afectar a la infección de *P. cinnamomi*. La actividad de *P. cinnamomi* también dependerá de la interacción con otros organismos propios de la rizosfera tales como las micorrizas y otros oomicetos. A través del presente trabajo, se pretende ahondar en la influencia de *P. cinnamomi* en el decaimiento de la encina y, cómo las propiedades físico-químicas del suelo, las ectomicorrizas y la humedad edáfica afectan la infección de las encinas por *P. cinnamomi*.

En este trabajo, se presentan tres tipos de estudios: (i) uno realizado a nivel extensivo cubriendo la región de Extremadura, (ii) otro realizado a nivel temporal en un número reducido de parcelas y (iii) otro experimento en invernadero. A nivel extensivo, se llevó a cabo el muestreo de 96 focos con decaimiento de encina ubicados en vaguada y ladera, diferenciándose en cada localización dos tipos de textura (gruesa o fina). En cada foco se escogieron tres encinas con síntomas de decaimiento y tres asintomáticas y se analizaron la presencia de los oomicetos *P. cinnamomi* y *Pythium spiculum*, la abundancia de

ectomicorrizas, varias propiedades del suelo (profundidad del suelo, espesor del horizonte Ah, textura, pH, potencial redox, compactación del suelo y concentraciones de $N\text{-}NH_4^+$ y $N\text{-}NO_3^-$) y la densidad radical de las encinas. El estudio a nivel temporal se realizó en cinco focos con decaimiento de encina donde se midieron mensualmente la humedad edáfica y el nivel freático, y estacionalmente la abundancia de ectomicorrizas, la presencia de *P. cinnamomi* y varios parámetros fisiológicos. Bajo condiciones de invernadero, se aplicaron distintos regímenes hídricos extremos a 140 plántulas de encina, que posteriormente fueron inoculadas con *P. cinnamomi*. Se evaluaron la mortalidad, el crecimiento, la biomasa y varios parámetros fisiológicos.

Los resultados mostraron que *P. cinnamomi* era el principal factor biótico implicado en el decaimiento de la encina. Se demostró que durante la época de mayor estrés hídrico la humedad edáfica era mayor bajo encinas decaídas que no decaídas. A pesar de ello, se observó una menor capacidad de retención de agua útil en el suelo bajo estos árboles decaídos probablemente relacionado con la textura más gruesa del suelo. El estrés hídrico podría ser un factor letal importante debido a que las encinas decaídas y las que estaban infectadas con *P. cinnamomi* tenían una menor densidad de raíces finas, y por tanto una menor capacidad de absorción de agua. La humedad edáfica jugaría un papel importante en el decaimiento de la encina cuando *P. cinnamomi* estuviese presente. Esto se demostró a través de las propiedades del suelo tales como la densidad del suelo, la textura y el espesor del horizonte Ah que influyen en los valores de humedad. Cuando los árboles estaban infectados, una menor densidad del suelo, una textura fina y espesores gruesos del horizonte Ah, aumentaban la severidad de los síntomas y la mortalidad. Respecto a los análisis de las propiedades edáficas usadas para detectar perturbaciones debido al manejo, solamente el ratio $N\text{-}NO_3^-/N\text{-}NH_4^+$ era mayor bajo encinas con síntomas de decaimiento. Por otro lado, las variaciones en el nivel freático no se relacionaban con el

decaimiento. Se demostró que los periodos de sequía prolongados, los cuales serán más frecuentes de acuerdo con las predicciones sobre el cambio climático, afectaban al crecimiento de la planta y aumentaban su susceptibilidad a posteriores infecciones por *P. cinnamomi*. Los resultados también mostraron una baja abundancia de ectomicorrizas, concentrándose durante las estaciones de primavera y verano, y una escasa diversidad, destacando la presencia de los morfotipos *Cenococcum geophilum*, *Tomentella* spp. y *Russula* spp. En general, se detectó un mayor número de ápices muertos bajo árboles decaídos que no decaídos, con independencia de la presencia de *P. cinnamomi*. También se observó un mayor número de ápices vivos no micorrizados en árboles no decaídos que decaídos. Se detectó una mayor abundancia de ectomicorrizas bajo árboles no decaídos que decaídos en el estudio de los 96 focos. Los morfotipos *Cenococcum geophilum* y *Tomentella* spp. mostraron una abundancia independiente del estado del árbol y de la presencia de *P. cinnamomi*, mientras que el patógeno alteró la abundancia de *Russula* spp. y otras ectomicorrizas en menor proporción entre árboles decaídos y no decaídos. Algunas propiedades del suelo se relacionaron con la abundancia de ectomicorrizas sólo cuando el árbol no estaba infectado, mientras que la relación entre los parámetros fisiológicos y la abundancia de *C. geophilum* o *Russula* spp. cambiaba en función del grado de decaimiento de la encina.

En general, la pudrición de raíces causada por la infección de *P. cinnamomi* en combinación con factores que influyen en la actividad del patógeno (como las propiedades edáficas relacionadas con la humedad) juegan un papel importante en el vigor de la encina. Las alteraciones en la abundancia y diversidad de ectomicorrizas también se relacionan con el decaimiento de la encina.

ÍNDICE GENERAL

INTRODUCCIÓN GENERAL

1. Decaimiento de *Quercus ilex*

A partir de finales de los años 80 y principios de los 90 se observa en toda Europa un deterioro generalizado de las masas de *Quercus*. En el caso de la encina (*Quercus ilex* L.), se registran casos de debilitamiento generalizado y mortalidad en países como Francia (Delatour, 1986; Robin et al., 1998; Hansen and Delatour, 1999; Marçais et al., 2004), Italia (Ragazzi et al., 2000; Vettraino et al., 2002) o Portugal (Sousa et al., 2007). En España este deterioro de la encina se observó en Castilla la Mancha (Tuset et al., 1996; Romeralo, 2008), Extremadura (Brasier et al., 1993; Tuset et al., 1996; Gallego et al., 1999; Rodríguez-Molina et al., 2005; Del Pozo, 2006; Solla et al., 2009) y Andalucía (Brasier et al., 1993; Tuset et al., 1996; Sánchez et al., 2002, 2003a, 2006), comunidades que incluyen la mayor concentración de encinares adehesados.

En España los grandes periodos de sequía acontecidos durante los años 80 y 90 fueron considerados inicialmente los principales responsables del debilitamiento de *Q. ilex* (Fernández y Montero, 1993; Lloret et al., 2004). Los síntomas asociados a este deterioro (defoliación, clorosis de hojas, muerte regresiva de ramas y brotes) eran similares a los descritos en plantas con un estado hídrico deficitario. Sin embargo, los patrones de distribución diferían de los típicos asociados a estrés hídrico, ya que las encinas decaídas aparecían aisladas o agrupadas en pequeños focos rodeadas de encinas asintomáticas. En ocasiones, estos focos también se distribuían a lo largo de puntos de escorrentía intermitente (valles o vaguadas) o en

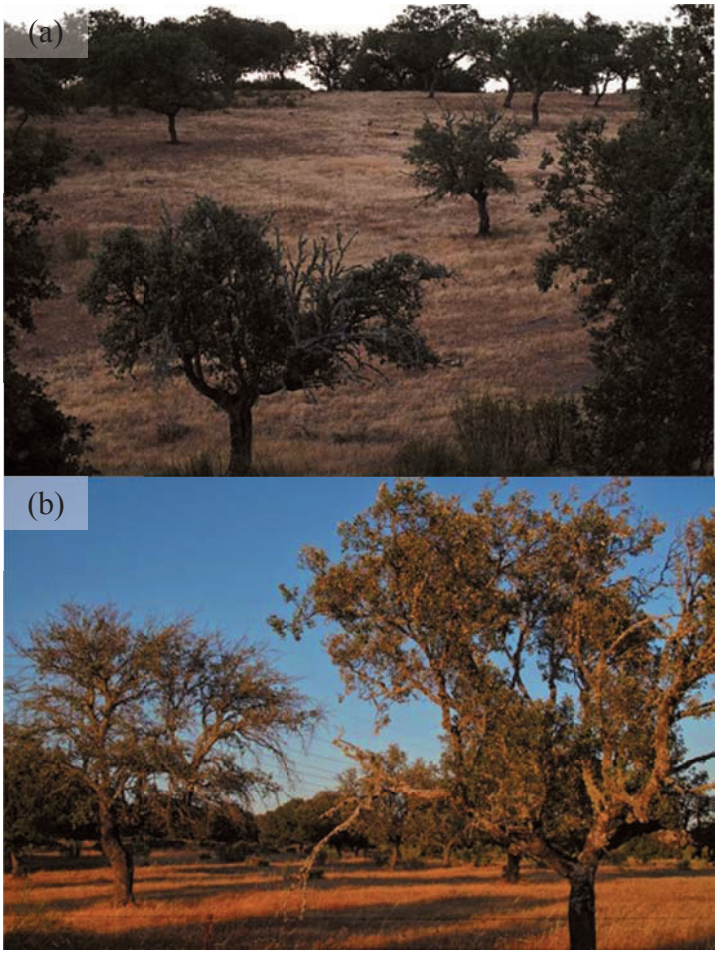

Fig. 1. Focos de encinas con síntomas de decaimiento en (a) ladera y (b) vaguada.

laderas (Fig. 1a y 1b), diferenciándose de otros grupos de árboles asintomáticos, lo que hacía sospechar la implicación de agentes bióticos. Mientras tanto, en Centroeuropa se comenzó a describir el debilitamiento en los *Quercus* como un decaimiento en el cual diferentes factores bióticos y abióticos se intercambiaban o interaccionaban, causando en ocasiones la muerte del árbol (Manion, 1991).

Estos factores involucrados en el decaimiento se podían agrupar en 3 tipos: (i) factores de predisposición, que actúan a largo plazo, incluyendo factores tipo bióticos y abióticos; (ii) factores incitantes, de corta duración, que suelen ser abióticos y dan lugar a la expresión de síntomas; y (iii) factores contribuyentes, que actúan a largo plazo en las últimas etapas del decaimiento, pudiendo provocar la muerte del árbol afectado. En España, el continuo aislamiento del oomiceto *Phytophthora cinnamomi* Rands en encinas y alcornoques decaídas, en muestreos realizados en 1991 y 1992 en Extremadura y Andalucía por Brasier et al. (1993), evidenciaron la posible implicación de este patógeno como un factor importante de mortalidad. Sin embargo, las bajas tasas de aislamiento de este patógeno observadas en sucesivos años condujeron a la búsqueda de otras causas.

De esta forma, se comenzó a explicar el deterioro, también conocido como "seca de la encina", con el modelo de decaimiento, el cual incluye los tres tipos de factores anteriormente mencionados (Manion, 1991). Los principales factores de predisposición son las prácticas selvícolas inadecuadas que se asocian al envejecimiento del arbolado, la falta de regeneración, la sobreexplotación ganadera y la presencia de heridas debidas a podas excesivas o a daños mecánicos (Naveiro et al., 1999). El factor de incitación más importante son las alteraciones climáticas, como una prolongación del periodo de sequía y el incremento de las temperaturas estivales, que además han caracterizado las últimas décadas. Estas variaciones climáticas podrían haber afectado al estado fisiológico de la encina y haber influido en un

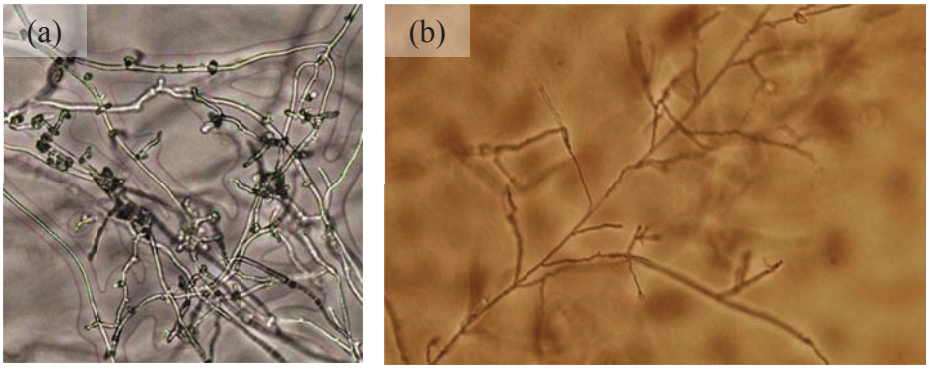

Fig. 2. Hifas de los oomicetos (a) *Phytophthora cinnamomi* y (b) *Pythium spiculum*.

aumento de los síntomas de decaimiento (Brasier, 1996). Los factores contribuyentes incluyen al oomiceto *P. cinnamomi* (Fig. 2a) (Brasier et al., 1993; Gallego et al., 1999; Sánchez et al., 2002) y al menos virulento *Pythium spiculum* (Fig. 2b) (Romero et al., 2007), ambos causantes de la podredumbre radical. También se han citado otras Phytophthoras involucradas (Vettraino et al., 2002), y otros agentes causantes de chancros como *Botryosphaeria* spp. (Sánchez et al., 2003b) y *Brenneria quercina* (Soria et al., 1997). De forma secundaria actuaría el hongo *Biscogniauxia mediterranea*, causante del chancro carbonoso en árboles ya debilitados (Carrasco et al., 2009), e insectos pertenecientes a los géneros *Cerambyx*, *Coroebus* y *Prinobius* (Romeralo, 2008; Carrasco et al., 2009).

La acción repetida de estos tres tipos de factores conduciría a la disminución del vigor del árbol y a la expresión de los síntomas de decaimiento (Fig. 3). Estos síntomas, que resultan inespecíficos, se pueden dividir en: (i) aéreos como defoliación, puntisecado de ramas, muerte de brotes, clorosis y marchitamiento de hojas, aparición de

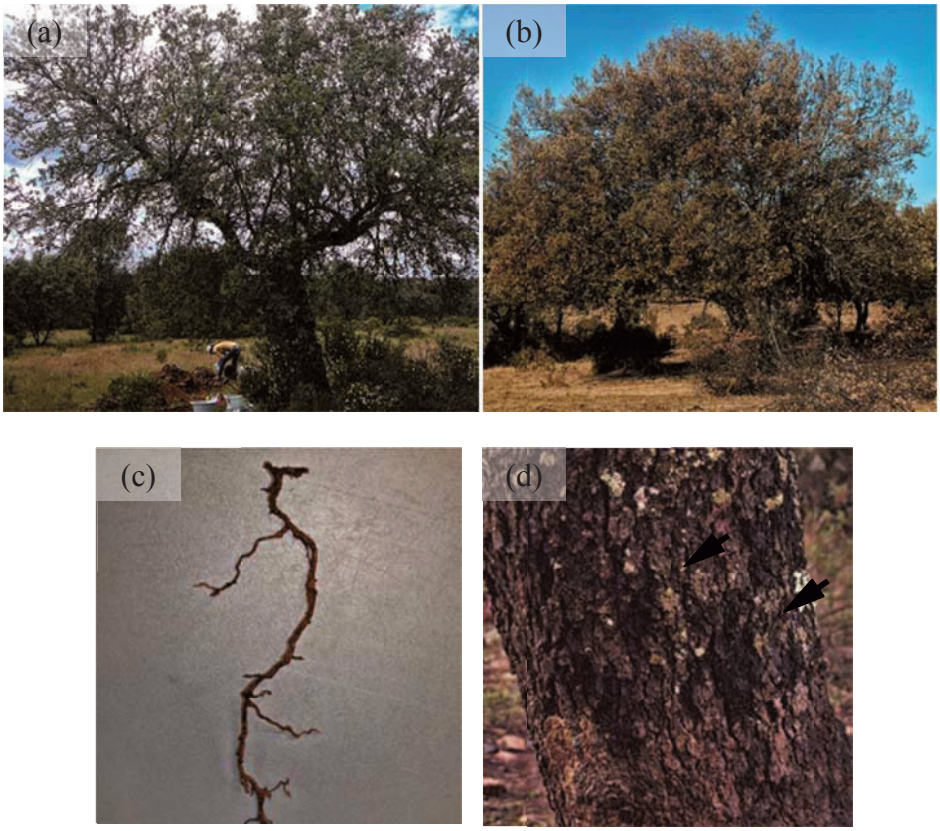

Fig. 3. Síntomas asociados al decaimiento de la encina: (a) defoliación progresiva; (b) muerte súbita; (c) necrosis de raíces finas; (d) chancro sangrante.

chancros, emisión de brotes adventicios; y (ii) subterráneos, como necrosis radical (principalmente de raíces finas y laterales), anillamiento de raíces y reducción de la micorrización. El decaimiento puede ser gradual, dando lugar a un proceso de muerte lenta, o puede estar asociado a procesos de muerte súbita, en los cuales todas las hojas se marchitan y permanecen adheridas al árbol moribundo.

En la actualidad, el debilitamiento y la mortalidad de las masas de *Q. ilex* en España se explican mediante patrones diferentes en el

espacio, ya sea por procesos de decaimiento o por enfermedades de etiología simple. De esta forma, en Andalucía occidental se asocia el debilitamiento de las encinas a la podredumbre radical producida por *P. cinnamomi* y por *Py. spiculum*, éste último con menor implicación (Sánchez et al., 2002, 2003b, 2006; Navarro et al., 2004). En cambio, en Andalucía oriental, el deterioro de las encinas viene explicado probablemente por la acción de la sequía (Sánchez et al., 2003b, 2006; Navarro et al., 2004). En la zona norte de Andalucía (Sierra Morena), los daños asociados a la encina se relacionan con un proceso de decaimiento en el que numerosos factores bióticos actúan, siendo el principal *P. cinnamomi* (Sánchez et al., 2006; Navarro et al., 2004).

A principios de los 90, en Castilla La Mancha, mediante las prospecciones realizadas en focos decaídos se detectó la presencia de *P. cinnamomi* (Cobos et al., 1993), aunque se apuntaba también como principal causa la escasez de precipitaciones (Fernández y Montoya, 1993). En el año 2007, se llevó a cabo en esta misma región un inventario para la localización y caracterización de focos de *Quercus* con síntomas de decaimiento. En dicho inventario se contabilizaron un total de 158 focos entre cuyas especies afectadas figuraba *Q. ilex* (Romeralo, 2008). En 2011, Solla et al. (datos no publicados) aislarían *P. quercina* y *P. psychrophila* en varias masas de encinas decaídas (Fig. 4). El aislamiento de estos patógenos hace sospechar su implicación en el deterioro de *Q. ilex* en ésta y otras regiones, tal y como se constata en Pérez-Sierra et al. (2013) para la Comunidad Valenciana.

Fig. 4. Ladera con encinas (*Quercus ilex*) mostrando síntomas de decaimiento localizada en Castellar de Santiago (Ciudad Real).

En Extremadura, los estudios realizados en las últimas décadas permitieron aislar *P. cinnamomi* y estudiar su patogeneidad (Gallego et al., 1999; Rodríguez-Molina et al., 2002, 2005). Estos estudios previos carecían de un análisis de todos los posibles factores abióticos y bióticos que podrían estar interaccionando con la actividad de *P. cinnamomi*. Con el objetivo de estudiar estos posibles factores, además de cuantificar y caracterizar los focos de *Quercus* con decaimiento en Extremadura, se realizó una prospección en 2003 y 2004 (Del Pozo, 2006). Los resultados del estudio mostraron la existencia de un total de 441 focos de *Quercus* sintomáticos, estableciendo relaciones con el tipo de suelo, la litología y los factores climáticos. De esta forma, surgió la necesidad de explorar más a fondo

estos agentes, así como su posible relación con *P. cinnamomi*. Precisamente, este estudio más detallado se realizaría en 2008 y 2009 sobre 96 focos (ver capítulos I, II, y IV). Además, la posible implicación de otras Phytophthoras en el decaimiento de la encina está siendo estudiado (Miranda et al., 2012; ver anexo I). Otro reciente trabajo realizado a través de imágenes IR ha mostrado un aumento en la región del número de focos con decaimiento hasta alcanzar los 5.017 (Cardillo et al., 2012).

2. *Phytophthora cinnamomi* Rands.

El género *Phytophthora* tiene una distribución mundial y es causante de más del 66 % de todas las enfermedades en raíces finas y más del 90 % de todas las necrosis de cuello del tronco (collar) (Jung, 2011). Esta amplia distribución se debe a la acción de vectores de dispersión a pequeña y gran escala (Fig. 5). A pequeña escala, encontramos vectores como los animales o los seres humanos a través de su desplazamiento o excreción (Krull et al., 2012). También, acciones antrópicas como los movimientos de tierra asociados a un uso agrícola o a la construcción de carreteras (Weste and Taylor, 1971) actúan como factores de dispersión. Por último, figuran los vectores ambientales como la escorrentía, las salpicaduras de lluvia, el viento o la lluvia arrastrada por el viento, en los cuales *Phytophthora* puede participar, tanto de forma activa como de forma pasiva. A gran escala destacan las masivas importaciones de plantas y sustratos infectados con *Phytophthora* spp. procedentes de viveros de todo el mundo. Probablemente, estas importaciones junto con prácticas sanitarias y de

Fig. 5. Vectores de dispersión de los propágulos de *Phytophthora cinnamomi*: (a) ganado presente en un foco de encinas infectadas con *P. cinnamomi* en la provincia de Cáceres; (b) parcela con encinas infectadas con *P. quercina* y *P. psychrophila* localizadas a ambos lados de un camino por el que discurren vehículos en la provincia de Ciudad Real; (c) plántulas de *Pinus pinaster* con presencia de *Phytophthora* spp. en vivero.

control inadecuadas han favorecido la dispersión de estos oomicetos a nivel mundial. Estos patógenos se verán más beneficiados en términos de dispersión si presentan un espectro grande de hospedantes como es el caso de *P. cinnamomi* (Brasier, 2008). Los muestreos más recientes realizados en Europa muestran la magnitud de esta problemática. Concretamente, un 93,7 % de los viveros presentan diferentes *Phytophthora* spp., y un 63,6 % de plantaciones están infectadas, lo

que supone una gran amenaza para la supervivencia de los bosques europeos (Jung et al., 2012).

El oomiceto *P. cinnamomi* fue hallado por primera vez en la isla de Sumatra (Indonesia) por Rands (1922) y fue descrito como agente causante del chancro en el cinamomo (*Cinnamomum burmannii*). Actualmente su origen se sigue asociando al sureste de Asia. Su presencia se extiende por los cinco continentes, incluyendo a más de 70 países y abarcando zonas de clima subtropical, tropical, mediterráneo y algunas ligeramente templadas (EPPO, 2011). Esta gran versatilidad ambiental se debe a sus estructuras de resistencia a corto y largo plazo, que le permiten sobrevivir bajo condiciones desfavorables. Está considerada una de las especies invasoras más devastadoras según la Unión Internacional para la Conservación de la Naturaleza (IUCN), con un amplio rango de huéspedes. Zentmyer (1980) identificó cerca de 1000 especies como posibles hospedantes, cifra que algunos autores han incrementado hasta 3000 (Grünwald et al., 2011) e incluso 5000 especies (Jung et al., 2013).

2.1 Taxonomía del género *Phytophthora*

El estudio del género *Phytophthora* comenzó en 1846 al descubrirse que la enfermedad del tizón tardío o mildiú de la patata, que causó la famosa gran hambruna irlandesa de la patata, estaba producida por un hongo. En 1876, tras numerosas descripciones de este hongo, el micólogo Anton de Bary sería quién le pondría el nombre definitivo, *Phytophthora infestans*, que procede del griego y significa “destructor (phthorá) de plantas (phytón)”.

El género *Phytophthora* pertenece al reino *Stramenopila*, clase *Oomycetes* que recientemente ha sido renombrada como Peronosporomycetes, orden Peronosporales, familia *Peronosporaceae* (Blair et al., 2008; Beakes and Sekimoto, 2009). Los oomicetos, aunque en un primer momento se incluyeron dentro del reino Fungi por poseer características similares, posteriormente se separaron debido a las diferencias en numerosos rasgos. Entre ellos, destaca su ciclo vital que es predominantemente diploide, a diferencia del de los hongos, que es haploide o dicariótico. Además, sus paredes celulares están compuestas de celulosa y β-glucanos y no de quitina, y sus hifas no tienen septas, como en el caso de los hongos (Judelson and Blanco, 2005). Otra característica esencial de *Phytophthora* es su dependencia de la toma externa de esterol, ya que no es capaz de sintetizarlo y resulta imprescindible para la formación de sus estructuras.

Según los datos moleculares más recientes, existen en la actualidad aproximadamente 121 especies de *Phytophthora* (Hardy et al., 2012). A este listado hay que añadir nuevas especies como es el reciente caso de *P. parvispora*, anteriormente clasificada como *P. cinnamomi* var. *parvispora* (Scanu et al., 2013). Para facilitar la identificación de *Phytophthora*, Waterhouse (1963) creó unas claves taxonómicas por las cuales dividía las especies de *Phytophthora* en seis grupos, atendiendo a características morfológicas. *P. cinnamomi* se encuadraba dentro del grupo VI. Los nuevos avances en filogenética molecular han permitido desarrollar diferentes técnicas de identificación basadas en secuenciación de ADN. Estos métodos han facilitado la creación de una nueva categorización, en la cual las

diferentes Phytophthoras se encuadran dentro de clados. Concretamente el estudio más reciente identifica 10 clados (Kroon et al., 2012). Además de la detección de nuevas especies, son más frecuentes los casos de hibridación entre especies geográficamente aisladas, como por ejemplo *P. alni* subspecie *alni* (Brasier, 2012) y otros híbridos (Burgess, 2012).

2.2 Morfología de *Phytophthora cinnamomi*

El oomiceto *P. cinnamomi* posee un micelio formado por hifas coraloides, hialinas y aseptadas, aunque, cuando son cultivos antiguos o cuando las hifas separan distintas estructuras, pueden estar septadas. Estas hifas producen grandes y abundantes hinchazones hifales que caracterizan a esta especie (Fig. 6a). Los hinchazones hifales tienen forma esférica, elipsoidal o angular, aunque también adquieren una forma irregular conocida como hinchazones botriosos, y pueden ser intercalares, terminales o estar aislados, formando cadenas o racimos. Este patógeno es capaz de producir un tipo de hifas especializadas llamadas haustorios, que se encargan de la asimilación de nutrientes de las células del hospedante (Crone et al., 2013). Los haustorios producen la invaginación de la célula sin destruir la membrana plasmática, de manera que se permite la continua absorción de nutrientes sin destruir las células (Hardham and Blackmam, 2010). Este tipo de adquisición de nutrientes es típico de organismos biótrofos, y aunque tradicionalmente se ha considerado a *P. cinnamomi* como necrótrofo, en la actualidad se discute la capacidad de este patógeno para pasar de una fase necrótrofa a una fase como

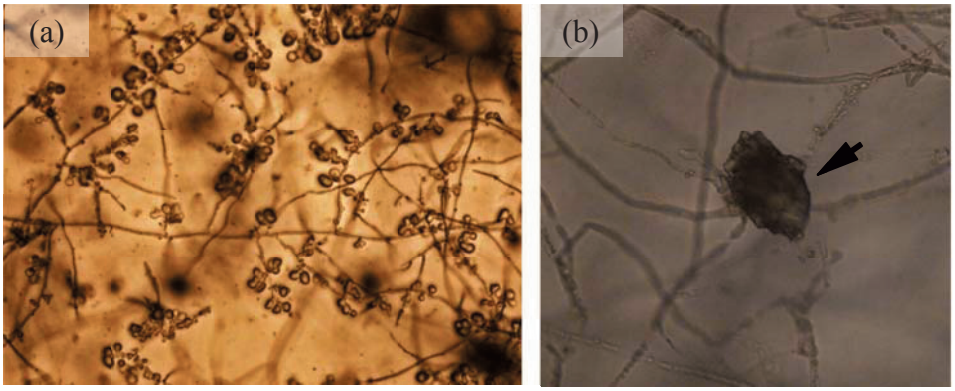

Fig. 6. Micelio de *Phytophthora cinnamomi* en el que se distinguen (a) los hinchamientos hifales con forma esférica y (b) las agregaciones de hifas similares a un estroma.

biótrofo o endófito, o al contrario, en función de los diferentes condicionantes ambientales (Hardham and Blackman, 2010; Crone et al., 2012, 2013). Otro tipo de formaciones son las agregaciones de hifas y de hinchamientos hifales que dan lugar a masas compactas parecidas a los estromas fúngicos (Fig. 6b) (Crone et al., 2013). Se ha observado, que estas agregaciones de hifas, además de hifas sueltas, podrían actuar como estructuras de resistencia a largo plazo cuando se localizaran en el interior de gruesas paredes celulares, papilas y depósitos de lignina de fragmentos de raíces (Jung et al., 2013).

Este oomiceto se considera una especie heterotálica, ya que para la formación de las estructuras sexuales es necesario el contacto de dos talos complementarios de la especie, conocidos con el nombre de A1 y A2. Al ponerse en contacto, por estimulación hormonal se crearán las estructuras sexuales conocidas como oogonios y anteridios. El talo A2 tiene una distribución mundial (Zentmyer,

1980), mientras que el talo A1 tiene un área muy limitada. Por otro lado, se ha demostrado que para la formación de estructuras sexuales no es necesario que A1 y A2 pertenezcan a la misma especie de *Phytophthora*. También existen casos de autofertilidad en A1 o A2 mediante otros estímulos. Esta autofertilidad se ha demostrado para A2 al ponerlo en contacto con *Trichoderma* spp. (Brasier, 1978), con raíces de *Acacia pulchella* o con suelo recogido bajo *A. pulchella* (Jayasekera, 2006; Jayasekera et al., 2007) y se ha observado para A1 en contacto con el medio de cultivo formado por harina de avena y agar (Ho et al., 1983). Otros casos de autofertilidad se han observado en plantas infectadas de forma natural (Crone et al., 2013; Jung et al., 2013).

Como se ha comentado, el oogonio y el anteridio son las estructuras sexuales que pueden ser unicelulares o bicelulares y aparecen delimitadas por septas. El oogonio suele ser globoso o subgloboso, con un tamaño variable y un diámetro medio de 40 μm (Erwin and Ribeiro, 1996). El anteridio mide de media 19 x 17 μm (Erwin and Ribeiro, 1996). Como especie heterotálica que es, el anteridio de *Phytophthora* es anfigino, de manera que durante la unión el oogonio crece a través del anteridio atravesándolo, y éste queda rodeando el pedúnculo del oogonio. Aún así, se han ratificado casos en los que *P. cinnamomi* ha producido anteridios paraginos (Hüberli et al., 1997). A través de la unión anteridio-oogonio se formará una oospora por meiosis en el interior del oogonio. Las oosporas tienen un tamaño de 19 a 54 μm (Erwin and Ribeiro, 1996) y poseen una pared celular gruesa que las protege y hace de ellas importantes estructuras

de resistencia a largo plazo. Jung et al. (2013) observó que la oospora constituía una estructura de supervivencia no tan inusual para el talo A2 de *P. cinnamomi* creada de manera autofértil en zonas de clima mediterráneo con sequías prolongadas.

Las estructuras reproductivas asexuales son los esporangios y las clamidosporas. Para la formación de las mismas y otras estructuras, *Phytophthora* depende de la toma de esteroles (Hendrix, 1965; Ponchet et al., 1999). Los esporangios se forman en unas hifas especializadas llamadas esporangióforos. Estos esporangióforos pueden estar aislados o formando ramificaciones simpodiales. El esporangióforo puede crecer en la base interna de un esporangio vacío dando lugar al nuevo esporangio (proliferación interna; Fig. 7a) o puede desarrollarse a través de este esporangio vacío a cierta distancia hasta formar el nuevo esporangio fuera de él (proliferación externa; Fig. 7b). Los esporangios maduran aproximadamente a las 24 horas de su formación (Jung, 2011, comunicación personal). Su tamaño es variable, siendo su valor medio de 75 x 40 µm (Erwin and Ribeiro, 1996). Estas estructuras se suelen formar en la superficie del material infectado debido a su dependencia de oxígeno para su formación (Zentmyer and Erwin, 1970). De manera directa o inducido por una repentina bajada de la temperatura (Tuset et al., 2001), los esporangios liberan las zoosporas, normalmente entre 8 y 40 (Fig. 7c y 7d). Las zoosporas son las esporas encargadas de infectar el material vegetal y de diseminar *P. cinnamomi*. Poseen 2 flagelos, uno liso y otro barbado que permiten su movilidad de forma activa por el agua libre del suelo, aunque también se desplazan de forma pasiva (Tuset et

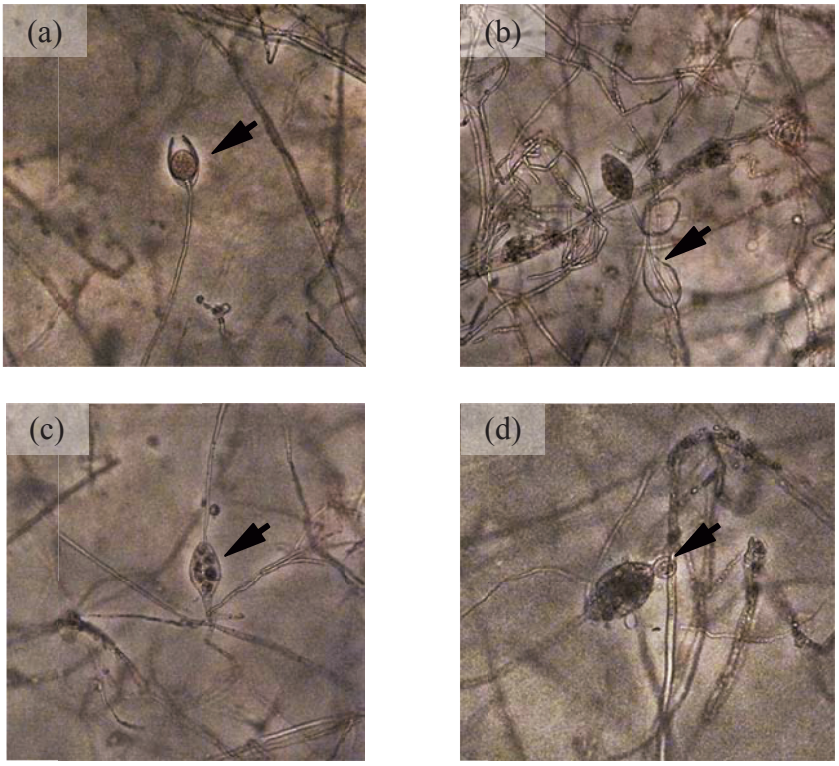

Fig. 7. Esporangios de *Phytophthora cinnamomi*: (a) formación mediante proliferación interna o (b) proliferación externa; (c) formación de zoosporas y (d) liberación.

al., 2001). Las zoosporas son atraídas quimiotácticamente por las exudaciones de las raíces (Weste, 1983) o bien a través de electrotaxis, autotaxis o autoagregación (Walker and Van West, 2007). Una vez en contacto con la raíz, pierden los flagelos y se enquistan, redondeándose y engrosando su pared celular (Hardham, 1987). Los quistes producen un tubo germinativo que penetra en la raíz y se ensancha formando una estructura similar a un apresorio que facilita la adhesión. Posteriormente se ramifica formando las hifas que dañaran los tejidos vasculares. Tienen una vida corta, de unas horas o

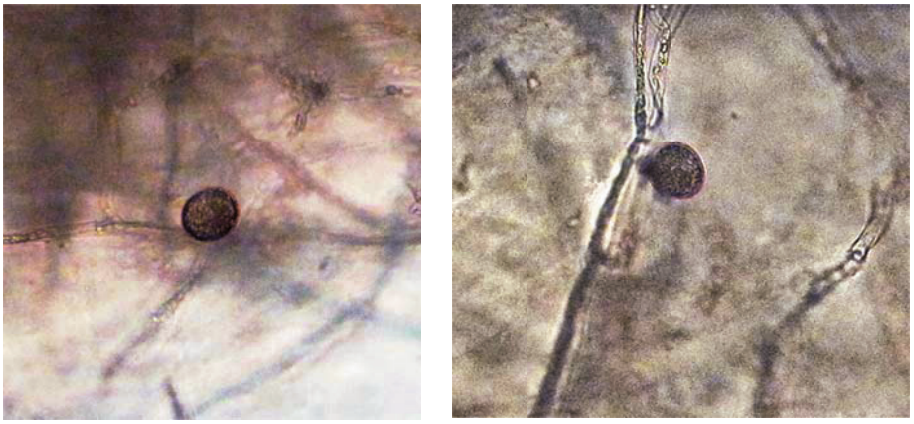

Fig. 8. Clamidosporas de *Phytophthora cinnamomi*.

incluso días, aunque como quistes pueden sobrevivir varias semanas dependiendo de la humedad (MacDonald and Duniway, 1979).

Las clamidosporas son estructuras de resistencia que se forman cuando las condiciones ambientales son desfavorables o ante la falta de nutrientes (Fig. 8). Como estructuras de resistencia que son, evitan la deshidratación y además impiden que otros microorganismos las destruyan. Tienen forma globosa y una pared fina, y se diferencian fácilmente de los hinchazones hifales porque se separan de las hifas a través de septas. Su tamaño medio ronda los 31-50 µm y se disponen de forma terminal o intercalar, formando frecuentemente racimos de tres a 10 clamidosporas. Las clamidosporas se han descrito como estructuras de supervivencia a corto plazo y no a largo plazo (Jung et al., 2013). Antiguos estudios mostraron la falta de viabilidad de las clamidosporas trascurridos varios meses (Mircetich and Zentmyer, 1966; Mackay et al., 1985). Por otro lado, Jung et al. (2013) no hallaron clamidosporas en muestras de raíces infectadas con *P.*

cinnamomi después de 6, 12 y 18 meses almacenadas bajo condiciones de aire seco.

2.3 Proceso de infección

Cuando las condiciones ambientales son desfavorables (bajas temperaturas y escasa humedad), *P. cinnamomi* se mantiene en el suelo o dentro de fragmentos de raíces necróticas en forma de estructuras de resistencia a corto plazo (clamidosporas) o largo plazo (oosporas, agregaciones de hifas e hifas) (Crone et al., 2012; Jung et al., 2013). La germinación de estas estructuras de resistencia se produce cuando las condiciones ambientales son favorables, es decir, cuando la temperatura ronda entre 24 y 28 ºC (Erwin and Ribeiro, 1996) o entre 26 y 30 ºC en el caso de aislados españoles (Sánchez et al., 2002), y cuando existe agua libre en el suelo, como ocurriría después de una lluvia otoñal o primaveral. Al germinar, producen esporangios que liberarán las esporas de infección, es decir, las zoosporas. Las zoosporas se desplazarán a través del agua libre y atraídas por las exudaciones de las raíces entrarán en contacto con éstas. Para infectar la raíz, este patógeno precisa que el tejido vegetal esté sano o recién herido, pero que no haya sido previamente invadido por otros organismos, ya que su baja capacidad competitiva hace de él un patógeno primario, no secundario. Inicialmente, suele infectar las raíces finas no suberizadas encargadas de la absorción del agua y posteriormente raíces laterales e incluso la raíz principal y el cuello (Robin et al., 1998). Al contacto con las raíces, las zoosporas se enquistan y se adhieren a ellas para germinar dentro de la raíz.

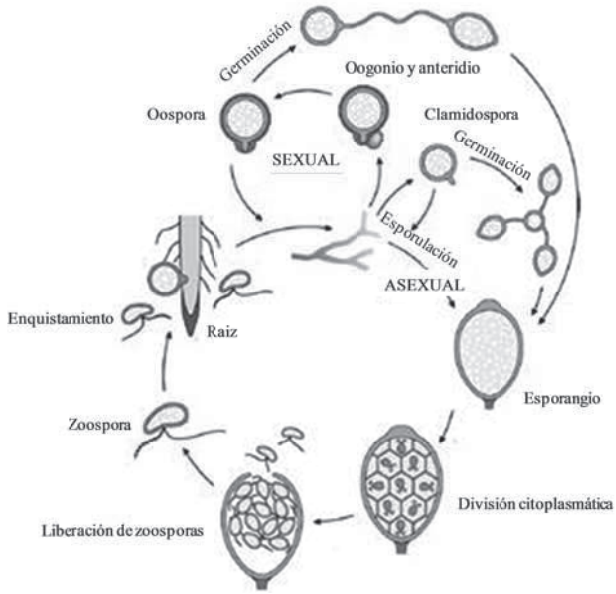

Fig. 9. Ciclo vital de *Phytophthora cinnamomi* (adaptación del diagrama del profesor A. Hardham, the Australian National University, Canberra, A.C.T.).

Una vez colonizadas las células de la raíz, el patógeno continúa expandiéndose inter y extracelularmente, infectando elementos del floema y xilema (Davison et al., 1994; Robin et al., 1998) y otras zonas de la raíz y además, comienza a formar clamidosporas. Redondo et al. (2012) observó que 7 días después de la inoculación de encinas con *P. cinnamomi* se formaban las clamidosporas, aunque en otros estudios con otras plantas se observan estas estructuras a las 48-72 horas después de la inoculación (Cahill et al., 1989). Al cabo de 24-48 horas se crearán esporangios en la superficie de la raíz infectada, que liberarán nuevas esporas infectivas (Cahill et al. 1989). El micelio del patógeno continuará creciendo y

expandiéndose a la vez que continuos ciclos de esporulación irán ocurriendo. El micelio de este patógeno se podrá descomponer por condiciones ambientales no óptimas, por la falta de nutrientes, por la competición ante la entrada de organismos antagónicos secundarios o por los mecanismos de defensa desarrollados por la planta contra *P. cinnamomi*. Se mantendrían, entonces, sólo intactas las estructuras de resistencia. Tras la descomposición de la raíz necrótica por hongos saprófitos, las esporas de resistencia son liberadas al suelo. Aquellos fragmentos de raíces no degradados servirán también de refugio a las estructuras de resistencia a corto y largo plazo. Por tanto, para la supervivencia del inóculo bajo condiciones ambientales desfavorables y ausencia de hospedantes se hacen imprescindibles las estructuras de resistencia. De hecho, Mircetich and Zentmyer (1966) confirmaron que *P. cinnamomi* pudo sobrevivir en un suelo húmedo después de 6 años en ausencia de hospedante. Al tratarse de un organismo de naturaleza multicíclica, cuando las condiciones ambientales sean de nuevo óptimas, el ciclo se repetirá con la germinación de estas estructuras aumentando el inóculo presente de *P. cinnamomi* (Fig. 9).

3. Complejidades en la relación de *Phytophthora cinnamomi* con el decaimiento de la encina.

La complejidad del estudio de la podredumbre radical causada por *P. cinnamomi* se debe a la existencia de numerosos factores que influyen en la patogeneidad de *P. cinnamomi* y en la susceptibilidad del árbol hospedante, los cuales pueden determinar el desarrollo y la evolución de la enfermedad.

3.1 Factores que afectan al vigor de *Quercus ilex*

En experimentos en campo (Ogaya and Peñuelas, 2007; Galiano et al., 2012), modelizaciones (Martínez-Vilalta et al., 2002) y en invernadero (ver capítulo V) se ha observado una sensibilidad de *Q. ilex* a la sequía prolongada, que produce un debilitamiento y muerte del arbolado, aún siendo una especie tolerante a este factor (Gimeno et al., 2009; Pinto et al., 2012).

La compactación del suelo dificulta el crecimiento y la expansión de las raíces y disminuye la tasa de infiltración del suelo, lo que influye en la encina a la hora de combatir situaciones de estrés hídrico (Cubera et al., 2009). Además, la compactación favorece el encharcamiento del suelo porque disminuye la conductividad hidráulica. Finalmente, el porcentaje de agua utilizable por las raíces será menor, ya que el agua se acumulará en poros de difícil acceso y zonas de escasa o nula presencia de raíces.

La escasez de suelo (Fig. 10a) se ha relacionado con la mortalidad de *Q. ilex* (Galiano et al., 2012). Suelos pocos profundos tienen numerosos efectos perjudiciales para las plantas ya que impiden el acceso de las raíces a niveles freáticos profundos. Al localizarse más superficialmente, las raíces son más susceptibles a daños mecánicos por manejo; además, estos suelos tienen más facilidad para sufrir encharcamiento y menos capacidad para almacenar agua en situaciones de déficit hídrico (Peñuelas et al., 2000).

Los suelos hidromorfos favorecen situaciones de anoxia y pudrición de raíces, asociándose a la ubicación de encinas decaídas

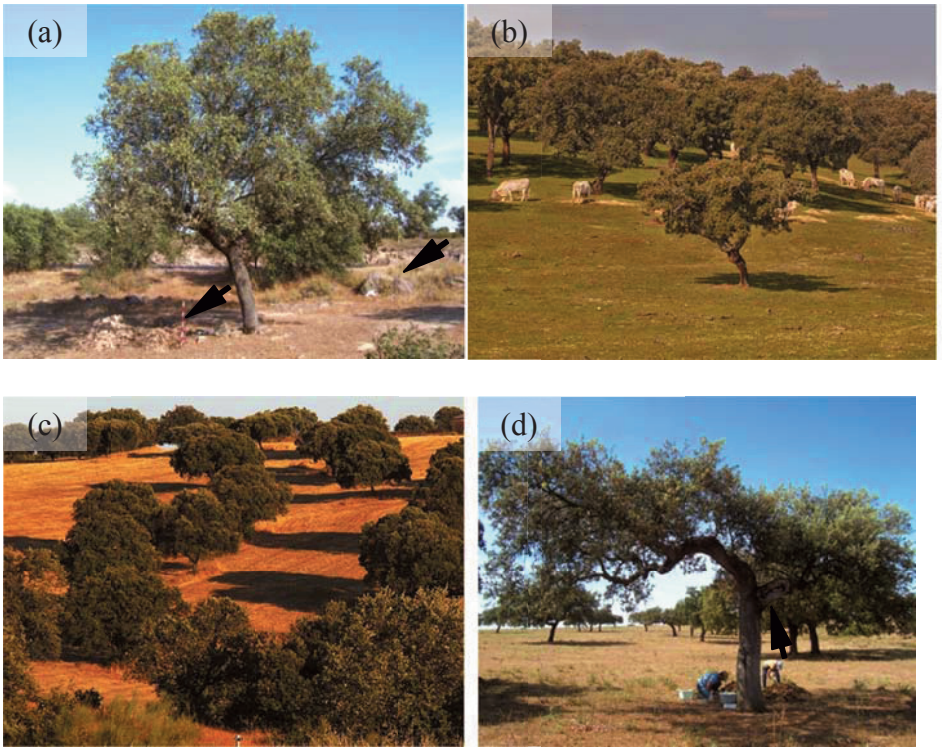

Fig. 10. Factores que afectan al vigor de la encina: (a) suelos poco profundos; (b) presencia de ganado asociado a un exceso de N y compactación; (c) siembra con uso de fertilizantes y vehículos pesados; (d) poda excesiva.

(Del Pozo, 2006). En ambientes encharcados se produce un cambio hacia la respiración anaerobia, lo que produce menos energía, la elongación de las raíces disminuye, y la toma de agua se reduce (Kreuzwieser et al., 2004; Davison, 2011). Sin embargo, hay estudios realizados en encina en los que no se han observado daños en el sistema radical tras un prolongado encharcamiento (Sánchez et al., 2005).

Las laderas, vaguadas o depresiones también se asocian a la existencia de un mayor número de árboles muertos o decaídos (Brasier et al., 1993; Fernández y Montero, 1993; Del Pozo, 2006). En las vaguadas, la mortalidad se explica porque los árboles tienen una mayor disponibilidad hídrica y, por tanto, presentan un menor desarrollo radical, lo que hace que en periodos de sequía intensa y/o prolongada, el árbol se vea afectado más severamente (Fernández y Montero, 1993). También se ha observado un aumento de la mortalidad y falta de vigor en árboles localizados en laderas con orientación sur posiblemente debido a una menor humedad edáfica (Costa et al., 2010; Brasier, 1996). Similarmente, encinas en zonas de mayor altitud se relacionan positivamente con un aumento de la mortalidad (Fernández y Montero, 1993; Galiano et al., 2012).

Los usos del suelo afectan igualmente a la disponibilidad de agua para la encina. Así, en sistemas adehesados se ha demostrado que la existencia de una abundante capa arbustiva disminuye la humedad edáfica y ejerce un efecto competitivo por los recursos hídricos con la encina, especialmente durante el verano (Cubera and Moreno, 2007). Un exceso de N asociado a la fertilización o al ganado (Fig. 10b y 10c), el cual tiene gran presencia en los sistemas adehesados, también resulta perjudicial para el árbol (Cubera et al., 2009). Concretamente, el nitrógeno mineral en forma de NH_4^+ asociado a los purines del ganado se suele concentrar bajo la copa de los árboles (Gallardo, 2003) y afecta negativamente el crecimiento de las raíces de las encinas (Cubera et al., 2009). El ganado, al igual que los trabajos mecánicos y el uso de vehículos pesados también

favorecerán la compactación del suelo y los problemas derivados de ésta. Un aclarado y una eliminación excesiva de los rebrotes repercute negativamente en las reservas de carbono de *Q. ilex* y por tanto en su vigor (Espelta et al., 2003; López et al., 2009). Otro tipo de manejos selvícolas como una poda única temprana, en contra de una poda de conservación o excesiva (Fig. 10d), es recomendada en el árbol ya que repercute en un mejor estado de salud en términos de defoliación (Jovellar et al., 2012).

En relación a la nutrición, la encina es considerada una especie que tolera suelos poco fértiles, como son los que caracterizan las dehesas del sur peninsular (Moreno and Obrador, 2007), debido a su estrategia más conservativa del uso de los nutrientes (Valladares et al., 2000). Por tanto, no cabe esperar una influencia importante en el estado del árbol.

También tienen un papel importante en el debilitamiento de la encina los xilófagos *Cerambyx* sp. y *Coroebus undatus* (Fabr.), el hongo *Botryosphaeria* spp. y la bacteria *Brenneria quercina*. En menor medida afectan el chancro carbonoso causado por *Biscogniauxia mediterranea*, el repilo producido por *Fusicladium quercus-ilicis* (Peglion) y la escoba de bruja causada por *Taphrina kruchii* (Vuill.) (Sánchez et al., 2003, 2006). Por el contrario, la abundancia de otros organismos como las micorrizas, ejercen un papel positivo en el vigor de las encinas ya que posibilitan una toma más eficiente de nutrientes y agua, como se ha confirmado en experimentos de invernadero (Domínguez et al., 2006; Oliveira et al., 2010). Estudios en focos de encinas decaídas han demostrado la

existencia de una relación entre la disminución de la simbiosis ectomicorrícica y el debilitamiento de encinas (Montecchio et al., 2004; ver capítulos II y III).

La falta de regeneración de *Q. ilex* es otro problema actual que repercute en la elevada edad del arbolado y amenaza la supervivencia de las dehesas (Moreno and Pulido, 2009; Plieninger et al., 2010). Esta falta de reclutamiento se debe a las prácticas de manejo inadecuadas como la falta de arbustos que protejan las plántulas de la depredación y la radiación (Plieninger et al., 2004; Rolo et al., 2013; Pérez-Ramos et al., 2013), los usos agrícolas (Plieninger et al., 2004), una excesiva carga ganadera acompañada del cambio de las prácticas trashumantes a pastoreo permanente (Carmona et al., 2013), la existencia de limitaciones relacionadas con la dispersión de bellotas (Pulido and Díaz, 2005) o la escasez de precipitaciones (Pérez-Ramos et al., 2013). Sin embargo, ni la producción de bellotas ni la depredación de éstas por insectos resultan factores limitantes en la regeneración (Leiva and Fernández-Alés, 2005).

3.2 Factores que afectan a *Phytophthora cinnamomi*

La disponibilidad de agua y la temperatura, como se ha descrito en apartados anteriores, son factores que tienen una gran influencia en el proceso de crecimiento y de infección de *P. cinnamomi*, por lo que variaciones en estas condiciones ambientales afectarán a la cantidad de inóculo presente (Shea et al., 1980; Shearer and Shea, 1987). Aunque se ha comentado anteriormente que el desarrollo de *P. cinnamomi* es favorable entre 24 y 30 ºC, la infección puede ocurrir

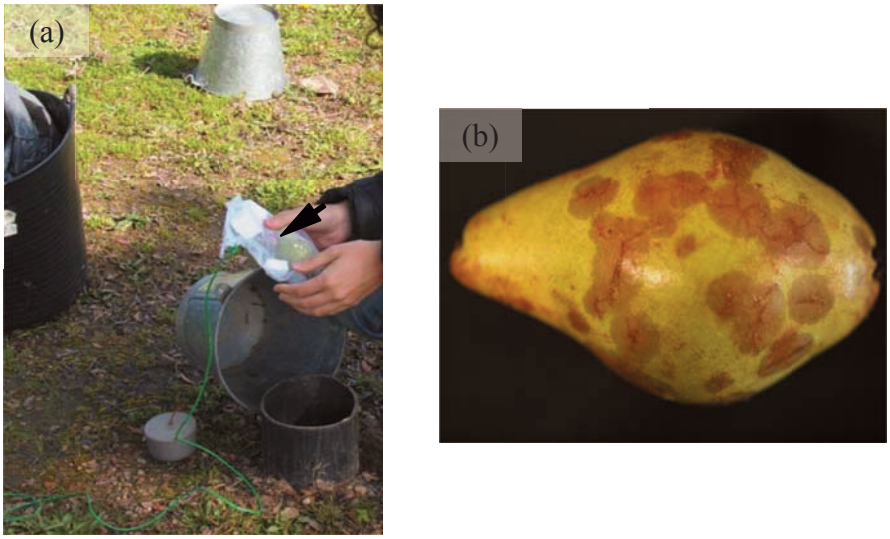

Fig. 11. Colocación de peras a modo de cebo (a) dentro de tubos de PVC utilizados para medir el nivel freático de las encinas y (b) posterior recogida de las peras infectadas con *Phytophthora cinnamomi* a partir de estos cebos.

incluso a los 15 ºC (Zentmyer, 1981), por tanto, nos encontramos ante un amplio rango que se corresponde con el otoño, la primavera y el inicio del verano. Incluso en invierno puede sobrevivir dentro del hospedante, que actúa como un buffer, o bien en suelos profundos (Shea et al., 1983), ya que en profundidad el suelo se mantiene a temperaturas relativamente más altas, lo que permitiría la germinación y esporulación de *P. cinnamomi* (Weste, 1983; Shearer and Tippett, 1989). En verano, el factor limitante es la humedad, que también se mantendría en profundidad y a niveles freáticos (Shearer and Tippett, 1989). De hecho, se pudo aislar *P. cinnamomi* usando cebos a una profundidad mayor de 2 m a través de piezómetros que contenían agua (Kinal et al., 1993). Igualmente, aprovechando los piezómetros colocados para el experimento del capítulo IV, se pudo detectar *P.*

cinnamomi en peras usadas como cebos flotantes en el agua a una profundidad de 5-6 m (Fig. 11). La lluvia sería clave para la dispersión de las zoosporas y otros propágulos en profundidad, arrastrándolos por capas profundas hasta alcanzar el agua freática, además de dispersarlos de forma horizontal. Por todo ello, *P. cinnamomi* es un patógeno que interacciona fuertemente con las condiciones climáticas del lugar, de manera que las alteraciones climáticas como el aumento de las lluvias intensas combinadas con sequías extremas o el aumento de las temperaturas derivadas del cambio climático podrían favorecer a la expansión de *P. cinnamomi* (Bergot et al., 2004).

La topografía del terreno, como las vaguadas y otro tipo de depresiones, benefician el encharcamiento, que a su vez favorece la dispersión de *P. cinnamomi* a través de las zoosporas, las cuales pueden llegar a sobrevivir más de tres semanas en suelos saturados (Tuset et al., 2001). De esta forma, el encharcamiento contribuye al aumento de las lesiones causadas por *P. cinnamomi* (Weste and Taylor, 1971; Shea et al., 1982; Brasier et al., 1993). Por otro lado, situaciones de anoxia derivadas del encharcamiento pueden dificultar la esporulación (Davison, 2011). Aún así, *P. cinnamomi* puede crecer a concentraciones bajas de oxígeno (Weste, 1983). En ladera, sin embargo, debido a una menor existencia de agua libre, *P. cinnamomi* solo actuaría durante cortos periodos de humedad asociado a precipitaciones (Brasier et al., 1993).

La textura del suelo, por su relación con la disponibilidad de humedad, y el pH influyen tanto en *P. cinnamomi* (ver capítulo I) como en las especies antagónicas de ésta. Los suelos de textura gruesa

pueden ejercer un efecto negativo en la abundancia de *P. cinnamomi* ya que tienen valores inferiores de capacidad de campo y menos facilidad para sufrir situaciones de encharcamiento. Ante periodos de sequía severos, este tipo de suelo afectará al ciclo biológico y a la capacidad de *P. cinnamomi* para infectar (Gómez-Aparicio et al., 2012; ver capítulo I). Respecto al pH, este patógeno tiene preferencias por un pH ácido, siempre que sean valores superiores o iguales a 3.8, lo que permitirá la producción de esporangios y, por tanto, la actividad de *P. cinnamomi* (Pegg, 1977; Benson, 1984).

La distribución espacial de este patógeno viene también determinada por la vegetación (Gómez-Aparicio et al., 2012). Ésta puede proporcionar un nicho y un hospedante para su multiplicación, por lo que influye en la abundancia de inóculo. Así, el tamaño del árbol (dbh) parece relacionarse de forma positiva con la abundancia del patógeno, explicado probablemente por una mayor capacidad para alojar inóculo (Gómez-Aparicio et al., 2012). Las plantas también podrán albergar un reservorio de inóculo de forma continua en aquellos casos en los que *P. cinnamomi* se comporte como biótrofo o endófito, manteniéndose la planta asintomática. Este hecho se ha observado en plantas como *Vicia sativa* (Serrano et al., 2012a), *Q. canariensis* (Gómez-Aparicio et al., 2012) u otras especies herbáceas y anuales (Crone et al., 2013). Por otro lado, las especies vegetales pueden ejercer efectos supresivos sobre el patógeno, como es el caso de *Olea europaea* (Gómez-Aparicio et al., 2012) mediante la liberación de exudaciones que producen la inhibición de la esporulación o el colapso de las clamidosporas (Jayasekera, 2006;

Jayasekera et al., 2007). También, las plantas pueden tener un papel estimulador, como es el caso de *Lupinus luteus* al incitar a la producción de zoosporas (Serrano et al., 2012a), o de *A. pulchella* var. *glaberrima*, *A. pulchella* var. *goadbyi* y *Persea americana*, al estimular la formación de oosporas (Zentmyer, 1983; Jayasekera et al., 2007).

Otros organismos también desempeñan un papel estimulador en *P. cinnamomi*, como algunas bacterias que incitan a la formación de esporangios (Ayers and Zentmyer, 1971), o el hongo *Trichoderma spp.* que estimula la formación de oosporas en el talo A2 (Brasier, 1978). La lisis del micelio de *P. cinnamomi* llevada a cabo por microorganismos puede también estimular la producción de esporangios (Malajczuk et al., 1983). Por otro lado, aquellos organismos antagónicos de *P. cinnamomi* ejercerán un papel regulador a través de la competencia o parasitismo de los propágulos. *P. cinnamomi* tiene poca habilidad saprófita debido a su incapacidad para competir con otros microorganismos. McCarren (2006) observó que este oomiceto era incapaz de crecer a través del suelo e infectar y desarrollarse dentro de materia orgánica muerta. Sólo en suelos donde la actividad microbiana es baja es probable contemplar cierta actividad saprófita en *P. cinnamomi* (Shearer and Smith, 2000; McCarren, 2006). Las micorrizas también pueden actuar como organismos antagónicos de *P. cinnamomi*, ejerciendo un rol de protección sobre la raíz con la cual establece la simbiosis (Fig. 12; ver capítulos II y III).

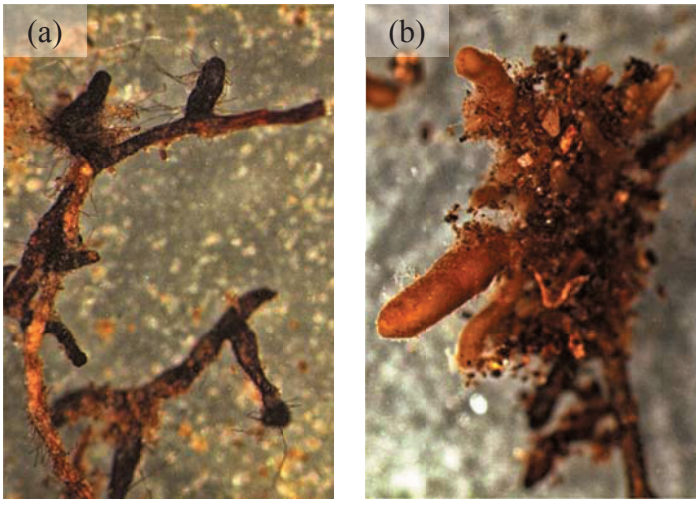

Fig. 12. Ectomicorrizas asociadas a encinas en focos con decaimiento: (a) el ascomiceto *Cenococcum geophilum*; (b) el basidiomiceto *Russula* spp.

Suelos con alta fertilidad, los cuales se asocian a un incremento de la materia orgánica, se relacionan con una mayor población microbiana que produce la lisis de las hifas o impiden la esporulación, y por tanto este tipo de suelos puede actuar como supresores de *P. cinnamomi* (McCarren, 2006). Sin embargo, sería recomendable estudiar estas densidades de población y los microorganismos que componen cada tipo de suelo, ya que se pueden observar tendencias contrarias (ver capítulo I). Por otro lado, Weste and Vinthanage (1978) observaron una desincronización entre el aumento en las concentraciones de microorganismos, que se daban en otoño e invierno, y el aumento de *P. cinnamomi* que tenía lugar durante la etapa primaveral y el inicio estival. Otro factor relacionado con la fertilidad es el contenido en nitrógeno del suelo en sus distintas formas (amonio, nitrato), el cual puede actuar también como inhibidor

de la germinación y esporulación de *P. cinnamomi* (Tsao and Oster, 1981).

Otros agentes que influyen en la dispersión y el aumento del inóculo son las prácticas de manejo en el área infectada, como se ha comentado previamente. Actividades como la agricultura y la ganadería ayudan a la dispersión de las esporas de *P. cinnamomi*, aumentando las probabilidades de infectar más árboles y de incrementar el inóculo presente (Brasier et al., 1993; Robin et al., 1998).

3.3 Factores que influyen la interacción *Phytophthora cinnamomi-Quercus ilex*

La complejidad del estudio de la infección y la severidad de la enfermedad viene determinada por las interacciones que tienen lugar entre los factores antes descritos.

Situaciones de sequía o de encharcamiento frente a condiciones óptimas de humedad pueden aumentar el daño que *P. cinnamomi* realiza a las raíces, y por otro lado pueden producir alteraciones fisiológicas en el árbol. Los experimentos de invernadero permiten simular distintas circunstancias de humedad en plántulas bajo densidades de inóculo probablemente más altas que en campo y sin la existencia en muchas ocasiones de una abundante flora microbiana antagónica. Bajo condiciones hídricas óptimas para la plántula infectada, se observan bajas mortalidades (Robin et al., 1998) y leves síntomas foliares (Maurel et al., 2001; Sánchez et al., 2002), lo

cual podría deberse a las condiciones óptimas de riego que permiten que la planta no sucumba.

Sin embargo, situaciones hídricas extremas tienen efectos visibles en la intensidad de la enfermedad. Situaciones de encharcamiento unidas a la presencia del patógeno dan lugar a un aumento en la severidad de la enfermedad (Sánchez et al., 2005) debido a que el estado fisiológico de la planta se puede alterar y la actividad del patógeno se ve favorecida. Esto trasladado a situaciones naturales, hace que en aquellas zonas o suelos con facilidad de encharcarse, la actividad de *P. cinnamomi* sea más severa, lo que combinado con un periodo de sequía podría provocar que el árbol, ya debilitado por el estrés hídrico, llegara a sucumbir (Sánchez et al., 2003a).

En situaciones de déficit hídrico, las encinas infectadas sufren una menor pudrición de raíces lo que repercute en un mejor estado aéreo (Maurel et al., 2001) y ecofisiológico (Robin et al., 2001), siempre y cuando ese déficit hídrico no sea muy prolongado. En este caso, la planta está sometida a un doble estrés hídrico, por un lado debido a la falta de agua y por otro lado debido a la reducción del sistema radicular que disminuye la capacidad de absorción de agua. En situaciones reales de campo con árboles adultos, un periodo de sequía severo puede conducir a efectos muy perjudiciales para la planta pudiendo desembocar en la muerte de ésta. Esto ocurriría, por ejemplo, durante una etapa de sequía en la cual el árbol se encuentra con escasas reservas de agua y por tanto con ajustes a nivel ecofisiológico (Gimeno et al., 2009) que se traducirían en una menor

disponibilidad de reservas de carbono (Galiano et al., 2012). Ante esta situación, un periodo corto de humectación (una lluvia puntual) sería aprovechado por *P. cinnamomi* para esporular y liberar zoosporas, ya que este patógeno responde rápidamente a cambios de humedad (Brasier et al., 1993; Sánchez et al., 2002). Sin embargo, para la planta ese periodo de humectación sería demasiado corto para recuperar las reservas de agua y de carbono y, de esta forma, producir nuevas raíces que reemplacen a las necróticas. Los efectos de la pudrición de raíces se agudizarían durante la época estival debido al estrés hídrico, conduciendo a un aumento de la mortalidad de las encinas como se observa en algunos estudios (Rodríguez-Molina et al., 2005).

Cuando la planta se expone a la alternancia de periodos de encharcamiento y sequía sucesivos (Fig. 13), los daños foliares y radicales por *P. cinnamomi* aumentan (Sánchez et al., 2005), dando lugar a una mayor mortalidad (Gallego et al., 1999). Hay que señalar que la gran mortalidad observada en campo durante los años 80 y 90 en España, coincidió con varios periodos de sequía prolongada combinados con lluvias esporádicas (Brasier et al., 1993; Brasier, 1996).

Otros factores importantes relacionados con la presencia de agua son la textura del suelo y la topografía. Suelos de textura arcillosa favorecerán la actividad de *P. cinnamomi* y podrán perjudicar a *Q. ilex* si se dan situaciones prolongadas de encharcamiento, como se ha comentado anteriormente. En ladera, el pátogeno se multiplica más lentamente, ya que estas zonas se asocian a déficit hídrico. Por tanto, sería solamente durante los cortos periodos de humectación

Fig. 13. Experimentos realizados en invernadero sometiendo a las plántulas inoculadas con *Phytophthora cinnamomi* a la alternancia de encharcamiento y sequía.

cuando *P. cinnamomi* se multiplicaría con mayor intensidad e infectaría al árbol de forma más severa, siendo el desarrollo de la enfermedad más lento en esta posición topográfica. En vaguadas, asociadas a periodos de encharcamiento más largos, la repetición del ciclo de germinación e infección de *P. cinnamomi* sería más rápido, con lo cual el debilitamiento del árbol se aceleraría, especialmente si coincide con una etapa de sequía severa.

El estado fisiológico y nutricional del árbol probablemente influya a la hora de enfrentarse a la infección de este patógeno y de mostrar los síntomas típicos (Jönsson, 2006). Nutrientes como Ca, Al o N afectan al crecimiento del patógeno y al árbol a la hora de combatir este patógeno. Así, en *Q. ilex* se ha demostrado que con una buena nutrición en Ca, el árbol se hace más tolerante a la infección por *P. cinnamomi* (Serrano et al., 2012b), debido seguramente al aumento de cohesión de la pared celular impidiendo la entrada del patógeno. Una vez que el árbol ha sufrido múltiples infecciones, se producen

efectos en términos fisiológicos, como la reducción de la conductancia estomática y fotosíntesis, reducción del potencial hidráulico y desequilibrios en el balance de nutrientes. Finalmente estos efectos se resumen en una alteración del balance de carbono y agua dentro de la planta (Jönsson, 2006). Efectos que son resultado directo e indirecto de la defoliación, del debilitamiento del sistema fotosintético de la planta, de la falta de raíces para absorber agua y nutrientes y de las interacciones entre ambos sistemas alterados (hojas y raíces). Si hay gran disponibilidad de agua y nutrientes, el árbol no sufrirá grandes alteraciones en términos fisiológicos y tendrá capacidad para producir carbono y destinarlo a la renovación de raíces, hasta que continuas infecciones vayan disminuyendo el carbono disponible para reponer raíces y hojas y mantener las defensas. Será entonces cuando disminuya aún más la toma de agua y nutrientes, y aumenten los ajustes fisiológicos, creándose un bucle de retroalimentación negativa (Jönsson, 2006).

Otro factor importante que influye en el progreso de la enfermedad es la presencia de otros organismos como las micorrizas que se asocian a los árboles de forma simbiótica e interaccionan de forma compleja con *P. cinnamomi* y la encina (ver capítulos II y III). Otros organismos con los que podría interaccionar *P. cinnamomi* son algunos basidiomicetos, también causantes de pudrición radical, que dan lugar a un aumento de los daños cuando estos patógenos actúan conjuntamente sobre el mismo hospedante (Marçais et al., 2011), aunque bajo condiciones controladas. Es probable que la introducción de otras especies de *Phytophthora*, además de *P. cinnamomi* (ver

anexo I), puedan aumentar los daños en la encina cuando actúen conjuntamente. Aunque se ha realizado algún estudio sin observar efectos sinérgicos (Miranda et al., 2012), una mayor exploración en este campo sería recomendable para obtener conclusiones.

La patogeneidad de *P. cinnamomi* también influirá en la severidad de la enfermedad, la cual depende del tipo de cepa (Robin et al., 1998). Robin and Desprez-Loustau (1998) hallaron diferencias en la virulencia de aislados de *P. cinnamomi* procedentes de ocho países. Sin embargo, ni las dos poblaciones diferentes halladas en el sur peninsular (Caetano et al., 2009) ni las cepas analizadas por Tuset et al. (2001) mostraron diferencias de virulencia. Por otro lado, algunos estudios han identificado en las distintas procedencias de *Q. ilex* diferencias en la susceptibilidad hacia el patógeno (Tapias et al., 2005; Moralejo et al., 2009; León, 2012), aunque en general *Q. ilex* está considerada una especie mucho más susceptible a *P. cinnamomi* que otros *Quercus* como así lo demuestran experimentos en invernadero (Robin et al., 1998; Tuset et al., 1996). La tolerancia de la encina es probable que también disminuya con el avance de la edad.

3.4 Factores que influyen en el diagnóstico del decaimiento y en la detección de *P. cinnamomi*

Los estudios relacionados con el decaimiento de la encina muestran la dificultad para determinar los agentes involucrados en el debilitamiento. En ocasiones, el debilitamiento del árbol es explicado como resultado de una enfermedad simple y otras veces es asociado a procesos de decaimiento, con varios factores interactuando o

sucediéndose. Por tanto, no existe un diagnóstico generalizado de las causas del decaimiento, sino que se requiere del estudio de las condiciones específicas de cada sitio. Para determinar de forma precisa los factores involucrados en el decaimiento es recomendable un seguimiento de las parcelas a medio plazo. El hecho de que los factores implicados puedan variar en el espacio y en el tiempo hace aún más imprescindible un seguimiento de las causas a lo largo de varios años. Así, desde 1999 en Andalucía se están realizando labores de seguimiento anual de focos de encina y alcornoque sintomáticos (Sánchez et al., 2006). Los experimentos realizados en los capítulos III y IV también incluyen mediciones mensuales en parcelas de encinas con síntomas de decaimiento (Fig. 14).

Existe también dificultad en atribuir visualmente el debilitamiento del árbol a la podredumbre radical por *P. cinnamomi*, ya que los síntomas asociados son inespecíficos, pudiendo ser también resultado de otras enfermedades o de estrés hídrico (Robin et al., 1998; Jung et al., 2000; Sánchez et al., 2006).

Por otro lado, las condiciones ambientales pueden contribuir para que un árbol se recupere de los síntomas de decaimiento (Sánchez et al., 2006), enmascarando a simple vista la posible presencia de *P. cinnamomi*. También, una actividad biótrofa o endófita de *P. cinnamomi* haría que el hospedante se mantuviera asintomático y se ocultase la presencia de éste (Crone et al., 2013). Por el contrario, puede ocurrir que la aparición de síntomas y la muerte de árboles sean rápidas cuando las condiciones ambientales son favorables. En general, se detecta un retraso entre la infección y la

Fig. 14. Muestra de parcelas con focos de decaimiento y presencia de *Phytophthora cinnamomi* donde se realizaron mediciones mensuales.

aparición de los síntomas, ya que se requiere de múltiples infecciones (Erwin and Ribeiro, 1996) que vienen condicionadas por las variables ambientales. Es preciso la pérdida de un porcentaje determinado de raíces para que aparezcan los síntomas (Tsao, 1990), y además ese porcentaje variará según la especie vegetal. De ello se deduce la necesidad de cuantificar la cantidad de daño en el sistema radical que se precisaría para la expresión de los síntomas aéreos (Jönsson, 2006). En los capítulos II y III se llevaron a cabo la cuantificación de raíces muertas y raíces vivas bajo árboles sintomáticos y asintomáticos y su relación con la infección de *P. cinnamomi*. El estudio de estos

síntomas radicales y foliares se ha realizado con frecuencia bajo condiciones de invernadero que favorecen la actividad del patógeno e impiden conocer las complejas interacciones con otros factores bióticos y abióticos y las dinámicas que se dan en el ecosistema. Esto impide obtener resultados realistas, y sólo algunos experimentos realizados en campo han permitido mostrar la complejidad del estudio de *P. cinnamomi* (Rodríguez-Molina et al., 2005; Gómez-Aparicio et al., 2012).

Con frecuencia, *P. cinnamomi* puede estar presente en el suelo o en el hospedante a niveles de inóculo tan bajos que resulta indetectable. Cuando estas concentraciones son bajas, un aumento de inóculo precisa que haya disponibilidad de raíces finas vivas susceptibles de ser infectadas y que se den condiciones ambientales adecuadas para la producción de esporangios y zoosporas que infecten el tejido (Shearer and Tippet, 1989). El hecho de que se precise una abundancia de raíces finas se pone de manifiesto en estudios como el de Gómez-Aparicio et al. (2012), en el cual se observó una menor abundancia de *P. cinnamomi* y *Pythium* spp. en árboles muertos.

Algunos estudios muestran una falta de relación entre síntomas y presencia de *P. cinnamomi* que puede venir asociado a causas relacionadas con la detección del patógeno. Según Davison and Tay (2005) es preciso la toma de 271 muestras de suelo para determinar con un 95 % de certeza la ausencia del patógeno dentro de un foco. También es recomendable la realización de estos muestreos de forma estacional, especialmente durante la primavera y el otoño (Sánchez et al., 2003a; Tuset et al., 2006), porque durante estas estaciones es

cuando tiene lugar la producción de raíces y la máxima actividad de *P. cinnamomi* (Shearer and Tippett, 1989; Brasier et al., 1993). Rodríguez-Molina et al. (2005) realizaron un seguimiento estacional de encinas plantadas en campo y detectaron una mayor mortalidad en primavera y verano atribuido a *P. cinnamomi.* En otro seguimiento a lo largo de distintas épocas, Sánchez et al. (2002) también observaron con el paso del tiempo un aumento de árboles con síntomas de podredumbre radical, además de una intensidad mayor en la defoliación. Otros estudios como los realizados por Shea et al. (1980) y Shearer and Shea (1987) describen muy bien la complejidad de la interacción de las variables ambientales de cada estación con la concentración de inóculo de *P. cinnamomi*.

El éxito de la detección de *P. cinnamomi* también está influido por el método de aislamiento utilizado (Robin et al., 1998), ya que el ciclo biológico de *P. cinnamomi* es muy específico y requiere de métodos concretos de aislamiento (Fig. 15). Por otro lado, el uso de estos métodos de aislamiento específicos para *P. cinnamomi* seguramente hayan impedido la detección de otras Phytophthoras presentes en el campo. Así, cada vez resultan más frecuentes las citas de otras especies en focos de encinas decaídas como *P. gonapodyides*, *P. drechsleri*, *P. megasperma*, *P. quercina*, *P. psychrophila*, *P. syringae* y una especie identificada como Clam-Phy (Sánchez et al., 2006; Pérez-Sierra et al., 2013; ver anexo I). Los métodos de aislamiento más eficaces suelen ser aquellos que intentan reproducir las condiciones ambientales naturales más favorables para la germinación y dispersión del patógeno, como es el método de uso de

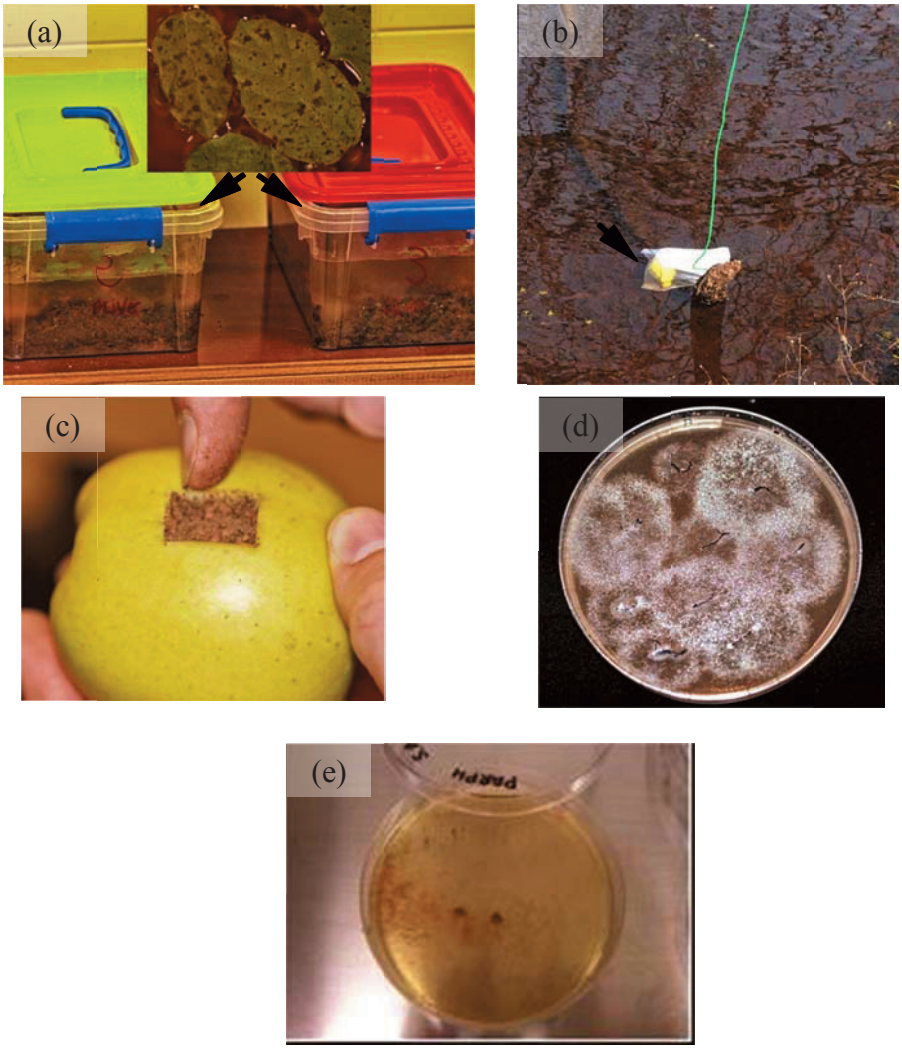

Fig. 15. Métodos de aislamiento de *Phytophthora cinnamomi* a partir de suelo, raíces y agua mediante (a) el uso de hojas, (b) peras y (c) manzanas como cebos; (d) aislamiento directo a partir de muestras de raíces sembradas en medio de cultivo selectivo NARPH; (d) técnica de extensión de suelo en placa.

cebos con muestras de suelo encharcado. Si las muestras se toman en primavera y otoño, posiblemente las probabilidades de éxito de aislamiento aumenten ya que el patógeno se encuentra activo (Tuset et

al., 2006). Durante la sequía estival es más difícil aislarlo debido a que estas condiciones restringen la esporulación de *P. cinnamomi* (Shearer and Tippett, 1989; Brasier et al., 1993). Asimismo, además de la temperatura media, la temperatura mínima también parece influir en el éxito de aislamiento (Sánchez et al., 2003a). En esos casos anteriores resulta recomendable humedecer la muestra de suelo sin encharcarla, manteniéndola en el laboratorio a temperaturas adecuadas, y posteriormente proceder a su aislamiento usando cebos (Jung, 2011, comunicación personal).

4. La simbiosis ectomicorrícica

Las ectomicorrizas constituyen un tipo de micorrizas de gran importancia en el ámbito forestal. Se definen como asociaciones simbióticas entre planta y hongo, en las cuales el hongo se beneficia de la toma de carbohidratos cedidos por la planta. El hongo, a su vez, contribuirá con una mejora en el crecimiento y la supervivencia de la planta a través de la captación más eficiente de nutrientes y agua, ejerciendo a su vez una mayor protección frente a factores estresantes como patógenos de raíz o periodos de sequía. La eficiencia en la toma de nutrientes está relacionada con el micelio extrarradical que poseen las ectomicorrizas, el cual proporciona una mayor superficie de absorción. Así, especies como el ascomiceto *Cenococcum geophilum* es capaz de aumentar en 28 veces la superficie de absorción de *Pinus taeda* (Rousseau et al., 1994). En la encina, se ha demostrado un aumento en la toma de P con la colonización de *Hebeloma mesophaeum* (Oliveira et al., 2010) y en la absorción de P, N y agua

con la colonización de *Tuber melanosporum* (Domínguez et al., 2006). Además, las micorrizas tienen la capacidad de mineralizar los nutrientes del suelo, de manera que la planta puede asimilar elementos en forma no disponible (Landeweert et al., 2001). Un mejor estado nutricional de la encina en simbiosis con las ectomicorrizas tiene efectos en el crecimiento. Así, la asociación de la encina con *H. mesophaeum* y con *T. melanosporum* implica un aumento de la altura del tallo (Oliveira et al., 2010) o un aumento del diámetro basal y de la altura y peso de tallo (Domínguez et al., 2006), respectivamente. Debido a la relación entre micorrizas y estado nutricional, y por tanto del vigor del árbol, la falta de micorrización ha sido en ocasiones asociada a ecosistemas en decaimiento (ver capítulos II y III). En estudios realizados con *Quercus* se han observado cambios en la riqueza de especies de ectomicorrizas en árboles decaídos, como en el caso de la encina (Montecchio et al., 2004). También, se ha observado una menor micorrización en árboles con síntomas de decaimiento que en árboles asintomáticos (Causin et al., 1996). Por otro lado, experimentos realizados mediante defoliación artificial han señalado que la defoliación afecta la simbiosis ectomicorrícica debido a una reducción en las reservas de carbono disponibles para la simbiosis (Saikkonen et al., 1999; Saravesi et al., 2008).

Un mejor estado nutricional de la planta, asociado a una mayor micorrización, probablemente contribuya a la reducción de la susceptibilidad de la planta a infecciones. Las ectomicorrizas también ejercen un papel importante como protector de la raíz frente a patógenos a través de diferentes mecanismos (Marx, 1972). Estos

mecanismos han demostrado detener la infección por *P. cinnamomi.* Entre ellos, destaca el manto fúngico y la red de Hartig que actúan como barrera física (Marx, 1970), la liberación de antibióticos (Marx, 1969), la estimulación por parte de las ectomicorrizas y los exudados de las raíces de una población microbiana protectora (Marx, 1973) o la estimulación de sustancias inhibidoras de patógenos liberadas por plantas colonizadas por ciertas ectomicorrizas (Marx, 1972). Atendiendo a los estudios realizados hasta la actualidad en el medio natural se observa una gran dificultad para explicar los cambios en la micorrización como una de las causas o efectos del decaimiento, lo cual pone de manifiesto la necesidad de más estudios.

5. Justificación de la Tesis Doctoral

La importancia del estudio del decaimiento de la encina en Extremadura deriva de la dominancia de la encina dentro de la dehesa, el sistema manejado más extenso de la región, que reporta grandes beneficios tanto económicos como ecológicos y sociales.

Hasta la actualidad, los estudios realizados en relación a las causas del decaimiento de *Q. ilex* tienden de manera generalizada al análisis de un número limitado de factores. Concretamente, los estudios se dirigen principalmente hacia el examen de factores bióticos (oomicetos, hongos, plagas) y climáticos (sequías, altas temperaturas) como posibles causas. Falta ahondar en las causas del decaimiento de la encina en relación a *P. cinnamomi*, y cómo las propiedades físico-químicas del suelo, las ectomicorrizas y la humedad edáfica afectan a la infección de las encinas por *P.*

cinnamomi. En esta tesis se abordan estos aspectos, estructurados en 5 capítulos interrelacionados. El capítulo I se enfoca hacia el análisis de la influencia de las propiedades edáficas, especialmente aquellas relacionadas con la fertilidad, la humedad y el manejo del suelo. En los capítulos II y III se estudian las alteraciones en la abundancia de ectomicorrizas a nivel extensivo y de forma estacional, respectivamente. En el capítulo IV se aborda el papel de la humedad edáfica. Finalmente, en el capítulo V se analiza la influencia de los eventos climáticos extremos en la susceptibilidad posterior de la planta a infecciones.

La influencia de las propiedades del suelo en el ciclo vital de *P. cinnamomi* ha sido origen de bastante investigación en laboratorio. Por el contrario, los estudios bajo condiciones naturales son escasos, destacando los realizados por Gómez-Aparicio et al. (2012) y Shearer and Crane (2011), aunque las especies vegetales estudiadas no eran las encinas. La influencia de las propiedades edáficas en la expresión de síntomas de decaimiento y en la interacción *P. cinnamomi*-árbol ha sido también poco explorado.

En relación a las ectomicorrizas, existe únicamente un estudio basado en la relación del decaimiento de la encina con la simbiosis ectomicorrícica (Montecchio et al., 2004), pero no existen estudios en campo que analizen la relación entre la infección de encinas con *P. cinnamomi* y el grado de micorrización, ni la relación entre estos dos factores y el grado de decaimiento.

Con el fin de estudiar la influencia de la humedad edáfica, se han realizado numerosos experimentos en invernadero donde se ha

demostrado una relación entre las condiciones de humedad y la infección de plántulas de *Q. ilex* con *P. cinnamomi* (Robin et al., 2001; Sánchez et al., 2002, 2005). Sin embargo, no existe en la actualidad ningún estudio en campo en el que se mida mensualmente la humedad edáfica y el nivel freático bajo encinas decaídas y no decaídas sometidas a la infección de *P. cinnamomi*.

El estudio del efecto del cambio climático en las masas forestales y en el comportamiento de los patógenos cuenta con un extenso número de referencias (por ejemplo, Santini et al., 2013; Bergot et al., 2004). Por el contrario, se desconocen los efectos que pueden tener en las plántulas eventos climáticos extremos (sequías prolongadas o precipitaciones abundantes), combinados o no, a la hora de enfrentarse a posteriores infecciones por *P. cinnamomi*.

Esta tesis doctoral se ha realizado en el marco del convenio "Prospecciones de focos de "seca de *Quercus*" con el fin de establecer su relación con las propiedades del suelo y la presencia de *Phytophthora cinnamomi* y de micorrizas en Extremadura" entre la Universidad de Extremadura y la Junta de Extremadura, y dentro de la acción COST "Established and emerging *Phytophthora*: increasing threats to woodland and forest ecosystems in Europe" (FP0801). Recibió financiación de la Junta de Extremadura (proyectos regionales III-PRI 08-A78 y IV-PRI IB10088), de los fondos europeos y del Ministerio de Ciencia e Innovación (AGL2007-64690/AGR y AGL2011-30438-C02-02).

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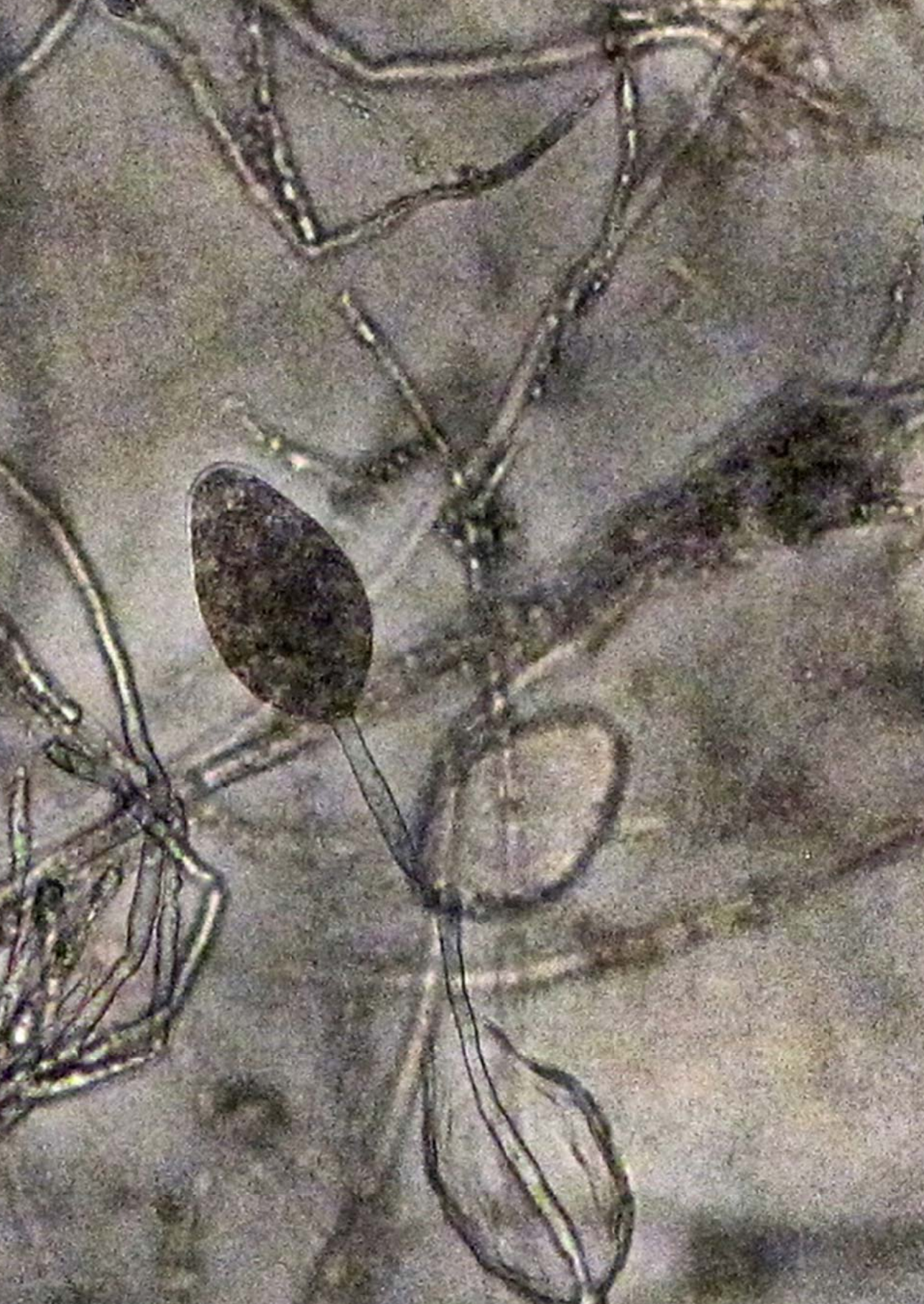

OBJETIVOS

El objetivo principal de esta tesis doctoral es realizar un estudio descriptivo de las causas del decaimiento de la encina en sistemas adehesados de Extremadura.

Los objetivos específicos son:

1. Analizar el rol de *Phytophthora cinnamomi* como agente biótico causante del decaimiento de la encina.
2. Estudiar la influencia de la humedad, del nivel freático y de las propiedades físicas y químicas del suelo en el decaimiento de la encina y en el proceso infectivo de *P. cinnamomi*.
3. Analizar la abundancia y diversidad de ectomicorrizas y su relación con la presencia de *P. cinnamomi* y el grado de decaimiento de la encina.
4. Estudiar la susceptibilidad de la encina a *P. cinnamomi* tras eventos climáticos extremos.

CAPÍTULO 1

Combined effects of soil properties and *Phytophthora cinnamomi* infections on *Quercus ilex* decline

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Abstract
The importance of soil properties as determinants of tree vitality and *Phytophthora cinnamomi* root infections was analysed. The study comprised 96 declining stands in western Spain, where declining and non-declining holm oak (*Quercus ilex* L.) trees were sampled. Soil properties (soil depth, Ah horizon thickness, texture, pH, redox potential, soil bulk density and $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ concentrations) and *P. cinnamomi* infections were assessed. Tree mortality rates increased with low soil bulk densities, which were also associated with more *P. cinnamomi*-infected trees. Occurrence of infected trees was higher in fine textured soils and in thick Ah horizons. Fine textured soils favoured trees, but with the presence of *P. cinnamomi* their health status deteriorated. Soil under declining trees had higher $N\text{-}NO_3^-/N\text{-}NH_4^+$ ratio values than under non-declining trees. Additional soil properties changes associated to grazing were not related to decline and *P. cinnamomi* infections. The implications of *P. cinnamomi* in holm oak decline and the influence of soil properties as contributors to pathogen activity were demonstrated. Fine soil textures and thick Ah horizons, usually favourable for vigour and vitality of trees growing in the Mediterranean climate, were shown to be disadvantageous soil properties if *P. cinnamomi* was present. Fine soil textures and thick Ah horizons are frequently related with higher levels of soil moisture, which increase the inoculum of the pathogen and favours root infection. Grazing does not seem to be directly linked to *Q. ilex* health status or *P. cinnamomi* root rot.

Keywords: silvopastoral systems; soil compaction; nitrogen deposition; soil texture; root-rot disease; oak decline

1. Introduction

Oak decline is widespread and has been explained by numerous concomitant factors made worse by the more frequent occurrence of climate extremes within the current global change context (Brasier, 1996), and with *Phytophthora* spp. as an important biotic factor involved (Jung et al., 1996; Corcobado et al., 2010; Pérez-Sierra et al., 2013), among others (Thomas et al., 2002; Camilo-Alves et al., 2013). The oomycete *P. cinnamomi* Rands is having a major impact in the Mediterranean region (Santini et al., 2013; Brasier, 1996), although during dry events water deficit also plays an important role (Lloret et al., 2004). Oak decline has also been explained as a consequence of long-term land use (Oszako and Delatour, 2000). In oak woodlands, grazing has been shown to have serious effects on tree population dynamics (Asner et al., 2004) and to enhance soil degradation (Evans, 1998). Grazing induces changes in nitrogen cycling and soil compaction (Singer and Schoenecker, 2003; Asner et al., 2004), and soil compaction affects soil properties such as porosity and water infiltration rate, and tree development with disruption in root growth (Kozlowski, 1999). Reduced root development due to compaction is especially critical in Mediterranean areas during summer, when trees depend on deep roots for water uptake (Alameda and Villar, 2009).

Ungulates and their grazing are major processors and regulators of N in the ecosystem, either accelerating or slowing N cycling (Singer and Schoenecker, 2003). Increased N supply in the form of NH_4^+-N excreted by ungulates through faeces and urine, especially when concentrated under tree patches used for feeding and shade

(Gallardo, 2003), has been suggested as a cause of plant toxicity with consequences for oak health (Jönsson et al., 2005; Cubera et al., 2009). Additionally, dung and urine deposition by grazing animals exerts a significant influence on microbial communities (Sankaran and Augustine, 2004). In the warm Mediterranean climate, soil organic matter is commonly quickly mineralised rather than humified (Aerts, 1997), which facilitates soil degradation in land managed a long time (Zhao et al., 2007). The nitrate to ammonium ratio has been proposed as an indicator of soil degradation, with higher ratios in disturbed soils (Wilson and Tilman, 1991).

The presence of *Phytophthora* spp. and their virulence (i.e. capacity to cause aerial and below-ground symptoms in infected trees) depends on soil water content and its dynamics (Zentmyer, 1980; Corcobado et al., 2013), given that *Phytophthora* species need free-water in soils for zoospore dispersal and consequent infection (Hardham, 1989). It has been suggested that the occurrence of *Phytophthora* spp. is associated with soil textures ranging from loamy to silty or clayey soils (Jung et al., 2000; Jönsson et al., 2005). This range of textures is associated with high soil water-holding capacities, which will favour moist conditions and *Phytophthora* sporulation (Gisi, 1983). Moreover, *Phytophthora* spp. require soil $pH_{(H2O)}$ values higher than 4.0 - 4.8 to stimulate sporangia production (Schmitthenner and Canaday, 1983; Jung et al., 2000), and nutrient-rich soils favour the presence of the pathogen (Schmitthenner and Canaday, 1983; Jönsson et al., 2005).

Holm oak (*Quercus ilex* L.) is the most dominant tree of many

Mediterranean ecosystems and is extremely vulnerable to infection by *P. cinnamomi* (Maurel et al., 2001). High mortality of *Q. ilex* in the Iberian Peninsula has been described for grazed open woodlands, known as dehesas in Spain and montados in Portugal (Brasier, 1996; Sánchez et al., 2002). The dehesa is the most common silvopastoral system in Europe and an outstanding example of biodiversity, listed as a high nature value farming, according to Oppermann et al. (2012). However, Iberian dehesas are experiencing progressive degradation caused by increased mechanisation and livestock density, among other inappropriate agroforestry practices (Moreno and Pulido, 2009). Consequences of these management practices include soil compaction, soil erosion (Schnabel et al., 2009), excess of N input (Morillas et al., 2012), maximum root depth decrease and fine root density reduction (Cubera et al., 2009).

Given the dependence of *Phytophthora* spp. activity on soil properties, it is hypothesised that changes in soil conditions from grazing would affect the ability of *Phytophthora* spp. to infect trees and influence the incidence and severity of disease symptoms. In this study, soil properties were analysed under declining and non-declining *Q. ilex* trees, infected with or free of *P. cinnamomi*. Soil depth, Ah horizon thickness, soil texture, redox potential and pH were assessed. Soil bulk density and mineral nitrogen content in the form of $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ were assessed as indicators of soil alteration from continuous livestock grazing or management. Through comparisons of soil properties associated with 288 symptomatic and 288 asymptomatic trees from 96 declining stands, the following

interconnected questions were addressed: (i) are the mortality and health status of *Q. ilex* trees related to soil properties?, (ii) do *P. cinnamomi* infections vary with soil properties?, and (iii) do soil properties interact with *P. cinnamomi* by modulating the intensity of oak decline? Answering these questions will make it possible to determine whether Iberian *Q. ilex* decline caused by *P. cinnamomi* is mediated by soil properties and processes of physical and chemical soil degradation caused by land use intensification.

2. Material and methods

Study area

The study was conducted in 96 declining *Q. ilex* stands in Extremadura (Western Spain), distributed throughout the region. Stands were selected at random from 420 declining oak forests (Del Pozo, 2006), after excluding stands where trees were infected by *Botryosphaeria corticola* or damaged by *Cerambyx* spp. Half the stands selected were located on stream banks and half were on slopes (8 to 20 degrees). The climate is typically Mediterranean with hot, dry summers (from June 21 to September 20) and cold, rainy winters (from December 21 to March 20). Stands were approximately 2-3 ha in size and composed of scattered *Q. ilex* trees (density 12-30 trees ha^{-1}) 6-10 m high, with annual species as understorey. Land use under most of the oak stands includes livestock rearing and cereal intercropping. Soils ranged from shallow (< 1 m depth) to moderately deep (1-1.5 m), mostly Cambisols and Luvisols, extending over acid

weathered rocks and derived sediments in a gently undulating landscape.

Tree selection and mortality assessment

The study was performed in 2008 and 2009, from late March to early June, the most favourable season for *Phytophthora* spp. isolation and tree status assessment in Spain. In each stand, decline intensity was obtained after rating and averaging the health status of 20 trees. The health status of a tree was assessed by visual estimation of the crown transparency, which referred to foliage loss. Crown or foliage transparency is defined as the additional amount of skylight visible through the crown compared to the amount of skylight visible through a fully foliated crown (UNECE, 2010). It is usually estimated in 5% classes based on the live, normally foliated portion of the crown. Dead branches, crown dieback and missing branches where foliage is expected to be missing were deleted from the estimate. Assessed by the same person during all the survey, crown transparency was scored as if the whole crown should be foliated (UNECE, 2010). A crown transparency of 0-14 % was rated class 1 and included non-declining trees; crown transparencies of 15-35, 36-55, 56-75 and higher than 75 % were rated class 2, 3, 4 and 5, respectively and included declining trees (Balci et al., 2007). Classification of the trees' health status was consistent with previous studies carried out in the Iberian Peninsula (Sánchez et al., 2000; Camilo-Alves et al., 2013). Mortality rates were obtained as the percentage of trees in classes 4 and 5. In each stand, three non-declining and three declining trees (classes 1 and 2,

respectively) were selected. Declining trees from the class 2 were selected because the probability of *P. cinnamomi* isolation under these trees is higher than under trees from classes 3 to 5. In conditions prevailing in SW Spain, once the threshold of 30 % of defoliation has been exceeded *Q. ilex* trees generally progress quickly into lower health status categories (Solla et al., 2009). To avoid the possibility of non-declining trees sharing parts of their rhizosphere with declining trees the distances between trees were above 50 m.

Soil sampling and analysis

One soil pit per tree (n = 576) was dug to allow sampling (see annex II.1). Soils pits were approx. 2.5 m wide, located 3-4 m down slope from tree trunks, and tangentially oriented to the crown. A hydraulic backhoe with a 40 cm-wide scoop was used for excavation. The maximum soil depth dug was 1.5 m, although in some cases the depth was lower due to the presence of hard rock, and this circumstance was recorded. Ah horizon thickness, defined visually *in situ* by the colour of the soil (change in value number in Munsell chart), was also recorded.

Soil redox status (Eh) was determined immediately in fresh samples from every pit at four depths (0.1, 0.5, 1.0 and 1.5 m) using the chemical tests proposed by Bartlett and James (1995). Tests involved qualitative measurements of tetramethylbenzidine oxidation colours, oxidation of added Cr ($CrCl_3$), reduced Fe (α,α'-dipyridyl), and easily reducible Fe (oxalic acid), combined with the detection of odor associated with anaerobic decomposition. This procedure

allowed soil layers to be classified into six soil redox status categories (*Superoxic, Manoxic, Suboxic, Redoxic, Anoxic*, and *Sulfidic*), which were related to quantitative values of redox potential for further statistical comparisons.

To study the possible compaction effect of livestock trampling on the uppermost soil layer, soil bulk densities were determined by extraction of two unaltered soil cores with a 100 cm^3 cylinder at depths of 0-5 cm and 5-10 cm. Soil bulk density was assessed by drying the volume of soil contained in the cylinders at 120 ºC for 48 h. After that, the soil was weighed and soil bulk density was calculated.

Mineral nitrogen as ammonium ($N\text{-}NH_4^+$) and nitrogen nitrate ($N\text{-}NO_3^-$) of the uppermost soil layer (0.1 m depth) were analysed in sieved fresh samples from the 96 stands and 576 trees. NH_4^+ was assessed by the semi-micro Kjeldahl method (Sparks, 1996) after extraction with a 2.0 M KCl solution. After extraction with a saturated $CaSO_4$ solution, NO_3^- was determined by the ultraviolet spectrophotometric method, following the second derivative approach proposed by Sempere et al. (1993).

Additional soil samples were taken from each pit at fixed depths (0.1, 0.5, 1.0 and 1.5 m, when available), air-dried and then sieved to soil particles lower than 2 mm diameter. pH was determined in all samples after dilution with distilled water at a ratio of 1:2.5, and soil texture was analysed in 29 stands: those in which samples from the six trees per stand and four depths per site were complete. Soil texture was determined by the pipette method, separating the soil mineral particles of the three size classes (sand, silt and clay) by

sedimentation (Dane and Topp, 2002). Soil was pretreated to disperse clay soil particles by adding sodium hexametaphosphate, following elimination of organic matter with hot hydrogen peroxide.

P. cinnamomi isolation

Occurrence of *P. cinnamomi* was tested in roots of the 576 *Q. ilex* trees. Fine roots of approximately 6 cm in length were collected at each of three depths (0.5, 1.0 and 1.5 m, when possible) from the two wide sides of the pit and washed under running tap water for two hours. Fifteen fine roots per tree and depth were cut into 6 mm segments (extreme segments discarded) and plated onto 3 separate selective PARPNH-agar plates (V8-agar amended with 10 µg m^{-1} pimaricin, 200 µg m^{-1} ampicillin, 10 µg m^{-1} rifampicin, 25 µg m^{-1} pentachloronitrobenzene (PCNB), 50 µg m^{-1} nystatin and 50 µg m^{-1} hymexazol). About 375 fine root segments per tree were examined. After 2-3 days of incubation at 22 ºC in the dark, the presence of *P. cinnamomi* in these segments was assessed. Developing colonies of *P. cinnamomi* were transferred to malt-agar medium (20 g/l agar agar, 15 g/l malt-agar and 900 ml distilled water) and their morphological features such as hyphal swellings, chlamydospores and sporangia typical for *P. cinnamomi* were checked.

Statistical analysis

The statistical analysis was performed at two levels: stand and tree. At the stand level, decline was characterised by the mortality rate. To analyse the influence of soil properties (mean values of soil depth, Ah

horizon thickness, texture, redox potential, pH, bulk density, and N-NH_4^+ and N-NO_3^- content) on the mortality rate, stepwise multiple regression was used. To assess the influence of soil properties on the occurrence of *P. cinnamomi*, two multivariate analyses were performed: non-metric multidimensional scaling (nMDS) and the multiresponse permutation procedure (MRPP). Texture was analysed separately from the other soil properties, as texture data were not available for all samples. To study whether *P. cinnamomi* presence determines tree decline, a two-way ANOVA was performed using *mortality rate* or *decline intensity* as the dependant variable and *P. cinnamomi infection* (parameterized as 0 or 1 if non-isolated or isolated from at least one root of the tree, respectively) and *topography* as fixed factors. To examine whether the effects of *P. cinnamomi* infection on tree mortality is soil-property dependent, an ANCOVA was performed using *mortality rate* as the dependent variable, *P. cinnamomi infection* as a fixed factor and each *soil property* as a covariate, with particular attention to *P. cinnamomi infection* x *soil property* interactions.

At the tree level, to assess the influence of soil properties on *P. cinnamomi* infection and tree decline, two-way ANOVAs were applied using each *soil property* (Ah horizon thickness, soil depth, bulk density, N-NO_3^-/N-NH_4^+ ratio, N-NH_4^+ and N-NO_3^- content) as the dependent variable, *topography* and either *tree status* or *P. cinnamomi infection* as fixed factors, and in some cases an additional *soil property* as a covariate. The model also considered the random effect of site nested in topography, which accounted for the

environmental variation within each type of topography. Given the possible interdependence of pH, redox status and texture values between different depths, multivariate analyses of variance (MANOVA) were applied, with *pH, redox status* and percentage of *sand, clay* or *silt* at different depths as dependent variables, and *topography* and either *tree status* or *P. cinnamomi infection* as fixed factors. The effect of *P. cinnamomi* infection on tree status was calculated through a Pearson's chi-squared test. The influence of soil properties on the relation between tree status and *P. cinnamomi* was assessed by three-way ANOVAs using each *soil property* as the dependent variable, *tree status*, *P. cinnamomi infection* and *topography* as fixed factors, and *site* nested in topography as a random factor. Normality and homoscedasticity of the data were checked by Kolmogorov-Smirnoff and Bartlett's tests. All analyses were performed with STATISTICA v.7 software, except the community-scale multivariate analyses, which were performed using the R statistical packages vegan, ecodist, BiodiversityR, and labdsv.

3. Results

Soil properties and Q. ilex decline

Mean decline intensity of trees per stand and mean percentage of tree mortality per stand were approx. 2.32 ± 0.05 (rated from 1 to 5) and 6.53 ± 0.79 %, respectively. The multiple regression output showed that the mean value of soil bulk density was significantly related to *mortality rate* of stands ($p < 0.001$; $r^2 = 0.138$), as *mortality rate* rose when soil bulk density decreased. Mortality also increased when clay

at 1.5 m depth increased (p = 0.010; r^2 = 0.220). Other soil factors showed no significant relation to *mortality rate*.

At the tree level, $N\text{-}NO_3^-/N\text{-}NH_4^+$ ratio was higher under declining than non-declining trees (1.66 ± 0.11 and 1.38 ± 0.11, respectively; F = 4.28, p = 0.039). Declining trees had similar $N\text{-}NH_4^+$ values than non-declining trees (11.86 ± 0.44 and 13.27 ± 0.73 mg/kg soil, respectively; F = 3.09, p = 0.080) and soil texture was also similar in both cases (Table 1). Results were independent of topography with the exception of redox potential, which was significant in the *tree status* × *topography* interaction (p = 0.036). On stream banks, lower redox potential values were observed under declining than non-declining trees, but similar values were detected on slopes (results not shown).

Table 1. Mean values (± SE) and comparison of percentages of sand, silt and clay for declining and non-declining trees in the two topography types using multivariate analyses of variance (MANOVA; data of four depths as dependent variables).

Topography		Sand	Silt	Clay	Clay/Sand
Stream bank	Non-declining (n=144)	54.06 (±1.65)	19.77 (±1.14)	26.17 (±1.07)	0.55 (±0.04)
	Declining (n=144)	53.37 (±1.65)	20.48 (±1.03)	26.15 (±1.04)	0.56 (±0.65)
Slope	Non-declining (n=141)	53.27 (±2.63)	19.23 (±1.51)	27.49 (±1.55)	0.59 (±0.07)
	Declining (n=141)	52.97 (±3.04)	18.79 (±1.47)	28.25 (±1.85)	0.64 (±0.09)
	Between tree status	W=0.15 p=0.965	W=0.89 p=0.470	W=0.43 p=0.784	W=0.45 p=0.770
	Between topography type	W=11.79 p<0.001	W=10.03 p<0.001	W=3.65 p=0.008	W=3.73 p=0.007
	Tree status × topography	W=0.81 p=0.520	W=0.18 p=0.950	W=1.00 p=0.413	W=0.04 p=0.997

Soil properties and P. cinnamomi infection

P. cinnamomi was isolated from roots of 100 of the 576 trees and infected 50 stands out of 96. MRPP showed that *P. cinnamomi* infection at the stand level was significantly related to soil properties (A = 0.018; p = 0.020). The spatial distribution of NMDS permitted clear separation of infected and non-infected stands, mostly explained by the Ah horizon thickness (i.e., deep Ah horizons were associated with *P. cinnamomi* infection) (Fig. 1). Presence of *P. cinnamomi* was also significantly conditioned by soil texture (Fig. 2), as higher sand content was linked to the absence of *P. cinnamomi* and higher silt and clay content were associated with *P. cinnamomi*-infected trees. Results were significant at 0.1 and 0.5 m depth (A = 0.056, p = 0.052

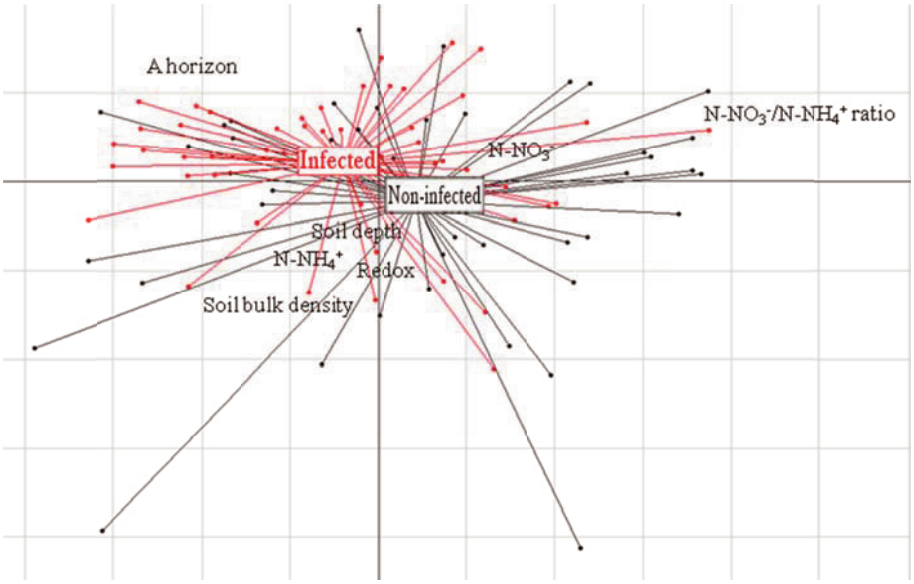


Fig. 1. Non-metric multi-dimensional scaling (nNMDS) ordination created from analysis of soil properties under *Quercus ilex* stands infected and non-infected with *Phytophthora cinnamomi*; n = 95 stands.

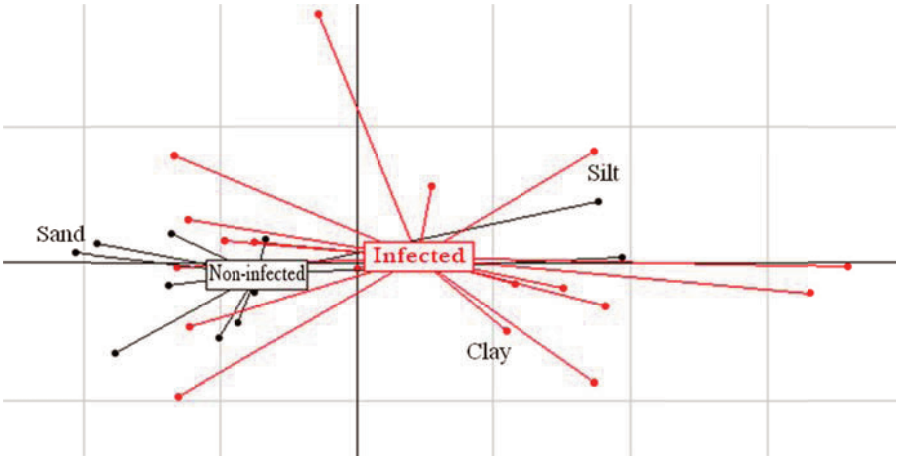


Fig. 2. Non-metric multi-dimensional scaling (nNMDS) ordination created from analysis of soil texture at 0.5 m depth under *Quercus ilex* stands infected and non-infected with *Phytophthora cinnamomi*; $n = 29$ stands.

and $A = 0.053$, $p = 0.047$) but not at 1 and 1.5 m depth ($A = 0.027$, $p = 0.102$ and $A = -0.008$, $p = 0.556$, respectively).

At the tree level, soil bulk density was lower under *P. cinnamomi*-infected trees than under *P. cinnamomi* non-infected trees (1.14 ± 0.02 and 1.17 ± 0.01 g/cm^{-3}, respectively; $F = 4.18$, $p = 0.041$). Soil depth was deeper under *P. cinnamomi*-infected trees than under *P. cinnamomi*-non-infected trees (1.19 ± 0.03 and 1.05 ± 0.02, respectively; $F = 9.99$, $p = 0.002$). Although not significantly, Ah horizon thickness was higher under infected than non-infected trees (27.15 ± 1.80 and 22.90 ± 0.98, respectively; $F = 3.27$, $p = 0.071$). Under infected and non-infected trees, sand percentages were 49.28 ± 2.42 and 54.52 ± 1.09 %, respectively ($p = 0.046$; Table 2). Minimal differences were also observed for clay/sand ratio ($p = 0.054$;Table 2).

Table 2. Mean values (± SE) and comparison of percentages of sand, silt and clay for *Phytophthora cinnamomi* non-infected trees and *P. cinnamomi*-infected trees in the two topography types using multivariate analyses of variance (MANOVA; data of four depths as dependent variables).

		Sand	Silt	Clay	Clay/Sand
Stream bank	Non-infected (*n*=227)	54.37 (±1.27)	19.61 (±0.84)	26.02 (±0.80)	0.53 (±0.03)
	Infected (*n*=61)	51.31 (±2.77)	22.02 (±1.79)	26.68 (±1.91)	0.62 (±0.09)
Slope	Non-infected (*n*=243)	54.83 (±2.09)	18.50 (±1.13)	26.68 (±1.24)	0.57 (±0.06)
	Infected (*n*=39)	41.17 (±3.62)	22.62 (±2.39)	36.21 (±1.77)	0.93 (±0.12)
	Between *P. cinnamomi* infection	*W*=2.52 *p*=0.046	*W*=0.40 *p*=0.809	*W*=2.14 *p*=0.081	*W*=2.41 *p*=0.054
	Between topography type	*W*=5.21 *p*=0.001	*W*=4.60 *p*=0.002	*W*=4.99 *p*=0.001	*W*=2.93 *p*=0.024
	P. cinnamomi infection × topography type	*W*=0.67 *p*=0.613	*W*=0.61 *p*=0.660	*W*=1.45 *p*=0.222	*W*=0.91 *p*=0.461

Table 3. Mean values (±SE) of pH, $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ concentrations (mg/kg soil) and $N\text{-}NO_3^-/N\text{-}NH_4^+$ ratios for declining and non-declining trees, *Phytophthora cinnamomi*-infected trees and *P. cinnamomi* non-infected trees.

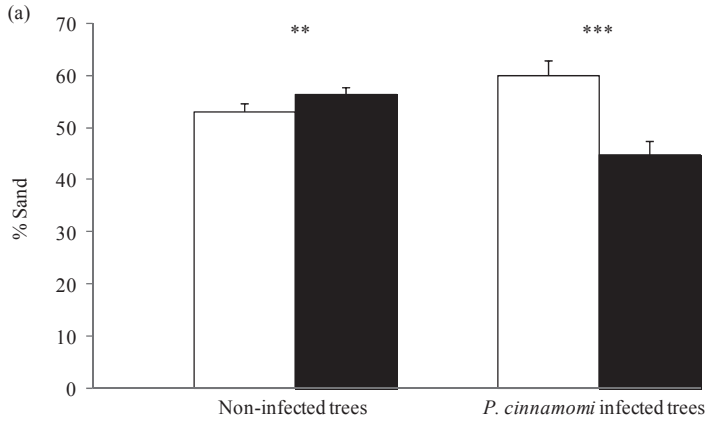
	$pH_{(H2O)}$	$N\text{-}NH_4^+$	$N\text{-}NO_3^-$	$N\text{-}NO_3^-/N\text{-}NH_4^+$ ratio
Non-declining	5.44 (± 0.06)	13.27 (± 0.73)	17.29 (± 1.14)	1.38 (± 0.11)
Declining	5.57 (± 0.06)	11.86 (± 0.44)	18.26 (± 1.04)	1.66 (± 0.11)
Non-infected	5.50 (± 0.05)	12.45 (± 0.49)	17.73 (± 0.85)	1.53 (± 0.08)
Infected	5.53 (± 0.08)	13.10 (± 0.75)	17.95 (± 1.83)	1.47 (± 0.17)

The influence of soil properties on *P. cinnamomi* infections was independent of topography (*P. cinnamomi infection* × *topography* interaction not significant; Table 2). Similar pH values, $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ concentrations and $N\text{-}NO_3^-/N\text{-}NH_4^+$ ratios under infected and under non-infected trees were observed (Table 3).

Relation of Q. ilex decline with interactions between P. cinnamomi infection and soil properties

At the stand level, mortality and decline intensity increased significantly if *P. cinnamomi* was present (8.8 and 4 % (F = 8.0, p = 0.006), and 2.5 and 2.1 (F = 9.1, p=0.003) for infected and non-infected stands, respectively). Similarly, *P. cinnamomi* infection within stands was observed more frequently under declining than non-declining trees (24.2 and 10.9 % of trees examined, X^2 = 12.383, p = 0.006).

Most soil properties showed no influence on the relation between *P. cinnamomi* infection and mortality at the stand level, except Ah horizon thickness (F = 6.75, p = 0.011), with an increase in tree mortality in *P. cinnamomi*-infected stands when Ah horizon thickness was higher. At the tree level, sand and clay showed a significant influence (F = 4.09, p = 0.045 and F = 6.00, p = 0.016, respectively). When trees were not infected, non-declining trees were associated with fine textured soils, but when trees were infected with *P. cinnamomi* the pattern was reversed (Fig. 3).



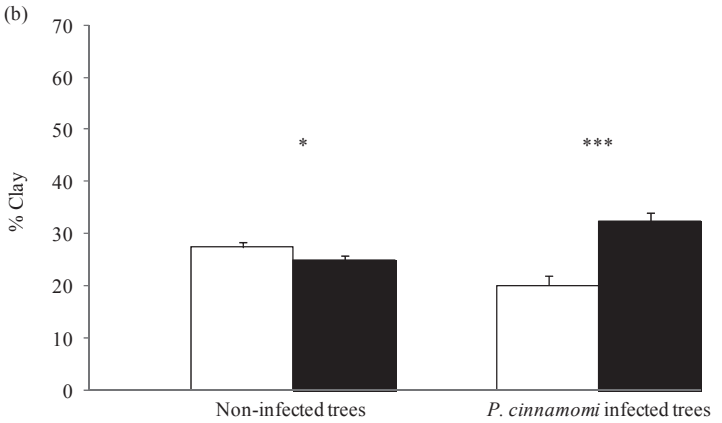


Fig. 3. Percentage of sand (a) and clay (b) under non-declining (□) and declining (■) *Quercus ilex* trees non-infected and infected with *Phytophthora cinnamomi*. Bars denote standard errors and asterisks indicate significant differences in values within non-infected and *P. cinnamomi*-infected trees at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***); $n=161$ trees.

4. Discussion

The role of soil quality in oak decline symptoms

A range of soil depths (from 30 to approx. 150 cm) were observed and soil depth is a primary determinant of soil water-holding capacity. No significant relationships were observed between soil thickness and tree status, which could indicate that episodic severe drought in SW Spain is not a primary cause of oak decline and mortality. Holm oaks have a very deep rooting system (> 5 m in the study area; Moreno et al., 2005) adapted to summer drought through tap roots with access to groundwater (David et al., 2004) or water stored in partially weathered deep rocks (Cubera and Moreno, 2007). During dry years, when groundwater cannot be reached by the roots or deep soil is not penetrated by rainfall, soil texture could be a determinant for tree survival. The results confirmed that under non-infected trees, soil

texture was slightly finer under non-declining than declining trees. In the absence of *P. cinnamomi*, declining trees had lower water-holding capacities than non-declining trees (Corcobado et al., 2013), which suggests the importance of soil texture and water reserves to cope with drought episodes and decline symptoms (Costa et al., 2010). Soil properties and oak decline interact with topography, as recently reported by Corcobado et al. (2013). The significant tree status × topography interaction observed in relation to the redox potential of soils seems to indicate that hydromorphy is a disadvantage for tree status if trees are located on stream banks. Hydromorphic conditions reduce the capacity of trees to replace dead rootlets (Thomas and Hartmann, 1998).

The important role of P. cinnamomi and its dependence on soil properties

The relation of tree mortality and aerial symptoms to *P. cinnamomi* root infections provides clear evidence of the involvement of *P. cinnamomi* in the decline of *Q. ilex* in SW Spain, consistent with other studies in the Iberian Peninsula (Moreira and Martins, 2005; Serrano et al., 2012). Interestingly, the association described here between soil texture and tree status changed when trees were infected or not with *P. cinnamomi*. In the presence of the pathogen, symptoms were associated mainly with finer-textured soils. Trees infected with *P. cinnamomi* are more abundant in fine textured than coarse textured soils (Jung et al., 2000; Jönsson et al., 2005; Gómez-Aparicio et al., 2012). In coarse textured soils, water depletes quickly, which is

detrimental for the production of sporangia and release of zoospores, while in fine textured soils water retention will favor the activity of the pathogen and provide conditions for inoculum increase (Zentmyer, 1980; Jung et al., 2000). Results obtained here for *Q. ilex* forests may not apply to other tree species and/or to other soil types present elsewhere. For example, in southwest Western Australia, a region also with a Mediterranean type climate, sandy soils were observed to be the most conducive for *P. cinnamomi* infections in *Banksia baxteri*, in contrast to soils with high clay and silt content (Shearer and Crane, 2011).

Some studies have recorded an association of oak decline and saturated soils, as insufficient aeration of soil affects tree respiration and growth (Thomas and Hartmann, 1998; Rozas and García-González, 2012) and low redox potentials are indicative of these situations of poor drainage and aeration. No significant differences of soil redox status between non-infected and *P. cinnamomi* infected trees were observed, and most of the soils assessed showed manoxic status irrespective of the presence or absence of the pathogen. Manoxic soils are highly oxidising and well drained soils, favourable for good root development, and are not generally associated with tree decline. Although additional measures such as oxygen concentration and oxygen diffusion rate could have given more conclusive results (Watson and Kelsey, 2006), our data suggest that *Q. ilex* decline was probably caused by increased activity of *P. cinnamomi* in fine textured soils. The increase of activity of *P. cinnamomi* causing more root rot in fine than in coarse textured soils would be related, as mentioned

before, with the capability of fine-textured soils to retain more water than coarse-textured soils. The finding of higher fine root loss under conditions of increased soil water content, which was detected close to stream banks rather than in intermediate or upper-slope positions (Corcobado et al., 2013), confirm this statement. Fine textured soils are known to stimulate sporangium production and zoospore release more than other soils (Shearer and Crane, 2011). Greater inoculum potential in fine textured soils has implications for hygiene management, as clayey or silty soils would more easily adhere to passing humans, cattle and vehicles, with higher risk of pathogen dispersal.

The results also suggest that Ah horizon thickness is a meaningful soil property which would have a role in root infections with *P. cinnamomi.* Both at stand and tree level, a thicker Ah horizon was associated with *P. cinnamomi* infections and tree mortality. Most organic matter and soil microorganisms concentrate in the Ah horizon. Increased organic matter usually enhances microbial population which could lead to *P. cinnamomi* suppression (Weste and Marks, 1987). Organic matter increases the water-holding capacity of soils, favours root development and consequently the number of root exudates per soil volume, which in turn would stimulate *P. cinnamomi* inoculum and infection (Khew and Zentmyer, 1973). Moreover, although some studies infer that *P. cinnamomi* is able to grow as a saprophyte through the soil (Weste and Marks, 1987, and references therein), the pathogen seems to benefit from stimulatory microbes, i.e. hyphal lysis

caused by these microbes correlates positively with sporangial production (Malajczuk et al., 1983; Shearer and Crane, 2011).

In the present study, both declining and non-declining trees and infected and non-infected trees were located on soils with pH above 5.0, which are within the tolerance range of sporangium production and zoospore survival of *P. cinnamomi* (Pegg, 1977; Benson, 1984) and other *Phytophthora* species (Schmitthenner and Canaday, 1983; Kong et al., 2012). Specific information is lacking on how pH and physical characteristics of water limiting soils influence *P. cinnamomi* sporulation, survival, dispersal and infection.

Grazing-associated soil property changes do not seem to accelerate oak decline

Grazing is common in many scattered-tree systems, including Iberian dehesas, and is thought to cause soil compaction (Asner et al., 2004). In the present study, bulk densities were lower under *P. cinnamomi*-infected trees than under non-infected trees and bulk density values were lowest in forest stands with the highest mortality rates. The inverse relationship of *P. cinnamomi* infection and tree mortality with bulk densities could be explained by the links between bulk density and organic matter (Heuscher et al., 2005) and between bulk density and the texture of soil. Lower bulk densities are related to higher organic matter content, which is mostly associated with Ah horizons, and consequently with the levels of *P. cinnamomi* infection and tree mortality mentioned above. It should be also taken into account that in the absence of compaction, fine textured soils show lower bulk

density values than coarse textured soils. Regardless of this, none of the bulk density values observed, even the highest (1.64 g/cm^{-3}), would negatively affect root development or soil drainage (Cubera et al., 2009). Earlier studies reported lower bulk densities under declining than non-declining *Quercus* stands, with values above 1.60 g/cm^{-3} under healthy trees (Thomas and Hartmann, 1998; Solla et al., 2009).

Grazing is also thought to cause alteration of the N cycle and availability (Singer and Schoenecker, 2003; Asner et al., 2004) and to increase the temporal and spatial heterogeneity of N in the soil at different scales (Gallardo, 2003). Large spatial variability for both N-NH_4^+ (3.15 to 118.65 mg/kg soil) and N-NO_3^- content (0.20 to 95.55 mg/kg soil) was observed, but no differences were obtained between declining and non-declining or infected and non-infected trees for N-NH_4^+ and N-NO_3^-. Excess N associated with grazing has been shown to be detrimental to some forests (Jung et al., 1996; Berger and Glatzel, 2001) and to be capable of increasing *Phytophthora* sporangial production (Jung et al., 2000). However, the results revealed a lack of negative effects of N content associated with wood pasture management on the health of *Q. ilex* forests. These findings are consistent with another study of *Q. robur* decline (Thomas and Büttner, 1998).

Although soil bulk density and mineral N (indicators of the effects of grazing on soil properties) failed to show any influence on tree status, the nitrate to ammonium ratio, interpreted as an indicator of soil degradation (Wilson and Tilman, 1991), reached higher values

under declining than non-declining trees. This finding is consistent with the progressive degradation of soils in Iberian dehesas (Schnabel et al., 2009) and other pasturelands (Asner et al., 2004). An examination of more stands, including totally healthy and unmanaged stands would have helped to elucidate further combined effects of soil properties and *P. cinnamomi* infections on *Q. ilex* decline.

5. Conclusions

The results described in this study showed a significant association between the declining status of trees and *P. cinnamomi* root infections and a significant effect of soil properties on the occurrence of *P. cinnamomi* and on the intensity of tree decline. Fine soil textures and thick Ah horizons, normally favourable soil properties for vigour and vitality of trees growing in the Mediterranean climate, contributed to decline if *P. cinnamomi* was present. Soil properties and topographic factors linked to an increase of the soil moisture led to mortality and decline of *Q. ilex* trees if *P. cinnamomi* was present. Results showed the independence of *Q. ilex* decline from soil compaction and mineral N content. The positive association of tree decline and the nitrate/ammonia ratio may be associated with progressive soil degradation caused by recent land use intensification in the *Q. ilex* forests studied.

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CAPÍTULO 2

Ectomycorrhizal symbiosis in declining and non-declining *Quercus ilex* trees infected or not with *Phytophthora cinnamomi*

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Abstract
Quercus ilex decline and the presence of the soil-borne pathogen *Phytophthora cinnamomi* are hypothesized to be associated with shifts in ectomycorrhizal fungi abundance. Soil properties may also influence the relation of this pathogen to ectomycorrhizae. Aiming to investigate such associations, 96 *Quercus ilex* declining stands were selected in western Spain and declining and non-declining trees were sampled. Soil properties (soil depth, Ah horizon thickness, texture, pH, redox potential, soil bulk density and $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ concentrations), *P. cinnamomi* root infections and ectomycorrhizal fungi abundance were assessed. The most dominant ectomycorrhizal morphotypes were *Cenococcum geophilum*, *Tomentella* spp. and *Russula* spp. Results showed a lower percentage of vital non-ectomycorrhizal and ectomycorrhizal tips in declining than in non-declining trees, and no significant differences between *P. cinnamomi* infected and non-infected trees. Tips colonized by *Russula* spp. and other less abundant ectomycorrhizal fungi increased in non-declining infected trees compared to declining infected trees. Trees growing on stream banks showed a lower abundance of ectomycorrhizal tips on fine than on coarse textured soils. Ectomycorrhizal tip abundance also varied with the thickness of the Ah horizon, irrespective of the tree decline status. Only in non-infected trees ectomycorrhizal tip abundance related to some soil properties. The presence of some interactions between ectomycorrhizal fungi abundance, soil properties, *P. cinnamomi* root infections and *Q. ilex* decline are reported together with a scarce ectomycorrhizal fungi diversity in declining stands.

Keywords: oak decline; root rot pathogen; ectomycorrhizae; soil fertility; Iberian dehesa

1. Introduction

The influence of environmental factors on ectomycorrhizal fungi community is evidenced in many studies (Lilleskov et al., 2002; Baier et al., 2006; Twieg et al., 2009). Crown defoliation has been demonstrated to induce changes in ectomycorrhizal fungi structure presumably because implies a decrease of the photosynthetic capacity of the tree, which would affect the tree ability to maintain the symbiosis (Kuikka et al., 2003; Saravesi et al., 2008). Decline of forests, which is the result of the combined action of biotic and abiotic factors and which causes defoliation among other symptoms, has been related to disturbances on the ectomycorrhizal fungi community (Power and Ashmore, 1996; Kovacs et al., 2000). Studies on the relation of oak decline to ectomycorrhizal fungi community are not scarce (Causin et al., 1996; Trevisani et al., 1999; Montecchio et al., 2004), and lower values of ectomycorrhizal roots and ectomycorrhizal diversity under declining trees than under non-declining trees have been reported (Causin et al., 1996; Montecchio et al., 2004).

Holm oak (*Quercus ilex* L.) is the most representative tree of forests from the south-western part of Europe. During the last three decades, decline has severely affected *Q. ilex* woodlands in Spain and Portugal (Brasier et al., 1993), and symptoms of declining trees include leaf discoloration and wilting, defoliation, root rot, dieback of branches and trunk exudations (Gallego et al., 1999). The soil-borne oomycete *Phytophthora cinnamomi* has been identified as the main biotic cause of Iberian *Q. ilex* decline (Brasier et al., 1993) due to its ability to cause fine root loss (Corcobado et al., 2013a). Infection of

Q. ilex roots by *P. cinnamomi* may depend on topography and several soil factors, most of them related to conditions favourable for pathogen survival and sporulation (Corcobado et al., 2013a, b). Due to its broad distribution, this pathogen is expected to coexist and possibly interact with the diverse soil ectomycorrhizal community. *Phytophthora* spp. is described as a poor competitor and a weak saprophytic colonizer (Malajczuk et al., 1983), in the way that soil microbes can easily suppress its activity and growth (Smith et al., 1990). Moreover, ectomycorrhizal fungi may confer protection to roots against this pathogen (Marx, 1972; Ross and Marx, 1972; Malajczuk, 1988).

Research of ectomycorrhizal symbiosis in *Phytophthora*-infected stands has been performed on *Castanea sativa*, *Eucaliptus* spp. and *Fagus sylvatica* (Blom et al., 2009; Anderson et al., 2010; Scattolin et al., 2012; Scott et al., 2012; Coince et al., 2013) but not on *Quercus* spp. Additionally, differences on the relative abundance of ectomycorrhizal tips between declining and non-declining *Phytophthora*-infected *Quercus* spp. trees are unknown. It has been studied how soil properties affect ectomycorrhizal structure in *Phytophthora*-infected stands (Coince et al., 2013), however how soil properties influence the relation between *Phytophthora* infection and ectomycorrhizal symbiosis remains unknown. In order to study possible tree decline × *P. cinnamomi* infection interactions on the abundance of the ectomycorrhizal community of *Q. ilex* woodlands and the role of soil properties on these interactions, an extensive field study was performed. Comparisons of the relative abundance of non-

vital, vital non-mycorrhizal and vital ectomycorrhizal root tips associated with 288 declining and 288 non-declining trees from 96 stands were undertaken and crossed with *P. cinnamomi* infection, topography and soil texture factors. Additional variables such as maximum soil depth, Ah horizon thickness, soil bulk density, soil N-NH_4^+ and N-NO_3^-, pH and fine root abundance were assessed and related to the abundance of ectomycorrhizal fungi. It is hypothesized that (i) ectomycorrhizal community depends on the soil properties, (ii) the relative abundance of ectomycorrhizal tips may differ between non-declining and declining trees and between *P. cinnamomi*-infected and non-infected trees, and (iii) relations between soil properties and ectomycorrhizal abundance may change depending if *Q. ilex* trees are declined, infected or not.

2. Materials and methods

Survey area and experimental design

The study was performed in Extremadura, western Spain, and included 96 woodland stands located throughout the region (Fig. 1). Stands were characterised by a scattered-tree layer of *Q. ilex* (dehesas) and an understory of pasture. Most sites were regularly grazed in spring and summer by cattle and in autumn and winter by Iberian pigs. The climate in Extremadura is continental Mediterranean with hot dry summers and cold winters. Climatic data for each stand were obtained from their nearest meteorological station (AEMET, Spanish Meteorological Agency). Annual precipitation and mean annual temperature are 654 mm and 15.9 ºC, respectively.

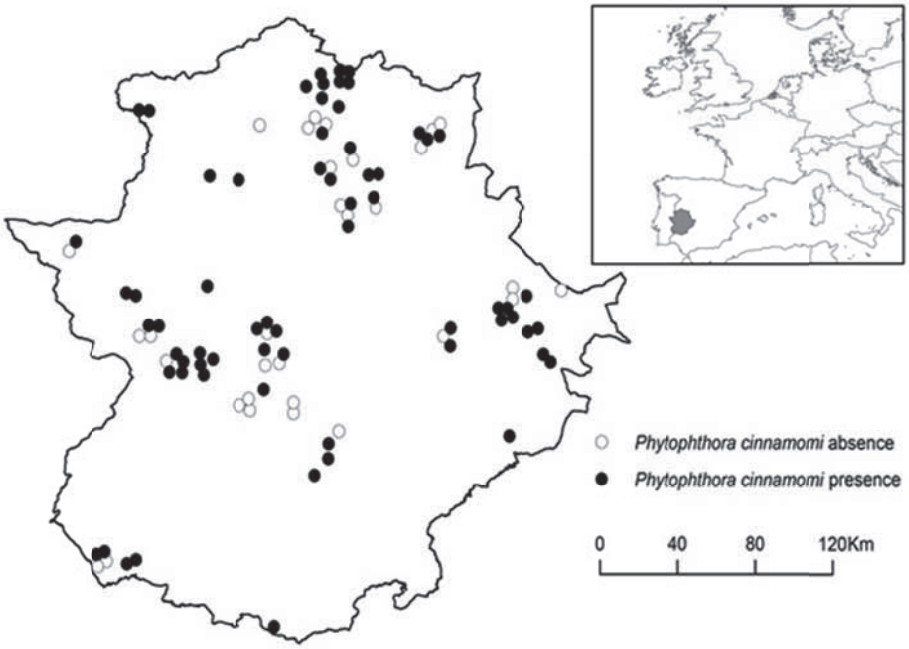

Fig. 1. Location of the 96 *Quercus ilex* stands surveyed in Extremadura region (SW Spain). Solid and empty circles refer to the presence and absence of *Phytophthora cinnamomi*, respectively.

Trees comprised three non-declining (0-14 % of crown transparency) and three declining *Q. ilex* trees (15-35 % of crown transparency) per stand (Fig. 2). Crown transparency referred to defoliation according to UNECE (2010). Inside each stand, trees were distanced more than 50 m to avoid the possibility of non-declining trees sharing parts of their rhizosphere with declining trees. Throughout the region, 48 stands were in or close to a stream bank, and 48 were on a mid slope (*topography* factor). The mean percentage of declining trees per stand (incidence) was approximately 57 % and the mean annual tree mortality rate of stands was approximately 5%.

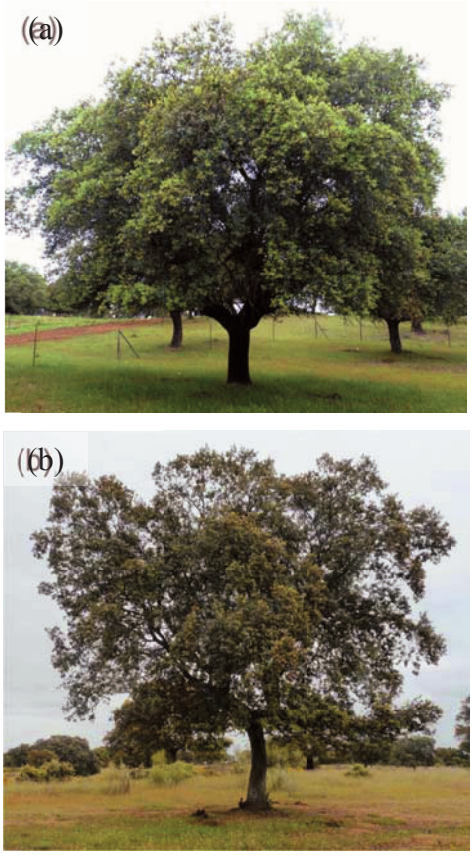

Fig. 2. Non-declining (a) and declining (b) *Quercus ilex* trees examined for soil properties, *Phytophthora* spp. root infections and ectomycorrhizal fungi community in Extremadura region (SW Spain). Note ≤ 10 and 15-35% of tree crown transparencies in (a) and (b), respectively.

Trees were 6–10 m high, 30–90 cm in trunk diameter at breast height and 7.5–12 m in crown diameter.

Ectomycorrhizal tip abundance, *P. cinnamomi* isolation and soil assessment of 576 *Q. ilex* trees were performed through examination of root and soil samples obtained from soil pits. One pit per tree was dug using a hydraulic backhoe with a 40 cm-wide scoop. Soil pits were approximately 2.5 m wide and 1.5 m deep (where

possible), 3–4 m downstream from the tree trunks, and tangentially oriented to the tree crown. Assessments were performed in spring, coinciding with the most favourable season for *P. cinnamomi* isolations in the Mediterranean area (Corcobado et al., 2013a).

Ectomycorrhizae assessment

Four-five soil cores per tree approximately 10 x 10 cm and randomly selected from the first 30 cm depth were collected. Soil cores were sealed in plastic bags, moistened with distilled water, kept in a portable cooler and stored at 4 ± 1 ºC in the laboratory and analyzed within 8 days (see annex II.2). Soil cores were kept in water 24 h before the assessment to facilitate the separation of the roots from the soil particles and then, grouped into the same tree sample. Only roots with a diameter < 2 mm were assessed. About 200 root tips per tree were examined, comprising circa 115,000 tips for the whole experiment. Tips were examined under an stereomicroscope (Olympus SZX10, Japan) and classified as 'non-vital' (NV, a scurfy surface and an easily detachable cortex, with or without the remnants of an ectomycorrhizal mantle), 'vital non-mycorrhizal' (NM, a well-developed, turgid and inflated tip, mantle lacking) or 'vital ectomycorrhizal' (EM, as above, but with an ectomycorrhizal mantle) according to specific literature (Montecchio et al., 2004; Scattolin et al., 2012). Each EM tip was then classified anatomically and morphologically (Agerer, 1987–2008; Goodman et al., 1996–1998; Agerer and Rambold, 2004–2013). The relative abundances of NV, NM and EM tips were expressed in percentages.

P. cinnamomi isolation

Occurrence of *P. cinnamomi* was tested in roots of the 576 *Q. ilex* trees. Fine roots of approximately 6 cm in length were collected at three depths (0.5, 1.0 and 1.5 m, when possible) from the two wide sides of the pit and washed under running tap water for two hours. Fifteen fine roots per tree and depth were cut into 6 mm segments (extreme segments discarded) and plated onto 3 separate selective PARPNH-agar plates (V8-agar amended with 10 µg m^{-1} pimaricin, 200 µg m^{-1} ampicillin, 10 µg m^{-1} rifampicin, 25 µg m^{-1} pentachloronitrobenzene (PCNB), 50 µg m^{-1} nystatin and 50 µg m^{-1} hymexazol) (Solla et al., 2009). About 375 fine root segments per tree were examined. After 2-3 days of incubation at 22 ºC in the dark, the presence of *P. cinnamomi* in these segments was assessed. Developing colonies of *P. cinnamomi* were transferred to malt-agar medium (20 g/l agar agar, 15 g /l malt-agar and 900 ml distilled water) and their morphological features such as hyphal swellings, chlamydospores and sporangia typical for *P. cinnamomi* were checked. According to the isolation success of *P. cinnamomi*, the plant material was grouped into non-infected non-declining, non-infected declining, infected non-declining and infected declining trees.

Soil analysis

In each soil pit of each of the 576 trees, maximum soil depth and Ah horizon thickness, defined visually *in situ* by the colour of the soil (change in value number in Munsell chart), were recorded. Soil bulk densities were determined by extracting four unaltered soil cores with

a 100-cm^3 cylinder at 0-10 cm depth, drying the soil at 120 ºC for 48 h, and weighing the soil. Mineral nitrogen as ammonium ($N\text{-}NH_4^+$) and nitrate ($N\text{-}NO_3^-$) were analysed in sieved fresh samples taken from the uppermost soil layer (0.1 m depth). NH_4^+ was assessed by the semi-micro Kjeldahl method after extraction with a 2.0 M KCl solution, and NO_3^- was determined by the ultraviolet spectrophotometric method, after extraction with a saturated $CaSO_4$ solution, following the second derivative approach proposed by Sempere et al. (1993). The nitrate to ammonium ratio was used as an indicator of soil degradation, with higher ratios in disturbed soils (Wilson and Tilman, 1991; Corcobado et al., 2013b). To assess fine root abundance a 0.1 m wide transparent grid was laid over the soil profile to count the roots (< 2 mm diameter) every 0.5 m of depth. Root abundance was expressed as the number of fine roots m^{-2}.

Additional soil samples (soil particles < 2 mm diameter) were taken from each pit at fixed depths (0-0.1, 0.1-0.5, 0.5-1.0 and 1.0-1.5 m, when available), air-dried and then sieved. pH was determined in samples from 0.1-0.5 m depth after dilution with distilled water at a ratio of 1:2.5, and soil texture was analysed in all samples by the feel method (Thien, 1979). To validate this method, soil texture of 29 stands was determined by the pipette method, separating the soil mineral particles of the three size classes (sand, silt and clay) by sedimentation. According to the average soil texture of samples, stands were grouped into fine- and coarse-soil-textured stands (*soil texture* factor).

Data analysis

The relation of tree decline status and *P. cinnamomi* infection with NV, NM and EM tips (ectomycorrhizal descriptors) was evaluated with a General Linear mixed Model using *NV*, *NM* and *EM tips* as the dependent variable, and *tree decline status*, *P. cinnamomi infection*, *topography* and *soil texture* as fixed factors. The model also considered the random effect of *stand* nested within *topography* and *texture* and two degree interactions, when $p \leq 0.10$. To compare averages, post-hoc tests were performed. The relationships between ectomycorrhizal descriptors and physical and chemical soil properties were examined by means of Pearson correlation coefficients and regression analysis. Normality and homoscedasticity of the data was checked by Kolmogorof-Smirnoff and Bartlett's tests, respectively, and data were transformed when necessary. All analyses were performed with Statistica v10 software (Stat Software Inc., Tulsa, OK).

3. Results

Non-vital (NV), vital non-mycorrhizal (NM) and vital ectomycorrhizal (EM) tips represented (mean ± standard error) 22.2 ± 10.2, 29.4 ± 11.3 and 48.3 ± 12.3 % of the total tips examined, respectively. The three most dominant ectomycorrhizal morphotype groups in the SW Iberian *Q. ilex* forests were *Cenococcum geophilum* (relative abundance of 57 %), *Tomentella* spp. (21 %) and *Russula*

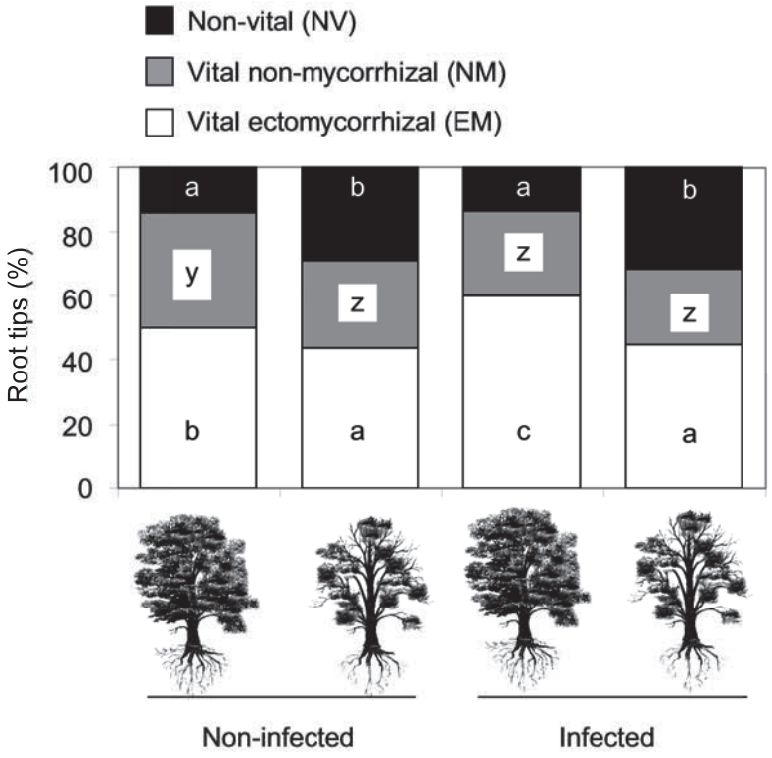

Fig. 3. Relative abundance of non-vital, vital non-mycorrhizal and vital ectomycorrhizal tips in non-infected non-declining, non-infected declining, *Phytophthora cinnamomi* infected non-declining, and *P. cinnamomi* infected declining *Quercus ilex* trees ($n > 63$). Within the same tip classification, different letters show significant differences ($p < 0.05$).

spp. (14 %). Additional identified groups with lower abundances were merged into a single group classified as 'other' EM tips (8 %).

Tree decline status, Phytophthora cinnamomi infection, and ectomycorrhizal symbiosis

Lower percentages of NV tips were observed in non-declining than in declining trees (14 *vs.* 30 %; Table 1), irrespective if trees were infected with *P. cinnamomi* ($p < 0.001$, Table 1, Fig. 3). On the

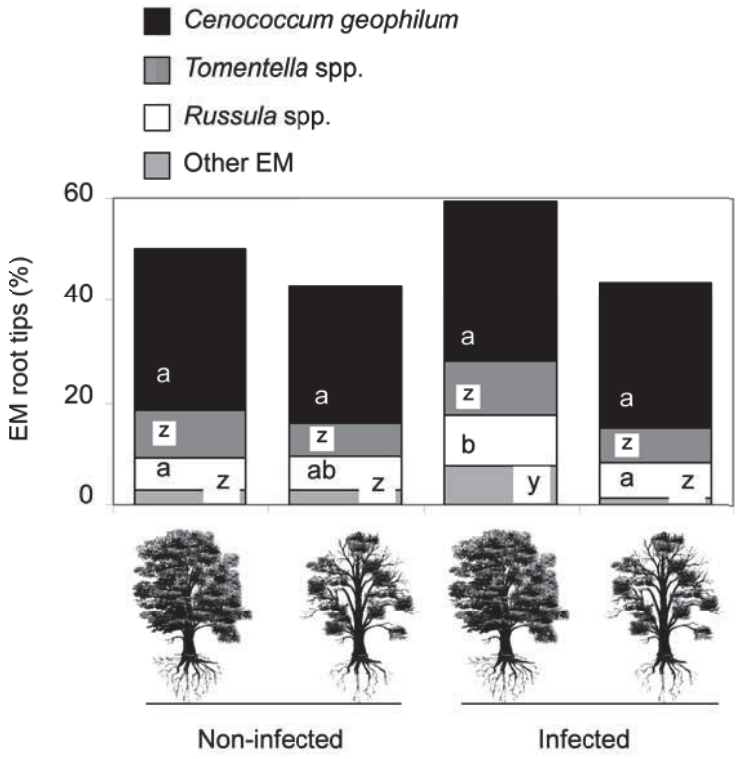

Fig. 4. Relative abundance of tips colonized with *Cenococcum geophilum*, *Tomentella* spp., *Russula* spp. and other morphotype groups in non-infected non-declining, non-infected declining, *Phytophthora cinnamomi* infected non-declining, and *P. cinnamomi* infected declining *Quercus ilex* trees ($n > 63$). Within the same ectomycorrhizal group, different letters show significant differences ($p < 0.05$).

contrary, higher percentages of NM and EM tips were observed in non-declining than in declining trees (34 *vs.* 25 % for NM tips, and 52 *vs.* 45 % for EM tips, respectively; Table 1) and differences among declining and non-declining for EM tips varied marginally if trees were infected with *P. cinnamomi* or not ($p = 0.090$ for *tree decline status* × *P. cinnamomi infection* interaction; Table 1, Fig. 3). Only *Russula* spp. and other EM tips varied among declining and non-

declining trees, infected or not with *P. cinnamomi* (significant *tree decline status* × *P. cinnamomi infection* interaction, Table 2). Interestingly, non-declining infected trees showed higher percentages of *Russula* spp. and other EM tips than declining infected trees (Fig.4).

Table 1. Results of the general linear mixed models for the analysis of the percentages of non-vital (NV), vital non-mycorrhizal (NM) and vital ectomycorrhizal (EM) tips observed in 576 *Quercus ilex* trees located in SW Spain. Degrees of freedom (DF) and F-ratios of fixed factors, and variance component (VarComp) and associated χ^2 of the random factor are shown.

	NV			NM			EM		
	DF or VarComp	*F* or χ^2	*p* value	DF or VarComp	*F* or χ^2	*p* value	DF or VarComp	*F* or χ^2	*p* value
Fixed factors									
Tree status [D]	1	97.7	< 0.001	1	14.3	< 0.001	1	17.6	< 0.001
P.cinnamomi infection [Phy]	1	1.6	0.199	1	2.3	0.130	1	0.19	0.659
Topography [T]	1	0.0	0.840	1	0.3	0.556	1	0.3	0.560
Texture [t]	1	0.2	0.673	1	0.8	0.363	1	1.3	0.244
D × Phy	1	0.4	0.482	1	2.3	0.124	1	2.8	0.090
Phy × t	1	0.2	0.673	1	4.2	0.043	1	2.0	0.154
T × t	1	3.7	0.056	1	0.1	0.745	1	4.8	0.029
Random factor									
Stand (T × t)[a]	654 ± 273	2.4	< 0.001	768 ± 368	2.1	< 0.001	745 ± 406	1.8	< 0.001

[a]The stand was nested within the topography and texture.

Table 2. Results of the general linear mixed models for the analysis of the percentages of vital ectomycorrhizal (EM) tips of *Russula* spp. and other morphotype groups observed in 576 *Quercus ilex* trees located in SW Spain. Degrees of freedom (DF) and F-ratios of fixed factors, and variance component (VarComp) and associated χ^2 of the random factor are shown.

	$EM_{Russula\ spp.}$			EM_{Other}		
	DF or VarComp	*F* or χ^2	*p* value	DF or VarComp	*F* or χ^2	*p* value
Fixed factors						
Tree decline status [D]	1	0.97	0.325	1	7.5	0.006
P. cinnamomi infection [Phy]	1	0.0	1.000	1	1.1	0.294
Topography [T]	1	2.3	0.130	1	1.1	0.278
Texture [t]	1	5.2	0.026	1	0.8	0.363
D × t	1	3.0	0.081	1	0.2	0.654
D × Phy	1	6.3	0.012	1	11.0	< 0.001
D × Phy × t	1	5.3	0.021	1	0.1	0.812
Random factor						
Stand (T × t) [a]	135 ± 76.7	1.7	0.004	127 ± 76	1.6	0.008

[a]The stand was nested within the topography and texture.

Site, topography, texture, and ectomycorrhizal symbiosis

Differences of ectomycorrhizal descriptors between stands were always significant (Tables 1 and 2). Descriptors were not influenced by topography and texture, but if both previous factors were combined they influenced the abundance of NV and EM tips of trees (marginally significant and significant *topography* × *soil texture* interactions for NV and EM tips, respectively; Table 1). Trees growing on mid slopes showed similar NV, NM and EM tips irrespective of the soil texture (Table 3), but trees growing on stream banks showed more NV (and less EM) tips on fine rather than on coarse textured soils (Table 3). In

non-infected trees, approximately 30 % of tips were NM tips, irrespective of the soil texture. In infected trees, however, NM tips were less if the soil texture was coarse rather than fine (23 and 31 %, respectively; $p < 0.05$; significant *P. cinnamomi infection* × *soil texture* interaction; Table 1).

The presence of *C. geophilum* did not vary within non-infected trees, and within infected non-declining trees (Figure 5a), but was significantly more abundant in infected declining trees growing in coarse textured soils than in infected declining trees growing in fine textured soils (Figure 5a) (significant *tree decline status* × *P. cinnamomi infection* × *soil texture* interaction; $F = 3.9$, $p = 0.049$). No other interactions were significant (results not shown). The presence of *Tomentella* spp. was not influenced by the tree decline status, *P. cinnamomi* infections, topography and soil texture (results not shown). The presence of *Russula* spp. was significantly more abundant in coarse than in fine textured soils (10.8 and 8 %, respectively, Table 2). The presence of *Russula* spp. was more abundant in non-declining

Table 3. Percentage values of non-vital (NV), vital non-mycorrhizal (NM) and vital ectomycorrhizal (EM) tips (± standard errors) of *Quercus ilex* trees located on mid slopes and stream banks of fine or coarse soil textures (n = 144 trees). Within the same column, different letters show significant differences ($p < 0.05$).

Topography	Soil texture	NV		NM		EM	
Mid slope	Fine	20.1 ± 1.4	a	31.5 ± 1.8	m	48.4 ± 2.1	yz
	Coarse	22.7 ± 1.7	ab	29.7 ± 1.6	m	47.6 ± 1.9	yz
Stream bank	Fine	25.7 ± 1.9	b	28.8 ± 2.0	m	45.5 ± 2.1	y
	Coarse	20.3 ± 1.5	a	27.8 ± 1.9	m	51.9 ± 2.0	z

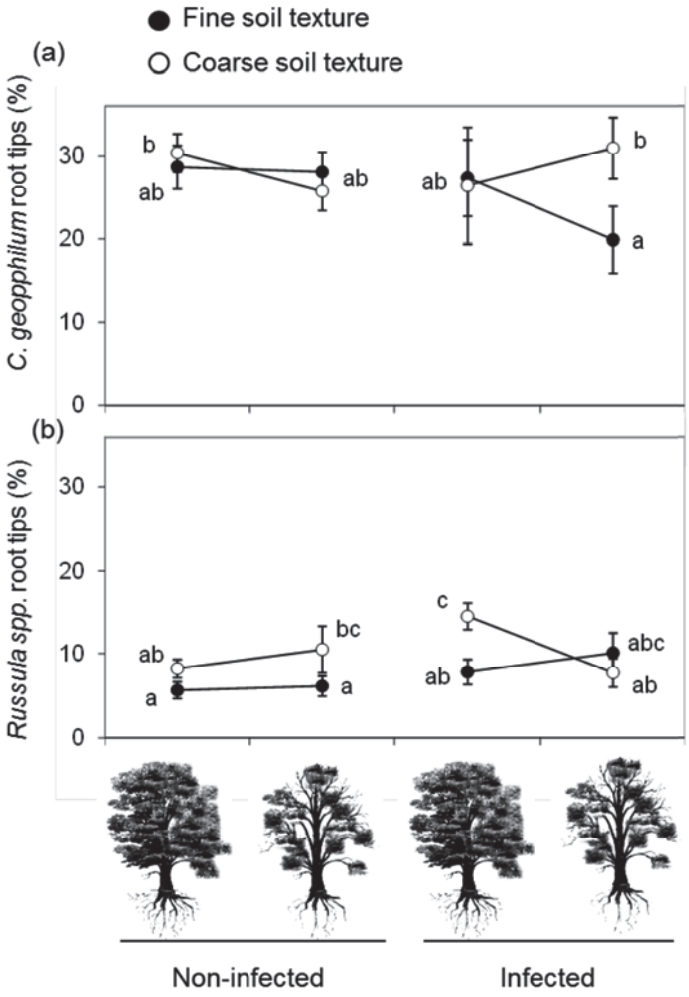

Fig. 5. Percentage values of tips colonized by *Cenococcum geophilum* (a) and *Russula* spp. (b) in non-infected non-declining, non-infected declining, *Phytophthora cinnamomi* infected non-declining, and *P. cinnamomi* infected declining *Quercus ilex* trees (n = 144) growing in soils of fine and coarse textures. Within the same ectomycorrhizal group, different letters show significant differences ($p < 0.05$).

Table 4. Pearson values from correlations between soil parameters and vital ectomycorrhizal (EM) tips of non-infected non-declining, non-infected declining, *Phytophthora cinnamomi* infected non-declining, and *P. cinnamomi* infected declining *Quercus ilex* trees. Asterisks indicate significance at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

	Non-infected		Infected	
	Non-declining	Declining	Non-declining	Declining
	$n \leq 223$	$n \leq 196$	$n \leq 63$	$n \leq 90$
Soil depth (m)	ns	ns	ns	ns
Ah horizon thickness (m)	0.35***	0.34***	0.23*	0.36***
Soil bulk density (g cm^{-3})	ns	ns	ns	ns
Ratio $N\text{-}NO_3^-$ /$N\text{-}NH_4^+$	-0.18**	-0.19**	-0.24*	ns
pH	0.29*	0.87***	ns	ns
Sand content (%)	-0.13**	-0.16**	ns	ns
Silt content (%)	ns	ns	ns	ns
Clay content (%)	0.14**	0.16**	ns	ns
Fine root abundance (n m^{-2})	0.12*	0.20**	ns	ns

infected than in declining infected trees (Fig. 4) but only for those trees growing in coarse textured soils (Figure 5b) (significant *tree decline status* × *P. cinnamomi infection* × *soil texture* interaction, Table 2).

Soil properties and ectomycorrhizal symbiosis

Ectomycorrhizal tip abundance did not vary with soil depth, soil bulk density or silt content, but with the thickness of the Ah horizon, irrespective of the tree status (Table 4). In non-infected trees,

abundance of EM tips was directly related to pH, clay content and fine root abundance, and inversely related with sand content (Table 4). In *P. cinnamomi*-infected trees, these relations were inexistent. $N\text{-}NO_3^-$ /$N\text{-}NH_4^+$ ratio values related negatively to EM tip abundance but signification disappeared under declining infected trees (Table 4).

4. Discussion

Quercus ilex decline was clearly related to the vitality of the root system and the abundance of ectomycorrhizal fungi. The increased abundance values of NV tips under declining than under non-declining trees corroborates previous research on Mediterranean (Trevisani et al., 1999; Montecchio et al., 2004) and non-Mediterranean *Quercus* declining forests (Blaschke, 1994; Jung et al., 2000). According to Scott et al. (2012), non-infected trees showed lower NM and EM tips in declining than in non-declining trees. Thus, *Q. ilex* decline may be associated with the NM and EM loss. Ectomycorrhizal fungi symbiosis would prevent trees to show decline symptoms (Scott et al., 2012) through enhancement of tree nutrition and therefore, of tree vigour (Landeweert et al., 2001). Ectomycorrhizal fungi would increase soil nutrients available for trees by mobilization of organic P and N and by their mineral weathering activity (Landeweert et al., 2001). Results also showed significant and positive relations between EM abundance and some soil properties associated to soil fertility (Ah horizon thickness, pH and clay content), which could indicate the need of nutrient accessibility to satisfy both fungi and host demands (Twieg et al., 2009). Ectomycorrhizal fungi

abundance has been reported to be influenced by environmental factors such as soil nutrients (Twieg et al., 2009), soil organic matter (Baier et al., 2006), nitrogen deposition (Lilleskov et al., 2002), soil type (Moser et al., 2005), soil compaction (Amaranthus et al., 1996), stand age (Kranabetter et al., 2005), land use practices (Azul et al., 2009), soil moisture (Cavender-Bares et al., 2009) or fire (De Roman and De Miguel, 2005).

Is decline (i.e. crown transparency) the cause or the consequence of EM tip loss? On one hand, crown transparency is usually associated with lower rates of photosynthesis per leaf area (Corcobado et al., 2013a). Reduced photosynthetic activity in declining trees affect negatively belowground carbon allocation to which ectomycorrhizal symbionts are dependent (Jönsson, 2006). Approximately 20 % of the carbon fixed by the host is assigned for the ectomycorrhizal symbiosis (Finlay and Söderström, 1992). Experiments based on artificial defoliation demonstrated that lower photosynthesis rates of defoliated trees resulted in decreased belowground carbon allocation, which originated reduced ectomycorrhizal fungi abundance and diversity (Gehring et al., 1997; Kuikka et al., 2003). In our study, declining trees would probably be unable to provide a great return of C investment to the ectomycorrhizal fungi symbiosis. On the other hand, soil and tree disturbances due to woodland management will probably reduce the amount of EM tips, which would cause in long term tree defoliation and subsequent decline. From descriptive studies, relationships of cause and effect are difficult to assess. However, in both

circumstances, a balanced feedback between leaf activity and ectomycorrhizal symbiosis is likely to occur, in which non defoliated trees would be able to maintain a more abundant ectomycorrhizal fungi community due to a possible higher C allocation to roots, and in consequence a positive nutritional effect due to a higher ectomycorrhizal fungi symbiosis will favour the tree crown. In order not to fall into speculation, it would be convenient to perform a study able to quantify in terms of energy the costs of tree ectomycorrhizal colonization and the subsequent implications for tree growth or defence.

Previous studies dealing with EM tips of declining *Q. ilex* forests did not consider distinction between *P. cinnamomi*-infected and *P. cinnamomi*-non-infected trees (e.g. Trevisani et al., 1999; Montecchio et al., 2004). Contrary to the notorious changes in ectomycorrhizal descriptors between declining and non-declining trees, the relationship between ectomycorrhizal fungi and *P. cinnamomi* infection seems to be much more complex. Such difficulties come from the fact that living organisms interact with a considerable number of biotic and abiotic factors, but limitations in studies hamper the consideration of all parameters. For example, relationship between *P. cinnamomi* and oak decline may be obscured by factors such as the validation of the pathogen presence (determined by the sampling, the isolation method and the environmental conditions), time lapse between infection and the appearance of decline symptoms (depending on the host, the pathogen activity/virulence and the environmental conditions), and phenological

and tolerance aspects related to oak trees, among others (Camilo-Alves et al., 2013). We did not find any significant relationship between *P. cinnamomi* and the proportion of NV and NM tips, but the presence of the pathogen altered the ectomycorrhizal abundance of some morphotypes (*Russula* spp. and other EM tips) between declining and non-declining trees. Among non-infected trees, declining trees lost a significant amount of NM and EM tips. Among infected ones, the loss of EM tips in declining trees was even higher. While among non-infected trees the EM tip community did not differ with decline, among infected trees, non-declining trees had a higher abundance of *Russula* spp. and other EM tips than declining trees. The presence of *P. cinnamomi* altered the proportion of the ectomycorrhizal fungi community probably due to changes in metabolic processes associated to root turnover and vitality (Scattolin et al., 2012). This may result in a shift in the cost:benefit ectomycorrhizal symbiosis balances which would mean that the presence of this invasive pathogen may represent an environmental threat for the tree. The presence of *P. cinnamomi* also blurred the relation (significant for non-infected trees) between soil properties and EM abundance (Table 4). Once roots are infected, the decreased ability to capture soil nutrients caused by root loss will reduce the vigour of the tree and would hamper C fixation and allocation to EM symbiosis.

Results revealed the existence of complex relations among EM community, soil properties, *P. cinnamomi* and *Q. ilex* decline (Table 4). Figure 5 illustrates this complexity too. The presence of *C.*

geophilum did not vary within non-infected trees, and within infected non-declining trees (Figure 5a), but was significantly more abundant in infected declining trees growing in coarse textured soils than in infected declining trees growing in fine textured soils (Figure 5a). The presence of *Tomentella* spp. was not influenced by the tree decline status, *P. cinnamomi* infections, topography and soil texture. Tips colonized by *Russula* spp. were more abundant in coarse- than in fine-textured soils except again for infected declining trees. Little is known about the effects of soil texture on mycorrhization. Gehring et al. (1998) reported that cinder soils and sandy-loam soils showed similar richness of ectomycorrhizal morphotypes but different ectomycorrhizal fungi composition. The importance of texture was conditioned by the topography position: in stream banks fine-textured soils were associated to significantly higher proportions of NV tips and lower proportions of EM tips. Finer-soil textures are usually related to a higher content in nutrients and moisture, influential factors in ectomycorrhizal symbiosis (Cavender-Bares et al., 2009). However, in our study, the higher occurrence of waterlogging events in fine-textured soils of stream-bank were associated to *Q. ilex* decline (Corcobado et al., 2013a) and likewise to the loss of ectomycorrhizal fungi. Accordingly, Corcobado et al. (2013b) showed that *Q. ilex* decline was more intense in fine-textured soils for infected trees in comparison with coarse-textured soils.

Studies related to the analyses of the ectomycorrhizal community abundance and diversity of holm oak forests were performed in non disrupted areas (Richard et al., 2005, 2009; Clavería

and De Miguel, 2005) and few were performed in disturbed sites (Montecchio et al., 2004; De Roman and De Miguel, 2005; Richard et al., 2011). It is known that disturbances such as the presence of pathogens, fertilization, fire, clear-cut, thinning and other management practices could lead to a shift in ectomycorrhizal diversity rather than a reduction in ectomycorrhizal abundance (Jones et al., 2008; Blom et al., 2009). Different physiological functions have been assigned to specific ectomycorrhizal morphotypes, and therefore a higher diversity of ectomycorrhizal fungi would imply more benefits to the tree associated (Goldbold, 2005). In this case, ectomycorrhizal symbiosis could indirectly reduce disease symptoms by enhancing tree nutrition, growth and other physiological reactions. A diversity of 112 ectomycorrhizal morphotypes was observed in a well preserved *Q. ilex* forest (Richard et al., 2005), while 62 morphotypes were detected in a declining *Q. ilex* forest (Montecchio et al., 2004). In this study three ectomycorrhizal fungi morphotypes were identified as the most abundant and rarely more than 5-6 morphotypes per stand were observed, what seems very poor in terms of diversity. Dehesas are generally experiencing a subtle to moderate process of soil degradation caused by management practices (Moreno and Pulido, 2009). The poor organic matter content of dehesa soils is one of the most worrying parameters (Schnabel et al., 2009). Corcobado et al. (2013b) showed the significant associations between tree decline and the nitrate/ammonia ratio, an indicator of soil degradation (Wilson and Tilman, 1991). Here a significant reduction of ectomycorrhizal abundance with the increase of nitrate/ammonia ratio was observed.

5. Conclusions

This study demonstrated the three hypotheses formulated. First, ectomycorrhizal community depended on the soil properties, increasing its abundance with soil properties associated with soil fertility, and conditioned by topography position. Second, root vitality and ectomycorrhizal abundance were higher in non-declining than in declining trees and root losses of non-infected trees were mostly associated with non-mycorrhizal tips, while root losses of *P. cinnamomi* infected trees were associated mostly with EM tips. Third, the presence of the pathogen weakened the relations usually observed in nature between soil properties and ectomycorrhizal abundance. Overall, ectomycorrhizal diversity was very poor in the declining *Q. ilex* stands studied what agrees with the increasing awareness of the progressive degradation of Iberian dehesas. Disentangling complex relations among ectomycorrhizal community and tree decline mediated by soil properties and altered by soil borne pathogens seems essential to incorporate mycorrhization programs within forest management planning.

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CAPÍTULO 3

Seasonal dynamics of ectomycorrhizal symbiosis in declining *Quercus ilex* woodlands: influence of tree health status and *Phytophthora cinnamomi* root infections

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Abstract
Ectomycorrhizal fungi symbiosis may vary with environmental changes and seasonality. *Quercus ilex* decline and *Phytophthora cinnamomi* infections may also influence ectomycorrhizal fungi along the seasons. Aiming to investigate such influences, five *Quercus ilex* declining stands were selected in western Spain and declining and non-declining trees were sampled. Ectomycorrhizal fungi abundance and *P. cinnamomi* root infections were assessed seasonally during two years. The physiological status of trees was also measured during the most stressful season. Results showed higher ectomycorrhizal abundance during spring and summer than during autumn and winter. The most dominant ectomycorrhizal morphotypes were *Cenococcum geophilum*, *Tomentella* spp. and *Russula* spp. The abundance of *Russula* spp. was higher in declining than in non-declining trees. However, the abundance of vital non-mycorrhizal tips was higher under non-declining than under declining trees. The relative presence of other ectomycorrhizal tips was surprisingly higher in infected than in non-infected trees. The significant difference of vital non-mycorrhizal tips observed between non-infected non-declining and declining trees became non-significant if trees were infected with *P. cinnamomi.* Relationships between the physiology of *Q. ilex* and the ectomycorrhizal community differed with tree decline status. *Q. ilex* decline and *P. cinnamomi* are accounted for being related to ectomycorrhizal changes and its scarce abundance and diversity.

Keywords: Oak decline; root rot pathogen; ectomycorrhizae; physiology; Iberian dehesa; land use

1. Introduction

Oak decline is the result of a complex interaction between different abiotic and biotic stresses (Jung et al., 1996; Camilo-Alves et al., 2013). Oak decline has been associated with changes in the ectomycorrhizal symbiosis, in the way that a lower abundance and diversity of ectomycorrhizal fungi in declining trees in comparison to non-declining trees have been reported (Blaschke, 1994; Causin et al., 1996; Lancellotti and Franceschini, 2012). Mycorrhizal symbiosis and associated networks are recognized as a critical interface between soil and plant communities (Leake et al., 2004; Finlay, 2008). The symbiotic ectomycorrhizal fungi assist their host plants in nutrient uptake, drought tolerance, and pathogen resistance (Smith and Read, 2008). For example, the ectomycorrhizal fungi community reveals to be diverse under drought conditions (Azul et al., 2010; Bingham and Simard, 2012) suggesting the critical role of ectomycorrhizal symbiosis in attending their hosts under non-favourable abiotic conditions. In parallel, environmental factors and its changes with seasonality may affect the ectomycorrhizal symbiosis (Koide et al., 2007).

Holm oak (*Quercus ilex* L.) is a broadleaved sclerophyllous tree from the Mediterranean region severely affected by declining processes (Robin et al., 1998; Sánchez et al., 2006; Corcobado et al., 2013b). Holm oak forms ectomycorrhizal associations with a vast diversity of Ascomycetes and Basidiomycetes, as reported through a unique sampling (Richard et al., 2004, 2005, 2009; Clavería and De Miguel, 2005) or a seasonal sampling (De Román and De Miguel,

2005; Richard et al., 2011). However, little is known about the ectomycorrhizal community related to declining *Q. ilex* stands (Montecchio et al., 2004). Seasonal changes of ectomycorrhizae in declining *Q. robur* trees have been studied (Mosca et al., 2007). However, there is a lack of studies on the seasonal patterns of ectomycorrhizal abundance and diversity in declining *Q. ilex* stands.

The soil-borne oomycete *Phytophthora cinnamomi* Rands was first associated to holm oak decline by Brasier et al. (1993) and it is considered the main biotic factor of *Q. ilex* decline in the Iberian Peninsula. This pathogen is responsible for multiple fine-root infections followed by substantial root girdling and tree death (Sánchez et al., 2002; Camilo-Alves et al., 2013). Fine root loss caused by *P. cinnamomi* contributes considerably to the impairment of tree water relations (Robin et al., 2001), particularly because fine roots are the root fraction with the highest water uptake efficiency (Thomas and Hartmann, 1998). In turn, ectomycorrhizal fungi have also been shown to protect trees against *P. cinnamomi* infections (Marx, 1972; Branzanti et al., 1999; Jönsson, 2006). Studies reporting interactions between ectomycorrhizae and *P. cinnamomi* refer mainly to *Castanea sativa*, *Eucalyptus* and *Pinus* hosts (Marx, 1972; Malajczuk, 1988; Branzanti et al., 1999). These interactions may change with the season, probably because the growth of ectomycorrhizal fungi is limited by low soil temperatures and low soil moisture (Breda et al., 2006), and *P. cinnamomi* needs temperatures ranging from 24 to 28 ºC to grow optimally, and waterlogging or high humid soil conditions to sporulate (Erwin and Ribeiro, 1996). Interactions involving

ectomycorrhizal fungi and *P. cinnamomi* root infections would also be affected by topography, since the location of trees in upper slopes or close to stream banks influences the soil water content (Corcobado et al., 2013a).

Disturbances in environmental conditions derived from oak woodland management may lead to shifts in the ectomycorrhizal communities. For example, studies on nitrogen fertilization (Avis et al., 2003), wildfire (De Román and De Miguel, 2005), thinning (Mosca et al., 2007) or type of exploitation (Azul et al., 2009, 2010) reported detrimental effects on ectomycorrizal fungi. A unique type of managed oak woodland in the southwestern Iberian Peninsula is the agrosilvopastoral system known as *dehesa* in Spain and *montado* in Portugal, which covers 3.1 million hectares (Díaz et al., 1997). Traditionally, this agrosilvopastoral system combines three functional levels of vegetation, an herbaceous layer with shrubs and scattered holm and cork oak trees. This system has been appreciated for maintaining an ecological sustainability. However, it has experienced intensification and abandonment processes since the second half of the last century, together with a high tree mortality associated to *Q. ilex* decline (Moreno and Pulido, 2009; Corcobado et al., 2013b). The progressive degradation of soils in Iberian dehesas (Moreno and Pulido, 2009) may have detrimental effects in ectomycorrhizal fungi community (Azul et al., 2010). Changes in the abundance of the ectomycorrhizal community or of a particular species caused by land uses in these systems may affect the vigour of *Q. ilex* trees. These effects may be explained by the influence of ectomycorrhizal

association in the P nutrition, water uptake and plant biomass, as shown by some studies performed in greenhouses and forest plantations (Domínguez et al., 2006; Oliveira et al., 2010). However, this amelioration may vary with the ectomycorrhizal specie, which deserves more research.

Plants inoculated with ectomycorrhizal fungi have been associated with an increase of stomatal conductance and net leaf photosynthesis in comparison to non-inoculated plants (Morte et al., 2000; Nardini et al., 2000). Most benefits provided by ectomycorrhizal symbiosis on trees at the physiological level have been reported from greenhouse experiments in which young seedlings were used. Field studies reporting those benefits would be desirable, especially within the tree decline context in which the ectomycorrhizal symbiosis would probably be essential for the survival of infected and declining trees, as the case of *P. cinnamomi* infected oaks. The objectives of this study were to describe and quantify the seasonal changes of the ectomycorrhizal community of *Q. ilex* trees in relation to tree decline status and *Phytophthora cinnamomi* root infections. A second objective was to relate, during the season of maximum water stress, the relative abundance of the ectomycorrhizal community to the water and photosynthetic status of *Q. ilex* trees. According to the objectives, it was hypothesised that (i) tree decline status and *Phytophthora cinnamomi* root infections may influence the seasonal changes of ectomycorrhizal fungi in *Q. ilex* trees, and (ii) relationships between the ectomycorrhizal community and the physiology of *Q. ilex* trees may differ if trees are declined, infected or not.

2. Materials and methods

Study site

The research was performed in Extremadura, western Spain, and included 5 oak woodlands (Table 1) infected with *P. cinnamomi*. Woodlands were characterised by a scattered-tree layer of *Q. ilex* trees combined with *Q. suber* trees and an understory of grazed pasture dominated by annual native species (i.e. dehesas). More than 50 % of trees of the five sites were declined. Information about land management over the last 25 years (Table 1) was obtained through questionnaires provided to landowners. The climate in the area is dry Mediterranean, with rain mainly from December to May and average annual rainfall of about 680 mm. Mean minimum and maximum temperatures are in January (7.4 ºC) and August (28.7 ºC), respectively. According to meteorological data from nearby stations, no substantial differences of temperature values among sites were observed. The study lasted two years (March 2009 to March 2011) and during the first year annual precipitation was lower than during the second year (778 and 865 mm, respectively). Mean annual precipitation for the 2-year study period was substantially higher in Haza than in Abadía (916 and 740 mm, respectively).

Experimental design

Three non-declining and three declining *Q. ilex* trees per site were selected attending to their defoliation degree (crown transparencies of 0-14 and 15–35 %, respectively, according to UNECE (2010)).

Table 1. Main characteristics of the five experimental sites.

	Stands				
	Abadía	Cuartón	Haza	San Esteban	Vegaviana
Location	40º15'N, 5º57'W	39º52'N, 6º02'W	39º50'N, 5º56'W	39º58'N, 6º05'W	40º00'N, 6º43'W
Altitude (m a.s.l.)	484	384	285	445	272
Geological substrate	Granite	Quartzite colluvium	Slate	Slate	Tertiary sediments
Soil type [a]	Endo-leptic Cambisol	Chromic Luvisol	Epi-leptic Cambisol	Epi-leptic Cambisol	Distric Regosol
P [b] (mm)	740	859	916	803	828
T [c] (ºC)	16.7	15.7	15.8	16.5	16.1
Crown transparency spring 2009 (%)	35	32	23	21	44
Crown transparency spring 2010 (%)	46	70	48	44	42
Extension (ha)	200	240	570	330	18.5
Exploitation regime	Extensive	Extensive	Extensive	Extensive	Extensive
Exploitation type	Silvopastoral	Silvopastoral	Agrosilvopastoral	Agrosilvopastoral	Silvopastoral
Land use	Natural pasture	Natural pasture	Natural pasture	Natural pasture/Sown pasture	Natural pasture
Livestock	Cattle/sheep/pigs	Sheep	Cattle	Cattle	Cattle
Pruning frequency	-	-	20 yr	15 yr	12 yr
Mushroom harvesting	No	No	No	Yes	No
Shrub control	Livestock/Cut	Livestock	Livestock/Cut with soil tillage	Livestock/Cut with soil tillage	Livestock/Cut
Last shrub control/yr[d]	Permanently/less than 1 yr	Permanently	Permanently/4 yr	Permanently/less than 1 yr	Permanently/6 yr
Last soil tillage control/yr[d]	No	No	4 yr (10 ha)	Less than 1 yr	No

Table 1. Continued

	Stands				
	Abadía	Cuartón	Haza	San Esteban	Vegaviana
Use of fertilizers[d]	No	No	Less than 1 yr	Less than 1 yr	2 yr
Use of pesticides	No	No	No	No	No
Pests	Yes	No	Yes	Yes	No
First decline observation (yr)	1999	1994	1995	1995	1988
Management of declining /dead trees	- /Cut and burning	Cut/Cut and burning	- /Stump removal and burning	Pest treatment/Stump removal and burning	- /Cut

[a] According to IUSS Working group WRB (2006).
[b] Mean annual precipitation for the 2-years period of study (March 1 2009 to February 28 2011).
[c] Mean annual temperature for the 2-years period of study (March 1 2009 to February 28 2011).
[d] Years that passed before 2009.

To avoid the possibility of non-declining trees sharing parts of their rhizosphere with declining trees, selected trees were separated at least 50 m. In each site, two trees were in or close to a stream bank, two were on a mid slope, and two were on a higher slope away from the stream bank. Pairs of trees at each topography position included one non-declining and one declining trees. Trees were 6–10 m high, 30–90 cm in trunk diameter at breast height and 7.5–12 m in crown diameter.

From March 2009 to March 2011 soil and roots from each of the 6 trees per site were sampled. Sampling was performed once a season and samples were used for *Phytophthora* spp. isolation and ectomycorrhizal fungi assessment (see annex II.3). Once a month, from July to September 2009 and 2010, coinciding with the driest season and the period in which declining *Q. ilex* trees usually show higher defoliation (Solla et al., 2009), the physiological status of all the 30 trees were assessed.

P. cinnamomi isolation

Approximately 25 fine roots per tree from the four cardinal points were sampled. Sampling was performed at distances of 1-2 m from the trunks and at depths of 10-50 cm. Roots were approximately 5-6 cm long and were immediately cut into 1 cm segments (extremes discarded), surface-sterilised (2 min in 1 % aqueous sodium hypochlorite), rinsed with sterile water, blotted dry and plated on petri dishes containing NARPH selective medium (Solla et al., 2009). About nine plates per tree and season containing about 10 fine root segments each were incubated in the dark at 24 °C, and after 2–3 days

selected isolates were transferred to a carrot agar (CA) medium. Colonies were identified by microscopic observations of distinctive structures such as clustered hyphal swellings, chlamydospores and sporangia. Isolation of *Phytophthora* from the soil was also successful using young leaves and green apples as baits according to Jung et al. (1996). Since isolations from the soil do not always confirm root infection, results of soil baiting were not included in this work.

Ectomycorrhizae assessment

Four-five soil cores per tree and season at 1 m of distance from the four cardinal points of the tree trunks were additionally collected. Cores were approximately 10 cm diameter and collected from 10 to 50 cm in depth. Cores were sealed in plastic bags, moistened with distilled water, kept in a portable cooler, stored at 4 ± 1 ºC in the laboratory and analyzed within 8 days. Monoliths were kept in water 24 h before the assessment to facilitate the separation of the roots from the soil particles and then, grouped into the same sample. Only roots with a diameter < 2 mm were assessed. On average 170 root tips per tree and season were examined, comprising circa 40,000 tips for the whole experiment. Tips were examined under an stereomicroscope (Olympus SZX10, Japan) and classified as 'non-vital' (NV, a scurfy surface and an easily detachable cortex, with or without the remnants of an ectomycorrhizal mantle), 'vital non-mycorrhizal' (NM, a well-developed, turgid and inflated tip, mantle lacking) or 'vital ectomycorrhizal' (EM, as above, but with an ectomycorrhizal mantle) according to specific literature (Montecchio et al., 2004; Scattolin et

al., 2012). Each EM tip was then classified anatomically and morphologically (Agerer, 1987–2008; Goodman et al., 1996–1998; Agerer and Rambold, 2004–2011), and ITS sequencing was performed in most morphotypes. To study the metabolic state of ectomycorrhizae (active or inactive) the methodology proposed by Harvey et al. (1976) was carried out. Active ectomycorrhizae were turgid while inactive ectomycorrhizae had a wrinkled mantle. Within this aim, during spring additional cores were collected under trees in three sites (n = 4 trees) and the number of active and inactive EM tips were assessed.

Tree physiology and soil water content measurements

Pre-dawn leaf water potential (Ψ_{pd}, MPa) was determined using a Scholander chamber (SKPM 1400, Skye Instruments Ltd., UK) on two terminal current-year twigs per tree collected from the outer mid portion of the crown. Stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) and net leaf photosynthesis (A, µmol CO_2 m^{-2} s^{-1}) were determined using a portable differential infrared gas analyser (IRGA) (LCi, ADC Bio Scientific Ltd., UK) connected to a broadleaf chamber. Measurements were taken from 9.30 to 11.00 h in three current-year leaves per tree exposed to the sun. Finally, maximum PSII photochemical efficiency (F_v/F_m) was determined with a Multi-Mode Chlorophyll Fluorometer OS5p (Multi-Mode Chlorophyll Fluorometer OS5p, Opti-science Inc., USA). Measurements were conducted between 8:30 and 10:00 h in ten current-year fully expanded leaves per tree. Leaves were previously covered with leaf clips during 30 min to assure fully dark adaptation.

Soil water content beneath the 30 trees was assessed using a portable probe (Diviner 2000, Sentek Technologies, Australia) inserted into PVC tubes (9 cm diam.) permanently installed to a depth of 3 m after soil drilling (Corcobado et al., 2013a).

Data analysis

The five sites were included as randomised blocks in the analysis. According to the presence or absence of *P. cinnamomi* colonies in the root samples, the trees were grouped into non-infected non-declining (n = 9), non-infected declining (n = 8), infected non-declining (n = 6) and infected declining (n = 7) trees. In each tree, NV, NM and EM tips were referred to the total number of tips examined and values expressed in percentages (ectomycorrhizal descriptors). In the same way, EM tips belonging to a particular morphotype were referred to the total number of tips examined and values expressed in percentages (ectomycorrhizal morphotype groups). Intrinsic water use efficiency (*iWUE*) was obtained from A/g_s. Normality and homocedasticity of the data was checked by Kolmogorof-Smirnoff and Bartlett's tests, respectively, and data was transformed when necessary. The influence of *P. cinnamomi* infection and tree health status upon the seasonal changes of ectomycorrhizal symbiosis were analysed through general linear mixed models, in which the *ectomycorrhizal descriptors* and *ectomycorrhizal morphotype groups* were used as dependent variables, *season*, *year*, *tree decline status*, *P. cinnamomi infection* and *topographic position* were used as fixed factors, and *site* was used as a random factor. Two degree interactions among the fixed factors

were included. Comparisons between means were made through multiple range tests with Fisher's least significant difference (LSD) intervals. To assess the relationship between active EM tips and tree status (declining or non-declining), t-student tests were applied, in which *the relative abundance of active EM tips* or *the relative abundance of active ectomycorrhizal morphotype groups* were used as dependent variables and *tree decline status* was used as a fixed factor. The relationships between descriptors and physiological parameters were examined by means of Pearson correlation coefficients. To study whether *tree decline status* and *P. cinnamomi infection* conditioned the relationships between descriptors and physiological parameters, Homogeneity-of-slopes models were run. All analyses were performed with STATISTICA v10 (Stat Software Inc., Tulsa, OK).

3. Results

The three most dominant EM morphotype groups observed were *Cenococcum geophilum* (EM_{Cg}) (relative abundance of 64.3 %), *Russula* spp. (EM_R) (13.0 %; including *Russula atropurpurea*, *Russula* sp. 1 and *Russula* sp. 2) and *Tomentella* spp. (EM_T) (12.6 %; including *Tomentella subtestacea*, *Tomentella* sp. 1 and *Tomentella* sp. 2). Additional identified groups were in lower proportions and merged into other EM tips (10.1 %; including *Lepista* sp. 1).

Site, topography and seasonal variations of ectomycorrhizal symbiosis

The mean relative abundances (± SE) of NV and EM tips were 35.6 ± 1.4 and 38.8 ± 1.3 %, and did not differ significantly between sites.

However, NM tips differed significantly between sites (Table 2), being Abadía and Haza the woodlands with the highest and lowest values, respectively (28.4 ± 2.1 and 21.4 ± 2.2 %, respectively). Differences of EM_{Cg} tips between sites were also significant (Table 3), being Haza and Abadía the forests with the highest and lowest values, respectively (33.6 ± 2.9 and 22.2 ± 2.6 %, respectively), although Abadía did not differ significantly from the other stands.

Tips classified as NM varied significantly with topographic position (Table 2), being their relative abundances higher in trees from the upper slopes than in trees close to stream banks (28.3 ± 2.5 and 23.5 ± 1.6 %, respectively, and 24.7 ± 1.8 % for mid-slopes). Trees close to stream banks showed higher relative abundances of EM_{Cg} tips in comparison with trees from mid and upper slopes (Table 3; Fig. 1).

Most ectomycorrhizal descriptors varied with year and season (Tables 2 and 3). Percentages of NV tips where higher in 2010 than in 2009 (Fig. 2), and showed the highest values in winter during both years (Fig. 2) and the lowest values in spring or summer depending on the year (significant *year* × *season* interaction; Table 2). Only during 2009 the abundance of NM tips varied seasonally, with higher values during the dry (summer and autumn) than during the wet (winter and spring) seasons. Percentages of EM tips were highest during spring 2009 and during summer 2010 (significant *year* × *season* interaction; Table 2; Fig. 3). Seasonal variations were higher for EM_T than for EM_R and EM_{Cg} as indicated by the F-ratios of the mixed models (Table 3; Fig. 3).

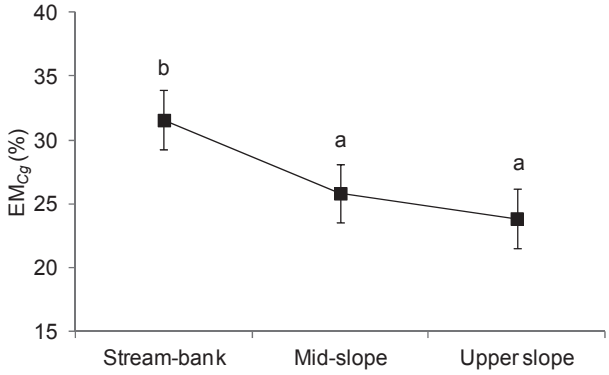

Fig. 1. Percentages of *Quercus ilex* root tips colonized by *Cenococcum geophilum* (EM_{Cg}) depending on the topographic position of trees. Bars are standard errors and different letters indicated significant differences at $p < 0.05$; $n = 10$ trees.

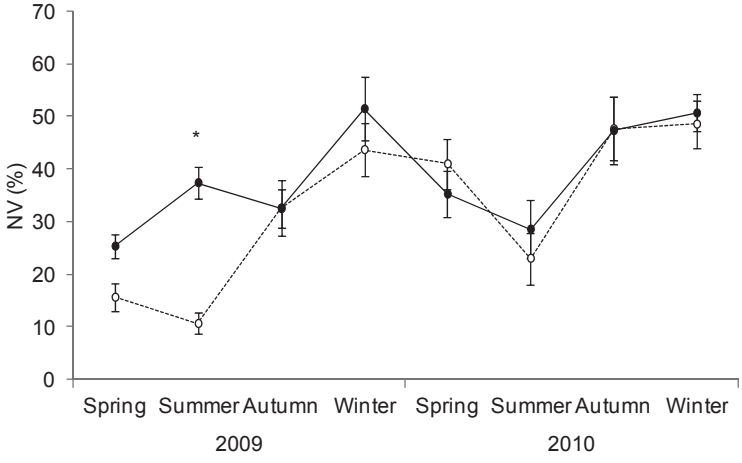

Fig. 2. Seasonal variations of the percentages of non-vital (NV) root tips of non-declining (○) and declining (●) *Quercus ilex* trees. Bars are standard errors and asterisk indicate significant difference at $p < 0.001$; $n = 12$ trees.

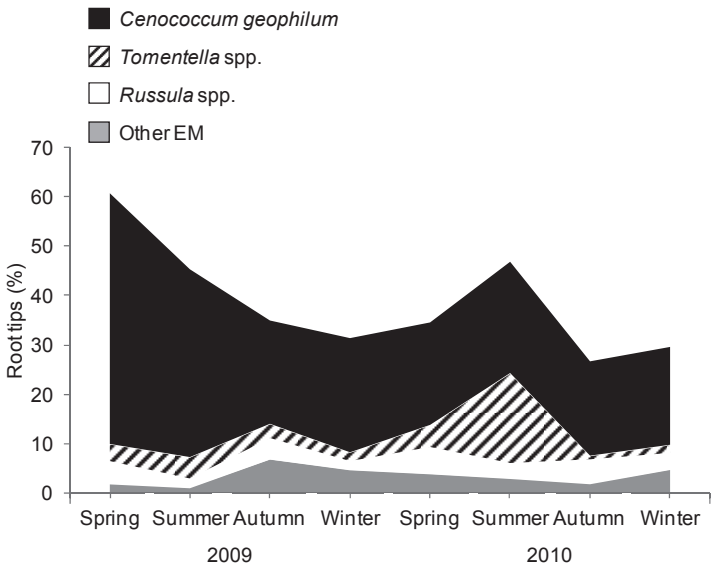

Fig. 3. Seasonal variations of the percentages of *Quercus ilex* root tips colonized by *Cenococcum geophilum* (EM_{Cg}), *Russula* spp. (EM_R), *Tomentella* spp. (EM_T) and other morphotypes; n = 30 trees.

Tree decline, Phytophthora cinnamomi infection and ectomycorrhizal symbiosis

A higher proportion of NV tips was observed under declining than under non-declining trees (Table 2; Fig. 4), with differences between the tree decline status especially notable during summer 2009 (significant *season* × *tree decline status* interaction; Table 2) (Fig. 2). Higher percentages of NM tips were observed under non-declining than under declining trees, while percentages of EM tips were similar under non-declining and declining trees (Fig. 4; Table 2). When considering morphotypes, EM_R tips were lower in non-declining than in declining trees (Fig. 5; Table 3). The relative abundance of active

Table 2. Results of the general linear mixed models for the analysis of the percentage of non-vital (NV), vital non-ectomycorrhizal (NM) and ectomycorrhizal (EM) tips of *Quercus ilex* trees. Degrees of freedom (DF) and F-ratios for the fixed factor, and variance component (VarComp) and its associated χ^2 value for the random factor are shown.

Effect	NV			NM			EM		
	DF or VarComp	*F* or χ^2	*p* value	DF or VarComp	*F* or χ^2	*p* value	DF or VarComp	*F* or χ^2	*p* value
Fixed factors									
Year [Y]	1	15.78	< 0.001	1	0.06	0.814	1	15.18	< 0.001
Season [S]	3	20.70	< 0.001	3	3.78	0.012	3	17.27	< 0.001
Tree status [D]	1	5.89	0.016	1	5.79	0.017	1	0.03	0.861
P.cinnamomi infection [P]	1	1.19	0.277	1	2.44	0.120	1	0.15	0.697
Topographic position	2	0.95	0.390	2	3.16	0.045	2	0.63	0.535
Y × S	3	3.42	0.019	3	2.21	0.089	3	7.69	< 0.001
S × D	3	2.85	0.039	3	1.85	0.139	3	0.46	0.708
S × P	3	0.40	0.751	3	0.45	0.716	3	1.72	0.165
D × P	1	1.93	0.167	1	4.97	0.027	1	0.53	0.466
Random factor									
Site	177.43 ± 251.03	0.71	0.549	628.07 ± 221.09	2.84	0.040	400.48 ± 232.82	1.72	0.165

EM tips was not affected by the tree decline status (31.3 ± 10.0 and48.1 ± 8.8 %, for declining and non-declining trees, respectively; t = -1.26, p = 0.253). When considering the percentage of active EM morphotype groups, the abundance of active EM_T tips was higher in non-declining trees than in declining trees (21.9 ± 4.0 and 1.2 ± 1.2 %, respectively; t = -4.95, p = 0.003).

Phytophthora cinnamomi was successfully isolated from fine roots of all declining stands and was more frequently isolated from

Table 3. Results of the general linear mixed models for the analysis of the percentage of *Quercus ilex* root tips colonized by *Cenococcum geophilum* (EM_{Cg}), *Russula* spp. (EM_R), *Tomentella* spp. (EM_T), and other morphotypes. Degrees of freedom (DF) and F-ratios for the fixed factor, and variance component (VarComp) and its associated χ^2 value for the random factor are shown.

Effect	EM_{Cg}		EM_R		EM_T		Other EM	
	F or χ^2	*p* value	*F* or χ^2	*p* value	*F* or χ^2	*p* value	*F* or χ^2	*p* value
Fixed factors								
Year [Y]	27.21	< 0.001	2.45	0.120	5.86	0.017	0.35	0.556
Season [S]	11.07	< 0.001	5.51	0.001	20.43	< 0.001	7.88	< 0.001
Tree status [D]	0.01	0.905	4.18	0.043	1.47	0.227	0.12	0.733
P.cinnamomi infection [P]	0.23	0.634	0.01	0.910	0.24	0.628	6.99	0.009
Topographic position	3.88	0.023	2.14	0.121	2.86	0.060	0.15	0.859
Y × S	7.18	< 0.001	0.27	0.845	13.30	< 0.001	9.65	< 0.001
S × D	1.12	0.344	0.55	0.650	0.37	0.772	1.44	0.232
S × P	0.97	0.406	0.51	0.676	0.79	0.504	0.99	0.400
D × P	0.36	0.550	0.00	1.000	0.02	0.885	2.53	0.114
Random factor								
Site	4.70	0.004	0.46	0.709	2.12	0.100	1.05	0.372

trees at Abadía, followed by San Esteban, than from trees at other stands (results not shown). *Phytophthora gonapodyides* was also isolated from a single tree at San Esteban (Corcobado et al., 2010). Isolation success was higher in spring than in autumn or winter and failed in summer. Infected and non-infected trees had similar percentages of NV, NM, EM, EM_{Cg}, EM_R and EM_T tips, but the relative presence of other EM tips was surprisingly higher in infected than in non-infected trees (Tables 2 and 3; Figs. 4 and 5). The

significant difference of NM tips observed between non-infected non-declining and non-infected declining trees (Fig. 4) became non-significant if trees were infected with *P. cinnamomi* (*tree decline status* × *P. cinnamomi* interaction for NM tips; Table 2).

Tree physiology parameters, soil water content and ectomycorrhizal symbiosis

Mean values of ψ_{pd}, g_s and A were significantly lower in declining than in non-declining trees (−2.6 ± 0.2 *vs*. −2.1 ± 0.2 MPa, 69 ± 7 *vs*. 93 ± 6 mmol H_2O m^{-2} s^{-1}, and 6.6 ± 0.5 *vs*. 8.1 ± 0.5 μmol CO_2 m^{-2} s^{-1}, respectively; $p < 0.05$), and similar in infected and non-infected trees(results not shown). Trees were more water stressed in 2009 than in 2010 ($p = 0.0003$), especially in August (ψ_{pd} = −3.1 ± 0.2 *vs* −2.7 ± 0.2 MPa, respectively; $p < 0.0001$), and if positioned at mid or upper slopes rather than close to stream banks ($p = 0.0009$; results not shown).

Percentages of NM tips were negatively related to soil water content (r = -0.21; $p = 0.045$). Homogeneity-of-slopes analysis revealed that NM tips related differently to soil water content depending on the tree decline status ($p = 0.026$), i.e. only non-declining trees related significantly. Within non-declining trees, percentages of other EM tips related positively to soil water content while within declining trees related negatively ($p = 0.008$).

No significant relations between any of the physiological variables monitored and NV, NM, EM tip percentages were observed, but with the different EM morphotypes. Percentages of EM_{Cg} tips

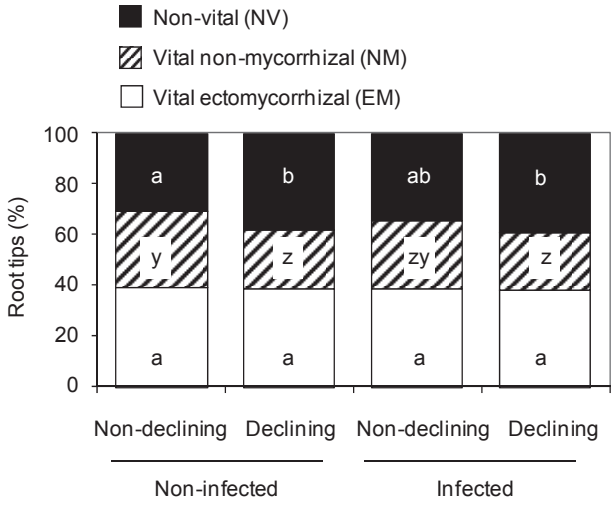

Fig. 4. Percentage of vital ectomycorrhizal, vital non-mycorrhizal and non-vital root tips under non-declining and declining *Quercus ilex* trees non-infected and infected with *Phytophthora cinnamomi*. Different letters denote differences between groups at $p < 0.05$; $n > 6$ trees.

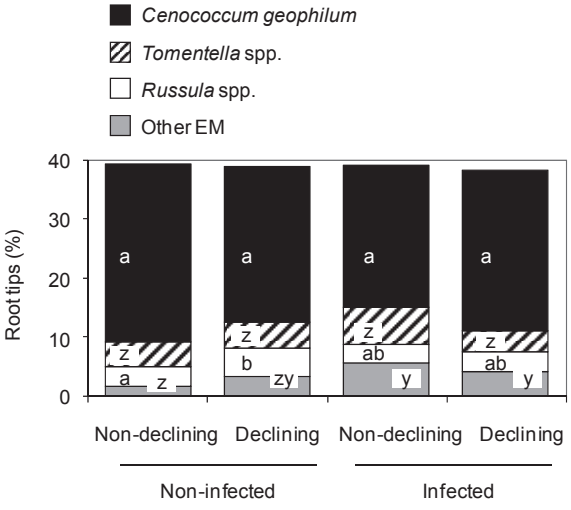

Fig. 5. Percentage of root tips colonized by *Cenococcum geophilum* (EM_{Cg}), *Russula* spp. (EM_R), *Tomentella* spp. (EM_T) and other morphotypes under non-declining and declining *Quercus ilex* trees non-infected and infected with *Phytophthora cinnamomi*. Different letters denote differences between groups at $p < 0.05$; $n > 6$ trees.

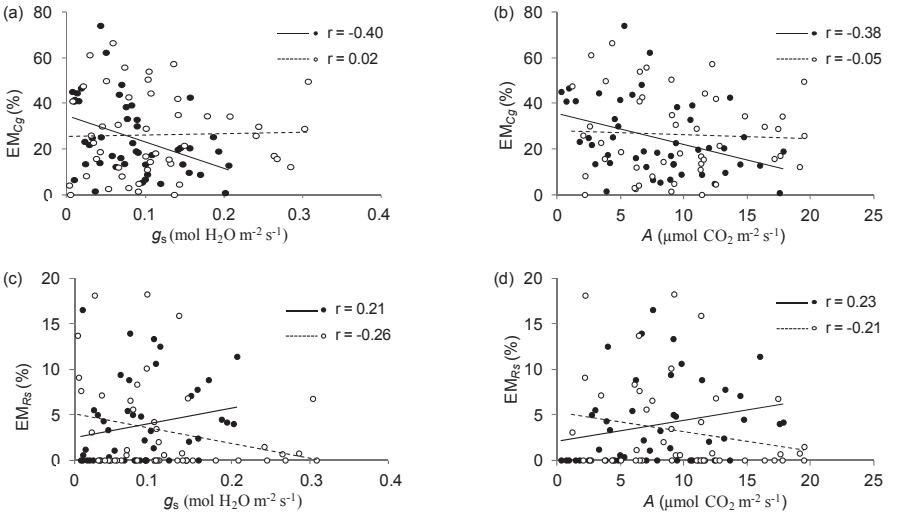

Fig. 6. Relationships between stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) and the percentage of root tips colonized by *Cenococcum geophilum* (EM_{Cg}), (a) or with *Russula* spp. (EM_R) (b), and between net leaf photosynthesis (A, µmol CO_2 m^{-2} s^{-1}) and these morphotypes (c and d, respectively) under declining (●) and non-declining (○) trees during summer 2009 and 2010.

were negatively related to g_s, A, and F_v/F_m (r = -0.23; p = 0.043; r = -0.19; p = 0.050 and r = -0.29; p = 0.012; respectively). Homogeneity-of-slopes analysis revealed that EM_{Cg} tips related significantly to g_s and A only within declining trees (Figs. 6a and 6b). Percentages of EM_T tips related positively to F_v/F_m (r = 0.23; p = 0.030) and percentages of EM_R tips related positively to Ψ_{pd} and *iWUE* (r = 0.24; p = 0.023 and r = 0.30; p = 0.003; respectively). Within declining trees, percentages of EM_R tips related positively to g_s and A, while within non-declining trees related negatively (Figs. 6c and 6d). Percentages of other EM tips related positively to Ψ_{pd}, A and *iWUE* (r = 0.24, p = 0.038; r = 0.09, p = 0.061; and r = 0.28, p = 0.001, respectively).

4. Discussion

Mediterranean areas are submitted to a marked seasonality mainly associated to precipitation and soil moisture changes, which are influential for the ectomycorrhizal fungi structure (Kennedy and Peay, 2007). Relative abundance of EM tips in our declining stands showed pronounced seasonal shifts, being EM tips significantly higher during spring and summer than during autumn and winter. Other studies also reported seasonal shifts in ectomycorrhizal fungi with maximum abundance in spring (Courty et al., 2008) and the highest richness in autumn (Mosca et al., 2007; De Román and De Miguel, 2005). Relative abundance of EM tips significantly diminished during the second year of assessment, coinciding with a relevant increase of tree defoliation in most stands (Table 1) and a significant increase of the abundance of NV tips (Fig. 2). The ascomycete *C. geophilum* was more represented during the first and driest year accounting for about 80% of the EM tips assessed in spring 2010 than during the second year (Fig. 3). Reported tolerance of *C. geophilum* to drought conditions (di Pietro et al., 2007; Kerner et al., 2012) may explain this shift in species abundance. It also seems that *C. geophilum* can adapt and colonize weakened trees as it has a high presence in declining oak stands (Montecchio et al., 2004; Mosca et al., 2007). *Russula* spp. was represented as a typical species in declining stands (Blaschke, 1994; Montecchio et al., 2004). The basidiomycete *Tomentella* spp. was present in all seasons sampled, with a significant higher representation during summer 2010 (Fig. 3). The presence of *Tomentella* species in other declining oak stands (Mosca et al., 2007) suggest putative

resistance to environmental stresses. *Year* × *seasonal* interactions of the abundance of EM tips may also be conditioned by the *year* × *seasonal* interactions of precipitation, significant here because in spring 2010 rained about 3 times more than in 2009 (209 and 62 mm, respectively). Under non-declining *Q. ilex* trees, Richard et al. (2011) reported Thelephoraceae and Russulaceae morphotypes to be more abundant during autumn and spring, respectively, while De Román and De Miguel (2005) did not find any seasonal variation in the relative abundance of the ectomycorrhizal fungi assessed.

Higher proportion of NV tips in declining than in non-declining trees is consistent with previous literature reporting fine root death as a consequence of decline (Jung et al., 1996; Sánchez et al., 2002; Corcobado et al., 2013a; Camilo-Alves et al., 2013). Differences were maximum during summer 2009 (Fig. 2), coinciding with the period in which trees were more drought-stressed. The influence of tree decline in the seasonal changes of NV tips confirms the first hypothesis of this study. During the second year, non-declining trees and declining trees showed a very similar proportion of NV tips, in comparison to the first year. This change may be explained by (i) the renewal of ectomycorrhizal roots by declining trees, probably implicating a depletion of C reserves (Jönsson, 2006) and a subsequent increased risk of decline (Brunner, 2001), (ii) by the worsening health status of non-declining trees or (iii) by both circumstances.

Declining trees had less proportion of NM tips than non-declining trees, in accordance to Scott et al. (2012). Other studies,

however, reported contrary results (Montecchio et al., 2004), or no relation between the crown status of trees and non-mycorrhizal fine root biomass (Power and Ashmore, 1996). Despite defoliation, declining trees were able to keep a similar proportion of ectomycorrhizal tips than non-declining trees. Previous studies showed no influence of defoliation on ectomycorrhizal abundance (Kuikka et al., 2003; Lancellotti and Franceschini, 2013), although other authors reported an association between defoliated trees and a lower abundance of ectomycorrhizal fungi (Power and Ashmore, 1996; Scott et al., 2012; Ishaq et al., 2013). Defoliation has been linked to a decrease of below ground carbon allocation to which ectomycorrhizal fungi are dependent (Jönsson, 2006), but this effect may be influenced by the defoliation intensity.

If a more advanced class of declining trees had been considered (i.e. crown transparency higher than 35 %, following UNECE, 2010), reported ectomycorrhizal abundance values would probably have been lower. In fact, decline intensity increase during 2010 was accompanied by a reduction of the relative abundance of EM tips. Nevertheless, it still remains unclear whether the impairment of mycorrhizal symbiosis is a cause or a consequence of decline.

Although being *P. cinnamomi* the main cause of tree decline of the sites studied (Corcobado et al., 2013a and b), this pathogen did not influence the relative abundance of any of the descriptors assessed in any of the seasons (non significant $S \times P$ interactions; Tables 2 and 3). In consequence, the first hypothesis stated in the introduction is not confirmed, but this should be taken with caution. It is important to

mention that the eight assessments carried out provided us with a snapshot of a long decline process (Camilo-Alves et al., 2013). The pathogen *P. cinnamomi* requires waterlogged soil to spread and temperatures ranging from 22 to 28 ºC for maximum root damage, conditions that are clearly influenced by seasonality. Soil moisture influences fine root loss caused by *P. cinnamomi*, and under greenhouse conditions *Q. ilex* symptom expression is maximum 7-8 weeks after infection, only when temperatures exceed 25 ºC (unpublished results). Apart of other factors, ectomycorrhizal composition and root flushing have to be considered into this complex equation. It is ignored if pathogen infection could have happened during or after the new roots were created or at rates faster than the ectomycorrhizal colonization and formation (Mukerji and Ciancio, 2007). Necrotic roots would then result invalid to be colonized by ectomycorrhizal fungi or certain ectomycorrhizal tips would be protected from pathogen penetration (Marx, 1972; Branzanti et al., 1999). Differences of seasonal changes in ectomycorrhizal composition between infected and non-infected *Q. ilex* trees were not detected probably because all the previous factors affected sequentially and simultaneously with *P. cinnamomi*.

The only significant *tree decline status* × *P. cinnamomi infection* interaction obtained corresponded with the proportion of NM tips, higher in non-infected non-declining than in infected declining trees. The soil water content may play at least a partial role in this interaction, in the way that if soil humidity is low the proportion of NM tips increases. From results obtained here, the proportion of NM

tips related inversely to soil water content. Non-declining trees showing lower soil water contents than declining trees (Corcobado et al., 2013a) gives support to this inverse relation. Besides, the direct influence of soil moisture in *P. cinnamomi* activity in terms of sporulation and dispersal (Corcobado et al., 2013a) may probably alter the proportion of NM tips.

High-productivity, old-growth forests with soils generally containing a high quantity of organic fractions (litter, humus, decayed wood) are associated with an increased proportion of active EM tips (Harvey et al., 1976), but this does not explain the higher proportion of active EM_T tips in non-declining than in declining stands. More research is desirable to explain this observation.

The low percentages of vital non-mycorrhizal root tips and ectomycorrhizal root tips, comparing to other studies in holm oak woodlands (Richard et al., 2005), suggest that our *Q. ilex* trees were suffering from an acute deterioration process and/or the sites were perturbed. The low percentage of vital root tips susceptible to be colonized by ectomycorrhizal fungi may be directly related to the atypical scarcity of the symbiosis in roots. It remains unclear whether ectomycorrhizal inoculum is scarce or the interaction with *P. cinnamomi* is the major agent affecting the colonization by ectomycorrhizal fungi, or if both circumstances occur.

The morphotype *C. geophilum* was the most abundant EM species, as confirmed by other studies on *Q. ilex* trees (Claveria and De Miguel, 2005) and on *Phytophthora* infected stands (Blom et al., 2009; Scattolin et al., 2012). The abundance of *C. geophilum* may be

explained by either its advantageous competition ability (Koide et al., 2004), especially under warm dry conditions or by the possibility of exerting some antibiosis, as Krywolap et al. (1964) demonstrated for *C. graniforme*. Despite its abundance, it is striking the lack of studies dealing with its role under different environmental stresses, especially on declining oak stands. The influence of the proximity of trees to stream banks to enhance the relative abundance of *C. geophilum* is reported here for the first time. No other EM morphotypes were influenced by topography, contrary to results showed by other studies with oaks (Scattolin et al., 2013; Zhang et al., 2013). Another abundant morphotype, *Russula* spp. have been also identified as a dominant type in *Phytophthora* infected stands (Scattolin et al., 2012) but little is known about its functionality. It is interesting to observe that in *P. cinnamomi* infected trees, other EM morphotypes were more abundant than in non-infected trees, in agreement with the studies of Blom et al. (2009) and Scattolin et al. (2012) related to *P. cambivora* infected trees. This would imply a shift of EM composition in response to *Phytophthora* infection.

The physiological status of trees in terms of $\Psi_{p,}$ g_s and A worsened with decline, as expected. The physiology of trees did not vary with *P. cinnamomi* infections, at least with the crown transparencies of trees selected here, contrary to results reported for *Q. ilex* seedlings (Robin et al., 2001) and for other mature plants (Manter et al., 2007). Physiological parameters were not significantly influenced by the abundance of EM tips but with some morphotypes. The positive role of EM tips in the physiological activity of plants is

well known (Breda et al., 2006), but neutral to negative relationships (e.g. Colpaert et al., 1992; Martin et al., 2003) and the distinctive role of different ectomycorrhizal morphotypes (e.g. Beckjord et al., 1985) have been also reported. Leaf water potential (Ψ_p) and water use efficiency (*iWUE*) increased with the abundance of EM_R and other EM tips. For photochemical efficiency (F_v/F_m) of leaves contradictory results were observed, being this parameter positively related to EM_T tips but negatively to EM_{Cg} tips abundances, and illustrating the complexity of the plant-ectomycorrhizal interactions (Grman and Robinson, 2013).

Relationships between the ectomycorrhizal community and the physiology of *Q. ilex* trees differed with tree decline status, confirming our second hypothesis. Abundances of EM_{Cg} tips related negatively to g_s and A only if trees were declining (Figs. 6a and 6b), and abundances of EM_R tips related negatively or positively depending on the health status (Figs. 6c and 6d). Our study suggests that the low vitality of declining oak trees influences negatively the colonization of roots by EM fungi, what could counteract the positive effects of symbiosis (Jönsson, 2006). The knowledge on this complex symbionts-pathogens relationship in nature has to be further clarified.

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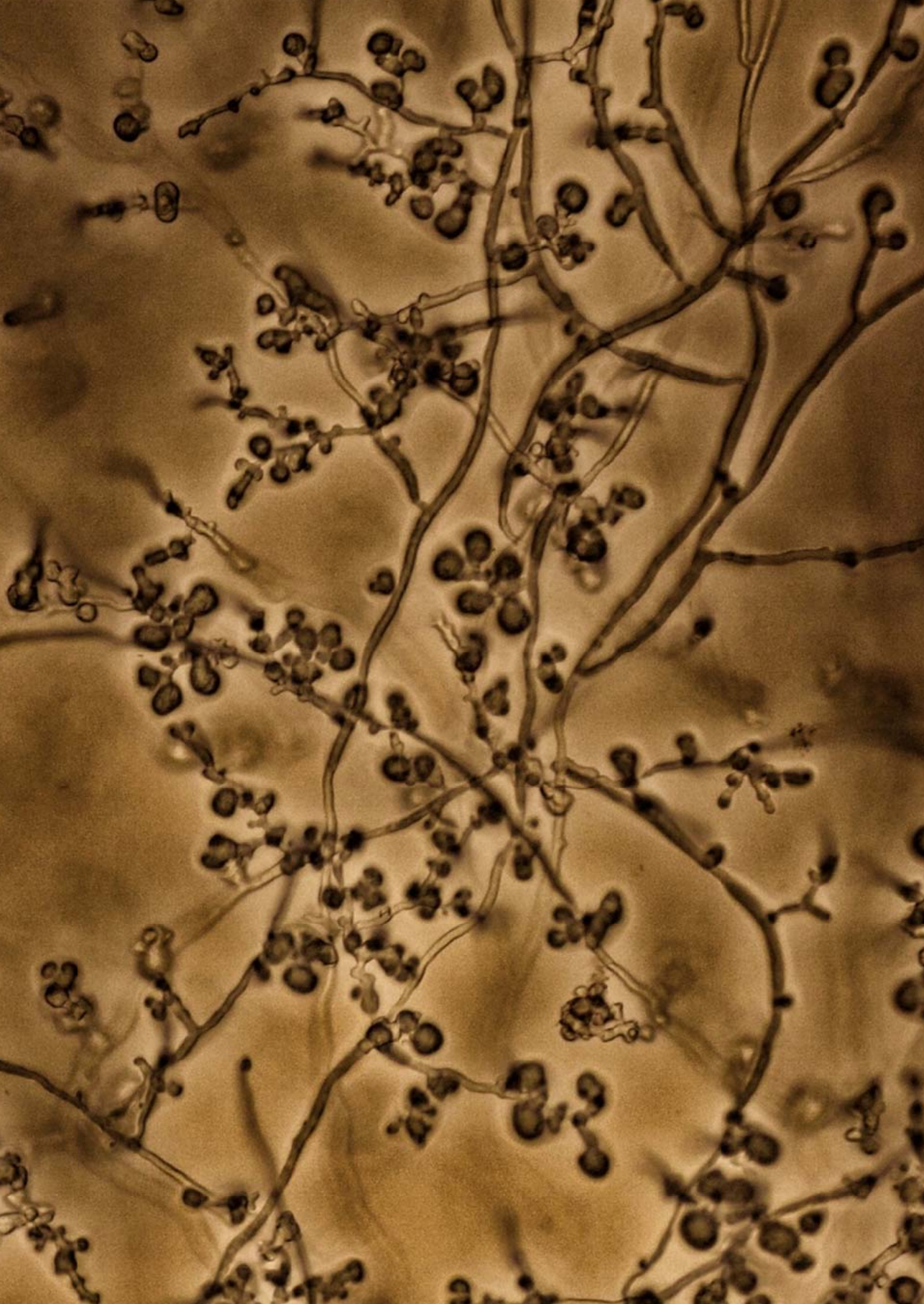

CAPÍTULO 4

Quercus ilex forests are influenced by annual variations in water table, soil water deficit and fine root loss caused by *Phytophthora cinnamomi*

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Abstract
It is hypothesised that major reductions in tree vitality are related to marked changes in soil water content, extremely wet winters followed by dry summers, and the presence of pathogenic organisms which take advantage of this situation. This study helps clarify the role of annual variations in water table, soil water content and fine root abundance in the decline of *Quercus ilex* forests, with special focus on trees growing in *Phytophthora cinnamomi*-infested soils. Conducted in western Spain, the study included 5 *Quercus ilex* dehesa stands in which tree water status and soil water dynamic were compared in declining and non-declining trees, and 96 additional stands in which fine root abundance and pathogen assessment were compared in declining and non-declining trees. Declining trees showed significantly lower values than non-declining trees for leaf water potential and stomatal conductance. The period of waterlogging (2 months maximum, fluctuating from -0.5 to -4.5 m), the soil water content values observed in summer (significantly higher in declining trees) and the similar presence of *Pythium spiculum* in declining and non-declining trees are not sufficient in themselves to explain tree health status. However, fine root density was 16.2 % lower in declining than non-declining trees and 42 % lower in *P. cinnamomi*-infected than non-infected trees. Root damage caused by *P. cinnamomi* in combination with periods of saturated soils favourable for the pathogen but unfavourable for the tree, coupled with small-scale differences in soil water-holding capacity, explain the symptoms and water status of declining trees. The combination of root damage and water stress explained above-ground symptoms of declining trees and will probably determine tree survival.

Keywords: saturated soil; water deficit; oak decline; root density; root-rot disease; open woodland

1. Introduction

Since the 1980s, oak decline has devastated forests in southern Spain and Portugal, primarily affecting the evergreen holm oak (*Quercus ilex*) and cork oak (*Q. suber*) (Brasier et al., 1993; Brasier, 1996; Moreira and Martins, 2005). Iberian oak decline is manifested through several above-ground symptoms such as crown transparency, leaf discoloration and wilting, branch dieback, exudations from the bark and root lesions (Brasier, 1996; Gallego et al., 1999; Sánchez et al., 2002), similar to those observed in declining oak species elsewhere (Jung et al., 2000; Balci et al., 2007, 2010; Kabrick et al., 2008). Mediterranean regions are particularly vulnerable to oak decline processes mainly because of the combined effect of water stress, soil disturbances and widespread distribution of exotic pathogens (Brasier et al., 1993). Trees and pathogens now cope with extreme weather conditions that are becoming more frequent every year (Giorgi and Lionello, 2008). Recent models and field observations confirm major reductions in tree growth and vitality following years with marked changes in soil water content or when extremely dry summers are followed by extremely wet winters and quick drying of soil (Fisher et al., 2011).

The soil-borne pathogen *P. cinnamomi* has been cited as the main biotic factor of oak decline in Spain and Portugal, and is responsible for multiple fine-root infections followed by substantial root girdling and death (Brasier, 1992; Brasier et al., 1993; Sánchez et al., 2002). *Pythium spiculum*, a recently described root rot pathogen of *Q. ilex*, is found in Spanish and Portuguese declining forests as

frequently as *P. cinnamomi* (Romero et al., 2007). At disease centres where no soil pathogen was isolated, water stress has been reported as responsible for oak decline, particularly in shallow, sandy and low water-holding soils (Thomas and Hartmann, 1998; Jung et al., 2000; Peñuelas et al., 2001; Costa et al., 2010) or coinciding with unseasonal or extended periods of drought (Brasier et al., 1993). Both biotic and abiotic causes of oak decline, pathogen's damage and water stress, separately and combined, cause the same symptoms in trees. This complex interaction could admit a further variable to explain the Iberian oak decline aetiology: exposure of trees to waterlogging. Periodic waterlogging has been reported to be particularly favourable for *P. cinnamomi* to colonise and infect woody plants, as this pathogen usually attacks roots via free-swimming zoospores in wet soils after rainfall (Ploetz and Schaffer, 1989; Brasier et al., 1993; Erwin and Ribeiro, 1996; Sánchez et al., 2002, 2005; Davison, 1998, 2011). In greenhouse conditions, continuous flooding has been reported to promote higher levels of root necrosis and foliar symptoms due to *P. cinnamomi* than periodic flooding (Sánchez et al., 2002, 2005; Romero et al., 2007). However, little is known about the combined effects of root rot pathogens, water stress and waterlogging on trees growing in more natural environments.

The dynamic of soil water seems to play a central role in the decline of Iberian oak forests (Brasier et al., 1993; Gallego et al., 1999; Sánchez et al., 2002) but remains little understood. Unanswered questions are: Is oak decline related to extreme variations of the water table depth? Is oak decline related to low soil water content values?

or, is oak decline related to fine root loss? This study answers these questions through two independent but complementary field experiments focusing on *Q. ilex*, the most dominant tree species in European Mediterranean ecosystems and the oak most susceptible to *P. cinnamomi* (Maurel et al., 2001; Sánchez et al., 2005). In relation to these questions, it was hypothesised that (i) declining *Q. ilex* trees are subjected to long periods of waterlogging and extreme fluctuations in the water table level, (ii) a soil water content deficit is directly involved in the decline of *Q. ilex*, and (iii) declining trees have fewer fine roots than non-declining trees because of the presence of root pathogens.

2. Materials and methods

Study area

The study was performed in Extremadura, western Spain, and included 101 declining forest stands located throughout the region. Stands were selected from 420 declining *Q. ilex* forests (Del Pozo, 2006), excluding those infested with *Botryosphaeria corticola*, damaged by *Cerambyx* sp. or mixed with abundant *Q. suber* trees. Stands were characterised by a scattered-tree layer of *Q. ilex* trees (dehesas) and an understory of pasture dominated by annual native species. Most sites were regularly grazed in spring and summer by cattle and in autumn and winter by Iberian pigs. The climate in the area is dry Mediterranean, with average annual rainfall of about 680 mm and rain mainly from December to May. Mean minimum and maximum temperatures are in January (7.4 ºC) and August (28.7 ºC).

Experimental design

Two experiments were performed (see annex II.4). The first included five stands (Table 1) selected at random from stands where *P. cinnamomi* was successfully isolated. It comprised 30 trees in which the water table depth, soil water content and presence of *Phytophthora* spp. were periodically assessed. The second experiment comprised 96 stands with 576 trees, on which root assessments and *P. cinnamomi* isolations were performed once only.

Plant material comprised three non-declining (≤ 10 % crown transparency) and three declining trees (15-35 % crown transparency) per stand, rated 1 and 2 following the oak decline classes defined by Balci et al. (2007). Inside each stand, trees had a scattered distribution to avoid the possibility of non-declining trees sharing parts of their rhizosphere with declining trees. In each stand, two trees were in or close to a stream bank, two were on a mid slope, and two were on a higher slope away from the stream bank. Pairs of trees at each *elevation* factor included one non-declining and one declining tree. The mean percentage of declining trees per stand (incidence) was approximately 57 % and the mean annual tree mortality rate of stands was approximately 5 %. Trees were 6-10 m high, 30-90 cm in trunk diameter at breast height and 7.5-12 m in crown diameter.

Table 1. Main characteristics of the five experimental sites.

Site	Location	Altitude (m a.s.l.)	Geological substrate	Soil type [a]	P [b] (mm)	T [c] (ºC)	Incidence [d] (%)	Mortality [e] (%)
Abadía	40º15'N, 5º57'W	484	Granite	Endo-leptic Cambisol	740	16.7	80	15
Cuartón	39º52'N, 6º02'W	384	Quartzite colluvium	Chromic Luvisol	859	15.7	90	15
Haza	39º50'N, 5º56'W	285	Slate	Epi-leptic Cambisol	916	15.8	65	5
San Esteban	39º58'N, 6º05'W	445	Slate	Epi-leptic Cambisol	803	16.5	55	5
Vegaviana	40º00'N, 6º43'W	272	Tertiary sediments	Distric Regosol	828	16.1	90	25

[a] According to IUSS Working Group WRB (2006)
[b] Mean annual precipitation
[c] Mean annual temperature for the 2-year study period (March 1 2009 to February 28 2011).
[d] Percentage of declining trees on March 1 2009.
[e] Percentage of dead trees on March 1 2009.
for the 2-year study period (March 1 2009 to February 28 2011)

Experiment 1

Variation in the water table depths of the five intensively studied stands was assessed through piezometers installed 3 m downstream from the base of each of the 30 tree trunks. Piezometers consisted of perforated PVC tubes (13 cm diam) inserted to a depth of 6 m after soil drilling. To avoid obstruction, the top of each tube was covered. Water table depths were recorded using a battery-operated water table meter (SI 30, Solinst Ltd., Canada) with a graduated tape and a probe which sounded in contact with water. Measurements were taken monthly from March 2009 to February 2011 on one day or two consecutive days for the five sites.

Soil water content (θ) of the 30 trees was assessed using a portable probe (Diviner 2000, Sentek Technologies, Australia) inserted into PVC tubes (9 cm diam) installed to a depth of 3 m after soil drilling. Tubes (one per tree) were located about 2 m from the piezometers. To ensure the tube was in good contact with the soil and to avoid air pockets, the space between the PVC tubes and the soil (thin film < 0.5 cm) was filled with kaolinite. Soil water content was measured monthly, from March 2009 to February 2011, on one day or two consecutive days for the five sites at depth intervals of 10 cm.

Once a month, from July to September 2009 and 2010, the plant water status of trees was checked. Pre-dawn leaf water potential (Ψ_{pd}) was determined using a Scholander chamber (SKPM 1400, Skye Instruments Ltd., UK) on two terminal current-year twigs per tree collected from the outer mid portion of the crown. Stomatal conductance (g_s) and net leaf photosynthesis (A) were determined

using a portable differential infrared gas analyser (IRGA) (LCi, ADC Bio Scientific Ltd., UK) connected to a broadleaf chamber. Three current-year leaves per tree with exposure to the sun from 9.30 to 11.00 h were analysed.

Soil and root samples were taken from each of the six trees per site. Approximately 300 ml soil and ~25 fine roots per tree were sampled at distances of 1-2 m from the trunks, at depths of 10-50 cm. The presence of *P. cinnamomi* and *Py. spiculum* in the soil samples was assessed following Romero et al. (2007). One millilitre aliquots of a soil–water agar suspension were plated on Petri dishes containing NARPH selective medium (Solla et al., 2009). Thirty plates per tree were used, and after two days of incubation in the dark at 24°C the *Phytophthora* spp. and *Pythium* spp. colonies growing on each plate were counted. Isolation was also attempted from root samples, which were cut into 1 cm segments, surface-sterilised (2 min in 1 % aqueous sodium hypochlorite), rinsed with sterile water, blotted dry and plated onto the selective medium. About nine plates per tree were incubated in the dark at 24 °C, and after 2–3 days, selected isolates were transferred to a carrot agar (CA) medium. Colonies were identified by microscopic observations of distinctive structures such as clustered hyphal swellings (Erwin and Ribeiro, 1996) or other structures (Paul et al., 2006). Sampling was performed every three months, from March 2009 to February 2011.

Experiment 2

Roots of 576 *Q. ilex* trees from 96 stands were assessed through examination of soil pits. One pit per tree was dug using a hydraulic backhoe with a 40 cm-wide scoop. Soil pits were approximately 2.5 m wide and 1.5 m deep (where possible), 3-4 m downstream from the tree trunks, tangentially oriented to the tree crown. A 0.1 m wide transparent grid was laid over the soil profile to count the fine (< 2 mm diam) and coarse roots (≥ 2 mm) every 0.5 m of depth. Root abundance was expressed as the number of roots m^{-2} and root density as the square root of root abundance raised to the third power m^{-3}. Root necrosis was not assessed. For pathogen isolation, soil and root samples were taken under each of the six trees per site, following the procedure described above. Root assessment and pathogen isolation were both performed in spring 2008 and 2009, coinciding with the most successful isolations in experiment 1.

Data analysis

For a more comprehensive analysis, *water table depth* and θ values were grouped into seasons, giving four readings per year. Within seasons, the absence of significant differences in *water table depth* and θ between months and between years was checked. The influence of *water table depth* on oak decline was analysed through a mixed linear model (ANCOVA type) using *water table depth* as the dependent variable, *tree status* and *elevation* as fixed factors, *site* as a random factor, and *season* as a repeated measure. Soil water content values were grouped into 0.5 m depth intervals, square-root

transformed to meet normality assumptions and analysed through a mixed linear model (ANCOVA type) using θ as the dependent variable, *tree status*, *elevation* and *soil depth* as fixed factors, *site* as a random factor and *season* as a repeated measure. Several factorial ANOVAs were performed, considering Ψ, g_s, A, *water table fluctuations* (difference between the maximum and minimum annual values of the water table depths), *fine roots*, *coarse roots* and *soil depth* as dependent variables, *tree status* and *elevation* as fixed factors, and *site* as a random factor. Tukey's tests were applied for multiple comparisons between all pairs of means when significant differences were observed. For the repeated-measure ANCOVAs, the Bonferroni correction was used and significance was adjusted to $p = 0.0062$. This significance level resulted from dividing 0.05 by 8, corresponding to the number of seasons into which *soil water content* and θ were grouped. *P. cinnamomi* and *Py. spiculum* presence was analysed through a generalised linear model assuming a binomial distribution and the logit function. The presence or absence (1/0) of these pathogens (roots or rhizosphere soil) was taken as the dependent variable, and *tree status*, *elevation* and *site* as factors. All analyses except the mixed linear models (SAS) were performed with STATISTICA v.7 software.

3. Results

Values of ψ_{pd}, g_s and A were significantly lower in declining than non-declining trees ($p < 0.05$; Fig. 1). Trees were more stressed in 2009

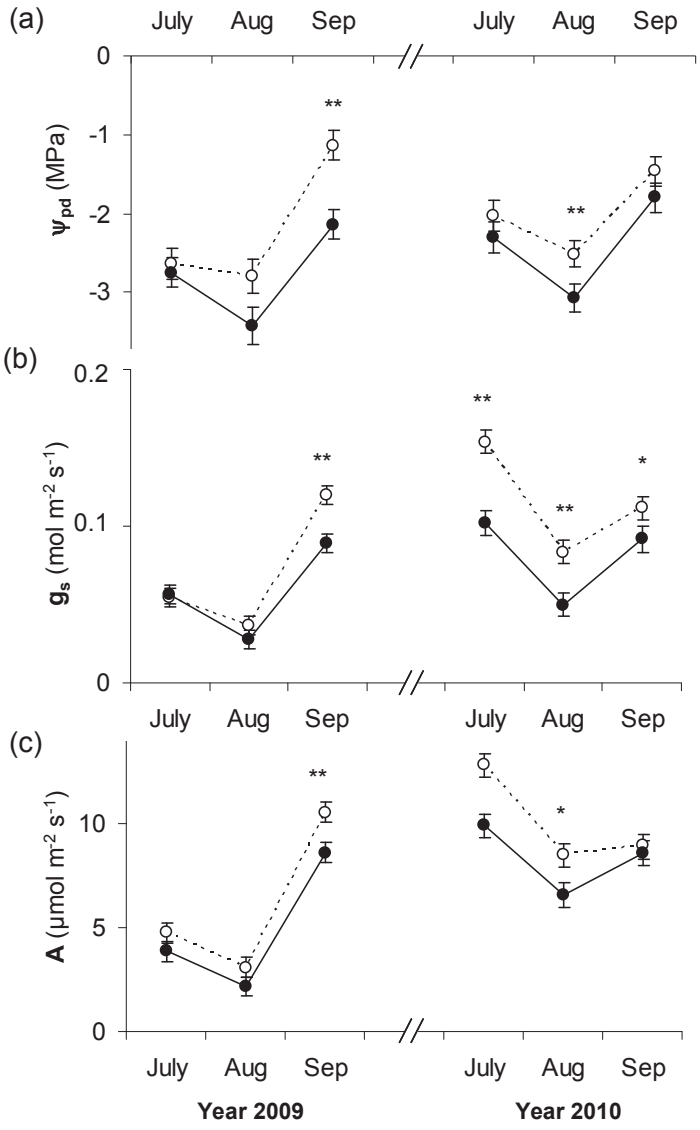


Fig. 1. Time evolution of (a) ψ_{pd}, (b) g_s and (c) A measured in declining (●; solid line) and non-declining (○; dotted line) trees in July, August and September 2009 and 2010 (n = 15 trees). Vertical bars are standard errors, and asterisks indicate significant differences in values within months at $p < 0.05$ (*) and $p < 0.01$ (**).

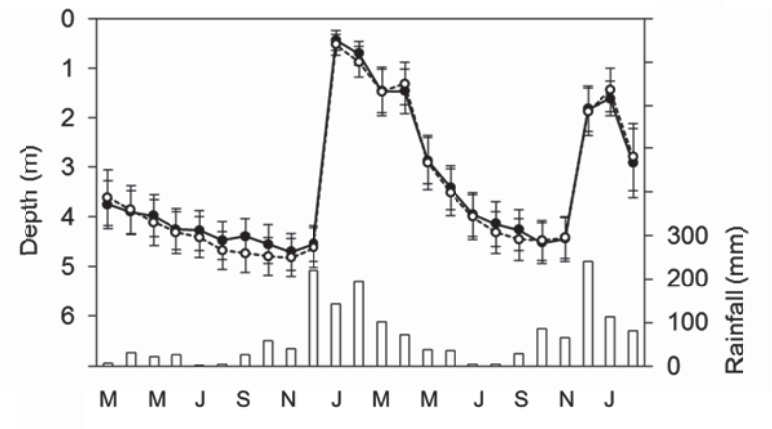


Fig. 2. Mean values of water table for declining (●; solid line) and non-declining (○; dotted line) trees from March 2009 to February 2011. Bars denote standard errors; n = 15 trees.

than in 2010 (p = 0.0003), especially in August ($p \leq 0.0001$; Fig. 1), and values of ψ_{pd} were significantly higher close to stream banks than in mid or upper slope positions (-1.76 ± 0.06, -1.89 ± 0.06 and -2.08 ± 0.05 MPa, respectively; p = 0.0009). Differences in ψ_{pd} between declining and non-declining trees were significantly higher on upper slopes than on mid slopes or close to stream banks (significant *tree status* × *elevation* interaction; p = 0.01; results not shown).

In 2009 and 2010 the water table level showed a typical alternation trend of wet and dry periods, accurately fitting the rainfall dynamic (Fig. 2). During January to February 2010 and during December 2010 to January 2011, the water table was near the soil surface in trees located at the stream banks. The mean water table level of declining trees (3.37 ± 2.07 m) was similar to that of non-declining trees (3.43 ± 2.16 m) and so was the mean SD of the water table values (1.6 ± 0.04 and 1.6 ± 0.04 m, respectively) and the

maximum annual variations of the water table (4.58 ± 0.12 and 4.62 ± 0.13 m, respectively). The water table level in Vegaviana was significantly deeper than in the other four sites (4.27 *vs* 3.18 m, respectively; $p = 0.050$). Moreover, the mean SD and the maximum annual variations of the water table in Vegaviana were significantly higher than in the other four sites (2.28 *vs* 1.44 and 5.72 *vs* 4.24 m, respectively; $p < 0.001$). The mean SD of the water table was significantly higher on upper slopes than on mid slopes or close to stream banks (1.81, 1.59 and 1.44 m, respectively; $p < 0.001$).

Table 2. Effect of *tree status*, *elevation* and *soil depth* factors, *season* (repeated measure) and their interactions (mixed linear model, ANCOVA type) on soil water content measured in *Quercus ilex* trees.

Factors	*F*	Degrees of freedom	*p*-value
Tree status	237.61	1	<0.0001
Elevation	798.09	2	<0.0001
Soil depth	307.71	5	<0.0001
Season	1133.82	3	<0.0001
Tree status × Elevation	281.35	2	<0.0001
Tree status × Soil depth	25.11	5	<0.0001
Tree status × Season	6.14	3	0.0004
Elevation × Soil depth	34.63	10	<0.0001
Elevation × Season	2.49	6	0.0586
Soil depth × Season	86.20	15	<0.0001
Tree status × Elevation x Soil depth	17.54	10	<0.0001
Tree status × Elevation x Season	51.12	6	<0.0001
Tree status × Soil depth x Season	6.34	15	<0.0001
Elevation × Soil depth x Season	2.12	30	0.0249

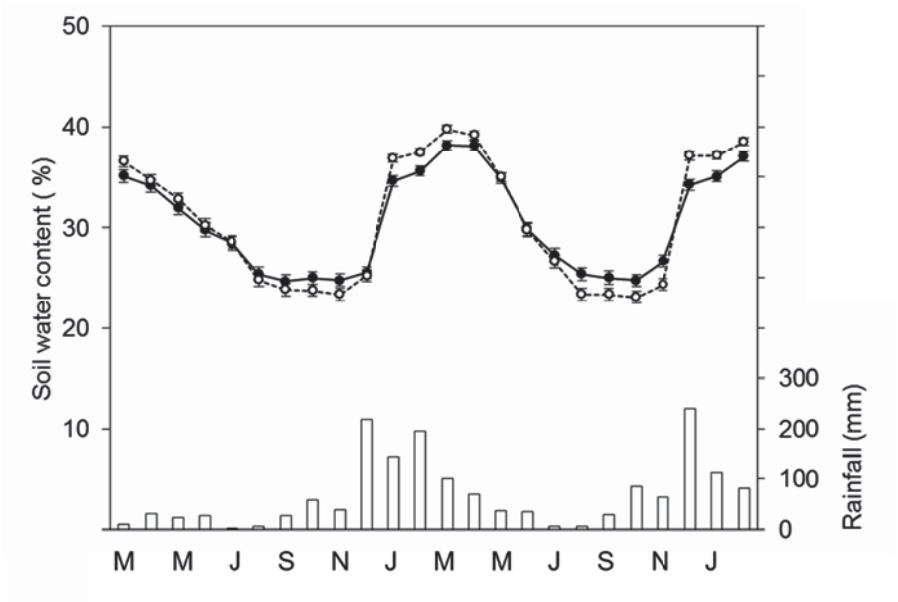


Fig. 3. Mean values of soil water content for declining (●; solid line) and non-declining (○; dotted line) trees from March 2009 to February 2011. Bars denote standard errors; n = 15 trees.

Soil water content (θ) differed slightly, but significantly, between declining and non-declining trees (mean annual values of 30.65 ± 0.11 % and 30.48 ± 0.12 % respectively; $p \leq 0.0001$; Table 2). Interestingly, differences in θ varied significantly with the season (significant *tree status* × *season* interaction; $p = 0.0004$; Table 2). In winter and spring, declining trees had lower θ values than non-declining trees ($p \leq 0.0001$), but in summer and autumn this tendency inverted ($p < 0.002$; Fig. 3). In summer and autumn, soil was significantly drier under non-declining trees than declining trees, particularly in the upper layers, although this was not observed on slopes or close to stream banks, or in winter or spring (significant *tree status* × *elevation* × *season* interaction; $p \leq 0.0001$; Table 2).

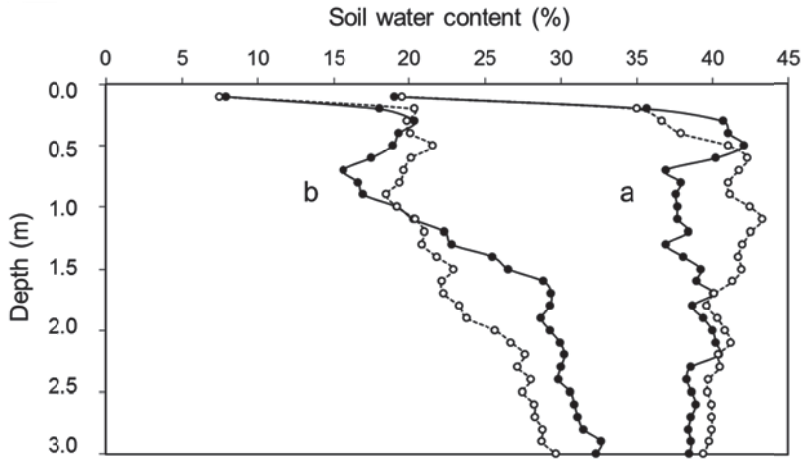


Fig. 4. Soil water content profiles during the wettest (a) and driest (b) months (March and October 2010, respectively) for declining (●) and non-declining (○) trees; n = 15 trees.

Mean θ values increased sharply with depth ($p \leq 0.0001$; Table 2), especially within the first 0.5 m of soil depth (Fig. 4). During the dry seasons (summer and autumn), this observation extended to the whole profile, but in winter and spring, θ was virtually constant as depth increased from 0.5 to 3 m (Fig. 4) (significant *soil depth* × *season* interaction; $p \leq 0.0001$; Table 2). Variations in θ with depth (and with season) differed between declining and non-declining trees (significant *tree status* × *season* × *soil depth* interaction; $p \leq 0.0001$; Table 2). For example, in winter the soil at intermediate depths (0.5-2.0 m) was drier under declining trees than under non-declining trees (p = 0.0004), whereas θ was similar for both groups of trees (p = 0.08) at greater depths (2.0-3.0 m) (Fig. 4). In autumn, soil was drier under declining than non-declining trees at intermediate depths (0.5-

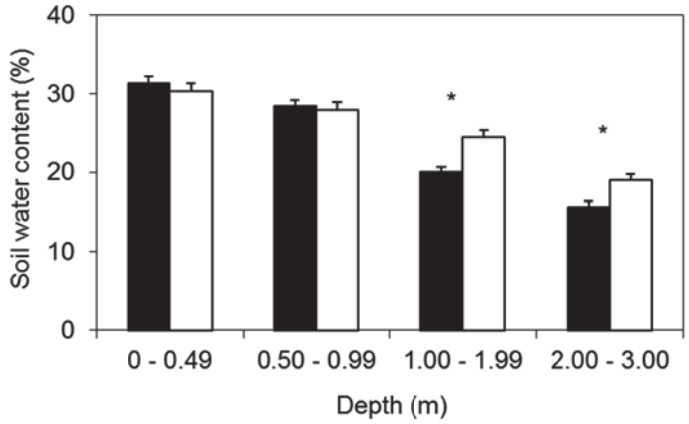


Fig. 5. Annual variation in soil water content (maximum – minimum values) at various depths for declining (■) and non-declining (□) trees. Bars denote standard errors and asterisks indicate significant differences at $p < 0.05$; n = 15 trees.

1.0 m), whereas soil was drier under non-declining than declining trees at greater depths (1.0-3.0 m) ($p \leq 0.0001$) (Fig. 4). Annual variations in θ were higher under non-declining than declining trees ($p = 0.0127$) and varied with depth (Fig. 5).

In experiment 1, *P. cinnamomi* was isolated from samples collected under 13 declining and 11 non-declining trees (n = 15). *Phytophthora gonapodyides* was also isolated from a single declining tree at San Esteban. Isolations were more successful in spring than in autumn or winter and failed in summer. *Pythium spiculum* was isolated under 14 declining and 10 non-declining trees (n = 15), although isolation success was not season-dependent.

In experiment 2, *P. cinnamomi* was isolated from 65 stands and its presence was significantly higher in declining than non-declining trees ($p = 0.008$; Table 3). Isolation success was also higher

for samples collected close to stream banks than for samples collected from mid or upper slope positions ($p < 0.001$; results not shown). *Pythium spiculum* was isolated from 46 stands and its presence was higher in declining than non-declining trees only when samples were collected close to stream banks (significant *tree status* × *elevation* interaction; $p = 0.012$; results not shown). Maximum soil depth and the number of coarse roots observed in the soil profiles were similar in declining and non-declining trees (Table 3), irrespective of the *elevation*. However, fine roots were significantly lower in declining than non-declining trees (73.7 and 82.9 roots m^{-2}, respectively; $p = 0.016$) and significantly lower close to stream banks than in mid or upper slope positions (73.1, 80.5, and 78.5 roots m^{-2} respectively; $p = 0.048$). Fine roots were also significantly lower in *P. cinnamomi*-infected than in *P. cinnamomi*-non-infected trees (59.1 and 84.8 roots

Table 3. Results obtained from assessing 576 *Quercus ilex* trees (Extremadura, SW Spain), half declining and half non-declining for a total number of observations of 288 for each measurement shown in table.

	Declining trees	Non-declining trees	Significance
Trees with *P. cinnamomi* present	90	63	$p = 0.008$
Trees with *Py. spiculum* present	38	31	$p > 0.1$
Maximum soil depth (m)	1.18	1.23	$p > 0.1$
Abundance or coarse roots (roots m^{-2})	7.9	6.7	$p > 0.1$
Abundance of fine roots (roots m^{-2})	73.7	82.9	$p = 0.008$

m^{-2}, respectively; $p < 0.001$), but similar between *Py. spiculum*-infected and *Py. spiculum*-non-infected trees (80.9 and 77.3 roots m^{-2}, respectively; $p = 0.6$).

4. Discussion

In answer to the first hypothesis, it cannot be concluded from experiment 1 that Iberian *Q. ilex* decline is directly related to long periods of waterlogging nor to extreme variations in the water table depth. In all five sites studied, annual variations of water table depth in declining trees were similar to variations in non-declining trees. Other field studies reported severe decline of oaks in sites suffering from high water table fluctuations (Oosterbaan and Nabuurs, 1991; Solla et al., 2009), similar as the one reported here in Vegaviana site. The effect of water table annual variations on tree health will indirectly depend on how long roots are exposed to conditions of anoxia, and whether lethal micro-organisms are favoured by annual variations. Anoxia inhibits tree growth, especially the roots, and also alters metabolism and various physiological parameters (Davison, 1988; Parent et al., 2008). After four weeks, continuous waterlogging of *P. cinnamomi*-infested soil causes 100 % mortality of *Q. ilex* seedlings (Sánchez et al., 2005). At the five sites studied here and assuming sinker roots growing deeper than 5 m as reported for *Q. ilex* in nearby stands (Moreno et al., 2005), about one third of the roots would have been waterlogged for four months in 2010 and two months in 2011. Results confirm *P. cinnamomi* had optimal conditions for root damage, but additional measurements such as soil redox

potential and oxygen diffusion rate (Sojka, 1992) would have provided better data on possible root damaging processes directly associated with waterlogging (Parent et al., 2008).

During the dry period, root water uptake relies on the deeper soil horizons and on the roots with direct access to the groundwater (David et al., 2004), and damage of the deepest roots due to prolonged waterlogging or *Phytophthora* spp. infestation will make it difficult for roots to obtain water. In disease centres in *Banksia* woodlands, viable inoculum of *P. cinnamomi* was found in groundwater 3–5 m below the soil surface (Shearer et al., 2010). It has been suggested that destruction of the deep sinker roots by *P. cinnamomi* has greater effects on tree water relations in summer than the loss of shallow roots (Crombie et al., 1987). This circumstance will be critical for *Q. ilex*, especially if drought continues into November or December (Fig. 2). When the water table is deep and out of the reach of roots, trees will survive only if θ is sufficiently high to allow water absorption by the roots. If θ is not high enough, sudden death will probably occur. It is presumed that the severe and rapid mortality observed in Vegaviana was related to this event.

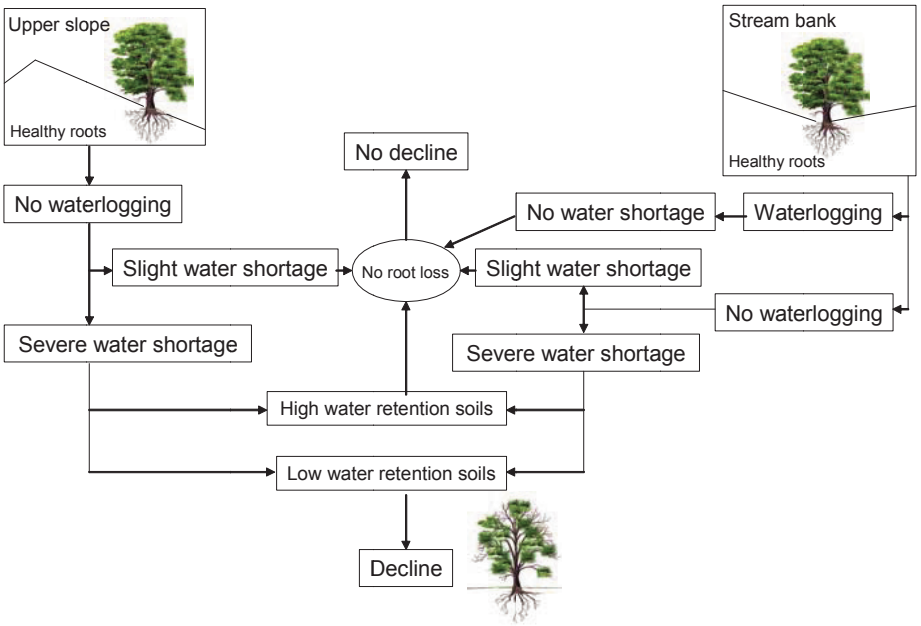
Under waterlogging, rapid long-distance dispersal of water-borne zoospores of *Phytophthora* occurs (Erwin and Ribeiro, 1996; Davison, 1988, 2011) and the effect of successive episodes of pathogen dispersal may be cumulative. Prolonged and repeated waterlogging, in addition to temperatures above 25 ºC, e.g. in April 2010, would explain the widespread distribution of *P. cinnamomi* observed in experiment 1, which was similar under declining and non-

declining trees, and the detection of *P. gonapodyides* (Corcobado et al., 2010), commonly present in streams or waterlogged sites. Seasonal variation in *P. cinnamomi* presence was also reported for *E. marginata* forests in Australia (Shearer and Shea, 1987) and *Q. alba* forests in southern Ohio (Balci et al., 2010), where recoveries decreased as rainfall dropped.

Is Iberian oak decline directly related to low θ values? The θ values observed here do not usually give rise to sufficient water stress to cause tree mortality (Fig. 6a). During 5-6-month drought periods in non-infested areas similar to the sites studied here, *Q. ilex* trees faced θ values of around 10 % (weight) at 1-2 m soil depth and ψ_{pd} values ranging from -0.3 to -1.0 MPa (Cubera and Moreno, 2007; David et al., 2007). The second hypothesis is invalidated by the higher θ values in declining than non-declining trees. This interpretation should be taken with caution, as discussed below, as declining trees had about 10 % fewer fine roots per square meter than non-declining trees. If fine root data were expressed in density (i.e. per cubic meter), declining trees would have 16.2 % fewer fine roots than non-declining trees.

In non-declining trees, θ values were higher close to stream banks than on upper slopes. In declining trees, however, θ was higher in trees on upper slopes than close to stream banks. Nagle et al. (2010)

(a) Mediterranean climate, absence of *Phytophthora* and *Pythium*



(b) Mediterranean climate, presence of *Phytophthora* and *Pythium*

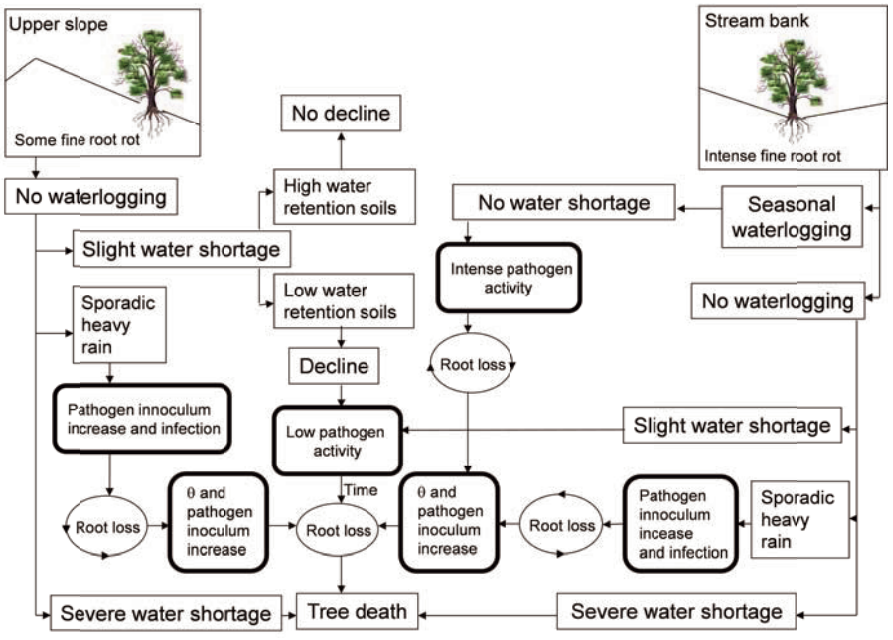


Fig. 6. Conceptual diagram of circumstances occurring in the Iberian Peninsula to *Quercus ilex* trees if root rot pathogens were absent (a) or present (b) in the soil. Under the first scenario (a), only severe water shortage of low water retention soils may originate oak decline. Under the second scenario (b), slight water shortage in low water retention soils at upper slopes, and slight water shortage at stream banks may originate decline. Tree death would be preceded by successive cycles of root loss, lower water consumption, increase of soil water content (θ), increase of inoculum, and again root loss.

found the same trend of increased soil moisture with elevation when declining and non-declining stands were compared. We hypothesise that the root density observed on upper slopes, 10 % higher than the root density observed close to stream banks, would explain this difference. This assumption could also explain the higher annual variation in θ observed in non-declining than declining trees. Differences in annual variations of θ are attributed to more active use of water by non-declining trees, particularly below 1 m, where the root system is more developed (Moreno et al., 2005). The higher values of θ observed in declining trees, in comparison to non-declining tress, may occur because of the reduction of the transpiration rates (increase of stomatal closure) of the declining trees due to fine root loss. Under greenhouse conditions, it was observed that θ of *Phytophthora*-inoculated *Q. ilex* seedlings remained high even under water shortage treatment, which was explained by the root damage caused by the pathogen (Maurel et al., 2001). In our forests, the lack of *Q. ilex* roots had a greater effect on θ than the topography, and results provide a good example of how a micro-organism indirectly changes θ around the rhizosphere. The impact of this

heterogeneity on the ecosystem, in combination with other sources of variability (Cubera et al., 2009, 2012), deserves further research, but the higher θ values occurring during the vegetative period beneath declining *Q. ilex* trees will probably favour *P. cinnamomi* more than the tree. Possible feedback consisting of root damage, lower water consumption, increased θ values, favourable conditions for *P. cinnamomi* (in terms of survival, inoculum increase and infection) and root damage is likely to occur (Fig. 6b). The creation of dynamic spatiotemporal niche refuges favourable to the pathogen through this type of feedback concurs with the pattern of *P. cinnamomi* inoculum occurrence observed in Western Australia disease centres (Shearer et al., 2010).

Reductions in g_s have been reported for artificially *P. cinnamomi*-infected oak seedlings (Luque et al., 1999; Maurel et al., 2001) and attributed to a pathogen-mediated hormonal imbalance. Cytokinins are synthesised in root tips, so diseased plants with fewer healthy root tips than uninfected plants are likely to have reduced cytokinin levels in the xylem (Cahill et al., 1986). This will trigger an increase in abscisic acid (ABA) concentration in buds and leaves, resulting in the typical droughting response of stomatal closure (Davison, 2011). A decrease of A in *Phytophthora*-infected seedlings has also been reported (Ploetz and Schaffer, 1989) and linked to the damage caused by the pathogen directly on the root system of plants or the release of phytotoxic peptides (elicitins) by the pathogen, which indirectly impaired plant photosynthesis (Fleischmann et al., 2005; Manter et al., 2007). Other studies reported similar effects of

Phytophthora spp. on the water relations of woody plants (Crombie et al., 1987; Crombie and Tippett, 1990).

Results from the extensive survey in experiment 2 support the hypothesis of fewer fine roots in declining than non-declining trees because of damage caused by *P. cinnamomi.* Earlier research reporting substantial fine root damage in oaks due to the presence of *Phytophthora* spp. in the forest (Jung et al., 2000; Balci et al., 2007, 2010) or after inoculations with *P. cinnamomi* in the greenhouse (Gallego et al., 1999; Maurel et al., 2001; Sánchez et al., 2005; Balci et al., 2008; Nagle et al., 2010) confirms our results. Under greenhouse conditions, the necessary root loss rate in a particular oak seedling to cause mortality was more than 60 % (Maurel et al., 2001; Balci et al., 2008). Because roots were not separated on the basis of infected or healthy by visual evaluation, the inclusion of infected or dead roots during the field survey certainly resulted in an underestimation of the degree of fine root loss. Moreover, if a more advanced class of declining trees had been considered (i.e. class 3 with crown transparency of 35-55 %, following Balci et al. (2007), reported root loss values would probably have been higher. A root abundance loss of 30 % in the *P. cinnamomi*-infected trees compared with non-infected trees (42 % when expressed as root density loss) may contribute considerably to the impairment of tree water relations, as fine roots host mycorrhizae and are the root fraction with the highest water uptake efficiency (Thomas and Hartmann, 1998). If combined with soil water shortage, moderate levels of root loss would severely affect tree water relations (Crombie et al., 1987; Robin et al.,

2001) and also play a critical role in tree survival, as suggested by Maurel et al. (2001).

In February 2012, two declining *Q. ilex* trees close to stream banks and two declining trees from slopes were recorded as dead. Before death, the declining trees probably coped with the following circumstances: (i) intense root rot combined with a slight water shortage and (ii) chronic and slight root rot combined with a severe water shortage. The first circumstance probably occurred close to stream banks, where soil is moist and waterlogging increases the severity of *P. cinnamomi* (Ploetz and Schaffer, 1989; Brasier et al., 1993; Robin et al., 2001; Sánchez et al., 2002, 2005; Balci et al., 2010). The second circumstance probably occurred in mid to upper slope positions, where *Phytophthora* inoculum is less abundant (Shearer et al., 2010), θ and water-holding capacity are usually lower, and deep water is further from the roots. Both scenarios (Fig. 6b) have been described for other declining oak forests (Kabrick et al., 2008; Costa et al., 2010) and explain why severe *Q. ilex* mortality has been reported simultaneously at the bottom of valleys and on slopes (Brasier et al., 1993; Sánchez et al., 2002; Moreira and Martins, 2005; Del Pozo, 2006).

Results reported here show that in winter, soils of non-declining trees were able to retain more water than declining trees (Figs. 3 and 4). The mixed distribution of both types of trees within the sites reflects a clear small-scale heterogeneity of soil water-holding capacities which influences tree health status. In consequence, declining *Q. ilex* forests in Spain and Portugal are likely the result of

trees, mostly infected, that will survive depending primarily on the amount of fine roots available, the soil water-holding capacity and the θ values to allow adequate water uptake. Tree survival will also depend on the strategies of each tree genotype to resist current or new *Phytophthora* infections, replace the fine roots damaged and tolerate drought (T. Jung, personal communication).

5. Conclusions

The present study provides circumstantial evidence that supports an association between the presence of *P. cinnamomi*, fine root loss and decline of *Q. ilex* trees. The association of *Py. spiculum* with Iberian oak decline was, however, not conclusive evidence of a cause-and-effect relationship. A time lapse between a variety of biotic and abiotic factors may have obscured the relationship between *Py. spiculum* and the observation of above-ground oak decline symptoms. Soil water content values detected in summer in *Q. ilex* trees in SW Spain are not low enough to induce holm oak decline or to explain the low ψ_{pd} values observed. However, if trees have a 16.2 % reduction in fine root density (42 % if *P. cinnamomi* was present), it is presumed that θ deficits in soils with low water-holding capacity would cause oak decline. Root loss, more severe close to stream banks than on upper slopes, significantly altered θ values. A feedback loop consisting of root damage, lower water consumption, increased θ values, favourable conditions for *P. cinnamomi* and again root damage is likely to occur (Fig. 6b). Finally, the combination of root damage and water stress will explain above-ground symptoms of declining

trees and determine tree survival. In the context of current spreading of *Phytophthora* spp. and unseasonal heavy rains and droughts in Spain and Portugal, increased *Q. ilex* decline is expected.

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CAPÍTULO 5

Drought events determine performance of *Quercus ilex* seedlings and increase subsequent susceptibility to *Phytophthora cinnamomi*.

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Abstract
More frequent weather extremes are expected to occur in the Mediterranean region within the present climate change context. These extremes may affect forests and plant diseases driven by pathogens. It is hypothesized that simulation of weather extremes during *Quercus ilex* growth will influence early performance and susceptibility to the pathogen *Phytophthora cinnamomi*. In 2010, 140 *Q. ilex* seedlings were submitted to three watering regimes under greenhouse conditions: waterlogging (W), water stress (S), and optimal watering regime for growth (C). During the second vegetative period, conditions were exchanged and the following scenarios were provided: WW, WS, SS, SW and CC. After the second vegetative period, plants were artificially infested with *P. cinnamomi*. Plant growth (stem height, number of leaves and aerial biomass), root system (root biomass, fine-to-total root ratio, root-to-shoot ratio and fine root-to-leaves ratio), physiological parameters (stomata conductance and rates of CO_2 assimilation) and mortality were assessed. In 2010, seedlings under W and S treatments showed lower values of physiological parameters and growth than seedlings under C. In 2011, SW plants had the lowest values in terms of physiology and number of leaves. Fine-to-total root ratio decreased for WS and SW plants. In 2010, the highest mortality occurred in W plants. In 2011, plants under S treatment in 2010 had the highest mortality rates while the lowest rates occurred in plants under CC and WW treatments. After *P. cinnamomi* infestations, maximum mortality rates were observed in plants under S conditions in 2011 (WS and SS) while minimum rates were found in CC, WW and SW plants. It appears that *Q. ilex* at seedling stage is more sensitive to prolonged drought events and this scenario increases its vulnerability to subsequent *P. cinnamomi* infestations.

Keywords: climate change; weather extremes; drought; flooding; oak decline; root rot pathogen

1. Introduction

Impacts of recent climate variability have been well illustrated with shifts in phenology, morphology, genetic frequencies, abundance and range of wild biota moving poleward or upward (Root et al. 2003; García-Mozo et al., 2010; Parmesan, 2006). Direct harm to biota caused by extreme climatic events has been also reported (Lloret et al., 2004). Considering the modern climate change scenarios predicted for the next century, novel environments will be expected with the subsequent difficulty to anticipate how species will respond to these changes (Visser, 2008; Fitzpatrick and Hargrove, 2009). Forests are susceptible to climate change, especially to climate extremes, as the long life-span of trees hamper their quick adaptation to these variations (Lindner et al., 2010). Plant diseases driven by pathogens are expected to exacerbate their damage with global warming, aggravating the negative effects of climate change on forests (Santini et al., 2013). Pathogen distribution and development is especially limited by temperature for overwintering or oversummering (Garret et al., 2006), but with higher winter temperatures under climate change, a range expansion is expected and latency stages may be reduced. In some cases, a higher occurrence of extreme events would allow them to increase their number of cycles per year. The Mediterranean region, already prone to extreme climatic events, is particularly considered a vulnerable area to global change according to climate model simulations, which project consistently a pronounced drying and warming (Giorgi and Lionello, 2008; Loarie et al., 2009) and more frequent extreme events (Lindner et al., 2010). In this region, the

soilborne pathogen *Phytophthora cinnamomi* is the main biotic factor involved in oak decline (Brasier, 1996), and new records of other *Phytophthora* species are regarded to oak decline recently (Corcobado et al., 2010; Pérez-Sierra et al., 2013). As a pathogen dependent of water and warm temperatures, warming together with weather extremes may probably enhance its development.

Tree resistance to drought and other disturbances can be enhanced during the seedling stage. In more mature phases, seedlings submitted to drought hardening have shown to acquire increased tolerance to water stress (Bañón et al., 2006; Villar-Salvador et al., 2012). Plant performance is also affected as a response to hardening practices showing increased root and shoot growth or a decreased shoot-to-root ratio (van den Driessche, 1992). The increased duration, intensity and frequency of drought events expected in Mediterranean areas could contribute to the hardening of the plant, as other studies have tested in relation to frost (Thorsen and Höglind, 2013). Possible implications would be associated to greater drought hardening stages and later dehardening, and improved plant performance (e.g. a better control of water loss or increased root regeneration) (Grossnickle, 2012). Such ameliorated plant performance and hardening could probably lead to acquire tolerance to other stressful factors such as root infections by *P. cinnamomi*, which causes symptoms and physiological disturbances similar to those of drought.

Upon a first infection by pathogens, it has been proved that plants can react with different cellular defence responses to subsequent infections. Cellular defense responses can be also induced

not only by biotic but also by abiotic agents (synthetic chemicals and plant extracts, heat shock, pH, salinity and proton stress) as demonstrated by many studies (Wiese et al., 2004; Walters et al., 2005). Water deficit is a common phenomenon in Mediterranean areas but its role as an inducer has been seldom reported (Kaya et al., 2006; Sun et al., 2010). Within a climate change context, the occurrence of more frequent drought and flooding phenomena make necessary the study of these elements as inducers of resistance. *Quercus ilex* is one of the most representative species in the Mediterranean region and its susceptibility to climate extremes followed by *P. cinnamomi* infections is ignored. It is hypothesized that simulation of weather extremes during *Q. ilex* seedling growth will influence early performance and susceptibility to *P. cinnamomi*. In this study, *Q. ilex* seedlings were exposed to changing watering conditions which reproduce weather extremes and then, infested with *P. cinnamomi*. Phenological and physiological assessments were accomplished within the goal of giving some elucidations to the following questions: (i) are *Q. ilex* seedlings able to adapt to prolonged drought and/or waterlogging events through growth and physiological adjustments?, (ii) which extreme events or alternation of extreme events are linked to higher mortality rates in *Q. ilex* seedlings?, (iii) will these extreme events affect seedlings responses to subsequent infections with *P. cinnamomi*?

2. Materials and methods

During their first vegetative period, *Q. ilex* seedlings were exposed to three scenarios of growth: waterlogging (W), water stress (S), and optimal watering regime for growth (C). The W and S watering regimes simulated an extreme precipitation event versus an extreme Mediterranean summer drought, respectively. During the second vegetative period, conditions were exchanged and the following WW, SS, WS, SW and CC scenarios were provided (Fig. 1). After the second vegetative period, all seedlings were artificially infested with *P. cinnamomi* (Fig. 1 and see annex II.5).

Plant material

The plant material originated from a *Q. ilex* savannah-like woodland (Malpartida de Plasencia, south-western Spain, 39º07'N, 7º29'W; 314 m asl), 300 m adjacent to a centre of declining trees (Corcobado et al., 2010). In November 2009, acorns of a single *Q. ilex* tree were collected and stored for three months in a cold chamber at 4º C. In February 2010, the acorns were germinated in trays with a mixture of peat and vermiculite (3:1) and watered near field capacity. A week later, acorns with emergent radicles were individually planted into 140 cylindrical PVC pots (approximately 16 L volume; 1.50 m high, 11.5 cm inner diameter; Fig. 2a) and packed with sand and peat (1:1, pH 5.5). Previous research carried out by Cubera et al. (2009) showed that the size of the pot used would provide seedlings with unrestricted root growth during the experiment. To avoid water loss, pots submitted to

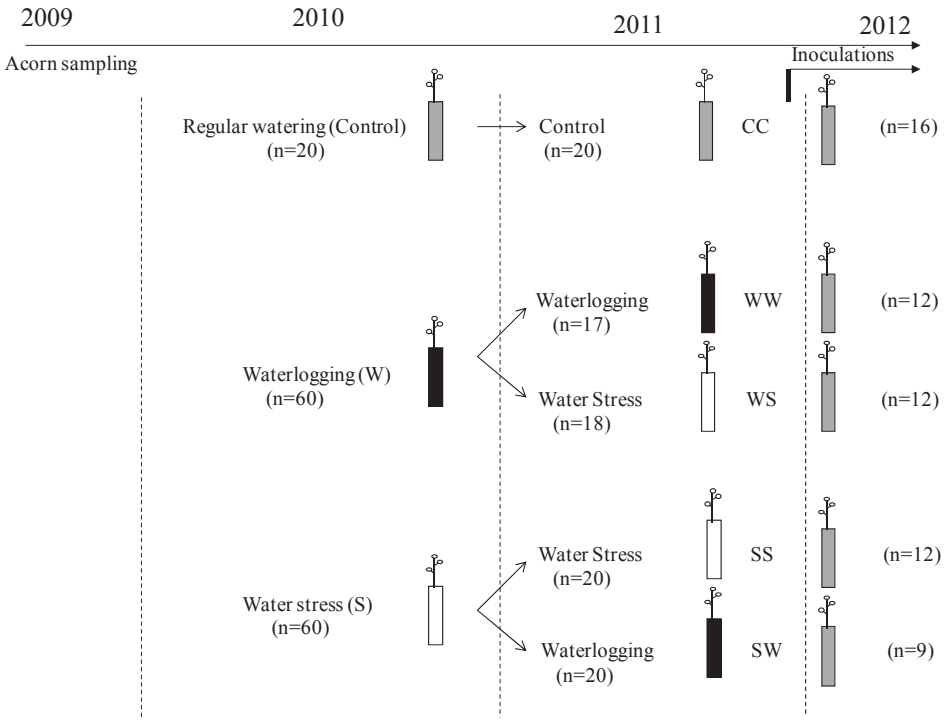

Fig. 1. Schematic representation of the experimental design showing the watering treatments applied to *Quercus ilex* seedlings in 2010 and 2011, and followed by the plant soil infestation with *Phytophthora cinnamomi* before 2012.

W were carefully sealed with long plastic bags, 150 cm in length. To avoid warming by the sun, the pots were painted in white colour. A plastic mesh was placed at the bottom of the S pots to prevent substrate movement and to facilitate, water draining and root air pruning. All pots were kept in natural daylight under a greenhouse shade that reduced solar radiation by 50 % at the Centro Universitario de Plasencia, Cáceres (40º02'N, 6º05'W; 374 m asl) (Fig. 2b).

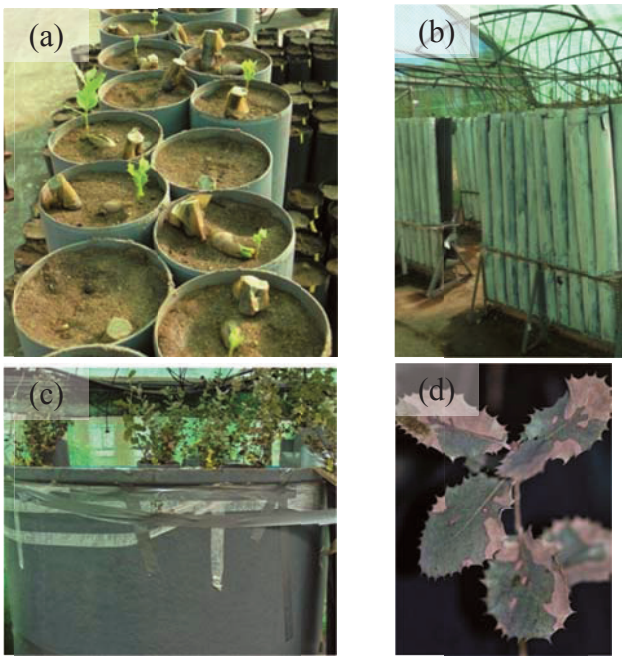

Fig. 2. Watering treatments experiment; (a**)** seedlings germination in pots; (b) watering treatments application to plants; (c) container with PVC tubes containing *Quercus ilex* seedling infested with *Phytophthora cinnamomi*; (d) above-ground symptoms of a *Q. ilex* seedling infected with *P. cinnamomi*.

Watering treatments

All pots were initially watered by hand to field capacity every 3 or 4 days until plants were well established. In May 2010, once seedlings had emerged after three months of growth, a drip-irrigation system was installed and pots were divided into W, S and C groups according to the three watering treatments (Fig. 2b). Seedlings submitted to different watering regime scenarios were distributed at random. Sixty pots were kept waterlogged during 2.5 months (W regime), 60 pots were not watered in 2.5 months except in the middle of August

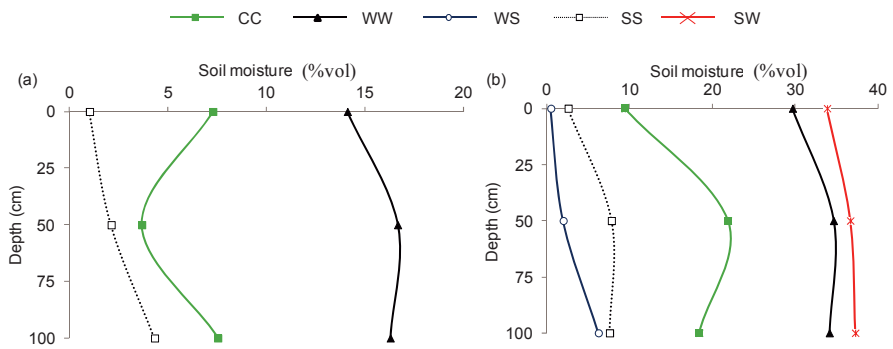

Fig. 3. Mean values of soil moisture under plants submitted to (a) 2010 and (b) 2011 watering treatments.

a single day with 2,000 mL (S regime), and 20 pots were watered with 200 mL (C regime) every two days. In May 2011, some plants subjected to waterlogging and water stress treatments changed into water stress and waterlogging treatments, respectively, and the following five treatments were established: CC, WW, WS, SW and SS ($n \geq 17$) during 2.5 months. Soil moisture was checked in all pots at the end of both 2010 and 2011 watering treatments at 0.50 and 100 cm depths with a Delta-Theta ML2X probe, by means of bores practiced in the wall of the PVC pots. Measurements confirmed differences between watering regimes (Fig. 3a and 3b). Increased values of soil moisture were observed under seedlings submitted to W and SW treatment in 2010 and 2011 than under seedlings submitted to other treatments ($F = 192.33$, $p > 0.001$ and $F = 135.72$, $p > 0.001$).

P. cinnamomi inoculum preparation and soil infestation

The *P. cinnamomi* strain used in the experiment was isolated from Badajoz during the survey performed by Corcobado et al. (2013).

Inoculum was prepared following the procedure of Jung et al. (1996), mixing and twice-autoclaving 455 cm^3 of fine vermiculite, 36.4 cm^3 of whole oat-grains and 318.54 ml of multivitamin juice broth (200 ml/l juice, 800 ml/l demineralized water amended with 3g/l $CaCO_3$) in 1l Erlenmeyer flasks. Then, individual pieces of *P. cinnamomi* plugs were added to the Erlenmeyer flasks containing the medium, and kept five weeks in the incubator at 20 ºC. Control flasks containing the same medium without *P. cinnamomi* isolates were also incubated. Before initiating the soil infestation process, the inoculum was rinsed with demineralized water to get rid of excess of nutrients.

The soil infestation process was carried out in October 2011 once *Q. ilex* plants were about 2 years old. Seedlings were carefully transplanted into bigger PVC tubes (15.5 cm in diameter) containing 25 L of substrate mixed at 2 % with the inoculum or with the uninfested medium (control tube). Tubes were introduced into a huge container (2000 L) to allow complete flooding. After transplanting, seedlings received a normal drainage and the next day were flooded. Seedlings were subjected to regular watering and waterlogged every 3 weeks for 72 hours until spring 2012 to stimulate *P. cinnamomi* sporulation and zoospore release.

In October 2012 *P. cinnamomi* was successfully reisolated from root samples collected from the artificially infested soil. Rootlets were cut into 1 cm segments, surface-sterilised (2 min in 1 % aqueous sodium hypochlorite), rinsed with sterile water, blotted dry and plated onto the selective medium. About 15 plates per tube were incubated in the dark at 24 ºC, and after 2–3 days selected isolates were transferred

to a carrot agar (CA) medium. Colonies were identified by microscopic observations of distinctive structures such as clustered hyphal swellings, chlamydospores and sporangia.

Plant measurements

In February 2010 before germination, acorns were individually weighed. Plant survival was registered monthly but, after the second vegetative period, a weekly monitoring of mortality was performed. Seedlings were recorded as dead when they had lost all their leaves, did not have any green and flexible leaves or when exhibited loss of stem flexibility (Valladares and Sánchez-Gómez, 2006). Plant height and number of leaves were assessed at the end of both 2010 and 2011. In August 2011 a subsample of plants were used for above and below-ground assessment. Four plants per treatment, all together 20 plants, were removed from the tubes, their shoots cut at the cotyledon insertion point, and shoot weight and length, number of leaves and leaf weight assessed. For the root system assessment, tubes were opened with a radial saw, and the cylinders of soil containing the plants were extracted and cut into six sections. Fine roots and coarse roots were collected with the help of a sieve and forceps and weighted. Stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) and net leaf photosynthesis (A, μmol CO_2 m^{-2} s^{-1}) were determined on approximately eight plants per treatment in summer 2010 and 2011 using a portable differential infrared gas analyser (IRGA) (LCi, ADC Bio Scientific Ltd., UK) connected to a broadleaf chamber. Gas exchange was measured from 9.30 to 11.00 h in one current-year leaf

per plant at saturating light (1500 mmol m^{-2} s^{-1}) with a red/blue light emitting diode (LC pro, ADC).

Data analysis

To assess the influence of watering treatments on physiological (g_s and A), growth (stem height, number of leaves and annual aerial growth) and root system (root biomass, fine-to-total root ratio, root-to-shoot ratio and fine root-to-leaves ratio) parameters, one-way ANOVAs were performed using each *physiological* and *growth* parameters as the dependant variable and *2010* or *2011 watering treatments* as the fixed factor. To analyse the relation between the parameters mentioned above, Pearson correlations were performed. To analyse how watering regimes affected the relationship between growth rates of successive years, homogeneity of slopes test was performed.

The final date of the experiment was the 48th week after the *P. cinnamomi* infestation date, coinciding with the beginning of autumn 2012, although some seedlings remained alive. To study time-to-death data (survival time) and determine survival probabilities of the seedlings submitted to the different watering regimes and the seedlings infested with *P. cinnamomi*, the Kaplan-Meier estimate was used in both cases. This survival analysis technique is a non-parametric procedure in which seedlings that were alive at the end of the experiment were considered censored since their time-to-death was unknown (Solla et al., 2011). To test the influence of acorn size, plant size or plant growth rate on seedling survival, generalized linear

models were performed using *mortality* (parameterized as 0 or 1 if the seedling was alive or dead, respectively) after the watering treatments or after *P. cinnamomi* infestations as the dependant variable, *2011 watering treatments* as the fixed factor and *stem height, stem growth* or *acorn weight* as covariates. The Likelihood type I test was used within this model. Normality and homoscedasticity of the data were checked by Kolmogorov-Smirnoff and Bartlett's tests. All analyses were performed with STATISTICA v.10 software.

3. Results

In 2010, seedlings submitted to W and S treatments showed marginally lower stomata conductance (g_s) than seedlings under C (F = 2.70, p = 0.079). Rates of CO_2 assimilation (A) were significantly lower in seedlings under W and S comparing to C treatments (3.1 ± 0.4, 3.4 ± 0.4 and 4.7 ± 0.4 μmol CO_2 m^{-2} $s^{-}1$, respectively; F = 4.77, p = 0.014). Mean stem height was reduced by 26.3 and 23.7 % in seedlings under S and W comparing to C treatments (F = 9.21, p < 0.001). Similarly, the number of leaves in seedlings subjected to S and W treatments was reduced by 19 and 14.5 % in contrast with seedlings under C treatment (F = 3.56, p = 0.031).

In 2011, higher values of g_s and A were observed in plants submitted to CC treatment comparing to the other treatments, and the minimum values were reached in plants under SW treatment (F = 8.34, p < 0.001 and F = 7.25, p < 0.001, respectively; Table 1). Photosynthetic activity correlated positively with plant growth. For instance, in 2011 A correlated positively with the number of leaves per

Table 1. Mean values (± standard error) and significances of parameters related with physiological activity, aboveground growth and root system of *Quercus ilex* seedlings growing under combined watering regimes.

	CC	WW	WS	SS	SW	*F*	*P* value
Physiology							
gs, mmol H_2O m^{-2} s^{-1}	0.11 ± 0.02 a	0.05 ± 0.01 c	0.05 ± 0.01 bc	0.08 ± 0.01 b	0.03 ± 0.01 c	8.34	< 0.0001
A, μmol CO_2 m^{-2} s^{-1}	6.4 ± 1.8 a	2.9 ± 0.7 bc	2.7 ± 0.5 bc	3.9 ± 0.4 b	1.6 ± 0.4 c	7.25	< 0.0001
Growth							
Stem height, cm	50.0 ± 4.5 a	41.2 ± 3.9 b	36.1 ± 2.2 bc	30.1 ± 1.6 c	31.9 ± 4.0 c	4.60	0.0023
Aerial growth, g	3.23 ± 0.35 a	2.32 ± 0.39 b	1.59 ± 0.39 bc	1.38 ± 0.39 c	1.34 ± 0.45 c	4.67	0.0020
Root system							
Root biomass, g	1.93 ± 0.39 a	1.93 ± 0.39 a	2.00 ± 0.39 a	2.06 ± 0.39 a	2.98 ± 0.39 a	1.60	0.196
Fine-to-total root ratio	0.57 ± 0.05 a	0.59 ± 0.05 a	0.46 ± 0.5 ab	0.55 ± 0.05 a	0.39 ± 0.04 b	3.14	0.025
Root-to-shoot ratio	0.54 ± 0.07 c	0.55 ± 0.07 c	0.71 ± 0.07 bc	0.80 ± 0.07 ab	0.93 ± 0.06 a	5.85	0.009
Fine root-to-leaves ratio	0.45 ± 0.6 a	0.47 ± 0.6 a	0.39 ± 0.6 a	0.60 ± 0.6 a	0.46 ± 0.5 a	1.66	0.181

plant ($r_{pearson}$ = 0.48; p = 0.002; n = 40) and with the aerial growth rate ($r_{pearson}$ = 0.38; p = 0.002; n = 40). In 2011, all treated plants had a decreased height if they were compared to CC plants (F = 4.60, p = 0.002; Table 1). Plants under SW treatment had a lower number of leaves (46.9 %) in comparison to WW and CC treatments (F = 3.41, p = 0.005). Considering together stem and leaves biomass, aerial growth in 2011 was higher in CC plants than in the other plants (Table 1). For CC plants aerial growth during 2011 related positively to aerial growth during 2010 ($r_{pearson}$ = 0.79; p < 0.0001; n = 20). This relation varied for the other watering regimes applied in 2011 (significant *watering treatments* × *aerial growth* interaction; F = 3.98; p = 0.006) and became negative for SS plants ($r_{pearson}$ = -0.60; p < 0.0149; n = 16; Fig. 4).

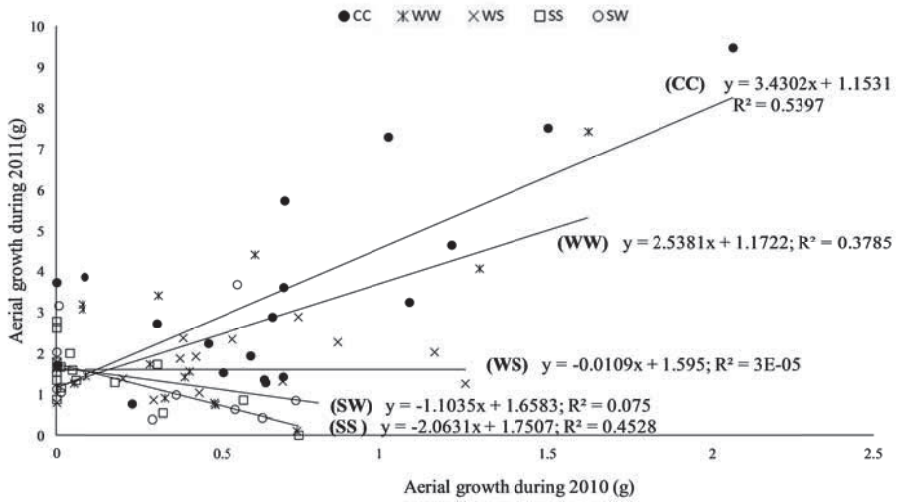

Fig. 4. Relation between aerial growth (stem + leaves biomass) rates in two consecutive years for seedlings under five different watering regimes. Homogeneity-of-slopes test resulted highly significant for *watering treatments* × *aerial growth* interaction ($F = 3.98$; $p = 0.006$; *d.f.* = 4 and 70).

In 2011, the biomass of the root system did not changed significantly between treatments ($F = 1.60$; $p = 0.196$; Table 1), but the proportion of fine root biomass respect to the total root biomass decreased for plants under alternation of water regimes compared to plants under constant watering conditions ($F = 3.14$, $p = 0.025$; Table 1). Root-to-shoot ratio and fine root-to-leaves ratio also varied significantly between watering regimes (Table 1).

Mortality of plants varied depending on the watering treatment. In 2010, mortality was higher in W (15 %) in comparison to S and C plants (1.7 and 0.0 % respectively; Table 2). In 2011 and regardless of the watering treatment during 2011, plants subjected to S treatment during 2010 had the highest mortality rates (Table 2). Mortality of SS and SW occurred at the end of the vegetative period,

and the lowest mortality rates occurred in plants exposed to CC and WW treatments (Table 2; Fig. 5). In 2011, plant mortality was independent of the acorn size (χ^2 = 0.10; p = 0.755, respectively) regardless of the watering regime (χ^2 = 0.21; p = 0.976 for *watering treatments* × *acorn weight* interactions).

After *P. cinnamomi* infestations, maximum mortality rates were observed in plants which were submitted to water stress treatment during 2011 (WS and SS) while minimum rates were observed under plants subjected to WW and SW treatments (Table 2; Fig. 6). Mortality showed independence of acorn size (χ^2 = 0.45; p = 0.502), regardless of the watering regime (χ^2 = 0.39; p = 0.942 for *watering treatments* × *acorn weight* interactions).

Table 2. Mortality of *Quercus ilex* seedlings submitted to control (C), waterlogging (W) and water stress (S) crossed watering treatments and subsequent soil infestation with *Phytophthora cinnamomi*.

Treatments	Mortality after 2010 watering treatments (%)	Mortality after 2011 watering treatments (%)	Mortality after both 2010 and 2011 watering treatments (%)	Mortality after *P. cinnamomi* infestation (%)[a]
CC	0	0	0	68.8
WW	15	5.9	20	66.7
WS		11.1	22	100
SS	1.7	30	30	91.7
SW		35	35	66.7
	χ^2= 11.15 p = 0.004	χ^2= 12.45 p = 0.014	χ^2= 8.31 p = 0.081	χ^2= 11.37 p = 0.010

[a] Calculated from plants which survived to previous watering treatments

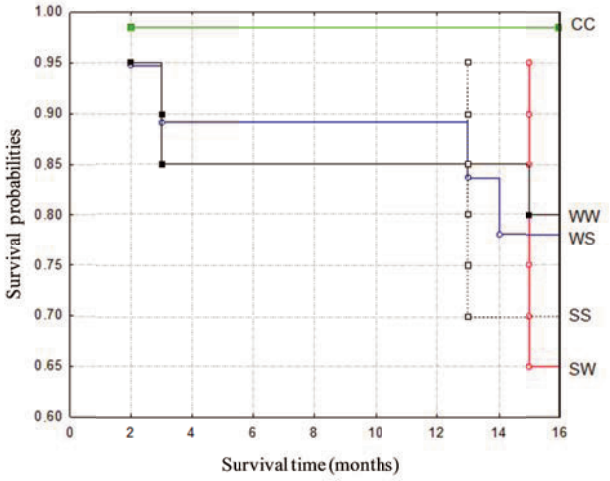

Fig. 5. Plot of survival probabilities using the Kaplan-Meier estimate, which showed differences in plant mortality rates after 2011 watering treatments (p= 0.037). *X* axis, months from the beginning of the watering treatments (May 2010); *Y* axis, proportion of surviving plants.

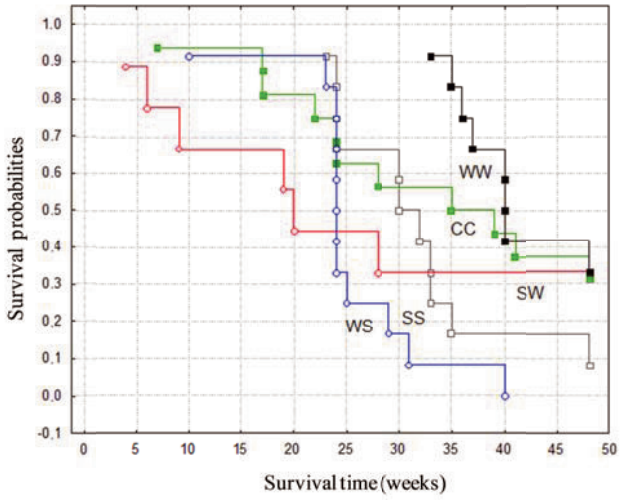

Fig. 6. Plot of survival probabilities using the Kaplan-Meier estimate, which showed differences in plant mortality rates between 2011 watering treatments after *Phytophthora cinnamomi* soil infestation (p = 0.009). *X* axis, weeks from the beginning of *P. cinnamomi* soil infestation (October 2011); *Y* axis, proportion of surviving plants.

4. Discussion

Response to water shortage

Holm oak has been characterized by its large phenotypic plasticity to adapt to adverse watering conditions (Valladares et al., 2004). Seedlings here responded to the suboptimal drought watering regimes through adjustments in morphology and physiology. Photosynthetic activity and aerial growth decreased respect to control plants, while root system remained constant, increasing the root-to-shoot ratio. This resulted in a low seedling mortality, especially during the first year. Physiological measurements showed some level of inhibition in reaction to water shortage and regulations in stomata conductance and CO_2 assimilation rate were detected in the two consecutive years with similar intensity. Stomatal closure is a strategy used by plants to diminish water transpiration and to prevent leaf water potential to drop critically, and is especially evident in drought-evader species such as *Q. ilex* (Valladares et al., 2004; Serrano and Peñuelas, 2005; Asensio et al., 2007). Through this regulation, the plant avoids xylem cavitation.

Growth reduction is a common mechanism used to compensate the shortage of water supply as a consequence of a decreased photosynthetic activity (Ogaya and Peñuelas, 2003). In our study, while photosynthesis was reduced 28 % in S respect to C during summer 2010, aerial growth was reduced 60 %, and similar reductions were observed during 2011. On the other hand, plants maintained their investment for the root system. Seedlings submitted to drought treatments invested their limited carbon savings in root development

rather than in aerial growth. This strategy may be explained by the importance of the root development for exploring a larger volume of soil. This mechanism enabled most plants to survive in the first year (2 % of mortality in 2010). However, mortality was higher in the second year (30 % in 2011) what may be interpreted as an accumulative negative drought effect. This is also confirmed by the negative relation shown between aerial growths between the two consecutive years (Fig. 4). It appears that *Q. ilex* at seedling stage is quite vulnerable to prolonged drought scenarios which can endanger its survival under the predicted warming and increased drought events.

Response to waterlogging

Q. ilex seedlings also responded to waterlogging treatments with changes in morphology and physiology. Under flooding scenarios, a drop in photosynthesis together with a decrease of growth has been observed here, which is typical of waterlogging scape species (Kreuzwieser et al., 2004; Kozlowski et al., 1991). When plants are predisposed to die, they usually undergo an inhibition of growth (McDowell et al., 2008; Mundo et al., 2010). Nevertheless, being the reduction of the CO_2 assimilation rate stronger for waterlogged plants, these plants showed a lower decrease of aerial growth than drought-stressed plants. This together with the lack of response in terms of root system could have caused the high mortality of waterlogged plants observed during 2010. However, results suggest some kind of adaptation to waterlogging, as most plants survived to the second year of waterlogging. The survival of plants submitted to anoxic conditions

caused by waterlogging relies on the change of roots from respiration to alcoholic fermentation (Kreuzwieser et al., 2004). Alcoholic fermentation reaction implies a lower yield energy, and consequently a decreased availability of energy which at long term affects to growth and survival of plants.

Studies usually refer to a reduction in aerial growth and root growth and an increased root necrosis which result in a higher shoot-to-root ratio (Glenz et al., 2006). Our results did not show any negative influence of waterlogging in root-related parameters and the expected loss of fine roots was not detected. Similarly, Sánchez et al. (2005) did not find any significant symptoms of root rot in non-inoculated plants under a continuous waterlogging treatment. Little is known about the sensitivity of *Q. ilex* to flooding which would deserve more research, as species of the same genera show different tolerances, i.e. *Q. petraea* and *Q. pubescens* are low-tolerant species while *Q. robur* is more capable of surviving to flooding.

Consequences of water regime alterations

The Mediterranean region is an adverse area in terms of water availability and is considered a climate change hotspot due to predicted reduction in precipitation, warming and increase in the frequency of extreme events (Giorgi and Lionello, 2008; Lindner et al., 2010). In this study, weather extremes scenarios and their combinations were simulated under greenhouse conditions. No differences in growth parameters or in physiological measurements were observed between SS and WS seedlings. However, mortality was

higher in seedlings under SS than under WS treatments (30 and 22 %, respectively), from which it seems that continuous drought may affect more negatively the survival of *Q. ilex* trees than drought preceded by a waterlogging event. If a double waterlogging scenario (WW) is compared with a combined drought and waterlogging scenario (SW), higher growth and survival is detected in seedlings under WW than under SW. On the whole, more negative effects seem to operate in seedlings under the combined treatment, as higher percentages of mortality were found (22 vs. 35 % for WS and SW, respectively). In general, *Q. ilex* seedlings were more vulnerable to scenarios comprising drought in the first year than to scenarios comprising waterlogging the first year. The delayed effect of the first year treatment in the subsequent drought/waterlogging treatment has been already proved (Lloret et al., 2004; Bigler et al., 2006), although some studies did not confirm this delayed effect. Gómez-Aparicio et al. (2008) reported that mortality of seedlings increased with increasing desiccation or waterlogging risk. They also showed that a wetter summer did not compensate a subsequent severe dry summer in terms of seedling survival.

Tolerance to adverse watering conditions also depends on the development stage of the plant material under monitoring. Considering the development stage, *Q. ilex* seedlings are considered more water spenders than adult trees, and a high seedling mortality has been reported as a consequence of this lower conservative water-use strategy (Lloret et al., 2004; Mediavilla and Escudero, 2004; Villar-Salvador et al., 2004). Similarly, other studies showed a lower

tolerance of young seedlings to waterlogging comparing to older ones (Siebel and Blom, 1998). Other crucial factors include timing, intensity or duration of such drought or waterlogging conditions (Kozlowski and Pallardy, 2002; Glenz et al., 2006; Dale et al., 2001). Thus, Martínez-Vilalta et al. (2002a) predicted that *Q. ilex* mortality increased acutely whether drought lasts more than 3 months. Regarding timing, late spring and summer are considered crucial stages, especially if waterlogging coincides with the end of the first flush of growth (Siebel and Blom, 1998). In this study, watering regimes were applied from the half of the growing season ongoing. Overall, *Q. ilex* seedlings showed a higher vulnerability to drought than to waterlogging, which is supported by the greater *Q. ilex* mortality registered during the episodes of extreme drought occurred in the 80's and 90's (Peñuelas et al., 2001; Lloret et al., 2004) rather than during rainy years. Within the predicted drought scenarios associated to climate change, our observations could have important implications in the survival and distribution of *Q. ilex* trees.

Seedlings response to P. cinnamomi infection under different water regimes

Seedlings previously exposed to drought usually increase their tolerance to stressful abiotic environments (van den Driessche et al., 1992; Kozlowski and Pallardy, 2002; Villar-Salvador et al., 2012), but there is a lack of knowledge about the effects of subsequent stress caused by biotic agents. *Phytophthora* spp. cause an alteration of physiological parameters which resemble those linked to water stress

(Manter et al., 2007). If trees were exposed to prolonged adverse watering conditions, *Phytophthora* infections are expected to impede the tree to recover from the previous physiological stress and probably accentuate the damage caused by such previous stress. From results, watering extremes including drought as the last event before infection exerted the most negative effect. It seems that the damage in plants caused by drought events and exacerbated by the stress related to *P. cinnamomi* infections caused a more rapid mortality of WS and SS than of WW and SW seedlings (Fig. 6). Interestingly, WW and SW treatments probably favored seedling strengthening to cope more efficiently with *P. cinnamomi* infections. It is ignored if WW and SW treatments could have strengthened seedlings through changes in plant performance or induced some kind of resistance against the pathogen. Abiotic factors can promote resistance to confer drought tolerance (Sun et al., 2010; Choi and Hwang, 2012), so it could be expected that resistance to this biotic stress could have been also induced.

5. Conclusions

Two-year old *Q. ilex* seedlings were more sensible to dehydration than to waterlogging, especially if during the first year they encountered a dry scenario. Similarly, exposure to drought events reinforced the mortality rates of seedlings after *P. cinnamomi* infections. Results on seedlings should not be extrapolated to mature *Q. ilex* forests, as different water-use strategies and growth rates occur (Mediavilla and Escudero, 2004; Pérez-Ramos et al., 2010), and seedlings may differ in susceptibility to *Phytophthora* spp. to mature trees (Greslebin and

Hansen, 2007). However results are especially important for their implication on regeneration and reforestation programs. Within the new climatic scenarios as a consequence of climate change, *P. cinnamomi* is expected to increase its potential range in the Mediterranean areas (Bergot et al., 2004). These shifts may have consequences on forest decline but uncertainty remains in modeling how pathogen activity will affect forests. The study aimed to provide some insight into this uncertainty. Summarizing, seedlings were more vulnerable to those scenarios which implied a first encounter with drought. It also occurred that drought conditions caused more *Q. ilex* mortality after infections with *P. cinnamomi* than other scenarios.

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CONCLUSIONES GENERALES

1. El oomiceto *Phytophthora cinnamomi* es el principal agente biótico involucrado en el decaimiento de *Quercus ilex* en la región de Extremadura.

2. Los valores de humedad edáfica registrados no son suficientemente bajos como para causar decaimiento en encina o explicar los bajos valores de potencial hídrico observados. Sin embargo, ante un 16,2 % de reducción en la densidad de raíces finas (un 42 % si *P. cinnamomi* estuvo presente), una baja capacidad de retención de agua útil en el suelo y/o un déficit de humedad edáfica puedan explicar el decaimiento.

3. Probablemente el decaimiento de la encina esté asociado a la existencia de una retroalimentación basada en la pudrición de raíces finas, un consumo menor de agua por parte del árbol, un aumento de la humedad edáfica, condición favorable para la proliferación e infección de *P. cinnamomi* y nuevamente la pudrición de raíces. La combinación de pérdida de raíces y el estrés hídrico explica los síntomas aéreos de los árboles decaídos y determina su supervivencia.

4. Se ha demostrado un efecto de las propiedades edáficas en la presencia de *P. cinnamomi* y en la intensidad del decaimiento. Texturas finas del suelo y espesores gruesos del horizonte Ah, propiedades edáficas normalmente favorables para el vigor y la vitalidad de los árboles bajo un clima mediterráneo, favorecen el decaimiento si *P. cinnamomi* está presente. Estas propiedades edáficas

en combinación con situaciones topográficas que mantengan una elevada humedad edáfica, benefician la actividad de *P. cinnamomi* y contribuyen al decaimiento.

5. El decaimiento de *Q. ilex* es independiente de la compactación del suelo y del contenido de N mineral (en los rangos estudiados), parámetros que están relacionados con el pastoreo. No obstante, la asociación positiva entre el decaimiento y el ratio nitrato/amonio podría estar asociada a la progresiva degradación del suelo causada por la reciente intensificación del manejo de las dehesas estudiadas.

6. El decaimiento de la encina estuvo claramente relacionado con la vitalidad del sistema radicular; no así con la abundancia de ectomicorrizas en parte de los focos muestreados. Se observó un menor porcentaje de ápices vivos no micorrizados bajo árboles decaídos que no decaídos.

7. La abundancia de ectomicorrizas fue mayor en primavera y verano que en otoño e invierno. Ni el estado de decaimiento de las encinas ni la presencia de *P. cinnamomi* influenció la variación estacional de la abundancia y diversidad de ectomicorrizas. El porcentaje de ápices muertos sí varió según las estaciones y el estado del árbol, siendo especialmente elevado en árboles decaídos frente a no decaídos durante el periodo estival.

8. La presencia del patógeno altera la abundancia de los morfotipos de ectomicorrizas menos abundantes entre árboles decaídos y no decaídos, siendo mayor en los árboles infectados no decaídos.

9. Se detectó un bajo porcentaje de ápices vivos, tanto micorrizados como no micorrizados, en comparación con otros estudios en encina. La diversidad ectomicorrícica fue también muy pobre en los focos estudiados, siendo *Cenococcum geophilum*, *Russula* spp. y *Tomentella* spp. los morfotipos más abundantes, lo cual es consistente con la degradación de las dehesas.

10. La relación entre la abundancia algunos morfotipos de ectomicorrizas y la fisiología de *Q. ilex* cambia en función del estado de decaimiento del árbol. La relación entre conductancia estomática o fotosíntesis y abundancia de *C. geophilum* se vuelve negativa para los árboles decaídos, mientras que con *Russula* spp. esta relación se vuelve positiva para los árboles decaídos y negativa para los árboles no decaídos.

11. La comunidad ectomicorrícica observada dependió de las propiedades del suelo, aumentando su abundancia con aquellas propiedades edáficas asociadas a fertilidad (espesor del horizonte Ah, pH y contenido en arcilla), y estuvo condicionada por la posición topográfica. Se observó que la presencia del patógeno debilitaba la relación natural entre las propiedades edáficas y la abundancia de ectomicorrizas.

12. Las plántulas de encina que se enfrentan a eventos prolongados o recientes de sequía seguidos de una posterior infección de *P. cinnamomi*, están más predispuestas a la muerte que aquellas que experimentan condiciones de mayor humedad, incluido el encharcamiento.

ANEXOS

ANEXO I

First report of *Phytophthora gonapodyides* involved in the decline of *Quercus ilex* in xeric conditions in Spain

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Keywords: *Phytophthora cinnamomi*; soil moisture; soil water table

Over the last three decades an intense dieback of holm oak (*Quercus ilex*) has been recorded in southwest Spain, with *Phytophthora cinnamomi* and water stress believed to be the major factors involved (Romero et al., 2007; Solla et al., 2009). In 2009, *P. cinnamomi* and *Pythium spiculum* were recovered during all seasons from soil and roots from trees showing characteristic symptoms in five declining *Q. ilex* stands in the province of Cáceres, Extremadura, SW Spain. In October 2009, a different *Phytophthora* species was isolated from roots and from rhizosphere soil of a single tree located in Malpartida de Plasencia (39º58'N 6º5'W, 443 m above sea level), using young *Q. robur* and *Q. ilex* leaves as baits and V8-PARPH agar as a selective medium (Jung et al., 1996). The heterothallic isolates formed irregularly branched hyphae, but no chlamydospores or hyphal swellings were observed. Nonpapillate elongated-ovoid to obpyriform sporangia (28-58 x 25-40 µm) with exit pores of 10-20 µm were produced by flooding one cm squares from the growing margin of a V8-agar culture for 24 h in non-sterile soil-extract. The colony pattern on V8 agar was stellate, and the average radial growth rates at 20, 25 and 30 °C were 2.5-2.7, 2.5-2.7 and 2.2-2.3 mm/day, respectively. All these features are typical of *P. gonapodyides* (Erwin & Ribeiro, 1996; Jung et al., 1996). The identity was confirmed by sequencing the internal transcribed spacer region of the rDNA with the primers ITS4/ITS6 (GenBank Accession No. GU724194).

Because *P. gonapodyides* causes root rot and stem lesions in *Q. robur* (Jung et al., 1996; Balci & Halmschlager, 2003), pathogenicity tests on one-year-old *Q. ilex* seedlings were performed. Thirty plants were grown on 250 ml pots containing a mixture of sand and peat (1:1). For inoculum preparation (Romero et al., 2007), the isolate was grown in Petri dishes containing 20 ml of carrot broth at 20 ºC in darkness. After 4 weeks of incubation, the liquid medium was discarded, and the mycelium was washed, added to sterile water, shaken and mixed for three minutes. Each pot was inoculated with the mycelium harvested from one petri dish. Plants were kept at an average temperature of 25 °C in natural daylight. Three months after inoculation, mortality of infected plants was 53 %, and mean survival time (± SD) of infected plants was 71 ± 15 days. For comparison, additional plants were inoculated in the same way with *P. cinnamomi*. After three months, mortality of *Q. ilex* seedlings was 94% and mean survival time 28 ± 7 days. The pathogens were consistently re-isolated from the roots of the dead plants. Control plants did not show any symptoms of disease.

To our knowledge, this is the first report of *P. gonapodyides* in Spain. This pathogen has always been associated with moist sites (Hansen & Delatour, 1999; Balci & Halmschlager, 2003), in contrast to our findings, in which mean volumetric soil moisture values at 30 and at 100 cm depth (loam soil) were 11.4 and 22.1 % respectively, and the mean soil water table depth was 4.6 m. Under field conditions, further research about the involvement of this pathogen in *Q. ilex* decline will be undertaken.

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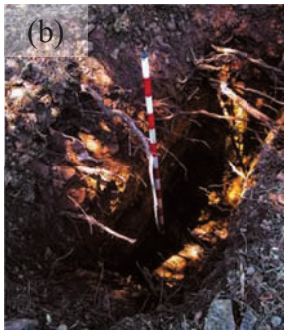
ANEXO II

Fotografías complementarias a los capítulos

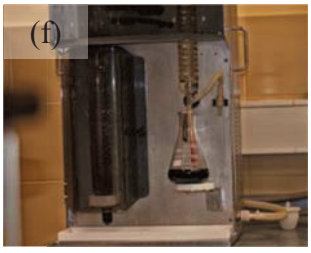

(a)


(b)


(c)


(d)


(e)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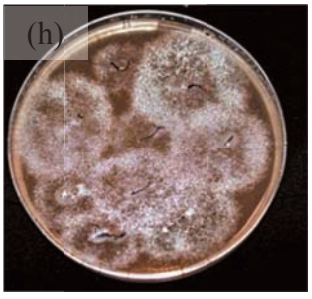
(g)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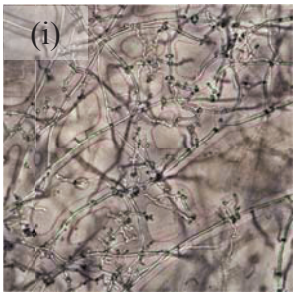

(i)

Anexo II.1. Fotografías complementarias al capítulo I: (a) excavación de una calicata de 1.5 m (cuando era posible) para la recogida de muestras bajo cada encina seleccionada de los 96 focos; (b) calicata en la cual se recogieron muestras de suelo y raíces a distintas profundidades para analizar la presencia de *Phytophthora cinnamomi* y *Pythium spiculum*, varias propiedades del suelo (profundidad del suelo, espesor del horizonte Ah, textura, pH, potencial redox, compactación del suelo y concentraciones de $N\text{-}NH_4^+$ y $N\text{-}NO_3^-$) y la densidad radical de las encinas; (c) muestras de suelo y raíces recogidas a profundidad de 50-99 cm; (d) extracción de 2 cilindros de 100 cm^3 a 0-5 cm y a 5-10 cm para medir la compactación del suelo; (e) muestras de suelo usadas para la determinación de la textura, pH, potencial redox y concentraciones de $N\text{-}NH_4^+$ y $N\text{-}NO_3^-$; (f) determinación de la concentración de NH_4^+ a través del método semi-micro Kjeldahl tras una extracción con 2,0 M KCl; (g) determinación de la concentración de NO_3^- mediante el método de espectrofotometría ultravioleta tras una extracción con una solución saturada de $CaSO_4$; (h) preparación de placas con medio selectivo NARPH para el aislamiento de *P. cinnamomi* y *Py. spiculum* a partir de muestras de raíces; (j) hifas de *P. cinnamomi*.


(a)

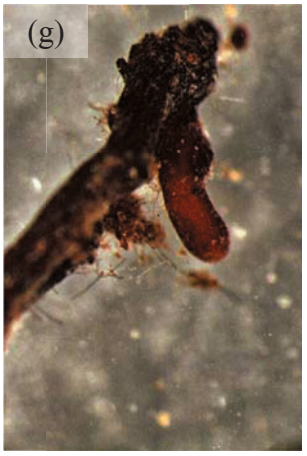

(b)


(c)


(d)

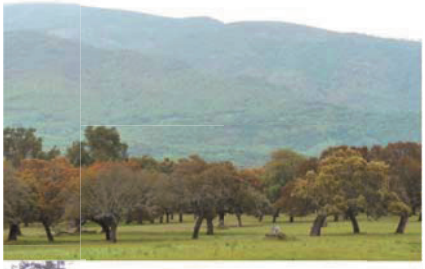

(e)


(f)


(g)

Anexo II.2. Fotografías complementarias al capítulo II: (a) Excavación de una calicata de 1.5 m (cuando era posible) para la recogida de muestras bajo cada encina seleccionada de los 96 focos; (b) recogida de muestras de raíces micorrizadas a profundidad de 10-50 cm y posterior almacenamiento en bolsas de plástico y humectación; (c) limpieza de la muestra tras 24 h mantenida en agua para facilitar la separación de las raíces y las partículas de suelo; (d) observación de las raíces bajo estereomicroscopio, cuantificación y clasificación en ápices muertos, ápices vivos no micorrizados y ápices vivos ectomicorrizados; (e) morfotipo de ectomicorriza más abundante, *Cenococcum geophilum*; (f) morfotipo *Russula* spp.; (g) morfotipo *Tomentella* spp.

(a)



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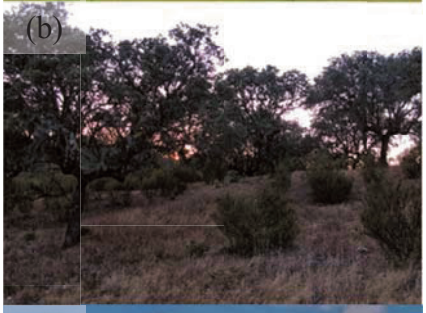

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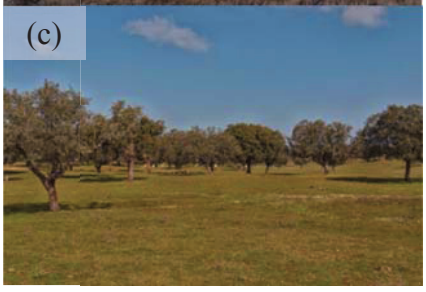
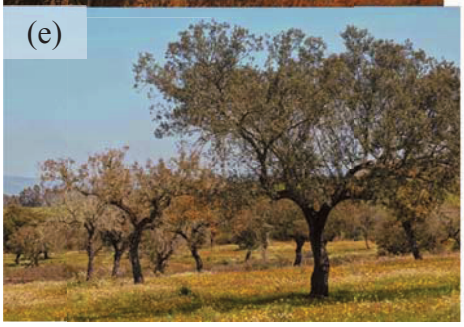
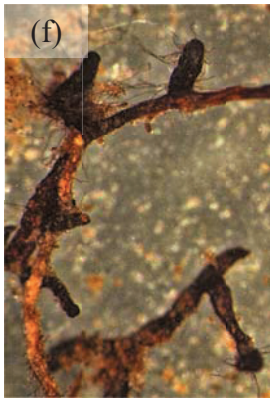


(d)

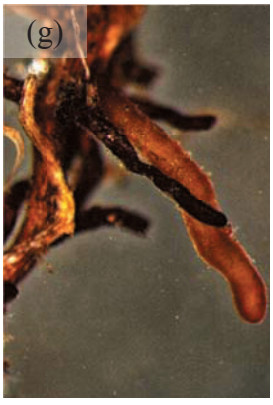
(e)



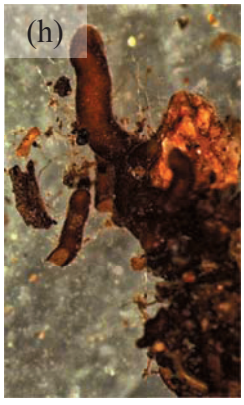
(f)



(g)



(h)



Anexo II.3. Fotografías complementarias al capítulo III: (a) foco de estudio en Abadía; (b) foco de estudio Cuartón; (c) foco de estudio Haza; (d) foco de estudio San Esteban; (e) foco de estudio en Vegaviana; (f) morfotipo más abundante *Cenococcum geophilum*; (g) morfotipo *Russula* spp.; (h) morfotipo *Tomentella* spp.


(a)


(b)


(c)


(d)

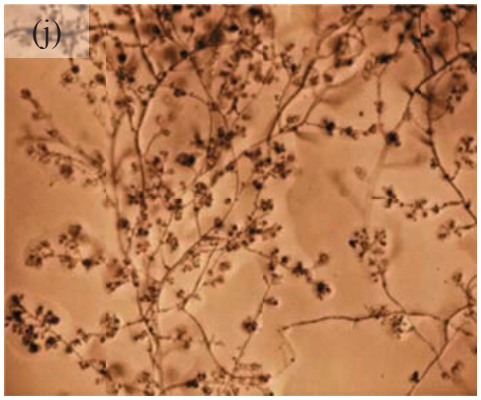
(e)

(f)

(g)

(h)


(i)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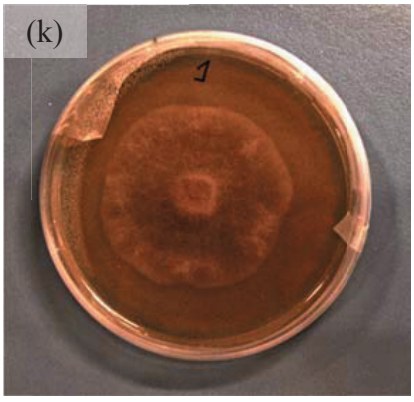
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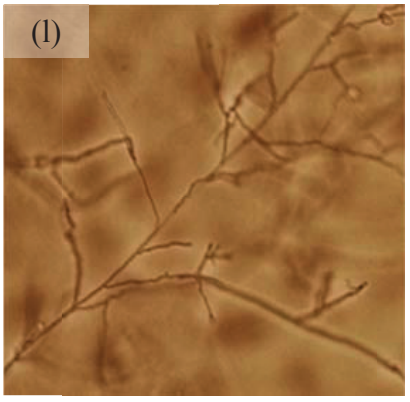
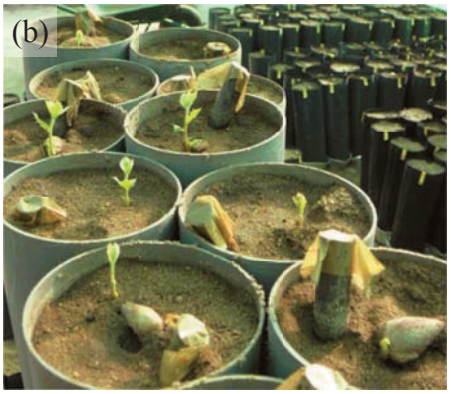

(l)

Anexo II.4. Fotografías complementarias al capítulo IV: experimento 1 con (a) instalación de tubos de PVC para la medición del nivel freático y de la humedad de los 30 árboles en los 5 focos de decaimiento mediante perforaciones a 6 y 3 m, respectivamente; (b) relleno del espacio libre entre el suelo y el tubo de PVC usado para medir la humedad con caolinita para evitar la formación de burbujas de aire; (c) medición mensual de la humedad a intervalos de 10 cm con una Diviner 2000 series II (Sentek, Australia); (d) medición mensual del nivel freático con una sonda que contiene un sensor, el cual emite una señal acústica en contacto con el agua; (e) medición de la fotosíntesis y la conductancia estomática mediante el aparato IRGA que consta de una cámara donde se introduce la hoja y se mide el intercambio gaseoso; (f) medición del potencial hídrico del tallo mediante la cámara de presión tipo Scholander conectada a una bombona de N_2. Experimento 2 con (g) excavación de una calicata de 1.5 m (cuando era posible) para la recogida de muestras bajo cada encina seleccionada de los 96 focos con el fin de detectar *Phytophthora cinnamomi* y *Pythium spiculum*; (h) recogida de muestras de suelo y raíces a profundidades de 10-49 cm, de 50-99 cm y de 100-150 cm; (i) preparación de placas con medio selectivo NARPH para el aislamiento de *P. cinnamomi* y *Py. spiculum*; (j) hifas e hinchamientos hifales de *P. cinnamomi*; (k) repicado y crecimiento del micelio de *P. cinnamomi* en V8 agar; (l) hifas de *Py. spiculum*.

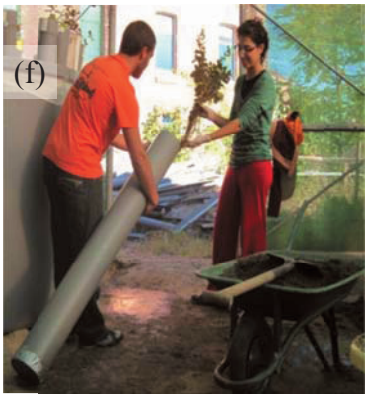

(a)


(b)


(c)


(d)


(e)


(f)


(g)

Anexo II.5. Fotografías complementarias al capítulo V: (a) instalación de los tubos de PVC, relleno con arena y turba y posterior colocación de las bellotas con la radícula emergente; (b) emergencia de plántulas de encina; (c) instalación de los regímenes hídricos de encharcamiento y sequía; (d) medición de la humedad edáfica con una sonda Delta-Theta ML2X; (e) levantamiento de algunas plantas para medir la biomasa radicular tras los tratamientos hídricos; (f) trasplante de las plantas a tubos de mayor tamaño; (g) preparación del inóculo de *Phytophthora cinnamomi* mediante la mezcla de vermiculita, avena, zumo de vegetales y un trozo de micelio de *P. cinnamomi*; (h) crecimiento del micelio de *P. cinnamomi* dentro de los matraces conteniendo la mezcla de vermiculita, avena y zumo de vegetales en la incubadora a 20 ºC durante aproximadamente 5 semanas; (i) encharcamiento de las plantas inoculadas con *P. cinnamomi* cada 3 semanas durante 72 horas; (j) síntomas foliares de la infección por *P. cinnamomi*.