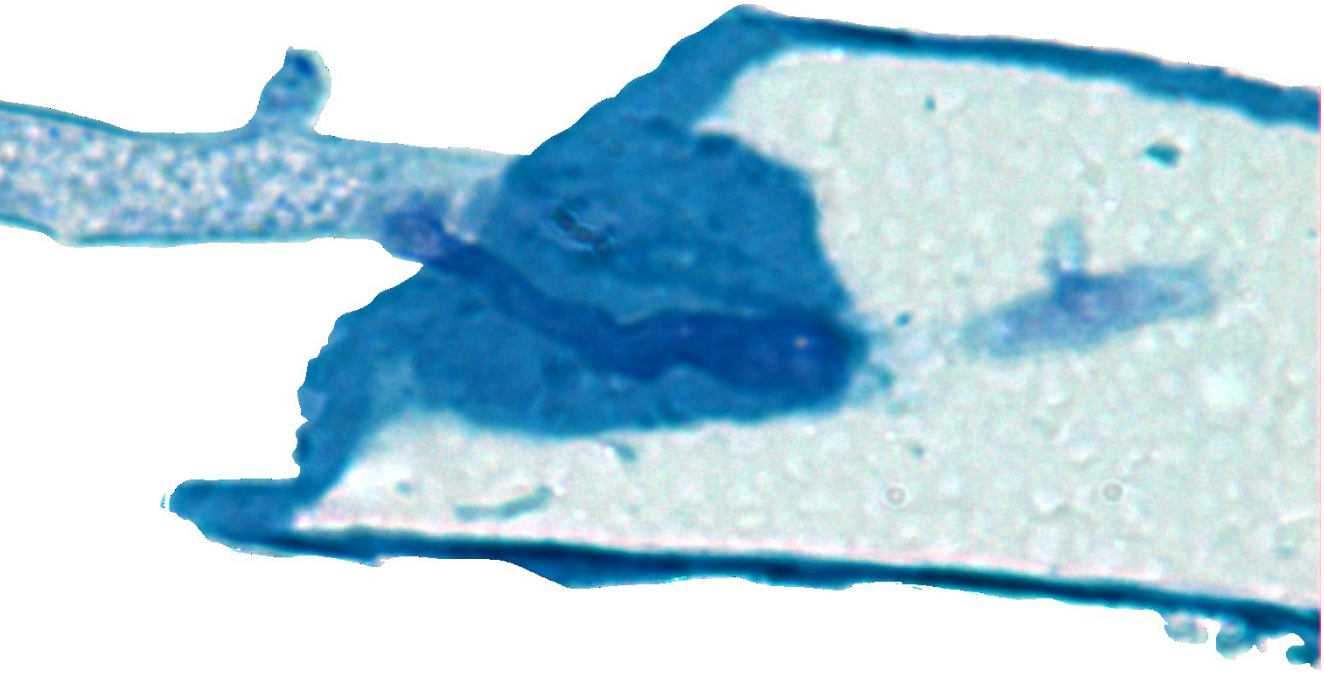
UNIVERSIDAD D CÓRDOBA

Departamento de Ingeniería Forestal
Área de Ingeniería Agroforestal

Study of the interaction between root rot oomycetes and *Quercus ilex* L.

"Estudio de la interacción entre oomicetos de podredumbre radical y* Quercus ilex *L."



Tesis Doctoral

Francisco José Ruiz Gómez

TITULO: *Study of the interaction between root rot oomycetes and Quercus ilex L.*

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Programa de Doctorado de Biociencias y Ciencias Agroalimentarias

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Directores:

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Tesis Doctoral presentada por

Francisco José Ruiz Gómez

para la obtención del título de

DOCTOR CON MENCIÓN INTERNACIONAL

POR LA UNIVERSIDAD DE CÓRDOBA

Córdoba, septiembre de 2018

Mención de Doctorado Internacional

La presente Tesis cumple con los requisitos establecidos por la Universidad de Córdoba para la obtención de la Mención de Doctorado Internacional:

- Estancia de 4 meses en el" Department for Innovation in Biological Agro-food and Forest systems (DIBAF)", de la Università degli studi della Tuscia, Vitrerbo (Italia), bajo la supervisión de la Dtr.ssa. Anna Maria Vetraino, en la que se realizó parte del trabajo del Capítulo 3.
- Estancia de 3 meses en el "Department of Phytopathology of Woody Plants", de la Technische Universität München, Freising (Alemania), bajo la supervisión del Dr. Wolfgang Oβwald, en la que se realizó el trabajo de análisis de laboratorio correspondiente al Capítulo 5.
- Evaluación previa de dos doctores con experiencia investigadora acreditada, pertenecientes a centros de investigación ó instituciones de educación extranjeros.
- Un miembro del Tribunal pertenece a un centro de investigación ó institución de educación superior extranjero.

TÍTULO DE LA TESIS:

"Estudio de la interacción entre oomicetos de podredumbre radical y *Quercus ilex* L."

DOCTORANDO: Francisco José Ruiz Gómez

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

El decaimiento de la encina (*Quercus ilex* L.) es uno de los principales problemas de las dehesas de la Península Ibérica. Su etiología es compleja, aunque se considera que el factor más importante es la podredumbre radicular asociada a algunos oomicetos patógenos, principalmente a *Phytophthora cinnamomi* Rands. El decaimiento y la pérdida del arbolado de la dehesa provocan pérdidas irreparables de tipo ecológico y socioeconómico, poniendo en riesgo la sostenibilidad de estos ecosistemas, así como el desarrollo de zonas rurales muy dependientes de su aprovechamiento. Es, por ello, que este problema es hoy en día uno de los principales temas de interés de las políticas ambientales, tanto a nivel autonómico como estatal y europeo.

Sin embargo, la mayor parte de los esfuerzos dirigidos a paliar los efectos del decaimiento del encinar se basan en trabajos empíricos, fundamentados en la observación de las causas, los síntomas y las consecuencias del decaimiento, pero los procesos subyacentes relacionados con la interacción entre el huésped y el patógeno son, en su mayoría, desconocidos.

Por todo ello, la presente tesis doctoral pretende contribuir a ampliar el conocimiento relacionado con esta interacción, mediante la aplicación de metodologías ya desarrolladas en otros patosistemas similares, como son el estudio histológico del ciclo infectivo, o la caracterización fisiológica de las plantas afectadas, y otras aproximaciones más novedosas, como el estudio de la influencia

del componente biológico del suelo en el decaimiento de la encina y la presencia de oomicetos de podredumbre radicular. El primer trabajo que se llevó a cabo propone una metodología para el análisis de secciones histológicas adaptada a las condiciones del sistema huésped-patógeno, ensayando las principales preparaciones utilizadas en histología vegetal, y proponiendo y evaluando índices que permiten la cuantificación de la infección en los tejidos.

Posteriormente, estas técnicas se aplicaron en un segundo trabajo, mediante el análisis de las raíces en un ensayo de inoculación. Los resultados de esta investigación han permitido describir el ciclo del patógeno en el interior de la raíz, así como los cambios en la estructura de los tejidos y las reacciones de la planta ante la colonización del patógeno, encontrando evidencias de la presencia de respuestas defensivas y fisiológicas de la planta ante el ataque del patógeno.

Los cambios producidos por la acción del patógeno en la raíz podrían relacionarse con la inducción de estrés hídrico, o con respuestas fisiológicas de la planta. En una segunda aproximación metodológica se estudia la respuesta de plantones de encina a la aplicación de un estrés combinado, mediante la inoculación de los plantones y la aplicación de un ciclo de sequía severa. Este trabajo presenta un gran interés debido a que la influencia del estrés hídrico en la respuesta del patógeno puede relacionarse con las consecuencias del cambio climático en las dehesas andaluzas, cuya área de distribución se prevé que sea una de las más afectadas por el calentamiento global.

El último trabajo que desarrolla la tesis es la evaluación de la estructura y funcionalidad de las comunidades de hongos y oomicetos presentes en las dehesas afectadas por procesos de decaimiento en Andalucía. Este trabajo se lleva a cabo mediante la aplicación de técnicas de multiplexión y secuenciación de ADN de última generación, así como técnicas bioestadísticas que permiten el análisis de grandes bases de datos moleculares a nivel regional. Los cambios en la funcionalidad y en la composición específica de la microbiota del suelo demuestran que la gestión del componente biológico es uno de los factores más prometedores en el tratamiento del decaimiento de la encina en las dehesas.

La Tesis se presenta como un compendio de 4 artículos científicos, dos de los cuales ya han sido publicados, y los otros dos se encuentran enviados a revistas

de impacto en el área de la tesis, encontrándose en el momento del registro de este trabajo en proceso de revisión:

1: **Ruiz-Gómez FJ**, Sánchez-Cuesta R, Navarro-Cerrillo RM, Pérez-de-Luque A. **2012**. A method to quantify infection and colonization of holm oak (*Quercus ilex*) roots by *Phytophthora cinnamomi*. *Plant Methods* **8**: 39. Índice de Impacto: 4,269. Q1 *Plant Scienes*, posición 16/222.

2: **Ruiz Gómez FJ**, Navarro-Cerrillo RM, Sánchez-Cuesta R, Pérez-De-Luque A. 2015. Histopathology of infection and colonization of *Quercus ilex* fine roots by *Phytophthora cinnamomi. Plant Pathology* **64**: 605-616. Índice de Impacto: 2,303. Q1 *Agronomy*, posición 16/87.

3: **Ruiz Gómez FJ**, Pérez-de-Luque A, Sánchez-Cuesta R, Quero JL, Navarro-Cerrillo RM. **2018**. Differences in the response to acute drought and *Phytophthora cinnamomi* Rands. infection in *Quercus ilex* seedlings. *Forests* (Submitted). Índice de Impacto: 1,956. Q2 *Forestry*, posición 18/66.

4: **Ruiz Gómez FJ**, Navarro-Cerrillo RM, Pérez-de-Luque A, Oβwald W, Vannini A, Morales-Rodríguez C. **2018**. Metabarcoding analysis of fungi and oomycetes in *Querus ilex* "dehesas": Functional and structural changes related to holm oak decline. *New Phytologist* (Submitted). Índice de Impacto: 7,433. Q1 *Plant Sciences*, posición 7/222.

Por otro lado, el candidato ha participado durante el desarrollo de la Tesis Doctoral en la publicación de 5 artículos en revistas científicas indexadas en el JCR:

- Duque Lazo J, Navarro-Cerrillo RM, **Ruiz Gomez FJ. 2018**. Assessment of the future stability of cork oak (*Quercus suber* L.) afforestation under climate change scenarios in Southwest Spain. *Forest Ecology and Management* **409**: 444-456. Índice de Impacto: 3.169. Q1 *Forestry*, posición 4/66.
- Loewe Muñoz V, Navarro-Cerrillo RM, Sánchez Lucas R, **Ruiz Gómez FJ**, Jorrin-Novo J. **2018**. Variability studies of allochthonous stone pine (*Pinus pinea* L.) plantations in Chile through nut protein profiling. *Journal of Proteomics* **175**: 95-104. Índice de Impacto: 3,722. Q1 *Biochemical Research Methods*, posición 17/79.
- Navarro-Cerrillo RM, **Ruiz Gómez FJ**, Cabrera-Puerto RJ, Sánchez-Cuesta R, Palacios Rodriguez G, Quero Perez JL. **2018**. Growth and physiological sapling responses of eleven *Quercus ilex* ecotypes under identical environmental

conditions. *Forest Ecology and Management* **415-416**: 58-69. Índice de Impacto: 3.169. Q1 *Forestry*, posición 4/66.

- Díaz-Lezcano MI, Navarro-Cerrillo RM, **Ruíz-Gómez FJ. 2018**. Morphoanatomy of *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. (Arecaceae) embryos. *Revista Chapingo Serie Ciencias Forestales y del Ambiente* **24**: 91-100. Índice de Impacto: 0.438. Q4 *Forestry*, posición 59/66.
- Chami M, Hajj A, Kahwaji J, Houssef H, Ghaith S, Fakih L, Smaha M, Nabbout R, El Riachy M, As-Sadi F, Al Zein M, **Ruiz Gomez FJ**, Palacios-Rodríguez G, Navarro-Cerillo R, Tous J, Chalak L. **2018**. Assessment of Ancient Carob Germplasm of Lebanon by Morphological Traits. *Journal of the American Pomological Society* **72**: 260-278. Índice de Impacto: 0.277. Q4 *Agronomy*, posición 82/87.

Adicionalmente, el candidato ha participado en la publicación de un artículo científico en una revista no indexada en JCR, y 5 aportaciones a congresos científicos, cuatro de ellas en congresos de relevancia internacional:

- Artículo**: Ruiz Gómez FJ**, Navarro-Cerrillo RM, Lara Gómez MA, Sánchez-Cuesta R. **2016**. Aislamiento e identificación de oomicetos en focos de podredumbre radical de Andalucía y Extremadura. *Cuadernos de la Sociedad Española de Ciencias Forestales* **43**: 363-376. IISSN: 1575-2410. Google Scholar Metrics Índice H5: 3. Ciencias Agrarias.
- Aportación a congreso: **Ruiz-Gómez FJ**, Sánchez-de-la-Cuesta R, Navarro-Cerrillo RM, Pérez-de-Luque A. **2012**. Quantification of infection and colonization of holm oak (*Quercus ilex*) roots by Phytophthora cinnamomi using histological methods. The Sixth Meeting of the International Union of Forest Research Organizations. IUFRO Working Party 7-02-09 *Phytophthora in Forests and Natural Ecosystems*. Córdoba (España), 9 de septiembre al 14 de septiembre 2012. Ámbito del congreso : Internacional. Tipo de comunicación : Póster.
- Aportación a congreso: Navarro-Cerrillo RM, Hernández-Clemente R, Quero-Pérez JL, Romero-Ramírez FJ; **Ruiz Gómez FJ**, [et al.]. **2013**. Early detection of dieback processes of Quercus ilex from spectral data at regional and local sites. *Oak forests coping with global change: ecology and management*. Universidad Internacional de Andalucía, Baeza (España), 30 de septiembre al 2 de octubre 2013. Ámbito del Congreso: Internacional. Tipo de presentación: Póster.
- Aportación a congreso: **Ruiz Gómez FJ**, Navarro-Cerrillo R, Lara-Gómez MA, Sánchez de la Cuesta R. **2015**. Aislamiento e identificación de oomicetos en focos de podredumbre radical de Andalucía y Extremadura. III Reunión Científica de Sanidad Forestal. Madrid, 7 y 8 de octubre 2015. Ámbito del congreso: Nacional. Tipo de presentación: Oral.

- Aportación a congreso: **Ruiz Gómez FJ,** Morales-Rodríguez C, Sánchez-Cuesta R, Pérez-de-Luque A, Vannini A, Navarro-Cerrillo R. **2016**. Molecular identification of oomycete biodiversity in soils from Los Alcornocales Natural Park (Cádiz, Spain). 8th IOBC-WPRS Working Group: *Integrated Protection in Oak Forests*. Córdoba (España), 23 de octubre al 28 de octubre 2017. Ámbito del congreso: Internacional. Tipo de presentación: Oral.
- Aportación a congreso: Hernández Clemente R, **Ruiz-Gómez FJ,** Sánchez de la Cuesta R, Quero JL. **2017**. Linking hyperspectral imaging and ecophysiological traits for detecting oak forest decline at an early stage. XIV MEDECOS & XIII AEET meeting. *Human driven scenarios for evolutionary and ecological changes*. Sevilla (España), 31 de enero al 4 de febrero 2017. Ámbito del congreso: Internacional. Tipo de presentación: Oral.

Por todo ello, se autoriza la presentación de la Tesis Doctoral "Estudio de la interacción entre oomicetos de podredumbre radical y *Quercus ilex* L.".

En Córdoba, 18 de septiembre de 2018

Firma de los directores

Fdo.: Dr. Rafael Mª Navarro Cerrillo.

Fdo.: Dr. Alejandro Pérez de Luque

"Daría todo lo que sé
por la mitad de lo que ignoro"

Descartes

Agradecimientos

Al final de esta etapa, se hace evidente que una Tesis es algo más que un trabajo académico. Esta Tesis forma parte de un proyecto personal, nacido del trabajo y la ilusión de un extraordinario grupo de personas. Estas personas me regalaron su idea para que la convirtiera en mi proyecto, pero además decidieron acompañarme y nunca me abandonaron. Por lo tanto, debo empezar agradeciendo a mis directores, Rafa Navarro y Alex Pérez, y junto a ellos a Rafa Sánchez, los integrantes del original "*Phytophthora* Team", su apoyo, su guía, su esfuerzo y, sobre todo, su amistad. La palabra Tutor se queda corta para ellos, y la palabra Equipo no llega a definir lo que somos. Espero que durante mucho tiempo seamos lo que somos, sea lo que sea...

Quiero acordarme de todos los que he ido encontrando durante este tiempo, porque todos formaron parte de mi proyecto. Empiezo por Rubén Delgado, Rafa Arias y Jesús Trujillo, compañeros desde los trabajos fin de carrera, con los que compartí las dudas ante lo desconocido y los "ruiditos" de Trujillo... Luego llegaron Rocío Hernández y David Ariza, con los que descubrimos que lo que parecía imposible sólo era difícil, frase que hice mía, y detrás José Luis Quero para corroborarlo, igual que Joaquín Duque, grandes investigadores y mejores personas, hoy en día mis amigos. Gracias por estar cuando os necesito. En el laboratorio y en el campo me han ayudado especialmente Rafa Sánchez, Roberto, Jesús Beira, Emidio y Andrés Walter. Sin ellos no hubiera podido sacar adelante tanto trabajo. Junto con otros que se fueron, como Kiko, José María Higuera, Fran Pereña, Paco, Raúl Sánchez,... y otros que aún están, como Guille, Ana, Inma, Lales, María Villa, Carmen, José Luis Vaca, Lourdes, Mari Ángeles, Cristina, Chelo, Alba, Antonio, Carlos, Andrés, Marta y Javi, he tenido la suerte de compartir el "*Grupo*", que ha sido el motor de mi proyecto. Gracias a ERSAF, a IDAF, gracias a todos.

Un proyecto como este requiere abrir fronteras, mentales y también geográficas. En Italia encontré a Maria Pía, Diana, Matteo..., y a la cabeza, el profesor Andrea Vannini, que más allá de cumplir con su parte, decidió seguir acompañándome en mi proyecto, igual que Carmen Morales, siendo profesores cuando los necesité, y amigos dentro y fuera de los laboratorios, personas con las que compartí los momentos de soledad lejos de la familia. Muchas gracias.

Además de un proyecto, la Tesis es, en palabras de mi director, "una mochila que te llevas a todos lados, también a casa". Si el proyecto es "personal", no hay nada más personal que tu familia, y por ahí voy a acabar, porque sin el apoyo y el cariño de los míos nunca hubiera conseguido nada, porque si el "*Grupo*" es el motor, la familia es la energía. Un pequeño soplo en el corazón se lo dedico a mi madre, que siempre creyó en mí, y aunque no ha podido acompañarme hasta el final, tiene una lágrima especial en mi recuerdo hoy. Tampoco olvido a mi padre, ni a la paciencia con la que contestaba una a una las miles de preguntas que le hacía de pequeño, forjando así mi carácter curioso, y enseñándome a ser yo mismo. De mis hermanos, aunque me acuerdo de todos, al que agradezco especialmente su afabilidad y su predisposición es a mi hermano Roberto, por su ejemplo en "algunas" cosas, y por su sonrisa permanente.

Muchas gracias también a Irene, una de las personas más sinceras y buenas que conozco, además de un ejemplo en lo profesional, con la que he compartido tanto y tan bueno que más que mi cuñada es mi hermana, y junto a ella le doy las gracias a Rafa, de igual categoría en lo moral y lo profesional, y una de las personas que más desinteresadamente me ha ayudado desde hace tiempo. Valéis *para un "moro" y para un descosío,* y espero que mantengamos la "Cofradía" por mucho tiempo. Tampoco me voy a olvidar de Juan Ramón, que nos ayudó a poner en pie la casa a la que llamamos hogar, y otra lágrima especial hoy es para Mari Paz, que también me enseñó lo que significa la palabra "familia".

Y si a alguien tengo que dar las gracias es a mi mujer. Tú me lo has dado todo. Confiaste en mí cuando otros me desahuciaron, me esperaste contra el consejo de algunos, me animaste a seguir el camino que quería, me apoyaste después, y me has dado lo que soy, y lo mejor que cualquier persona puede tener, nuestra familia y nuestros hijos Fran y Cecilia, a los que agradezco la fuerza que me dan todos los días, aunque no lo sepan. Te agradezco que me hayas enseñado el verdadero significado de las palabras "esfuerzo", "constancia", "apoyo", "entrega", "humildad", "compañía" y, sobre todo, de la palabra "amor". En lo material te agradezco las horas que me has regalado en este último año, y sobre todo en el último e intenso mes de la redacción de esta Tesis, así como tu pragmatismo, que ha sido fundamental para levantarme de los reveses, que haberlos los hubo. Quiero decirte que esta Tesis es tan tuya como mía, y que lo que yo he puesto en ella no hubiera sido posible sin ti.

Al final de estas líneas siento que aquí no acaba este proyecto personal, vital y profesional, solo acaba una etapa, y empieza otra, en la que espero contar con todos vosotros, así que gracias por vuestra ayuda, pasada, presente y futura.

Colaboración institucional

Los trabajos que forman parte de la presente Tesis Doctoral se han llevado a cabo con ayuda de los proyectos DECOVA (AGL2009-12243-C02-02), DIVERBOS (CGL2011-30285-C02-02), QUERCUSAT (CGL2013-40790-R), ENCINOMICA (BIO2015-64737-R) y ESPECTRAMED (CGL2017-86161-R), financiados por el Ministerio de Economía, Industria y Competitividad.

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Los autores de los trabajos quieren agradecer a la Consejería de Medio Ambiente y Ordenación del Territorio (Junta de Andalucía) su colaboración en los muestreos de campo, la toma de datos, el cultivo de plántulas y el suministro de información del territorio. También agradecen al Campus de Excelencia Internacional en Agroalimentación CeiA3, el soporte recibido, especialmente a través del Servicio Centralizado de Apoyo a la Investigación (SCAI) por sus servicios de análisis.

Summary

Forest decline is nowadays a major challenge to management and sustainability of natural ecosystems worldwide. This syndrome is a multifactorial disease influenced by several biotic and abiotic agents such as alien invasive pathogens, changes in land use and management policies, population dynamics driven by economic and politic changes, and climatic perturbations. All these factors changing due to anthropogenic influence, together with others, conformed the so-called global change.

Since the 1990's decade, the oak decline has been identified as one of the most important ecological problems in Europe, affecting deciduous and evergreen *Quercus* species from the continental forests on Central and North Europe, to temperate forests of the Mediterranean basin. In the case of Iberian Peninsula, holm oak (*Quercus ilex* L.) and cork oak (*Quercus suber* L.) decline has been detected since the 1980's decade. These two species covered most of the forest surface of the south and central part of the Iberian Peninsula, mainly through "dehesas" and "montados" formations. This area is considered to be one of the worst affected regions in the world by climate change, worsening the effects and the consequences of oak decline in "dehesas".

"Dehesas" are Mediterranean savanah-like ecosystems, which provide several economic yields and ecological services. In turn, the holm oak is the most representative tree in the Iberian Peninsula, and the main species conforming the tree layer on the Spanish "dehesas". The loss of this tree layer is a major ecological and economical constraint. The holm oak decline is mainly associated to the action of soil-borne pathogens, especially *Phytophtora cinnamomi*. Despite the influence of other factors in the decline, there is a strong association between root rot caused by oomycetes and the death of trees. *Phytophtora cinnamomi* is an aggressive alien plant pathogen widely widespread worldwide, which has been associated with the disease, die-off and death in a large list of different

plant hosts. It is able to change trophic relationships with their hosts, becaming biotroph in asymptomatic hosts and hemibiotroph or necrotroph in susceptible hosts. It is considered that holm oak is the most susceptible *Quercus* sp. to the action of this pathogen. Additionally, other *Phytophthora* spp. and *Pythium* spp. have been recorded associated with the holm oak decline in Spain, Portugal, Italy and France.

Many scientific efforts have focused to study this important host-pathogen system, obtaining great results and increasing the knowledge of the causes and effects of the interaction, improving management techniques to limit the spread and the symptoms. However, most of the reviewed works are based on empirical approaches, being the underlying mechanisms regulating the interaction between both species mostly unknown.

This PhD Thesis covers part of this lack of basic knowledge, focusing on the host-pathogen interaction at histological and physiological level, and exploring the influence of the soil biota in the severity of the disease symptoms. For this purpose, the work was structured in seven chapters.

Chapter 1 provides the framework in which the present PhD Thesis has been developed, and the general and specific objectives.

Chapter 2 presents the methodology developed to evaluate the colonization and infection of *Phytophthora cinnamomi* in *Quercus ilex* seedling through the semi-automated quantification of pathogen structures present in histological sections of fine roots. A workflow was tuned-up testing different fixing solutions, embedding substances and staining methods, and the results allowed the clear differentiation of pathogen structures from host tissues. Furthermore, different indices based on structure location, host tissue classification and specificity of pathogen structures were evaluated to find the easiest and statistically robust indices that are able to explain the progress of the oomycete into the root.

In chapter 3, inoculation experiments in growth chamber were carried out to describe the pathogenesis of the *Quercus ilex-Phytophthora cinnamomi* interaction. Longitudinal sections were analysed for epidermal, cortex, parenchymatous tissue of central cylinder and vascular tissue after 1, 3, 7 and 14 days after inoculation. Total oomycete structures area, intracellular structures area, extracellular structures area, and specific structures area of the pathogen were quantified. The analysis of these data results in the description of the colonization/infection cycle of the pathogen, classified in three different stages related with their trophic behaviour. Moreover, histological changes of the root tissues as a result of the presence of defence responses and the action of the pathogen were described.

In Chapter 4, the differential responses of holm oak seedlings to the inoculation with *P. cinnamomi*, the acute drought and combination of both stressors were assessed. Six-months old seedlings were inoculated and mock-inoculated, and half of each inoculation treatment plans were subjected to acute drought meanwhile the others were well irrigated. Photosynthesis, stomatal conductance and fluorescence were measured weekly, and total biomass and biomass allocation parameters were quantified at the end of the experiment. The resulting data showed differences in the response of seedlings to drought and inoculation, and the influence of the additive effect of both stressors in the seedlings die-off.

In Chapter 5, soil samples of "dehesas" were collected and total DNA was extracted and analysed through metabarcoding techniques, to evaluate the specific composition and diversity of the fungal and oomycete communities, and to study their relationship with the disease symptoms. The fungal community included a wide range of pathogens and abundance of ectomycorrhizal key taxa. *Phytophthora* spp. dominated the oomycete community, but the species related to root rot did not appear as the most abundant, nor were they related directly to defoliation levels. A particular Operational Taxonomic Unit (OTU) belonging to the genus *Trichoderma* was strongly correlated with the scarcity of pathogenic *Phytophthora* spp.

The differences in defoliation were related to changes in the functionality of soil microbiota and diversity levels of pathogenic species.

Chapter 6 presents the general discussion of the Thesis, including some aspects limiting the results of the works carried out, ad new work lines deriving from this Thesis, and finally Chapter 7 contains the conclusions of the work.

Changes in roots as a consequence of *P. cinnamomi* inoculation, including defence responses, and the differential response identified with pathogen colonization/inoculation, leads to new insights about the causes of tree death. Holm oak responds to the attack of the pathogen, and their physiological changes differ from the ones caused by water stress, allowing the recovery of plants if no additional stress is present. Moreover, the study of soil microbiome in declined "dehesas" showed the influence of the microbial diversity in the health status of trees, and also presented new species of oomycetes and fungi that must be considered in the management of holm oak decline in Andalusian "dehesas".

Resumen

El decaimiento forestal es hoy en día uno de los mayores desafíos para el manejo y la sostenibilidad de los ecosistemas naturales en todo el mundo. Dicho síndrome es una enfermedad multifactorial en la que intervienen diversos agentes bióticos y abióticos como los patógenos invasores, los cambios en los usos del territorio y las políticas de recursos, o las perturbaciones climáticas. El cambio producido en todos estos factores debido a la influencia del hombre entre otros motivos es lo que se ha dado en llamar cambio global.

Desde la década de los 90 del siglo XX, el decaimiento de los robles se ha identificado como uno de los problemas ecológicos más relevantes en Europa, afectando a masas de *Quercus* caducifolios y perennifolios desde los bosques continentales de Centro Europa y Norte Europa, hasta los bosques templados de la cuenca mediterránea. En el caso de la Península Ibérica, el decaimiento de la encina (*Quercus ilex* L.) y el alcornoque (*Quercus suber* L.) se identificó en los años 80 del siglo XX. Estas dos especies cubren la mayor parte de la superficie forestal del sur y centro de la Península Ibérica, principalmente formando sistemas de dehesa y "montados". Dicha área geográfica está considerada como una de las regiones a nivel mundial que se verán peor afectadas por el cambio climático, lo que agravaría las consecuencias y los efectos del decaimiento de la encina en las dehesas.

Las dehesas son ecosistemas mediterráneos semejantes a la sabana, que proveen de diversos beneficios económicos y servicios ambientales. Por su parte, la encina es el árbol más representativo de la Península Ibérica, y la especie principal del estrato arbóreo de las dehesas. La pérdida de este estrato es un problema ecológico y económico de gran relevancia. El decaimiento de la encina está asociado principalmente a la acción de patógenos de suelo, especialmente *Phytophthora cinnamomi*. Sin olvidar la influencia de otros factores en el síndrome, existe una fuerte asociación

entre la podredumbre radicular causada por los oomicetos y la muerte del arbolado. *Phytophthora cinnamomi* es un patógeno invasor muy agresivo, ampliamente distribuido por todo el mundo, que ha sido asociado con la enfermedad, la decadencia y la mortalidad de una larga lista de diferentes especies vegetales. Es una especie capaz de cambiar su relación trófica con el huésped, comportándose como un organismo biótrofo en huéspedes asintomáticos, y como hemibiótrofo ó necrótrofo en huéspedes susceptibles. Se considera que la encina es la especie del género *Quercus* más susceptible a la acción del patógeno. De forma adicional, otras especies de los géneros *Phytophthora* y *Pythium* se han encontrado asociadas con el decaimiento de la encina en España, Portugal, Italia y Francia.

Para estudiar este sistema patógeno-huésped se han llevado a cabo grandes esfuerzos científicos, que han obtenido resultados muy meritorios y han incrementado el conocimiento de las causas y los efectos de la interacción, mejorando las técnicas de gestión para limitar la dispersión y los síntomas del decaimiento. Sin embargo, la mayoría de los trabajos revisados se basaron en aproximaciones empíricas, desconociéndose gran parte de los mecanismos subyacentes que controlan la interacción entre las dos especies.

Esta Tesis cubre parte de esta falta de conocimiento básico, centrándose en la interacción entre el huésped y el patógeno a nivel histológico y fisiológico, así como explorando la influencia de la biota del suelo en la severidad de los síntomas de la enfermedad. Para dicho propósito, el trabajo se estructuró en 7 capítulos.

El Capítulo 1 proporciona el marco teórico en el que se desarrolla esta Tesis Doctoral, así como los objetivos generales y específicos.

El Capítulo 2 muestra la metodología desarrollada para evaluar la colonización e infección de *Phytophthora cinnamomi* en plántulas de *Quercus ilex* a través de la cuantificación semiautomática de las estructuras del patógeno presentes en secciones histológicas de raíces finas. El flujo de trabajo fue puesto a punto probando diferentes soluciones de fijación,

sustancias de inclusión y métodos de tinción, y los resultados permitieron la diferenciación clara de las estructuras del patógeno y de los tejidos del huésped. Además, distintos índices basados en la localización y especificidad de las estructuras del patógeno y en el tejido del huésped, se evaluaron con el fin de buscar la manera más sencilla y estadísticamente robusta de explicar el progreso del oomiceto en la raíz a través de índices.

En el capítulo 3, se llevaron a cabo experimentos en cámara de crecimiento para describir la patogénesis de la interacción entre *P. cinnamomi* y *Q. ilex*. Se analizaron secciones longitudinales de epidermis, córtex, tejido parenquimático del cilindro central y tejido vascular, a los 1, 3, 7 y 14 días después de la inoculación. Se cuantificó el área total de estructuras, estructuras intracelulares, estructuras extracelulares y estructuras específicas del patógeno. El análisis de estos datos proporcionó una descripción del ciclo de colonización/infección del patógeno, clasificada en tres etapas diferentes relacionadas con su comportamiento trófico. Asimismo se describieron los cambios histológicos resultantes de la presencia del patógeno o de las respuestas desencadenadas por la planta.

En el capítulo 4 se analizó la respuesta diferencial de plántulas de encina ante la inoculación con *P. cinnamomi*, ante la sequía severa y ante ambos estreses combinados. Plántulas de seis meses de edad fueron inoculados y sometidos a falsa inoculación, y la mitad de cada uno de estos tratamientos fue sometida a sequía severa, mientras que el resto fue regada de manera óptima. Semanalmente se midieron los valores de fotosíntesis, conductancia estomática y fluorescencia, y la biomasa total así como la compartimentación de la biomasa fueron cuantificadas al final del experimento. Los datos resultantes mostraron la existencia de diferencias en la respuesta de las plántulas ante la sequía y la inoculación, así como el efecto aditivo de ambos estreses en la muerte de las plántulas.

En el Capítulo 5 se colectaron muestras de suelo de dehesas y se extrajo el ADN total, que se analizó a través de técnicas de metabarcoding [1],

[1] Multiplexión de secuencias mediante la metacodificación de productos de PCR

con el fin de evaluar la composición específica y la diversidad de las comunidades fúngica y de oomicetos, y para estudiar sus relaciones con los síntomas de la enfermedad. La comunidad fúngica presentó una gran variedad de patógenos y abundancia de taxones clave de ectomicorrizas. *Phytophthora* spp. apareció como el taxón dominante dentro de la comunidad de oomicetos, pero las principales especies relacionadas con la podredumbre radicular no fueron las más abundantes, ni presentaron relación directa con los niveles de defoliación. Una unidad taxonómica operacional (OTU) particular, perteneciente al género *Trichoderma*, presentó correlaciones significativas con la escasez de especies patógenas de *Phytophthora* spp. Las diferencias en defoliación se correlacionaron con cambios en la funcionalidad de la microbiota del suelo y con los niveles de diversidad de las especies patógenas.

El Capítulo 6 presenta la discusión general de la Tesis, incluyendo algunos aspectos que limitan los resultados de los trabajos realizados, y nuevas líneas de trabajo que se derivan de esta Tesis, y finalmente, el Capítulo 7 contiene las conclusiones del trabajo.

Los cambios que se producen en la raíz a consecuencia de la inoculación con *P. cinnamomi*, incluyendo las respuestas defensivas, así como la respuesta diferencial identificada con la colonización/infección, conducen a nuevas apreciaciones sobre la causa de la muerte del arbolado. La encina responde al ataque del patógeno, presentando variaciones en la fisiología diferentes de las causadas por el estrés hídrico, las cuales permiten la recuperación de las plantas si no se superpone un estrés adicional a la inoculación. Adicionalmente, el estudio del microbioma del suelo en dehesas con decaimiento del encinar mostró la influencia de la diversidad microbiana en el estado sanitario del arbolado, así como mostró nuevas especies de oomicetos y hongos que deben tenerse en consideración en el manejo del decaimiento de las dehesas de encina en Andalucía.

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Chapter 1.- Introduction

1.1.- What and Why of "dehesas"

"Dehesa" is a specific term referred to a particular savannah-like ecosystem present in the south-west of the Iberian Peninsula (called "Montados" in Portugal). In general, "dehesa" is a traditional land use in areas excluded from agriculture, occupying a large extension of territory in areas mostly characterized by a typical Mediterranean climate, with marked seasonal drought periods and punctual episodes of intense rainfall, and soils usually shallow, acidic and deficient in nutrients (Marañón, 1988).

This ecosystem is mainly composed by open oak woodlands with an annual grassland understory (Figure 1.12), being defined as "*Multifunctional livestock and/or hunting system in which at least 50% of the surface is occupied by grassland, with scattered adult trees producing acorns and with a canopy cover between 5 and 60%*" [1] (Pulido & Picardo, 2010).

Figure 1.1. Example of Andalusian "dehesa". Typical "dehesa" with a mix holm oak-cork oak tree layer and pastures for livestock feeding. Source: Author's archive.

[1] Original version (Spanish): "*Sistema de explotación ganadera y/o cinegética de carácter multifuncional en que al menos el 50% de la superficie está ocupado por pastizal con arbolado adulto disperso productor de bellotas y con una fracción de cabida cubierta entre el 5 y el 60%*".

This description fits with similar ecosystems that can be found in other parts of the world, like genuine savannah ecosystems, *Quercus* savannah in California, North of Africa and other Mediterranean countries, and also similar open woodlands with other species such as carob tree (*Ceratonia siliqua* L.) on the Mediterranean area (Batlle Caravaca & Tous Marti, 1997), or argan tree (*Argania spinosa* L.) on the North of Morocco (Costa Pérez *et al.*, 2006). Other similar ecosystems can be found in Brazil ("cerrados"), Argentina, Australia and New Zealand (Martins & Batalha, 2006; González-Roglich *et al.*, 2015; Veldman *et al.*, 2015; Thrippleton *et al.*, 2018).

Holm oak (*Quercus ilex* L.) and cork oak (*Quercus suber* L.) are the main species dominating "dehesas" and "montados" tree layer. Both species together cover an estimated surface over 4.6 mill ha in the Iberian Peninsula (Figure 2), including 1.42 mill ha of holm oak natural Mediterranean forests (Rodá *et al.*, 2009), over 193,000 ha of *Quercus suber* forests (Díaz Estéban *et al.*, 2009), and over 2.2 mill ha of "dehesas" rangeland ecosystems of *Q. ilex* and *Q. suber* in Spain, plus 869,000 ha in Portugal (Díaz Esteban & Pulido Díaz, 2009).

This tree layer plays a fundamental role in "dehesas" and "montados", stabilizing the system and providing socio-economical uses and ecosystem services or indirect benefits (Olea & San Miguel-Ayanz, 2006). Oaks in "dehesa" contribute to the production with acorns, browse and grassland improvement under crown, providing food and shadow for livestock, and other products such as cork, fuelwood, edible fungus and pollen, contributing decisively to the economical sustainability of farms (Table 1.1).

It also provides for other sociocultural uses and many ecosystem benefits, such as shelter for a high degree of biodiversity (soil microbiota and animals) or soil erosion prevention, being considered a first line barrier against desertification (Horta *et al.*, 2010).

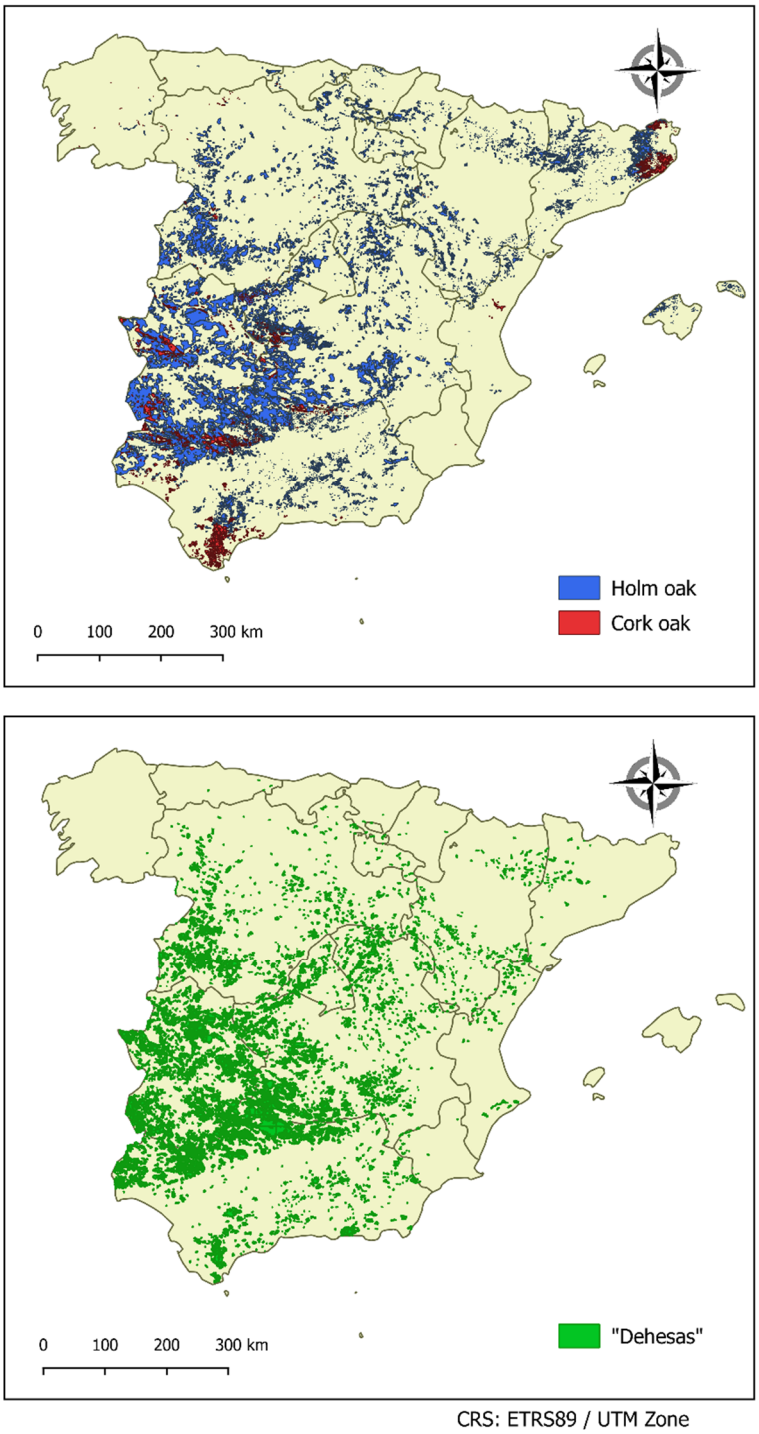

Figure 1.2. Distribution of *Quercus ilex*, *Quercus suber* and "dehesas" in the Spanish territory.

Table 1.1. Major characteristics of tree production in "dehesas". Adapted from Olea and San Miguel Ayanz (2006).

Product	Characteristics	Range
Fuelwood	Pruning, each 10-15 years	8000-5000 kg ha^{-1} $rotation^{-1}$ (DM)
Browse	Pruning or direct browsing	400-1500 kg ha^{-1} (DM)
Acorn	*Q. ilex* and *Q. suber* "dehesas"	200-600 kg ha^{-1}
Cork	*Q. suber* debarking, each 9-12 years	500-1500 kg ha^{-1} $rotation^{-1}$

In general, tree layer on "dehesas" are composed by mature to old trees, with a density ranging between 20 and 100 trees ha^{-1}, which means around 10-50% of canopy cover, being the most representative species – by order – *Q. ilex* subsp. *ballota*, *Q. suber*, *Q. faginea* and *Q. pyrenaica* (Olea & San Miguel-Ayanz, 2006). There are schlerophyllous and perennial or semi-deciduous species, belonging to the Fagaceae family, and presenting strong and very widespread root system, with almost tree growth, but also shrub and bush depending on management practices (Ruiz de la Torre, 2006).

The main threats in "dehesas" ecosystem are related to the loss of tree canopy, due to several causes, such as the lack of regeneration resulting of agricultural intensification and overgrazing, and the effects of global warming, the occurrence of phytophagous plagues, and the presence of new invasive pathogens. Moreover, the problems caused by the abandonment of highly anthropized ecosystems, overexploitation of resources, and others, derived from inadequate management, are linked to the appearance and proliferation of diseases, often derived from the increase in the traffic of people and goods at present (Guzmán Álvarez, 2016). In this sense, since the 1990's decade, the holm oak decline is considered a major threat for the "dehesas" sustainability. It is considered that root rot causes important economic and ecological losses on those ecosystems, estimated in 500,000 trees and over 1 mill € per year (Senado, 2010).

- The "dehesas" in Andalusia

From the over 3 mill ha of "dehesas" present in the Iberian Peninsula, more than 1.2 mill ha belong to Andalusia (Fig. 3) (Costa Pérez *et al.*, 2006), accounting for over 14% of its territory – 23% of the Andalusian forest land (Fernández Rebollo *et al.*, 2014). The "dehesas" are mainly located in the northern part of the provinces of Jaén, Córdoba and Sevilla, the province of Huelva, the Ronda Mountains and North-west of Cadiz.

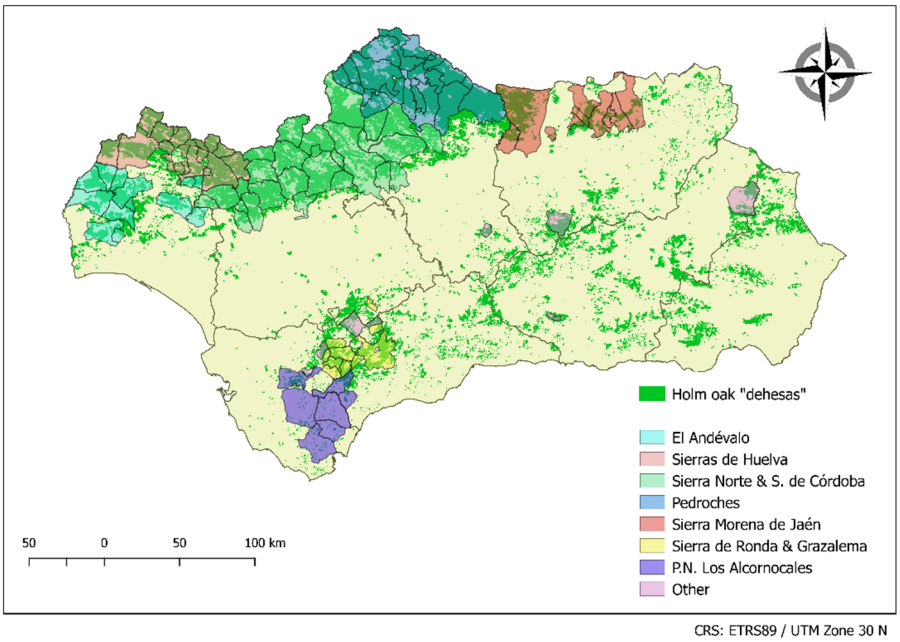

Figure 1.3. Distribution of "dehesa" territory in Andalusia with "Dehesa counties" grouped by geographical zones. Modified and actualized from CAP (2008).

The relevance of "dehesas" in Andalusia is highlighted on a specific area, comprising the north of Sevilla and Córdoba provinces, Huelva and "Los Alcornocales" and "Sierras de Ronda y Grazalema", located between Málaga and Cádiz provinces, and in less extent, some areas in the north of Jaén. Those areas conforming the so-called "Dehesa Counties[2]", in which "dehesas" occupy more than 25% of the county territory (CAP, 2008). These

[2] *"Municipios de la dehesa"*

counties account for the 27% of the total surface of Andalusia, but only the 6.5% of the total population (data not actualized), presenting a population density below 15 habitants km^{-2}. Therefore, these areas are considered rural zones with high risk of population loss, being "dehesas" the major provider of economic benefits, and the main tool to ensure the sustainability of the rural economy.

The main uses of the farms and yields in the Andalusian "dehesas" is the livestock farming, with some important agricultural crops, but "dehesas" are generally characterized by a wide list of services and other yields provided, which includes - by relevance order - hunting, fuelwood, cork in the case of cork oak "dehesas", beekeeping and mushroom harvesting, as the commonest. Also, provides many social and cultural uses, being associated the "dehesas" and the holm oak to a lot of folkloric cultural and religious expressions, as for instance "Romerías". Related to their natural values and cultural traditions, tourism is considered other promising activity for the future sustainability of many "dehesas" (Costa Pérez *et al.*, 2006).

Regarding ecosystem services, "dehesas" showed special relevance on this area as a biodiversity reservoir, and barrier against desertification. The high number of different plant and animal species presents in the Andalusian "dehesas", and the role of trees on landscape heterogenicity, providing resources and refuge for many species, are reasons to consider the Andalusian "dehesas" as resilient ecosystems with a high value as biodiversity reservoir (Moreno *et al.*, 2018). On the other hand, it is considered that Andalusia and overall the south of Iberian Peninsula is one of the European territories that might be the most severely affected by Climate Change (Navarro-Cerrillo *et al.*, 2018). The extreme climate variability associated to anthropogenic global warming is considered responsible of irreversible landscape changes (Watson *et al.*, 2018), being directly related to tree mortality (Allen *et al.*, 2010, 2015). On this sense, the character as drought-tolerant species of *Quercus ilex* L. (Quero *et al.*, 2011),

and the tree layer structure of "dehesas", converts this resilient ecosystem into the first barrier against desertification in Andalusian territory.

However, since the 1960's decade, the rural area of "Dehesa Counties" have been influenced by the decline of the rural population, the abandonment of agriculture, and the intensification of livestock and agricultural practices, prevailing the search of economic benefit over the preservation of natural and traditional values. These factors are the origin of important changes in management practices of "dehesas", which have been related to the rise in phytosanitary problems (CAP 2008).

1.2.- The holm oak decline

- *Phytophthora* spp. are emerging forest pathogens

Alien-invasive species and climate change are nowadays major threats for ecosystems sustainability around the world. The current ease of people mobility and the increase of international trade and transport of goods have facilitated the spread of pathogenic species in locations far from their original distribution areas (Prospero & Cleary, 2017). It is possible to found examples of different die-back syndrome of woody species devastating natural or semi natural ecosystem in all the continents because of both factors. One of the best-known examples is the destructive chestnut canker caused by *Cryphoectria parasitica* (Murrill) Barr. in North America and Europe, or the Dutch elm disease caused by *Ophiosthoma ulmi* (Buisman) Melin & Nannf. and *O. novo-ulmi* Brasier (Liebhold *et al.*, 2017). Climatic change could influence forest die-back in different ways, through the change of environmental conditions, displacing species out of their normal distribution range, as it is the case of *Pinus sylvestris* and *Pinus nigra* decline in South Spain (Navarro-Cerrillo *et al.*, 2016), or interacting with forest pathogens, increasing host susceptibility, and the host range of some pathogens (Sturrock *et al.*, 2011).

In the last decades, different pathogenic *Phytophthora* spp. and *Pythium* spp. are emerging as important invasive pathogen species, considering the environmental losses they cause (Liebhold *et al.*, 2017; Sena *et al.*, 2018). Some examples are the case of *Phytophthora ramorum* Werres on Sudden Oak Death, affecting tanoak (*Notholithoarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S.H.Oh) and several oak species in California (Rizzo & Garbelotto, 2003), *Phytophthora cinnamomi* Rands. on eucalyptus and Jarrah ecosystem in Australia (Burgess *et al.*, 2017), or *Phytophthora cryptogea* Pethybr. & Laff., *Phytophthora cambivora* (Petri) Buisman and *P. cinnamomi* causing ink disease on chestnuts worldwide (Vettraino *et al.*, 2005).

Among the most relevant diseases caused on European woody species by *Phytophthora* spp., root rot has been identified since the 1990's affecting oaks (Thomas *et al.*, 2002). Sessile and pedunculate oaks (*Quercus petraea* (Matt.), Liebl. and *Quercus robur* L.) from North and Central Europe are threatened by some *Phytophtora* spp. (Jung *et al.*, 2000), and in the Mediterranean basin, the decline of holm oak (*Quercus ilex* L., subsp. *ballota* in Spain and Portugal, and subsp. *ilex* in central Italy) and cork oak due to root rot was identified since the early 1980's, affecting wide forest areas in Spain, Portugal, France and Italy (Brasier, 1996; Vettraino *et al.*, 2002; Navarro Cerrillo *et al.*, 2004).

In the case of *Quercus ilex* subsp. *ballota*, it is considered that root rot caused by *P. cinnamomi* is the main factor of the decline (Brasier *et al.*, 1993; Sánchez *et al.*, 2002), being holm oak the most susceptible species to the attack of this pathogen among *Quercus* spp. (Moralejo *et al.*, 2009). *Phytophthora cinnamomi* is a very widespread invasive plant pathogen, considered as one of the major threats for many different ecosystems and agricultural crops around the world (Burgess *et al.*, 2017). It presents a wide host range, with approximately 5000 different host species, including woody, shrubs and perennial or annual herbaceous plants (Hardham & Blackman, 2018).

The lifecycle of *P. cinnamomi* presents both asexual and sexual reproductive cycle, with two mating types. It can growth in infested soils feeding on litter and colonizing asymptomatic hosts, persisting as chlamydospores, and oospores (Figure 1.4) when mating types A1 and A2 were present (Hardham, 2005), and growing in plant tissue of asymptomatic annual herbaceous plants, releasing chlamydospores and stromata to the soil after natural senescence of plants (Crone *et al.*, 2013). When conditions are optimal, pathogen structures encyst, germinating sporangia and infecting new hosts through biflagellate zoospores. Its asexual lifecycle can be repeated several times in a short lapse, depending on the host, greatly amplifying the inoculum concentration in the soil on local climatic episodes.

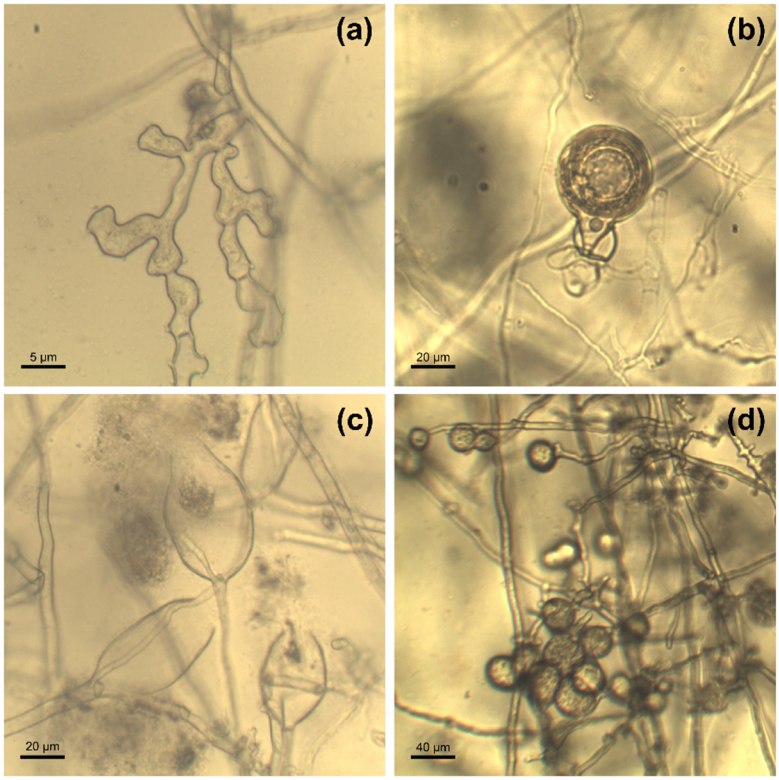

Figure 1.4. *Phytophthora cinnamomi* structures under optic microscopic examination. (a) Coraloid hyphae on soil extract liquid (SE) medium. (b) Oospore with amphigynous antheridia on potato dextrose agar (PDA) medium. (c) Sporangia after zoospore release in SE medium with nested proliferations. (d) Chlamydospores cluster on carrot agar (CA) medium. Source: Author's archive.

It can survive in adverse conditions through resistance structures over 6 years, and interacts with plant hosts in different ways: as biotroph in asymptomatic hosts, hemibiotroph or necrotroph in susceptible hosts, and saprotroph feeding on litter in wet soils (Hardham, 2005; Cahill *et al.*, 2008; Crone *et al.*, 2013). All these conditions promote the long-term stability of soil inoculum presence, even in soils with extreme moisture conditions, explaining the relationships of severity symptoms in holm oak decline with extreme climatic events, both severe drought or rainfall episodes, which often occurs in the Mediterranean climate (De Rigo & Cadullo, 2016).

Apart from *P. cinnamomi*, other oomycete species have been linked with holm oak root rot in Spain. *Phytophthora quercina* T. Jung and T.I. Burgess , *Phytophthora psychrophilla* Jung and *Phytophthora cactorum* (Lebert & Cohn) J. Schröt, have been isolated on declining stands of holm oak in the east of the Iberian Peninsula, related to the root rot (Pérez-Sierra *et al.*, 2013; Català *et al.*, 2017). Additionally, *Pythium spiculum* Paul, *Pythium irregulare* Buisman, *Phytophthora gonapodyides* (H.E. Petersen) Buisman and *P. cryptogea* have been isolated on declining stands of "dehesas" from Andalusia and Extremadura, associated with high severity of decline symptoms (Sánchez *et al.*, 2005; Romero *et al.*, 2007a; Corcobado *et al.*, 2010; Ruiz Gómez *et al.*, 2016).

- The holm oak decline in the "dehesas" ecosystems

Forest decline is a complex multifactorial disease influenced by several external biotic and abiotic stressors (Manion & Lachance, 1992). Agreeing with this definition, it might be considered that extreme climatic variations, incorrect management practices and alien invasive pathogens are agents involved in the holm oak decline. The environmental and economic losses due to holm oak decline are amplified in the "dehesa" rangeland ecosystem, due to the confluence of factors affecting their sustainability, such as: i) intensification of agricultural practices and livestock farming, which includes soil erosion, and overgrazing; ii) land withdrawal, lack of regeneration of trees and shrub invasion; and iii) the

direct effects of Climate Change, which can cause the displacement of holm oak and cork oak from certain distribution areas in the south-west of the Iberian Peninsula (CAP, 2008; Gea-Izquierdo *et al.*, 2013; Gil-Pelegrín *et al.*, 2017). Despite the low canopy cover and the subsequently discontinuity in the tree layer of "dehesas" promotes defence against plagues, reducing severity of damage and increasing resilience, in the last decades the holm oak decline threatened the sustainability of Spanish "dehesas" (Serrada, 2008).

The holm oak is considered an emblematic species in the Iberian Peninsula, with high cultural and ecological value. Together with the socioeconomical importance of "dehesas", these are enough reasons to carry out important efforts on the study of the holm oak decline. An increasing number of scientific works have been published in the topic about this interaction since 1990. The main scientific efforts have been focused on experimental issues, including: i) the study of the causal agents involved in root rot and their pathogenicity; ii) the evaluation of the severity of holm oak decline and the consequences on the environment; iii) the prediction of the widespread and future habitat projection for the host and the pathogen; and iv) the influence of specific management practices on the symptoms and the spread of disease (Gallego *et al.*, 1999; Sánchez *et al.*, 2005; Romero *et al.*, 2007b; Gómez-Aparicio *et al.*, 2012; Ríos *et al.*, 2018; Cardillo *et al.*, 2018; Duque-Lazo *et al.*, 2018).

Other works have been carrying out different approaches, studying the influence of ectomycorrhizal fungi (EcM) on the root rot (Corcobado *et al.*, 2014, 2015) or using proteomic and genomic studies to identify signalling or molecular markers related to susceptibility or tolerance (Turco *et al.*, 2004; Horta *et al.*, 2010; Sghaier-Hammami *et al.*, 2013). But in general, the main works found in the reviewed literature are empirical approaches based on the observed symptoms of holm oak decline, which are mostly unspecific (chlorosis and wilting of foliage, defoliation, epicormic trunk sprout, cankers…) and are visible only when the root rot is widely spreaded.

1.3.- Effects and causes of holm oak root rot: host-pathogen interaction

Defining symptoms and degrees of severity in oak decline caused by root rot is a challenge. The root rot shows a high variety of symptoms degree, depending even on the season and climatic conditions. Root rot could drive to the death of trees through a slow decline process – regressive death – or in some cases through the collapse of the tree in a short period – sudden death (Figure 1.5) (Navarro Cerrillo *et al.*, 2004), but also, recovering of high defoliation degree trees after favourable climatic conditions have been noticed.

- Mechanisms of the interaction at histological level

Little is known about the specific interaction between *Q. ilex* and *P. cinnamomi* at microscopic, metabolomic and molecular level. The underlying infection mechanisms altering the plant metabolism, growth patterns, or biomass and photosynthates reallocation on infected plants (which are identified in other susceptible hosts) remain unknown in this case.

In the study of fungal phytopathogens, it is common to evaluate pathogenicity through histological techniques. Also, indices such as the number of spores, survival structures, stem or trunk lesions, pustules, and other reproductive structures that are present in the affected tissues are often used to evaluate the different degrees of severity of the disease or virulence of the pathogen (Emeran *et al.*, 2011; Jagger *et al.*, 2011).

Although several histology works can be found for other species of the same genus (Cahill, 1989; Blaschke, 1994), they are not found for *Quercus ilex*, despite their socioeconomical and ecological importance. Plant histology techniques might be a tool to identify some unspecific defensive responses of plants such as the accumulation of substances in xylem vessels, cell-wall thickening and the accumulation of phenolic compounds in tissues, which are common responses of vascular plants against root colonization, being observed in other *Quercus* spp. (Oßwald *et al.*, 2014).

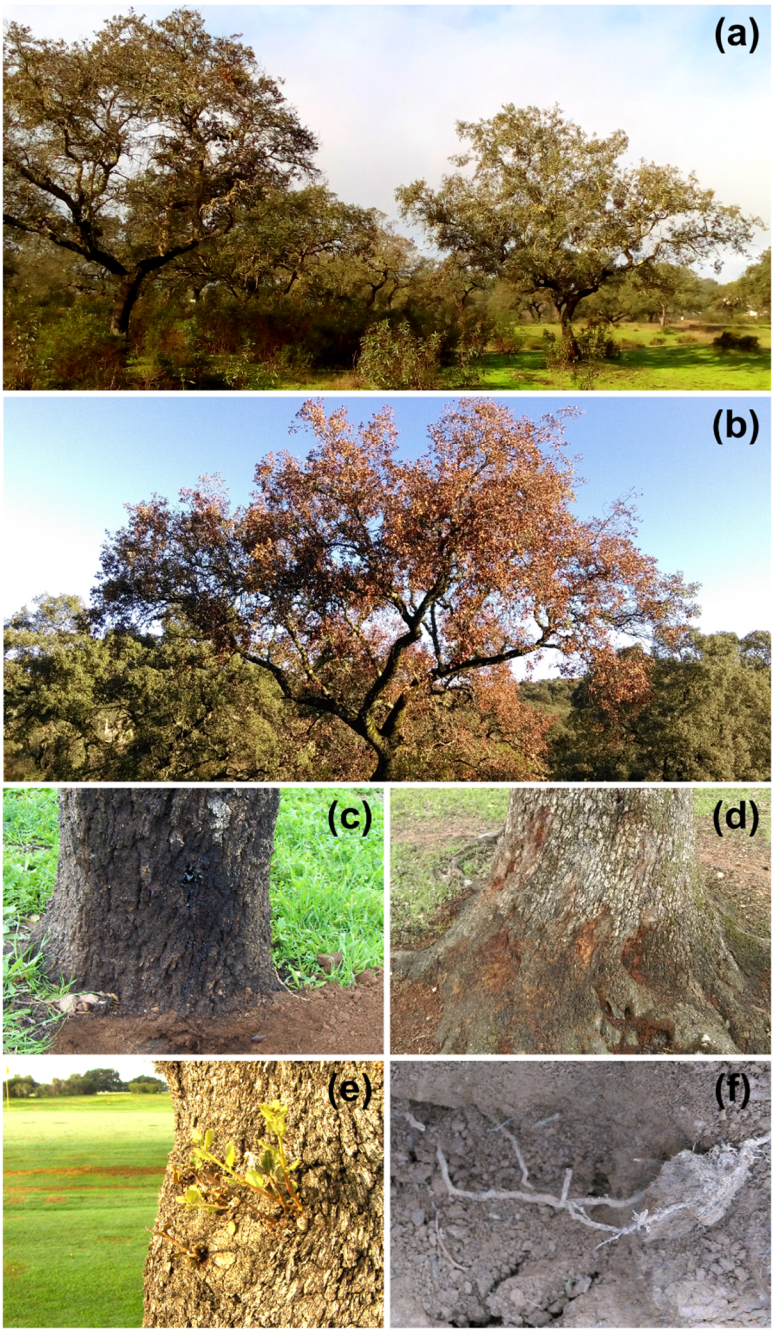

Figure 1.5. Holm oak main decline symptoms. (a) regressive decline with progressive defoliation. (b) sudden death. Blighted foliage remains on the tree crown. (c) canker at the base of the trunk. (d) galleries of wood drilling insects with sawdust at the base of the trunk. (e) epicormic sprouts on the trunk. (f) fine root loss on superficial absorbent roots. Source: Author's archive.

These general responses can occur after root colonization by *Phytophthora* spp., mediated by other signals, for example osmotic imbalance of plants (Oßwald *et al.*, 2014), and might be a useful way to assess tolerance of plants to root rot.

Moreover, holm oak remains considered an orphan species mostly at the molecular level (López-Hidalgo *et al.*, 2018). On the last decades some works based on -omics approaches have been carried out to assess the intraspecific variability of this species (Valero Galván *et al.*, 2012; Jorrin-Novo & Navarro Cerrillo, 2014; Guerrero-Sanchez *et al.*, 2017; Fernández i Marti *et al.*, 2018), but there is only one work found in the reviewed literature including *P. cinnamomi* inoculation as a factor in the analysis of the molecular response of *Q. ilex* subsp. *ballota* (Sghaier-Hammami *et al.*, 2013).

- Alterations on plant physiology due to root rot

The symptoms of root rot are often associated with water stress, mainly due to the loss of absorbent fine roots (Oßwald *et al.*, 2014; Hardham & Blackman, 2018), altering tree physiology, reducing stomatal conductance and thus, photosynthesis rate (Sghaier-Hammami *et al.*, 2013; Swiecki & Berndhart, 2017; Corcobado *et al.*, 2017). Although non-specific immune responses are recognized to date on the interaction between holm oak and *P. cinnamomi*, some signs have been identified in the physiological response of holm oak to root rot, which indicates the existence of specific mechanisms altering plant physiology. It has been shown that the hydric response of plant is decoupled of the photosynthetic one, often presenting inoculated trees high water use efficiency (Oßwald *et al.*, 2014).

Moreover, there is a high variability of results in the evaluation of physiological changes in holm oak seedlings as a response to *P. cinnamomi* infection, depending on the experimental conditions. Some reports have shown strong differences in photosynthesis and water potentials between inoculated and mock-inoculated plants (Corcobado et al. 2014; Sghaier-Hammami et al. 2013), whereas others found no significant effects of

inoculation on the final physiological status (León et al. 2017; Maurel et al. 2001; Turco et al. 2004), depending on whether the plants were subjected to continuous or occasional waterlogging, drought stress, or only slight stress (both drought and flooding). This variety of results includes high differences in the die-off rate of saplings in controlled condition tests.

On the other hand, not only drought, but also waterlogging treatments have shown significant effects on the mortality of infected plants (Corcobado et al. 2013, 2014; Swiecki and Berndhart 2017). Apart from the positive effect that waterlogging has in the *Phytophthora* reproduction cycle, flooding might be an important agent altering tree physiology and health status of the plant. It has been demonstrated that oxygen deprivation under flooding conditions reduced fine root growth and volume, taproot rot and toxic substances accumulation in other *Quercus* spp. (Bourgeade *et al.*, 2018).

Briefly, it is difficult to characterize the physiological response of the plant to the pathogen infection avoiding mixed effects of external factors, even under controlled conditions, if the underlying process of the interaction remains unknown. However, all the possible alterations in seedling physiology should involve a sensible change in the growth patterns, which may be useful tools to characterize plant responses to different stressors, in particular, biomass allocation as a key factor (Poorter *et al.*, 2012).

Related to this, differences in root traits have been linked to key processes that vary in changing environmental conditions (photosynthesis and photosynthates allocation, drought tolerance strategies, water potentials, transpiration rates…) (Lopez-Iglesias *et al.*, 2014; Chen *et al.*, 2017). The root functional traits are often used to analyse ecological, ontological or physiological differences among species, ecotypes or different stress conditions (Olmo *et al.*, 2014; Bongers *et al.*, 2017).

1.4.- Environmental factors influencing holm oak decline: The biological component of the soil

Once recognized the influence of environmental factors as agents of holm oak decline, different approaches can be used to evaluate their impact. For spatially distributed climatic, edaphic and topographic variables, spatial distribution models and habitat prediction models are powerful tools to evaluate the probability of disease and to implement accurate management policies (Duque-Lazo *et al.*, 2018). Additionally, the plant community diversity and specific composition is linked with the probability of *Phytophthora* root rot occurrence (Rodríguez-Molina *et al.*, 2005; Gómez-Aparicio *et al.*, 2012; Avila *et al.*, 2017).

It is recognized the influence of soil microbiota in the health status of plant community in several ways, interacting as commensal, pathogenic, or mutualistic organisms. Plant community is closely related to soil fungal community (Heijden *et al.*, 2007; Chen *et al.*, 2018), being influenced by soilborne plant pathogens and also beneficial ones, such as EcM and arbuscular mycorrhizal (AM) species, which enhance water and nutrient uptake of roots; saprobes improving soil fertility and nutrient availability; fungal, insect and nematode parasites controlling plant pests; and other endophytes acting as antagonist of plant pathogens(Pérez-de-Luque *et al.*, 2017; Tamayo-Vélez & Osorio, 2018).

Particularly, the presence and abundance of EcM and AM species are important below-ground characteristics related with fine root abundance or the establishment of seedlings in deciduous and semi-deciduous oak forests (Dickie *et al.*, 2004; Mosca *et al.*, 2017). In the case of holm oak, it has been demonstrated that EcM fungi are linked with changes in health status of trees in declining stands (Corcobado *et al.*, 2014, 2015)..

On the other side, regarding the relationships of soil community and *Phytophthora* spp., two principal effects have been identified, aligned in first place with the niche competence, and in second place, through the presence of antagonist or parasite species. Despite the ability of *P.*

cinnamomi to adapt to changing conditions, it is known that it is a poor competitor under saprophytic conditions (Dunstan *et al.*, 2010). It can be hypothesized that in soils with high variability of species root rot caused by oomycetes might be less severe.

Several antagonist and parasitic relationships have been identified between *Trichoderma* spp. and other endophytes with many *Phytophthora* spp. *Trichoderma* spp. are opportunistic, avirulent plant symbionts with recognized parasitic or antagonist functions against phytopathogens, being one of the most frequently isolated fungus in soils. Their effects in the biocontrol strategies are due to several effects: i) competence with other microbial and fungal species in soil community; ii) plant root colonization; iii) plant growth promotion; and iv) induction of plant-defence responses. Also, the genus *Trichoderma* include some species with the ability to act as mycoparasites (Vinale *et al.*, 2008).

Trichoderma asperellum Samuels, Lieckf. & Nirenberg, *Trichoderma hamatum* (Bonord.) Bain. and *Trichoderma virens* (J.H. Mill., Giddens & A.A. Foster) Arx. have been isolated from holm oak roots (*Q. ilex* subsp. *ilex*) in nurseries of Italy, being demonstrated their effect in different combinations, reducing root rot of seedlings in a controlled experiment including *Phytophthora nicotianae* Breda de Haan and *P. cinnamomi* (Aleandri *et al.*, 2015). *Trichoderma asperellum* has also been proved as antagonist of *Phytophthora ramorum* Werres, De Cock. & Man. in't Veld sp. nov. in *Viburnum tinus* L. (Widmer & Shishkoff, 2017) and *P. nicotianae* in sweet pepper (*Capsicum annum* L.) (Ros *et al.*, 2017), and *T. hammatum* was effective on the control of *Phytophthora capsici* Leonian, in Chili pepper (Chemeltorit *et al.*, 2017). *Trichoderma harzianum* Rifai reduced mycelial growth of *Phytophthora infestans* (Mont.) de Bary in laboratory tests (García-Núñez *et al.*, 2017) and leaf fall caused by *Phytophthora palmivora* Butler in rubber trees (*Hevea brasiliensis* Muell. Arg.) (Promwee *et al.*, 2017). Moreover, it is proved their antagonistic effect in numerous fungal pathogenic species (Studholme *et al.*, 2013; Siddaiah *et al.*, 2017; Kong & Hong, 2017; Haddad *et al.*, 2017).

1.5.- Thesis objectives and structure

The high plasticity of the *Phytophthora* spp. behaviour depending on host and environment conditions, together with the singularities of the holm oak and the "dehesa" ecosystem, and the evidence of the influence of many different oomycete species, encourages the deep study of the pathogen-host system to better understand the triple interaction between host-pathogen-environment. Histopathology of infection and colonization process, and the physiological and biomass allocation responses are aspects that remain unclear. Likewise, the scarcely information about the influence of soil biota in the disease, together with the evidence of antagonism and parasitic relationship between several endophytes and *Phytophthora* spp. encourages the study of the soil fungal and oomycete communities structure and functionallity of declining "dehesas".

Basic knowledge is necessary not only to understand complex processes, but also to drive scientific efforts to answer the right questions. Knowledge of the interaction at tissue level and growth responses are useful tools to better evaluate the interaction between holm oak and *P. cinnamomi*. To guarantee the preservation of the genetic resources of the species it is necessary to promote the adaptation ability searching for the favourable characteristics in front of the different biotic and abiotic stressors (Jorrin-Novo & Navarro Cerrillo, 2014), being fundamental to know the interaction mechanisms between the host and the pathogen, but also the influence of other environmental factors for future susceptibility studies and management strategies to mitigate tree decline.

- Thesis objectives

This work is focused on the study of the interaction between *Quercus ilex* L. and the oomycetes causing root rot –mainly *Phytophthora cinnamomi* Rands. – at histological and physiological level, exploring the influence of biological components of soil in the severity of the disease symptoms. For this purpose, four specific objectives were stated:

1. Set up of histology techniques and image analysis to assess evolution and severity of *Phytophtora cinnamomi* colonization and infection in fine roots of *Quercus ilex* subsp. *ballota* seedlings.
2. Analysis of the histopathological process of *Phytophthora cinnamomi* colonization and infection of fine roots in *Quercus ilex* subsp. *ballota* seedlings.
3. Study of the physiological response and the biomass allocation parameters of *Quercus ilex* subsp. *ballota* seedlings under combined acute drought and *Phytophthora cinnamomi* infection.
4. Evaluation of diversity and composition of soil fungal and oomycete communities, and their relationship with decline symptoms in declining "dehesas" of Andalusian territory.

- Structure of Thesis document

To present the results related with the objectives of this work, the present thesis document is structured in seven different chapters:

Chapter one is an introductory section, which presents in first place a brief description of the main characteristics and problems of holm oak decline in the Andalusian "dehesas". Also, the main results of the published scientific work involving *P. cinnamomi* and holm oak were reviewed, focusing in the basic aspects of the interaction that remains unknown or unclear.

In the **second chapter**, plant histology techniques were adapted to the host-pathogen system, new image processing methodology was implemented, and results were statistically analysed to develop new histological indices, which allowed us to describe the colonization and infection process of holm oak fine roots by *P. cinnamomi*. Seedlings were inoculated, and root samples were taken at 1, 2, 3, 4, 7 and 14 days post inoculation (dpi). Samples were fixed with different solutions and embedded both in paraffin and histology resin, and sections of different

thickness were analysed to find the best resolution to identify pathogen structures without altering plant tissue structures. The results of this work were published in a methodology paper in Plant Methods (ISSN: 1746-4811; Q1 "Plant Sciences").

Chapter three describes a controlled-conditions experiment in growth chamber, in which holm oak seedlings were inoculated with *P. cinnamomi*, and histological methods developed in Chapter 2 were used to describe the process of colonization and infection in fine roots through the evaluation of proposed indices in different plan tissues. Also, unspecific defence responses were identified using different staining methodologies. The results allowed us to propose a conceptual model for the infection cycle into the roots, leading to new hypothesis about the physiological responses in infected plants. This work has been published in Plant Pathology (ISSN: 0032-0862, Q1 "Agronomy").

Chapter four studies the physiologic response of *Quercus ilex* seedlings to acute drought and root rot, both factors analysed individually and combined in a complete multifactorial greenhouse experiment. Seedlings were inoculated, and mock inoculated with *P. cinnamomi*, and photosynthesis, stomatal conductance and fluorescence were measured for 30 days. At the end of the experiment, roots were evaluated and scanned in a densitometer, to analyse root traits using WinRHIZO Pro 2004a, and biomass allocation parameters of above and belowground plant organs were calculated to assess differential response to each stress. The results were sent to FORESTS (ISSN: 1999-4907, Q2 "Forestry") for their consideration to be published.

On **chapter five** was described a survey of "dehesas" biological soil component. Plots were chosen within the "Red SEDA" Environmental Monitoring Network (ICP Forest, level I) along the main distribution area of holm oak "dehesas" in Andalusia. Soil samples were taken for declining holm oak stands and were analysed through metabarcoding and NGS sequencing techniques - Illumina MiSeq - to evaluate the diversity and

specific composition of fungal and oomycete communities. Moreover, the different Functional Guilds were classified, and both taxonomy and functionality of soil community were analysed comparing between different ecological areas and defoliation degrees. Also, specific key taxa were found, and antagonist relationships between *Trichoderma* spp. and *Phytophthora* spp. abundance were described. The work has been sent to the New Phytologist journal (ISSN: 0028-646X, Q1 "Plant Sciences") for their publication.

The synthesis and the general discussion of the works that compose this PhD Thesis are presented in **Chapter six**, including considerations to explain key host-pathogen interaction aspects, and exploring new ways to management in affected areas.

Finally, **Chapter seven** correspond to the list of conclusions derived of the works composing this PhD Thesis.

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Chapter 2.- New methodology to evaluate infection and colonization of *P. cinnamomi* on *Q. ilex* roots

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Abstract

Phytophthora cinnamomi Rands. is an important root rot pathogen widely distributed in the north hemisphere, with a large host range. Among other diseases, it is known to be a principal factor in the decline of holm oak and cork oak, the most important tree species in the "dehesa" ecosystem of south-western Spain. Previously, the focus of studies on *P. cinnamomi* and holm oak have been on molecular tools for identification, functional responses of the host, together with other physiological and morphological host variables. However, a microscopic index to describe the degree of infection and colonization in the plant tissues has not yet been developed. A colonization or infection index would be a useful tool for studies that examine differences between individuals subjected to different treatments or to individuals belonging to different breeding accessions, together with their specific responses to the pathogen. This work presents a methodology based on the capture and digital treatment of microscopic images, using simple and accessible software, together with a range of variables that quantify the infection and colonization process.

Resumen

Phytophthora cinnamomi Rands. es un importante patógeno de podredumbre radicular ampliamente distribuido en el hemisferio norte, con un gran número de huéspedes. Entre otras causas, es conocido por ser el principal factor en el decaimiento de encinas y alcornoques, las especies arbóreas más importantes de los ecosistemas adehesados del suroeste de la Península Ibérica. Previamente, el objetivo de los estudios sobre *P. cinnamomi* y la encina se han centrado en la identificación a través de técnicas moleculares, las respuestas funcionales del huésped, junto con otros estudios de variables morfológicas y fisiológicas del huésped. Sin embargo, no se ha desarrollado ningún tipo de índice microscópico que describa el grado de infección y colonización en los tejidos de la planta. Un índice de colonización o infección podría ser una herramienta útil para los estudios que examinan las diferencias entre individuos sometidos a diferentes tratamientos o procedentes de diferentes cultivares, junto con la respuesta específica hacia el patógeno. Éste trabajo presenta una metodología basada en la captura y el tratamiento digital de imágenes de microscopía, utilizando software sencillo y accesible, así como un conjunto de variables capaces de cuantificar el proceso de colonización e infección.

Keywords: *Phytophthora*; Holm oak decline; Microscopy; Histology; Root rot; Infection degree.

2.1.- Introduction

Phytophthora cinnamomi Rands. is an oomycete belonging to the family *Peronosporaceae*[1] (EPPO, 2004) that causes root rot in many woody, herbaceous, and shrub species. This pathogen is one of the most common phytopathogens in nature, with the number of potential hosts estimated at more than 3000 (Hardham, 2005). *Phytophthora cinnamomi* plays a role as one of the main factors participating in the decline syndrome of *Quercus* (Brasier *et al.*, 1993; Brasier, 1996; Gallego *et al.*, 1999; Sánchez *et al.*, 2002, 2005; Navarro Cerrillo *et al.*, 2006; Romero *et al.*, 2007).

Holm oak (*Quercus ilex* L.) is the most common forest species in the Mediterranean basin and its surrounds (Ruiz de la Torre, 2006), and is the most widespread species in Spain covering an area of over 4 million hectares (Pérez *et al.*, 2009). This oak is the main species of the "dehesa" forest systems in the south-western portion of the Iberian Peninsula, which is of vital importance to animal husbandry in these ecosystems. Moreover, holm oak is one of the *Quercus* species with the highest susceptibility to *P. cinnamomi* (Robin *et al.*, 2001; Maurel *et al.*, 2001; Moralejo *et al.*, 2009), which accounts for the importance of studying the interaction between these species, especially considering the growing number of cases of declining holm oaks that have been detected in the "dehesas" since the 1990s.

Previous studies have used different approaches to explore pathogen-host relationships, that could be classified as (i) basic determination techniques, (ii) molecular techniques, (iii) morphophysiologic variable measurements, and (iv) experiments based on survival under stressful conditions. Basic techniques include the microscopic observation of isolated pathogens from infected tissue (Brasier, 1996; Sánchez *et al.*, 2002; Sánchez Hernández *et al.*, 2003; Romero *et al.*,

[1] The original version of published article mentions *Pythiaceae*, information provided by EPPO in 2012.

2007). Currently, pathogen detection trough molecular analysis is an important field of study. ELISA, PCR or isozyme patterns have recently been used in this approach (Hardham, 2005). Morphophysiological studies include experiments on sapling development under controlled conditions (Sánchez *et al.*, 2005; Serrano *et al.*, 2010), and have assessed the effectiveness of potential treatments, such as phosphonates in the presence or absence of the pathogen (Navarro Cerrillo *et al.*, 2006; Pérez *et al.*, 2009; Solla *et al.*, 2009). Moreover, it is relevant to highlight practical-focused experiments, which are based on the management and modification of novel and traditional cultural practices (Navarro Cerrillo & Fernández, 2000; Navarro Cerrillo *et al.*, 2004; Rodríguez-Molina *et al.*, 2005).

Several studies have been conducted to assess the pathogen-host interaction at a histological level (Cahill, 1989; Blaschke, 1994; Brummer *et al.*, 2002; Pires *et al.*, 2008), but no quantitative index that assesses the extent of *P. cinnamomi* infection and colonization at the tissue level has been presented for any forest species, as the morphological characteristics of oomycetes and their way of life render it difficult to apply this type of index (EPPO, 2004; Hardham, 2005).

In the study of fungal phytopathogens, it is common to use indices such as the number of spores, survival structures, stem or trunk lesions, pustules, and other reproductive structures that are present in the affected tissues (Rubiales & Moral, 2011; Emeran *et al.*, 2011; Jagger *et al.*, 2011). These indices assess the level of infection in tissues and can reveal differences between individuals subjected to specific treatments or that exhibit different levels of resistance or susceptibility to the pathogen. An alternative method for quantifying the level of tissue infection and colonization by pathogens includes a combination of microscopic techniques and digital analysis of images, and includes the application of stereological techniques in microscopy (Stark *et al.*, 2011; Tschanz *et al.*, 2011). The use of stereological techniques in histological analysis requires the use of many equations and specialized statistical processing, which entails a highly specialized staff and a substantial investment of time and

resources (Tschanz *et al.*, 2011). However, certain plant tissues present a level of structural simplicity that makes the precise digitalization of their structures easy through the use of simple computer tools. This allows for the transformation of the structures that result from the interactions between the host and the pathogen into quantitative data.

The objective of this study was to establish a specific methodology to determine the level of *P. cinnamomi* infection and colonisation in *Q. ilex* root tissues by applying computer techniques to process microscopic images, allowing us to obtain quantitative data and indices.

2.2.- Material and Methods

- Plant material and growing conditions

The plant material was comprised of 6 month-old saplings of *Q. ilex* L. subsp. *ballota* (Desf.) Samp., grown from acorns gathered at "La Jarosa" farm (Córdoba, Spain. The Universal Truncated Mercator coordinates were 37,893-37,924 N, 4,945-4,901 W). The acorns were sterilized (5 minutes in a 1 % NaClO solution), and germinated on perlite in a growth chamber (24 °C, 70 % relative humidity (RH), 12-h photoperiod). Seven days after germination, they were transferred to 32 cell seed trays containing 400 cm^3 perlite and placed in a growth chamber (22 °C, 60 % RH, photoperiod 14 h of light/10 h in the dark), and watered twice weekly with Hoagland's solution (Hoagland & Arnon, 1950).

- Inoculation

Prior to inoculation the perlite was gently removed from the roots by washing in water, the roots of the samples were then submerged in a solution of liquid V8 medium that contained an A2-type isolate of *P. cinnamomi* (Pe-90), provided and certified by NBT laboratories (New Biotechnics, Sevilla, ISO 9001/17025). The pathogen was grown in Petri dishes containing 20 ml of V8 solution, in a growth chamber at 20 °C in

darkness. After 15 days the dishes were superficially washed and the mycelium in V8 medium was crushed in a mixer with distilled water (33 ml per dish). The final concentration of millet inoculum was adjusted counting chlamydospores as a reference and diluting it to 25 IU/μl.

After 10 minutes in the V8 medium, the samples were returned to the 32 cell seedling trays containing sterilized perlite, and were maintained in the growth chamber as described above. The control plants were not inoculated, but there roots were immersed in sterilized water.

- Sample processing

The inoculated plants were examined at 1, 3, 7, and 14 days after inoculation (*dai*) by taking samples from the absorbing roots (thin secondary rootlets of $\varnothing \leq 2$ mm) that would exhibit the symptoms of infection described for root rot caused by *P. cinnamomi* (Cahill, 1989; Blaschke, 1994; Sánchez *et al.*, 2002, 2005; Balci *et al.*, 2008). At each sampling time, root samples from three inoculated plants and three control plants were harvested, avoiding root tips and lignified tissues. Twenty four plants were sampled in total (four sampling times, two treatments and three biological replicates). The sampled material was fixed in a paraformaldehyde-glutaraldehyde solution (Karnovsky solution), which is routinely used for ultrastructural microscopy studies (Ruzin, 1999; Pérez-de-Luque *et al.*, 2007), and was subsequently infused with synthetic resin using the Leica Historesin kit according to the protocols for dehydration and infusion with resin recommended by the manufacturer (Leica Microsystems GmbH, Wetzlar, Germany)[2].

The sampled root material was sectioned with a Leica RM 2245 Microtome (Leica Microsystems GmbH, Wetzlar, Germany) with carbon-tungsten knives Leica TC-65 (Leica Biosystems Nusloch GmbH; Geschäftsfürher, Germany). We obtained 2-μm thick longitudinal sections of the roots in a longitudinal disposition, which were then stained in a 0.1 % toluidine blue-O solution in citrate buffer (pH 5) (Ruzin, 1999). The

[2] Additional file with protocols is available at https://plantmethods.biomedcentral.com

sectioning thickness was chosen in order to obtain the maximum resolution and to minimize tissue destruction due to mechanical effects produced by the sectioning process. The samples were then mounted on a slide for optic microscopy with Entellan® synthetic resin for quick assembly (Merck KGaA, Darmstadt, Germany).

- Image capture and processing

From the pooled prepared samples, random subsamples were selected. The images were taken with lenses from the Plan Fluor series by Nikon Instruments Inc., through a Nikon DS-Fi1 digital optic device used with a Nikon Eclipse 50i optic microscope and connected to a PC through the Nikon DS-U2 control unity (Nikon Instruments Inc., Melville, NY, USA). The capture and storage of the images were performed with NIS-Elements F3.22.00 Build 710 computer software (Nikon Instruments Inc., 2008). A total of 160 images of 2560 x 1920 pixel were captured at 400X magnification.

The images were treated with the CorelDRAW Graphics Suite 12 software package (Corel Corporation, 2003). The images were transformed into area maps of different colors using subtractive masks. On these maps, each type of structure was represented by a color (Figure 2.1).

The areas of the image belonging to the structures of the pathogen were differentiated from the rest of the plant tissue by the tones of the colors. We systematically scanned the images with a 22.58 x 22.58 pixel mesh (300 sectors), marking the pathogen structures present in each quadrant. We used a mask-editing tool included in the Corel Photo Paint software that identified the groups of pixels exhibiting color homogeneity within a determined range. This range was established in a 16-color interval for the standard 32-bit *Cyan, Magenta, Yellow & Key* (CMYK) color model. The outlines that were selected by this procedure were manually corrected when they exhibited a change in color or structure in contact with the cellular walls.

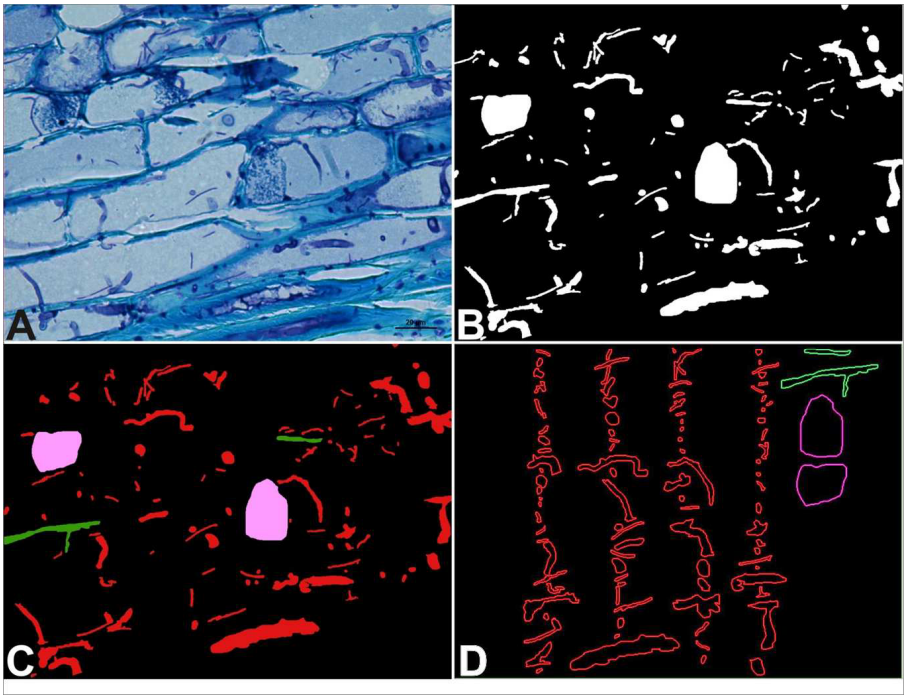

Figure 2.1. Acquisition of measurable regions of pathogen colonization through image treatment. A, Captured image from microscopy analysis. B, First representation map obtained from the subtractive masks, which represents the TSA. C, Color map after transformation (see Table 2.1 for Representative area color). D, Measurables structures in CAD files

We thus obtained maps with five colors that were vectorized using a JPG image transformation tool with raster archives.

Finally, the area of each of the pathogen structures in the image was obtained by processing the maps that were vectorized with assisted design software (AutoCad 2009, AutoDesk Inc.). Thus, the following parameters shown in Table 2.1 were measured:

- Total oomycete structure area (TSA): the area of all of the structures belonging to the pathogen and present in the sampled section.
- Intracellular structure area (ISA): the area of the structures of the pathogen that were found within the cellular space.
- Extracellular structure area (ESA): the area belonging to the structures of the pathogen that were not found inside the cells.

- Specific structure area (SSA): the area of structures that are different from somatic hyphae and haustoria-like structures, which were not present before 7 dai. In this work, with Phytophthora cinnamomi, these include swollen hyphae, botryose hyphae, and chlamydospores. It does not include sporangiophores or oogonia because they were not found.

Table 2.1. Variables and Kolmogorov-Smirnov test results

Variable	Representative area color[1]	Normality test	
		Kolmogorov -Smirnov	Sig
Total oomycete structures area (TSA)	White	0.229	<0.001
Intracellular structures area (ISA)	Red	0.283	<0.001
Extracellular structures area (ESA)	Green	0.268	<0.001
Specific structures area (SSA)	Violet	0.424	<0.001

[1]: See Figure 2.1C to D

- Statistical processing

The normality of the studied variables was verified by the Kolmogorov-Smirnov test. Afterwards, an analysis of variance was conducted to determine the existence of significant differences and an LSD test of average grouping, establishing the significance level at 5 %. The analysis was conducted with the SPSS 17 statistics package (SPSS Inc.).

2.3.- Results

- Oomycete localization

The staining method resulted in clearly differentiated pathogen structures, which stained to blue-violet, while most of the plant structures dyed dark blue. *Phytophthora cinnamomi* was observed in all of the sections

for each sampling period, while it was not present in the control plant roots (Figure 2.2).

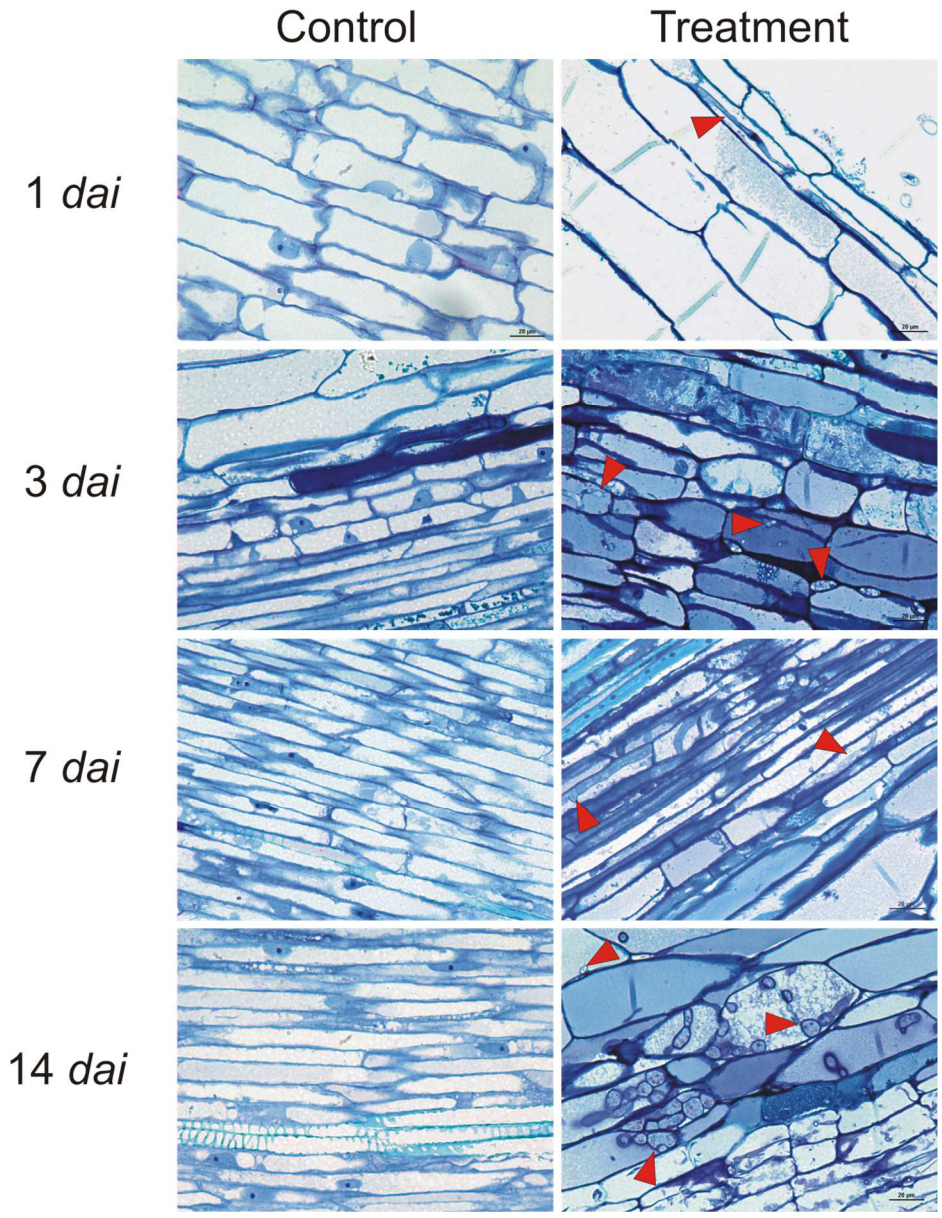

Figure 2.2. Comparison of images of treated and control root sections over time (1–14 *dai*). All the sections represent vascular tissue, except the images from 1 *dai*, which belong to the outer cortex (at this sampling time, the pathogen was not found in other sections). Some pathogen structures are indicated by red arrowheads in all the treated sections

We found aseptate hyphae with diameters ranging from 6 to 9 μm. From 3 *dai* (days after inoculation), it was common to find well developed hyphae in the cortex, in which cytosolic material was clearly distinguished and where we could observe nuclear material (Figure 2.3A, B, D). Where the pathogen could be found, we observed a type of hyphal structure inside the cells with a diameter ranging from 2 to 3 μm, that could resemble filiform haustoria, or fibrous finger-shaped haustoria.

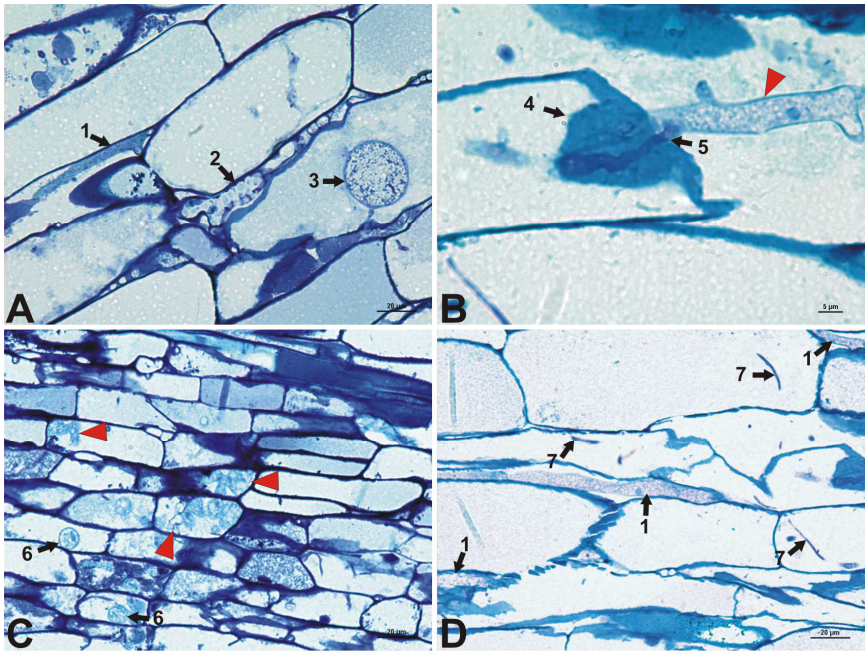

Figure 2.3. Pathogen structures identified in longitudinal root sections. A, Root cortical tissue. Several structures can be identified, such as primary hyphae (1), botryose hyphae (2), and survival structures such as chlamydospores (3). B, Hyphae observed at high magnification (x1000) (arrowhead). The pathogen penetrates the host cell through the cellulose wall. Accumulation of well stained cell wall material (4) (possibly papillae) is observed surrounding the penetration hyphae (5). C, Invasion of the central cylinder parechymatic tissue of the root by the pathogen 14 days after inoculation. Botryose hyphae that grow haphazardly were found inside the cells (arrowheads). At this time, we found immature or small chlamydospores in the parenchymatous cells (6). D, Several hyphae (1) and structures resembling finger-like haustoria (7) in cortical cells

These structures, despite their small size, were easily distinguishable because of their uniform coloration, more purple than blue (Figure 2.3D). We also localized chlamydospores, which were frequent in the cortex of 14-*dai* samples (Figure 2.3A). At this time, a large number of specific structures of *P. cinnamomi* were localized inside the parenchyma cells of the vascular cylinder. These structures might be identified as botryose hyphal swellings or incipient chlamydospores (Figure 2.3C). We did not find structures for sexual reproduction (gametangia) or sexual spores (oospores).

In the sample sections from 1 *dai*, the oomycete was found almost entirely in the most external areas of the cortex and in the epidermis of the root, whereas only a few scattered structures in the parenchyma cells of the stele were found. Structures within the fibrotracheids of the xylem of the stele were not observed. However, at 3 *dai*, the oomycete was already present throughout the section, with a considerable increase in the structures in the cortex, although still rare within the fibrotracheids.

From 7 *dai* onwards, the pathogen was abundant in all sections, with immature survival structures being identified in the cortex and parenchyma cells of the central cylinder at 7 *dai*, and mature chlamydospores separated from the somatic hyphae in cortex tissues and in the central cylinder at 14 *dai*. The oomycete in the cortex was found both in the apoplast and the symplast, whereas in the stele, the structures of the pathogen were found almost entirely inside the cells.

- Total oomycete structure area (TSA)

The analysis of variance depending on the sampling time showed highly significant ($F = 199$; $P < 0.001$) differences for this variable. The LDS analysis did not show significant differences between 1 and 3 *dai*, whereas there were significant ($P < 0.001$) differences at 7 and 14 *dai* (Figure 2.4).

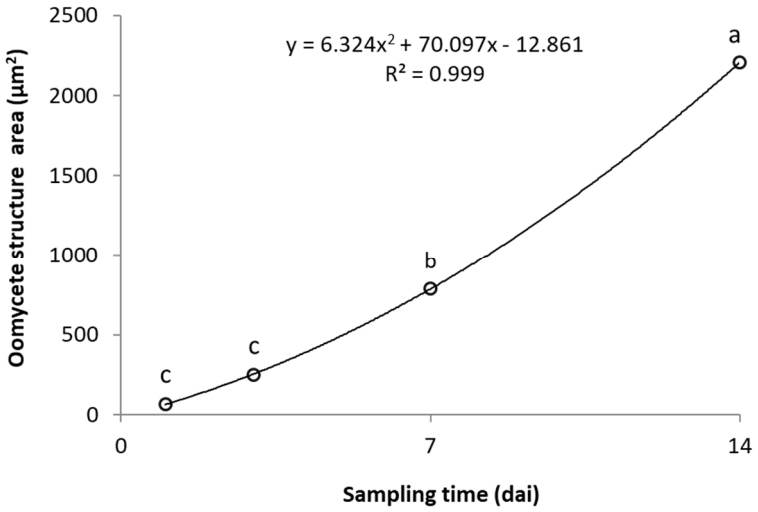

Figure 2.4 Total oomycete structure area (TSA), with tendency curve. Values with the same letter do not differ significantly ($P < 0.001$)

The graphic representation of TSA showed a gradual positive increase in the slope. A quadratic equation ($F = 102.959$; $P < 0.001$) was shown to best fit the data (Figure 2.4).

- Area by type of structure

Significant differences were found for the following variables depending on the sampling time: *Intracellular Structure Area* (ISA) ($F = 107$; $P < 0.001$), *Extracellular Structure Area* (ESA) ($F = 80$; $P < 0.001$), and *Specific Structure Area* (SSA) ($F = 27$; $P < 0.001$).

The ISA index for all sampling times was up to 80 % of the TSA value, and its evolution throughout the sampling time was similar (Figure 2.5). The graphic representation of the ESA was linear, with limited slope, in which the test of class grouping only showed differences at 14 *dai* (Av = 183.44 µm²; $P < 0.001$). SSA does not show a value above zero until 7 *dai*, although after this time its increase was significant and reached similar values to ESA at the end of experience (14 *dai*).

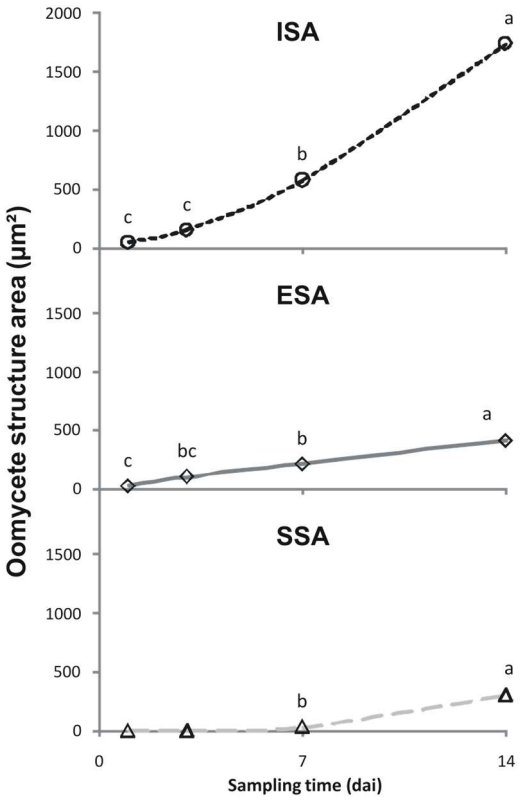

Figure 2.5 Oomycete structure area, represented by different structure types (ISA, ESA and SSA). Values with the same letter within a graph do not differ significantly ($P < 0.001$)

2.4.- Discussion

The morphology of the hyphae matched the description reported for *P. cinnamomi* by the *European and Mediterranean Plant Protection Organization* (EPPO, 2004). The description of the haustoria-like structures agrees with published results by other authors (Blaschke, 1994; Brummer *et al.*, 2002). Blaschke (1994) observed the presence of this type of structure for *P. cinnamomi* in *Quercus robur* L. using *Scanning Electron Micrographs* (SEM). In a recent study, Brummer *et al.* (2002) performed a *Transmission Electron Microscopy* (TEM) study and identified *Electronic Dense Material* (EdM) around the haustoria-like structures for *Phytophthora quercina* T. Jung in *Q.*

robur. In both cases the visual description of these structures agrees with the results presented in the current study. However, in our case, further SEM and/or TEM studies are required to confirm whether these structures are haustoria or not. The specific structures and chlamydospores we describe are therefore similar to those described in other similar studies (Sánchez Hernández *et al.*, 2003; EPPO, 2004).

For all sampling times, extracellular and intracellular structures were detected, agreeing with the observations made by other authors (Cahill, 1989; Blaschke, 1994; Brummer *et al.*, 2002; Pires *et al.*, 2008). Similar to the work conducted on *Corymbia calophylla* Lindl. (formerly known as *Eucalyptus calophylla* Lindl.) by Cahill *et al.* (1989), the presence of the pathogen in the stele was detected at 24 h; however, contrary to the work conducted on *Q. ilex* and *Q. suber* L. by Pires *et al.* (2008), in which they found a generalized invasion of the stele after this time, we only found scattered instances of hyphae in these tissues. However, details about the inoculum concentration or the growing conditions are not provided in the study of Pires *et al.* (2008). Cahill *et al.* (1989) indicate the presence of immature survival structures at 72 h after inoculation, whereas in our study, these structures were not detected until 7 *dai*.

The aspect of septate hyphae of the intracellular structures in a parenchimatous tissue belonging stele of the root is due to an artifact. The sequence of images from consecutive sections allows the third-dimensional reconstruction of aseptate hyphae (Supplementary Material Figures S.2.1 and S.2.2).

Clear pictures were obtained and the pathogen structures were sharp and well differentiated, so that both the sample preparation and the staining method used proved to be adequate for digital processing. The pathogen and host structures appeared to be well conserved for all of the sampling times. The differentiation between the pathogen and host structures were clear enough to allow for a selection of structures based on color gradient.

In the parenchymal tissues, the definition of the outline of the structures was more complex, due to the lower contrast in the captured images. The thickness of the transition of the outline varied by approximately 0.01 μm, whereas the smaller areas that were detected varied between 6 and 30 μm^2, with the overall average area per structure being 45.8 μm^2. Compared with the calculated areas, the potential variation between the micrographs and the computer generated structure images introduces an error of less than 0.01 % of the overall average and was considered to be acceptable for this reason.

Regarding the measured variables (TSA, ISA, ESA and SSA), the appropriate adjustment of the normal distribution shows the absence of a bias. This observation indicated that the distribution of the variables is consistent with a random model, enabling us to conduct parametric tests, which is an important aspect for giving the methodology statistical robustness. Such tests revealed clear differences that explain, in a manner consistent with the visual observations, the evolution of infection and colonization in the root tissues. The exponential evolution of the TSA variable seems similar to the characteristic tendency of the growth of mycelial organisms (Coelho *et al.*, 2007; Taskin *et al.*, 2011). This variable was the first to be obtained in our analysis and provides useful preliminary information about how the pathogen colonizes the root.

Consistent with the observations reported by other authors (Cahill, 1989; Blaschke, 1994; Pires *et al.*, 2008), we localized the oomycete invading the root by 24 h post-inoculation, spreading from the cortex through both the apoplast and the symplast by means of primary hyphae, in search of parenchymal tissues. From 3–7 *dai* on, colonization occurred more rapidly, as was previously observed in various *Quercus* species (Blaschke, 1994; Brummer *et al.*, 2002). Once the oomycete recognizes these tissues, it increases its development, mainly growing through intracellular structures (hyphae and presumably, haustoria), which explains why the ESA index presents a lower growth rate in the advanced stages of colonization, relative to the increase in ISA. High levels of ESA alongside low levels of

ISA show a pattern of tissue exploration by the pathogen. Low levels of ESA accompanied by a high ISA ratio show that the pathogen colonizes this tissue in order to obtain nourishment and to reproduce. Therefore, both indices, if they are examined together, can be useful for studying pathogen behavior in different plant tissues. The SSA index presents an important evolution between 7 and 14 *dai*, as it reaches, in half the time, the same level of presence in the root that it has in the extracellular structures. All of these data indicate that the pathogen during the advanced stages of colonization does not explore the tissue in a disorganized way but focuses on invading the parenchymal cells, to feed on them and to develop survival structures to complete its life cycle. The study of the SSA index may reveal the development status of the pathogen inside plant tissues. We might consider that the pathogen starts the development of chlamydospores and other specific structures when the colonization of tissue has extended and the oomycete expands into new areas of the roots.

2.5.-Conclusions

The determination of area parameters when studying the pathogen-host interaction in the root provides solid evidence that may help us quantify infection and colonization stages at the tissue level. This method provides a precise tool to evaluate hyphal colonisation and spore production in control and toxicity tests, using histological and microscopy techniques. In biological experiments, it is advisable to avoid techniques involving an added obstacle to analyze the results. The methodology presented here does not introduce higher levels of complexity, but provides results with an adequate statistical rigor.

The methodology presented in this study is readily accessible to staff with a basic knowledge of microscopy, histology and office automation, no complex mathematical models are required. Nevertheless, this approach generates results supported by rigorous statistical analyses and provides quantitative data to accompany the evolution that is observed

in a qualitative manner during the development of infection and colonisation.

It must be noted that this method can be readily performed with the material that is available in a histology laboratory, as almost any image capturing equipment can be used, including a simple digital camera used together with a microscope.

The current method avoids using specific programs for histological analysis provided by firms that manufacture capturing devices, as these programs represent a considerable expense and often require specific training. Instead, the variables that are determined in this technique can be obtained using freely available image processing software, such as Wintopo Standard (Soft Soft Ltd, 2007, UK) or GIMP 2 (Free Software Foundation, Inc. 2008, USA), and assisted design programs based on CAD, such as FreeCAD (Juergen Riegel, Werner Mayer & Yorik van Harve, 2001–2011).

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Chapter 3.- Histological study of the interaction between *P. cinnamomi* and *Q. ilex*

This chapter has been published in:

- **Ruiz Gómez FJ, Navarro-Cerrillo RM, Sánchez-Cuesta R, Pérez-De-Luque A. 2015**. Histopathology of infection and colonization of *Quercus ilex* fine roots by *Phytophthora cinnamomi. Plant Pathology* **64**: 605-666.

Impact Factor (JCR): 2.303. Q1 *Agronomy*, rank 16/87

Abstract

Quercus ilex is the dominant tree species over large areas of natural forest ecosystems in the Western Mediterranean Basin. This species is one of the most susceptible hosts to the oomycete *Phytophthora cinnamomi*, which causes root rot. This is a main factor in holm oak disease, a particularly serious problem in the "dehesas" ecosystem of south western Iberian Peninsula. This work describes the pathogen-host interaction of *Q. ilex* and *P. cinnamomi*, using new infection indices at tissue level. Six-month-old saplings were inoculated with *P. cinnamomi*. Fine roots were collected and prepared for optical microscopy. A random pool of images was analyzed in order to calculate different indices based on the measurement of the area of pathogen structures. In the early stages of invasion, *P. cinnamomi* colonizes the apoplast and grows haustorial structures in cortex cells. When the pathogen reaches the parenchymatous tissues of the central cylinder, it starts to develop different reproduction and resistance structures inside the cells and then expands through the vascular system of the root. We identified some host responses, such as cell wall thickening, accumulation of phenolic compounds in the middle lamella of schlerenchyma tissues, and mucilage secretion blocking vascular cells. The main physiological effect of morpho-anatomical root changes is a severe reduction in water supply and related water stress, which causes drought induced mortality. Host responses fail due to rapid expansion of the pathogen once it reaches the central cylinder, and due to a change in the behaviour of the pathogen from biotrophic to necrotrophic.

Resumen

Quercus ilex es la especie arbórea dominante a lo largo de grandes áreas forestales en la cuenca mediterránea occidental. Esta especie es uno de os huéspedes más susceptibles frente al oomiceto causante de podredumbre radicular *Phytophthora cinnamomi*, siendo uno de los factores principales en el decaimiento de la encina, problema especialmente severo en los ecosistemas adehesados del suroeste de la Península Ibérica. El presente trabajo describe la interacción patógeno-huésped entre *Q. ilex* y *P. cinnamomi*, mediante el empleo de nuevos índices de infección a nivel de tejidos. Plantones de 6 meses de edad fueron inoculados con *P. cinnamomi* y raíces finas fueron colectadas y preparadas para microscopía óptica. Se analizó un conjunto de imágenes seleccionadas al azar con el fin de calcular diferentes índices basados en la medida del área ocupada por las estructuras de patógeno. En las etapas tempranas de la invasión, *P. cinnamomi* coloniza el apoplasto y genera estructuras haustoriales en células del córtex. Cuando el patógeno alcanza los tejidos parenquimáticos del cilindro central, comienza a desarrollar diferentes estructuras de reproducción y de resistencia en el interior de las células y se expande a través del sistema vascular de la raíz. Se identificaron algunas respuestas del huésped, como el engrosamiento de las paredes celulares, la acumulación de sustancias fenólicas en la lámina media de los tejidos de esclerénquima, y secreciones mucilaginosas bloqueando las células vasculares. El efecto principal de los cambios morfoanatómicos sobre la fisiología es la reducción en el suministro de agua, causando mortalidad por la sequía inducida. Las respuestas del huésped no resultan efectivas debido a la rápida expansión del patógeno una vez que alcanza el cilindro central, y debido al cambio en el comportamiento del patógeno de biotrófico a nectrotrófico.

Keywords: Histopathlogy, oak decline, *Quercus ilex*, *Phytophthora cinnamomi*, root rot, infection index.

3.1.- Introduction

Quercus spp. forests in the south of the Iberian Peninsula are of great value, because of their high biodiversity and role in defending against desertification (Horta *et al.*, 2010). "Dehesa" (savanna-like forest) is a Mediterranean agroforest system in which holm oak is the main tree species; it provides important economic benefits (Gaspar *et al.*, 2009) and high sociocultural value (Campos *et al.*, 2008). Holm oak (*Quercus ilex* subsp. *ballota* [Desf.] Samp.) is one of the dominant tree species in natural forest ecosystems over large areas of the Western Mediterranean Basin (Pulido *et al.*, 2001), covering an approximate area of 3 MHa. Since the early 1980s, a severe decline of *Quercus* spp. has been reported across the Mediterranean region (Brasier, 1996, Pérez-Sierra *et al.*, 2013).

In dehesa ecosystems, the decline of *Quercus* spp. has three main causes: i) overgrazing and shrub invasion (CAP, 2008); ii) the effects of climatic change, which can cause the displacement of some forestry species (Fernández-Cancio *et al.*, 2012; Gea-Izquierdo *et al.*, 2013); and iii) oak mortality related to the interaction of biotic and abiotic factors (Thomas *et al.*, 2002; Jönsson, 2006). It is well recognised that forest decline is a complex multifactorial disease related to stress caused by adverse abiotic and biotic factors (Thomas *et al.*, 2002). However, *Phytopthora cinnamomi* root rot is considered one of the main factors responsible for oak decline (Brasier *et al.*, 1993; Brasier, 1996; Sánchez *et al.*, 2002, 2005).

Holm oak is the most susceptible *Quercus* species to *P. cinnamomi* root rot (Maurel *et al.*, 2001; Robin *et al.*, 2001; Moralejo *et al.*, 2009). In host plants, root colonization by *P. cinnamomi*, causes root lesions, necrosis and loss of fine roots, water deficiency symptoms, reduced stomatal conductance and transpiration, and leaf lesions such as yellowing and wilting (Jung *et al.*, 1996; Gallego *et al.*, 1999; Moralejo *et al.*, 2009). The level of fungal damage depends on host species, population, plant age and

environmental conditions (Rodriguez Molina *et al.*, 2005; Moralejo *et al.*, 2009).

Histopathology is often used in the study of root rot because it allows the observation of tissues and pathogenesis in different root structures. These techniques have been applied to study *P. cinnamomi* damage in woody species such as *Vaccinium* spp. (Millholand, 1975), *Castanea sativa* Mill. (Maia *et al.*, 2012), *Q. suber* (Medeira *et al.*, 2012), *Eucalyptus* spp., *Acacia* spp., *Xanthorrhoea australis* R. Br. (Cahill *et al.*, 1989) and *Fagus sylvatica* L. (Portz *et al.*, 2010). There are no existing studies using histological techniques to describe the interaction between *Q. ilex* and *P. cinnamomi*. The most similar pathosystem description using a histopathological approach was conducted in *Quercus robur* L. roots (Blaschke, 1994, Brummer *et al.*, 2002).

The aim of this work was to describe the pathogen-host interaction of *Q. Ilex* and *P. cinnamomi* using histology methods, and to describe the progression of colonization through the application of a new image treatment methodology (Ruiz-Gómez *et al.*, 2012). We addressed the following specific objectives: i) evaluation of the temporal process of tissue infection to better understand pathogen development over time; ii) description of the differences in pathogen behaviour in each root-tissue structure and their consequences for invasion and colonization; iii) clarification of when, where and how the pathogen can be found on the holm oak root.

3.2.- Material and Methods

- Plant material and growing conditions

Acorns of *Quercus ilex* subsp. *ballota* were collected from healthy trees located in a natural forest (Córdoba, Spain, 37°53'34.80"-37°55'26.40"N, 4°56'42.00"-4°54'3.60"W). Seeds were sterilized (5 minutes in a 1% NaClO solution) and germinated in a growth chamber (15 days, 24°C, 70% relative

humidity, 12h photoperiod). Seedlings were grown in 32-cell seed trays (400 cm^3, perlite as substrate, 22°C, 60% relative humidity, photoperiod 14h light/10h dark) and watered twice weekly with Hoagland's nutrient solution (Hoagland and Arnon, 1950) for 6 months before inoculation. The six-month old seedlings (average height 13.9 ± 5.2 cm and basal diameter 3.41 ± 0.75 mm, mean values and standard error, N=40) were placed in 1.5 L pots containing perlite.

- Inoculation

P. cinnamomi isolate P90 (mating type A2, NBT Laboratories New Biotechnics, Sevilla, Spain, ISO 9001/17025) was grown in petri dishes containing carrot agar medium (CA) in a growth chamber at 20°C in darkness. After 15 days, the surfaces of the dishes were washed and the CA medium containing the mycelium was crushed in a mixer with distilled water (33 ml per dish). The final concentration of liquid inoculum was adjusted by counting chlamydospores as a reference and diluting it to 25 IU μl^{-1} (Infection Units per microliter).

After carefully removal of perlite by washing with deionized water, roots were immersed for 10 min in the inoculum solution, before the plants were placed in sterilized perlite.

- Sample processing

3-5 mm sections of fine roots (secondary rootlets of Ø≤2mm) were obtained at 1, 3, 7 and 14 days after inoculation (*dai*). Plants were extracted from the pots, and perlite was gently removed from the roots. Root samples were collected from three different plants per treatment, avoiding lignified zones and the root tips. In the case of inoculated plants, sections were collected from parts showing symptoms of root rot, as described (Blaschke, 1994; Sánchez *et al*., 2005). Samples were then fixed in Karnovsky solution (Pérez-de-Luque *et al*., 2007) and embedded in synthetic resin (Historesin, Leica Microsystems GmbH, Wetzlar, Germany).

Next, both longitudinal and cross-sections of 2 μm thickness were obtained from the embedded roots, using a microtome (Leica RM 2245, Leica Microsystems GmbH, Wetlzar, Germany) equipped with carbon-tungsten blades (Leica TC-65, Leica Microsystems GmbH, Geschäftsfürher, Germany). Slides containing the samples were stained, either in 0.1% toluidine blue-o solution in citrate buffer (pH 0.5) (TBO) (Ruzin, 1999) or in 0.05% ruthenium red/distilled water solution (RhRed) (Vallet *et al*., 1996). Slides were mounted with synthetic resin for quick assembly (Entellan, Merck KGaA, Darmstadt, Germany).

- Capture and image processing

Pictures from sections were taken through a digital optical device (Nikon DS-Fi1, Nikon Instruments Inc., Melville, USA) incorporated in an optical microscope (Nikon Eclipse 50i, Nikon Instruments Inc., Melville, USA) and connected to a PC through the control unit Nikon DS-U2 (Nikon Instruments Inc., Melville, NY, USA).

Images were at 400x magnification for longitudinal sections and at 100x, 200x, 400x and 1000x magnification for cross sections. For each sampling time, a random set of longitudinal sections was chosen through the generation of random number series. This set of images was divided into four sub-sets and a different tissue type was assigned to each one: outer cortex containing epidermal cells (EP), inner cortex close to the central cylinder (C), parenchymatous tissue of the central cylinder (stele) (S1) and prosenchymatous tissue (xylem, metaxylem and protoxylem cells) of the stele (S2). One image of the selected tissue was taken in each selected section. Finally, a total of 160 pictures of 2560 x 1920 pixel were captured (4 sampling times x 4 tissue types x 10 technical replicates).

Treatment of images was carried out according to the methodology described by Ruiz Gómez *et al*. (2012). Images were treated with the CorelDRAW ® Graphics Suite 12 software package (Corel Corporation, 2003). The area of each pathogen structure in the image was obtained by processing the vectored maps with computer-assisted design

software (AutoCad® 2009, AutoDesk Inc.).The following parameters were measured:

- Total oomycete structure area (TSA): the area of all the structures belonging to the pathogen and present in the sampled section.
- Intracellular structure area (ISA): the area of the pathogen structures found within the cellular space.
- Extracellular structure area (ESA): the area of the pathogen structures found outside the cells.
- Specific structure area (SSA): the area of the pathogen structures other than somatic hyphae, which were not present before 7 *dai*. For this purpose, haustoria-like structures, stromata aggregations and chlamydospores were considered specific structures in this work. Sporangiophores, hyphal swellings and oogonia have not been included because these were not found.
- Percentage of infected cells: the ratio between infected cells and all cells present in the image. To calculate this index, edge cells (those whose cell walls were not completely included in the image) were only considered when their area was greater than 1% of the total area of the picture. In this way, we avoided the error that would be caused by the relative rarity of pathogen structures inside infected edge cells in the early stages of colonization.

- Statistics

Histological variables were compared between treatments with a one-way ANOVA. Post hoc comparisons between pairs of treatments were identified with Tukey's test based on adjusted levels of significance (Sokal & Rohlf, 1995). The assumptions of normality and homogeneity of variance of the residuals were examined using the Kolmogorov–Smirnov and Levene tests, respectively. The percentages of infected cells was subjected to arcsen[$\sqrt{(X/100)}$] transformation to improve normality (Milton & Tsokos, 2007). Null hypotheses were rejected at the $P < 0.05$ level, and all analyses

were run with statistic package SPSS V.8 (IBM España, S.A., Madrid, Spain. *http://www-01.ibm.com/software/analytics/spss/*).

3.3.- Results

- Plant symptoms

Roots from inoculated plants showed necrotic and rot symptoms 1 day after inoculation (*dai*). The first symptoms were observed in lateral and thin absorbent roots (Ø<2 mm) and consisted of dark brown colouration, root softness and dark lesions in the growing apex. Additionally, loss of secondary roots occurred over time, being most noticeable from 7 *dai* and almost complete at the end of the experiment (14 *dai*) (Figure 3.1).

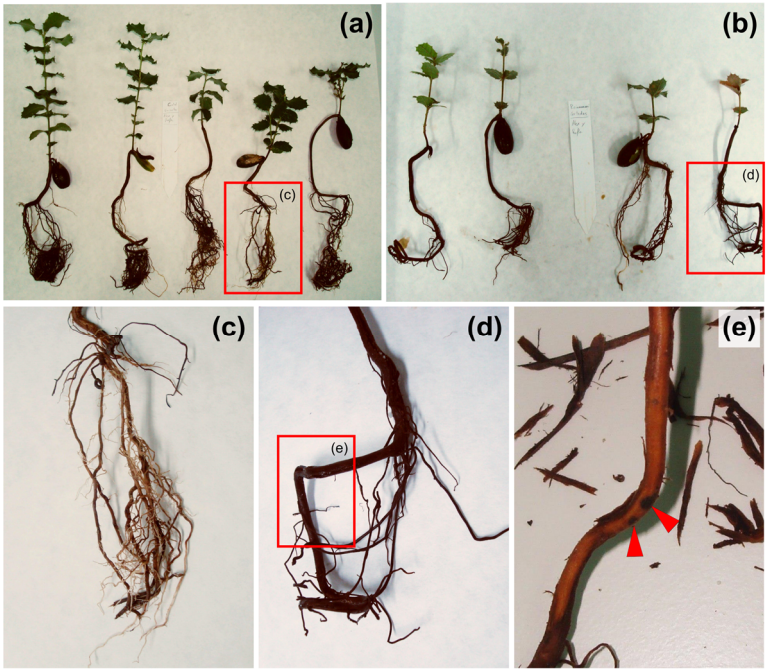

Figure 3.1. Early symptoms and differences between inoculated and control plants. (a) Control plants 14 *dai*. Scale bar = 5 cm. (b) Inoculated plants 14 *dai*. Scale bar = 5 cm. (c) Detail from (a), showing the root of a healthy control plant. (d) Detail from (b) showing the root of an infected plant. (c) and (d) clearly differ in colour and presence of secondary absorbing roots. (e) Detail of lignified root from (d) after outer bark detachment. Necrotic lesions can be observed at insertion points for lateral roots (arrowhead).

Other identified symptoms were generally slower plant development and exfoliation of the external surface of lignified roots exposing small necrotic lesions underneath (Figure 3.1e). At the end of the assay, the aerial part of inoculated plants showed discolouration and partial defoliation, whereas control plants lacked such symptoms (Figure 3.1a and b).

- Identification of pathogen structures

Pathogen structures were present in all samples of inoculated plants, showing intracellular and/or intercellular structures, for all tissues and sampling times, and were absent from all observed sections corresponding to control plants. Hyphae from 6 to 9 μm thickness were found in cortical tissues at all sampled times (Figure 3.2). In addition, starting from 3 *dai*, densely purple stained hyphae appeared inside parenchyma cells (Figure 3.2d, e) together with thicker hyphae of botryose aspect in the cortical intercellular space (Figure 3.2c).

Frequently ramified thin (2-3 μm) hyphal structures were present in all sections containing the pathogen, except inside xylem and protoxylem cells (Figure 3.2, red arrowheads). In S1 sections, some hyphal structures occasionally showed thickening and ramifications with vesicular appearance, and the staining was more intense (darker blue) in the outlines of the structures and fainter further inside. (Figure 3.2d, e). Lignitubers were identified inside cortical cells and parenchymatous cells in the central cylinder at 14 *dai* (Figure 3.2e).

Starting from 7 *dai*, we observed single hyaline globular structures occupying cells in vascular tissues, in addition to other tissues. These structures were circular with a diameter of between 20 and 30 μm, were surrounded by a thin wall, showed a concentration of grainy material inside, and were linked to intercellular hyphae through an appendix that crossed the plant cell wall. Such structures were identified as chlamydospores (Figure 3.2f).

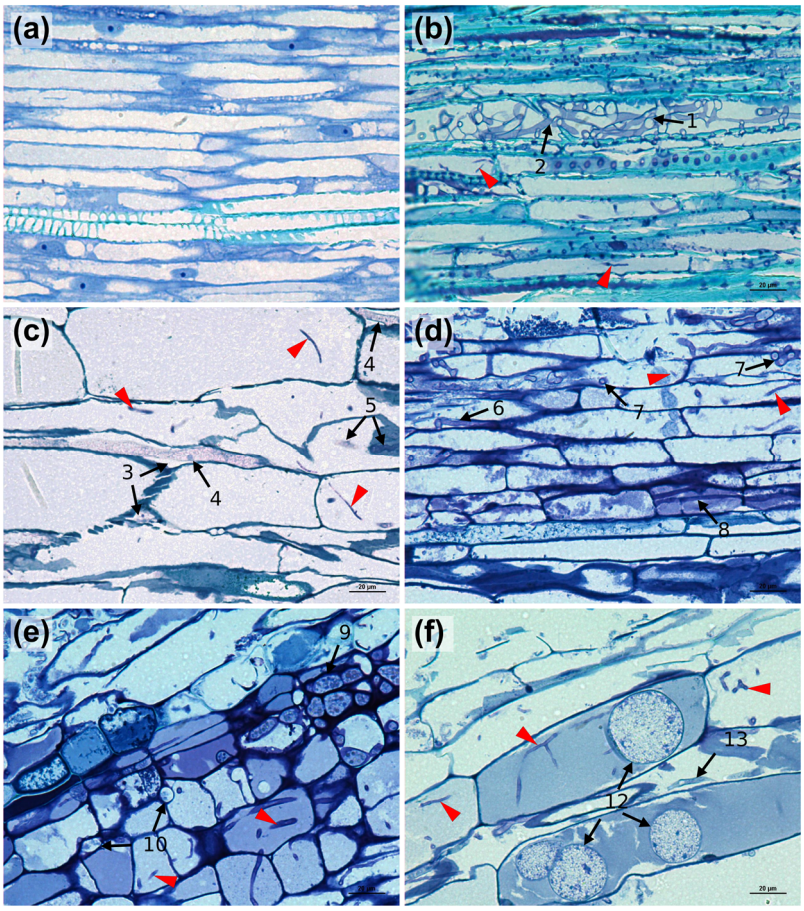

Figure 3.2. Identification of pathogen structures. (a) Longitudinal section of the vascular core at 14 *dai* (type S2) for control treatment. (b) S2 section of inoculated plant at 14 *dai*. An intense proliferation of vegetative hyphae inside xylem vessels can be observed (1). The hyphae cross xylem vessels through sieve plates (2) (c) Longitudinal cortex section (type C) 7 *dai*. Intercellular spaces (3) increased in number and size, and botryose hyphae (4) can be observed. Structures penetrating the cell walls were identified (5), showing accumulation or thickening of the cell wall around them (see also Fig. 7A). (d) Longitudinal section of the central cylinder (type S1) 14 *dai*. Pathogen structures were mostly inside the host cells. Proliferation of intracellular hyphae (6) and hyphal swellings with vesicular aspect next to the host cell walls (7) was observed. Lignitubes can be identified crossing some host cells (8). (e) Longitudinal section of the central cylinder (type S1) 14 *dai*. In several of these sections, circular or ellipsoid structures appeared clustered inside host cells (9) together with others showing hyaline vesicular aspect at the intercellular spaces (10). (f) Longitudinal section of the outer cortex (type EP) 14 *dai*. Other specific structures (chlamydospores) have developed in the cytosolic space of non-lysed host cells (12) as well as vegetative hyphae next to the chlamydospores (13). From (b) to (f) Arrowheads indicate intracellular haustorial hyphae.

Spherical or semi spherical corpuscles completely filled some parenchyma cells from the stele 14 *dai* (Figure 3.2e). These corpuscles corresponded to an accumulation of hyphal structures growing in a disordered way inside the cells. Furthermore, vesicles were present in the intercellular space of the parenchyma cells.

No structures for sexual reproduction, such as oospores or gametangia, were found at any time or in any sample.

- Pathogen structure localization

Starting from 1 *dai*, oomycete intra- and intercellular hyphae were identified in the external root cortex (EP). At this time, single oomycete structures were visible in internal cortical tissues (C) and there was no colonization of parenchyma cells from the central cylinder (S1), protoxylem or xylem vessels (S2).

Figure 3.3 shows that by 3 *dai* the pathogen had colonized all root tissues, although it was rare in sections from S2 type tissues. The oomycete had infected the inner cortex (C) with intra- and intercellular hyphal structures, and hyphae and wall thickenings were visible in parenchyma cells from the central cylinder (S1). Only a few sections from S2 tissues showed intracellular hyphae.

The infection of host tissues was ubiquitous in all samples at 7 and 14 *dai*, which showed a significant increase in colonization of protoxylem and xylem cells (S2) by hyphae with 6-12 μm diameter (Figure 3.2b). Such an increase was obvious in all S2 samples and consisted of proliferation of intracellular structures, mainly vegetative hyphae with a botryose appearance. However, unlike the other tissue types, S2 cells showed no thickening of intracellular hyphae near the cell wall (Figure 3.2d) and several parenchymal cells showed no clustered structures at 14 *dai* (Figure 3.2e).

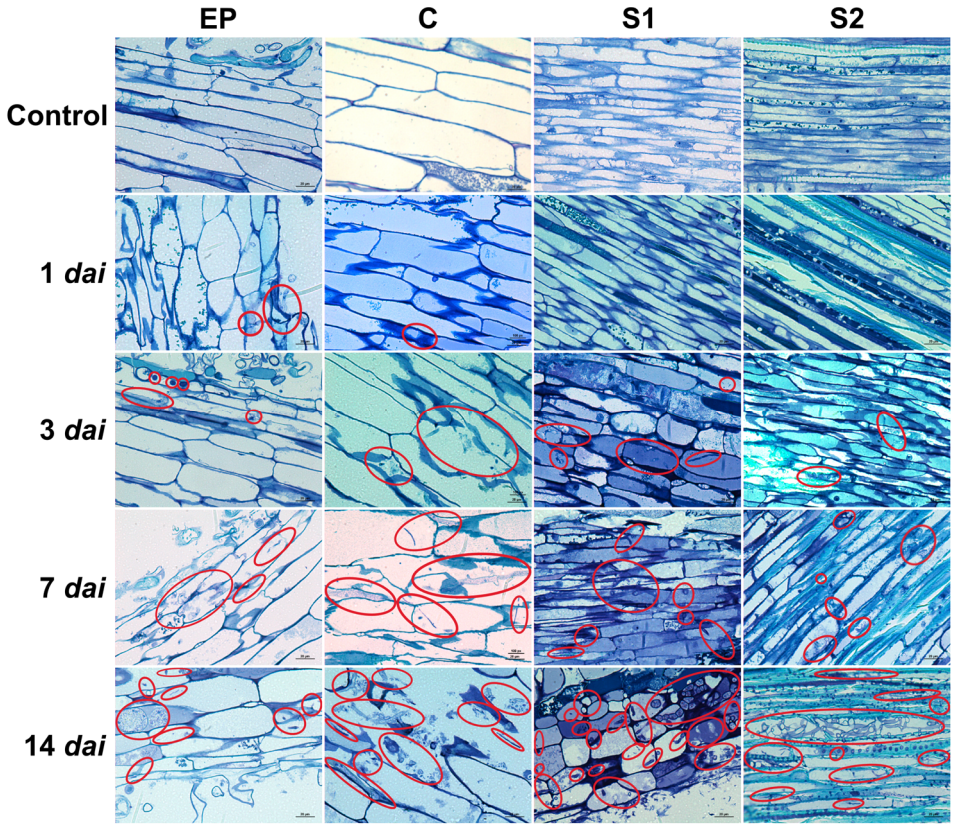

Figure 3.3. Pathogen localization over sampling time. Selected images are the closest to the mean values of TSA for each type and sampling time. Surrounded areas indicate presence of pathogen structures in each section type. The pathogen was absent only in control sections and in those corresponding to the stele (S1 and S2) at 1 *dai*. The increase in size and number of surrounded areas shows the highest colonization levels in sections from the inner cortex (C) and parenchymatous tissue from the central cylinder (C1) at 14 *dai*.

Most chlamydospores appeared 14 *dai* in the inner cortex (C) near disorganised tissue areas and destroyed cells, although some incipient hyaline structures identified as chlamydospores were observed at 7 *dai*.

No pathogen structures were ever identified inside cells from the Casparian strip.

- Quantification of infection and colonization process

The percentage of infected cells showed significant differences in sampling time ($F = 155.6$, $P < 0.05$) and in the interaction between sampling time and type of section ($F = 10.6$, $P < 0.05$), whereas no differences were found when considering type of section alone ($P = 0.87$). The progression of cell colonization was different in EP and C type sections compared with S1 and S2 type (Figure 3.4): we observed that for EP and C type sections the percentage of infected cells increased at a slower rate between 7 and 14 *dai*, but this did not occur for sections from S1 and S2 (central cylinder). In sections from types EP and C, the significant change in percentage of infected cells occurred between 3 and 7 *dai*.

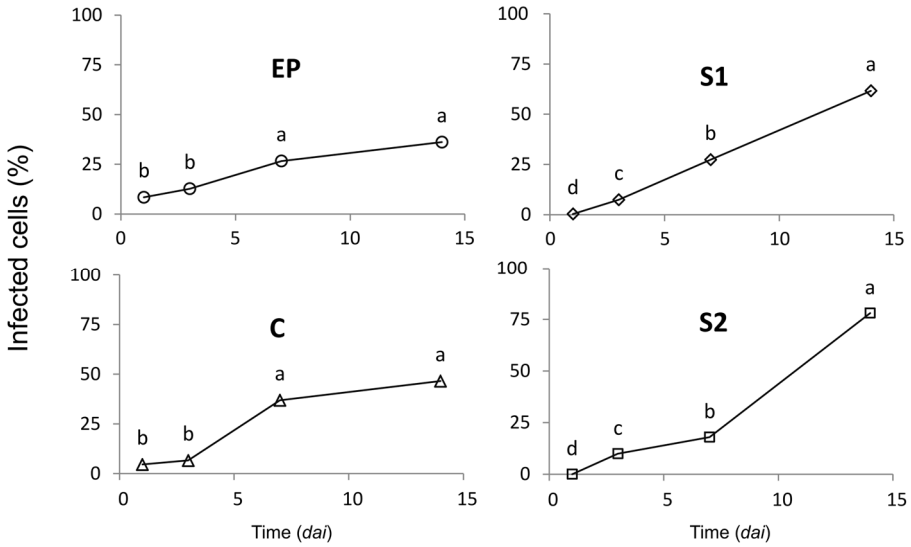

Figure 3.4. Proportion of infected cells in each section type. Values with the same letter do not differ significantly ($P<0.05$).

All variables for pathogen structure area showed significant differences when considering time evolution, whereas only the extracellular structures area (ESA) and the intracellular structures area (ISA) varied by type of sampled section (Table 3.1). The analysis of time

evolution by tissue type confirmed significant differences in all cases except for the specific structure area (SSA) index in C type sections (middle-inner cortex).

Table 3.1. Analysis of variance of the pathogen area indices with respect to time and double interaction. F: Fisher's parameter; P: probability value; dai: days after inoculation; TSA: total oomycete structure area; ISA: intracellular structure area; ESA: extracellular structure area; SSA: specific structure area; EP: outer cortex containing epidermal cells; C: inner cortex close to the central cylinder; S1: parenchymatous tissue of the central cylinder (stele); S2: prosenchymatous tissue (xylem: metaxylem and protoxylem cells) of the stele.

			TSA	*ISA*	*ESA*	*SSA*
Total variation	*Time (dai)*	F	68.25	40.85	17.06	21.063
		P	<0.001	<0.001	<0.001	<0.001
	Section type	F	2.65	5.53	8.41	2.17
		P	0.051†	<0.05	<0.001	0.093†
EP	*Time (dai)*	F	11.94	6.62	5.61	3.87
		P	<0.001	<0.05	<0.05	<0.05
C	*Time (dai)*	F	12.75	5.22	10.85	1.72
		P	<0.001	<0.05	<0.001	0.18†
S1	*Time (dai)*	F	78.93	54.59	5.27	14.73
		P	<0.001	<0.001	<0.05	<0.001
S2	*Time (dai)*	F	84.91	60.53	9.78	4.95
		P	<0.001	<0.001	<0.001	<0.05

†: Data do not differ significantly in respect to time

The progression of total structures area (TSA) showed differences between cortical tissues (EP and C type sections) and those from the central cylinder (S1 and S2 type) (Figure 3.5). At the end of the experiment, TSA values were significantly higher for tissues from the stele (S1 and S2) than for cortical tissues (EP and C) ($F = 208.6$, $P < 0.001$).

The ISA values were similar to or even lower than the ESA values in C and EP type tissues, whereas in S1 and S2 type tissues ISA values reached more that 80% of TSA. ESA values were higher than ISA only in C type sections at the last sampling times and were almost negligible in stele tissues (S1 and S2). The low ESA values accompanied a general appearance

of disorganized cell areas and greatly increased intercellular spaces. The trend for ESA and ISA was similar in EP and C tissues, but varied in S1 and S2 tissues.

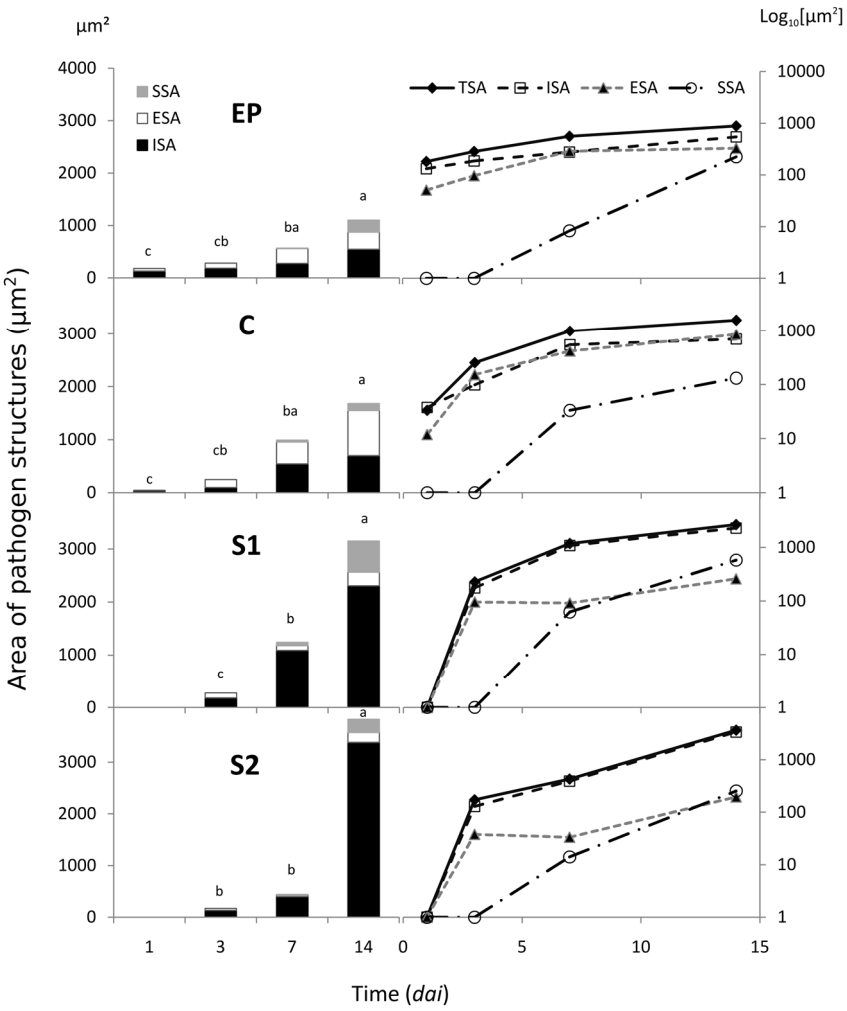

Figure 3.5. Pathogen structures area. Left side/axis: bar graphs corresponding to accumulated area by tissue type and time. Bars with the same letters within the same tissue type are not significantly different (LSD test, $P<0.05$) for the total structure area index (TSA). Right side/axis: graph showing the different areal indexes in each tissue types over time, in logarithmic scale. SSA: specific structure area; ESA: extracellular structure area; ISA: intracellular structure area; TSA: total oomycete structure area; EP: outer cortex containing epidermal cells; C: inner cortex close to the central cylinder; S1: parenchymatous tissue from the central cylinder (stele); S2: prosenchymatous tissue (xylem, metaxylem and protoxylem cells) from the stele.

The only significant increase of TSA in EP and C tissues was observed between 1 and 7 *dai*, after the point at which the first pathogen structures appeared inside the central cylinder (3 *dai*). Most of the specific pathogen structures appeared in type S1 sections by the end of the experiment.

Tukey grouping revealed no clear differences for indexes in EP and C type sections, except for SSA in EP tissues between 7 and 14 *dai* ($P<0.05$) (Table 3.2). For S1 type sections, groupings were only significant for ISA and SSA ($P<0.05$). In S2 type sections, differences appeared between 7 and 14 *dai*, and were highly significant for both ISA and SSA ($P < 0.001$).

Table 3.2. Tukey HSD (Honestly Significant Difference) analysis of the area indices for the interaction Time × Section Type. dai: days after inoculation; ISA: intracellular structure area; ESA: extracellular structure area; SSA: specific structure area; EP: outer cortex containing epidermal cells; C: inner cortex close to the central cylinder; S1: parenchymatous tissue of the central cylinder (stele); S2: prosenchymatous tissue (xylem: metaxylem and protoxylem cells) of the stele;

		1 *dai*	3 *dai*	7 *dai*	14 *dai*
EP	*ISA*	b	b	ba	a
	ESA	c	cb	ba	a
	SSA	-	-	b	a
C	*ISA*	b	b	ba	a
	ESA	b	b	b	a
	SSA	-	-	a	a
S1	*ISA*	-	c	b	a
	ESA	-	a	a	a
	SSA	-	-	b	a
S2	*ISA*	-	b	b	a
	ESA	-	b	b	a
	SSA	-	-	b	a

Interactions with the same letter do not differ significantly ($p<0.05$)
(-) = Index mean equal to zero

- Host response and pathogenic interaction

Cross sections observed under 100x magnification showed differences in the intensity of staining of inoculated and control individuals (Figure 3.6). TBO stained the tissues of inoculated roots with an intense deep blue; under 100x magnification, inoculated roots showed thicker cell walls and an increase in the number and size of intracellular spaces in the central cylinder (Figure 3.6d) compared with the control. Similarly, ruthenium red stained the same areas in inoculated root sections a strong red. In addition, a discontinuity between the cortex and the central cylinder, destroying the Casparian strip, was observed in inoculated samplings at 14 *dai* (Figure 3.6d, h).

Observation under 200x magnification revealed an accumulation of substances inside the cells of the vascular cylinder at 7 *dai*, which were stained with both TBO (Figure 3.7b) and ruthenium red (Figure 3.7f). A general thickening of the metaxylem and protoxylem cell walls (Figure 3.7b) was also detected at 3 *dai*. When observing the longitudinal cuts of the S2 sections, accumulation of a dark blue stained substance inside some xylem vessels was detected that blocked the border pits of those cell walls (Figure 3.7h).

Under 400x magnification, differences in cell wall thickness between treatments for all tissues and sampling times were observed when compared with the control (Figure 3.7g). Accumulation of materials was also localized in areas of the cell wall that were in contact with the pathogen, being higher at points of penetration of hyphae in the intracellular space. These materials were stained darker by TBO than the rest of the cell material (Figure 3.7a). Greenish-coloured accumulations were also identified in the middle lamella of the vascular tissues with this staining method at 7 *dai* (Figure 3.7d).

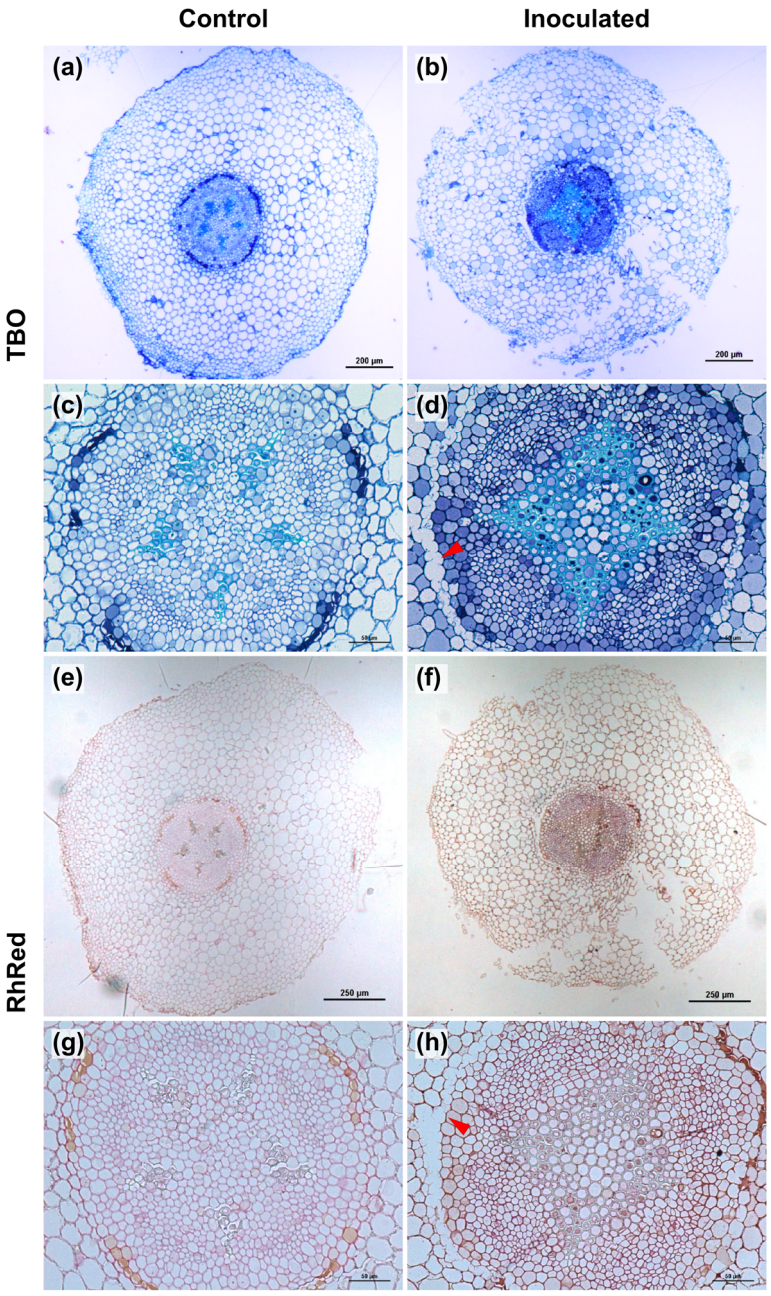

Figure 3.6. Differences between cross sections of control and inoculated roots stained with Toluidine blue O (TBO) and Ruthenium red (RhRed). (a) and (e) Cross section of control root at 14 dai, under 100x magnification. (b) and (f) Cross section of inoculated root at 14 dai, under 100x magnification. (c) and (g) Detail of central cylinder of (a) and (b) respectively, under 200x magnification. (d) and (h) Detail of central cylinder of (e) and (f) respectively, under 200x magnification. Red arrowheads indicate discontinuity between cortex and central cylinder.

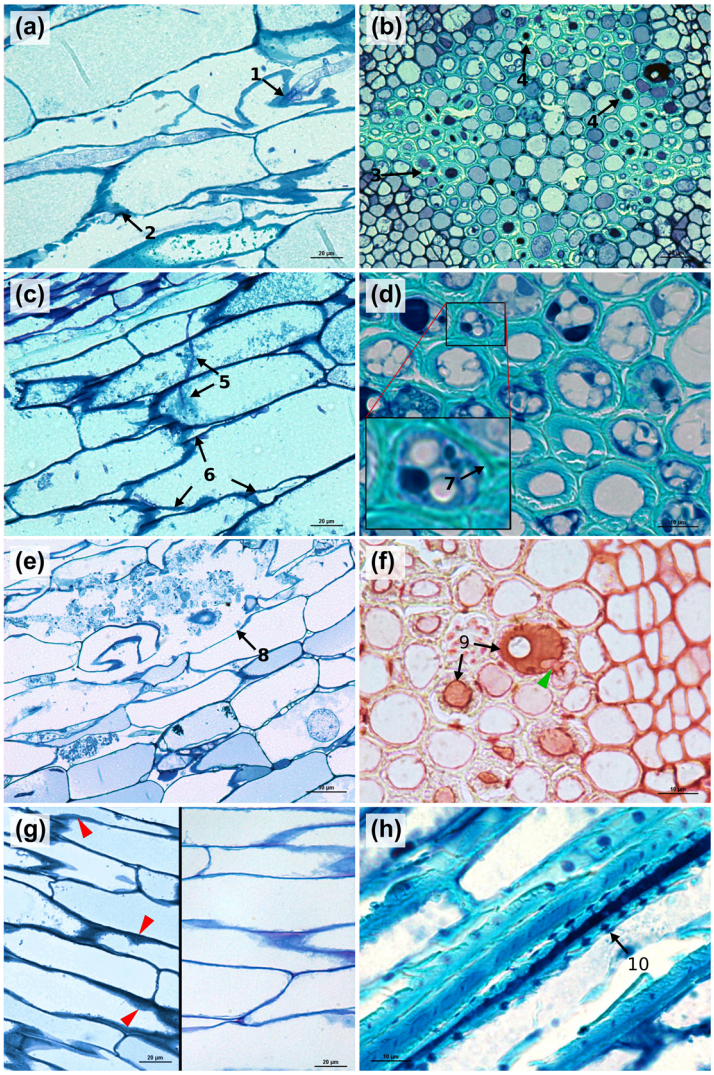

Figure 3.7. Details of the pathogenesis process. (a) Longitudinal section of the inner cortex (tissue type C) at 3 *dai*. Accumulation of material can be observed at the contact point of the hyphae with the host cell (black arrow 1) in addition to a thickening of the host cell walls (black arrow 2). (b) Cross section of the stele at 7 *dai*. There is thickening of xylem vessels cell wall (black arrow 3) and accumulation of cytosolic material stained strongly dark with TBO (black arrows 4). (c) Section of the inner cortex (type C) at 14 *dai*. The presence of granular material in the cytoplasm around pathogen structures can be observed (black arrows 5). In addition, an increase in number and size of intercellular (apoplastic) spaces is detected (black arrows 6). (d) Detail of a cross section from the stele at high magnification (1000x) at 7 *dai*. The presence of a greenish stained material in the middle lamella of xylem and protoxylem vessels was detected (black arrow 7). (e) Section of the inner cortex (type C) at 14 *dai*. Disorganized areas with cell disruption was frequent (black arrow 8) for both cortex and central cylinder tissues. (f) Detail of a RhRed stained cross section from the stele at high magnification (1000x), at 7 *dai*.

Accumulation of pectic substances can be observed inside vessels (black arrows 9). Some pathogen structures were also stained with different intensity (green arrowhead). (g) Comparison between longitudinal sections of the cortex in inoculated and control roots at 1 *dai*. In the inoculated root (left), despite no visible oomycete structures, there is an increase in the thickening of the cell walls (arrowheads) compared with the control root (right). (h) Detailed longitudinal section of xylem vessels at 7 *dai*. Xylem vessels have very thick cell walls. Complete sealing of some vessels by a dark stained substance can be observed (black arrow 10). This substance can be seen also across the xylem pits.

An increase in the intercellular spaces of the cortex tissue was observed at 3 *dai*, compared to the control at 3 *dai* and to the inoculated tissue at *1 dai*. Areas of cellular disorganization and destruction were found in the tissue from 7 *dai* onwards (Figure 3.7e) and in the tissue of the central cylinder at 14 *dai*. Sections of the cortex sampled at 7 and 14 *dai* showed cells with accumulation of granular substances that stained with TBO and formed clusters around intracellular pathogen structures (Figure 3.7c).

3.4.- Discussion

- Root rot

In this study, we inoculated *Q. ilex* roots with *P. cinnamomi*. The symptoms we describe are consistent with reported studies of *P. cinnamomi* inoculation in woody plant species. The lesions that appeared on the tip of the lateral roots have been described for other species (Cahill and McComb, 1992; Vettraino *et al*., 2003) as well as discolouration, softening of lateral roots, exfoliation of the main root bark and appearance of necrotic stains under the bark (Cahill, 1989; Cahill and McComb, 1992; Blaschke, 1994; Jung and Blaschke, 1995; Vettraino *et al*., 2003; Larach *et al*., 2009). Here, these symptoms were found after only one day following infection. Several studies have reported that *Q. ilex* seedlings inoculated with *P. cinnamomi* show higher water stress levels than non-inoculated seedlings under different water regimes (Robin *et al*., 2001; Turco *et al*., 2004) and field conditions on trees of different ages (Corcobado *et al*., 2013). Loss of lateral

roots and arrest of root growth seem to be the cause of early water stress levels that lead to early defoliation of the underdeveloped seedlings.

According to the study of Cahill *et al.* (1989), from 24 to 48 h after inoculation total root growth arrest occurs in the affected plants due to the damage of fine rootlets and the reduction of supply of water and nutrients to the plant. In *Q. ilex*, root growth arrest may also be associated with early disruption of the root tip, which disables the root meristem and prevents the longitudinal growth of the root.

- Pathogen detection and biological significance

For all sampling times, the oomycete was observed invading tissues via the apoplast and cell plasmalemma. Asexual reproductive structures (chlamydospores) were found inside the cortex cells. In other studies, the accumulation of spherical or hemispherical bodies inside the parenchymal cells of the central cylinder were identified after 14 *dai* (Ruiz-Gomez *et al.*, 2012; Jung *et al.*, 2013). These structures have recently been identified as stromata, which are hyphal aggregations confined to one cell or distributed among several cells that function as a survival structure when complete necrosis of the root occurs (Crone *et al.*, 2013; Jung *et al.*, 2013). These structures were considered in this study as specific pathogen structures (SSA), and their proliferation was responsible for the significant increase in this index between 7 and 14 *dai* in type S1 sections.

The pathogenicity of P. cinnamomi in holm oak has been established by Koch's Postulates (Gallego *et al.*, 1999, Romero *et al.*, 2007). The presence of chlamydospores and stromata inside the cells, the discontinuity between the cortex and central cylinder, and the display of areas of cell destruction, provide further evidence of the susceptibility of holm oak to pathogen infection.

Intracellular hyphal structures of less than 3 μm in diameter, in addition to the branches and vesicles that appeared near them and the cell wall (Figure 3.2b and red arrowheads), could form part of filiform

haustoria, such as those identified by Blaschke (1994) by scanning electron microscopy (SEM) in *Q. robur*, and recently by transmission electron microscopy (TEM) and visible light microscopy by Crone *et al.* (2013) for *P. cinnamomi* in several species.

It is thought that *P. cinnamomi* may behave as a saprophyte by proliferating in soils with dead organic matter (Shea *et al.*, 1980) or as a necrotroph by invading plant tissues (Hardam, 2005), but the existence of haustoria indicates that it could also behave as a biotrophic organism (Attard *et al.*, 2010; Crone *et al.*, 2013). The evidence found in this study indicates that *P. cinnamomi* behaves as a biotroph in the early stages of root invasion in holm oak, but when the amount of infection reaches a threshold value, the processes that cause root rot begin. From this point forward, the pathogen acts as a necrotroph.

- Pathogen development inside the root

The infection indexes clearly described the pathogen behaviour over time. Figure 3.8 shows a scheme of the pathogen development pattern inside fine roots. Infection starts when a zoospore, chlamydospore or another infective body (i.e. stromata or hyphae) is brought into contact with the surface of a suitable host root, passing through intercellular spaces of epidermal tissue and invading cells and intercellular spaces of most external cortex cells (C).

Previous studies based on *P. cinnamomi* infection have remarked that the time required by the pathogen to invade the central cylinder varies according to the host and pathogen species, ranging from 24 h after inoculation (Cahill *et al.*, 1989) to 48-72 h, when root colonization is at an advanced stage (Blaschke, 1994; Brummer *et al.*, 2002).

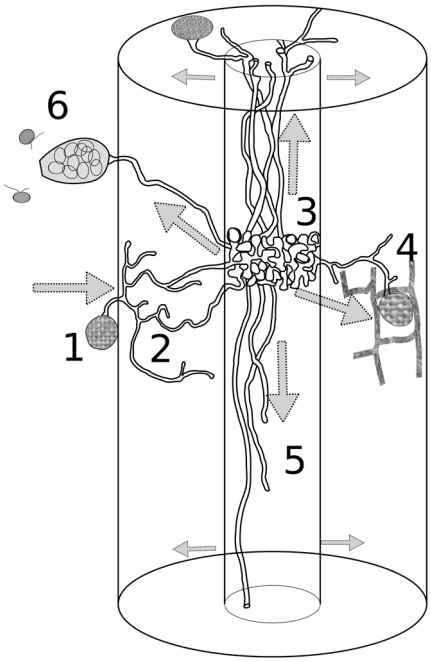

Figure 3.8. Proposed model for the development of the pathogen inside absorbent roots over time. 1: The infective structure contacts the epidermis and germinates. 2: Hyphae grow through cortex tissue, aiming to reach parenchymatous cells inside the central cylinder. 3: Development of functional structures in parenchymatous tissue of the central cylinder, such as finger-like haustoria and functional intracellular hyphae. When the invasion of this tissue is complete, the oomycete starts to create stromata and expands again transversally towards the cortex developing chlamydospores (4) and along the root longitudinally through the xylem vessels (5). Finally, it expands towards the epidermis (6) to sporulate.

In this study, pathogen structures did not appear in xylem before 3 *dai*, and during this early stage invasion occurred with a few hyphae. When the pathogen reached the central cylinder and colonized the symplast of parenchymatous cells, an exponential growth of pathogen structures took place, as shown by the evolution of the indexes of pathogen structure area. The increase of ISA in stele (S1 and S2) tissues entailed a decrease in the rate of ISA and ESA growth in cortical (EP and C) tissues. Growth of TSA at this time (from 3 *dai* onwards) was due to SSA increment.

We observed a decreasing trend in the percentage of infected cells for the cortical sections data (EP and C) around 3 and 14 *dai*. Pathogen development was focused on the central cylinder tissues (S1 and S2), primarily developing its structures in the intracellular spaces. In S1 and S2 sections, we found significant growth differences at all sampling times, corresponding to increase of ISA and ESA indexes.

Unlike random growth patterns in other necrotrophic root pathogens, *P. cinnamomi* is able to differentiate between tissues, and focuses its growth on the appropriate kind of structures for better root colonization. In parenchymatous tissues, haustorial structures such as hyphae and stromata developed, whereas in the cortex chlamydospores appeared. Vegetative hyphae were detected in all tissues, but this structure was the only one existing inside xylem and protoxylem cells. Thus, it seems that the pathogen expands longitudinally through the vascular tissue and colonizes other areas of the root from the central cylinder, feeding on inner tissues and completing its reproductive stage outward. Hence, this appears to be the principal cause of early collapse of the vascular system.

Towards the end of the experiment, ESA and ISA indexes increased in EP and C sections. Once the degree of colonization of parenchymatous tissues of the central cylinder was sufficient to allow the pathogen to complete its cycle, the growth pattern changed from the inner to the outer parts of the root, where it again colonised cortical tissues. The pathogen developed chlamydospores within the intracellular spaces of the inner cortex (C) following the apoplast path towards the outer cortex to produce sporangia. Other authors have reported presence of sporangia of *P. cinnamomi* in the surface of fine roots of susceptible plants at 2 or 3 *dai* (Cahill *et al*., 1989; Hardam, 2005; Jung *et al*., 2013) and chlamydospore formation in cortex tissues (Cahill *et al*., 1989; Blaschke, 1994; Crone *et al*., 2013; Jung *et al*., 2013).

Significant changes in the development indexes in inner cortical (C) tissues were linked to both the presence of pathogen structures in central cylinder (between 3 and 7 *dai*), and the stage in which the pathogen growth increased significantly. At that time (7 *dai*) cell necrotic areas were absent and discontinuity between cortex and central cylinder was rarely found. Once the pathogen reached parenchymatous cells of central cylinder, its supply of nutrients was increased, allowing for faster growth and, finally, expansion towards new unexplored root areas through vascular tissues.

- Host response

Sections of inoculated plants showed specific structural characteristics not present in mock-inoculated plants. Cellular necrotic areas or increase in intercellular spaces due to pathogen activity in the root have been previously reported (Cahill *et al*., 1989; Blaschke, 1994). However, other phenomena such as increased cell wall thickness or accumulation of different substances in the intracellular space of metaxylem and protoxylem cells, even blocking the pits of cell wall, have been described as host responses to the invasion of *P. cinnamomi* (Cahill *et al*., 1989; Blaschke, 1994).

It is possible that greenish accumulations in the middle lamella correspond to phenolic-like compounds (Cahill *et al*., 1989). Some phenolic substances, including gallic acid, ferulic acid or p-cumaric acid, alongside their structural function in cell walls, play an important role as bactericides or fungicides, as well as in the protection of the plant against oxidative stress (Li *et al*., 2010). Brummer *et al*. (2002) identified the presence of electronic dense material in 90% of intercellular and wall accumulation observed in roots of *Q. robur* inoculated with *P. quercinia* by TEM. This accumulation of electronic-dense material suggests the presence of a phenolic matrix.

The intense ruthenium red staining in cell walls and apoplast of infected tissues indicates the presence of de-esterified pectins (Vallet *et al*., 1996). It is well known that pectin-methyl estearase (PME), pectin liase and polygalacturonase 2 (PG2) are involved in de-esterification of pectic substances, and the genes that code these enzymes have been identified in diverse *Phytophthora* species (LeBerre *et al*., 2008; Attard *et al*., 2010). The high degree of de-esterification shown in the advanced stages of invasion indicates less consistency of cell walls, which could result in an increase in the number and size of intercellular spaces, and contribute to the discontinuity between cortex and central cylinder. Furthermore, it facilitates the colonization of intracellular spaces by the pathogen, allowing

development of chlamydospores or stromata without the destruction of cell walls. In general, the presence of de-esterified pectins can be related to the flaccidity of fine roots, which is a symptom described in this and previous histopathology studies (Cahill *et al.*, 1989; Cahill y McComb, 1992). Discontinuity between cortex and stele contributes to reduced water uptake, increasing plant drought stress.

The accumulation of pectidic substances identified with ruthenium red in vessels of vascular tissues and schlerenchyma cells represents a defence mechanism against pathogen expansion through the vascular system (Jung and Blaschke, 1995; Oh and Hansen, 2007). In most cases, obstructed and sealed cells did not show pathogen structures and the cell wall pits were sealed (Figure 2B; Figure 7H). However, it is important to note that these accumulations differ from tyloses, which were not identified in any sample, contrasting with their description as part of the symptoms in other studies (Cahill *et al.*, 1989; Blaschke, 1994; Jung and Blaschke, 1995).

3.5.- Conclusions

We showed that the main morpho-anatomical effects caused by *P. cinnamomi* invasion in the secondary root system are related to: i) blockage of vessels produced by hyphae accumulation and mucilage secretions of neighbouring cells, leading to collapse of the vascular system; ii) disruption of meristematic development, which stops the growth of new fine roots; iii) softening of fine root tissues caused by de-esterification and cell wall degrading processes; iv) discontinuity between cortex and central cylinder; and v) loss of fine roots due to the above described effects and to the necrotrophic behaviour of the pathogen in advanced colonization stages.

The main physiological effect of these morpho-anatomical root changes is a severe reduction of water supply and induction of water stress, which causes drought plant mortality (Corcobado *et al.*, 2013). *Q. ilex* trees, adults or saplings, affected by *P. cinnamomi,* always show higher values of

xilematic water potential than healthy plants (Corcobado *et al.*, 2013; Sghaier-Hamammi *et al.*, 2013). In Mediterranean climate conditions, where seasonal drought and water availability are important limiting factors, those differences are the main factor in tree mortality caused by *P. cinnamomi*.

Regarding host responses and possible resistance-tolerance mechanisms, we identified tissue defence reactions in the host plant, but such responses failed to prevent pathogen development. It is well known that *P. cinnamomi* quickly expands to other root areas through the vascular system and changes its trophic relation with the host, becoming biotrophic or necrotrophic under different conditions. Such capabilities allow the pathogen to overcome the host defences against colonization.

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Chapter 4.- Physiological responses and growth changes of seedlings to *P. cinnamomi* inoculation

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Abstract

The sustainability of "dehesas" and "montados" ecosystems is nowadays threatened by the holm oak decline. The effects of root rot on plant physiology vary depending on external stress factors, being plant growth and biomass allocation useful tools to characterize differences in the response to drought and infection. The study of physiologic responses together with growth patterns will clarify how and to what extent root rot is able to severely damage the plant. A full factorial experiment, including drought and *P. cinnamomi* infection as factors, was carried out with *Quercus ilex* L. seedlings in a 30-days greenhouse experiment. Photosynthetic parameters were measured at 0, 7, 15, 22 and 30 dpi; additionally, biomass allocation and root traits were calculated at the end of the experiment. Photosynthetic variables responded differently to drought and infection over time. The root mass fraction showed changes due to infection, supporting our hypotheses. *Phytophtora cinnamomi* root rot altered mainly the growth patterns of plants, while the plants could not recover from the physiological effects of infection only when the root rot coincided with water stress. Without additional stressors, the strategy of our seedlings in the face of root rot was to reduce the biomass increment and reallocate resources.

Resumen

La sostenibilidad de los ecosistemas de dehesa y montados se encentra hoy en día amenazada por el decaimiento de la encina. Los efectos de la podredumbre radicular en la fisiología de la planta varían dependiendo de los factores externos de estrés, presentándose el crecimiento y la compartimentación de biomasa como herramientas útiles para caracterizar las diferencias en la respuesta ante la sequía y la infección. El estudio de las respuestas fisiológicas junto con los patrones de crecimiento explicará cómo y en qué medida la podredumbre radicular es capaz de dañar severamente a la planta. Se llevó a cabo un experimento en invernadero con plántulas de *Quercus ilex*, considerando la sequía y la inoculación con *P. cinnamomi* como factores. Se midieron los parámetros relacionados con la fotosíntesis a los 0, 7, 15, 22 y 30 dpi; asimismo, se calcularon los valores de materia seca y compartimentación de biomasa al final del experimento. Los parámetros de fotosíntesis respondieron de forma diferencial a la sequía y la infección con respecto al tiempo. La fracción de biomasa radicular presentó cambios debido a la infección, de acuerdo con nuestras hipótesis. La podredumbre radicular causada por *P. cinnamomi* alteró principalmente los patrones de crecimiento de las plántulas, aunque estas únicamente no pudieron recuperarse de los efectos de la inoculación cuando la podredumbre radicular coincidió con el estrés hídrico. Sin a presencia de dicho estrés adicional, la estrategia de nuestras plántulas para hacer frente a la podredumbre radicular fue la de reducir el incremento de biomasa y relocalizar recursos.

Keywords: Drought, biomass allocation, dehesa, oak decline, plant traits, root rot.

4.1. Introduction

Holm oak (*Quercus ilex* L.) is a native species, widely distributed in the central-western part of the Mediterranean basin. This tree can be considered a key species due to its ecological and socioeconomic importance, and it shows a high rate of phenotypic plasticity in relation to conditions such as temperature, elevation, and soil composition (De Rigo & Cadullo, 2016). It is considered a drought-tolerant species and thus can play an important role against desertification (Gil-Pelegrín *et al.*, 2017). The most representative examples of its socioeconomic relevance are the "dehesas" and "montados" agroforestry systems, present mainly in Spain and Portugal (Guzmán Álvarez, 2016; Pinto-Correia & Azeda, 2017).

Since the 1990s, oak decline has been recorded in Europe (Thomas *et al.*, 2002), holm oak being the species most affected in the Mediterranean area. In south-western Europe (Spain and Portugal), rangeland management practices, climatic change, and biotic agents are the main factors triggering tree death and ecosystem loss, making the ecosystems here some of the most vulnerable in the Mediterranean area (CAP, 2008; Gea-Izquierdo *et al.*, 2013).

An increase in the extremity of rain episodes and in the extent of drought periods, alongside the rise in mean temperatures, might play an important role in the decline of holm oak, as indicated by habitat projection models for some specific areas of the south-west of the Iberian Peninsula (Gea-Izquierdo *et al.*, 2013; Gil-Pelegrín *et al.*, 2017).

The severity of root rot and decline symptoms in *Q. ilex*, including mortality, has been related to water stress, both drought and waterlogging, which, depending on microsite conditions, can occur sequentially (De Rigo & Cadullo, 2016; Swiecki & Berndhart, 2017). Regarding biotic factors, root rot oomycetes, mainly *Phytophthora cinnamomi* Rands., are considered a causal agent or triggering factor in holm oak decline (Brasier *et al.*, 1993;

Brasier, 1996; Hardham & Blackman, 2018). *Quercus ilex* is considered the most susceptible species of the genus to the root rot (Moralejo *et al.*, 2009).

Although some unspecific responses were detected in previous histological studies of infected roots (Ruiz Gómez *et al.*, 2012, 2015), the main changes in plant status caused by *P. cinnamomi* infection are related not to defensive responses (PAMP-triggered immunity, effectors-triggered immunity, HR, hormonal signaling…), but to physiological ones (Sghaier-Hammami *et al.*, 2013; Oßwald *et al.*, 2014; Corcobado *et al.*, 2014; Hardham & Blackman, 2018). The relationship between root rot and its associated water stress and mortality of trees and seedlings remains unclear in some aspects. Not only drought, but also waterlogging treatments have been demonstrated to have significant effects on the mortality of infected plants (Corcobado *et al.*, 2013, 2014; Swiecki & Berndhart, 2017). Little has been published on physiological changes in holm oak plants as a response to *P. cinnamomi* infection, with high variability of results depending on the experimental conditions. Some reports have shown strong differences in photosynthesis and water potentials (Corcobado *et al.*, 2014; Sghaier-Hammami *et al.*, 2013), while others have shown no significant effects (León *et al.*, 2017; Maurel *et al.*, 2001; Turco *et al.*, 2004), depending on whether the plants were subjected to continuous and acute waterlogging, drought stress, or only slight stress. All of the above highlights the relevance of studying the possible differences in the responses of holm oak to water stress and pathogen infection.

Variables related to plant morphology and biomass have been demonstrated to be powerful tools in the analysis of ecological, ontological, or physiological differences among species, ecotypes, or stress conditions (Bongers *et al.*, 2017; Poorter *et al.*, 2015). Plant growth changes are related to physiological responses to stress conditions; in particular, biomass allocation is a key factor (Poorter *et al.*, 2012). Root traits were found to be related to different key processes that vary in changing environmental conditions (photosynthesis and photosynthates allocation, drought

tolerance strategies, water potentials, transpiration rates…) (Chen *et al.*, 2017; Lopez-Iglesias *et al.*, 2014; Olmo *et al.*, 2014; Roumet *et al.*, 2016).

The main objective of this study was to characterize the effects of acute water stress and *P. cinnamomi* infection in seedlings of *Quercus ilex* L. subsp. *ballota*, focusing on physiological, growth, and biomass allocation changes. We hypothesized that the effects and plant responses would differ according to whether the seedlings were water stressed, inoculated, or both, and that there would be significant variation in the magnitude of the responses, depending on the type of stress. To reach our main objective, we defined three specific goals: i) to describe changes in physiological plant status due to either *P. cinnamomi* infection or drought; ii) to evaluate plant growth changes related to drought stress and *P. cinnamomi* infection, analyzing biomass allocation, and iii) to determine and characterize differences in the effects of water stress (drought) and *P. cinnamomi* infection on *Q. ilex* roots. This will clarify how and to what extent root rot is able to severely damage the plant, thereby helping the development of more accurate strategies to palliate the consequences of holm oak decline.

4.2. Materials and Methods

- Plant material

Seedlings of *Quercus ilex* subsp. *ballota* were used in this experiment. Acorns were collected in a warm-temperate holm oak forest in Arenas del Rey (Granada, Spain, ETRS89, UTM 30N: 417 586, 4 095 930, elevation 490 m.a.s.l.). The site is characterized by dry summers and wet, mild winters: the mean temperatures over the last 30 years are 24.7 °C (warmest month) and 11.5 °C (coldest month), and the average annual rainfall is 489.3 mm. This parental tree was chosen because it was considered drought tolerant in previous studies (Navarro Cerrillo *et al.*, 2018).

The acorns were sown in a peat-perlite-vermiculite growth medium (4-2-1 by vol.), in black plastic containers (2.5 L). These were placed in a

growth chamber (25 ºC, 60 % RH, photoperiod 14/10 light/dark) until acorn germination. Then, the seedlings were placed in a greenhouse at the University of Córdoba-Campus Rabanales (Córdoba, Spain; ETRS89, UTM 30N: 348 360, 4 198 200), in semi-controlled conditions (25 ± 7 ºC, 60 ± 10 % RH), and were watered twice a week to saturate the growth medium. For three months, artificial light (HPS lamps, 400 W, 48000 Lm, ≥600 μmol (photons) m^{-2} s^{-1} at 50 cm above the table surface) was provided, to extend the photoperiod to 12 h and ensure that the photosynthetic photon flux density (PPFD) exceeded 1000 μmol (photons) m^{-2} s^{-1}. This value is considered to be enough to light-saturate *Q. ilex* leaves (Quero *et al.*, 2006). Before the experiment, seedlings were selected that were homogeneous in morphology (height, H=31.79 ± 0.92 cm; diameter at the root collar, Øi=6.98 ± 0.16 mm; total leaf area, LAt=202.6 ± 9.3 cm^2; mean values with standard error), instantaneous chlorophyll fluorescence, and photosynthetic efficiency of photosystem II (Ft=3205 ± 409; QY=66.4 ± 5.2%; FluorPen FP100 fluorescence analyzer, Photon Systems, spol. s.r.o., Drásov, Czech Republic).

- Experimental design and inoculation

The experiment had a completely randomized design in which inoculation (2 levels: with and without) and watering (2 levels: with and without) were the main factors, resulting in four different treatments: *Control* (pots watered and mock-inoculated), *Inoculation* (pots watered and inoculated with *P. cinnamomi*), *Drought* (Pots non-watered and mock-inoculated), and *I×D* (Pots non-watered and inoculated with *P. cinnamomi*). Each of the four treatments had 10 replicates, providing a total of 40 experimental units. The pots were placed in black plastic trays, five pots of the same treatment in each tray, and the trays were distributed randomly in the greenhouse.

Inoculation was carried out with carrot agar (CA) liquid inoculum at a concentration of >30 Infective Units (IU)/μL of *P. cinnamomi* chlamydospores (Scholander *et al.*, 1965). The *P. cinnamomi* strain was

isolated from *Q. ilex* roots in a previous survey in Puebla de Guzman (Huelva, Spain). The pathogen was grown in 9-cm-diameter Petri dishes containing CA medium for 15 days. Prior to inoculum preparation, the surface of the CA containing the pathogen mycelium was well rinsed and the CA was mixed with demineralized water. The concentration of IU (chlamydospores) was evaluated using a Neubauer chamber. A false inoculum was made by mixing the same number of CA plates, but without *P. cinnamomi*, with water. The methodology used for the inoculation treatment was adapted from the work of Turco *et al*. (2004). Three holes were made in the substrate of each pot, using a 10-mL syringe with a trimmed end. Then, approximately 15 mL of inoculum or false inoculum were placed in each hole (45 mL in each pot) and subsequently covered with the substrate extracted previously with the trimmed syringe.

Before inoculation, the pots were watered to substrate saturation, the plants were inoculated, and the pots were subsequently weighed to obtain their weight at the field capacity of the substrate. The watering regime of the control and inoculation treatment consisted of a first manual watering 72 h after inoculation, and subsequent watering every 48 h with 100 mL of water. To boost the induction of water stress in the treatments that did not include watering, the environmental conditions of the greenhouse were altered to increase the evapotranspiration rate of the plants (T=28±3 ºC; RH=40±10 %).

- Parameters measured

The physiological status of the plants was evaluated through the stomatal conductance and the rate and efficiency of photosynthesis, at the beginning of the experiment (0 days post- inoculation – dpi) and at 7, 15, 22, and 30 dpi. Thirty days after inoculation, the plants were harvested and their water status, aboveground and belowground fractions, and root distribution parameters were characterized. The description and units of each measured or calculated variable are shown in Table 4.1.

Table 4.1. Description of studied variables

Variable	Abv.	Units	Description
Water status			
Volumetric Water content	θ	$cm^3\ [cm]^{-3}$	Relative humidity of pot substrate
Midday Water potential	Ψ_m	MPa	Water potential of leaves at midday (at solar noon)
Dry Matter of root	*DMr*	$g\ [100g]^{-1}$	Dry matter of root relative to 100 g of fresh weight
Plant growth and biomass allocation			
Net Photosynthesis	*A*	$\mu mol\ CO_2 m^{-2} s^{-1}$	Mean estimate of photosynthetic rate of leaves
Stomatal Conductance	*Gs*	$mmol\ H_2O\ m^{-2} s^{-1}$	Mean estimate leaf stomatal conductance
Photosynthetic Efficiency	*QY*	...	Max. quantum efficiency of photosystem II (Fv/Fm)
Root Dry Weight	*RDW*	g	Weight of root system after oven drying (root biomass)
Stem Dry Weight	*SDW*	g	Weight of stem after oven drying (stem biomass)
Leaf Dry Weight	*LDW*	g	Weight of leaves after oven drying (leaves biomass)
Root Mass Fraction	*RMF*	$g\ [g]^{-1}$	Root biomass relative to total plant biomass
Stem Mass Fraction	*SMF*	$g\ [g]^{-1}$	Stem biomass relative to total plant biomass
Leaf Mass Fraction	*LMF*	$g\ [g]^{-1}$	Leaves biomass relative to total plant biomass

The volumetric water content (θ) of the pot substrate was measured during the experiment, in five pots per treatment, using time domain reflectometry probes (Decagon ECH2O Ec-5 - Decagon Devices, Inc., Washington USA), previously calibrated for the specific pot substrate composition (accuracy ±1%, resolution 0.1% of θ). Prior to harvest, the midday stem water potential (Ψm) of three leaves per pot was measured using an SKPM 1400 pressure chamber (Skye Instruments, Ltd.; Llandrindod Wells, Powys, UK) (Scholander *et al.*, 1965). The minimum Ψm considered was -6 MPa, due to the technical limitations of the pressure chamber; in *Q. ilex* the minimum values of Ψm before sap flux failure range between -4 and -6 MPa (Tognetti *et al.*, 1998).

The maximum quantum efficiency of photosystem II (QY) was measured using a Hansatech PEA portable chlorophyll fluorimeter (Hansatech Instrument, Ltd.; King's Lynn, Norfolk, UK), for dark-adapted leaves after covering them for 20 min with portable leaf clips (Hansatech

Instrument, Ltd). Five fully-expanded leaves per plant were measured at 0, 7, 15, 22, and 30 dpi.

The net photosynthesis rate (*A*) and stomatal conductance (*Gs*) were measured in three fully-expanded leaves of each plant, using a portable infrared CO_2 gas analyzer (LiCor Li6400XT, Li-Cor, Inc.; Lincoln, NE, USA) fitted with a 6-cm^2 leaf cuvette. The measurements were taken using a CO_2 concentration of 390±1.7 ppm, a flow of 300±1.2 cm^3 min^{-1}, and PPFD>1000 μmol (photons) m^{-2} s^{-1}. When a leaf did not fit completely in the leaf cuvette, a photograph of it was taken using a digital camera (HP Photosmart R827, Hewlett Packard Inc., Palo Alto, CA, USA). In order to correct the measurements according to the actual leaf area, the photographs taken at the moment of each measurement were analyzed using ImageJ image analysis software (Schneider *et al.*, 2012). All the measurements of physiological variables were taken at 11:20-13:20 h UTC, considering a 2-h window around the solar noon (13:20-15:20 h CET).

After the physiological measurements, the stems and leaves were excised from the root collar and the root ball was extracted from each pot. A subsample of fine roots was collected for pathogen isolation and, subsequently, the root ball was carefully washed with tap water on a 0.5-mm sieve, avoiding the loss of fine roots (Phillips & Hayman, 1970). The fine and very fine roots lost in this process were recovered from the detached substrate using tweezers; subsequently, the root fraction was scanned in a Regent LA1600+ densitometer (Regent Instruments Inc., Quebec, Canada).

The plant biomass was estimated for the stem, leaves, and roots. The stems and leaves were dried immediately after excision (85 ºC for 48 h; JP Selecta Conterm, Barcelona, Spain), in paper bags of known weight. The root fraction was dried after scanning, following the same procedure conducted for the aboveground biomass. All the dried samples were cooled in a desiccator at room temperature for 30 min prior to weighing. All the

biomass fractions were expressed as dry biomass (stem dry weight, *SDW*, leaves dry weight, *LDW*, and root dry weight, *RDW*, in g).

- Pathogen isolation

To confirm the presence or absence of *P. cinnamomi* in the root system, a representative subsample of fine roots was collected for each plant, prior to root-ball detachment. Fifty pieces of fine and very fine roots (Ø<2 mm), approximately 1 cm in length, were excised randomly from different regions of the root-ball. They were surface-disinfected by immersion in 70% ethanol for 10 s, washed in sterilized-deionized water, trimmed, and placed in 9-cm Petri dishes containing the PARPBH selective medium (Jeffers, 1986). They were stored at room temperature, in darkness, for 14 days and were assessed every 48 h.

The colonies that grew were sub-cultured and sown in PARPBH and CA to obtain axenic cultures. The colonies were observed under a microscope to identify the genus or species according to their morphology, following the indications of Erwin and Ribeiro (Erwin & Ribeiro, 1996). The pathogen *P. cinnamomi* was recovered and isolated from fine roots of all the samples of the inoculation treatments and was not isolated from any of the studied roots from mock-inoculated plants. No other oomycete species was isolated from the samples.

- Data analysis

The scanned images of roots were analyzed with WinRHIZO Pro 2004a software (Regent Instruments Inc., Quebec, Canada).

The effects of the factors on the root variables were calculated, according to Olmo *et al.* (2014), as the ratio of the mean value of a root trait under the treatment to its mean value under control conditions. A ratio greater than 1 means that the treatment increased the value of this trait and thus was considered (+), and (-) for values between 0 and 1.

The plasticity index (*Pi*) of each variable was calculated as described by Valladares and Sánchez-Gómez (2006), with the expression

$$Pi=(Xmax-Xmin)/Xmax \quad (1)$$

The *Pi* varies between 0 and 1. The mean root *Pi* was calculated as the average plasticity of all the root variables, to compare treatments.

The normality and homoscedasticity of the variables were assessed using the Shapiro-Wilk and Levene tests, respectively. Variables that did not fit a normal distribution were transformed by applying log(x) or 1/x (Milton & Tsokos, 1983), and the normality of the transformed variables was re-analyzed. After the normality test, two-way ANOVA was carried out, considering inoculation and watering as the independent factors. When no interaction between factors was detected in the two-way ANOVA, the Student's t test was used to study the effects of inoculation and watering on biomass variables. Repeated measures ANOVA (RMANOVA) was carried out for the physiological variables and θ, using dpi as the repeated measure and considering the watering and inoculation treatments as between-subject factors. To avoid test failure due to noncompliance with the ANOVA assumptions, Greisser-Greenhouse correction of the degrees of freedom was used for univariate within-subjects' analysis. The post-hoc comparison of treatments, in all other cases, was carried out using Tukey's HSD test. Null hypotheses were rejected at the $p<0.05$ level. All the statistical analyses were performed using IBM SPSS Statistics 19 (IBM Corp., Armonk, NY, USA).

4.3. Results

- Plant and root symptoms

Plants of the *Drought* and combined (*I*×*D*) treatments presented strong chlorosis and wilting of the leaves at the end of the assay. In the *Inoculation* treatment (watered), the symptoms were more variable - with

chlorosis and partial wilting in several plants, while others had only slight symptoms in the upper part of the stem. No plants from the *Inoculation* (watered) treatment were dead after 30 days. No chlorosis or wilting was seen in plants from the *Control* treatment (Figure 4.1).

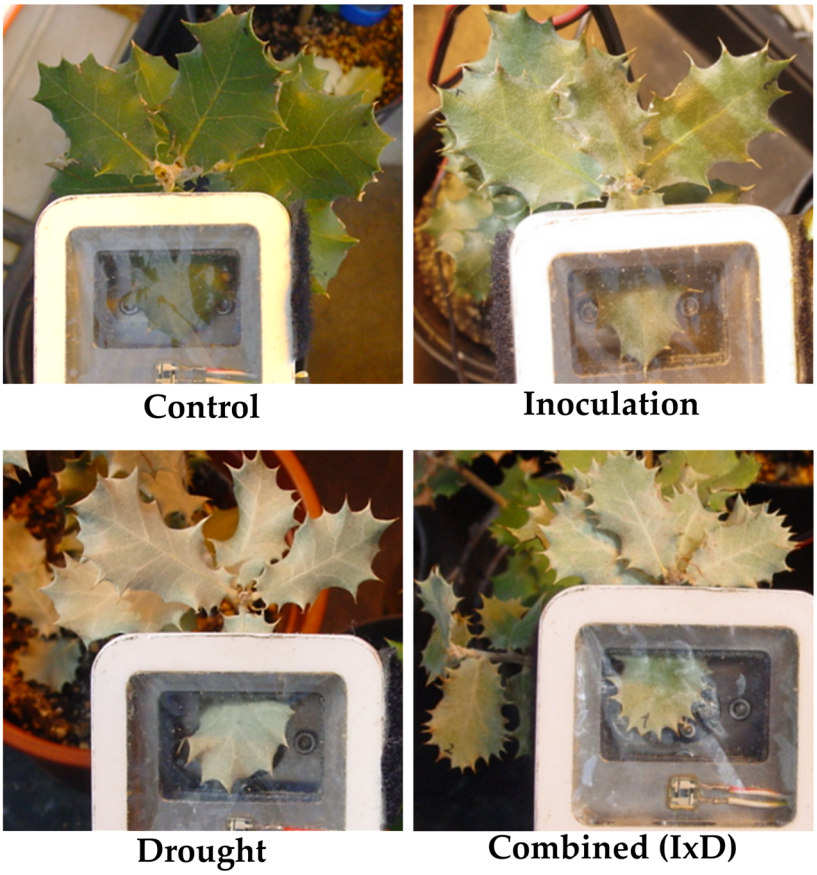

Figure 4.1. Leaf symptoms for the different treatments after 30 days.

The root system of plants from the *Drought* and combined (*I×D*) treatments exhibited taproot necrosis and root frailty. The plants from the Inoculation treatment (watered) showed root rot symptoms - consisting of dark-brown coloration, necrotic lesions in tips, and root softness - when compared with control plants. Another important symptom was the lack of fine lateral roots on the coarse roots (Ø>2mm).

- Water status

The RMANOVA showed significant differences in the volumetric water content of the substrate (θ) regarding watering (F=91.7; p<0.001) and inoculation (F=6.5; p<0.05). Time (dpi) strongly influenced θ (F=373.8; p<0.01) (Table 4.2, Figure 4.2a), with a significant effect of the interaction between dpi and the watering treatment (dpi×D; F=60.9; p<0.001).

Table 4.2. Repeated-measures ANOVA (RMANOVA) results for the effect of inoculation and water stress on physiological variables and volumetric water content. The degrees of freedom (df), type III sum of squares and significance level of effects are shown for each variable. Geisser-Greenhouse adjusted probabilities were used for the within-subject analysis. Values of sum of squares are highlighted in bold type when significant (*p<0.05; **p<0.01; ***p<0.001).

	df	A	Gs	QY	θ
Between-subjects source					
Inoculation (I)	1	**31.531*** **	**0.016** **	**0.183** **	**0.047***
Drought (D)	1	**86.041*** **	**0.010***	0.001	**0.660** **
I×D	1	0.394	0.000	**0.351*** **	0.002
Error	16	31.698	0.023	0.304	0.058
Within -subjects source					
Days (dpi)	4	22.821	0.007	**0.262*** **	**0.901*** **
dpi×I	4	3.254	0.003	0.015	0.002
dpi×D	4	**77.014*** **	0.007	**0.162** **	**0.147*** **
dpi×I×D	4	8.279	0.001	0.051	0.001
Error	64	139.518	0.051	0.472	0.02

The differences due to inoculation were not influenced by dpi. At the end of the experiment, water stressed plants (*Drought* and *I×D* treatments) presented values of θ around 0.1 cm3 cm-3, and midday water potential (Ψm) values below -6 MPa, with differences only between watered and non-watered plants (Supplementary material, Table S4.1).

The root dry matter content (*DMr*) was influenced by a significant interaction between the watering and inoculation treatments (Table 4.3). Although no significant differences in *DMr* due to the inoculation factor were found at 30 dpi, plants of the *Inoculation* (watered) treatment had a

significantly lower value than *Control* plants, without differences between the *I×D* and *Drought* treatments (Figure 4.3).

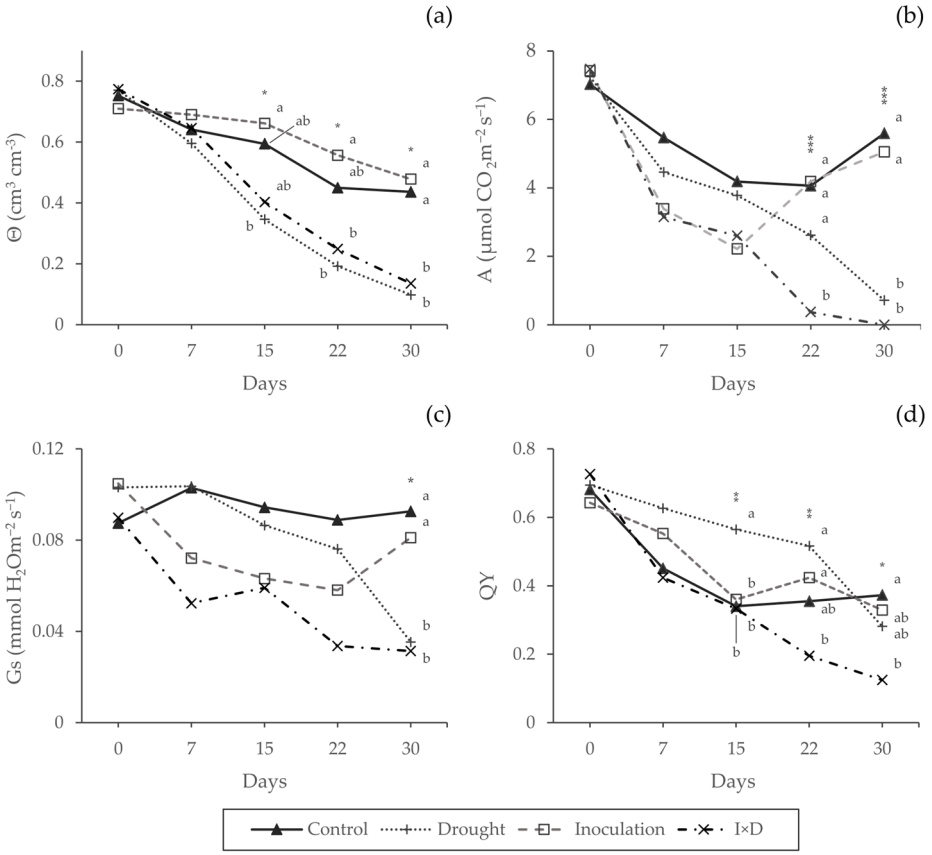

Figure 4.2. Physiological characterization of *Quercus ilex* seedlings during the experiment. (a) Volumetric water content. (b) Net photosynthesis. (c) Stomatal conductance. (d) Photosynthetic efficiency of Photosystem II. Points with the same letter are not significantly different for the analyzed dpi ($p>0.05$). Differences between values of the same days are represented only when significant at $p<0.05$ (*), $p<0.01$(**) and $p<0.001$(***).

- Photosynthesis

At 30 dpi, only the watering treatment had a significant effect on the net photosynthesis rate (*A*) and *Gs* (Figure 4.2bc; Supplementary material, Table S4.1), while *QY* differed significantly only between the *Control* and *I×D* treatments (Figure 4.2d). However, significant effects of both

inoculation and watering on *A* and *Gs* were found when the time-trend was analyzed (RMANOVA, Table 4.2).

Table 4.3. Two-way ANOVA results for variables measured 30 dpi. Values of F statistic are highlighted in bold type when significant (*p<0.05; **p<0.01; ***p<0.001). R^2 represents corrected ANOVA model adjustment. (+) or (–) mean that the factor has a positive or negative effect, respectively.

	Factors			
Variable	Inoculation (I)	Drought (D)	I x D	R^2
Ψ_m	2.4	**6763.7 ***(-)**	2.4	0.99 ***
DMr	0.0	**25.1***(+)**	**8.0 ****	0.61 ***
RDW	0.7	**4.3 *(+)**	0.0	0.24 **
SDW	**5.0 *(-)**	3.3	**5.5 ***	0.46 ***
RMF	**10 **(-)**	**41.7 ***(+)**	0.0 n/s	0.76 ***
SMF	**5.0 *(-)**	**12.4 **(-)**	**7.6 ****	0.61 ***
LMF	**18.8 ***(+)**	**8.8 **(-)**	3.9 n/s	0.66 ***

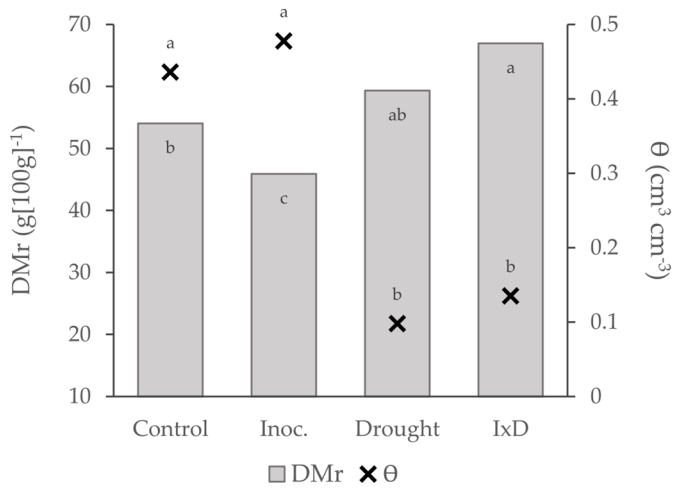

Figure 4.3. Mean volumetric water content (θ) of soil (points) and mean dry matter of roots (columns) at 30 dpi. Treatments with the same letter for each variable are not significantly different (p>0.05).

The time after inoculation did not influence *A* or *Gs*, but *A* varied significantly among the dpi depending on the water stress level, as shown by the significant interaction between dpi and the watering factor for this variable. No significant differences in *A* were found between treatments until day 22, when the value was lower for the *I×D* plants, coinciding with the lower values of *Gs*. At this time, a significant change in the trend was found in all physiological variables for the Inoculation treatment (inoculated and watered plants) (Figure 4.2). The *QY* was not affected significantly by the watering treatment, but it was by the inoculation and the interaction between factors, being significantly influenced by dpi and, as in the case of *A* as well, by the interaction between dpi and drought. The *QY* values for the *I×D* treatment at 15 and 22 dpi were significantly lower than those of the *Drought* treatment, for which *QY* and *Gs* did not decrease until the end of the experiment, when the substrate showed values of θ around 0.1 cm^3 cm^{-3}.

- Growth and biomass allocation

The root biomass (*RDW*) was higher in water-stressed plants ($t=2.2$, $df=28$, $p<0.05$) but did not differ significantly when inoculation was considered (Figure 4.4a; Table 4.3). However, despite a lack of statistical significance, inoculation gave the lowest values of *RDW*, for both watering treatments (Figure 4.4a; Supplementary material, Table S4.1).

Aboveground biomass (leaves plus stem) did not differ significantly among treatments, but the factor inoculation had a negative influence on *SDW*, with a significant interaction between the two experimental factors (Table 4.3). The relative proportion of *SDW*, regarding aboveground biomass (Figure 4.4b), was lower for the *I×D* treatment, *SDW* representing, on average, 45.6% of the total aboveground biomass in mock-inoculated plants (*Control* and *Drought*) and 27.2% for the inoculated ones. For the plants of the *I×D* treatment, on average, both *SDW* ($F=4.6$; $p<0.01$) and relative *SDW* ($F=3.6$; $p<0.01$) were significantly lower than for the *Drought* treatment plants.

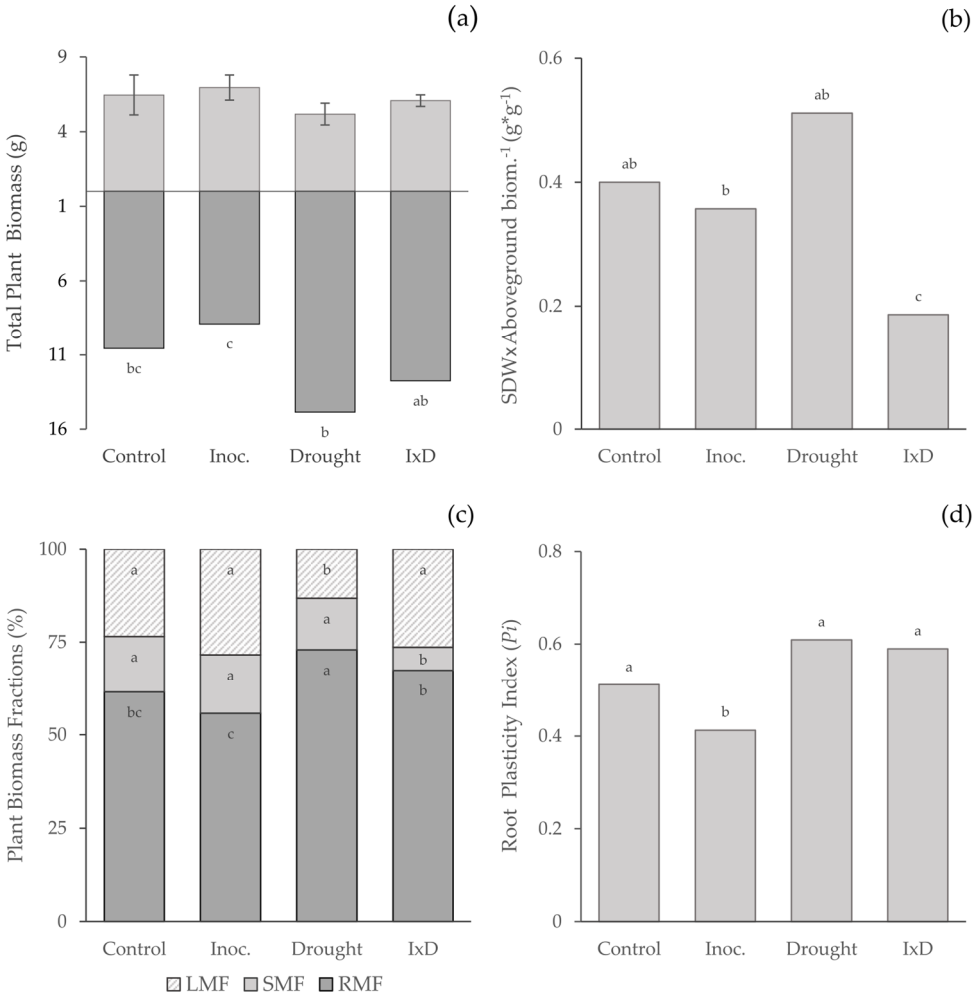

Figure 4.4. Plant biomass analysis (a) Biomass distribution per treatment. Upside mean aboveground (Stem and leaves) biomass. Downside mean belowground (root) biomass. (b) Relative stem biomass respect to total aboveground biomass, per treatment. (c) Plant fractions for each treatment. LMF= Leaf mass fraction. SMF= Stem Mass Fraction. RMF=Root Mass Fraction. (d) Average plasticity index for each treatment. Error bars show ±SE. Bars with the same letter are not significantly different ($p>0.05$).

The biomass fractions (*RMF*, *SMF*, and *LMF*) were significantly influenced by both inoculation and watering, but the interaction between them was only significant for *SMF* (Table 4.3). The *RMF* decreased due to

the effect of inoculation ($t=-2.7$, $df=38$, $p<0.05$) and increased due to water stress ($t=5.4$, $df=38$, $p<0.001$), while *LMF* increased significantly due to inoculation ($t=3.4$, $df=38$, $p<0.01$). The graphical comparison of fractions and treatments (Figure 4.4c) shows different trends in the aboveground (*SMF*, *LMF*) and root (*RMF*) fractions. All the biomass allocation parameters differed between the *Drought* and *I×D* treatments. The *Drought* treatment gave the maximum value of *RMF* (0.73±0.02) and the minimum value of *LMF* (0.13±0.01).

The minimum value of *RMF* occurred in the *Inoculation* treatment (0.56±0.01), but without differences from the *Control* treatment. The values of *RMF* and *SMF* for the *I×D* treatment were lower than those of the *Drought* treatment, the plants of the *I×D* treatment showing a clear decrease in *SMF* ($F=8.3$; $p<0.001$). The average *Pi* for all the studied variables shows that the roots of watered plants had lower plasticity ($t=4.5$, $df=110$, $p<0.001$). When the means of the *Pi* were compared for each of the four treatments in a one-way ANOVA, significant differences appeared ($F=7.87$; $p<0.001$), the mean *Pi* of the *Inoculation* treatment having the lowest value (Figure 4.4d).

4.4. Discussion

Although previous authors have proposed this idea as a hypothesis (Turco *et al.*, 2004; Corcobado *et al.*, 2013; Oßwald *et al.*, 2014), to the best of our knowledge this is the first work to evidence differences in the physiological response of *Quercus ilex* to the stress combining *P. cinnamomi* and drought, in comparison with each stress applied separately. All the studied variables and traits responded to the experimental conditions in a manner aligned with our main hypothesis, confirmed by the recovery of our seedlings inoculated with *P. cinnamomi* if no additional stress was induced and by the differential responses of physiological parameters, growth, and biomass allocation to the influence of inoculation and water supply, without interaction between them in most cases.

- General symptoms and stress indicators

The plant symptoms as a result of *P. cinnamomi* inoculation described here were similar to those reported for leaves (Hardham & Blackman, 2018; Sghaier-Hammami *et al.*, 2013) and roots (Oßwald *et al.*, 2014; Ruiz Gómez *et al.*, 2015) of holm oak seedlings in previous work. The root rot symptoms were differentiated from water stress damage in roots mainly by the color and general aspect of the root ball, the aboveground symptoms being less specific (i.e., chlorosis and wilting) (Figure 4.1).

Regarding water balance, θ was expected to be high in those pots which contained plants less able to take up water, being influenced by the inoculation. Time-trend analysis showed this significant effect, but only non-watered plants underwent water stress. However, the high *DMr* of the *I*×*D* treatment plants seems to be in conflict with this hypothesis (Figure 4.3). Previous works have reported the early accumulation of pectidic and mucilaginous materials in xylem vessels of fine *Q. ilex* roots as a result of *P. cinnamomi* infection, and also the invasion of pathogenic structures in xylem cells in advanced stages of infection (Ruiz Gómez *et al.*, 2015), this obstruction of conductive vessels causing the reduction of xylem conductance. In small plants, when water availability alternates between low and high, blocked vessels display the two stages characteristic of active vessels, alternating between embolism due to evaporative stress and refill by reverse osmosis (Canny, 1997). In well-irrigated plants, the refill of blocked vessels in coarse roots is related to higher water potential (Ψ), and could be the cause of a high moisture content in root tissue (Borghetti *et al.*, 1993), the blockage of vessels causing different effects in water stressed plants, in which reverse osmosis did not occur.

Leaf water potential is often used as a water stress indicator (Urban *et al.*, 2017). This variable was only affected significantly by drought in our study (Tables 4.2 and 4.3), agreeing with Turco *et al.* (2004) - who found that Ψ in *Q. ilex* seedlings was not changed by *P. cinnamomi* inoculation. Holm oak is considered an isohydric species (Quero *et al.*, 2011), the

detection of the first drought stress symptoms resulting in quick stomatal closure. However, in this work, inoculated seedlings responded to the root rot with early partial stomatal closure and a reduction in their photosynthetic activity at 7 dpi, at which time the pathogen would not have invaded to a significant extent the xylem vessels (Ruiz Gómez *et al.*, 2015). At this time, growth cessation and loss of root uptake ability due to root rot occurred (Oßwald *et al.*, 2014; Ruiz Gómez *et al.*, 2015). This imbalance might be related to alterations in plant osmoregulation, and thus to the increase in root tissue water content in the Inoculation treatment plants, agreeing with previous studies in other species (Aigbe & Remison, 2010). Oßwald et al. (Oßwald *et al.*, 2014) indicated that the infection of woody plants by *Phytophthora* spp. could trigger a generalized dysfunction in plant water status related to hormonal changes, with an alteration in the balance between ABA and other plant hormones involved in stomatal regulation.

- Physiological changes

Water stress produced significant reductions in *A*, *Gs*, and *QY*, agreeing with the expected response of *Q. ilex* to water stress (Quero *et al.*, 2011), but inoculated plants responded in a different way, the watering treatment influencing only photosynthetic efficiency (*QY*), but not *A* or *Gs*. The early reduction in photosynthetic activity as a result of inoculation, supported by the results of RMANOVA, might be related to the lower values of root biomass and root proportion in inoculated plants and agrees with other results which evidenced an early cessation of root growth, only 24 h after inoculation (Ruiz Gómez *et al.*, 2015). At 15 dpi, Inoculated plants had recovered their photosynthetic activity, their *Gs* and *QY* being equal to those of control plants at 30 dpi, but plants of the *I×D* treatment did not exhibit photosynthetic activity after 22 dpi. Other works obtained similar results when inoculated plants were not subjected to acute water stress, without important changes in physiology, nor mortality of seedlings (Tuset *et al.*, 1996; Corcobado *et al.*, 2014; León *et al.*, 2017). Turco *et al.* (2004) did not find changes in the final physiological status of *Q. ilex* seedlings due to

P. cinnamomi infection under slight drought conditions. In their work, inoculated plants (both watered and water-stressed) recovered their water status after 14 dpi, only being water-stressed after 42 days.

Root rot caused by *P. cinnamomi* intensifies the damage resulting from physiological stress (Corcobado *et al.*, 2014), probably due to changes in the metabolism of seedlings, which increase the production of secondary metabolites related to drought tolerance, thus reducing the storage of carbon compounds in roots under water stress (Simova-Stoilova *et al.*, 2015). However, when the normal metabolism of plants was only altered by pathogen infection, the responses detected in roots (Ruiz Gómez *et al.*, 2015; Redondo *et al.*, 2015) could be considered as evidence of a set of physiological and morphological changes which led to a recovery of our seedlings after the first infection cycle.

The effects of *P. cinnamomi* root rot are not homogeneous throughout the root system; they depend on root diameter distribution (Blaschke, 1994) and other factors such as availability of inoculum. In infected plants, some functional roots could still be active, or new fine roots could grow as a response to root uptake reduction (Tuset *et al.*, 1996), taking up water and preventing total stomatal closure in well-irrigated plants. Previous work with *Q. ilex* described the maintenance of root growth independently of soil moisture and the ability to increase the growth of smaller-diameter fractions, leading to a tendency towards a thinning of roots, as a response to water deficiency in the plant (Manes *et al.*, 2006). All this evidence might explain the recovery of plants receiving the Inoculation treatment in our experiment at 15 dpi. According to our physiological data (*A*, *Gs*, and *QY*), the alterations of plant physiology produced by the Inoculation treatment were uncoupled from water stress; this indicates that root rot by itself was not enough to seriously disturb the functionality of the vascular system in our infected and well-watered seedlings, at least up to 30 dpi.

- Biomass allocation

The root volume and biomass increments of *Q. ilex* seedlings in response to water stress are consistent with other results (Leiva & Fernández-Alés, 1998; Villar-Salvador *et al.*, 2004). These changes in response to drought are considered an adaptative response of drought tolerant plants (Poorter *et al.*, 2012; Olmo *et al.*, 2014; Bongers *et al.*, 2017), such as holm oak (Turco *et al.*, 2004). Hence, if we consider that root rot due to *P. cinnamomi* infection reduces water uptake as a consequence of fine roots loss (Hardham & Blackman, 2018; Oßwald *et al.*, 2014), a response to water stress similar to the one triggered by drought could be expected in inoculated plants – increasing fine root turn over and root biomass in secondary roots. But, if no water stress signaling was triggered, the fine root "rot/growth" rate would have altered this response in inoculated plants.

Detailed evaluation of the aboveground biomass showed a decrease in the proportion of stem, relative to the overall aboveground biomass, and a lack of differences in leaf biomass, confirmed by the effects of the combined treatment (*I×D*) (Figure 4.4bc). Evaluating the plant fractions, it might be considered that the main effects of the treatments were observed in roots - the aboveground biomass not being sensitive to the effects of either treatment, except in the case of stem growth decline, which agreed with the lower *SMF* in *I×D* plants. Jönsson (2004) reached a similar conclusion for *Quercus robur* L. infected with *Phytophthora quercina* and *P. cactorum*, since the aboveground biomass showed no significant response to the fine root loss produced.

One of the main symptoms associated with the root rot caused by *P. cinnamomi* is the lack of lateral fine roots (Cahill, 1989; Blaschke, 1994; Sánchez *et al.*, 2002, 2005; Vettraino *et al.*, 2003; Ruiz Gómez *et al.*, 2012, 2015), as described in this work. However, our data do not show a significant reduction of fine roots or effects on related traits in the inoculation treatments (*Inoculation* and *I×D*). One of the main objectives of

our work was to assess biomass allocation differences in plants, since we recovered all the fine roots found in the substrate. We considered that 30 days is too short a time for the significant disappearance of excised or rotted roots in the pot substrate, neither with controlled watering (without flooding) nor without watering. Nevertheless, root rot symptoms and lateral root excisions were clearly identified in the Inoculation treatment, which might explain the significant increment in the number of tips in coarse roots (roots of >2 mm Ø, data not shown).

The *Pi* is frequently calculated to assess differences in the tolerance of stress factors between species or phenotypes, and to indicate the variability of different root traits in limiting conditions (Olmo *et al.*, 2014; Valladares & Sánchez-Gómez, 2006). In our case, *Pi* might explain some of the differences in the root changes, agreeing with the idea of the reduction of the plant's ability to explore the soil due to *P. cinnamomi* infection. The *Inoculation* treatment gave lower *Pi* values, statistically different from the rest, with the maximum value corresponding to *Drought* (Figure 4.4d). High plasticity levels are correlated with tolerance of stress factors; Bongers *et al.* (2017) found, for functional traits correlated with a drier climate and stress conditions, that greater phenotypic plasticity was related to traits associated with rapid recovery and growth after drought. Stress caused by *P. cinnamomi* inoculation provoked lower plasticity of root traits than water stress, agreeing with the high susceptibility of *Q. ilex* to *P. cinnamomi* (Moralejo *et al.*, 2009) and the high tolerance of this species to hydric stress (Quero *et al.*, 2011). Thus, it can be hypothesized that, without additional stressors, the strategy of our seedlings in the face of root rot was to reduce the biomass increment and reallocate resources to the equilibration of osmotic and hormonal imbalances, enabling them to recover their physiological status after the first infection cycle. On the other hand, changes in physiology and decreases in root plasticity caused by root infection could reduce the drought tolerance of *Q. ilex* plants, this being another possible cause of tree decline.

4.5. Conclusions

This work evidences that the responses of *Quercus ilex* to *Phytophthora cinnamomi* infection and water stress are different. *Phytophthora cinnamomi* root rot altered mainly the growth patterns of plants, while the plants could not recover from the physiological effects of infection only when the root rot coincided with water stress. The differing responses of the roots to drought and infection were reflected in the early reduction of photosynthetic activity and relative changes in biomass allocation under water deprivation; the effects of the pathogen, without additional stress, focused on the reduction of plant growth and of the ability of the root system to explore the substrate, confirmed by the low *Pi*.

The infection of *Q. ilex* seedlings by *P. cinnamomi* was not enough, in this case, to kill the plants or to cause permanent damage to their physiological status. The differing degrees of susceptibility among provenances (Navarro Cerrillo *et al.*, 2018) should be considered as one of the causes of this observation, but, doubtless, the pathogen aggravated the consequences of hydric stress, since the results provide no evidence to support the induction of acute water stress by root rot.

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Chapter 5.- Analysis of soil biota in declining "dehesas" through metabarcoding

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- **Ruiz Gómez FJ**, Navarro-Cerrillo RM, Pérez-de-Luque A, Oβwald W, Vannini A, Morales-Rodríguez C. **2018**. Metabarcoding analysis of fungi and oomycetes in *Querus ilex* "dehesas": Functional and structural changes related to holm oak decline. *New Phytologist* (Submitted).

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Abstract

The decline of holm oak in "dehesas" represents a serious threat to the sustainability of these ecosystems. Although *Phytophthora cinnamomi* is considered the main agent of holm oak decline, little is known about the relationship between diversity of soilborne microbial community and the decline syndrome of holm oak. Soil samples of "dehesas" were collected and total DNA was extracted and analyzed through metabarcoding techniques, to evaluate the specific composition and diversity of the fungal and oomycete communities and to study their relationship with the severity of the disease symptoms. The fungal community included a wide range of pathogens and abundance of ectomycorrhizal key taxa. *Phytophthora* spp. dominated the oomycete community, but the species related to root rot did not appear among the most abundant, nor were they related directly to defoliation levels. A particular OTU belonging to the genus *Trichoderma* was strongly correlated with the scarcity of pathogenic *Phytophthora* spp. The diversity and composition of both communities were key factors related to the severity of the decline symptoms. The differences in defoliation were related to changes in the functionality of soil microbiota and diversity levels of pathogenic species.

Resumen

El decaimiento de las dehesas de encina representa una seria amenaza para la sostenibilidad de estos ecosistemas. Si bien se considera que *Phytophthora cinnamomi* es el principal agente causante del decaimiento de la encina, existe un escaso conocimiento sobre las relaciones entre la biodiversidad del suelo y el síndrome de decaimiento de la encina. Se colectaron muestras de suelo en dehesas con decaimiento y se extrajo el ADN total, que fue analizado mediante técnicas de metabarcoding, con el fin de evaluarla composición específica y la diversidad de las comunidades fúngica y de oomicetos, así como su relación con la severidad de los síntomas de la enfermedad. La comunidad fúngica presentó un amplio rango de patógenos y abundancia de ciertos taxones de ectomicorrizas de interés. *Phytophthora* spp. se presentó como el género dominante en la comunidad de oomicetos, pero las especies que han sido relacionadas con la podredumbre radical no fueron las más abundantes, ni tampoco se encontró relación directa de su abundancia con la defoliación. La abundancia de una unidad taxonómica operacional (OTU) identificada como *Trichoderma* sp. presentó una relación consistente con la escasez de las especies patógenas de *Phytophthora* spp. La diversidad y la composición de ambas comunidades fueron factores clave en relación a la severidad de los síntomas de decaimiento. Las diferencias en defoliación estuvieron relacionadas con cambios en la funcionalidad de la microbiota del suelo y con los niveles de diversidad de las especies petógenas.

Keywords: Illumina, *Phytophthora* spp., root rot, soil biodiversity, tree dieback and mortality, *Trichoderma* spp.

5.1.- Introduction

"Dehesas" represent ecosystems with relevant ecological and socio-economic importance covering 23% of the forested area in Spain (around 14% of the total area), accounting for over 3 mill ha in the Iberian Peninsula (Díaz Esteban & Pulido Díaz, 2009). Tree mortality of holm oak (*Quercus ilex* L.) associated with root rot is a serious threat to the sustainability of these ecosystems. *Phytophthora cinnamomi* Rands. is considered the main agent of this disease (Brasier *et al.*, 1993; Oßwald *et al.*, 2014), but the influence of other biotic and abiotic factors - such as the increasing frequency of extreme drought episodes and decreasing precipitation associated with global warming - is also recognized (Gea-Izquierdo *et al.*, 2013). Research efforts on holm oak decline have been focused on the interaction between the host plants and the pathogen – *Q. ilex*/*Q. suber* and *P. cinnamomi* – (Gómez-Aparicio *et al.*, 2012; Corcobado *et al.*, 2014a; Ruiz Gómez *et al.*, 2015), and on their relationships with environmental and human factors, including socio-economic and management ones (Corcobado *et al.*, 2015; Cardillo *et al.*, 2018; Duque-Lazo *et al.*, 2018). However, the relationship between the plant community and oomycetes and the spatial patterns of *Pythium* spp. and *Phytophthora* spp. were shown to be relevant factors influencing the dynamics of declining Mediterranean forests (Gómez-Aparicio *et al.*, 2012). Moreover, the implication of other *Phytophthora* spp. in the decline of *Q. ilex* and other *Quercus* spp. in the Iberian Peninsula is recognized (Corcobado *et al.*, 2010; Català *et al.*, 2017).

In addition, little is known about the role of the soil fungal community in the decline syndrome of holm oak, or about the relationship between the latter and the presence pathogenic species of oomycetes. Different authors have highlighted the importance of the interactions among communities of soil taxa, mainly focusing on bacterial and fungal communities (Gdanetz & Trail, 2017; Deveau *et al.*, 2018). The soil fungal community interacts with other communities of biota in different ways,

acting as commensal, pathogenic, anthagonistic or mutualistic organisms. In the case of the plant community, its interaction is mainly associated with beneficial species such as ectomycorrhizal and arbuscular mycorrhizal fungi and other soil saprobes which improve soil fertility and structure (Tamayo-Vélez & Osorio, 2018) and enhance plant defenses and health status (Pérez-de-Luque *et al.*, 2017). Ectomycorrhizal and endophytic species, such as *Russula* spp. or some *Trichoderma* spp., have been found to be related to changes in the tree health status of *Q. ilex* (Corcobado *et al.*, 2014b; Aleandri *et al.*, 2015; Corcobado *et al.*, 2015). On the other hand, pathogenic relationships between fungal species and the plant community, including asymptomatic ones, are also related to disease expression in some cases (Malcolm *et al.*, 2013).

Oomycete communities are composed of a wide range of plant pathogens, some of which are host-specific and others with a wide range of hosts. Their lifestyle can be either biotrophic, necrotrophic and hemibiotrophic (Lee & Rose, 2010; Fawke *et al.*, 2015). *Phytophthora* spp. community has often been found to be the dominant one in forest soils with a known history of *Phytophthora*-related diseases (Vannini *et al.*, 2013), but little is known about the relationship between the oomycete community and soil fungal or bacterial communities. Sapkota & Nicolaisen (2018) published the first study of the soil community diversity, including the taxa of fungi and oomycetes, associated with cavity spot of carrot, but in the literature, there are no studies describing fungal diversity or the relationship between oomycetes and fungi in "dehesas" rangeland.

Nowadays, new methodologies allow the macroscale analysis of soil community diversity (Bálint *et al.*, 2014; Sapkota & Nicolaisen, 2015). The isolation of living cultures was the traditional way to assess oomycete or fungal diversity in soil (Martin *et al.*, 2012), but this approach seems to be insufficient due to the time and specialized scientific knowledge required, considering the potentially enormous number of different species in a high number of samples. In this sense, next-generation sequencing (NGS) techniques are an alternative methodology. This technology is able to

detect the presence of a very high number of different species in a single sample: successful examples include pyrosequencing (Vannini *et al.*, 2013; Tedersoo *et al.*, 2014) and second-generation techniques, such as metabarcoding based on Illumina de novo sequencing (Veach *et al.*, 2017; Chen *et al.*, 2018; Sapkota & Nicolaisen, 2018).

In this context, the main objective of our work was to assess and describe the diversity and structure of fungal and oomycete communities in the holm oak "dehesas" rangeland ecosystems of Andalusia (southern Spain) affected by oak decline, using a metabarcoding approach. To reach this goal, the following specific objectives were addressed: i) to compare the fungal and oomycete diversity, and the defoliation levels in holm oak stands, of different study areas; ii) to identify the predominant functional guilds of the fungal and oomycete communities and their relationships; and iii) to identify key taxa which might be related to holm oak decline. These results should contribute to a better understanding of the influence of soil community associations on tree health status and to the identification of important fungal and oomycete taxa related to holm oak decline in "dehesas" ecosystems.

5.2.-Material and Methods

- Study area

This study was focused on holm oak (*Quercus ilex* subsp. *ballota*) "dehesas" of north-west Andalusia. The studied area was divided into four zones - Andévalo (Andv), Sierra de Aracena (Arac), Sierra Norte de Sevilla and S. Morena (S.Nor) and Valle de los Pedroches (Pedr) - located in the provinces of Huelva, Sevilla and Córdoba, representing the core area of the holm oak "dehesa" ecosystems of Andalusia (Figure 5.1). This division was made based on differences in environmental factors (Table 5.1) and silvicultural characteristics (Costa Pérez *et al.*, 2006).

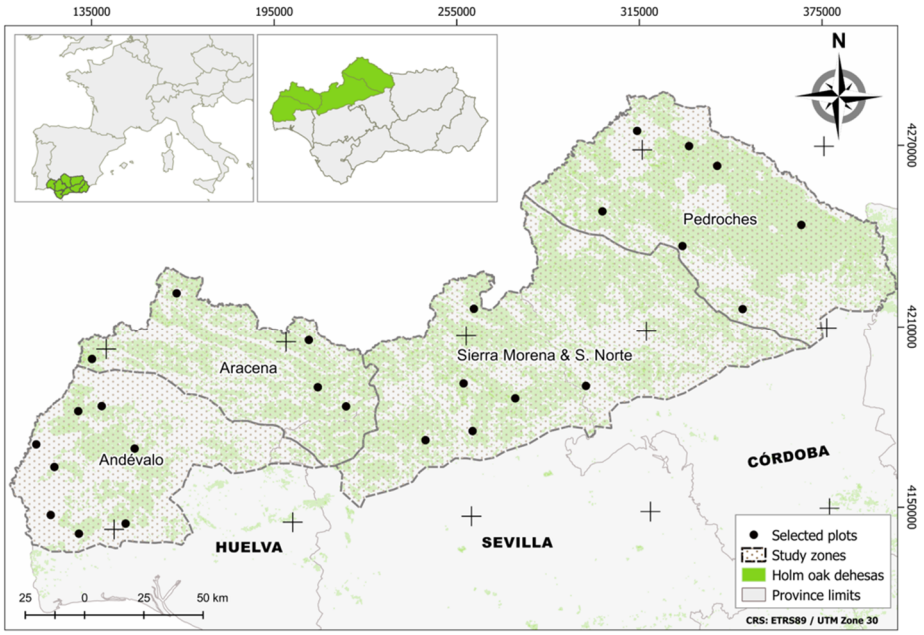

Figure 5.1: Map of sampled plots of *Quercus ilex* "dehesas" of the Andalusian Network for Damage Monitoring in Forest Ecosystems.

A total of 26 plots were selected among the permanent environmental monitoring points of the Andalusian Network for Damage Monitoring in Forest Ecosystems (Junta de Andalucía, 2015 ICP Forest, EC Network level I - www.juntadeandalucia.es). The plots were selected based on the following criteria; i) >85% of the trees were *Q. ilex*, ii) a positive record of *P. cinnamomi* and iii) the tree mortality rate (Figure 1, Supporting Information Table S5.1). All the trees of the plots (24 trees x 26 plots) were classified in defoliation classes (C1=defoliation<25%; C2=25%<defoliation<75%; C3=defoliation≥75%; Ferretti & Sánchez Peña, 1994). The mean defoliation (%), calculated using the individual defoliation of the 24 trees per plot, ranged between 13.3% and 46.5% (defoliation classes C1 and C2) (Supporting Information, Table S5.1) and showed significant differences among the study zones (χ^2=11.1; DF=3; $p<0.05$), the highest value corresponding to Pedr.

Table 5.1: Mean values of environmental and silvicultural parameters considered for the selection of the study zones (m.a.s.l.: meters above sea level). Average values for the period 1969-2000, taken from the Andalusia Environmental Information Network - REDIAM; Consejería de Medio Ambiente, Junta de Andalucía (http://www.juntadeandalucia.es/medioambiente/site/rediam/portada/). The average tree cover, the dominant tree age and the prevalent class of dehesa were determined as the trend of all the visited plots for each zone, following the criteria of Costa Pérez *et al.* (2006).

	Andevalo	Aracena	Sierra Morena & S. Norte	Pedroches
Mean Precipitation (mm)	675.7	829.2	712.1	572
Mean Annual temperature (°C)	17.4	16.2	16.1	15.8
Mean Evapotranspiration (mm)	1445	1396	1458	1443
Max elevation (masl)	293	1046	936	943
Mean elevation (masl)	191	434	429	570
*Average cover**	Low	Medium	High	Low
*Dominant tree age***	Young to Mature	Mature	Young	Old to very old
Class of dehesa	Cultivated dehesa	Normal tree cover with shrubs and pasture	High tree cover with shrubs and pasture	Pastures

*: Low= N<50 tree ha^{-1}; Medium=50<N<80 tree ha^{-1}; High= N>80 tree ha^{-1}

**: Young trees= 50-90 years / 20-40 cm Ø; Mature trees= 110-210 years / 50-80 cm Ø; Old trees= 210-250 years / 90-110 cm Ø; Very old= >250 years / Ø>110 cm.

- Soil sampling

In each plot, soil samples were collected under the crown of a tree with defoliation degree C1 or C2, and under the crown of a tree with defoliation degree C3. To the two trees selected per plot (52 in total), three were added to ensure spatial representability at three specific locations, giving a final total of 55 trees. For each tree selected, sub-samples were taken under the crown for two orientations (North and South) and two distances from the trunk (0.5 m and 1-2 m), making four sub-samples per tree. Then, the sub-samples of each tree were bulked and stored at 4 °C until processing. The soil samples were dried at room temperature for 24 h

and well-mixed. Litter and roots were separated, and 20 g of the fine fraction was collected after passing the soil through a 1-mm sieve.

- DNA extraction, ITS1 amplification, library generation, and sequencing

Total DNA was extracted from soil using the MoBIO Power Soil DNA Isolation kit (MoBIO Laboratories, Inc., CA, USA). Five replicates of fresh soil (0.33 g each) were taken from each soil sample. The aliquots were mixed together, and total DNA was purified and concentrated using a DNA purification kit (Wizard SV Genomic DNA Purification System; Promega Corporation, WI, USA). The DNA concentration was quantified, with a Qubit dsDNA BR Assay kit (Thermo Fisher Scientific Inc., MA, USA), prior to amplification.

Additionally, two mock communities were created by pooling DNA from a pure culture of 13 different species of oomycetes and another with seven species of fungi; one community had the same concentration of each species and the other had different concentrations. These were used as controls and were processed in the same way as the samples, following the methodology of Morales Rodriguez (Unpublished).

The internal transcribed spacer 1 (ITS1) was amplified in a multiplexing PCR using a set of barcoded primers: ITS1F and 2 (Gao *et al.*, 2010) for fungi, and ITS6 and 7 for oomycetes (Cooke *et al.*, 2000). The primers were tagged with different barcodes to distinguish different samples.[1]

The PCR reaction mixture consisted of 25 μl of Maxima Hot Start PCR Master Mix (Thermo Fisher Scientific, USA), 2 μM of each primer, 4 μl of 2 mg ml^{-1} BSA and 3 μl of DNA template diluted to 50 ng μl^{-1}, in a total volume of 50 μl The thermal cycle was an initial 5-min step at 95 °C, followed by cycles (30 for fungal ITS and 35 for oomycete ITS) of 40 s of denaturation at 95 °C, 2 min of annealing (58 °C for fungal ITS amplification and 60 °C for oomycete ITS) and 1 min of extension at 72 °C,

[1] Supporting Information File S2 in the submitted article version

with a final extension step of 7 min at 72 °C. For each sample and the controls, three replicates of PCR for fungal ITS and five for oomycetes ITS were carried out and the products were pooled.

Amplicons were purified using the MagJet NGS Cleanup and Size Selection kit (Thermo Fisher Scientific Inc., MA, USA) and the final concentration of purified PCR product was quantified using a Qubit dsDNA HS Assay kit (Thermo Fisher Scientific Inc., MA, USA). The resulting amplicons were pooled in equal amounts to give a total volume of 40 μL, with a concentration of 200 ng μL^{-1}, and were sent to Eurofins Genomics AT GmbH (Vienna, Austria), to be sequenced in an Illumina MiSeq 2x300 bp platform. The reads are available in the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl) under the project name "NGS_Dehesa" and accession number PRJEB28448.

- Sequence processing, OTU selection, and abundance matrix construction

A bioinformatic pipeline was adapted from Bálint *et al.* (2014) and Sapkota & Nicolaisen (2015). The primers were debarcoded and classified by Eurofins Genomic AT GmbH (Vienna, Austria). The sequences were joined (22 774 511 sequences) and reads with mismatches or ambiguous bases were unassigned (1 246 294 sequences). Finally, only paired sequences in which the 5′ barcode and forward primer and 3′ barcode and reverse primer were found, were used in the bioinformatic analysis (Table 5.2). Singletons were excluded from the analysis.

The forward and reverse sequences of paired reads were trimmed, the primers were eliminated and sequences were overlapped and paired using CLC Genomics 3.6.5 (https://www.qiagenbioinformatics.com/) (Supporting Information Table S5.2). The ITS1 region was extracted with the FungalITSextractor (Nilsson *et al.*, 2010), sequences were clustered in operational taxonomic units (OTUs) and the chimeric sequences were filtered - de-novo chimera filtering for oomycete files - using USEARCH 8 (Edgar, 2010) with a similarity level established at 98%. The consensus sequences of the OTUs were identified with the GenBank nucleotide

database (nr/nt) of NCBI, using the Megablast algorithm, and the results were parsed and the sequences retained in MEGAN 6.10.8 (Huson *et al.*, 2016) to assign taxonomic identifications to each OTU (Supporting Information Table S5.3). Afterward, abundance tables of OTUs for oomycete and fungal files were constructed using USEARCH 8 with a level of identity of 98%.

Table 5.2. Illumina de novo sequencing results of soil total DNA library, from soil samples of *Quercus ilex* dehesas of the Andalusian Network for Damage Monitoring in Forest Ecosystems. A: Statistics of the sequencing project referred to the whole raw data. B: Resume of debarcoded sequences to the fungi and oomycete dataset after debarcoding and grouping of ITS1F/2 and ITS6/7 identified tagged primers.

A

Type	%Q30	MeanQ	#Cluster
Illumina MiSeq 2x300	84.34%	33.52	14 732 937

B

		Debarcoded Seqs.	
Features	Project	ITS1F/2	ITS6/7
Forward sequence only	3 639 702	2 375 465	1 264 259
Reverse sequence only	1 805 367	1 456 828	348 535
Paired sequences	8 041 574	6 769 397	1 272 177
Mean freq. Per sample	101 366.60	90 593.20	9025.70
Sign. OTU's	4193	3912	281
Mean OTU freq	1378.00	1343.10	1862.90

The amplified ITS sequences from all the species included in the internal controls were correctly identified. The mean frequency of debarcoded paired sequences did not differ between oomycetes and fungi (χ^2=0.727; p=0.394), with strong correspondence between the abundance of OTUs and the estimated amount of DNA included in each control. Prior to downstream analysis of the abundance matrix, control samples and OTUs with less than 10 reads were eliminated from both the fungal and oomycete feature tables.

- Fungal and oomycete community analysis

After the abundance matrix filtering, functional guilds of the identified taxa were characterized using FunGuild (Nguyen *et al.*, 2016). Only OTUs classified with a confidence level of "Probable" or "Highly Probable" were used. The functional guilds of the remaining OTUs, classified as "Unassigned" or "Possible", were revised based on the literature; those that were confidently classified were added to the analysis[2]. The rest of the unassigned OTUs and those assigned with low confidence were placed in the "Unclassified" category. Taxa classified as soil saprotrophs, plant saprotrophs, litter saprotrophs or wood saprotrophs were grouped in a unique *Soil-Plant Saprotroph* category. Those taxa that were confidently classified in more than one guild were assigned to the class *>1 Guild*, except in the case of taxa that were confidently assigned to four or more guilds, including pathogen and saprotroph guilds; these were classified in the *Opportunistic Pathogenic Species* class.

To analyze the diversity of oomycetes and fungi, abundance matrices were processed using QIIME2 version 2017.12.1 (Caporaso *et al.*, 2010). Rarefaction curves, Good's coverage, Shannon H', OTUs abundance and Pielou evenness vector and the Bray-Curtis distance matrix were calculated using the plugin Diversity 2017.12.0 (Chen *et al.*, 2012). The 10 most-abundant OTUs for fungi and oomycetes were selected from the core classified OTUs using the Feature-table plugin (McDonald *et al.*, 2012), considering only the OTUs present in over 50% of samples. The relative abundance of the top 10 core features with respect to the number of paired sequences was calculated separately for each dataset (oomycetes and fungi). Rarefaction curves (Supporting Information, Figure S5.1) were evaluated and Good's Coverage was calculated to estimate the adequate depth of sampling in the abundance matrices, the abundance matrices being rarefied with an even sampling depth for each dataset, with 10 iterations to avoid bias due to uneven sequencing depth. The Shannon (H')

[2] Supporting Information File S5 in the submitted article version

diversity index, OTUs diversity and Evenness vector indices were calculated to study the diversity of the fungal and oomycete communities in each sample and the Bray-Curtis dissimilarity matrix was calculated; the β-diversity was analyzed to find the differences in community composition among the study zones.

- Statistical methods

The differences among zones or defoliation levels were tested through ANOVA, together with the Bonferroni mean differences test, in the case of normally-distributed variables, or the Kruskal-Wallis rank sum test with the Bonferroni correction, when variables did not show normality. A Spearman correlation matrix was calculated for the functional guilds present in five or more samples of all zones, thus excluding the Lichen Parasite and Endophyte guilds. Unclassified features were excluded from the correlation matrix to avoid artifacts in the analysis. The Pearson or Spearman correlation test was used, depending on whether the two variables in each pair were distributed normally or not. Null hypotheses were rejected in all cases when $p \leq 0.05$.

Alpha-diversity indices were subjected to the Kruskall-Wallis rank sum test with the Bonferroni correction, to compare the diversity levels of fungal and oomycete communities in the distinct study areas and according to the mean defoliation class of the plots. The Bray-Curtis dissimilarity matrix was used in non-metric multidimensional scaling analysis (NMDS, *vegan* package, Oksanen *et al.*, 2018), together with Analysis of Similarity (ANOSIM, 999 permutations), to determine the influence of the study location on the composition of the fungal and oomycete communities.

All the statistical analyses were performed in the environment R Studio V 1.0.143 (RStudio Team, 2015) running under R 3.4.0 (R Core Team, 2014). Other packages used were *dunn.test*, *nortest*, *ggplot2*, *ggpurb*, and *devtools* (https://CRAN.R-project.org/).

5.3.-Results

- OTUs clustering and taxonomy

After bioinformatic treatment of the raw data, 3912 OTUs were clustered for the fungal community and 281 for oomycetes (Table 5.2). From all the assigned taxa, 1789 fungal OTUs and 178 oomycete OTUs were confidently assigned to the genus or species level.

In the taxonomic revision, it was difficult to differentiate between certain species. Some *Trichoderma* spp. share an identical ITS1 sequence, making the identification of fungal OTU#51 at the species level impossible. Fungal OTU#2, which was initially assigned to the *Fusarium oxysporum* Schltdl. taxon, was considered as *Fusarium* spp. after the taxonomic review. There are many species in this genus which could not be differentiated by the sequence of the ITS1. After the revision of the taxonomy, the metabarcoding analysis showed enough accuracy to study the diversity and composition of the fungal and oomycete communities, clearly differentiating between different genera in all cases.

Among the fungal taxa, Basidiomycota was the most abundant division (46.8%) followed by Ascomycota (45.1%) and Zygomycota (7.4%). The other divisions accounting for less than 1% of the total reads. The class Agaricomycetes was the most abundant (42.87%) and, together with Sordariomycetes, Eutriomycetes, Mortierellomyetes, and Pezizomycetes, represented the five dominant fungal classes, comprising more than 80% of the total fungal OTUs (Figure 5.2a). The class Agaricomycetes had its maximum relative abundance in the S.Nor zone, with low abundance in greatly-defoliated plots. The opposite tendency was shown by Mortierellomycetes (Figure 5.3a, b). The abundance of Eurotiomycetes differed significantly among zones, being lowest in S.Nor and Andv ($F=3.30$; $p<0.05$). Other relevant classes of Ascomycota were Dothideomycetes (5.36%) and Leotiomycetes (1.15%); the abundance of the former was highest in C2 plots ($F=3.86$; $p<0.05$).

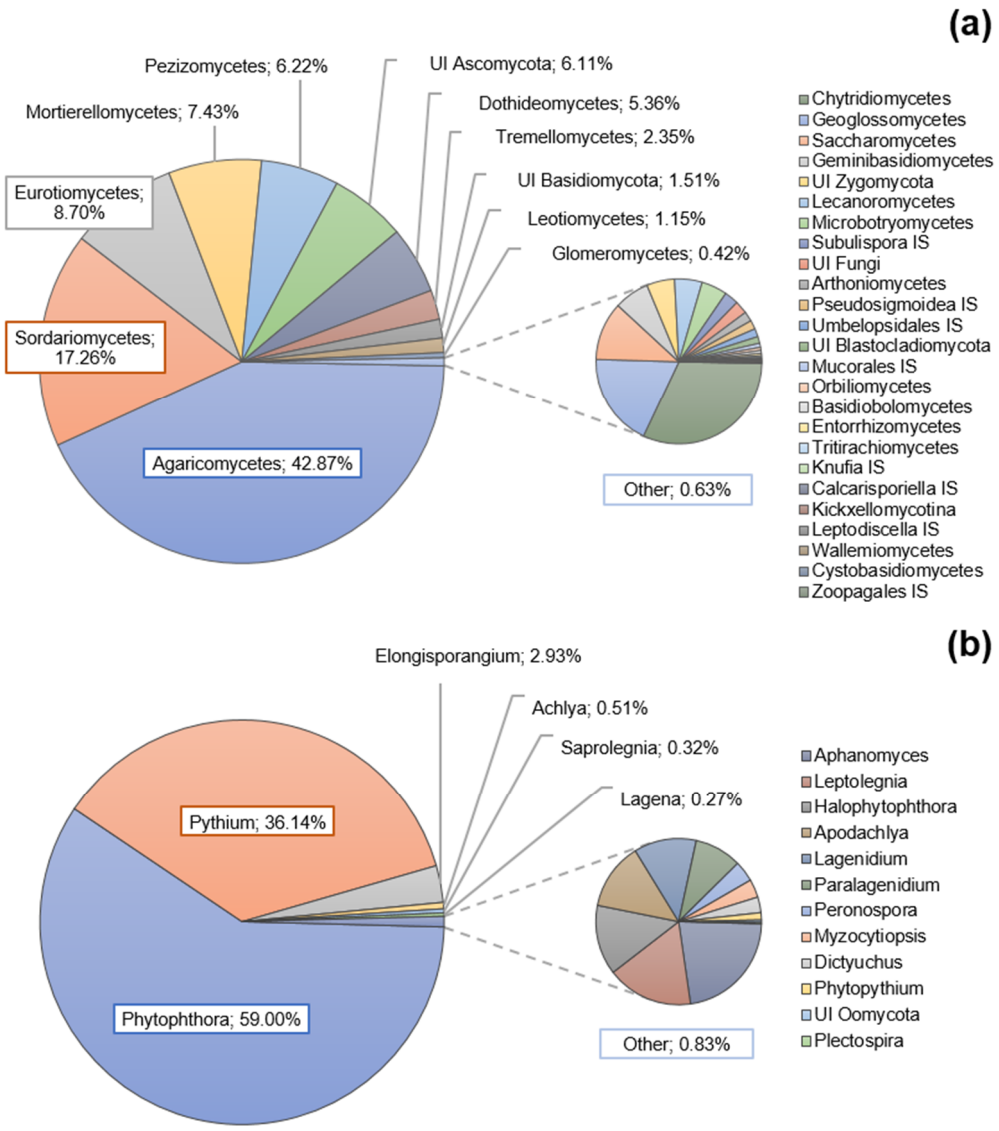

Figure 5.2. Mean relative OTU's abundance in 26 plots of *Quercus ilex* "dehesas" of the Andalusian Network for Damage Monitoring in Forest Ecosystems. A: Abundance for fungi OTU's *classified* by "class" taxonomic level. B: Abundance for oomycete OTU's classified by "genus" taxonomic level. Legends are referred to "Other" detail graph (left) ordered by frequency. Ul: Unidentified (for those OTU's which classification at the chosen taxonomic level was impossible). IS: Incertidae Sedis

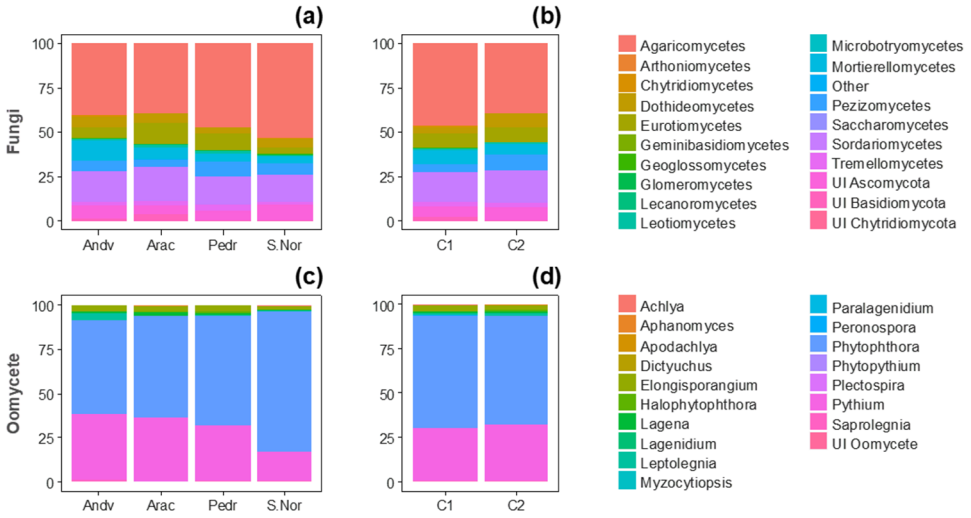

Figure 5.3. Relative abundance of oomycete and fungal community across study zones and mean defoliation class on the 26 studied *Quercus ilex* "dehesas" of the Andalusian Network for Damage Monitoring in Forest Ecosystems. Vertical axis represents % of frequencies. Fungal abundance for zone (A, %) and mean defoliation class (B, %) is represented for taxonomic level 3 (class). Oomycete abundance for zone (C, %) and mean defoliation class (D, %) is represented for taxonomic level 6 (genus). Andv: Andévalo. Arac: Aracena. S.Nor: Sierra Morena & Sierra Norte. Pedr: Pedroches. UI: Unidentified taxon

The identified species of Leotiomycetes present in the samples were classified mostly as saprotrophs, except in the case of the plant pathogen *Cadophora* spp., which was present with notable abundance in the Pedr and S.Nor zones. True truffles (*Tuber* spp.), *Terfezia* spp., *Geophora* spp. and *Peziza* spp. were the most abundant Pezizomycetes - which were present in all the studied zones, with high relative frequency in Pedr soils (8.06%), but without significant differences among zones or defoliation levels (ANOVA, $F=0.59$, $p>0.05$).

Regarding oomycetes, species of the genera *Phytophthora* and *Pythium* represented over 95% of the total clustered sequences, followed by *Elongisporangium* spp. (*E. undulatum*), with almost 3% of the total sequence abundance, all of them corresponding to plant pathogen species (Figure 5.2b). Moving eastward across the plots, the abundance of *Phytophthora* spp. increased and that of *Pythium* spp. decreased, with a significant inverse correlation between the relative abundances of the two taxa (Spearman ϱ=-

0.966; $p<0.001$). However, none of the analyzed oomycete taxa presented significant differences in abundance among the defoliation levels (Figure 5.3c, d).

Up to 350 fungal species were present in almost half of the soil samples, and the 10 most-abundant fungal species were present in more than 95% of the samples (Table 5.3). The fungal OTU#3, corresponding to *Mortierella elongata* Linnem., was the most abundant, representing over 4.4% of the total number of sequences. Also, OTU#11, identified as *M. elongata*, was among the 10 most abundant species, the two OTUs accounting for 6.1% of the total number of frequencies. Up to 75 different fungal OTUs were assigned to the genus *Mortierella*, with 15 different species identified. Fungal OTU#2, *Fusarium* sp., was the second-most-abundant fungus of the core biome; it was present in all the samples analyzed and represented over 3.4% of the total frequency, being the most-abundant sequence identified in S.Nor. The third-most-abundant taxon was fungal OTU#8, assigned to *Russula praetervisa* Sarnari. This OTU was present in all the samples of Andv and S.Nor, but not in all the samples of Arac, in which it represented only 0.4% of the total abundance, and only in 75% of the samples of Pedr. Its abundance varied significantly with the mean defoliation ($F=3.38$; $p<0.01$), being higher in trees suffering little defoliation. OTU#24, classified as the fifth-most-abundant phylotype, accounted for 1.1% of the total sequences and was identified by its consensus sequence, using BLAST, as *Solicoccozyma* spp. fungus; ITS1 analysis could not distinguish between *S. phenolicus* and *S. terreus*.

Two species of the genus *Trichoderma* were among the 10 most-abundant species of the core biome. OTU#51, identified as *Trichoderma* sp., appeared in 98.1% of samples and had its maximum frequency in samples of Arac, coinciding with the lowest frequency of *Trichoderma hamatum* (Bonord.) Bainier, which was present in 94.2% of the samples and represented over 1% of the total number of sequences in samples of Andv and S.Nor.

Table 5.3. Ten most abundant oomycete and fungi OTUs found in 26 *Quercus ilex* dehesas of the Andalusian Network for Damage Monitoring in Forest Ecosystems, ordered by relative frequency (from core-features of each abundance matrix calculated among OTUs present in at least 50% of samples).

				Andevalo (n=15)		S. Aracena (n=13)		S. Norte (n=13)		Pedroches (n=14)		Total (n=55)	
	OTU ID	Taxon	Functional Guild	OTU rel. Freq.	% of samples	OTU rel. Freq.	% of samples	OTU rel. Freq.	% of samples	OTU rel. Freq.	% of samples	% of Total Freq.	% of Tot. samples
Oomycete	OTU#1	*s_Phytophthora plurivora*	Plant Pathogen	11.10%	100.00%	17.50%	100.00%	19.70%	100.00%	7.10%	100.00%	15.20%	100.00%
	OTU#6	*s_Pythium paroecandrum*	Plant Pathogen	6.00%	66.70%	21.40%	84.60%	0.50%	61.50%	0.40%	71.40%	7.80%	70.90%
	OTU#10	*s_Phytophthora psychrophila*	Plant Pathogen	1.10%	73.30%	5.80%	38.50%	11.50%	76.90%	1.80%	57.10%	6.00%	61.80%
	OTU#3	*s_Phytophthora quercina*	Plant Pathogen	5.50%	100.00%	2.70%	84.60%	6.10%	76.90%	4.00%	85.70%	4.70%	87.30%
	OTU#9	*s_Phytophthora citrophthora*	Plant Pathogen	1.50%	73.30%	2.90%	53.80%	6.70%	76.90%	0.40%	100.00%	3.50%	76.40%
	OTU#17	*s_Pythium heterothallicum*	Plant Pathogen	5.20%	60.00%	4.10%	84.60%	1.40%	76.90%	1.30%	85.70%	3.20%	76.40%
	OTU#15	*s_Pythium irregulare*	Plant Pathogen	8.70%	86.70%	1.70%	53.80%	0.70%	84.60%	0.10%	64.30%	3.00%	72.70%
	OTU#7	*s_Elongisporangium undulatum*	Plant Pathogen	3.90%	100.00%	4.60%	76.90%	0.40%	84.60%	1.30%	100.00%	2.60%	90.90%
	OTU#4	*s_Phytophthora cinnamomi*	Plant Pathogen	2.00%	66.70%	1.90%	30.80%	2.10%	46.20%	3.50%	64.30%	2.20%	52.70%
	OTU#16	*g_Pythium sp.*	Plant Pathogen	1.40%	80.00%	3.20%	30.80%	1.40%	69.20%	0.10%	57.10%	1.70%	60.00%
Fungi	OTU#3	*s_Mortierella elongata*	Soil-Plant Saprot./Endophyte	7.50%	100.00%	4.70%	100.00%	2.80%	100.00%	1.80%	100.00%	4.40%	100.00%
	OTU#2	*g_Fusarium sp.*	Plant Pathogen/Soil-Plant Saprot.	4.20%	100.00%	3.70%	100.00%	3.70%	100.00%	1.90%	100.00%	3.40%	100.00%
	OTU#8	*s_Russula praetervisa*	Ectomycorrhizal	2.80%	100.00%	0.40%	92.30%	2.30%	100.00%	1.10%	78.50%	1.60%	94.50%
	OTU#11	*s_Mortierella elongata*	Soil-Plant Saprot./Endophyte	2.80%	100.00%	1.10%	84.60%	2.00%	76.90%	6.00%	100.00%	1.30%	90.90%
	OTU#24	*g_Solicoccozyma sp.*	Unassigned	1.00%	100.00%	0.90%	100.00%	0.80%	100.00%	1.70%	100.00%	1.10%	100.00%
	OTU#9	*g_Thelonectria sp.*	Soil-Plant Saprotroph	1.40%	100.00%	1.10%	100.00%	0.80%	100.00%	0.60%	100.00%	1.00%	100.00%
	OTU#25	*g_Penicillium sp.*	Soil-Plant Saprotroph	0.60%	100.00%	1.30%	100.00%	0.70%	100.00%	0.70%	100.00%	0.90%	100.00%
	OTU#51	*s_Trichoderma sp.*	Soil-Plant Saprot./Endophyte	0.60%	100.00%	1.10%	100.00%	0.50%	92.30%	0.70%	100.00%	0.70%	98.20%
	OTU#48	*s_Clonostachys rosea*	Soil-Plant Saprotroph	0.60%	100.00%	1.30%	100.00%	0.20%	92.30%	0.30%	100.00%	0.70%	98.20%
	OTU#15	*s_Trichoderma hamatum*	So Soil-Plant Saprot./Endophyte	1.10%	93.30%	0.20%	100.00%	1.20%	92.30%	0.50%	92.80%	0.70%	94.50%

Both OTUs had lower frequency rates in Pedr. The consensus sequence of OTU#51 was unable to differentiate among four different *Trichoderma* spp. (*T. koningiopsis, T. koningii, T. paraviridescens* and *T. viride*). *Clonostachys rosea* (Link) Schroers (OTU#48) was the ninth-most-abundant fungal OTU, with a total frequency similar to those of OTUs #51 and #15 and being the third-most-abundant species in Arac.

The most-abundant oomycete OTU, present in all the samples with important differences in abundance, was *Phytophthora plurivora* T. Jung & T.I. Burgess (oomycete OTU#1) - which accounted for 15.2% of the total frequencies, except in the case of Arac, where OTU#6 (*Pythium paroecandrum* Drechsler) was the most abundant, representing over 21% of the total frequencies in this zone. The fourth-most-abundant oomycete was OTU#3, identified as *Phytophthora quercina* T. Jung & T.I. Burgess, whose presence was greatest in the Andv zone. *Phytophthora cinnamomi* (OTU#4) was the ninth-most-abundant OTU, accounting for 2.2% of the total sequences and identified in 18 of the 26 selected plots. Its presence was greatest in Pedr, where it was the third-most-abundant species (3.5% of the total frequency), and in Andv: in both cases, it showed a substantial difference in abundance from *P. plurivora* and a lower frequency value than *P. quercina*.

Two OTUs were identified as *Pythium spiculum* Paul. (OTU#21 and OTU#235), but they were present in only 21.8% of samples (12 samples, in 10 plots), with low abundance (1.4% in total).

Spearman's correlation analysis showed a significant negative correlation between the abundance of *Trichoderma* spp. (fungal OTU#51) and that of 10 different OTUs of the oomycete core biome (Table 5.4), including most of the *Phytophthora* spp., except for OTUs #3 (*P. quercina*), #48 (*Phytophthora* sp.) and #12 (*P. syringae*). Regarding *Pythium* spp., only OTUs #6 (*Py. paroecandrum*) and #2 (*Pythium* sp.) presented significant negative correlation.

Table 5.4. Significant correlations between the abundance of OTU#51 (*Trichoderma* sp.) and the abundance of the core biome OTU's of oomycete dataset at 26 *Quercus ilex* dehesas of the Andalusian Network for Damage Monitoring in Forest Ecosystems. Rank: Order of the OTU by abundance (1 for the most abundant, to 20 for the less abundant). ρ: Spearman's Rho value. *: Significant correlation at p<0.05; **: Significant correlation at p<0.01.

Rank	OTU's	Assigned Taxon	ρ
1	OTU#1	*P. plurivora*	-0,392**
2	OTU#6	*Py. paroecandrum*	-0,263*
3	OTU#10	*P. psychrophila*	-0,272*
5	OTU#9	*P. citrophthora*	-0,378**
8	OTU#7	*E. undulatum*	-0,325*
9	OTU#4	*P. cinnamomi*	-0,345**
12	OTU#5	*P. cactorum*	-0,277*
13	OTU#8	*P. gonapodyides*	-0,308*
14	OTU#2	*Pythium* sp.	-0,395**
15	OTU#14	*P. cambivora*	-0,379**

- Functional guilds

Of the OTUs confidently classified in an ecological guild (Table 5.5), the *Soil-Plant Saprotroph* guild was the most abundant in all cases (over 15%), except in S.Nor, where *Plant Pathogen* was the most-abundant guild (over 21%). However, when the frequencies of all the putative plant pathogenic OTUs were summed (including *Plant Pathogen*, *Opportunistic Pathogenic Species* and those OTUs classified in *>1 Guild*, which includes *Plant Pathogen* as one of those guilds), their abundance was greater than that of the rest of the guilds, in all the zones studied.

Five functional guilds showed significant differences among zones, the *Ectomycorrhizal* (EcM) and *Endophyte* guilds exhibiting opposing trends, with the minimum frequency values of EcM species corresponding to the zone with maximum values for *Endophyte*. In the Pedroches zone (Pedr) the maximum values were found for the *Endophyte*, *Animal Pathogen*, *Dung*

Saprotroph, and *Soil-Plant Saprotroph* functional guilds, with a significantly-lower value for EcM.

Table 5.5. Mean±SE relative abundance of functional guilds between study zones of *Quercus ilex* dehesas of the Andalusian Network for Damage Monitoring in Forest Ecosystems. Functional guilds which presented significant differences (ANOVA with Bonferroni mean differences test, or Kruskal-Wallis rank sum test) were written in Italic. Andv: Andévalo. Arac: Aracena. S.Nor: Sierra Morena & Sierra Norte. Pedr: Pedroches.

Functional Guild	Andv	Arac	S.Nor	Pedr	Overall
>1 Guild designation	3.3±0.1	3.6±0.2	3.8±0.2	4.0±0.3	$3.6±0.1^{n/s}$
Animal Pathogen	$0.9±0.1^{b}$	$1.0±0.1^{ab}$	$0.8±0.1^{b}$	$1.3±0.1^{a}$	1.0±0.1**
Arbuscular Mycorrhizal	3.4±0.5	3.3±0.4	5.1±0.9	3.5±0.9	$3.8±0.3^{n/s}$
Dung Saprotroph	$1.0±0.1^{ab}$	$0.7±0.1^{c}$	$0.8±0.1^{bc}$	$1.1±0.1^{a}$	0.9±0.0***
Ectomycorrhizal	$9.9±0.7^{a}$	$9.8±0.9^{a}$	$9.4±0.4^{a}$	$5.5±0.6^{b}$	8.7±0.4***
Endophyte	0.2†	$0.14±0.02^{b}$	$0.12±0.02^{b}$	$0.4±0.1^{a}$	0.22±0.04**
Fungal Parasite	0.24±0.02	0.31±0.04	0.4±0.1	0.29±0.04	$0.30±0.02^{n/s}$
Lichenized	0.3±0.0	0.25±0.04	0.24±0.03	0.19±0.04	$0.25±0.02^{n/s}$
Opp. pathogenic species	3.7±0.2	3.4±0.2	3.9±0.2	4.1±0.3	$3.8±0.1^{n/s}$
Plant Pathogen	10.0±1.0	14.1±5.9	21.7±8.4	13.4±6.1	$14.5±2.8^{n/s}$
Soil-Plant Saprotroph	$15.7±0.5^{ab}$	$15.4±0.5^{ab}$	$14.0±0.5^{b}$	$17.1±0.7^{a}$	15.6±0.3**
Unclassified	51.7±0.7	50.5±3.4	45.2±4.9	51.4±4.0	$49.8±1.7^{n/s}$

†= Only one sample of Andevalo presented confident identification of endophyte organism

ANOVA (or Kruskall-Wallis for not-normal distributed functional guilds) significance level: *= p≤0.05; **= p≤0.01; ***= p≤0.001; n/s= p>0.05. Values with the same letter are not significantly different (p>0.05).

The Spearman´s correlation matrix between functional guilds (Figure 5.4a) shows a significant, positive correlation of *Soil-Plant Saprotrophs* with *Animal Pathogen* abundance (ρ=0.491, p<0.01) and a negative one with *Plant Pathogens* (ρ=-0.356, p<0.05) (Figure 4b-1 and 2). On the other hand, EcM was negatively correlated with *Soil-Plant Saprotrophs* (ρ=-0.559, p<0.01; Figure 5.4b-3), *Animal Pathogen* (ρ=-0.492, p<0.01) (Figure 4b-4), *Dung Saprotrophs* (ρ=-0.384, p<0.01), *Opportunistic Pathogenic Species* (ρ=-0.348, p<0.05) and *>1 Guild*, (ρ=-0.343, p<0.01). The *Dung Saprotroph* guild was positively correlated with *Soil-Plant Saprotrophs* (ρ=0.332, p<0.01) and with the species assigned to more than one guild (*>1 Guild*, ρ=0.392,

p>0.01). Other significant correlations appeared between >*1 Guild* OTUs, *Lichenized* and *Opportunistic Pathogenic Species*.

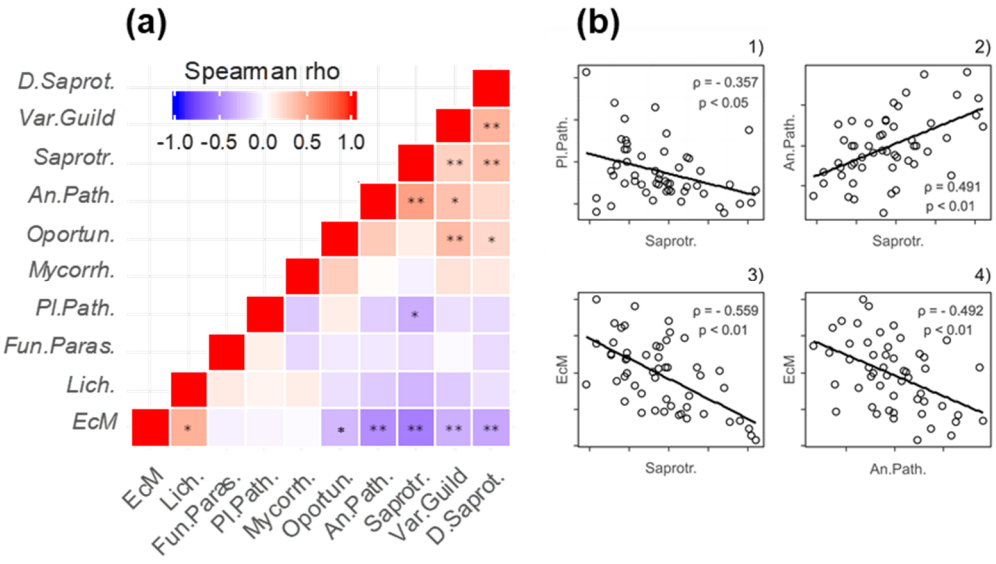

Figure 5.4. Relationships between functional guilds on the 26 studied *Quercus ilex* "dehesas" of the Andalusian Network for Damage Monitoring in Forest Ecosystems. A: Correlation matrix between functional guilds relative abundance in each sample. *: Significant correlation at p<0.05; **: Significant correlation at p<0.01. B: Scatterplots of some significant correlations between Functional Guilds: 1) Plant Pathogen/Soil-Plant Saprotroph; 2) Animal Pathogen/Soil-Plant Saprotroph; 3) Ectomycorrhizal/Soil-Plant Saprotroph; 4) Ectomycorrhizal/Animal Pathogen. ρ: Spearman's Rho value. p: Significance level of correlation.

- Fungal and oomycete diversity

The Good's Coverage indices for the selected thresholds were high for the rarefied matrices of the oomycetes (G=0.98) and fungi (G=0.99), indicating that over 98% of the identified OTUs were sampled in the rarefaction process and thus included in the α-diversity analysis.

The Shannon H' and Pielou Evenness α-diversity indices of the oomycetes exhibited similar trends according to the longitude, their abundances decreasing from west to east (Figure 5.5), with significant differences between Pedr and Andv ($H_{Shannon}$: 5.69, p<0.01; $H_{Evenness}$: 6.06; p<0.01), but without differences in OTUs richness.

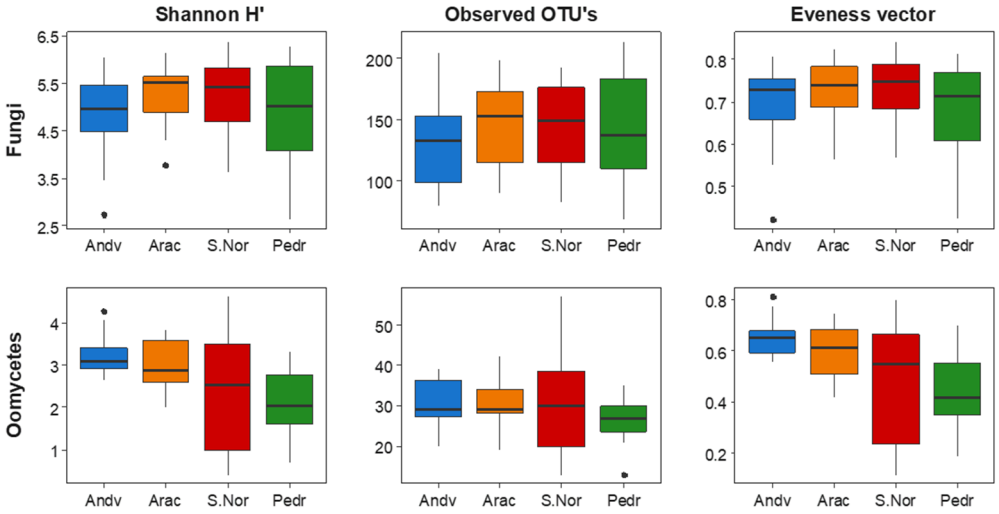

Figure 5.5. Analysis of Shannon H' diversity, (left), OTU's richness (centre) and Pielou-e eveness vector (right) by geographical zones, for the studied *Quercus ilex* "dehesas" of the Andalusian Network for Damage Monitoring in Forest Ecosystems, for fungi (up) and oomycete (down).

The fungal community did not show significant differences for the α-diversity indices. When these indices were compared between the mean defoliation levels for both communities (Figure 5.6), only oomycete evenness was lower for the greatly-defoliated plots (χ^2:4.6416, DF:1, $p<0.01$); the fungal community did not show differences.

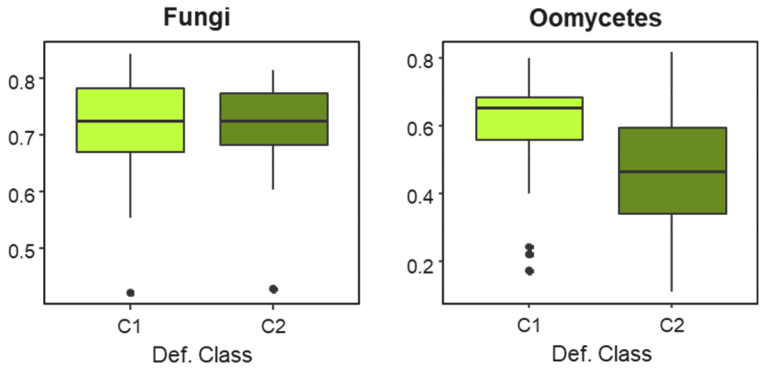

Figure 5.6. Comparison of Pielou-e eveness vector between defoliation classes of sampled plots at the studied *Quercus ilex* "dehesas" of the Andalusian Network for Damage Monitoring in Forest Ecosystems. C1= defoliation ≤ 25%; C2= 25% < defoliation ≤ 75%. There was no plot with an average degree of defoliation above 75% (C3).

Regarding β-diversity, ANOSIM shows that the composition of the fungal community was influenced by site (R=0.167; p<0.001), whereas for the oomycete community (R=0.078; p<0.05) differences were only found between Pedr and Andv (R=0.214; p<0.01) and between Pedr and S.Nor (R=0.125; p<0.05). The non-linear metrics (NMDS) show a more-aggregated zonal cluster for the fungal community, in comparison with the oomycetes (Figure 5.7), but also a clearer separation of the highly-defoliated plots for the oomycete community. Oomycete OTU#1 (*P. plurivora*) had the vector which most clearly influenced the separation between the degrees of defoliation.

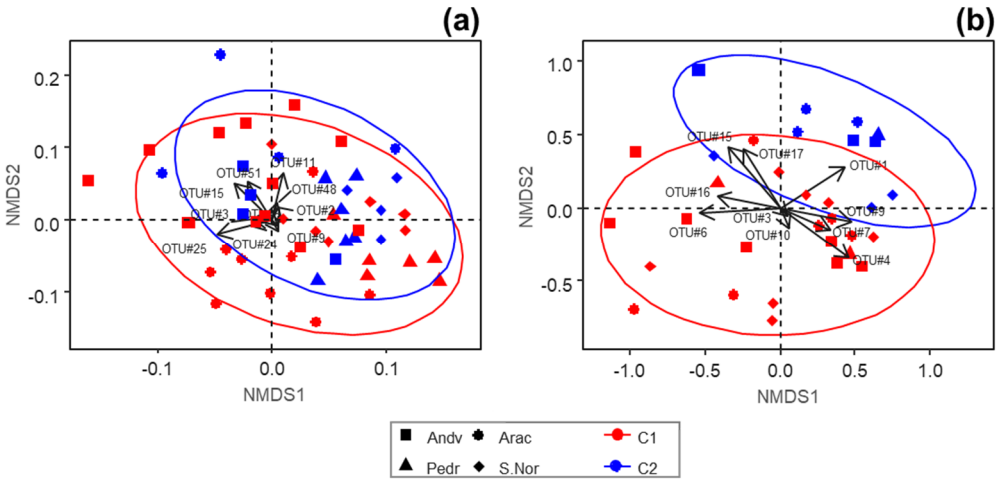

Figure 5.7: Non-metric multidimensional scaling analysis of OTU's abundance influence in defoliation levels and site location, for the 26 studied plots on *Quercus ilex* "dehesas" of the Andalusian Network for Damage Monitoring in Forest Ecosystems. A) Analysis for fungi dataset. B) Analysis for oomycete dataset. Ellipsoid indicates normal probability of point distribution at 90% confidence level for mean defoliation classes of plots. Arrows show influence vectors for the ten most abundant OTU's of fungi and oomycete dataset.

5.4.-Discussion

This work is, to our knowledge, the first analysis of the diversity of soil biota in declining holm oak "dehesas" at the regional level, exploring the interaction between fungal and oomycete communities in these soils. It

reveals high levels of diversity in the mycobiota and relationships between taxa, influenced by location and crown defoliation severity.

- The fungal community of "dehesas" has a wide range of plant pathogens and abundance of EcM

On average, in the studied soils, the sub-kingdom Dikarya (the phyla Ascomycota and Basidiomycota) dominated the fungal community, with more than 88% of the OTUs and over 91% of the total number of sequences. This observation agrees with the general composition of the soil fungal community in diverse forest ecosystems around the world (Tedersoo *et al.*, 2014; Veach *et al.*, 2017; Chen *et al.*, 2018; Jimu *et al.*, 2018). No similar works related to fungal diversity in *Q. ilex* "dehesas" were found in the literature reviewed.

Some pathogens present in our samples have been found in "dehesas" as part of the endophytic mycobiota of pasture species (Lledó Gómez, 2016). However, there are no references to the presence in "dehesas" of other like *Alternaria alternata* and *Pyrenochaeta cava*. These species were isolated from *Quercus robur* roots by (Kwaśna *et al.*, 2016), with significant changes in abundance according to the degree of defoliation. The increment in Dothideomycetes abundance might be related to a higher severity of decline due to the pathogenicity of some species, but also to changes in the functionality of mycobiota.

Mortierella elongata is a saprotroph often isolated in natural soils, being related their abundance with soil improvement in nutrient quality and fertility (Nordberg *et al.*, 2014; Tamayo-Vélez & Osorio, 2018). It plays an important role in the degradation of several compounds, including toxic ones (Li *et al.*, 2018), and its abundance can be considered an indicator of the resilience level of "dehesas" ecosystems.

Of the symbiotic species, *Russula* was the most-abundant genus, with 76 different OTUs classified within it, including the third-most-abundant species, *R. praetervisa*. Ectomycorrhizal abundance and

biodiversity have been related to fine root vitality in *Quercus robur* forests, being considered the most-important below-ground trait related to their decline (Mosca *et al.*, 2017). Also, its abundance has been related to benefits for the tree health status and the establishment of *Quercus* spp. seedlings (Dickie *et al.*, 2004). In our work, we did not find a relationship between Russulaceae abundance and the individual pathogenic *Phytophthora* spp,, but statistically-significant differences were found when OTU#8 abundance was analysed in relation to defoliation, both results agreeing with other studies in *Q. ilex* forests (Corcobado *et al.*, 2014b, 2015).

In our search for plant pathogens within the core biome of the fungal community, the genus *Fusarium* was the most relevant. This genus includes several soil-borne plant pathogens, related to forest and agricultural plant diseases in woody, shrub and herbaceous hosts (Aoki *et al.*, 2014). The *F. oxysporum* species complex causes vascular wilts, damping-off and crown and root rot in a wide range of species, being able to produce the death of holm oak seedlings in a pathogenicity test (Gallego *et al.*, 1999). The role of *F. eumartii* in the decline of *Q. robur*, *Q. cerris* and *Q. pubescens* in Italy has been recognised (Ragazzi *et al.*, 1993, 2001), and other species of this genus have been linked with diseases in other *Quercus* spp. (Lynch *et al.*, 2013; Wit *et al.*, 2015; Salvatore Moricca *et al.*, 2018).

In addition to *Fusarium* spp., 39 more OTUs within the fungal community core biome were assigned to plant pathogenic species, including some species known to be pathogens of oaks - such as some *Peniophora* spp. (Pecoraro *et al.*, 2014) (among which, OTU#816 was identified in 98% of samples), *Pyrenochaeta* spp., *Thelonectria* spp., *Alternaria alternata* (Kwaśna & Szewczyk, 2016), *Sporothrix inflata, Pezicula radicicola* (Kwaśna *et al.*, 2016) and *Phomopsis* spp. (Omari *et al.*, 2001). The genus *Cadophora*, present in more than 50% of the samples of S.Nor and Pedr, is an important genus of plant pathogens associated with diseases in different woody species (Prodi *et al.*, 2008; Travadon *et al.*, 2015). The abundance of fungal pathogenic species in zones with a low H' index for oomycetes, such as Pedr and S.Nor, coincided with a lower presence of beneficial fungal

species, such as strict saprotrophs, AM or EcM. These circumstances could lead to an environment that is ideal for triggering the decline of trees.

- *Phytophthora* spp. dominate the oomycete community in declining "dehesas"

Phythophthora was the dominant genus, but the most-abundant species among the oomycetes was *P. plurivora* - instead of those related to holm oak root rot, according to the literature (Pérez-Sierra *et al.*, 2013; Oßwald *et al.*, 2014; Jung *et al.*, 2016). Particularly, *Pythium spiculum*, considered a trigger of holm oak decline and mortality (Jiménez *et al.*, 2008; Serrano *et al.*, 2012), was excluded from the oomycete core biome. Among the oomycete pathogens causing root rot in *Quercus ilex*, *P. psychrophila*, by its abundance, and *P. quercina*, by its frequency of appearance, were the most-important taxa, *P. cinnamomi* appearing as the ninth-most-abundant oomycete, present in 52.7% of samples.

Phytophthora plurivora is a very-common soil-borne pathogen that has been isolated from soils of many natural forests in Europe. It was recorded as the most-abundant species in a survey of oak stands in Italy (Vettraino *et al.*, 2002) and in chestnut forests of central Italy, in a study of soil *Phytophthora* biodiversity through metabarcoding (Vannini *et al.*, 2013). Its pathogenicity on *Quercus alba* (McConnell & Balci, 2013), *Q. robur*, *Q. petraea*, *Q. rubra* (Jung & Burgess, 2009) and *Q. cerris* (Jung *et al.*, 1996) is recognised. However, to our knowledge, there is no evidence of a direct pathogenic relationship with *Q. ilex* root rot, or of its involvement in the decline syndrome in "dehesas", the work of Vettraino *et al*. (2002) being the only one that recorded this species (designated as *P. citricola*) under *Q. ilex* trees (subsp. *ilex*). The high abundance of this species in our samples, together with its influence in the NMDS aggregation of defoliation classes, encourages the study of *P. plurivora* involvement in holm oak decline.

Most of the *Phytophthora* taxa found in our samples were also recorded in soil samples of *Q. ilex* from natural forests in eastern Spain (Català *et al.*, 2017), where *P. psychrophila* and *P. quercina* were the most-relevant taxa; they appeared, respectively, as the third- and fourth-most-

abundant oomycete species in our study. Both taxa have been confirmed as *Q. ilex* root pathogens, causing root rot and tree death (Pérez-Sierra *et al.*, 2013). However, in the work of Català *et al.* (2017), no match was found for *P. plurivora* and *P. cinnamomi*. The overall specific composition of the *Phytophthora* spp. community in our samples was more similar to that described by Vettraino *et al.* (2002), who found *P. plurivora* (previously designated as *P. citricola*), *P. quercina*, *P. cinnamomi* and *P. cactorum* – in that order – as the most-frequent *Phytophthora* spp. isolated in *Q. ilex* stands.

Regarding the severity of symptoms associated with the oomycete community, no significant differences in taxa abundance or community composition were found among the defoliation categories, neither for the individual trees nor for the plot average, agreeing with the work of Corcobado *et al.* (2014b, 2015). Notwithstanding, defoliation and the severity of root rot should be related; in our case, this relationship was detected for the overall structure and functionality of the oomycete community (diversity and evenness), including the influences of the fungal community (EcM abundance).

Given our results, the involvement in the oak decline of all the plant pathogens present in the "dehesa" soils should be considered, not only that of *Py. spiculum* and *P. cinnamomi*, but also other pathogenic *Phytophthora* species - such as *P. plurivora*, *P. quercin*a and *P. psychrophila* - and other fungal plant pathogens such as *Fusarium* spp. or members of the Dothideomycetes.

- High diversity and changes in community structure are associated with the severity of symptoms

In comparison with recent analyses of the mycobiota in soils of different ecosystems (Veach *et al.*, 2017; Chen *et al.*, 2018; Jimu *et al.*, 2018), the "dehesas" had a high Shannon H' index (greater than 4.5 in all the studied zones) and a high value of evenness (exceeding 0.7). This shows a high diversity and good equilibrium of the mycobiota, but with a high proportion of plant pathogens and significant changes in the specific

composition of the soil community among zones, agreeing with the results of ANOSIM. No relationship of the studied parameters with plant community changes was found, agreeing with other authors who reported that changes in the diversity of mycobiota were decoupled from plant α-diversity (Veach *et al.*, 2017; Chen *et al.*, 2018).

The values of the α-diversity indices of the oomycete community were, in general, higher than those found by (Català *et al.*, 2017) in natural forest soils of eastern Spain, and were similar to (slightly higher than) those reported by (Vannini *et al.*, 2013) for chestnut forests of Italy. The lowest values of the Shannon index and evenness vector for the oomycete community occurred in Pedr, the most-defoliated zone, an observation that agrees with the significantly-lower values of evenness in the highly-defoliated plots. Although a direct relationship between specific oomycete taxa, or their abundance, and the severity of symptoms was lacking, the β-diversity analysis showed aggregation of highly-defoliated trees regarding the oomycete community. Similar results were obtained by (Linaldeddu *et al.*, 2011) when evaluating the diversity of the fungal endophytic community in declining *Quercus suber* L. trees. The severity of crown symptoms, therefore, seems to be related more to the functional structure of the oomycete community – driven by the diversity - than to its specific composition, the most-influential drivers being the change in the specific composition of the fungal community and the reduction in competition between root pathogens.

- EcM and AM are key guilds related to shifting soil microbiota functionality in "dehesas" ecosystems

The dominance of *Plant Pathogen* among the functional guilds - including *Plant Pathogen, Opportunistic pathogenic species* and *>1 Guild*, which includes *Plant Pathogen* - agrees with the great number of phytopathogenic taxa identified among the fungal and oomycete OTUs. On the other hand, the *Ectomycorrhizal* (EcM) and the *Arbuscular Mycorrhizal* (AM) guilds, the third- and fourth-most-abundant functional guilds,

respectively, had lower abundance rates overall when compared with other works in soils of different natural ecosystems (Veach *et al.*, 2017; Chen *et al.*, 2018). A significant reduction in EcM was found in Pedr, which also showed significant increments in pathogens and saprotrophs abundance, in line with its status as the most-highly-defoliated zone. Corcobado *et al.* (2014b) identified non-mycorrhizal root tips of *Q. ilex* as vulnerable points for pathogen invasion. Beneficial mycorrhizae, including AM and EcM, can potentially induce higher pathogen tolerance (Dawkins & Esiobu, 2017) through stimulation of the plant immune system (Pérez-de-Luque *et al.*, 2017), agreeing with our finding that higher EcM abundance was correlated with lower pathogens and saprobes abundance, and coincided with the areas of low defoliation.

Some studies have correlated the abundance of pathogenic species and reductions in EcM and AM with a rise in the N concentration and other changes in the soil chemical composition (Veach *et al.*, 2017; Chen *et al.*, 2018). The *Animal Pathogen*, *Soil-Plant Saprotrophs*, and *Dung Saprotroph* guilds had their maximum values in Pedr, which showed the lowest α-diversity values for oomycetes and an increment in the abundance of *Phytophthora* spp., with good values for the diversity of the fungal community, but lower EcM abundance. This is another example of the greater influence of the community structure and functionality, in comparison with the specific composition. It also supports the existence of a triple relationship among the abundance of EcM and other beneficial fungi, the plant pathogens and the tree health status, agreeing with other works (Corcobado *et al.*, 2014b, 2015).

- The abundance of *Pythium* spp. and *Trichoderma* spp. in relation to *Phytophthora* spp. dominance

The abundances of *Pythium* and *Phytophthora* influenced each other, but it remains unclear how the changes were influenced by location or by a natural equilibrium between the taxa. Our results show a significant decrease in oomycete α-diversity in soils of highly-defoliated stands,

coinciding with an increase in the abundance of *Phytophthora* spp. Other authors have demonstrated that the mixture of *P. cinnamomi* with different species of *Pythium* or other pathogenic fungi (such as *Fusarium oxysporum*) did not increase the severity of symptoms (Gallego *et al.*, 1999), supporting the hypothesis that, in soils with less diversity of oomycetes, *P. cinnamomi* and other pathogenic *Phytophthora* species have an increased ability to infect roots and affect the health status of the tree, increasing the severity of visible symptoms.

Another key relationship between taxa was the significant inverse correlation between OTU#51 abundance (*Trichoderma* sp.) and most of the core biome taxa of oomycetes (Table 5.4). The effects of *Trichoderma* spp. in biocontrol strategies are well known (Vinale *et al.*, 2008). *Trichoderma asperellum*, *T. hamatum,* and *T. virens* have been isolated from holm oak roots (*Q. ilex* subsp. *ilex*) and reduced the root rot of seedlings in a controlled experiment including *P. nicotianae* and *P. cinnamomi* (Aleandri *et al.*, 2015). Other *Trichoderma* spp. have also been proved to be antagonists of diverse *Phytophthora* spp. (García-Núñez *et al.*, 2017; Chemeltorit *et al.*, 2017; Promwee *et al.*, 2017; Widmer & Shishkoff, 2017). The evidence of the multiple effects of *Trichoderma* spp. against multiple phytopathogenic species should encourage further investigation of this relationship in the holm oak "dehesas".

As a conclusion, the complexity revealed by our data should encourage deeper research into the relationship between environmental factors and the whole soil community, beyond the simple picture of the single plant-pathogen interaction.

5.5.- References

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Chapter 6.- General discussion

6.1.- Interactions and defence responses of the host-pathogen system

Forest decline is currently a challenge for the conservation of ecosystems worldwide. In the case of holm oak decline of Andalusia "dehesas", it is aggravated by global change, including climate change and other factors such as the spread of alien invasive pathogens and the intensification or abandon of agricultural practices (Costa Pérez *et al.*, 2006; Gea-Izquierdo *et al.*, 2013; Duque-Lazo *et al.*, 2018). These topics seem to be far from the basic knowledge on the host-pathogen interaction, but in this work, we have demonstrated that the way in which *Phytophthora* spp. interacts with the host, and with the rest of soil biota, influences the effects of future control treatments and the spread of the disease, and thus, the management policies.

The holm oak root rot has been identified as one of the major threats to "dehesas" sustainability (Costa Pérez *et al.*, 2006), being *P. cinnamomi* considered as the main factor involved in tree death (Brasier, 1996; Sánchez *et al.*, 2002; Duque-Lazo *et al.*, 2018). Despite the evidence of the implication of other *Phytophthora* spp. in the oak decline in the Iberian Peninsula (Corcobado *et al.*, 2010; Pérez-Sierra *et al.*, 2013; Ruiz Gómez *et al.*, 2016), it has been considered that *P. cinnamomi* cause the most severe damages in holm oak fine roots compared with other *Phytophthora* spp. (Sánchez *et al.*, 2005; Moralejo *et al.*, 2009). However, the mechanisms of colonization and infection of root rot oomycetes are considered to be very similar for the main *Phytophthora* soilborne pathogens (Oßwald *et al.*, 2014), and no pathogen-specific immune responses have been identified between *Q. ilex* and any of the main root rot oomycetes associated with holm oak decline. Thus, we can consider that most of the interaction mechanisms, defensive histological responses and induced physiological changes described in this

work might be common to all the *Phytophthora* root rot pathogens. Further research must be required to confirm this statement, but it is consistent through the different approaches carried out in this research, and with the trends observed for the oomycete community in the analysis of the "dehesas" soil biodiversity.

- Histological methods and colonization / infection indices

Histopathology is considered a powerful technique to assess plant susceptibility and to evaluate the different plant responses to pathogen attacks (Pérez-de-Luque *et al.*, 2006; Jagger *et al.*, 2011). The adaptation of plant histology techniques to the *Quercus ilex* root tissue included the consideration of some important facts, which allowed us to a suitable quantification of infection indices in roots.

It is important to consider the influence of the different embedding methodologies in the fixation process, and staining methods. Fixation with Karnovsky solution presented optimal results, together with Toluidine blue-O (TBO) and Ruthenium Red (RhRed) stains with resin embedding, due to the tissue differences on the holm oak fine roots sections. The developed methodology was adequate to the analysis of root sections up to 4 mm in diameter and with a rather high degree of lignification. Also, the paraffin embedding methodology presented good results regarding sample conservation and availability for different preparations, with lower costs, but in coarser sections, the tissue integrity was often damaged during the microtomy step due to the lower consistency of the embedding material.

On the other hand, the development of a semi-automated methodology to analyse sections searching for pathogen development indices presented some constraints. Severity indices are often constructed based on specific reproductive or infective structures (haustoria, appressorium, sporangia, stromata…) (Rubiales & Moral, 2011; Emeran *et al.*, 2011). This approach is based on the link between the main effects of fungal plant pathogens with their reproduction or infective cycle. In the case of our experimental system, specific structures of the pathogen inside

the root tissues were scarce, because *Phytophthora* root pathogens complete their asexual cycle by germinating sporangia outside the roots, being only identified some chlamydospores in the cortex, haustorial structures at the early stages of infection, and resistance stromata-like structures at 14 dpi in the central cylinder. Moreover, it was difficult to establish an automated method to differentiate between the specific pathogen structures by their shape, which was highly influenced by the structure of intra and intercellular spaces of different root tissues, or by their colour, which did not present differences between somatic and specific structures with tested stains, except in the case of haustoria.

However, the general colour of pathogen structures was clearly differentiated for the host tissues with TBO stain; and the single consideration of pathogen structure localization, differentiating between intra and intercellular structures, was enough to describe the intensity of colonization and infection, and to divide the pathogen development cycle into the root in different stages.

- The physiological recovery of inoculated seedlings

The study of physiological responses in plants is indissolubly linked to plant growth and/or plant biomass allocation parameters. This relationship should be considered when studying the effects of *Phytophthora* root rot. Hemibiotroph and biotroph plant pathogens often develop competitive metabolic sinks leading to complex signalling pathways, which alters assimilates allocation (Freeman & Beattie, 2008; Zhang & Sonnewald, 2017), triggering the unspecific defensive responses and influencing the plant growth and biomass allocation under stress. These changes are subtle in adult trees, but our results showed important differences between young seedlings in a short time.

Moreover, plant physiology under stress conditions is highly dependent on phenotype and plant variability, but also environmental conditions (Mediavilla *et al.*, 2016). Experimental trials under controlled conditions eliminate the environmental source of variability and limits the

range of intraspecific variability, which is known to be really wide in holm oak (Jorrin-Novo & Navarro Cerrillo, 2014). However, in the reviewed literature, a wide variability of results was found regarding plant mortality and physiological changes, depending on the experimental conditions (Turco *et al.*, 2004; Sánchez *et al.*, 2005; Corcobado *et al.*, 2014a; León *et al.*, 2017).

In our experiment, we tried to homogenize the variability source to obtain a clearer definition of changing parameters because of experimental factors, selecting the genetic material as homogeneous as possible. Our results bring new insights about the differential physiological response to combined stress, leading to better understanding of the underlying processes regulating seedlings die-off. However, further research is necessary to understand if the identified recovery of seedlings after the first inoculation cycle was due to the chosen genotype. Subsequent works have confirmed the low rate of die-off for this genotype, and the general recovery of seedling physiology when plants were not subjected to flooding or drought (Ruiz-Gómez, unpublished data).

- Linking host-plant interaction and soil biota influence

In summary, we have showed that plant defence mechanisms against *Phytophthora cinnamomi* root rot are different from those related to drought stress. The combination of drought and pathogen stress in plants is considered one of the most complex and important stress affecting crops and natural ecosystems worldwide (Pandey *et al.*, 2017). The interaction between these two stressors might lead to several different scenarios, changing plant tolerance or susceptibility, resilience of plant affecting long-term growth rates, or influencing predisposition against other factors including other plant pathogens.

The unspecific nature of defensive responses identified in the histological evaluation, and the physiological changes that confirms the relevance of these responses in plant recovery, lead us to consider the possible influence of soil microbiota in the root rot, mainly linked to the

induction of plant immune responses due to endophytes colonization (Freeman & Beattie, 2008). Once hypothesized the influence of fungal community in root rot, it is unavoidable to consider other aspects such as the influence of EcM or AM fungi in plant health status, the identification of antagonist species for *Phytophthora* spp. root rot pathogens, or the presence of other important plant pathogens associated to tree decline.

Second-generation mass sequencing techniques allows the semi-quantitative evaluation of species abundance for fungi and oomycete divisions through *de novo* multiplexed sequencing – such as Illumina MiSeq platform (Bálint *et al.*, 2014; Sapkota & Nicolaisen, 2015). This analysis was questioned for the quantification accuracy as a consequence of multiple biological and methodological issues such as PCR amplification (Deagle *et al.*, 2014). However, other authors have found that the number of reads for a given species positively correlates with its relative abundance (Diaz-Real *et al.*, 2015; Sapkota & Nicolaisen, 2018), even using RT-PCR to quantify *in situ* presence of oomycetes (Català *et al.*, 2017). To avoid the discussion about methodology biases, our work was the first metabarcoding analysis that included internal DNA custom standards as a reference, validating our results through the comparison with the quantification of fungal and oomycete communities generated *in vitro* with a known specific composition and DNA relative abundance.

Not only specific composition of soil biota was related with the tree decline status –referred both to defoliation degree and the abundance of pathogenic oomycetes– but also diversity parameters showed significant trends, revealing that the holm oak decline is influenced by complex interactions between soil microorganisms.

6.2.- Plant responses influencing plant physiology

Holm oak seedlings presented early response (e.g. 24 h after inoculation) in fine root tissues as a result of pathogen colonization, with

cell wall thickening, de-esterification of cell walls and increased size of intracellular spaces (even in tissues in which pathogen structures were not present), and 3 dpi, thickening of cell wall was extended to the xylem and protoxylem cells of central cylinder, presenting some individuals blockage of xylem vessels at this time. Also, accumulation of phenolic compounds was observed in the contacts between pathogen and cell walls, and inside the cells, granular compounds were accumulated around intracellular structures of the pathogen. All these reactions directly alters the transport of water and solutes, and must be related with important changes in the metabolic activity of seedlings, affecting in the long term to the net photosynthesis and carbon compounds translocation.

Net photosynthetic assimilation (*A*) seems to be reduced in inoculated plants at 7 dpi, as well as stomatal conductance (*Gs*), but photosynthetic efficiency (*QY*) did not present significant differences in inoculated plants respect to the control. However, combined stress led to a significant reduction on this parameter, agreeing these results with other works involving *P. cinnamomi* and woody plant hosts (Oßwald *et al.*, 2014; Reeksting *et al.*, 2014). Some secondary metabolism pathways, such as the proline redox cycle and the stimulated oxidative pentose phosphate pathway, drive to the phenylpropanoid pathway increment, diverting carbon skeletons from primary to secondary metabolism to produce phenolic compounds in plants under biotic or abiotic stress, and thus increasing CO_2 concentration in leaves (Caretto *et al.*, 2015). The increment of the secondary metabolism activity may alter the carbon balance in leaves, being possible that high *QY* together with low A and partial stomatal closure indicates the increment of secondary metabolism production in leaves. These changes could be linked to the hydric imbalance in plants. The high moisture content in fine roots showed by inoculated plants could be related with the blockage of xylem vessels and the reverse osmosis mechanism (Borghetti *et al.*, 1993; Canny, 1997), which favours the sap flow from leaves to root, and thus the transport of defence compounds, on the first stages of root colonization.

Two different substances were identified blocking xylem vessels. When pathogen invades all the different root tissues and turns into necrotrophy –appearing cellular degradation areas and disruption of the Casparian strip– the xylem and parenchyma cells blockage increased exponentially. Pectidic substances blocking xylem vessels seems to be produced *in situ* by parenchymatous cells from the vascular cylinder, probably activated by the presence of foreign enzymes secreted by the pathogen like polygalacturonase, which is demonstrated that *P. cinnamomi* produces (Le Berre *et al.*, 2008). These substances de-esterify cell wall pectins (mainly homogalacturonan and xylogalacturonan) of parenchyma and metaxylem cell walls surrounding xylem vessels, producing mucilage composed by pectic oligosaccharides that move into and block vessels (Pérez-de-Luque *et al.*, 2006). Moreover, pectic oligosaccharides are known to elicit the accumulation of phytoalexins and protease inhibitors, and also promote lignification (Voragen *et al.*, 2009).

Other xylem vessels were blocked by large amounts of substances, filling large xylem cells and accumulating in cell pits. These compounds would be allocated into the central cylinder of fine roots due to reverse osmosis. This mechanism also agrees with the osmotic severe imbalance observed in infected plants for other interactions between woody hosts and *Phytophthora* spp. (Oßwald *et al.*, 2014). During the first 7 dpi, cell degradation was not identified, and most of the pathogen structures were present in the cortex and scarcely in the intracellular spaces of the central cylinder. Although some xylem vessels appeared blocked by mucilage, generalized vessels blockage appeared only after 7 dpi.

Additional data comparing plants subjected to acute drought, inoculated and mock inoculated, showed differences in the status of cell tissues regarding inoculation (Figure 6.1) (Ruiz-Gómez, unpublished data). Cell turgor loss and collapse of central cylinder parenchyma and xylem cells have been identified in absorbent roots as a consequence of water stress, agreeing with the observations for other species (Mayek-Pérez *et al.*, 2002; McElrone *et al.*, 2003; Mostajeran & Rahimi-Eichi, 2008).

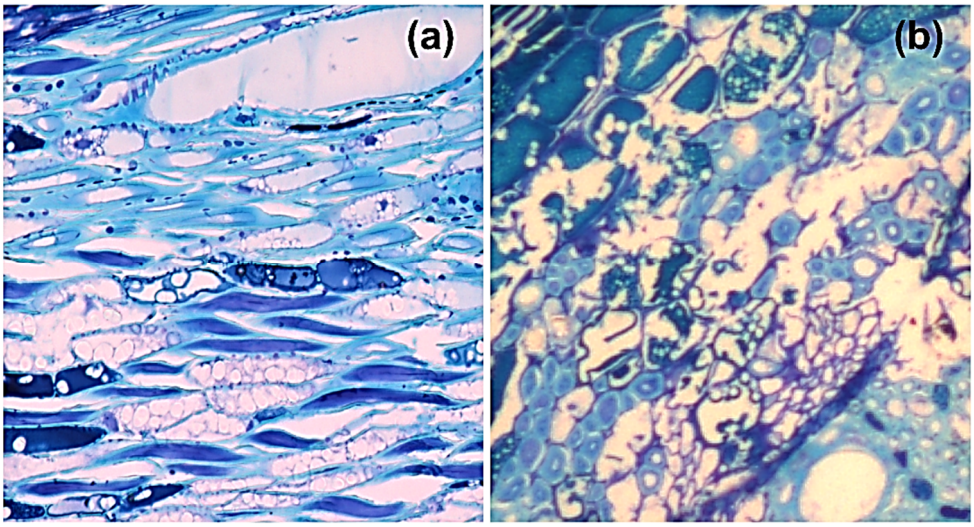

Figure 6.1. Effects of water stress on sections of mock-inoculated plants. (a) longitudinal section of vascular tissue from fine roots of inoculated plants subjected to acute drought. Generalized accumulations of mucilage and phenolic compounds into xylem and protoxylem cells, and blockage of cell pits was identified. No cell collapse was identified. (b) Detail of central cylinder in transversal section of mock-inoculated plants subjected to acute drought, showing turgor loss and collapse of xylem, metaxylem and parenchymatous cells.

Moreover, in early stages of infection cycle, not all the absorbent root are affected by root rot, depending on the proportion of the inoculum concentration in soil and root diameter distribution (Blaschke, 1994; Tuset *et al.*, 1996). As a result, it might be considered that before 7 dpi seedlings did not suffer water shortage, due to the action of *Phytophthora cinnamomi* in roots. However, inoculated plants close stomata even before than only water stressed ones.

A coherent explanation to all these changes would be linked by complex carbon compound signalling pathways in plants (Figure 6.2). When root infection raises root demand for assimilates, a "debit message" signal could occur, attracting stressed roots molecules from the shoot (Jackson, 2002). This signal might trigger the induction of secondary metabolite production in leaves, increasing intercellular CO_2 concentration (Else et al., 2009), which is considered a promoter of stomatal closure. The stomatal closure as a consequence of plant signals could favour reverse osmosis processes and thus, secondary metabolites reallocation from leaves

to roots. Further research must be developed to confirm this hypothesis, which could have important consequences in the evaluation of susceptibility of plants to root rot.

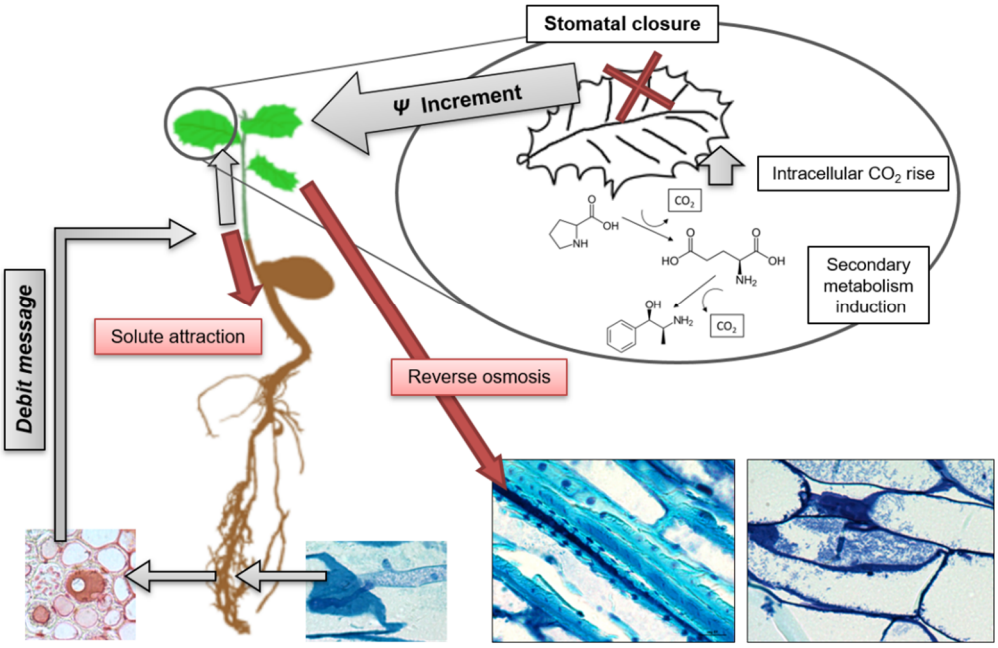

Figure 6.2. Proposed carbon compounds-mediated model for seedling responses to *P. cinnamomi* root infection. When pathogen colonizes and infects roots, structural and chemical changes in tissue trigger the "debit message", mainly driven by the ceasing of root uptake and the presence of high concentration of low-weight carbon compounds. The solute attraction from stem activates several secondary metabolic pathways producing phenolic and nitrogen defence compounds, and carbon dioxide levels rise into intracellular spaces of leaves, triggering stomatal closure, which favours reverse osmosis and metabolite transport into the root.

The significant reduction in stem biomass identified in our work as a consequence of inoculation agrees with this hypothesis (Figure 4.4.b) Furthermore, high CO_2 concentration in leaves might avoid the damage of the photosynthetic apparatus that has been identified as a result of stomatal closure in water stressed plants (Pandey *et al.*, 2017; Zhang & Sonnewald, 2017), allowing the recovery of seedlings when the demand for assimilates in roots decrease.

6.3.- Plant growth and biomass allocation are influenced by pathogen colonization

Although the high photosynthetic efficiency showed by inoculated plants, seedling growth suddenly stops after inoculation treatments, both in root tips and aboveground organs of the plant. Many authors have reported this cessation of growth as a consequence of *P. cinnamomi* colonization and infection of roots (Cahill, 1989; Blaschke, 1994; Solla *et al.*, 2009).

The increased size of intracellular spaces, the accumulation of phenolic compounds in cell walls, the Casparian strip discontinuity and the cortex cell necrosis, are changes reducing water and nutrient uptake of roots, due to loss of tissue conductivity both via apoplast and symplast (Vettraino *et al.*, 2003; Balci *et al.*, 2008; Oßwald *et al.*, 2014), and might be related with the ceasing of root tip growth identified in *Q. robur* only 24 h after inoculation (Blaschke, 1994). Pectic oligosaccharides detected in root sections as a result of cell wall de-esterification may also have an important role in the cessation of taproot growth (Goldberg *et al.*, 1996). However, no important changes were observed in the multifactorial experiment on total plant biomass (dry matter), both in belowground and aboveground organs, due to the inoculation treatment, but biomass allocation parameter was significantly influenced by this factor, mainly root mass fraction (*RMF*) but also stem fraction (*SMF*).

Water stress reduces significantly growth, gas exchange and photosynthesis of holm oak, presenting trees under severe drought low metabolic rates (Forner *et al.*, 2018). In our case, when plants are not subjected to additional stress, inoculation seems to be not enough to kill seedlings, and histological studies showed several changes in roots that indicate high rates of secondary metabolism. Thus, photosynthesis rate might be less influenced by root changes due to inoculation that for acute drought, being the increment of phenolic compounds production the cause of the reduction in net photosynthetic assimilation (*A*). Plant invests its

resources in defensive responses reducing growth, and promoting biomass allocation changes (Caretto *et al.*, 2015).

On the other hand, *SMF* reduction in inoculated plants may be consequence of the putative "debit message" of infected roots (Jackson, 2002). Changes in stem biomass on woody species as a response of pathogen attacks are nor reported in the reviewed literature, but holm oak seedlings present high plasticity and growth rates, being possible high rates of carbon compounds and photosynthates reallocation between different tissues.

6.4.- Influence of soil fungal and oomycete community in holm oak decline.

Several authors have highlighted the influence of soil biocenosis under differential ecosystem factors, or vice-versa, mainly focused on bacterial and fungal communities (Gdanetz & Trail, 2017; Deveau *et al.*, 2018). The analysis of soil fungal and oomycete communities in declined "dehesas" showed a great number of interesting relationships, not only between key taxa or specific composition, but also with the diversity and functional structure of both communities regarding plant health status and environment differences, in agreement with other authors (Tedersoo *et al.*, 2014; Coleman-Derr *et al.*, 2016; Veach *et al.*, 2017; Chen *et al.*, 2018; Jimu *et al.*, 2018).

Fitting our hypothesis, fungal-plant interactions showed effects in both pathogenic *Phytophthora* spp. abundance and tree defoliation used as health status indicator. Competition between species occupying the same ecological niche has been showed when plant pathogens abundance presented significant negative correlation with saprotrophs. More interesting for our case, *Pythium* spp. abundance was related with lower abundance of *Phytophthora* spp., and it is recognized the low ability of some soil-borne *Phytophthora* spp. to compete under saprophytic conditions (Dunstan *et al.*, 2010). Our results showed that the highest defoliation levels

were present under the low oomycete diversity indices. It might be considered that *Phytophthora* spp. is a weak competitor, which agreed with the described colonization process of roots by *P. cinnamomi*, in which pathogen behaves like a biotroph on the first stages, turning into necrotroph when it reaches parenchymatous tissues of the central cylinder, that is supposed to be rich in assimilates such as sugar, starch and other photosynthates. Therefore, when competence is reduced, the effects of pathogen activity in roots are more evident.

On the other hand, one particular OTU identified as *Trichoderma* sp. presented significant antagonist relationship with the main pathogenic *Phytophthora* spp. Therefore, the specific composition of fungal community could have significant effect in the plant health status, probably inducing defence mechanisms. Endophytes like *Trichoderma* spp. might promote plant defence against root pathogens inducing phytoalexins accumulation or cell wall lignification (Vinale *et al.*, 2008). The changes in cell walls of fine roots might be an important barrier against *P. cinnamomi* colonization in the biotrophic stage, avoiding or limiting the invasion of cortex cells by somatic hyphae or haustoria.

The main effects of EcM fungi in tree decline might be related with the enhancement of water and nutrient uptake (Shahin *et al.*, 2013), increasing tree health status and therefore, rising the possibility of plant defence against root colonization. The Russulaceae family dominates EcM communities in temperate forests (Avis *et al.*, 2003), being identified in *Quercus ilex* roots, and related with lower defoliation rates (Corcobado *et al.*, 2015), agreeing with our results. Another possible interaction that should be considered regarding the presence of EcM and AM fungal species would be the occupation of the absorbent root surface in mycorrhized plants, which would physically prevent the fixation of zoospores, at least partially, reducing infection rates in localized climatic episodes.

In view of the results of our work, acting on the composition and functional structure of fungal community might have significant influence on the oomycete community, and consequently, in the root rot disease. *Trichoderma* spp. are the most promising taxa regarding this strategy (Vinale *et al.*, 2008), but also saprobes (Tamayo-Vélez & Osorio, 2018; Li *et al.*, 2018), which presented high abundance levels among our samples, but low diversity, and EcM enhancing holm oak health status (Corcobado *et al.*, 2014b, 2015).

6.5.- Limitations of the work and future research lines

This research fulfils the thesis objectives, providing new insights about the host-pathogen interaction. It has been developed a conceptual model based in tissue invasion indices, which explains the infection cycle of *P. cinnamomi* into the roots, the differences to the plant responses facing drought and root rot were evidenced, and the biological compound of the soil has been described and key relationships with the biotic community were identified. However, some limitations should be considered to better understand the results and to improve future research.

- The characterization of the physiological response to combined stress must include the species range of susceptibility

The methodology used to assess biomass allocation and root traits is very expensive in time and needs a careful treatment of roots. These conditions limit the number of samples that can be managed in a controlled conditions experiment. Also, the selection of plant material for our experiment requires the adequate characterization of genetic resources for an accurate explanation of symptoms. The chosen plant ecotype was identified belonging to a drought-tolerant population in a previous work assessing plant expression in a common garden experiment (Navarro Cerrillo *et al.*, 2018). Our results showed differences due to inoculation in water stressed plants, but the drought tolerance did not explain the

recovery of seedlings after 14 dpi. On this sense, future research must include other populations, increasing variability and testing physiological changes among different susceptibility levels to confirm some of the hypothesis drawn from our work. Subsequent works have been carried out on the last months before the publication of this thesis, including different plant material origin and therefore, higher genetic variability, and the preliminary results confirms our hypothesis regarding physiological changes, and the recovery of seedlings after the first 14 dpi when plants were not subjected to water stress or periodic flooding, but with significant differences between the origin of plant material.

- The influence of soil pathogen community in tree decline must consider relationship evidences

The analysis of taxa and divisions of fungal community described in the soils of "dehesas" showed a very wide number of different fungal species, some of them plant pathogen related with other host species or agricultural crops which could be present in "dehesas" ecosystems (Lledó Gómez, 2016; Kwaśna *et al.*, 2016). Moreover, only from the analysis of abundance of pathogenic *Phytophthora* spp. we cannot conclude pathogenic relationships with holm oak without additional studies.

Regarding the metabarcoding analysis, the next logical step is to compartmentalise the sample. Other authors have analysed the differences between the bulk soil, the rhizosphere and the endosphere of plants, obtaining evidences of plant colonization and taxa relationships differences for bacterial and fungal communities (Coleman-Derr *et al.*, 2016; Gdanetz & Trail, 2017). The main constraint about this approach in woody species is the difficulty to perform DNA extraction of endophytes or pathogens into roots due to the interference of phenolic compounds in the isolation of DNA molecules. Also, it should be considered the scarce proportion of fungal and oomycete DNA in fine roots regarding total DNA amount from the host. However, some tests have been carried out with samples from our work, with acceptable DNA yields. It is planned to carry out the library

construction and the metabarcoding analysis of the root endosphere DNA extracted from those samples.

On the other hand, classical methodologies of culture isolation and pathogenicity tests must be taken into account, but the previous analysis of the metabarcoding data could focus this work, selecting the most relevant species, and therefore facilitating one work that is almost always arduous and requires highly qualified staff (Martin *et al.*, 2012).

- Other future research lines

Re-emphasizing the importance of the characterization of genetic resources, the methodologies developed, and the results obtained in this research, allows for a better knowledge for the implementation of accurate methodologies assessing *Q. ilex* seedlings tolerance to *P. cinnamomi*, or to other *Phytophthora* spp. Particularly, the quantification of colonization indices through histopathology would represent an accurate way to quantify and classify tolerance ranges. Also root traits, mainly *RMF*, and seedling recovery must be considered, although the analysis of different responses in a wider range of susceptibility is necessary to confirm this last point.

Other future work is the study of the relationships between soil biota shifts and environmental conditions. The chemical composition and structure of the soils is closely related to the soil community composition, and other environmental factors such as precipitation, temperatures, exposure and management have demonstrated their influence on the spread of the disease (Duque-Lazo *et al.*, 2016, 2018). The study of the relationships between these factors along a gradient of disease severity would lead to the identification of specific soil biota community structures related with different tree decline status. This analysis must consider also bacterial microbiota and compartmental analysis.

Finally, we consider that the most promising research line deriving from our results is the consideration of the biological components of the

soil in the management of declined areas. After the deep analysis of fungal and oomycete community for each specific situation, it may be feasible to act on the factors affecting radical rot, modifying the specific composition or the abundance of some species. "Tailor-made bio-solutions" including *Trichoderma* spp., EcM, saprobes, or even species of the Saprolegniaceae and Pythiaceae family could be used in management strategies as amendments.

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Chapter 7.- Conclusions

- Plant histology has demonstrated to be a useful tool to assess the severity of oomycete colonization and/or infection in fine roots of *Quercus ilex* L. The workflow described, including fixing solution, embedding material, staining method and semiautomated image analysis, allowed a clear differentiation between pathogen and host structures, making possible to estimate quantitative infection indices based on the location and abundance of intra- and intercellular pathogen structures.

- The histological study of the interaction between *Phytophthora cinnamomi* and *Q. ilex* allowed the description of colonization and infection cycle of the pathogen inside fine roots, showing three main stages: i) Biotrophic colonization and haustoria invasion of cortex tissue; ii) Behaviour shift to hemibiotrophic and necrotrophic, feeding on parenchymatous cells when pathogen structures reached central cylinder; and iii) Longitudinal spread through vascular tissue, asexual cycle completion with the sporangia germination outside the root, and creating chlamydospores and stromata-like aggregations inside the root.

- General and unspecific defence responses of the host were identified as a consequence of the inoculation with *P. cinnamomi*, but early biotrophic growth of the pathogen, rapid spread through vascular system and the ability to change its trophic relationship allows it to overcome the host defences.

- The main responses identified in fine roots of *Quercus ilex* seedlings against *P. cinnamomi* colonization and infection were: i) thickening of cell walls; ii) accumulation of different substances around contact zones between pathogen and cell walls, and around intracellular pathogen structures; iii) blockage of vessels with pectidic mucilage and substances rich in

polyphenols; iv) partial stomatal closure accompanied with reduction in photosynthetic net assimilation rates, but without significant decrease of photosynthetic efficiency; and v) biomass allocation changes as a result of assimilates translocation. These responses allowed the recovery of seedlings after the first infection cycle when plants were not subjected to an additional stress.

- The main effects of *P. cinnamomi* colonization and infection in fine roots of *Quercus ilex* seedlings were: i) increment of intercellular spaces and softening of tissues caused by the de-esterification and degradation of cell walls; ii) discontinuity between cortex and central cylinder in Casparian Strip; iii) cell degradation and necrosis areas in parenchymatous tissues of central cylinder and cortex; iv) blockage of xylem vessels due to the accumulation of hyphae, leading to collapse of the vascular system; iv) ceasing of meristematic development and plant growth; v) loss of fine absorbent roots. Although these effects seem to lead to water shortage in plants, hydric stress was not identified in seedlings inoculated and properly irrigated.

- *P. cinnamomi* root rot reduced significantly the ability of roots to explore substrate, presenting inoculated seedlings lower root plasticity and *RMF*, but the infection of *Q. ilex* seedlings with this pathogen didn't kill the seedlings in our case, neither caused permanent damage to their physiological status, without additional water stress. Moreover, when water stress was present, the effects of both biotic and abiotic stressors were additive.

- The analysis of soil fungal and oomycete communities revealed the high diversity of microbial community in *Quercus ilex* "dehesas", and the relationships of the location and the defoliation level with the composition of both communities. High α-diversity and medium species richness for fungi were accompanied with significant differences in the specific composition and changes in abundance of species among study zones.

Functionality of soil fungal and oomycete communities were related with defoliation and with *Phytophthora* spp. abundance, and specific composition was mainly influenced by location.

- Defoliation was related with changes in the specific composition of fungal community and the reduction of competition between the main root rot pathogens, revealed by the low oomycete α-diversity, without changes in the specific composition of the last one. Ectomycorrhizal (EcM) abundance – mainly *Russula* spp. – and saprophytic species were related with changes in tree health. Both guilds, together with endophytes and *Pythium* spp. abundance, influenced pathogenic *Phytophthora* spp. abundance.

- Specific competence, improvement of soil conditions, enhancing tree health status, systemic defence induction and antagonism were the possible interactions identified between fungi, oomycete and plants, resulting this analysis from the significant trends presented by pathogens, saprotrophs, EcM and endophytes. The presence of significant opposite correlations between a particular *Trichoderma* sp. and most of the pathogenic *Phytophthora* spp., as well as the relationships between EcM and tree health status, lead us to the possibility of including soil microbiome management in the control strategies of holm oak decline.

Capítulo 7.- Conclusiones

- Las técnicas de histología vegetal demostraron ser una herramienta útil para evaluar el grado de colonización y/o infección en raíces finas de *Quercus ilex* L. La metodología descrita, desde la elección de la solución de fijación, pasando por el material de inclusión de muestras y el método de tinción, hasta el análisis semiautomático de imágenes, permitió la estimación cuantitativa de índices de infección basados en la localización y la abundancia de las estructuras intra- e intercelulares del patógeno.

- El estudio histológico de la interacción entre *Phytophthora cinnamomi* y *Q. ilex* permitió la descripción del proceso de colonización e infección en las raíces finas, estableciéndose tres etapas diferentes: i) etapa biotrófica del patógeno, colonizando los tejidos del córtex y el interior de las células de dicho tejido mediante estructuras haustoriales; ii) cambio de la relación trófica, etapa hemibiótrofa y/o necrotrófica, alimentándose de las células parenquimáticas una vez que el patógeno invade el cilindro central; y iii) finalización del ciclo de infección, con la dispersión del patógeno a través del tejido vascular, la finalización del ciclo de reproducción asexual con la germinación de esporangios en la superficie de la raíz, y la aparición de estructuras de resistencia como clamidosporas y agregaciones estromáticas en los tejidos de la raíz.

- Los plantones de encina presentaron respuestas de defensa de tipo genérico ante el ataque de *P. cinnamomi*, pero el comportamiento biotrófico del patógeno en las etapas tempranas del ciclo, la habilidad de modificar su relación trófica con el huésped, y su capacidad de dispersarse rápidamente a través del sistema vascular, permitieron al patógeno superar las reacciones defensivas del hospedante.

- Las principales reacciones defensivas identificadas en raíces finas de plantones de *Quercus ilex* frente a la colonización e infección de *P.*

cinnamomi fueron: i) engrosamiento generalizado de las paredes celulares; ii) acumulación de sustancias de distinta naturaleza en zonas de contacto del patógeno con la pared celular, y alrededor de las estructuras intracelulares del patógeno; iii) bloqueo de vasos en el tejido conductor mediante la acumulación de sustancias mucilaginosas pectídicas y sustancias ricas en polifenoles; iv) cierre estomático parcial, acompañado de la reducción de la asimilación fotosintética neta, pero sin una reducción significativa de la eficiencia fotosintética; y v) cambios en la compartimentación de la biomasa como resultado de la traslocación de compuestos asimilados por la planta. Estas respuestas permitieron la recuperación de los plantones tras el primer ciclo de infección del patógeno, cuando los plantones no fueron sometidos a un estrés adicional.

- Los principales efectos debidos a la colonización e infección de *P. cinnamomi* en raíces finas de plantones de *Q. ilex* fueron: i) Aumento de los espacios intercelulares y reblandecimiento de los tejidos causados por la deesterificación y degradación de las paredes celulares; ii) discontinuidad entre córtex y cilindro central en la Banda de Caspari; iii) presencia de zonas necróticas y de degradación celular en tejidos parenquimáticos del cilindro central y del córtex; iv) bloqueo de los vasos de xilema debido a la acumulación de hifas, dando lugar al colapso del sistema vascular; iv) detención del desarrollo meristemático y del crecimiento de la planta; v) pérdida de raíces finas absorbentes. Aunque estos efectos parecen conducir a la deficiencia de agua en la planta, los plantones inoculados que fueron regados correctamente no presentaron síntomas de estrés hídrico.

- La podredumbre radicular causada por *P. cinnamomi* reduce significativamente la capacidad de las raíces para explorar el sustrato, presentando los plantones inoculados menor plasticidad y *RMF,* pero la infección de plantones de *Q. ilex* con este patógeno, en nuestro caso, no provocó su muerte, ni causó daño permanente en su estado fisiológico, sin la presencia de estrés hídrico adicional. Además, cuando se combinaron el

estrés hídrico y la inoculación, los efectos de ambos factores resultaron aditivos.

- El análisis de las comunidades fúngicas y de oomicetos en el suelo reveló el alto grado de diversidad de la comunidad microbiana en las dehesas de *Quercus ilex*, y las relaciones de la localización y el nivel de defoliación con la composición de ambas comunidades. Los altos valores de α-diversidad y una riqueza de especies de tipo medio en la comunidad fúngica estuvieron acompañados con diferencias significativas en la composición específica y cambios en la abundancia de las especies entre zonas de estudio. La funcionalidad de las comunidades fúngica y de oomicetos se relacionó con la defoliación y con la abundancia de *Phytophthora* spp., y la composición específica se vio influenciada principalmente por la localización.

- La defoliación estuvo relacionada con los cambios en la composición específica de la comunidad fúngica y con la reducción de la competencia entre los principales patógenos de raíz, revelada por la baja α- diversidad de oomicetos, sin cambios en la composición específica de los mismos. La abundancia de EcM, principalmente *Russula* spp., y de especies saprófitas, estuvo relacionada con cambios en el estado sanitario del arbolado. Ambos gremios funcionales, junto con los hongos endófitos y la abundancia de *Pythium* spp., presentaron influencia sobre la abundancia de *Phytophthora* spp.

- La competencia específica, la mejora de las condiciones del suelo, la mejora del estado sanitario de los árboles, la inducción de respuestas sistémicas y la relación de antagonismo, fueron las posibles interacciones entre los hongos, los oomicetos y las plantas, identificadas como resultado de analizar las tendencias significativas que presentaron los patógenos, saprótrofos, EcM y endófitas. La presencia de correlaciones inversas, significativas, entre una especie de *Trichoderma* y la mayoría de las especies patogénicas de *Phytophthora* identificadas, así como la relación entre las EcM y el estado sanitario del arbolado, conducen a considerar la

posibilidad de incluir el manejo del microbioma del suelo en las estrategias de control del decaimiento de la encina.

APPENDIX I: Supplementary materials

A.I.1.- Supplementary Figures

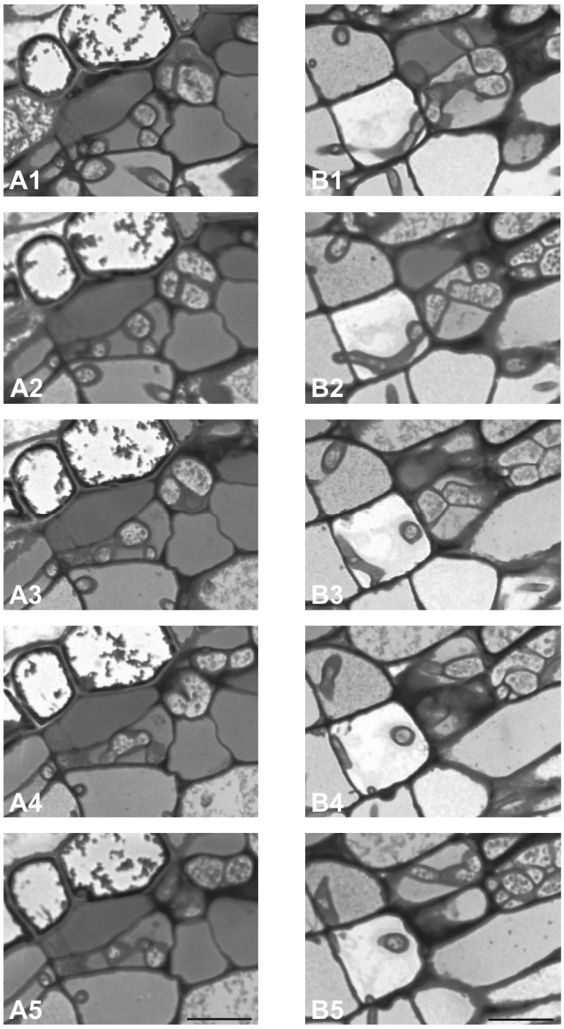

Figure S.2.1. Two series (A1-A5, B1-B5) of consecutive sections from vascular tissue of inoculated samples 14 dai. Observation of consecutive sections allows a three-dimensional reconstruction of the intracellular pathogen structures and confirms that the appearance of septate hyphae in a single section is an artefact. Bars present in figures A5 and B5 show 20 µm.

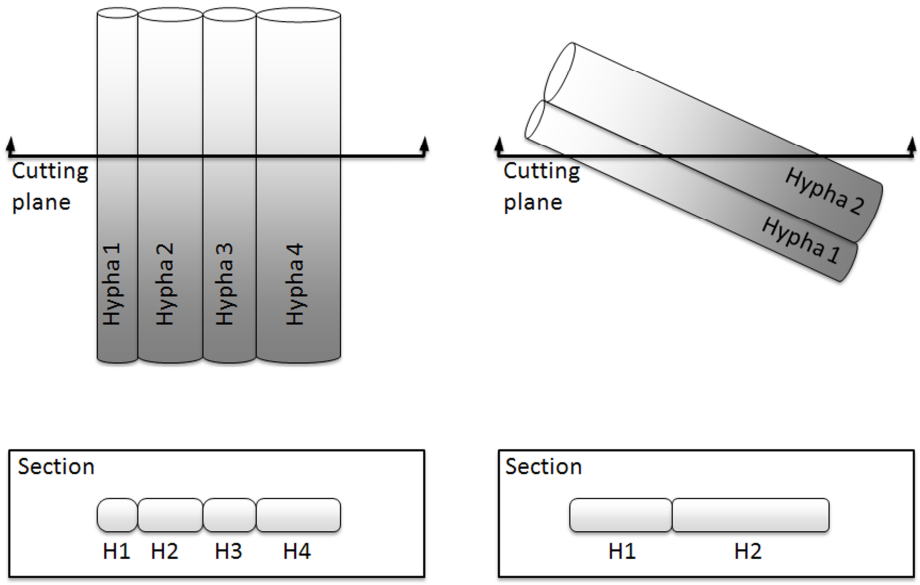

Figure S.2.2. Schematic representation for the sectioning of several neighboring hyphae. When observed in a slide, a single section could be confused with a septated hypha. For that reason, several consecutive sections must be checked in order to get a three dimensional representation of the tissues. H1, Hyphae 1; H2, Hyphae 2; H3, Hyphae 3; H4, Hyphae 4.

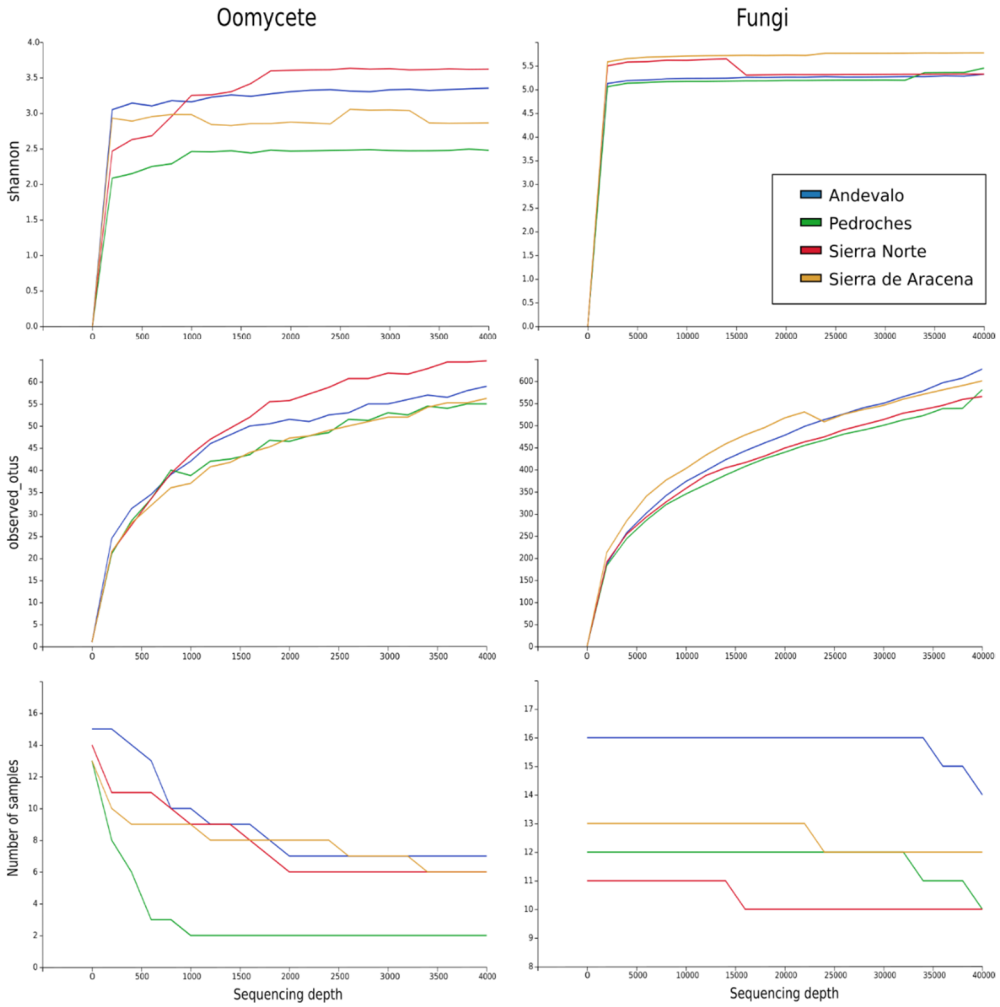

Figure S5.1 Rarefaction analysis of oomycete (left) and fungi (right) raw datasets of soil total DNA library, from soil samples of Quercus ilex dehesas of the Andalusian Network for Damage Monitoring in Forest Ecosystems

A.I.2.- Supplementary Tables

Table S4.1. Means of all the studied variables with standard error at 30 dpi. Treatments with the same letter did not differ significantly (HSD Tukey's test or Kruskal-Wallis rank of sum test, $p<0.05$).

Var.	Control	Inoculation	Drought	IxD	F
θ	0.436 ± 0.024^{a}	0.478 ± 0.039^{a}	0.098 ± 0.013^{b}	0.135 ± 0.024^{b}	8.0^{**}
Ψ_m	-0.76 ± 0.17^{a}	-0.95 ± 0.13^{a}	$<-6^{b}$	$<-6^{b}$	232.6^{***}
DMr	54.03 ± 1.54^{b}	45.89 ± 1.66	59.31 ± 1.79^{ab}	66.93 ± 2.16^{a}	11.0^{***}
A	4.70 ± 0.53^{a}	4.69 ± 0.65^{a}	0.74 ± 0.49^{b}	-0.13 ± 0.18^{b}	16.7^{***}
Gs	0.074 ± 0.023^{a}	0.079 ± 0.025^{a}	0.032 ± 0.010^{b}	0.031 ± 0.009^{b}	4.2^{*}
QY	0.409 ± 0.033^{a}	0.345 ± 0.020^{ab}	0.230 ± 0.057^{ab}	0.102 ± 0.036^{b}	6.5^{**}
RDW	10.56 ± 1.42^{bc}	8.89 ± 0.95^{c}	14.86 ± 1.01^{a}	12.75 ± 1.83^{ab}	7.6^{*}
SDW	2.44 ± 0.38^{a}	2.47 ± 0.33^{a}	2.62 ± 0.34^{a}	1.08 ± 0.27^{b}	4.6^{**}
LDW	4.02 ± 1.05	4.49 ± 0.58	2.56 ± 0.48	5.00 ± 0.57	--
RMF	0.615 ± 0.023^{bc}	0.558 ± 0.014^{c}	0.730 ± 0.018^{a}	0.674 ± 0.016^{b}	17.2^{***}
SMF	0.150 ± 0.017^{a}	0.158 ± 0.011^{a}	0.138 ± 0.014^{a}	0.062 ± 0.018^{b}	8.3^{***}
LMF	0.235 ± 0.035^{a}	0.284 ± 0.014^{a}	0.132 ± 0.012^{b}	0.263 ± 0.014^{a}	10.4^{***}

Table S5.1. Plots selected for the study among points in *Quercus ilex* dehesas of the Andalusian Network for Damage Monitoring in Forest Ecosystems, and their main characteristics. X, Y coordinates in ETRS89 / UTM Zone 30

Plot_code	UTMX	UTMY	Zone	Province	Defoliation (%)	Root rot presence	*Q. ilex* (%)	Elevation (m.a.s.l.)	Annual temperature T (°C)	Evapotranspiration (mm)	Annual Precipitation (mm)
CO1004	313548	4276464	Pedr	Cordoba	35.2	Yes	100%	487	15.94	1481.88	524.17
CO1013	330568	4271091	Pedr	Cordoba	30.6	Yes	100%	494	16.16	1509.15	546.29
CO1023	339741	4264392	Pedr	Cordoba	33.5	Yes	100%	534	16.11	1472.18	527.86
CO1029	301451	4250271	Pedr	Cordoba	25	Yes	100%	594	15.51	1453.79	497.68
CO1055	327694	4238224	Pedr	Cordoba	26.5	Yes	100%	643	15.47	1433.67	584.06
CO1060	367208	4244448	Pedr	Cordoba	22.1	Yes	100%	648	15.51	1409.91	640.99
CO1101	347254	4217003	Pedr	Cordoba	23.1	No	100%	243	17.06	1538.49	591.68
CO1121	294529	4192414	S.Nor	Cordoba	19.4	Yes	92%	208	16.91	1538.55	677.9
HU1001	159238	4227729	Arac	Huelva	46.9	Yes	85%	344	16.29	1452.9	767.31
HU1007	130122	4206909	Arac	Huelva	23.3	Yes	100%	199	16.94	1458.21	693.05
HU1025	202588	4210264	Arac	Huelva	13.3	Yes	100%	581	15.8	1363.7	791.76
HU1027	124740	4189836	Andv	Huelva	25	Yes	100%	209	17.1	1521.43	677.72
HU1028	132652	4191150	Andv	Huelva	37.1	No	100%	298	16.66	1423.43	751.12
HU1040	110198	4179458	Andv	Huelva	21.9	Yes	100%	154	17.62	1545.53	657.76
HU1052	205011	4194513	Arac	Huelva	20	Yes	100%	330	17.13	1438.48	687.37
HU1060	143033	4176569	Andv	Huelva	22.9	Yes	96%	171	17.39	1476.12	708.56
HU1069	214215	4187820	Arac	Huelva	23.8	Yes	100%	299	17.14	1414.34	725.28
HU1082	113946	4155755	Andv	Huelva	18.1	Yes	100%	144	17.8	1499.95	568.35
HU1094	123107	4149092	Andv	Huelva	20.4	Yes	100%	106	18.03	1463.71	588.43
HU1096	138927	4151764	Andv	Huelva	31.9	Yes	100%	116	17.95	1396.96	617.99
PULIDOS	115904	4171666	Andv	Huelva	18.1	Yes	100%	148	17.67	1515.93	596.59
SE1005	257837	4219069	S.Nor	Sevilla	14.8	Yes	100%	663	15.16	1360.79	706.77
SE1024	253659	4194150	S.Nor	Sevilla	25.2	Yes	100%	433	16.2	1451.89	740.25
SE1040	270746	4188715	S.Nor	Sevilla	18.5	No	100%	380	16.22	1424.86	765.73
SE1048	240366	4175736	S.Nor	Sevilla	21.9	Yes	100%	283	16.99	1483.04	684.96
SE1050	256188	4178282	S.Nor	Sevilla	21.7	Yes	92%	170	17.35	1472.05	665.11

Table S5.2. Parameters of sequence processing in CLC Genomics Workbench 3.6.5.

Input	Value
Trimming limit	0.5
Ambiguous bases threshold	0
Minimum read size	100 bp
Maximum read size	500 bp
Overlapping mismatch	1
Overlapping gap cost	3
Minimum score	50

Table S5.3. LCA Parameters of taxonomy processing in MEGAN 6.10.8

Input	Value
Minimum score threshold	170
Minimum sequence similarity	99%
Minimum LCA percent	50%

APPENDIX II: Supplementary works

A.II.1.- Aislamiento e identificación de oomicetos en focos de podredumbre radical de Andalucía y Extremadura.

- **Ruiz Gómez FJ**, Navarro-Cerrillo RM, Lara Gómez MA, Sánchez-Cuesta R. **2016**. Aislamiento e identificación de oomicetos en focos de podredumbre radical de Andalucía y Extremadura. *Cuadernos de la Sociedad Española de Ciencias Forestales* **43**: 363-376.

IISSN: 1575-2410. Google Scholar Metrics Índice H5: 3. Ciencias Agrarias.

Resumen

El decaimiento de los *Quercus* sigue siendo uno de los principales problemas de sanidad forestal en España y Portugal. Es difícil cuantificar los ingentes recursos económicos y humanos destinados a la búsqueda de soluciones, sin que se haya podido atajar el avance de la mortalidad en las dehesas. Aunque se señala a *Phytophthora cinnamomi* como agente causal de los episodios más graves, otros oomicetos han sido identificados como agentes relacionados con estos procesos de mortalidad, o como patógenos oportunistas o acompañantes. La complejidad de los sistemas forestales influye en la diversidad de patógenos de podredumbre radical en el suelo, por lo que se debe tener en cuenta el efecto agregativo de la interacción de dichos organismos en la podredumbre radical. En el presente trabajo se colectaron muestras de suelo en dehesas de Córdoba, Sevilla y Cáceres, en áreas de afección por decaimiento. El daño del arbolado se evaluó mediante una escala asimilada de Ferreti. Las muestras se cultivaron en medio

selectivo, y se identificaron morfológicamente 26 especies diferentes de oomicetos. *Phytohthora cinnamomi* fue el patógeno que mayor número de aislamientos presentó. El número de especies aisladas en función del grado de daños mostró diferencias significativas, siendo los árboles con grado 2 aquellos en los cuales se identificó el mayor número de especies. La presencia de *P. cinnamomi* no estuvo asociada significativamente con la diversidad de otras especies.

Palabras clave: Decaimiento, interacción *Phytophthora, Pythium, Quercus ilex*

A.II.2.- Growth and physiological sapling responses of eleven *Quercus ilex* ecotypes under identical environmental conditions

- Navarro-Cerrillo RM, **Ruiz Gómez FJ**, Cabrera-Puerto RJ, Sánchez-Cuesta R, Palacios Rodriguez G, Quero Perez JL. **2018**. Growth and physiological sapling responses of eleven *Quercus ilex* ecotypes under identical environmental conditions. *Forest Ecology and Management* **415-416**: 58-69.

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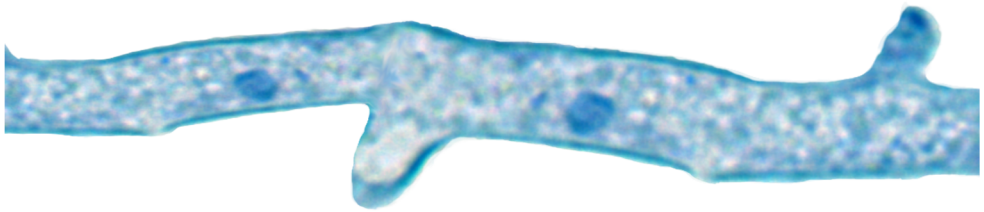
Abstract

Studies with holm oak indicate that genetic variation may result in substantial differences in drought tolerance among its ecotypes. However, few trials have studied this variation under common environmental conditions. This study aimed to assess physiological and morphological responses of holm oak saplings for 11 ecotypes that represent a longitudinal transect across south-central Spain (Andalusia). Drought resistance was assessed by measuring growth, xylem water potential, chlorophyll fluorescence, and photosynthesis. Possible relationships among morphological and physiological responses across ecotypes were determined using Pearson productmoment correlations and multiple linear regressions. The response variables were used in multivariate analyses including discriminant function analysis, principal component and cluster analyses. Last, we used sparse Partial Least Squares regression (sPLS) to analyse the relationships between the morpho-physiological responses and biophysical parameters of the parent locations. Our results indicate that Q. ilex ecotypes growing in a common garden setting exhibited substantial variation in morphological and physiological traits. At the end of the growth trial (65 months post-planting), basal diameter, leaf area, and

midday water potential were higher in *Q. ilex* ecotypes from western sites compared to eastern sites across Andalusia. PCA and clustering revealed clear morphological and physiological differentiation in response to gradients of geographical and ecological variation in ecotype origin. Variables that were related to the water regime of the ecotypes, such as seasonal precipitation and evapotranspiration, showed stronger correlations with ecotype responses. Consequently, eastern ecotypes were more likely to spread in response to projected increases in temperatures and declines in summer precipitation; however, western ecotypes would likely decrease in response to hotter and drier summers.

Keywords: Drought tolerance, Provenance, Chlorophyll fluorescence, Water potential, Holm oak, Afforestation.



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EVALUACIÓN Y RESTAURACIÓN DE SISTEMAS AGRÍCOLAS Y FORESTALES