UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

Respuesta química defensiva de *Quercus* mediterráneos frente al estrés biótico

Manuela Rodríguez Romero

Programa de Doctorado en Ciencia y Tecnología de los Sistemas
Agroforestales (R022)

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Conformidad de los directores;

"La conformidad del director/es de la tesis consta en el original en papel de esta Tesis Doctoral"

Fdo. Fernando J. Pulido Díaz (Director)	Fdo. Ramón Santiago Beltrán (Codirector)

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El Dr**. Fernando Javier Pulido Díaz**, Profesor Titular del Área de Producción Vegetal del Departamento de Ingeniería del Medio Agronómico y Forestal de la Universidad de Extremadura, como Director de Tesis.

El Dr. **Ramón Santiago Beltrán**, Ingeniero de Montes Investigador del Centro de Investigaciones Científicas y Tecnológicas de Extremadura (CICYTEX), Instituto ICMC, perteneciente a la Consejería de Economía, Ciencia y Agenda Digital de la Junta de Extremadura, como Codirector de Tesis.

INFORMAN:

Que la memoria titulada "Respuesta química defensiva de *Quercus* mediterráneos frente al estrés biótico" que presenta la Ingeniera de Montes Manuela Rodríguez Romero, ha sido realizada bajo su dirección y que, a su juicio, reúne todas las condiciones para poder optar al Grado de Doctora.

Los resultados que se han ido generando durante este trabajo de investigación se han expuesto y discutido en los foros y congresos científicos nacionales e internacionales que se detallan a continuación:

Comunicaciones presentadas a Congresos Internacionales

1. **Rodríguez-Romero M,** Godoy B, Neno J, Calha IM, Passarinho JA, Moreira AC (2019). Chemical characterization of the phenolic and volatile compounds from roots extracts of three Brassicaceae species and their anti-*Phytophthora* activity. 9th meeting of IOBC Integrated Protection in Oak Forests. Oeiras (Portugal) 8th-11th October 2019. 1 comunicación oral.

2. **Rodríguez-Romero M**, Calha IM, Passarinho JA, Moreira AC (2019). Susceptibility of the herbaceus species used as pastures in dehesa/montado to *Phytophthora cinnamomi.* 9th meeting of IUFRO Working Party 7.02.09: *Phytophthora* in Forests and Natural Ecosystems. La Maddalena, Sardinia (Italy) 17th-25th October 2019. Comunicación oral y póster. Awarded for the best poster contribution to the scientific programme of the IUFRO Meeting.

3. **Rodríguez-Romero M**, Godoy B, Conrad AO, Pulido F, Bonello P (2019). Characterization of constitutive and induces chemical defenses of *Quercus ilex* against infection by *Phytophthora cinnamomi.* 9th meeting of IUFRO Working Party 7.02.09: *Phytophthora* in Forests and Natural Ecosystems. La Maddalena, Sardinia (Italy) 17th-25th October 2019. Comunicación oral y póster. Awarded for the best poster contribution to the scientific programme of the IUFRO Meeting.

4. **Rodríguez-Romero M,** Godoy B, Neno J, Calha IM, Passarinho JA, Moreira AC (2019). Characterization of the volatile compounds from three Brassicaceae species and their effects in the presence of *Phytophthora cinnamomi.* 9th meeting of IUFRO Working Party 7.02.09: *Phytophthora* in Forests and Natural Ecosystems. La Maddalena, Sardinia (Italy) 17th-25th October 2019. Comunicación oral y póster. Awarded for the best poster contribution to the scientific programme of the IUFRO Meeting.

Comunicaciones presentadas a Congresos y Jornadas Nacionales

5. **Rodríguez-Romero M** (2017). Diferencias en las defensas constitutivas e inducidas de encinas (*Quercus ilex*) de diversas procedencias ante dos agentes causantes de estrés biótico: daños

mecánicos por herbivoría e infección con el patógeno *Phytophthora cinnamomi.* Congreso multidisciplinar de Jóvenes Investigadores (Cáceres). Comunicación oral.

6. **Rodríguez-Romero M,** Gallardo A, Pérez A, Pulido F (2017). Study of the geographical variation in the constitutive and induced levels of biotic defenses in oak (*Quercus ilex*). 7ºCongreso Forestal Español (Plasencia). 1 comunicación y publicación en el Libro de Actas. ISBN: 978-84-941695-2-6. Edita: Sociedad Española de Ciencias Forestales.

7. **Rodríguez-Romero M**, Godoy B, Calha IM, Passarinho, JA, Moreira AC (2018). Evaluation of allelopathic potential of Brassicaceae root extracts on *Phytophthora cinnamomi* activity obtained by three extraction methods. Congreso de la Sociedad Española de Fitopatología (Toledo). 1 comunicación y publicación en el Libro de Actas. Edita: Sociedad Española de Fitopatología.

8. **Rodríguez-Romero M (2018).** Búsqueda de resistencia a la Seca de los *Quercus* en la Dehesa. Jornada de Divulgación Científica II Semana de la Ciencia (Escuela de Ingenierías Agrarias, Badajoz). Programa de Doctorado en Ciencia y Tecnología de los Sistemas Agroforestales. Comunicación oral.

9. **Rodríguez-Romero M (2019).** El decaimiento del arbolado en la dehesa: La Seca. Jornada sobre la Regeneración de suelos y conservación del arbolado en la dehesa (Finca Castro Enríquez, Aldehuela de la Bóveda, Salamanca). Organizada por la Asociación de Ganaderos 19deAbril. 1 Comunicación oral.

10. **Rodríguez-Romero M (2019).** Diferencias intraespecíficas individuales para aumentar la resiliencia de la masa: el caso de *Phytophthora cinnamomi* en la dehesa. Seminarios de Investigación en el Instituto del Corcho, la Madera y el Carbón Vegetal (ICMC-CICYTEX, Mérida). Programa de Doctorado en Ciencia y Tecnología de los Sistemas Agroforestales. 1 Comunicación oral.

11. **Rodríguez-Romero M (2019).** Resistencias y alelopatías frente a la seca de los *Quercus*. Jornada de Sanidad Forestal dirigida a técnicos y agentes del medio natural de la Junta de Castilla La Mancha (Talavera de la Reina). 1 Comunicación oral.

y han dado lugar a las siguientes publicaciones:

Artículos publicados en revistas indexadas (SCI, Science Citation Index)

12. **Rodríguez-Romero M,** Gallardo A & Pulido F (2020). Geographical and within-population variation of constitutive chemical defences in a Mediterranean oak (*Quercus ilex*). Forest Systems 29 (1), e011, 12 pages. https://doi.org/10.5424/fs/2020292-16943. JCR 1.178. Q2 (Forestry).

13. **Rodríguez-Romero M,** Gallardo A, Pérez A & Pulido F (2020). Interactive effects of biotic stressors and provenance in chemical defence induction by holm oak (*Quercus ilex*). Enviado a Trees, Structure and Function. En revisión. JCR 2.125. Q1 (Forestry).

14. **Rodríguez-Romero M**, Godoy-Cancho B, Calha IM, Passarinho JA & Moreira AC (2020) Allelopathic effects of three herb species on *Phytophthora cinnamomi*, a pathogen causing severe oak decline in Mediterranean wood pastures. Enviado a Forests. En revisión. JCR 2.221. Q1 (Forestry).

15. **Rodríguez-Romero M**, Cardillo E, Santiago R & Pulido F (2020). Susceptibility to *Phytophthora cinnamomi* of six holm oak (*Quercus ilex*) provenances: are results under controlled vs. natural conditions consistent? Enviado a Forest Systems. En revision. JCR 1.178. Q2 (Forestry).

Capítulos de libro

16. Pulido F, Gallardo A, Morcuende D, Moreno G, **Rodríguez-Romero M,** Solla A, Fernández-Rebollo P, Quesada A (2019). "Determinantes de la resistencia al estrés biótico en una especie forestal modelo: la encina (*Quercus ilex*) en los Parques Nacionales". En: "Proyectos de investigación en parques nacionales 2013-2017". Naturaleza y Parques Nacionales. Serie Investigación en la Red. Pep Amengual (editor). Organismo Autónomo Parques Nacionales (España) ISBN:978-84-8014-924-2. Depósito legal: M-33646-2019

17. **Rodríguez-Romero, M.** (2020). "Buscando resistencia a la seca de los *Quercus* en la dehesa: el fascinante mundo de las interacciones planta-patógeno". En: Catálogo de Investigación joven en Extremadura. Volumen III. Universidad de Extremadura. Servicio de Publicaciones.

Además, para completar su formación, la doctoranda realizó las siguientes estancias de 3 meses de duración en el extranjero:

18. Instituto Nacional de Investigação Agrária e Veterinária (INIAV, IP.). Oeiras, Portugal. Año 2017.

Estudio de alelopatías entre diversas especies de la flora mediterránea y el patógeno *Phytophthora cinnamomi* (cultivo de las plantas, del patógeno y de bacterias asociadas). Siembra y cuantificación de estructuras del Oomycete. Extracción DNA bacterias para estudio del microbioma asociado. 3 meses.

19. The Ohio State University (OSU). Columbus, Ohio, USA. Año 2018.

Estancia formativa en USA (trabajo de laboratorio en Universidad de Ohio y de campo en Universidad de Berkeley) para el desarrollo de biomarcadores químicos de resistencia mediante cromatografía líquida de alta eficacia. 3 meses.

20. Institut National de la Recherche Agronomique (INRAE). Unité Mixte de Recherche (UMR) BIOGECO (BIOdiversité, GÊnes & COmmunautés). Bordeaux, La France. Año 2019.

Desarrollo de análisis moleculares para la determinación de la hibridación en especies del género *Quercus* y su tolerancia a *Phytophthora cinnamomi*. 3 meses.

Y para que todo ello conste a efectos legales, firman el presente documento en Plasencia, a 11 de enero de 2021.

"La conformidad del director/es de la tesis consta en el original en papel de esta Tesis Doctoral"

Fdo. Fernando J. Pulido Díaz	Fdo. Ramón Santiago Beltrán
(Director)	(Codirector)

Manuela Rodríguez Romero ha disfrutado de una beca FPI-INIA del Ministerio de Ciencia, Innovación y Universidades adscrita a CICYTEX.

GOBIERNO DE ESPAÑA
MINISTERIO DE CIENCIA, INNOVACIÓN Y UNIVERSIDADES
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INIA
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria
CICYTEX
CENTRO DE INVESTIGACIONES CIENTÍFICAS Y TECNOLÓGICAS DE EXTREMADURA

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CERTIFICADO

MANUELA RODRÍGUEZ ROMERO, aluna da Universidade da Extremadura e bolseira FPI-INIA do Centro de Investigaciones Científicas y Tecnológicas de Extremadura (CICYTEX) participou num estágio no INIAV, I.P., Oeiras, Portugal, entre 1 de Outubro e 31 de Dezembro de 2017, e entre 22 de Janeiro e 7 de Março de 2018. Participou nas linhas de investigação sob o tema "selecção de plantas da flora natural com efeito alelopático para *Phytophthora cinnamomi* e avaliação do seu grau de susceptibilidade ao patogénio" no âmbito do projecto GO- Declínio do Montado no Alentejo- PDR2020-101-031496 que tem como principal objectivo a mitigação do declínio do montado.

Durante este período participou nas seguintes actividades:

1. Ensaio *in vivo* de susceptibilidade ao patogénio *Phytophthora cinnamomi* de 15 espécies de plantas herbáceas pertencentes às famílias: Poaceae e Fabaceae.

2. Estudio *in vitro* do efeito dos extractos radiculares de *Diplotaxis tenuifolia, Eruca vesicaria* e *Raphanus raphanistrum* na actividade de *Phytophthora cinnamomi* (crescimento micelial e produção de estructuras de infecção e resistência); Comparação de três métodos de extracção e dos extractos radiculares com stress hídrico.

3. Ensaio *in vivo* do efeito alelopático de *Diplotaxis tenuifolia* e *Eruca vesicaria* sobre o patogénio *Phytophthora cinnamomi* em *Quercus suber, Quercus rotundifolia* e *Quercus faginea* (em curso).

4. Estudo do microbioma associado aos extractos radiculares das plantas seleccionadas.

Oeiras, 8 de Março de 2018

A investigadora responsável do estágio

Ana Cristina Moreira PhD
Protecção de Plantas

THE OHIO STATE UNIVERSITY

Department of Plant Pathology

201 Kottman Hall
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E-mail bonello.2@osu.edu
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July 31, 2018

To Whom It May Concern:

This is to state that Manuela Rodríguez Romero, a PhD student at the University of Extremadura and a recipient of a FPI-INIA grant from the Scientific and Technological Research Center of Extremadura (CICYTEX), conducted research as a Visiting Scholar in my laboratory at the Ohio State University (Columbus, Ohio, USA) from 1st May to 31st July 2018.

During this period, she became familiar with chemical analysis of tree tissues by way of Ultra Performance Liquid Chromatography – Mass Spectrometry (UPLC-MS). Manuela learnt procedures ranging from tissue extraction to data interpretation, with the ultimate goal of identifying biochemical markers of oak resistance to a very important soil-borne pathogen, *Phytophthora cinnamomi*, which is involved in acute and chronic oak decline in the Iberic peninsula and other Mediterranean areas. This work is based on prior discoveries by my lab of biomarkers of oak resistance in the sudden oak death system in California that were made in collaborative work with colleagues at the University of California, Berkeley. As part of her US experience, Manuela also had an opportunity to travel to California to learn about sudden oak death affecting *Quercus agrifolia* directly in the field while assisting in reevaluation of permanent plots we have in Briones Regional Park, Contra Costa County, California.

Should you have any questions, please do not hesitate to let me know.

Sincerely,

Pierluigi Bonello

Pierluigi Bonello
Professor of Plant Pathology

biogeco
université de BORDEAUX
INRA SCIENCE & IMPACT

Arndt Hampe
Directeur de Recherche
Institut National de la Recherche Agronomique (INRA)
UMR 1202 Biodiversité, Gènes & Communautés (BIOGECO)
arndt.hampe@inra.fr

Cestas, 14/12/2019

To whom it may concern,

I hereby certify that Manuela Rodriguez Romero, PhD student at the University of Extremadura, Plasencia (Spain), interned under my supervision between 15st September and 14st December 2019 at BIOGECO. Her research stay enabled her to complete the genetic characterization of oak (*Quercus*) trees tolerant and susceptible to infection by *Phytophthora cinnamomi* as originally scheduled.

Arndt Hampe

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UMR BIODIVERSITE, GENES ET COMMUNAUTES (BIOGECO)
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TESIS DOCTORAL

Respuesta química defensiva de *Quercus* mediterráneos frente al estrés biótico

▪

PhD THESIS

Defensive chemical response of Mediterranean *Quercus* against biotic stress

▪

TESE DE DOUTORAMENTO

Resposta química defensiva dos *Quercus* mediterrâneos contra o *stress* biótico

Manuela Rodríguez Romero

2021

De acuerdo con la mención de Doctorado Internacional
In agreement with the International Doctorate Mention
De acordo com a Menção de Doutorado Internacional

Agradecimientos

Durante la realización de esta tesis que aún me parece mentira estar cerrando, he descubierto para mí un nuevo mundo que me ha ido atrapando con cada pregunta que nos hacíamos y en cada experimento que diseñábamos. De todo ello he aprendido mucho, pero, sobre todo, he tomado conciencia de lo importante que es la gente que te rodea y te enseña cada día. Por eso, y aunque los últimos meses no han sido nada fáciles, no quería dejar pasar esta oportunidad para agradecer a todas esas personas que de una u otra forma se embarcaron y desembarcaron junto a mí a lo largo de estos años haciendo posible este documento.

En primer lugar, agradecer a mi director, Fernando Pulido, la confianza depositada (mucho mayor que la que yo misma tenía) y su paciencia para enseñar a quién no sabe, ejemplo para mí de la verdadera sabiduría. Gracias a mi codirector, Ramón Santiago, porque hace fácil lo difícil y así el trabajo es mucho más gratificante. Y a Tomás Rodríguez, mi tutor, porque siempre estuvo ahí para todas las cuestiones académicas que iban surgiendo.

Los cuatro años de trabajos que recoge este trabajo los pasé a medio camino entre el Centro Universitario de Plasencia (Universidad de Extremadura) y el Instituto para la Promoción del Corcho, la Madera y el Carbón Vegetal de Mérida (Centro de Investigaciones Científicas y Tecnológicas de Extremadura). Así, las personas con las que empecé trabajando se han convertido hoy día en verdaderos amigos que me han apoyado cuando todo marchaba, y también cuando el proceso se complicaba. De Plasencia vienen a mi mente los buenos ratos y las horas de invernadero con Javi, Álvaro, Quisco, Javi Miranda y Yoni, las conversaciones siempre instructivas de Tara, Álvaro y Guadalupe y el apoyo del resto de profesores e investigadores del Grupo de Investigación Forestal, especialmente de Alejandro Solla.

De Mérida, mi compañera de despacho Celes me hizo las horas más amenas y Belén mucho más divertidas, además de ser mi apoyo más fiel en todo el trabajo de laboratorios. Almudena le dio sentido a muchas horas de trayecto hasta el centro de trabajo cada mañana a las 6.30h…Pablo, Raúl, Jose, Adrián, Mónica, Anabel, Luis, Rocío, Alba, Guadalupe, Máximo, Mª José, Paloma, Raquel, Ana, Tomás, Matilde, Paco, Santi...¡gracias! Especial mención quisiera hacer a Carmen y a Carlos, porque siempre

confiaron en mí y me apoyaron en todo lo que iba surgiendo; y a Enrique, porque ha estado ahí desde el principio y hasta el final, contagiándome la pasión que siente por la investigación y que tan bien transmite. Eusebio, Emi y nuestras horas en el laboratorio de fitopatología… siempre os estaré agradecida porque además de ayudarme en todo, me aportasteis ese componente humano que tantas veces hace falta. Y por supuesto, no sería nadie sin las risas y el trabajo de campo compartido con Pedro, Rubio, Pablo, Ángel, Juan Ángel y Luis, al que siempre tendré muy presente. De La Orden, Carlos, Fernando, Ignacio y Sandra me acercaron a la agronomía y me mostraron el valor del trabajo en equipo; al igual que Mariqui y Pablo, del CAEM, siempre dispuestos a ayudar, ¡incluso en los ámbitos más insospechados!

Y aunque no entraba en mis planes, la Facultad de Veterinaria de Cáceres y en concreto el Departamento de Genética y Bioquímica se convirtió en la pieza clave de este trabajo. Nada de esto hubiera sido posible sin la ayuda de Gloria, Ana, María, David Morcuende (Iprocar) y Alberto Quesada, pero sobre todo, de mi gran maestro, Álex. Él ha sido, sin pretenderlo, el elemento imprescindible de esta tesis. ¡Gracias por aguantarme y por compartir tan generosamente tus conocimientos!

Además, la beca FPI-INIA me dio la posibilidad de disfrutar de estancias formativas en el extranjero que se terminaron convirtiendo en experiencias vitales. Del INIAV de Portugal me quedo con el trabajo en equipo y cercano de sus miembros, especialmente de Ana Cristina Moreira, que además me ayudó con el resumen en portugués de este trabajo, con Isabel Calha, José Antonio Passarinho, Paula Sá y ese gran equipo de “bolseiros” que nos concentrábamos en el “despacho del fondo a la izquierda”. De la Universidad de Ohio, Enrico Bonello, Anna Conrad, Carrie Erwin, Marie Laborde, Cat, Emile… ¡cuánto bien hacen esas lecciones de gran profesionalidad a la par que compañerismo y planes divertidos los fines de semana! Enrico me permitió también conocer la problemática de *Phytophthora ramorum* en California y participar en el trabajo de campo con el equipo de la Universidad de Berkeley, además de conocer a grandes personas como Brice McPherson y David Wood. Todos ellos hicieron que me sintiera en casa entre aquellos montes de *Quercus* tan parecidos a los nuestros (también en las temperaturas calurosas del verano). Del INRA francés tengo que agradecer a Arndt Hampe su ayuda y generosidad desde el primer momento. Y su buen hacer en cada detalle, hasta para integrarme en el resto del grupo, lo cual no fue difícil por el gran equipo que forman Elena, Santiago, Kata, Marina, Erwan, Olivier, Emilie…

El trabajo de campo e invernadero tampoco hubiera sido posible sin la incondicional disponibilidad y ayuda de Juan Antonio. Las horas que echamos en aquellas parcelas son difíciles de calcular. Por supuesto, gracias a todos los que alguna vez me acompañaron en la recogida de bellotas, a Santi, con quien pasé grandes ratos recorriendo las dehesas del sur de Badajoz. A Raúl Bonal, que también contribuyó en esta colecta ibérica, a Demetrio, a los hermanos Romero-Aragüete y seguramente a muchas otras personas que me estoy dejando en el tintero sin intención, espero que puedan perdonarme.

Pero sobre todo, tengo que agradecer a mi familia, los verdaderos artífices de mi mundo de sueños e ilusiones y mis ganas por embarcarme en proyectos muchas veces apasionantes pero que terminan absorbiéndome por completo y para los cuales, sin embargo, me apoyan sin condición, como siempre, desde siempre.

¡GRACIAS!

Agradecimientos a entidades colaboradoras

Esta tesis doctoral ha sido financiada por una beca FPI-INIA del Ministerio de Ciencia, Innovación y Universidades adscrita al Centro de Investigaciones Científicas y Tecnológicas de Extremadura (CICYTEX).

TABLE OF CONTENTS

Abstract

Plants have several defence mechanisms against natural enemies that may compromise their survival. However, given the current scenario of changing biotic and abiotic conditions, in-depth knowledge of these mechanisms is essential to face future challenges. The plant response and their ability to survive in these new scenarios will be key to the conservation of the ecosystems like oak wood pastures (Spanish dehesas).

This Thesis analyzes the production of chemical defences, mainly phenolic, in the plant-biotic stressor interaction to assess possible geographical and individual differences. In Chapter 1, it is analyzed whether constitutive levels of total phenols (Tp) and condensed tannins (Ct) acting as chemical defences in the holm oak (*Quercus ilex)* vary across Iberian regions, populations and genotypes. Significant differences were found for the concentration of Tp and Ct among regions and among genotypes, but not among populations within regions. The lowest levels of constitutive defences were found in the northern Iberian Peninsula. Also, the defensive phenolic content was significantly higher in regions with acidic soils. Heritability values (Tp 0.37±0.08 and Ct 0.48±0.36) were lower than those obtained for total tannins in a previous study.

In Chapter 2, the ability of holm oaks from the six Iberian provenances to respond to multiple biotic damages was analysed through their induced chemical defence patterns. Biotic damage was induced at the root level (by infection with the widespread pathogen *Phytophthora cinnamomi –Pc-*) and at the above-ground level (by mechanical defoliation). The levels of constitutive and induced Tp, total tannins (Tt) and Ct were measured. Results showed that (1) the defensive chemical patterns show significant local and geographical variation; (2) survival to stress is more related to constitutive defences than induced ones; (3) the induced response is stressor-specific; and (4) there is an interactive effect among stressors and the induction or inhibition depends on the provenance. These findings on the biotic stressor effects on the chemical defences and survival of holm oak may facilitate the development of genetic material selection programs in the integrated control of the widespread *Quercus* decline.

This oak decline is closely associated with *Pc.* The wide distribution of this soil-borne pathogen and its exacerbation of damage by climate change in the Mediterranean region raise the need for breeding programs based on resistant and tolerant material to the

disease. Chapter 3 aims to determine if holm oaks planted in four field plots with highly Pc-infected soils show differences in mortality according to their provenances after a four-year monitoring. It also evaluates whether the most tolerant provenances in the field are those with the greatest constitutive chemical defences measured in the previous greenhouse experiment. In general, there was a high mortality (56.0-80.5%) but provenances with higher constitutive chemical defences better tolerate the pathogen both in greenhouse conditions and in the field. These provenances show a higher probability of survival and also a better growth. In the southern provenances, some families with higher tolerance and, therefore, candidates for use in reforestation programs in areas infected by Pc, were identified.

Additionally, the ability of some herbs from Iberian wood pastures to reduce the Pc pathogen populations through allelopathic relationships was also studied in Chapter 4. Three herbaceous plants (*Diplotaxis tenuifolia* (L.) DC., *Eruca vesicaria* L. and *Raphanus raphanistrum* L.) and their aqueous root extracts (AREs) were analyzed *in vitro* conditions. Their inhibitory capacity was evaluated on mycelial growth and production of Pc reproductive structures. Finally, the effect of these herbaceous plants on seedlings of other two typical Mediterranean oaks (*Q. suber* and *Q. faginea*) with those grown in Pc-infested soil was evaluated *in vivo*. Results show a strong inhibitory capacity of AREs on Pc activity *in vitro* and a protective effect of these herb species on *Quercus* plants against Pc *in vivo*. *D. tenuifolia* would be especially suited for biological control in the pathogen spread.

These laboratory, greenhouse and field tests allowed us to identify some sources of holm oaks with high tolerance to the pathogen that could be used in future breeding programs. Furthermore, verifying that the constitutive condensed tannins of this species can be used as indicators of greater tolerance and that it is a heritable trait shows that improvement is possible. On the other hand, the finding of allelopathic effects of some Mediterranean herb species that can control the dispersion of the pathogen *in vitro* and reduce stress on cork oaks and gall oaks *in vivo* (greenhouse) opens a new study line focused on the specific composition of grasslands sown in Mediterranean agrosilvopastoral systems. The enrichment of these meadows with allelopathic species could reduce the effect of this stressor pathogen, considered key in the current worldwide decline of *Quercus*.

Resumen

Las plantas tienen diversos mecanismos para hacer frente a los enemigos naturales que puedan comprometer su supervivencia. Sin embargo, dado el escenario actual de condiciones cambiantes bióticas y abióticas, un mayor conocimiento de estos mecanismos es fundamental para afrontar retos futuros. La respuesta de las plantas y su capacidad para sobrevivir en estos nuevos escenarios será clave para la conservación de ecosistemas como las dehesas españolas y los montados portugueses.

Esta Tesis analiza la producción de defensas quimicas, principalmente fenólicas, en la interacción planta-estresor biótico para evaluar posibles diferencias geográficas e individuales. En el Capítulo 1 se analiza si los niveles constitutivos de fenoles totales (Tp) y taninos condensados (Ct) que actúan como defensas químicas en la encina (*Quercus ilex*) varían entre regiones, poblaciones y genotipos en la Península Ibérica. Se encontraron diferencias significativas en la concentración de Tp y Ct entre regiones y entre genotipos, pero no entre poblaciones dentro de regiones. Los niveles más bajos de defensas constitutivas se encontraron en el norte de la Península Ibérica. Además, el contenido fenólico defensivo fue significativamente mayor en regiones con suelos ácidos. Los valores de heredabilidad (Tp 0.37 ± 0.08 y Ct 0.48 ± 0.36) fueron inferiores a los obtenidos para taninos totales (Tt) en un estudio previo.

En Capítulo 2 se analizó la capacidad de las encinas de las seis procedencias ibéricas para responder a múltiples daños bióticos a través de sus patrones de defensas químicas inducidas. El estres biótico fue inducido a nivel de raíz (mediante la infección con el patógeno generalizado *Phytophthora cinnamomi* -Pc-) y en la parte aérea de la planta (por defoliación o tronchado mecánico). Más tarde se midieron los niveles constitutivos e inducidos de Tp, Tt y Ct. Los resultados mostraron que (1) los patrones químicos defensivos muestran una variación local y geográfica significativa; (2) la supervivencia al estrés está más relacionada con las defensas constitutivas que con las inducidas; (3) la respuesta inducida es específica del estresor; y (4) hay un efecto interactivo entre los factores estresantes y la inducción o inhibición de defensas químicas depende de la procedencia. Estos hallazgos sobre los efectos estresantes bióticos en la producción de defensas químicas y su relación con la supervivencia de la encina pueden

facilitar el desarrollo de programas de selección de material genético en el control integrado del declive generalizado de los *Quercus*.

Este decaimiento está íntimamente ligado a la presencia de Pc en el suelo. La amplia distribución de este patógeno del suelo y su exacerbación de los daños por el cambio climático en la región mediterránea plantean la necesidad de programas de mejora basados en el empleo de material resistente y tolerante a la enfermedad. El Capítulo 3 tiene como objetivo determinar si las encinas plantadas en cuatro parcelas de campo sobre suelos altamente infectados por Pc muestran diferencias en la mortalidad según sus procedencias al final de un seguimiento de cuatro años. También evalúa si las procedencias más tolerantes en el campo fueron aquellas mayores defensas químicas constitutivas medidas en el ensayo de invernadero anterior. En general, hubo una alta mortalidad (56.0-80.5%) pero las procedencias con mayores defensas químicas constitutivas toleraron mejor al patógeno tanto en condiciones de invernadero como en el campo. Estas procedencias muestran una mayor probabilidad de supervivencia y también un mejor crecimiento. En las procedencias del sur se identificaron algunas familias con mayor tolerancia y, por tanto, candidatas a ser utilizadas en programas de reforestación en áreas infectadas por Pc.

Por otro lado, en el Capítulo 4 se estudió la capacidad de algunas herbáceas de los pastos y formaciones arboladas ibéricas para reducir las poblaciones del patógeno Pc a través de relaciones alelopáticas. Se analizaron *in vitro* tres especies de herbáceas *(Diplotaxis tenuifolia* (L.) DC., *Eruca vesicaria* L. y *Raphanus raphanistrum* L.) y sus extractos acuosos radiculares (ARE). Se evaluó su capacidad inhibitoria sobre el crecimiento micelial y la producción de estructuras reproductivas de Pc. Finalmente, se analizó *in vivo* el efecto de estas plantas herbáceas sobre las plántulas de otros dos robles típicos mediterráneos (*Q. suber* y *Q. faginea*) cuando crecen junto a ellos en suelo infestado por Pc. Los resultados muestran una fuerte capacidad de los AREs para inactivar a Pc *in vitro* y un efecto protector de estas hérbaceas en las plántulas de *Quercus* frente a Pc *in vivo*. *D. tenuifolia* sería especialmente adecuada para el control biológico de la propagación del patógeno.

Estas pruebas de laboratorio, invernadero y campo nos permitieron identificar algunas procedencias y familias de encinas con alta tolerancia al patógeno que podrían ser utilizadas en futuros programas de mejora. Además, comprobar que los Ct constitutivos de esta especie pueden utilizarse como indicadores de mayor tolerancia y que su contenido es un rasgo hereditario demuestra que la mejora de la especie es posible. Asimismo, el hallazgo de efectos alelopáticos de algunas especies herbáceas mediterráneas que pueden controlar la dispersión del patógeno *in vitro* y reducir el estrés en alcornoques y quejigos *in vivo* (en condiciones de invernadero) abre una nueva línea de estudio centrada en la composición específica de pastizales sembrados en sistemas agrosilvopastorales mediterráneos. El enriquecimiento de estos pastos con especies alelopáticas podría reducir el efecto del patógeno, considerado clave en el actual decaimiento de los *Quercus*.

Resumo

As plantas possuem inúmeros e diversos mecanismos de defesa contra os inimigos naturais que podem comprometer a sua sobrevivência. No cenário atual de mudanças das condições bióticas e abióticas, o conhecimento aprofundado desses mecanismos é essencial para enfrentar futuros desafios. A resposta das plantas e a sua capacidade de sobreviver nesses novos cenários será a chave para a conservação dos ecossistemas como os montados portugueses e as dehesas espanholas.

Esta Tese analisa a produção de defesas químicas, principalmente fenólicas, na interação planta-indutor biótico de *stress* para avaliar possíveis diferenças geográficas e individuais. O primeiro capítulo analisa se os níveis constitutivos de fenóis totais (Tp) e taninos condensados (Ct) que atuam como defesas químicas na azinheira (*Quercus ilex*) variam entre regiões, populações e genotipos na Península Ibérica. Diferenças significativas foram encontradas na concentração de Tp e Ct entre as regiões e entre os genótipos, mas não entre as populações dentro das regiões. Os níveis mais baixos de defesas constitutivas foram encontrados no norte da Península Ibérica. Além disso, o conteúdo fenólico defensivo foi significativamente maior em regiões com solos ácidos. Os valores de herdabilidade (Tp 0.37 ± 0.08 e Ct 0.48 ± 0.36) foram inferiores aos obtidos com os taninos totais (Tt) num estudo anterior.

No segundo capítulo foi analisada a capacidade das azinheiras das seis proveniências ibéricas para responder a múltiplos danos bióticos através dos seus padrões de defesa química induzidos. O dano biótico foi induzido na raiz (através da infecção com o patogénio *Phytophthora cinnamomi* Rands -Pc) e na parte aérea (por desfolha mecânica). Posteriormente, foram medidos os níveis constitutivos e induzidos de Tp, Tt e Ct. Os resultados mostraram-que (1) os padrões químicos defensivos indicam variações locais e geográficas significativas; (2) a sobrevivência ao *stress* está mais associada às defesas constitutivas do que às induzidas; (3) a resposta induzida é específica para o indutor biótico de *stress*; e (4) existe um efeito interativo entre os indutores, e a indução ou inibição das defesas químicas depende da origem das plantas. Esses resultados sobre os efeitos bióticos dos indutores de stress na produção de defesas químicas, e a sua relação com a sobrevivência da azinheira, podem facilitar o desenvolvimento de programas de seleção de material genético no controle integrado do declínio de *Quercus*.

Considera-se que o declínio dos *Quercus* está intimamente ligada à presença de Pc no solo. A sua ampla distribuição no solo e o agravamento dos prejuízos causados pelas alterações climáticas na região do Mediterrâneo conduz à necessidade de se desenvolver programas de melhoramento baseados no uso de material resistente e tolerante à doença. O objetivo do terceiro capítulo é, ao final de quatro anos, determinar se as azinheiras plantadas em quatro parcelas de campo com solo muito infestado pelo patogénio apresentam diferenças na mortalidade, de acordo com a sua origem. O estudo avalia ainda se as procedências mais tolerantes em campo foram as que apresentam as defesas químicas constitutivas mais elevadas, avaliadas em estufa. Em geral, observou-se elevada mortalidade (56.0-80.5%). Contudo, as procedências com defesas químicas constitutivas mais elevadas, toleraram melhor o patogénio tanto em estufa como em campo. Essas procedências apresentam uma maior probabilidade de sobrevivência e também um melhor crescimento. Nas procedências do sul foram identificadas algumas famílias com maior tolerância e, portanto, candidatas a serem utilizadas em programas de reflorestamento em áreas infestadas por Pc.

Por outro lado, no quarto capítulo foi estudada a capacidade de algumas plantas herbáceas utilizadas em pastagens e presentes na vegetação natural em florestas ibéricas poderem reduzir a população do patogénio *Pc* através de relações alelopáticas. Assim, analisou-se *in vitro* o efeito inibitório de três espécies herbáceas (*Diplotaxis tenuifolia* (L.) DC., *Eruca vesicaria* L. e *Raphanus raphanistrum* L.) através dos seus extratos aquosos de raízes (ARE). Foi avaliada a capacidade inibitória dos AREs no crescimento micelial e na produção de estruturas reprodutivas do patogénio. Finalmente, foi avaliado *in vivo,* o efeito dessas plantas herbáceas em outros dois carvalhos típicos do Mediterrâneo (*Q. suber* e *Q. faginea*) quando cultivadas em simultâneo com os *Quercus* em solo infestado com Pc. Os resultados mostram, *in vitro,* uma forte capacidade das AREs para inativar o Pc e, *in vivo,* um efeito protetor dessas herbáceas nas jovens plantas de *Quercus* contra a infeção por Pc. A *D. tenuifolia* mostrou potencial para reduzir a actividade do patogénio e consequentemente a sua disseminação.

Esses testes de laboratório, estufa e campo permitiram identificar algumas procedências e famílias de azinheiras com alta tolerância ao patogénio que poderiam ser usadas em futuros programas de melhoramento. Além disso, verificar que os Ct constitutivos desta espécie podem ser usados como indicadores de maior tolerância e que o seu conteúdo é um traço hereditário mostra que, o melhoramento da espécie é possível.

Por outro lado, a constatação de efeitos alelopáticos de algumas espécies herbáceas mediterrânicas que podem controlar a dispersão do patogénio *in vitro* e reduzir o *stress* em sobreiro e carvalho-português *in vivo* (na estufa) abre uma nova linha de estudo centrada na composição específica de pastagens semeadas em sistemas agrossilvipastoris do Mediterrâneo. O enriquecimento dessas pastagens com espécies alelopáticas poderia reduzir o efeito do patogénio, considerado chave no atual declínio dos *Quercus*.

INTRODUCTION

INTRODUCTION

Plants have a wide variety of different mechanisms to defend themselves against external agents which could threaten their survival. Among these mechanisms, the chemical defence production is a field of prolific bibliography in which, however, many aspects are still unknown. The production of secondary metabolites is one of the main chemical defences. Secondary metabolites are organic compounds not essential for the life but that contribute to their survival. These compounds have a high specificity due to their coevolution with natural enemies in different environments (Freeman & Beattie 2008, Agrawal & Heil 2012). This specificity has been widely studied in their antagonistic interaction with herbivores. To prevent their damage, plants can vary the defensive response depending on their provenance and the impact of the damage (Thompson 2005, Becerra et al. 2009). In species of wide geographical distribution, the variability of the response is even greater and can affect the plant survival (Solla *et al.* 2014). However, geographical variation has been scarcely studied as a source of intraspecific variability in the chemical defence diversity (but see Solla *et al.* 2016, Moreira *et al.* 2018a, López-Goldar *et al.* 2019). Altitude and latitude are two of the geographical aspects that influence defensive variability across many plant taxa (Abdala-Roberts *et al.* 2016, Stevens *et al.* 2016, Moreira *et al.* 2017, Abdala-Roberts *et al.* 2018, Bogdziewicz *et al.* 2019, Moreira *et al.* 2019). A classic ecological paradigm argues that at low temperatures the herbivore pressure and also the production of chemical defences increase (Rasmann & Agrawal 2011, Pearse & Hipp 2012, Moreira *et al*. 2014, Anstett et al. 2016). However, some studies in the last decade have been published showing a positive relationship between latitude and chemical defence production (Stevens *et al.* 2016, Moreira *et al.* 2017, Moreira *et al.* 2018a). The greater the geographical distribution area of the species, the more likely it is that the climatic and edaphic conditions in which it develops (pH, moisture, temperature…) will be greater too. In fact, the effect that these factors have on the generation and variability of chemical defences in trees has been evaluated in various studies (Kraus *et al.* 2004, Salminen & Karonen 2011, Moreira *et al.* 2014, Abdala-Roberts *et al.* 2016, Abdala-Roberts *et al.* 2018).

Chemical defences in plants are divided into two groups according to the time period in which they occur: constitutive and induced defences. The constitutive ones are characteristic of the organism and correspond to plant defensive traits that are relatively stable or whose variation depends on internal factors (Schultz 1988). They are genetically controlled, show important individual and geographical differences (Dicke & Hilker 2003, Solla *et al.* 2016, Gallardo *et al.* 2019) and seem to be printed by phylogeographical signals (Eckert *et al.* 2008). Constitutive defence action prevents the stressor in the first place, while induced defence typically shortens the attack period (Boots & Best 2018). Both contribute to the plant survival. On the other hand, the plant response to the attack can vary among different possibilities (intolerance, tolerance and resistance). The plant is intolerant to stress when it dies from its action. If the plant shows symptoms but overcomes the attack, it is tolerant. If the stressor´s action does not harm the plant, it is resistant. Plant tolerance is understood as the "host's ability to reduce the stress factor damage” and increasing evidence indicates that tolerance is a host defence strategy against several pathogens as widespread and successful as resistance (Pagán & García-Arenal 2020). However, this mechanism is still quite unknown and difficult to distinguish experimentally from quantitative resistance, with which it is often confused. The susceptibility to the stressor shows evident interspecific and intraspecific differences.

This Thesis focuses on the geographical variation of chemical defences in three *Quercus* species and especially, in the holm oak (*Quercus ilex*), due to its wide genetic diversity and distribution. The holm oak is an evergreen oak well adapted to drought and distributed throughout the Mediterranean basin (Rodá *et al.* 2009). The main chemical defences in *Quercus* are phenolic compounds, especially high molecular weight tannins, both hydrolyzable (Ht, derived from gallic and ellagic acids) and condensed (Ct, also known as proanthocyanidins). These compounds play an important role as a defence mechanism against biotic stress in plants, although their role is controversial (Moctezuma *et al.* 2014). They are strong inhibitors of digestive proteases, which explains why they accumulate in the leaves, stems and roots of *Quercus* for their defence against herbivores (Haslam 2007, Moctezuma *et al.* 2014). In addition to their effect on herbivores, they also cause metabolic alterations in insects (Barbehenn *et al.* 2009), inhibit the activity of root pathogens (Oliva *et al.* 1999, Kraus *et al.* 2003, Baptist *et al.* 2008) and have a high antimicrobial activity (Scalbert 1991, Daglia 2012). Pre-infective levels of certain phenolic compounds allow the identification of trees resistant to other pathogens such as

Phytophthora ramorum in American oaks (Conrad *et al.* 2017), but this mechanism is still poorly understood in the Mediterranean context.

Oak stands are suffering a worldwide distributed decline related to the increase in average temperatures and the invasion of very aggressive invasive pathogens (Dukes *et al.* 2009, Santini *et al.* 2013, Pérez-Ramos *et al.* 2014). The increase in biotic stress driven by climate change and especially the invasion of oomycetes from the *Phytophthora* genus has been widely reported. Several *Phytophthora* species are associated with oak decline all over the world (Jung *et al.* 1996, Balci & Halmschlager 2003, Pérez-Sierra *et al.* 2013). In the Mediterranean Basin the soil pathogen *Phytophthora cinnamomi (Pc)* has been reported in several countries, including France, Portugal, Italy and Spain (Brasier *et al.* 1993, Robin *et al.* 1998, Sánchez *et al.* 2006, Corcobado *et al.* 2013, Jung *et al.* 2013, Scanu *et al.* 2013). Its distribution is expected to be favored by global warming, moving towards the poles (Burgess *et al.* 2017). The action of these pathogens, together with the lack of tree regeneration, are the main threats to the Mediterranean *Quercus* conservation (Moreno & Pulido 2009, Plieninger *et al.* 2010, Jorrín-Novo & Navarro-Cerrillo 2014).

In the Iberian Peninsula, and mostly within its southwestern area, some *Quercus* species such as holm oak (*Q. ilex*) and cork oak (*Q. suber*) are agrosystems known as “dehesas” in Spain and “montados” in Portugal. In areas of Atlantic influence with milder and more humid climate, these two species are also accompanied by the gall oak (*Q. faginea*). The stands of these species are a good example of "grazed wood pastures", common in the Mediterranean region (Den Herder *et al.* 2017). It is the main agrosilvopastoral system in Europe, with more than 4.5 million hectares in the Iberian Peninsula (Moreno & Pulido 2009). They have been exploited by humans throughout history and they constitute the economic base of much of the Western Iberian rural world (Escribano & Pulido 1998, Campos 2004, Pereira *et al.* 2004). In fact, these socio-ecological systems are considered by the European Union as High Natural and Cultural Value Systems (HNCV; Moreno *et al.* 2018).

Nevertheless, these Iberian agrosilvopastoral ecosystems are currently suffering an increasing decline with serious impact on oak species due to several factors, among them, those generated by biotic stressors. Besides the natural herbivory, livestock grazing also occurs, with densities that sometimes exceed the land´s carrying capacity (García *et al.*

2010). Several works have also studied the incidence of defoliation exerted by numerous insects on *Quercus*, many of which are prone to population outbreaks (Humphrey & Swaine 1997, Solla *et al.* 2016, Tiberi *et al.* 2016). Generally, defoliation negatively affects the holm oak growth (Schmid *et al*. 2017) and compromises its regeneration (Carbonero *et al.* 2004, Olmo *et al*. 2017). In recent decades, another stress factor adds to the aforementioned intense defoliation: several pathogen infections, among which *Pc* is the primary causal agent of the *Quercus* decline in the Iberian Peninsula (Brasier 1992, 1996, Sánchez *et al.* 2006, Corcobado *et al*. 2014). This root rotting oomycete is considered one of the 100 worst invasive alien species on Earth (Lowe *et al.* 2000). It is a soil-borne pathogen que affects more than 5000 trees, shrubs and herbs species in the world (Hardham & Blackman 2018) and causes root rot and death of several *Quercus* species. Its eradication from the soil in field is very complex due to the durability of its resistance structures and the easy spread by different pathways (but see Dunstan *et al.* 2010). Today, it is assumed that the control is only possible from an integrated perspective given its wide dispersion and high range of hosts. Especially susceptible are holm oak and cork oak, which are main tree components of the Iberian wood pastures. Its mitigation in dehesas is a high priority task that requires isolation of affected areas to avoid pathogen spread, elicit the vigor of healthy plants and, in the long term, selection and implementation of resistant plants (Camilo-Alves *et al.* 2013). It is expected that the combination of several biotic stress factors will further weaken these forests, already aged (Plieninger *et al.* 2010). Although the importance of its effects varies between regions, over time, the persistence of forests and dehesas could be at risk, with direct negative implications on resilience and associated biodiversity (Pulido *et al.* 2001, Plieninger *et al.* 2015, Duque-Lazo *et al.* 2018). However, and despite the fast advance of *Quercus* decline, the differences among individuals, populations and provenances in susceptibility to the involved biotic stressors have hardly been studied. Recent studies have addressed the analysis of the constitutive chemical defences of some species of the *Quercus* genus (Abdala-Roberts *et al.* 2018) and the geographical variation in the defences of another common oak in Central Europe, *Q. robur* (Moreira *et al.* 2018a). But very few studies have addressed these defensive differences in Mediterranean *Qu*ercus (but see Solla *et al.* 2016), despite the high value and socioeconomic importance of these species for the rural world.

To date, this Thesis is the first considering the combined attack at the root and aerial level and their possible interactions in the constitutive and induced chemical defences of *Q. ilex*. Studies focused on the effects of combined biotic stress on tree chemical defences and their geographic variability are still scarce. The tolerance to biotic stress will depend, among other factors, on the genes involved in the production of these chemical defences (Gallardo *et al.* 2019). Then, it is expected the existence of different levels of tolerance associated with genetic polymorphism and local adaptation processes (Sork *et al.* 1993, Pautasso *et al.* 2012). The high genetic diversity of the holm oak, in addition to its wide distribution, a priori facilitates the existence of different patterns of chemical response whose heritability has been assessed (Solla *et al.* 2016). Furthermore, the natural selection, genetic drift and local adaptation favor intraspecific diversity in tolerance to stressors (Eguiarte *et al*. 2013), facilitating the occurrence of tolerant and even resistant individuals to certain stressors, which could be very useful in genetic improvement programs. This diversity in the response of plants to stress-causing agents allows the selection of those individuals, populations and/or provenances with greater tolerance. Forest managers need this plant material to regenerate areas seriously affected by pests and diseases or with difficulties for natural regeneration, among others. To achieve this, it is essential to study the chemical pattern of defences in the species and identify later what compounds could grant greater tolerance. Advances in this field are needed to develop selection programs of forest material for reproduction, because of their involvement in the adaptation and response to the environmental stress factors and, therefore, against future scenarios in changing environmental conditions (Isabel *et al.* 2019). Tests of geographical variation in susceptibility to the stressor or stressors are very useful to find them. But stressor tolerance experiments are often carried out under very controlled conditions and even in greenhouses, therefore, very far from the natural environments affected by various combined stressors.

Previous works under greenhouse conditions yielded high mortality rates but also revealed the existence of more tolerant provenances and genotypes against *Pc* (Cuenca *et al.* 2018, León 2013). León (2013) and Cuenca *et al.* (2018) found a high variability in the genotype response to *Pc* infection. Cuenca *et al.* (2018) also demonstrated that the tolerance of holm oak to infection by *Pc* –and also to water stress- is genetically controlled and, therefore, susceptible to improvement. However, these results are always under controlled greenhouse conditions and with short-term experiments, so they should be treated with caution. A study carried out in the field with cork oak (Moreira *et al.* 2018b) determine the existence of some genotypes tolerant to the disease and capable of growing in infected areas ten years after planting. However, no published results of tolerant holm oaks from different provenances to the pathogen in the field are known. Detected the need for field experiments to assess the geographical and local variability in the defensive response of holm oaks, this Thesis included a four-year field experiment on areas naturally affected by the root pathogen Pc, in addition to tests of laboratory and greenhouse.

But in addition to the defensive response of the holm oak against its stressors, companion species of oaks in the Mediterranean wood pastures can favor or harm the effect of stressors by allelopathic relationships. Plants can influence the composition of microbial communities around their roots through the exudation of carbohydrates and other chemical compounds. Allelopathy is a naturally occurring ecological phenomenon of interference among organisms by which a plant produces one or more biochemical compounds that influence the development of other organisms (bacteria, fungi or other plants), either negatively or positively (Molisch 1937, Rice 1984, Farooq *et al.* 2011). Some exudates present bactericidal and fungicidal activity and can affect the growth, survival and/or reproduction of various microorganisms and among them, some oak stressors. The allelochemicals may be located in different parts of the plant, such as leaves, branches or roots (Ferguson *et al.* 2013). They are usually released directly into the aqueous phase of the soil, or from volatile gaseous substances in the surrounding air (Rizvi *et al.* 1992). The allelochemical release is influenced by the soil, climatic conditions and the plant itself (Singh *et al.* 2001).

In addition to the susceptible holm oak and cork oak mentioned above, the gall oak (*Q. faginea*) is also susceptible facing *Pc*, although less than the first two (Rodríguez-Molina *et al.* 2002, Sánchez *et al.* 2005, Pérez-Sierra *et al.* 2013). On the other hand, there are species that instead of being susceptible to the pathogen, show resistance to it. The release of their allelochemicals can even inhibit the infective capacity of some pathogens (Kong *et al.* 2019). In the Mediterranean flora there are various species with allelopathic effects against *Pc* (Sampaio 2017, Ríos *et al.* 2016a 2016b, Moreira *et al.* 2018c, Rodríguez-Molina *et al.* 2018). Their allelopathic property can be observed in plants used for biofumigation such as *Brassica carinata* and *B. juncea* (Ríos *et al.* 2016a 2016b, Rodríguez-Molina *et al.* 2018) and in other native flora still under study (Ríos *et al.* 2016a, Sampaio 2017, Moreira *et al.* 2018c). The family to which they all belong, *Brassicaceae*, stands out for its high concentration of glucosinolates and its fungicidal effect in certain concentrations and conditions (Fenwick *et al.* 1983, Dunne 2004, Ríos *et al.* 2016a). Glucosinolates are sulfur compounds with proven fungicidal and biocidal capacity (Bialy *et al.* 1990, Wolf *et al.* 1984). Their defensive properties are generated by an enzymatic hydrolysis that releases volatile compounds, among which are isothiocyanates, nitriles, thiocyanates and oxazolidines, depending on the structure of the hydrolyzed glucosinolate (Fahey *et al.* 2001). The studies by Sampaio (2017) and Moreira *et al.* (2018c) offered interesting screening of herbaceous species from the rangeland's native flora with potential allelopathic effect facing *Pc* that this Thesis also addresses, in order to characterize *in vitro* its inhibitory ability on *Pc*. Finally, the defensive chemical response of *Q. faginea* and *Q. suber* to *Pc* stressor was also analysed, evaluating the possible protection of allelopathic herbaceous plants *in vivo* as a biological control tool within the integrated fight against oak decline. Among the different management strategies against diseases, biological control still has an underdeveloped alternative, but with certain advances in agricultural systems that could be the basis for the development of a forestry strategy (FAO, 2001). But to achieve this, a deeper understanding of plant-pathogen interactions and the possible allelopathic relationships among them is essential.

Objectives

The overall aim of this Thesis is, therefore, to deepen the knowledge of the chemical defences of some Mediterranean oaks to facilitate an improvement strategy and defence of the agrosilvopastoral systems that they make up. And thus, contribute to their conservation facing the growing biotic stressors that threaten them. By combining laboratory, greenhouse and field tests with analytical chemical techniques and forest pathology work, this Thesis addresses geographical and individual diversity in the defensive response to some biotic stressors to support the selection of tolerant families that can be used in restoration of affected areas. In addition, the identification of allelopathic species in the field is carried out to strengthen this restoration strategy, adding to the use of tolerant plant material, the chance of taking advantage of the allelopathic relationships of some herb plants in Mediterranean wood pastures to reduce the infective capacity of the pathogen *Pc*, a biotic stressor associated with oak decline. Specifically, the main objectives of this Thesis were to:

1. Study the intraspecific variation of secondary metabolites depending on the different sources of genetic material in holm oak (*Q. ilex*)
2. To analyse the defensive chemical response to two types of biotic stress in holm oak (*Q. ilex*): infection of the root pathogen *Pc* and mechanical defoliation.
3. Field verification of the results obtained under controlled greenhouse conditions. Identification of families tolerant to root rot disease.
4. Analyse the allelopathic effect of some herb plants on the induced defences of other Mediterranean *Quercus* infected by *Pc* (*Q. suber* and *Q. faginea*).

References

Abdala-Roberts L, Moreira X, Rasmann S, Parra-Tabla V, Mooney KA (2016). Test of biotic and abiotic correlates of latitudinal variation in defenses in the perennial herb *Ruellia nudiflora*. J Ecol 104: 580-590.

Abdala-Roberts L, Galmán A, Petry W, Covelo F, De la Fuente M, Glauser G, Moreira X (2018). Interspecific variation in leaf functional and defensive traits in oak species and its underlying climatic drivers. PloS One 13(8) e0202548.

Agrawal AA & Heil M (2012). Synthesizing specificity: multiple approaches to understanding the attack and defense of plants. Trends Plant Sci 17: 239-242.

Anstett DN, Nunes KA, Baskett C, Kotanen PM (2016). Sources of controversy surrounding latitudinal patterns in herbivory and defense. Trends Ecol Evol 31: 789–802.

Balci Y & Halmschlager E (2003). Incidence of *Phytophthora* species in oak forests in Austria and their possible involvement in oak decline. Forest Pathol 33:157–174.

Baptist F, Zinger L, Clement JC, Gallet C, Guillemin R, Martins JM, Sage L, Shahnavaz B, Choler Ph, Geremia R (2008). Tannin impacts on microbial diversity and the functioning of alpine soils: a multidisciplinary approach. Environ Microbiol 10:99–809.

Barbehenn RV, Jaros A, Lee G, Mozola C, Weir Q, Salminen JP (2009). Tree resistance to *Lymantria dispar* caterpillars: importance and limitations of foliar tannin composition. Oecologia. 159: 777-788.

Becerra JX, Noge K, Venable DL (2009). Macroevolutionary chemical escalation in an ancient plant-herbivore arms race. Proc Natl Acad Sci USA 106:18062–6.

Bialy Z, Oleszek W, Lewis J, Fenwick GR (1990). Allelopathic potential of glucosinolates (mustard oil glycosides) and their degradation products against wheat. Plant and Soil 129: 277-281.

Bogdziewicz M, Espelta JM, Bonal R (2019). Tolerance to seed predation mediated by seed size increases at lower latitudes in a Mediterranean oak. Annals of Botany 123: 707-714.

Boots M & Best A (2018). The evolution of constitutive and induced defences to infectious disease. Proceedings of the Royal Society B: Biological Sciences 285(1883), 20180658.

Brasier CM (1992). Oak tree mortality in Iberia. Nature 360, 539.

Brasier CM (1996). *Phytophthora cinnamomi* and oak decline in Southern Europe. Environmental constraints, including climate change. Ann For Sci 53: 347-358.

Brasier CM, Robredo F, Ferraz JFP (1993). Evidence for *Phytophthora cinnamomi* involvement in Iberian oak decline. Plant Pathol 42:140–145.

Burgess TI, Scott JK, McDougall KL, Ota N, Hardy GESJ (2017). Current and projected global distribution of *Phytophthora cinnamomi*, one of the world's worst plant pathogens. Global Change Biology 23(4): 1661-1674.

Camilo-Alves CSP, Da Clara MIE, Ribeiro NA (2013). Decline of Mediterranean oak trees and its association with *Phytophthora cinnamomi*: a review. Eur J For Res 132:411–432.

Campos P (2004). Towards a sustainable global economics approach for Mediterranean agroforestry systems. In: Schnabel, S., Ferreira, A. (eds.) Sustainability of agrosilvopastoral systems. Advances in GeoEcology, vol 37. Catena Verlag, Reiskirchen, Germany, pp 13–28.

Carbonero MD, Blázquez A, Fernández-Rebollo P (2004). Acorn production and defoliation as vigour signs in *Quercus* spp. Connections with edaphic conditions. In: Reunión Científica de la Sociedad Española para el Estudio de los Pastos, 44. Salamanca (España).

Conrad AO, McPherson BA, Wood DL, Madden LV, Bonello P (2017). Constitutive phenolic biomarkers identify naïve *Quercus agrifolia* resistant to *Phytophthora ramorum*, the causal agent of sudden oak death. Tree Physiol 26: 1–11.

Corcobado T, Solla A, Madeira MA, Moreno G (2013). Combined effects of soil properties and *Phytophthora cinnamomi* infections on *Quercus ilex* decline. Plant and Soil 373: 403-413.

Corcobado T, Cubera E, Juarez E, Moreno G, Solla A (2014). Drought events determine performance of *Quercus ilex* seedlings and increase their susceptibility to *Phytophthora cinnamomi.* Agric For Meteorol 192-193:1–8.

Cuenca B, Dorado FJ, Camisón A, Luquero L, Ocaña L, Solla A (2018). Selección de brinzales de *Quercus suber* y *Q. ilex* tolerantes a *Phytophthora cinnamomi* y al estrés hídrico. En: Libro de Resúmenes XIX Congreso de la Sociedad Española de Fitopatología, Toledo (España).

Daglia M (2012). Polyphenols as antimicrobial agents. Curr. Opin. Biotechnol 23(2):174-181.

Den Herder M, Moreno G, Mosquera-Losada RM, Palma JH, Sidiropoulou A, Freijanes JJS, Crous-Duran J, Paulo JA, Tomé M, Pantera A, Mantzanas K, Pachana P, Papadopoulos A, Plieninger T, Burgess PJ (2017). Current extent and stratification of agroforestry in the European Union. Agr Ecosyst Environ 241: 121-132.

Dicke M & Hilker M (2003). Induced plant defences: from molecular biology to evolutionary ecology. Basic & Appl Ecol 14: 3–14.

Dukes JS, Pontius J, Orwig D, Garnas JR, Rodgers VL, Brazee N, Cooke B, Theoharides KA, Stange EE, Harringto R, Ehrenfeld J, Gurevitch J, Lerdau M, Stinson K, Wick R, Ayres M (2009). Responses of insect pests, pathogens, and invasive plant species to climate change in the forests of northeastern North America: what can we predict? Can J For Res 39:231–248.

Dunne C (2004). Control of sudden death in cultivated proteas from the South west of Western Australia. PhD Thesis. Murdock University, Australia.

Dunstan WA, Rudman T, Shearer BL, Moore NA, Paap T, Calver MC, Dell B, Hardy GESJ (2010). Containment and spot eradication of a highly destructive, invasive plant pathogen (*Phytophthora cinnamomi*) in natural ecosystems. Biological Invasions, 12, 913–925.

Duque-Lazo J, Navarro-Cerrillo RM, Van Gils H, Groen TA (2018). Forecasting oak decline caused by *Phytophthora cinnamomi* in Andalusia: Identification of priority areas for intervention. Forest Ecology and Management 417, pp. 122-136.

Eckert CG, Samis KE, Lougheed SC (2008). Genetic variation across species' geographical ranges: the central-marginal hypothesis and beyond. Mol Ecol 17:1170–88.

Eguiarte LE, Aguirre-Liguori JA, Jardón-Barbolla L, Aguirre-Planter E, Souza V (2013). Genómica de poblaciones: nada en evolución va a tener sentido si no es a la luz de la genómica y nada en genómica tendrá sentido si no es a la luz de la evolución. Rev Esp Cienc Quim Biol 16(1).

Escribano M & Pulido FJ (1998). La dehesa en Extremadura. Estructura económica y recursos naturales. SIDT Junta de Extremadura, Mérida.

Fahey JW, Zalcmann AT, Talalay P (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry, 56: 5-51.

FAO (2001). Protecting plantations from pests and diseases. Report based on the work of W.M. Ciesla. Forest Plantation Thematic Papers, Working Paper 10. Forest Resources Development Service, Forest Resources Division. FAO, Rome. (http://www.fao.org/forestry)

Farooq M, Jabran K, Cheema ZA, Wahid A, Siddique KH (2011). The role of allelopathy in agricultural pest management. Pest Management Science 67(5): 493-506.

Fenwick RG, Heaney RK, Mullin WJ (1983). Glucosinolates and their breakdown products in food plants. CRC. Crit Rev Food Sci Nutr 18:123-201.

Ferguson JJ, Rathinasabapathi B, Chase CA (2013). Allelopathy: How plants suppress other plants. University of Florida, 1–5. Retrieved from http://edis.ifas.ufl.edu/hs186

Freeman BC, Beattie GA (2008). An overview of plant defenses against pathogens and herbivores. The Plant Health Instructor.

Gallardo A, Morcuende D, Solla A, Moreno G, Pulido FJ, Quesada A (2019). Regulation by biotic stress of tannins biosynthesis in *Quercus ilex*: crosstalk between defoliation and *Phytophthora cinnamomi* infection. Physiol Plant 165: 319-329.

García A, Perea J, Acero R, Angón E, Toro P, Rodríguez V, Castro AGG (2010). Structural characterization of extensive farms in Andalusian dehesas. Archivos de zootecnia 59(228):577-588.

Hardham AR, Blackman LM (2018). *Phytophthora cinnamomi*. Mol Plant Pathol. 19(2):260-285.

Haslam E (2007). Vegetable tannins-lessons of a pythochemical lifetime. Phytochemistry 68: 2713-2721.

Humphrey J & Swaine M (1997). Factors affecting the natural regeneration of *Quercus* in Scottish oakwoods. Insect defoliation of trees and seedlings. J Appl Ecol 34(3): 585-593

Isabel N, Holliday JA, Aitken SN (2019). Forest genomics: advancing climate adaptation, forest health, productivity and conservation. Evol Appl 13: 3-10.

Jorrín-Novo J, Navarro-Cerrillo RM (2014). Variabilidad y respuesta a distintos estreses en poblaciones de encina (*Quercus ilex* L.) en Andalucía mediante una aproximación proteómica. Ecosistemas 23(2):99-107

Jung T, Blaschke H, Neumann P (1996). Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. Eur J Forest Pathol 26:253–272.

Jung T, Vettraino AM, Cech T, Vannini A (2013). The impact of invasive *Phytophthora* species in European forests 146–158 in Lamour, K. (ed.) *Phytophthora*: A global perspective. CABI international, Wallingford, UK.

Kong CH, Xuan TD, Khanh TD, Tran HD, Trung NT (2019). Allelochemicals and signaling chemicals in plants. Molecules 24(15): 27-37.

Kraus TEC, Dahlgren EC, Zasoski RJ (2003). Tannins in nutrient dynamics of forest ecosystems - a review. Plant and Soil 256: 41–66.

Kraus TEC, Zasoski RJ, Dahlgren RA (2004). Fertility and pH effects on polyphenol and condensed tannin concentrations in foliage and roots. Plant and Soil 262: 95-109.

León IM (2013). Selección de progenies de encina (*Quercus ilex* L. spp. ballota) y alcornoque (*Quercus suber* L.) tolerantes al patógeno *Phytophthora cinnamomi*. Universidad de Huelva, Tesis Doctoral.

López-Goldar X, Villari C, Bonello P, Borg-Karlson AK, Grivet D, Sampedro L, Zas R (2019). Genetic variation in the constitutive defensive metabolome and its inducibility are geographically structured and largely determined by demographic processes in maritime pine. J. Ecol. 107, 2464–2477.

Lowe S, Browne M, Boudjelas S, De Poorter M (2000). 100 of the World´s Worst Invasive Alien Species. A selection from the Global Invasive Species Database. The IUCN Invasive Species Specialist Group (ISSG). IUCN. 12 pp.

Moctezuma C, Hammerbacher A, Heil M, Gershenzon J, Méndez-Alonzo R, Oyama K (2014). Specific polyphenols and tannins are associated with defense against insect herbivores in the tropical oak *Quercus oleoides*. J Chem Ecol 40:458–467.

Molisch H (1937). Der Einfluss einer Pflanze auf die andere Allelopathie. Jena, Fischer.

Moreira X, Mooney KA, Rasmann S, Petry WK, Carrillo-Gavilan A, Zas R, Sampedro L (2014). Trade-offs between constitutive and induced defences drive geographical and climatic clines in pine chemical defences. Ecol Lett 17 (5): 537– 546.

Moreira X, Glauser G, Abdala-Roberts L (2017). Interactive effects of plant neighbourhood and ontogeny on insect herbivory and plant defensive traits. Sci Rep 7: 4047.

Moreira X, Castagneyrol B, Abdala-Roberts L, Berny-Mier y Teran JC, Timmermans BGH, Bruun HH, Covelo F, Glauser G, Rasmann S, Tack AJM (2018a). Latitudinal variation in plant chemical defences drives latitudinal patterns of leaf herbivory. Ecography 41 1124–1134.

Moreira X, Abdala-Roberts L, Henrik-Bruun H, Covelo F, De Frenne P, Galmán A, Gaytán A, Jaatinen R, Ten Hoopen JPJG, Pulkkinen P *(*2019). Latitudinal variation in seed predation correlates with latitudinal variation in seed defensive and nutritional traits in a widespread oak species. Ann Bot mcz207.

Moreira AC, Tapias R, Fernandes L, Rodriguez A (2018b). Field susceptibility of cork oak trees with different provenances to *Phytophthora cinnamomi*. Forest Pathology 48(5) e12461.

Moreira A, Calha I, Passarinho JA, Sampaio A (2018c). Anti-P*hytophthora* activity of root extracts from herbaceous species. Revista das Ciências Agrárias 41 (Especial): 39-47.

Moreno G & Pulido FJ (2009). The functioning, management, and persistente of dehesas. In: Agroforestry Systems in Europe. Current Status and Future prospects. Riguero-Rodriguez A, Mosquera-Losada MR, McAdam J. (eds.). Advances in Agroforestry Series, Springer Publishers pp 127-161.

Moreno G, Aviron S, Berg S, Crous-Duran J, Franca A, de Jalón SG, Hartel T, Mirck J, Pantera A, Palma JHN, Paulo JA, Re GA, Sanna F, Thenail C, Varga A, Viaud V, Burgess PJ (2018). Agroforestry systems of high nature and cultural value in Europe: provision of commercial goods and other ecosystem services. Agrofor syst 92(4): 877-891.

Oliva A, Lahoz E, Contillo R, Aliota G (1999). Fungistatic activity of *Ruta graveolens* extract and its allelochemicals. Chem Ecol 25: 519-526.

Olmo M, Andicoberry S, García-Moreno AM, Caño AB, Gómez-Giráldez PJ, Carbonero MD, Zamora-Rojas E, Fernández-Rebollo P, Villar R, Guerrero-Ginel JE (2017). Producción de bellota y defoliación en el arbolado de las dehesas del proyecto Life+bioDehesa. In: 7º Congreso Forestal Español. Plasencia (España).

Pagán I & García-Arenal F (2020). Tolerance of plants to pathogens: a unifying view. Annual Review of Phytopathology 010820-012749.

Pautasso M, Doring TF, Garbelotto M, Pellis L, Jeger MJ (2012). Impacts of climate change on plant diseases-opinions and trends. Eur J Plant Pathol 133:295-313.

Pearse IS, Hipp AL, 2012. Global patterns of leaf defences in oak species. Evol 66: 2272– 2286.

Pereira HM, Domingos T, Vicente L (Eds.) (2004). Portugal Millennium Ecosystem Assessment: State of the Assessment Report. Centro de Biologia Ambiental de la Faculdade de Ciências, Universidade de Lisboa.

Pérez-Ramos IM (2014). El milagro de regenerar en especies mediterráneas de *Quercus* ¿Cómo serán los bosques del futuro? Ecosistemas 23(2):13-17.

Pérez-Sierra A, López-García C, León M, García-Jiménez J, Abad-Campos P, Jung T (2013). Previously unrecorded low-temperature *Phytophthora* species associated with *Quercus* decline in a Mediterranean forest in eastern Spain. For Pathol 43:331–339.

Plieninger T, Rolo V, Moreno G (2010). Large-scale patterns of *Quercus ilex, Quercus suber* and *Quercus pyrenaica* regeneration in Central-Western Spain. Ecosystems 13: 644-660.

Plieninger T, Hartel T, Martín-López B, Beaufoy G, Bergmeier E, Kirby K, Montero MJ, Moreno G, Otero-Rozas E, Van Uytyanck J (2015). Wood-pastures of Europe: Geographic coverage, social–ecological values, conservation management and policy implications. Biol Conserv 190: 70-79.

Pulido F, Díaz M, Hidalgo de Trucios S (2001). Size structure and regeneration of Spanish holm oak *Quercus ilex* forests and dehesas: effects of agroforestry use on their long-term sustainability. Forest Ecol Manag 146: 1-13.

Rasmann S & Agrawal AA (2011). Latitudinal patterns in plant defence: evolution of cardenolides, their toxicity, and induction following herbivory. Ecol Lett 14: 476– 483.

Rice EL (1984). Allelopathy. 2nd ed., New York, Academic Press.

Ríos P, Obregón S, González M, de Haro A, Sánchez ME (2016a). Screening brassicaceous plants as biofumigants for management of *Phytophthora cinnamomi* oak disease. For Path 46(6): 652-659.

Ríos P, Obregón S, Haro A, Fernández-Rebollo P, Serrano M, Sánchez ME (2016b). Effect of *Brassica* biofumigant amendments on different stages of the life cycle of *Phytophthora cinnamomi*. Journal of Phytopathology 164 (9): 582-594.

Rizvi SJH, Haque H, Singh UK, Rizvi V (1992). A discipline called allelopathy. In: Rizvi SJH, Rizvi H (Eds.). Allelopathy: Basic and applied aspects. London, Chapman & Hall, p.1-10.

Robin C, Desprez-Loustau M, Capron G, Delatour C (1998). First record of *Phytophthora cinnamomi* on cork and holm oaks in France and evidence of pathogenicity. Ann Sci For 55(8):869–883.

Roda F, Vayreda J, Ninyerola M (2009). 9340 Encinares de *Quercus ilex* y *Quercus rotundifolia*. En: VV.AA., Bases ecológicas preliminares para la conservación de los tipos de hábitat de interés comunitario en España. Madrid: Ministerio de Medio Ambiente, y Medio Rural y Marino. 94 p.

Rodríguez-Molina MC, Serrano-Pérez P, Santiago R, Cardillo E, Godoy B, De Santiago A (2018). Efecto de la biofumigación con pellets de *Brassica carinata* en la progresión de la enfermedad causada por *Phytophthora cinnamomi* en *Lupinus luteus*. En Libro de Resúmenes XIX Congreso de la Sociedad Española de Fitopatología, Toledo (España).

Rodríguez-Molina MC, Torres-Vila LM, Blanco-Santos A, Nunez EJP, Torres-Álvarez E (2002). Viability of holm and cork oak seedlings form acorns sown in soils naturally infected with *Phytophthora cinnamomi*. Forest Pathology, 32(6): 365-372.

Salminen JP & Karonen M (2011). Chemical ecology of tannins and other phenolics: we need a change in approach. Funct Ecol. 25:325–338.

Sampaio AR (2017). Selecção de plantas com efeito alelopático para controlar *Phytophthora cinnamomi* Rands. Dissertação para obtenção do grau de Mestre em Engenharia Agronómica-Protecção de Plantas. ISA/UL, 71 pp.

Sánchez ME, Andicoberry S, Trapero A (2005). Pathogenicity of three *Phytophthora* spp. causing late seedling rot of *Quercus ilex* ssp. *ballota*. Forest Pathology, 35(2): 327-334.

Sánchez ME, Caetano P, Romero MA, Navarro RM, Trapero A (2006). *Phytophthora* root rot as the main factor of oak decline in southern Spain. In: Brasier C, Jung T, Oßwald W, eds. Progress in Research on Phytophthora Diseases of Forest Trees. Farnham, Surrey, UK: Forest Research, 149-54.

Santini A, Ghelardini L, De Pace C, Desprez-Loustau ML, Capretti P, Chandelier A, Cech T, Chira D, Diamandis S, Gaitniekis T, Hantula J, Holdenrieder O, Jankovsky L, Jung T, Jurc D, Kirisits T, Kunca A, Lygis V, Malecka M, Marcais B, Schmitz S, Schumacher J, Solheim H, Solla A, Szabó I, Tsopelas P, Vannini A, Vetrraino AM, Webber J, Woodward S, Stenlid J (2013). Biogeographical patterns and determinants of invasion by forest pathogens in Europe. New Phytol 197(1): 238-250.

Scalbert A (1991). Antimicrobial properties of tannins. Phytochemistry 30: 3875–3883.

Scanu B, Linaldeddu BT, Franceschini A, Anselmi N, Vannini A, Vettraino AM (2013). Occurrence of *Phytophthora cinnamomi* in cork oak forests in Italy. For Pathol 438(4): 340–343.

Schmid S, Palacio S, Hoch G (2017). Growth reduction after defoliation is independent of CO_2 supply in deciduous and evergreen young oaks. New Phytol 214(4): 1479-1490.

Schultz J (1988). Plant responses to herbivores. Trends Ecol Evol 3: 45:49.

Singh H, Batish D, Hohli R (2001). Allelopathy in agroecosystems: an overview. The Haworth Press, Inc, 1-42.

Solla A, López JC, Martín JA, Gil LA (2014). Genetic variation and heritability estimates of *Ulmus minor* and *Ulmus pumila* hybrids for budburst, growth and tolerance to *Ophiostoma novo-ulmi*. "Iforest", v. 8; pp. 422-430.

Solla A, Slobodan M, Gallardo A, Bueno A, Corcobado T, Cáceres Y, Morcuende D, Quesada A, Moreno G, Pulido FJ (2016). Genetic determination of tannins and herbivore resistance in *Quercus ilex*. Tree Genet Genom 28: 743–751.

Sork VL, Stowe KA, Hochwender C (1993). Evidence for local adaptation in closely adjacent subpopulations of northern red oak (*Quercus rubra* L.) expressed as resistance to leaf herbivores. American Naturalist 142:928–936.

Stevens MT, Brown SC, Bothwell HM, Bryant JP (2016). Biogeography of Alaska paper birch (*Betula neoalaskana*): latitudinal patterns in chemical defense and plant architecture. Ecology 97: 494–502.

Thompson JN (2005). The Geographic Mosaic of Coevolution. Chicago: University of Chicago Press.

Tiberi R, Branco M, Bracalini M, Croci F, Panzavolta T (2016). Cork oak pests: a review of insect damage and management. Ann For Sci 73:219-232.

Wolf RB, Spencer GF, Kwolek WF (1984). Inhibition of velvet leaf (*Abutilon thephrasti*) germination and growth by benzyl isothiocyanate, a natural toxicant. Weed Science 32: 612–615.

CHAPTER 1

Geographical and within-population variation of constitutive chemical defences in a Mediterranean oak (*Quercus ilex*)

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Published in Forest Systems 29(1), e011, 11 pages (2020)

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Abstract

Aim of study: to assess whether constitutive levels of total phenols and condensed tannins acting as chemical defences in *Quercus ilex* vary across regions, populations and genotypes.

Area of study: acorns from six Spanish regions with natural presence of *Q. ilex* were collected for later sowing in the greenhouse of the University of Extremadura at Plasencia, western Spain.

Materials and methods: 1176 acorns from 12 populations (2 per region) were sown according to a nested design (6 regions x 2 populations/region x 7 genotypes/population). After five months, 588 emerged seedlings were harvested for phenol extraction from their leaves. Quantification of total phenols through the Folin-Ciocalteu method and condensed tannins by Porter's reagent method was carried out.

Main results: total phenolics and condensed tannins correlated positively. Significant differences were found for the concentration of total phenolics and condensed tannins among regions and among genotypes, but not among populations within regions. The lowest levels of constitutive defences were found in the northern Iberian Peninsula. Also, the defensive phenolic content was significantly higher in regions with acidic soils. Heritability values (total phenols 0.37±0.08 and condensed tannins 0.48±0.36) were lower than those obtained of total tannins in a previous study.

Research highlights: constitutive levels of heritable chemical defences in holm oak significantly depend on their geographical origin and genotype.

Introduction

Chemical defences in plants have a high specificity due to their coevolution with natural enemies in different environments (Freeman & Beattie, 2008; Agrawal & Heil, 2012). This chemical diversity has been extensively studied in antagonistic interactions with herbivores. These organisms can vary the defensive response of plants according to their origin and impact (Thompson, 2005; Becerra *et al*., 2009). In addition, the pattern of constitutive chemical defences seems to be printed by phylogeographical signals (Eckert *et al.,* 2008).

In species with wide geographical distribution this diversity is even greater and its variability in response to different sources of stress affects plant survival (Solla *et al*., 2014; Ivetić *et al*., 2016, 2017; Martín *et al*., 2019). However, and despite its importance, geographical variation has been barely studied as a source of intraspecific variability of chemical defences (but see Solla *et al*., 2016; Moreira *et al*., 2018; López-Goldar *et al.,* 2019). Geographical factors such as latitude or altitude have been shown to influence this defensive diversity across many plant taxa (Abdala-Roberts *et al.,* 2016; Stevens *et al.,* 2016; Moreira *et al.,* 2017; Abdala-Roberts *et al*., 2018; Bogdziewicz *et al.,* 2019; Moreira *et al.,* 2019). Regarding latitude, a classic paradigm in ecology argues that at lower latitudes the herbivore pressure and also the production of plant defences increase (Rasmann & Agrawal, 2011; Pearse & Hipp, 2012; Moreira *et al.,* 2014; Anstett *et al.,* 2016). Nevertheless, recent studies have observed a positive relationship between latitudinal increase and defensive chemical levels of plants (Stevens *et al.,* 2016; Moreira *et al.,* 2017; Moreira *et al.,* 2018). A wide geographical distribution can also encompass greater differences in the edaphic variables (pH, moisture, temperature, porosity...) and climatic conditions. The potential of these environmental factors to modify basal phenolic levels has also been reported (Kraus *et al*., 2004; Salminen & Karonen, 2011; Moreira *et al.,* 2014; Abdala-Roberts *et al.,* 2016; Abdala-Roberts *et al*., 2018).

This study addresses the geographical variability in chemical defences of a widely distributed species in the Mediterranean basin, the holm oak (*Quercus ilex*). The main chemical defences in this species, as in other oaks, are based on phenolic compounds, especially high-molecular-weight tannins, both hydrolyzables (Ht, derivatives of gallic and ellagic acids) and condensed (Ct, also known as "proanthocyanidins"). These compounds reduce the digestibility of plant tissues in herbivores (Haslam, 2007), cause metabolic alterations in insects (Barbehenn *et al.,* 2009) and inhibit the activity of root pathogens (Oliva *et al*., 1999; Kraus *et al*., 2003). Other studies have shown a potent antimicrobial activity (Scalbert, 1991) and suggest that pre-infection levels in plant tissues might condition the interaction between certain pathogens such as *Phytophthora* and their hosts (Conrad *et al*., 2017), although this resistance mechanism is still poorly known in the Mediterranean context.

The constitutive resistance levels correspond to defensive traits of plants that are relatively stable or whose variation depends on internal factors (Schultz, 1988). They are genetically controlled and show extensive variation between genotypes and provenances (Dicke & Hilker, 2003; Solla *et al*., 2016; Gallardo *et al*., 2019).

Recent studies have approached the interspecific diversity in the constitutive defences of several species of the genus *Quercus* (Abdala-Roberts *et al.,* 2018), or the geographical component in the defensive chemical variability of species such as *Quercus robur* (Moreira *et al*., 2018). But there are few known studies on the intraspecific variation of these secondary metabolites in holm oak (but see Solla *et al*., 2016) despite the declining status this species experiences in certain valuable habitats such as dehesas. The diversity in the response of plants to stress-causing agents allows the selection of those individuals, populations and/or provenances of greater tolerance. To achieve this, it is essential to study the chemical pattern of constitutive defences in the species and identify later what compounds could grant greater tolerance.

The presence and variations of chemical defences in holm oak according to the region of origin are still quite unknown (Solla *et al.,* 2016). Advances in this field are needed to develop selection programs of forest material for reproduction, because of their involvement in the adaptation and response to the environmental stress factors and, therefore, against future scenarios in changing environmental conditions (Isabel *et al*., 2019).

The holm oak is the most abundant forest species in the Iberian Peninsula and the dominant one in “dehesas”, which constitute a High Natural and Cultural Value System (HNCV) thus considered by the European Union. Dehesas have been exploited by humans throughout history and they constitute the economic base of much of the Iberian rural world (Escribano & Pulido, 1998; Campos, 2004; Pereira *et al.,* 2004). Nevertheless, they are experiencing symptoms of decay in recent decades due to various factors (Brasier, 1992, 1996; Sánchez *et al*., 2006; Corcobado *et al*., 2013) whose consequences are becoming increasingly intense. Although the importance of its effects varies between regions, over time, the persistence of forests and dehesas could be at risk, with direct negative implications on resilience and associated biodiversity (Pulido *et al.,* 2001; Plieninger *et al.,* 2015; Duque-Lazo *et al.,* 2018).

In this study, the constitutive chemical defences of holm oak in six regions of Spain are analysed to answer the following questions:

(i) Does the secondary metabolite concentration in holm oak show significant geographical and local variation?
(ii) Does variation in defences depend on latitude or soil pH?
(iii) To what extent this chemical variation is heritable?
(iv) Which provenances are likely candidates to selection programmes?

Materials and methods

Plant material

Plant material from six Spanish regions with natural presence of holm oaks (located in National Parks and their surroundings) was collected. Two natural populations were selected at random in each region (Fig. 1). The studied morphologies were always from *Q. ilex* subsp. *rotundifolia* in Cabañeros (C), Guadarrama (G), Monfragüe (M), Ordesa (O) and Sierra Nevada (S). In Picos de Europa (P), acorns were collected from a population subsp. *rotundifolia* and another one of *Q. ilex* subsp. *ilex.* To determine whether differences are genetically-based, acorns from these regions were grown under a common environment through a greenhouse experiment.

Acorns were collected between November and December 2015 in the six natural regions and sown at the University of Extremadura greenhouses in Plasencia (UTM Zone 29N X: 748862; Y: 4435709; 395 meters above sea level). Within each region, mature acorns without signs of infestation were collected. In order to evaluate their viability, acorns were subject to flotation before sowing.

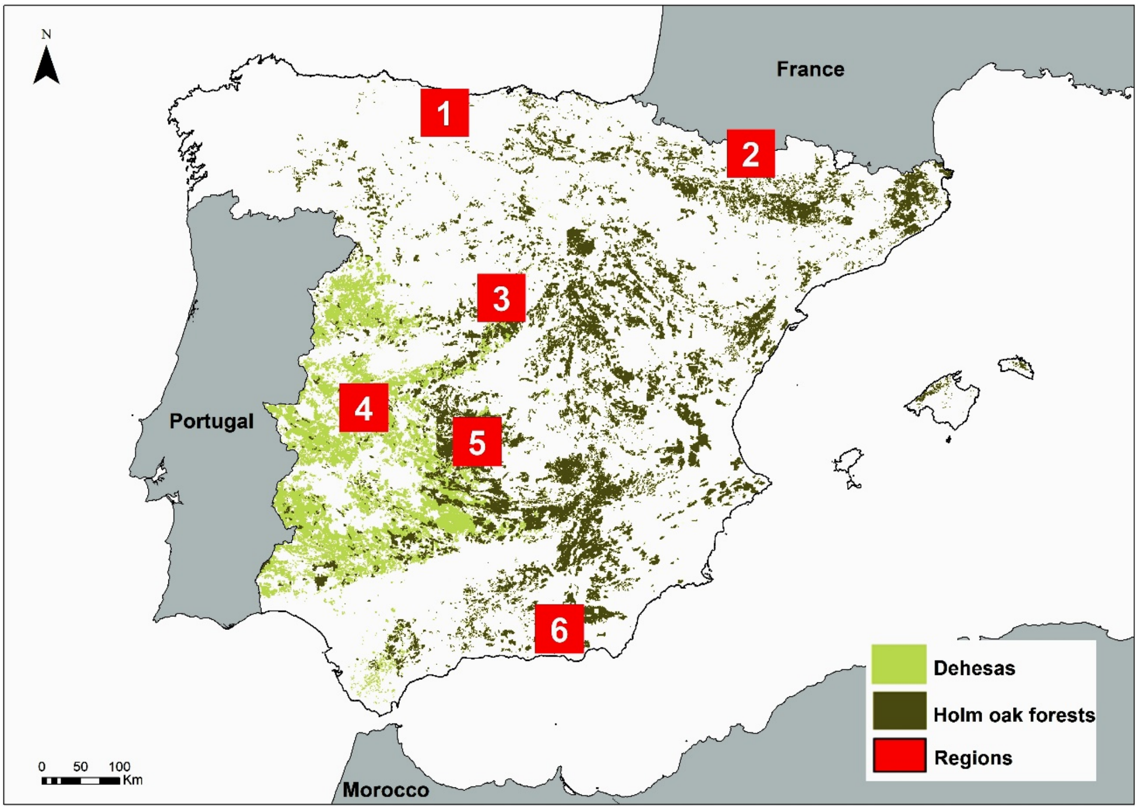

Fig.1. Distribution range of sclerophillous grazed dehesas with evergreen *Quercus* spp. (light green area), Mediterranean sclerophyllous *Quercus ilex* and *Quercus rotundifolia* forests (dark green area) and location of the studied regions in Spain (numbered red squares) according to Díaz & Pulido (2009) and Rodà *et al.* (2009): 1. Picos de Europa, 2. Ordesa, 3. Guadarrama, 4. Monfragüe, 5. Cabañeros and 6. Sierra Nevada.

Experimental design

A nested type design with a total of 588 seedlings (6 regions x 2 populations/region x 7 genotypes/population x 7 seedlings/genotype) was implemented at the greenhouse. Before sowing, acorns were placed in trays with sterilized sand for germination. Once the radicles emerged, the collected acorns were planted at the end of December in 28-cell plastic trays, 450 ml in volume, containing sand and peat (1:1, pH: 5.5). In order to maximize seedling availability, 2 acorns were planted per alveolus, selecting later the first emerged. The emergence took place in February and in general seedlings grew successfully.

Chemical analysis

Five months later, when the seedlings were at least 20 cm in height, three samples were harvested per each mother tree studied, removing the substrate carefully to avoid plant damage and subsequently processing the sample for freezing at -80ºC. Frozen seedlings were weighed and 5-6 leaves per plant were extracted for determination of phenolic content as described by Gallardo *et al.* (2019). These leaves were lyophilized using a Telstar LyoQuest lyophilizer (temperature -55ºC and 0.001-0.002 mbar pressure) and ground to a fine particle size. Once the milling was completed, tissue sampling was stored at -80ºC until analysis. The phenolic content of the plants was extracted from lyophilized material with 70% (v/v) aqueous methanol for 60 minutes in an ultrasonic bath at room temperature. The crude extracts were centrifuged at 10000 rpm for 5 minutes at 4ºC and the supernatant was collected and stored at -80ºC.

The total phenolics content (Tp) of the extract was determined by the Folin-Ciocalteu method (Makkar *et al*., 2003). Crude extracts were mixed with 50 volumes of 10% Folin-Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) and 40 volumes of 7.5% (w/v) sodium carbonate. In the control tube, the extract volume was replaced by methanol. The mixture was stirred gently and maintained in the dark and at room temperature for 45 minutes. After incubation, the absorbance was measured at 725 nm. Gallic acid (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used as standard and results were expressed as milligrams of gallic acid equivalents (G.A.E.) per grams of lyophilized sample.

The butanol–HCl assay (Porter *et al*., 1986) was used to quantify condensed tannins (Ct) using procyanidin B2 (Sigma) as a reference compound. Briefly, crude extracts were mixed with 100 volumes of n-butanol/acetone 1:1 (46% each) plus HCL (1.85%) and ferric ammonium sulphate (0.04%). In the control tube, the extract volume was replaced by methanol. Samples were heated at 70º. After 45 minutes of incubation, the samples were cooled and the absorbance at 550 nm was measured, with final results expressed as milligrams of procyanidin B equivalents (PB.E.) per grams of lyophilized sample.

Statistical analyses

The effects of region, population (nested in region) and genotype (nested in population) were analysed through a general linear model (GLM). Region, population and genotype were used as random factors and the Tp content (expressed in mg GAE/g sample) and Ct content (expressed in mg PBE/g sample) were used as dependent variables. Seedling weight was added to this model as a covariate in the Ct analysis to account for size effects on chemical defences. Data were analysed to check normality (by Kolmogorov-Smirnov test) and homoscedasticity (through Levene´s test). The relationships between both dependent variables as well as the influence of the germination time and seedling size (measured from their weight during the harvest of samples) on the constitutive defences were analysed with Pearson´s linear correlation.

Heritability of chemical traits ($\hat{h}^2$) was calculated following the procedure described by Solla *et al.* (2016) for the same species, as the additive genetic variance (VA) divided by the phenotypic variance (VP). The $\hat{h}^2$ estimate was corrected for a generalized selfing rate of 1 to 3% in native holm oak stands (Ortego *et al.,* 2014), using an r coefficient of 0.27 because half-sibs have a quarter of their alleles in common (¼VA). The following equation was used:

$$\hat{h}^2 = \mathrm{VA/VP} = \frac{\left(\sigma^2 \mathrm{f(gen)}\right) * (1/r)}{\left(\sigma^2 \mathrm{f(gen)}\right) + (\sigma^2 \mathrm{r}) + (\sigma^2 \mathrm{e})}$$

where σ^2f(gen) is the variance component among families (genotypes), σ^2r the among-regions variance and σ^2e the error variance. Standard errors were obtained following Jayaraman (1999). The quantitative genetic differentiation (Q_{ST}) was estimated as described by Leinonen *et al.* (2013):

$$\mathrm{Qst} = \frac{(\sigma^2 \mathrm{r})}{\left(2 * \sigma^2 \mathrm{f(gen)}\right) + (\sigma^2 \mathrm{r})}$$

Statistical analyses were performed in Statistica v10 software.

Results

Total phenolics and condensed tannins depend on region and genotype

Highly significant differences were observed in the concentration of Tp and Ct according to the region ($p<0.001$ for both compounds) and genotypes ($p<0.05$ for both compounds, Table 1). By contrast, there were no significant effects of the population.

Table 1. Effect of region, population (nested in region) and genotype (nested in population) on total phenolics (Tp) and condensed tannins (Ct) content in *Quercus ilex* leaves.

Effect	df	Total phenolics		Condensed tannins	
		F	*p*	F	*p*
Region	5	10.873	0.000	4.293	0.000
Population	6	0.748	0.611	1.430	0.208
Genotype	54	1.489	0.036	1.470	0.042

There was a significant relationship ($r=0.589$, $p<<0.001$, $r^2=0.347$) between the Tp obtained by the Folin-Ciocalteu method and the accumulation of Ct measured by the Porter method. The percentages of variance explained by region, population, and genotype for Tp were 21.37%, 1.61% and 30.72%, respectively. For Ct figures were 17.21%, 0.004% and 32.00%, respectively (% variance calculated from results of GLM).

Variation of defences with latitude and soil

Concentrations of Tp and Ct showed high and negative correlations with latitude, though they did not reach statistical significance due to low sample sizes (N=6 regions). Correlation values were $r=-0.681$, $p=0.136$ and adjusted $r^2=0.464$ in Tp, and $r=-0.236$, $p=0.653$ and adjusted $r^2=0.055$ in Ct. Analysed these levels against the increase in soil pH with the same sample size (N=6 regions), there was also a strong negative but not significant correlation ($r=-0.584$, $p=0.223$ and adjusted $r^2=0.341$ in Tp, and $r=-0.467$, $p=0.351$ and adjusted $r^2=0.212$ in Ct).

By grouping the northern regions (P, O and G) and the southern regions (M, C and S), higher and significant averages were obtained in the defensive levels of the southern area versus the northern one for Tp ($F(1; 186)=16.17$, $p<0.001$, Fig.2), and also higher for Ct ($F(1; 183)=3.23$, $p=0.07$, Fig.3). Mean and standard error values were 37.73±1.11 Tp and 62.24±2.68 Ct in the northern regions, and 44.05±1.09 Tp and 68.58±2.32 Ct in the southern ones. With the total number of analysed genotypes grouped in north and south regions and therefore, increasing the sample size (N=186 in Tp, N=183 in Ct), the correlation between chemical defences and latitude was negative and very significant in Tp ($r=-0.293$, $p<<0.001$ and adjusted $r^2=0.086$) but not significant in Ct ($r=-0.132$, $p=0.074$ and adjusted $r^2=0.017$).

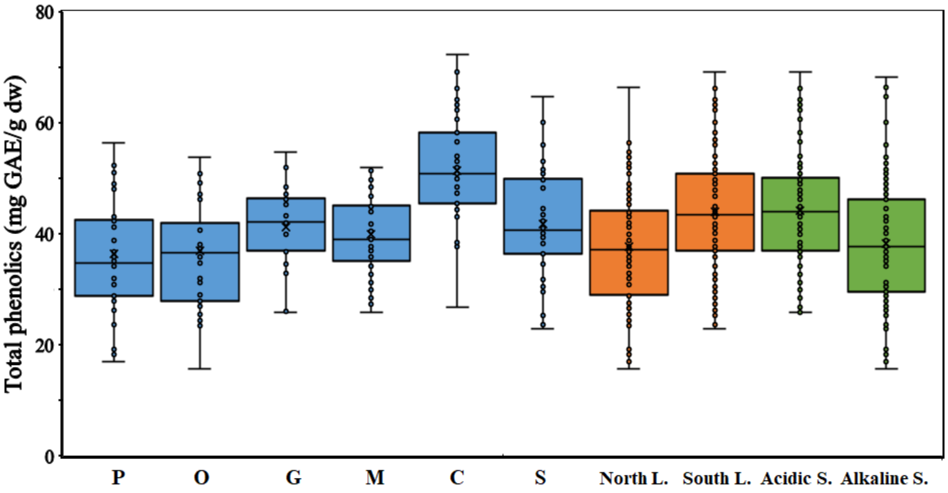

Fig.2. Constitutive levels of total phenolics (expressed as milligrams of gallic acid equivalents (G.A.E.) per grams of lyophilized sample) in leaves of *Quercus ilex* according to the region of origin (ordered from highest to lowest latitude): Picos de Europa (P), Ordesa (O), Guadarrama (G), Monfragüe (M), Cabañeros (C) and Sierra Nevada (S). The last four boxes are the averages grouping the studied data according to north (P, O, G) and south latitude (M, C, S), and according to the region of origin soil type in two groups, acidic (G, M, C) and alkaline soil (P, O, S). Bar height represents the mean and whiskers are standard errors. Statistics: Regions (F test (5, 179)=8.725, *p*=0.000 and Kruskal Wallis test (5, 185)=34.099, *p*=0.000). Latitude (F test (1; 186)=16.173, *p*=0.000 and Kruskal Wallis test (1; 185)=14.593, *p*=0.000). PH soil (F test (1; 186)=13.462, *p*=0.000 and Kruskal Wallis test (1; 185)=12.227, *p*=0.000).

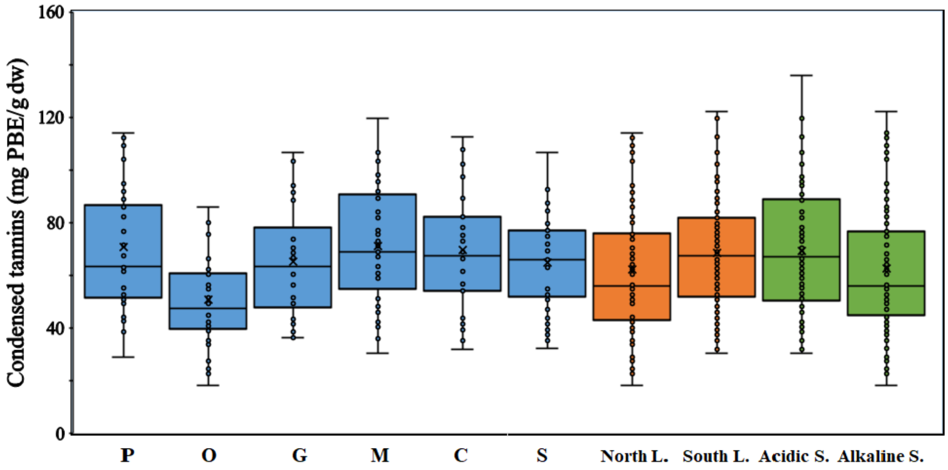

Fig. 3. Constitutive levels of condensed tannins (expressed as milligrams of procyanidin B equivalents (PB.E.) per grams of lyophilized sample) in leaves of *Quercus ilex* according to the region of origin (ordered from highest to lowest latitude): Picos de Europa (P), Ordesa (O), Guadarrama (G), Monfragüe (M), Cabañeros (C) and Sierra Nevada (S). The last four boxes are the averages grouping the studied data according to north (P, O, G) and south latitude (M, C, S), and according to the region of origin soil type in two groups, acidic (G, M, C) and alkaline soil (P, O, S). Bar height represents the mean and whiskers are standard errors. Statistics: Regions (F test (5; 179)=3.435; *p*=0.005 and Kruskal Wallis test (5; 185)= 16.645; *p*=0.005. Latitude (F test (1; 183)=3.229, *p*=0.074 and Kruskal Wallis test (1; 185)=4.627, *p*=0.031). PH soil (F test (1; 183)=3.537, *p*=0.061 and Kruskal Wallis test (1; 185)=4.124, *p*=0.042).

Grouping the regions according to the type of soil, two major groups are distinguished: acidic soils (G, M and C) and alkaline soils (P, O and S). Significant differences were obtained for Tp (F (1; 186)=13.462, p<0.001) but not in Ct (F (1; 183)=3.537, p=0.06). Regions with acidic soils showed higher defensive phenolic levels than alkaline ones (Fig. 2 and 3). Mean and standard error values were 38.4±1.09 Tp and 62.55±2.42 Ct in the alkaline soils, and 44.22±1.15 Tp and 69.19±2.56 Ct in the acidic ones. The relationship between chemical defences of all the genotypes analysed (N=186 in Tp, N=183 in Ct) and the increasing soil pH was negative and significant in Tp (r=-0.27, p<<0.001 and adjusted r^2=0.073) but not significant in Ct (r=-0.138, p=0.061 and adjusted r^2=0.019).

Heritability of total phenols and condensed tannins

Defences showed significant narrow-sense heritability across populations ($\hat{h}^2$) both for Tp (0.37±0.08) and Ct (0.48±0.36). The quantitative genetic differentiation (Q_{ST}) was 0.51 and 0.25, respectively (Table 2).

Table 2. Variance components (σ^2), narrow-sense heritabilities ($\hat{h}^2$) and quantitative genetic differentiation among regions (Q_{ST}) for phenolic defences (Tp and Ct) in *Quercus ilex* leaves.

Statistic	Total phenolics	Condensed tannins[a]
σ^2f(gen)	12.7	75.69
σ^2r	26.84	38.51
σ^2e	88.9	473.96
$\hat{h}^2$	0.37±0.08	0.48±0.36
Q_{ST}	0.51	0.25

[a] Variance components were adjusted for seedling weight covariate.

Discussion

This study shows significant among regions and genotypes differences in baseline heritable secondary metabolite patterns in holm oak for a significant portion of the species´ range. Generally, higher levels of constitutive defences were observed at lower latitudes and on acidic rather than basic soils. Furthermore, total phenols and condensed tannins showed a positive correlation. Their narrow-sense heritability was moderate.

Defences in holm oak show significant geographical and local variation

Significant differences were found for the concentration of total phenolics and condensed tannins among regions and among genotypes, but not among populations within regions. The non-significant differences between the populations within each region indicate that our estimates characterize defence levels of the region of origin. Previous studies have analysed total phenols and condensed tannins in acorns of *Q. ilex* and *Quercus suber* (Cadahía *et al.*, 1993) and in leaves of several oak species (Faeth, 1986; Dawra *et al.*, 1988). However, geographical variation is still poorly known (but see Solla *et al.,* 2016). This work analyses for the first time the geographical variation of total phenols and condensed tannins in *Q. ilex* leaves. Nonetheless, a previous study had already estimated the concentration of total tannins in the same species. Solla *et al.* (2016) showed that total tannin content did not differ significantly among regions, but it differed significantly among families. Moreira *et al.* (2018) analysed the variation of condensed tannins in another oak species, *Q. robur*, in 38 populations along an 18º latitudinal gradient, obtaining significant differences. This disparity in the estimates of the different phenolic defence groups shows the need to continue studying chemical patterns in *Quercus.*

Regarding the evaluated phenolic groups, we found a positive correlation between total phenols and condensed tannins. Therefore, both traits could be used as regional indicators of holm oak leaf defences. Finally, for the Picos de Europa region, our results for Tp and Ct were consistent with those found previously in a similar study performed using the same methodology (Gallardo *et al.,* 2019).

Effects of latitude and soil pH on defences

Though our sample sizes were limited, we found that the lowest levels of constitutive defences correspond to regions in the northern Iberian Peninsula and in regions with alkaline soils. Theory predicts that chemical defences should decrease as latitude increases (Rasmann & Agrawal, 2011; Abdala-Roberts *et al.,* 2016). The results of this work are consistent with such prediction. However, recent studies have found a positive relationship between latitude and phenolic compounds in species such as *Q. robur* (Moreira *et al.,* 2018) or *Pinus pinaster* (López-Goldar *et al.,* 2019). Our average values showed an increase in chemical defences with decreasing latitude. This trend could be linked to underlying factors associated with latitudinal variation such as climate and soil (Moreira *et al.,* 2018). Cabañeros and Monfragüe regions stood out especially above the rest for their high defensive levels and could be of interest for genotype selection.

On the other hand, soil type as described by pH also showed an apparent effect on phenolic defences. Seedlings from acidic-soil regions (G, M and C) have higher phenolic contents than those of alkaline soils (P, O and S). Soil pH influences the availability of nutrients for plants and may therefore be the cause of deficiency or toxicity (Benton, 2003). Tannins may induce allelopathic responses at the ecosystem level, including toxicity against pathogens (Kraus *et al.,* 2003). One of the most widely spread and aggressive pathogens in recent decades is *Phytopthora cinnamomi*, the soil-borne oomycete that causes root rot disease and is identified as the primary causal agent of *Quercus* decline in the Iberian Peninsula (Brasier, 1992, 1996; Sánchez *et al.,* 2006; Corcobado *et al.,* 2014). This pathogen develops better in acidic than basic soils (Blaker & MacDonald, 1983; Dixon, 1984; Duvenhage & Kotzé, 1995). In fact, calcium mineral nutrition raises the soil pH and increases the tolerance of *Q. ilex* to *Phytophthora* root disease (Serrano *et al.,* 2013). Assuming the important function that tannins exert on soil pathogens (Scalbert, 1991; Stong *et al.,* 2013) plants in basic soil would not need to produce high levels of these compounds, as we see in our study, because the pathogen does not develop well at high pH. However, in acidic soils, this greater production of phenolic compounds could be the defensive response to a higher pressure by the pathogen.

Heritability of constitutive defences

The heritability of total phenols and condensed tannins in *Q. ilex* leaves was moderate. In the context of tree improvement determining the narrow-sense heritability is a useful tool to know how much of the observed variation of a certain quantitative character is due to the additive genetic component. In our study the narrow-sense heritability was moderate and likely to allow selection (Eriksson *et al.,* 2013; Alcaide *et al.,* 2019, 2020). Nevertheless, these results must be taken with caution due to the low number genotypes evaluated (Jensen & Barr, 1971; Visscher & Goddard, 2015).

Heritability values were lower than those obtained in a previous work in the same species but for the total tannin content (Tt) with fewer families sampled (0.37 in Tp and 0.48 in Ct versus 0.83 in Tt; Solla *et al.,* 2016). However, this could enable short-term evolutionary change in response to selection and makes the selection of material from regions with higher constitutive levels meaningful in future species improvement programmes.

Genetic differentiation by quantitative traits (Q_{ST}) was moderate for Ct (0.25) and higher in Tp (0.51) in the six regions analysed. Solla *et al.* (2016) studied the Q_{ST} in total tannins, observing a lower genetic differentiation between the *Q. ilex* regions (0.12). There is a general lack of knowledge regarding this variation in chemical defences. However, this value allows Q_{ST}-F_{ST} comparisons and thus, discrimination between natural selection and genetic drift as differentiation mechanisms in traits (Leinonen *et al.,* 2013). Therefore, this study offers a first insight into the characterization of variations that *Q. ilex* secondary metabolites undergo in the Mediterranean area. Nevertheless, these results should be taken with caution owing to the relatively small size of sampled families. In addition, it would be very interesting to study the fixation index (F_{ST}) to find out if there is divergent selection in the species and thus, confirm whether the different genotypes are favoured under different environmental conditions (Merilä & Crnokrak, 2001).

Applicability for selection programmes

The lowest phenol concentrations were found in the northern region on alkaline soils (Ordesa and Picos de Europa), while the highest values were found in Cabañeros (highest Tp) and Monfragüe (highest Ct), in the southern region and on acidic soils. Likewise, the heritability of the evaluated characters was moderate but enough to show genetic control and opportunities for artificial selection (Eriksson *et al.,* 2013; Alcaide *et al.,* 2019). The important variability observed among chemical defences patterns from regions may be a source of useful ecotypes in areas under high levels of biotic stress and, especially, in forests affected by the infection with pathogens of the genus *Phytophthora*, which are known to suffer from tannin inhibition (Stong *et al.,* 2013). This suppressive ability of tannins is a key factor, since it is expected that plants with elevated tannin levels will develop better under unfavourable environmental conditions.

The basic unit to distinguish the forest reproductive materials (FRM) is the region of provenance, and in Spain these have been established for *Q. ilex* according to their distribution and patterns of variation (Alía *et al.,* 2019). Southern areas on acidic soils with higher levels of constitutive defences seem to be candidates for possible selection of FRM against widespread pathogens such as *P. cinnamomi*. In particular, Cabañeros and Monfragüe consistently showed the highest levels of defensive compounds. However, in a widely distributed species such as the holm oak, other factors should be taken into account because individuals that could be good candidates for their high defensive chemical levels, may not adapt suitably to other types of soil, climatic conditions or phenological conditions, among others. Confirmatory studies are needed in order to establish a sound basis for future selection.

References

Abdala-Roberts L, Moreira X, Rasmann S, Parra-Tabla V, Mooney KA, 2016. Test of biotic and abiotic correlates of latitudinal variation in defenses in the perennial herb *Ruellia nudiflora*. J Ecol 104: 580-590.

Abdala-Roberts L, Galmán A, Petry W, Covelo F, De la Fuente M, Glauser G, Moreira X, 2018. Interspecific variation in leaf functional and defensive traits in oak species and its underlying climatic drivers. PloS One 13(8) e0202548.

Agrawal AA, Heil M, 2012. Synthesizing specificity: multiple approaches to understanding the attack and defense of plants. Trends Plant Sci 17: 239-242.

Alcaide F, Solla A, Cherubini M, Mattioni C, Cuenca B, Camisón Á, Martín MA, 2020. Adaptive evolution of chestnut forests to the impact of ink disease in Spain. Jnl of Sytematics Evolution.

Alcaide F, Solla A, Mattioni C, Castellana S, Martín MA, 2019. Adaptive diversity and drought tolerance in *Castanea sativa* assessed through EST-SSR genic markers. Forestry 92 (3): 287-296.

Alía R, García del Barrio JM, Iglesias S, Mancha JA, De Miguel J, Nicolás JL, Pérez F, Sánchez de Ron D, 2009. Regiones de Procedencia de especies forestales en

España. Ministerio de Medio Ambiente y Medio Rural y marino. Organismo Autónomo Parques nacionales. 363 pp. Madrid.

Anstett DN, Nunes KA, Baskett C, Kotanen PM, 2016. Sources of controversy surrounding latitudinal patterns in herbivory and defense. Trends Ecol Evol 31: 789–802.

Barbehenn RV, Jaros A, Lee G, Mozola C, Weir Q, Salminen JP, 2009. Tree resistance to *Lymantria dispar* caterpillars: importance and limitations of foliar tannin composition. Oecologia. 159: 777-788.

Becerra JX, Noge K, Venable DL, 2009. Macroevolutionary chemical escalation in an ancient plant-herbivore arms race. Proc Natl Acad Sci USA 106:18062–6.

Benton JJ, 2003. Agronomic handbook: Management of crops, soils and their fertility. CRC press LLC. USA.

Blaker NS, MacDonald JD, 1983. Influence of container médium pH on sporangium formation, zoospore reléase and infection of rhododendron by *Phytophthora cinnamomi*. Plant Disease 67: 259-263.

Bogdziewicz M, Espelta JM, Bonal R, 2019. Tolerance to seed predation mediated by seed size increases at lower latitudes in a Mediterranean oak. Annals of Botany 123: 707-714.

Brasier CM, 1992. Oak tree mortality in Iberia. Nature 360, 539.

Brasier CM, 1996. *Phytophthora cinnamomi* and oak decline in Southern Europe. Environmental constraints, including climate change. Ann For Sci 53: 347-358.

Cadahía E, Conde E, Cañellas I, García-Vallejo MC, 1993. Variación del contenido de taninos en las bellotas de *Quercus suber* L. y *Quercus ilex* L. durante el proceso de maduración. Congreso Forestal Español, Lourizán. Tomo 11. 485.

Campos P, 2004. Towards a sustainable global economics approach for Mediterranean agroforestry systems. In: Schnabel, S., Ferreira, A. (eds.) Sustainability of agrosilvopastoral systems. Advances in GeoEcology, vol 37. Catena Verlag, Reiskirchen, Germany, pp 13–28.

Conrad AO, McPherson BA, Wood DL, Madden LV, Bonello P, 2017. Constitutive phenolic biomarkers identify naïve *Quercus agrifolia* resistant to *Phytophthora ramorum*, the causal agent of sudden oak death. Tree Physiol 26: 1–11.

Corcobado T, Solla A, Madeira MA, Moreno G, 2013. Combined effects of soil properties and *Phytophthora cinnamomi* infections on *Quercus ilex* decline. Plant and Soil 373: 403-413.

Corcobado T, Cubera E, Juarez E, Moreno G, Solla A, 2014. Drought events determine performance of *Quercus ilex* seedlings and increase their susceptibility to *Phytophthora cinnamomi.* Agric For Meteorol 192-193:1–8.

Dawra RK, Makkar HPS, Singh B, 1988. Total phenolics, condensed tannins and protein-precipitable phenolics in young and mature leaves of oak species. J. Agric. Food Chem. 36: 953-957.

Díaz M, Pulido FJ, 2009. 6310 Dehesas perennifolias de *Quercus* spp. En: VV.AA., Bases ecológicas preliminares para la conservación de los tipos de hábitat de interés comunitario en España. Madrid: Ministerio de Medio Ambiente, y Medio Rural y Marino. 69 p.

Dicke M, Hilker M, 2003. Induced plant defences: from molecular biology to evolutionary ecology. Basic & Appl Ecol 14: 3–14.

Dixon G, 1984. Plan pathogens and their control in horticulture. Horticultural crops.

Duque-Lazo J, Navarro-Cerrillo RM, Van Gils H, Groen TA, 2018. Forecasting oak decline caused by *Phytophthora cinnamomi* in Andalusia: Identification of priority areas for intervention. Forest Ecology and Management 417, pp. 122-136.

Duvenhage JA, Kotzé JM, 1991. The influence of calcium on saprophytic growth and pathogenicity of *Phytophthora cinnamomi* and on resistance of avocado to root rot. South African Avocado Growers'Association Yearbook 14: 13-14.

Eckert CG, Samis KE, Lougheed SC, 2008. Genetic variation across species' geographical ranges: the central-marginal hypothesis and beyond. Mol Ecol 17:1170–88.

Eriksson G, Ekberg I, Clapham D, 2013. Genetics applied to forestry. Department of Plant Biology and Forest Genetics, SLU. Uppsala, Sweden.

Escribano M, Pulido FJ, 1998. La dehesa en Extremadura. Estructura económica y recursos naturales. SIDT Junta de Extremadura, Mérida.

Faeth SH,1986. Indirect interactions between temporally separated hervibores mediated by the host plant. Ecology 67: 479-454.

Freeman BC, Beattie GA, 2008. An overview of plant defenses against pathogens and herbivores. The Plant Health Instructor.

Gallardo A, Morcuende D, Solla A, Moreno G, Pulido FJ, Quesada A, 2019. Regulation by biotic stress of tannins biosynthesis in *Quercus ilex*: crosstalk between defoliation and *Phytophthora cinnamomi* infection. Physiol Plant 165: 319-329.

Haslam E, 2007. Vegetable tannins-lessons of a pythochemical lifetime. Phytochemistry 68: 2713-2721.

Isabel N, Holliday JA, Aitken SN, 2019. Forest genomics: advancing climate adaptation, forest health, productivity and conservation. Evol Appl 13: 3-10.

Ivetic V, Dvetakovic J, Nonic M, Stankovic D, Sikacic-Nikolic M, 2016. Genetic diversity and forest reproductive material - from seed source selection to planting. iForest 9: 801-812.

Ivetic V, Devetakovic J, 2017. Concerns and evidence on genetic diversity in planted forests. Reforesta 3:196-207.

Jayaraman K, 1999. A statistical manual for forestry research. FORSPA-FAO Publication, Bangkok.

Jensen EL, Barr GR, 1971. Standard errors of heritability estimates calculated from variance component analysis of a two-way classification. J. Anim. Sci 32 (6): 1069-1077.

Kraus TEC, Dahlgren EC, Zasoski RJ, 2003. Tannins in nutrient dynamics of forest ecosystems - a review. Plant and Soil 256: 41–66.

Kraus TEC, Zasoski RJ, Dahlgren RA, 2004. Fertility and pH effects on polyphenol and condensed tannin concentrations in foliage and roots. Plant and Soil 262: 95-109.

Leinonen T, McCairns RJS, O´Hara RB, Merilä J, 2013. Q_{ST}–F_{ST} comparisons: evolutionary and ecological insights from genomic hetero-geneity. Nat Rev Genet 14:179–190.

López-Goldar X, Villari C, Bonello P, Borg-Karlson AK, Grivet D, Sampedro L, Zas R, 2019. Genetic variation in the constitutive defensive metabolome and its inducibility are geographically structured and largely determined by demographic processes in maritime pine. J. Ecol. 107, 2464–2477.

Lowe S, Browne M, Boudjelas S, De Poorter M, 2000. 100 of the World´s Worst Invasive Alien Species. A selection from the Global Invasive Species Database. The IUCN Invasive Species Specialist Group (ISSG). IUCN. 12 pp.

Makkar HPS, 2003. Measurement of total phenolics and tannins using Folin-Ciocalteu method. In: Quantification of tannins in tree and shrub foliage. Springer, Dordrecht, the Netherlands, pp 49–51.

Martín JA, Sobrino-Plata J, Rodríguez-Calcerrada J, Collada C, Gil L, 2019. Breeding and scientific advances in the fight against Dutch elm disease: Will they allow the use of elms in forest restoration? New Forests 50: 183.

Merilä J, Crnokrak P, 2001. Comparison of genetic differentiation at marker loci and quantitative traits. J. Evol. Biol 14: 892-903.

Moreira X, Mooney KA, Rasmann S, Petry WK, Carrillo-Gavilan A, Zas R, Sampedro L, 2014. Trade-offs between constitutive and induced defences drive geographical and climatic clines in pine chemical defences. Ecol Lett 17 (5): 537– 546.

Moreira X, Glauser G, Abdala-Roberts L, 2017. Interactive effects of plant neighbourhood and ontogeny on insect herbivory and plant defensive traits. Sci Rep 7: 4047.

Moreira X, Castagneyrol B, Abdala-Roberts L, Berny-Mier y Teran JC, Timmermans BGH, Bruun HH, Covelo F, Glauser G, Rasmann S, Tack AJM, 2018. Latitudinal variation in plant chemical defences drives latitudinal patterns of leaf herbivory. Ecography 41 1124–1134.

Moreira X, Abdala-Roberts L, Henrik-Bruun H, Covelo F, De Frenne P, Galmán A, Gaytán A, Jaatinen R, Ten Hoopen JPJG, Pulkkinen P *et al.,* 2019. Latitudinal variation in seed predation correlates with latitudinal variation in seed defensive and nutritional traits in a widespread oak species. Ann Bot mcz207.

Oliva A, Lahoz E, Contillo R, Aliota G, 1999. Fungistatic activity of *Ruta graveolens* extract and its allelochemicals. Chem Ecol 25: 519-526.

Ortego J, Bonal R, Muñoz A, Aparicio JM, 2014. Extensive pollen immigration and no evidence of disrupted mating patterns or reproduction in a highly fragmented holm oak stand. J Plant Ecol 7:384–395.

Pearse IS, Hipp AL, 2012. Global patterns of leaf defences in oak species. Evol 66: 2272– 2286.

Pereira HM, Domingos T, Vicente L (Eds.), 2004. Portugal Millennium Ecosystem Assessment: State of the Assessment Report. Centro de Biologia Ambiental de la Faculdade de Ciências, Universidade de Lisboa.

Plieninger T, Hartel T, Martín-López B, Beaufoy G, Bergmeier E, Kirby K, Montero MJ, Moreno G, Otero-Rozas E, Van Uytyanck J, 2015. Wood-pastures of Europe: Geographic coverage, social–ecological values, conservation management and policy implications. Biol Conserv 190: 70-79.

Porter LJ, Hirstich LN, Chan BG, 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochem 25: 223-230.

Pulido F, Díaz M, Hidalgo de Trucios S, 2001. Size structure and regeneration of Spanish holm oak *Quercus ilex* forests and dehesas: effects of agroforestry use on their long-term sustainability. Forest Ecol Manag 146: 1-13.

Rasmann S, Agrawal AA, 2011. Latitudinal patterns in plant defence: evolution of cardenolides, their toxicity, and induction following herbivory. Ecol Lett 14: 476– 483.

Roda F, Vayreda J, Ninyerola M, 2009. 9340 Encinares de *Quercus ilex* y *Quercus rotundifolia*. En: VV.AA., Bases ecológicas preliminares para la conservación de los tipos de hábitat de interés comunitario en España. Madrid: Ministerio de Medio Ambiente, y Medio Rural y Marino. 94 p.

Salminen JP, Karonen M, 2011. Chemical ecology of tannins and other phenolics: we need a change in approach. Funct Ecol. 25:325–338.

Sánchez ME, Caetano P, Romero MA, Navarro RM, Trapero A, 2006. *Phytophthora* root rot as the main factor of oak decline in southern Spain. In: Brasier C, Jung T, Oßwald W, eds. Progress in Research on Phytophthora Diseases of Forest Trees. Farnham, Surrey, UK: Forest Research, 149-54.

Scalbert A, 1991. Antimicrobial properties of tannins. Phytochemistry 30: 3875–3883.

Schultz J, 1988. Plant responses to herbivores. Trends Ecol Evol 3: 45:49.

Serrano MS, Fernández-Rebollo P, De Vita P, Sánchez ME, 2013. Calcium mineral nutrition increases the tolerance of *Quercus ilex* to *Phytophthora* root disease affecting oak rangeland ecosystems in Spain. Agroforestry Systems, 87(1): 173-179.

Solla A, López JC, Martín JA, Gil LA, 2014. Genetic variation and heritability estimates of *Ulmus minor* and *Ulmus pumila* hybrids for budburst, growth and tolerance to *Ophiostoma novo-ulmi*. "Iforest", v. 8; pp. 422-430.

Solla A, Slobodan M, Gallardo A, Bueno A, Corcobado T, Cáceres Y, Morcuende D, Quesada A, Moreno G, Pulido FJ, 2016. Genetic determination of tannins and herbivore resistance in *Quercus ilex*. Tree Genet Genom 28: 743–751.

Stevens MT, Brown SC, Bothwell HM, Bryant JP, 2016. Biogeography of Alaska paper birch (*Betula neoalaskana*): latitudinal patterns in chemical defense and plant architecture. Ecology 97: 494–502.

Stong RA, Kolodny E, Kelsey RG, González-Hernández MP, Vivanco JM, Manter DK, 2013. Effect of plant sterols and tannins on *Phytophthora ramorum* growth and sporulation. Chem. Ecol 39: 733-743.

Thompson JN, 2005. The Geographic Mosaic of Coevolution. Chicago: University of Chicago Press.

Visscher PM, Goddard ME, 2015. A general unified framework to assess the sampling variance of heritability estimates using pedigree or marker-based relationships. Genetics 199: 223-232.

CHAPTER 2

Interactive effects of biotic stressors and provenance on chemical defence induction by holm oak (*Quercus ilex*)

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Submitted to Trees, Structure and Function (under revision)

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Abstract

Quercus forests are suffering serious decline worldwide, closely linked to the consequences of climate change. The increase of biotic stressors threatens the survival of the holm oak (*Quercus ilex)*, a dominant tree species in the Mediterranean Basin. So, it is urgent to unveil its resistance mechanisms for a better control of oak decline. In this work, the ability of holm oaks from six Iberian provenances to respond to multiple biotic damages is studied through the analysis of their induced chemical defence patterns.

Using 2016 seedlings established in a common garden trial (6 regions x 12 families/region x 7 seedlings/family x 4 treatments), biotic damage was induced at the root level (by infection with the widespread pathogen *Phytophthora cinnamomi*) and at the aerial level (by mechanical defoliation). The levels of constitutive and induced total phenols, total tannins and condensed tannins were measured. Results showed that (1) the defensive chemical patterns show significant local and geographical variation, (2) survival to stress is more related to constitutive defences than induced ones, (3) the induced response is stressor-specific and (4) there is an interactive effect among stressors whose sign (induction/inhibition) depends on the provenance. These findings on the biotic stressor effects on the chemical defences and survival of holm oak may facilitate the development of genetic material selection programs in the integrated control of the widespread *Quercus* decline.

Key Message

The patterns of induced chemical defences in *Quercus ilex* leaves are specific to the biotic stress factor that causes them. Interactive effects between stressors depend on provenance.

Introduction

Plants have different strategies to deal with external biotic attacks. One of them is the production of constitutive and induced chemical compounds. The constitutive resistance levels correspond to defensive traits of plants that are relatively stable or whose variation depends on internal factors (Schultz, 1988). They are genetically controlled and show extensive variation among genotypes and provenances (Dicke and Hilker 2003, Solla *et al.* 2016, Gallardo *et al.* 2019). Constitutive defence prevents infection in the first place, while induced defence typically shortens the infectious period (Boots and Best 2018).

The main chemical defences in *Quercus* are phenolic compounds. Among them, tannins play an important role as a defensive mechanism against biotic stress in plants, although their role is controversial (Moctezuma *et al.* 2014). These secondary metabolites are strong inhibitors of digestive proteases, which explains why *Quercus* species accumulate them in leaves, stems and roots to protect themselves from damage by herbivores (Moctezuma *et al.* 2014). In addition to reducing digestibility in herbivores (Haslam 2007), they cause metabolic alterations in insects (Barbehenn *et al.* 2009), inhibit the activity of root pathogens (Oliva *et al.* 1999, Kraus *et al.* 2003, Baptist *et al.* 2008) and have a high antimicrobial activity (Scalbert 1991, Daglia 2012). Pre-infection levels of some chemical defences in plant tissues allow the identification of trees naturally resistant to *Phytophthora* (Conrad *et al.* 2017).

Oak forests are suffering a worldwide decline related to the rise in average temperatures and the invasion of very aggressive alien pathogens (Dukes *et al.* 2009, Santini *et al.* 2013, Pérez-Ramos *et al.* 2014). The increase in biotic stress driven by climate change and especially the invasion of oomycetes of the *Phytophthora* genus has been widely reported. Several *Phytophthora* species are associated with oak decline all over the world (Jung *et al.* 1996, Balci and Halmschlager 2003, Pérez-Sierra *et al.* 2013).

The Mediterranean basin is not exempt from these pathogens and the soil-borne *Phytophthora cinnamomi (Pc)* has been reported in several Mediterranean countries, including France, Portugal, Italy and Spain (Brasier *et al.* 1993, Robin *et al.* 1998, Sánchez *et al.* 2006, Corcobado *et al.* 2013, Jung *et al.* 2013, Scanu *et al.* 2013). Its distribution is expected to be favored by global warming, moving towards the poles (Burgess *et al.* 2017). The action of these pathogens, together with the lack of tree regeneration, are the main threats to the Mediterranean *Quercus* conservation (Moreno and Pulido 2009, Plieninger *et al.* 2010, Jorrín-Novo and Navarro-Cerrillo 2014).

In the Iberian Peninsula, and mostly within its southwestern area, some *Quercus* species such as holm oak (*Q. ilex*) and cork oak (*Q. suber*) are agrosystems known as "dehesas" in Spain and "montados" in Portugal. They are a good example of "grazed wood pastures", common in the Mediterranean region (Den Herder *et al.* 2017). These socio-ecological systems are considered by the European Union as High Natural and Cultural Value Systems (HNCV; Moreno *et al.* 2018) but they are affected by several biotic stressors. Besides natural herbivory, livestock grazing also occurs, with densities that sometimes exceed the land´s carrying capacity (García *et al.* 2010). Several works have also studied the incidence of defoliation exerted by numerous insects on *Quercus*, many of which are prone to population outbreaks (Humphrey and Swaine 1997, Solla *et al.* 2016, Tiberi *et al.* 2016). Generally, defoliation negatively affects the holm oak growth (Schmid *et al.* 2017) and compromises its regeneration (Carbonero *et al.* 2004, Olmo *et al.* 2017). In recent decades, another stress factor adds to the aforementioned intense defoliation: several pathogen infections, among which *Pc* is the primary causal agent of the *Quercus* decline in the Iberian Peninsula (Brasier 1992, 1996, Sánchez *et al.* 2006, Corcobado *et al.* 2014). This root rotting oomycete is considered one of the 100 worst invasive alien species on Earth (Lowe *et al.* 2000) and its mitigation in dehesas is a high priority task that requires isolation of affected areas to avoid pathogen spread, elicit the vigor of healthy plants and, in the long term, selection and implementation of resistant plants (Camilo-Alves *et al.* 2013). It is expected that the combination of several biotic stress factors will further weaken the forests, already aged (Plieninger *et al.* 2010).

To date the study of oak decline has not considered the differences among individuals, populations and regions in susceptibility to biotic stressors. While some studies have been published on geographic variability in the induction of certain defensive chemical traits in holm oak against the biotic attack (Solla *et al.* 2016, Pulido *et al.* 2019), this is the first considering the combined attack at the root and aerial level and their possible interactions. Studies focused on the effects of combined biotic stress on tree chemical defences and their geographic variability are still scarce. The tolerance or "host´s ability to reduce the stress factor damage" (Pagán and Arenal 2020) to biotic stress will depend, among other factors, on the genes involved in the production of these chemical defences (Gallardo *et al.* 2019). Then, it is expected the existence of different levels of tolerance associated with genetic polymorphism and local adaptation processes (Sork *et al.* 1993, Pautasso *et al.* 2012). The high genetic diversity of the holm oak, in addition to its wide distribution, a priori facilitates the existence of different patterns of chemical response whose heritability has been assessed (Solla *et al.* 2016, Rodríguez-Romero *et al.* 2020). Furthermore, the phenomena of geographic and local adaptation facilitate the occurrence of tolerant and even resistant individuals to certain stressors, which could be very useful in genetic improvement programs.

In this study, a common garden trial was carried out with holm oak seedlings from six provenances to which combined biotic stress treatments (*Pc* infection and mechanical defoliation) were applied to answer the following questions:

(1) Is there an induction of defences by the applied treatments?

(2) Is the induced variation in chemical response stressor-specific?

(3) Is there an interactive effect of stressors?

(4) Does induction have consequences for plant survival?

Materials and methods

Plant material, growth conditions and experimental design

Acorns from natural holm oak forests in six Spanish areas were collected between November and December 2015. The sampled regions, located within or nearby Spanish National Parks (Fig. 1), covered a large part of the species distribution range. Ordered from highest to lowest latitudinal range, these regions were: Picos de Europa (P), Ordesa (O), Guadarrama (G), Monfragüe (M), Cabañeros (C) and Sierra Nevada (S).

After checking their viability by flotation, acorns were sown under a common environment at the greenhouse on the Faculty of Forestry of the University of Extremadura (Plasencia campus; UTM Zone 29N X: 748862; Y: 4435709; 395 meters above sea level). Collected acorns were sown in 28-well plastic trays, 450 ml in volume, filled with a soil substrate consisting of mixed peat and sand (1:2, pH: 5.5). Pots were placed on metal trays with a 2-cm deep water line to keep the substrate at field capacity (seedlings did not suffer water stress). In order to ensure germination success of seedlings, two acorns were sown per alveolus, selecting later the first emerged (Fig. S1 from supplementary information).

We used a complete randomized and nested type experimental design, with a total of 2016 seedlings (6 regions x 12 families/region x 7 seedlings/family x 4 treatments). The emergence took place in March and seedlings grew successfully under optimal conditions until the end of May, when the treatments were applied.

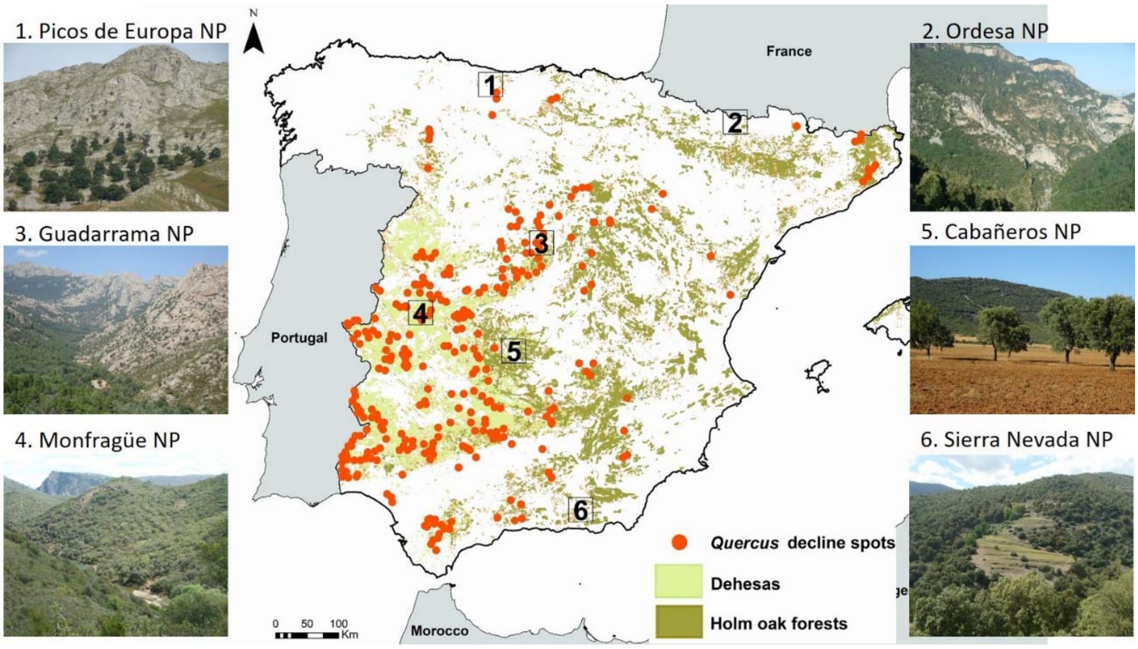


Fig.1. Distribution range of dehesas (light green area), holm oak forests (dark green area) and location of the studied regions in Spain (numbered black squares) according to Díaz & Pulido (2009) and Rodà *et al.* (2009): 1. Picos de Europa, 2. Ordesa, 3. Guadarrama, 4. Monfragüe, 5. Cabañeros and 6. Sierra Nevada. Sampling points of the European Forest Damage Monitoring Network with presence of *Quercus* decline (orange points) due to water stress and/or infection by pathogens (mainly *Phytophthora cinnamomi*). Modified from Fernández-Cancio *et al.* (2004) with the most recent data on the *Phytophthora cinnamomi* presence (CICYTEX-INIAV 2020).

Application of treatments and symptom assessment

Once a minimum development was reached (8-12 leaves per plant, 15 cm high), seedlings were subjected to four treatments to test for the inducibility of chemical defences against biotic stressors:

-control treatment (c), without exercising any damage.

-inoculation with the soil-borne pathogen *Phytophthora cinnamomi* (*Pc*)

-mechanical wounding or mechanical defoliation of the upper third of the seedling (d), mimicking the effect of herbivory on chemical defences (Pardo *et al.* 2018, Gallardo *et al.* 2019).

-double treatment (dPc), combining pathogen infection with mechanical defoliation.

Treatments were applied in equal parts (84 plants per region and treatment). The pathogen used in the inoculation was isolated by the Forestry Research Group (FRG) of the University of Extremadura from a holm oak in Valverde de Mérida, SW Spain (Corcobado *et al.* 2013). The high virulence of this *Pc* strain (UEx1; an A2 mating type) has been demonstrated in several studies of the FRG (Corcobado *et al.* 2017). Nonetheless, this *Pc* strain has been reactivated every year and kept cold at 10ºC. For this test, it was activated again by using it as inoculum to cause infective lesions in the root system of 1-month-old *Q. ilex* and *Castanea sativa* seedlings. Thus, two weeks before making the inoculum for the experiment´s infection, the pathogen was reactivated.

The inoculum was prepared inside Erlenmeyer flasks following the procedure described by Jung *et al.* (1996) with some plugs of the UEx 1 strain in V8 multivitamin juice broth, whole oat grains and fine vermiculite, and incubated for five weeks at 22ºC (Fig. S1 supplementary information). Plant infection was facilitated by inoculating the soil with 20 cc of the mycelial culture into each cell. After inoculation, seedlings were lightly watered and flooded the following day in chlorine-free water to stimulate sporangia production and release of infective zoospores.

The simulation of the mechanical damage that an herbivore could cause on the seedling was made from cut off of the upper third of the plant. Mechanical damage does not always have the same effect on chemical defence induction as natural herbivory (Baldwin 1990), but it allows to control both the timing and type of damage, independent of any putative inducing or inhibitory effects of specific herbivore-derived elicitors (Pardo *et al.* 2018, Moctezuma *et al.* 2014). In this work, aimed at analyzing intra and inter-population differences in response to damage applying the same treatment, this artificial herbivory represents a suitable approach.

The control treatment provides the constitutive defences of the studied seedlings and the double treatment offers the possibility of analyzing interactions between both biotic damages. The development of external symptoms (chlorotic, wilted or dead foliage) and evolution of mortality and resprouting were recorded daily for each plant during four months.

Sampling and secondary metabolites quantification

Three weeks after the application of treatments, when the symptoms were already evident in all the treatments and mortality began to increase, sampling was performed for the quantification of secondary metabolites. Three samples per family of still-living seedlings (864 plants in total) were harvested, removing the substrate carefully to avoid plant damage and subsequently processing the sample for freezing at -80ºC. To confirm Koch´s postulates, from the fine roots of plants infected with *Pc,* the pathogen was successfully re-isolated in selective medium NARPH (Nystatin-Ampicillin-Rifampicin-Pentachloronitrobenzene-Hymexazol) corn meal agar, a modified medium of PARPH (Jeffers and Martin, 1986). As expected, *Pc* was not found in seedlings from control treatment and mechanical defoliation. The timing for the experiment was decided to assess the induced systemic response of the seedlings, thus giving enough time to the whole plant to activate the response at distal sites.

Previous studies on chemical characterization of defensive compounds in holm oak (Pulido *et al.* 2019) concluded that the leaf phenolic content is the most stable throughout the year (stems, roots and acorns oscillate depending on the season), so the extraction of phenols in leaves was the chosen method. Frozen seedlings were weighed and five to six leaves per plant of similar developmental stage were extracted for determination of phenolic content as described by Gallardo *et al.* (2019). Leaf samples were then freeze-dried (Telstar LyoQuest lyophilizer, temperature -55ºC and 0.001-0.002 mbar pressure) and ground to a fine particle size. Once the milling was completed, tissue sampling was stored at -80ºC until analysis. The phenolic content of the plants was extracted from lyophilized material with 70% (v/v) aqueous methanol for 60 minutes in an ultrasonic bath at room temperature. The crude extracts were centrifuged at 10000 rpm for 5 minutes at 4ºC and the supernatant was collected and stored at -80ºC.

The total phenolics content (Tp) of the extract was determined by the Folin-Ciocalteu method (Makkar, 2003). Crude extracts were mixed with 50 volumes of 10% Folin-Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) and 40 volumes of 7.5% (w/v) sodium carbonate. In the control tube, the extract volume was replaced by methanol. The mixture was stirred gently and maintained in the dark and at room temperature for 45 minutes. After incubation, the absorbance was measured at 725 nm. Gallic acid (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used as standard and results were expressed as milligrams of gallic acid equivalents (G.A.E.) per gram of lyophilized sample.

The total tannins content (Tt) was determined by the agarose gel radial diffusion method, measuring the ring-area of the precipitation with bovine serum albumin (BSA; Hagerman 1987). Gel Petri dishes were prepared using 1% agarose (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), containing a solution of 50 mM acetid acid, 60 µM ascorbic acid and 0,1% BSA. Uniform wells were made on plates containing the same volumen of solidified agarose and constant volumes of samples were loaded and incubated at 30ºC for 120 h. A calibration was performed with tannic acid (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and results were expressed as milligrams of tannic acid equivalents (T.A.E.) per gram of lyophilized sample.

The butanol–HCl assay (Porter *et al.* 1986) was used to quantify condensed tannins (Ct) using procyanidin B2 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) as a reference compound. Briefly, crude extracts were mixed with 100 volumes of n-butanol/acetone 1:1 (46% each) plus HCL (1.85%) and ferric ammonium sulphate (0.04%). In the control tube, the extract volume was replaced by methanol. Samples were heated at 70º. After 45 minutes of incubation, the samples were cooled and the absorbance at 550 nm was measured, with final results expressed as milligrams of procyanidin B equivalents (PB.E.) per gram of lyophilized sample.

Statistical analysis

First, the effects of region, population (nested in region), family (nested in population) and treatments on secondary metabolite patterns were analysed through a general linear mixed model (GLMM). The effects of germination time and seedling size (measured from their weight during the harvest of samples) on chemical defences were analysed with Pearson's linear correlation. To test for inducibility upon mechanical damage and inoculation with *Pc*, differences between constitutive defences (control treatment) and those induced by each treatment were calculated. Region, population, family and treatment were used as random factors and the Tp content, Tt content and Ct content were used as dependent variables. Data were analysed to check normality (by Kolmogorov-Smirnov test) and homoscedasticity (through Levene's test). Interactions between treatment and region, treatment and population, and treatment and family were also included in the model. The components of the variance were also calculated for each measured variable from GLMM to analyse the amount of variation for each one. The effect of treatments was assessed by t-test using a two-tailed alternative.

The stress tolerance was estimated by survival recorded weekly. To analyse the survival time of plants after the application of treatments, the Kaplan-Meier estimate was used. Gehan's Wilcoxon test was used to assess differences among treatments in survival. Survival was analysed with the "Survival Analysis" module. The relationship between dependent variables and tolerance was calculated by Pearson´s linear correlation. Statistical analyses were performed in Statistica v10 software.

Results

Chemical defence induction by treatments

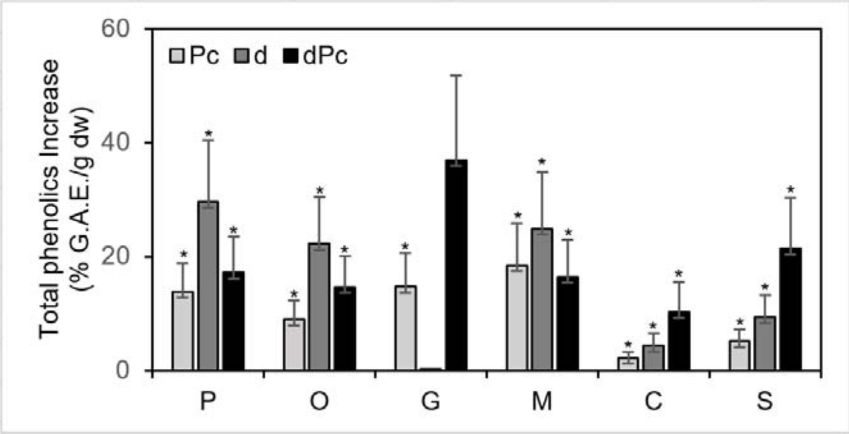
Highly significant differences among treatments, regions and families were found in the defensive chemical levels of holm oak leaves ($p<0.001$, Table 1, Fig. 2 and Fig. S2 for supplementary information). The Ct levels showed very significant differences ($p<0.001$) in the treatment/region interaction thus indicatinggeographical variation of the response to treatments. The local variation in the response to treatments showed very significant differences in Tt production ($p<0.001$).

All the applied treatments resulted in significant induction (Tukey's test $p<0.05$, Fig. 2). The induced response was based on the increase of their metabolic levels except in the Tt variable. In general, mean values of the three phenolic groups were always lowest in the control treatment and highest in the combined treatment for Tp and Ct, and they were highest for the defoliation treatment in Tt. The Folin-Ciocalteu analysis method yielded Tp concentrations in *Q. ilex* leaves between 36.44-56.98 mg GAE/g dw. Tt concentrations fluctuated more and were between 68.3-103.36 mg TAE/g dw, while Ct was the variable with the greatest range of variation, with values between 50.17-119.74 mg PBE/g dw.

The constitutive defences (control treatment) did not show significant correlations with those induced by the different treatments in all cases (Table S1 from supplementary information). The existence of correlation varied according to the region, the induction treatment and the phenolic group. The significant correlations were always negative. Regions with the highest significant and negative correlation between constitutive and induced defences were P and S.

The percentages of variance explained by treatment, region and family for Tp were 4.3%, 14.8% and 12.4% respectively. They were 0%, 20.4% and 14.3% respectively for Tt; and 11%, 8.6% and 8.8% for Ct, respectively (% variance calculated from results of GLMM, Fig. 3).

I)



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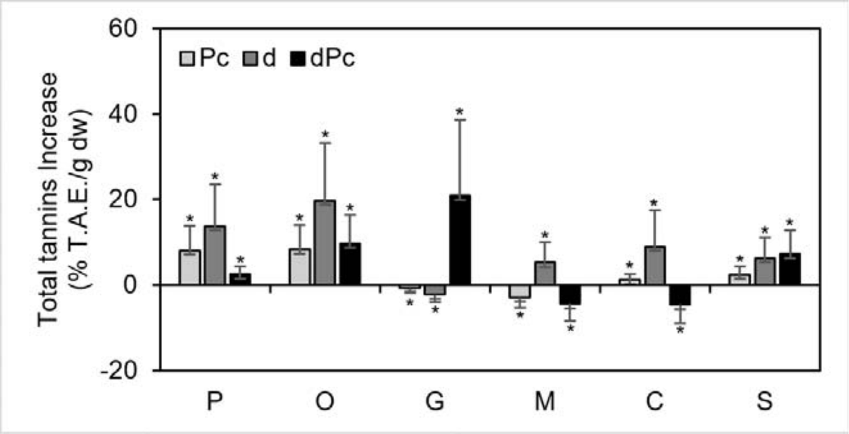

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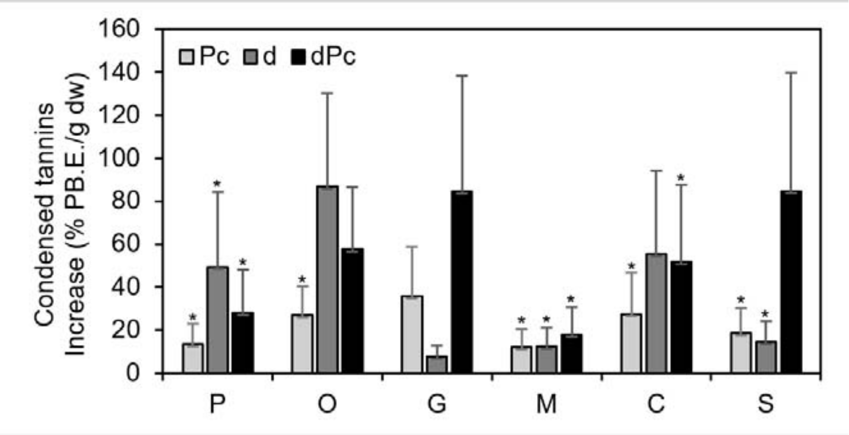


Fig. 2. Phenolic compounds induction in leaves of *Quercus ilex* (percentage increased compared to the control treatment) after biotic stress according to the region of origin (ordered from highest to lowest latitude): Picos de Europa (P), Ordesa (O), Guadarrama (G), Monfragüe (M), Cabañeros (C) and Sierra Nevada (S). (I) Total phenolics, (II) Total tannins, (III) Condensed tannins. Applied treatments: inoculation with *Phytophthora cinnamomi* (*Pc*), mechanical defoliation (d) and double treatment pathogen infection/mechanical defoliation (dPc). Absolute values of constitutive chemical defences (control treatment) and induced ones (by *Pc*, d and dPc treatments) are shown in Fig. S2 of the

"Supplementary information". Bar height represents the mean and whiskers are standard deviations.* Statistically significant differences between constitutive (control treatment) and induced defences.

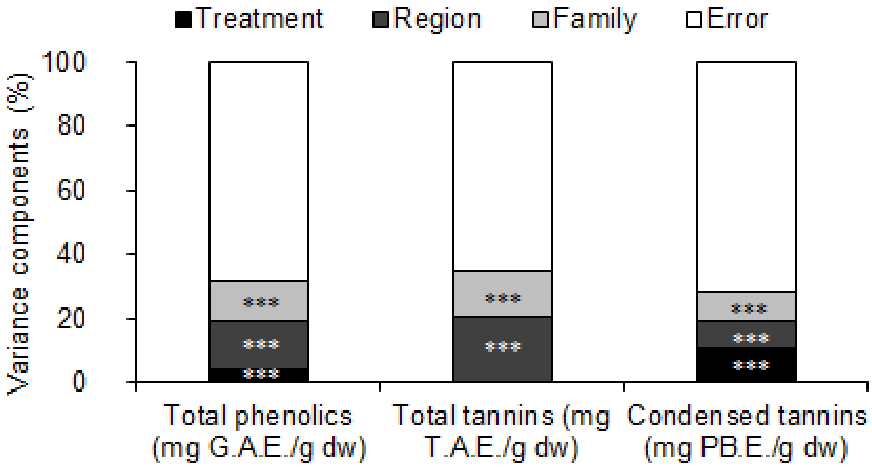


Fig 3: Components of the variance of total phenols, total tannins and condensed tannins among treatments, regions and families, expressed as percentages of the total variance. The error value corresponds to the differences among individuals within each family. The statistical significance of the major represented components is shown within the bars *** p<0.001.

Induced responses by stressors

The induced response showed significant differences and specific to the stressor that causes it (t-test $p<0.05$ in Tp, Tt and Ct, Table 1, Fig. 2). Mechanical defoliation and double treatment induced the greatest significant increases with respect to the control in Tp and Ct. The Tt variable showed in some regions a reduction instead of induction of defences, with decreases whose magnitude differed among treatments and regions. Grouping regions according to treatments, the production of Tp was minimal in the control treatment, it increased with infection by the pathogen, even more with defoliation and reached the highest values with the combined application of both treatments (r = 0.502, $p = 0.013$; Fig. S2 from supplementary information). Following the same sequence of treatments from lowest to highest induction of defences, the relationship between the Tt and the applied treatments was low and not significant (r = 0.195, $p = 0.361$). The maximum production of Tt resulted from the application of mechanical defoliation. On the contrary, Ct was the dependent variable with highest relationship to applied treatment in that sequence (r = 0.718, $p = 0.000$). As expected because Tt and Ct are part of Tp, there was a high and significant relationship among the Tp obtained by the Folin-Ciocalteu method, the Tt obtained by the BSA method and the accumulation of Ct measured by the Porter method (r from 0.67 to 0.82, p<0.001). The specific response to the stressor varied geographically and locally (Table 1).

Table 1. Effect of treatment, region, and family (nested in region) on total phenolics, total tannins and condensed tannins content in *Quercus ilex* leaves.

Effect	df	Total phenolics	Total tannins	Condensed tannins
Treatment	3	18.86***	6.47***	30.83***
Region	5	26.31***	36.52***	5.87***
Family (region)	58	2.58***	2.04***	2.48***
Treatment x region	15	1.92*	1.27	4.14***
Treatment x family (region)	160	1.12	1.45***	0.91

F-values are shown along with statistical significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns $p > 0.05$.

The induced response by combined biotic stress showed significant differences with respect to the treatments applied separately (Fig. 2 and Fig. S2 from supplementary information). There were significant differences between the defensive patterns induced by the pathogen infection and the double treatment (t-test $p<0.05$) and significant differences between mechanical defoliation and the double treatment (t-test $p< 0.05$). As described before, the double treatment was the main inducer of Tp and Ct in three of the six studied regions. However, the Tt variable showed reductions by the double treatment in two regions (Fig. 2). Combined stressors neutralized the production of defences in some regions, being intermediate between that induced by the pathogen and by mechanical defoliation. In other regions, it enhanced the response, further increasing the defences induced by the pathogen or by mechanical defoliation separately. The effect of the double treatment, therefore, differed according to regions and the sign of the interaction (neutralization or enhancement) varied geographically.

Induction and survival

Induction did not show a significant correlation with survival (p>0.05, Table S2 from supplementary information). Only in the O and S regions there was a significant correlation between Ct production and survival (r = 0.37 in O, r = -0.55 in S, p<0.05). Neither the absolute defence values (sum of constitutive and induced) showed a significant relationship with survival (p>0.05, Table S2 from supplementary information). Thus, only P, O and S regions showed a significant correlation between Ct production and survival (r = 0.39 in P, r = 0.43 in O, r = -0.59 in S, p<0.05). However, the relationship between constitutive defences and survival was positive and significant in Tp (r = 0.31, p = 0.011) and in Tt (r = 0.44, p = 0.0002). The constitutive Ct did not show a significant relationship (r = -0.07, p = 0.585).

All applied treatments reduced the probability of seedling survival (Gehan's Wilcoxon test $p<0.001$, Table 2, Fig. 4). Infection with the root pathogen was the most damaging (74.78% survival versus 91.4% of the control). *Pc* inoculation was the most aggressive biotic stress factor in the northern regions (minimum survival probability of 57.5% in P). In the southern area, M was more affected by defoliation (85.11% survival), C by infection with *Pc* (80% survival) and S by combined treatment (83.72% survival). In general, southern regions showed higher survival rates facing stressors than northern regions, highlighting M and S regions (survival probabilities greater than 80%). C showed high tolerance to defoliation but not so much to *Pc* infection and double treatment. It was observed that mortality due to defoliation increases when combined with *Pc* infection, that is, *Pc* increases the susceptibility of seedlings that also suffer herbivory. But on the other hand, defoliation combined with root infection caused lower mortality than when *Pc* acted alone (*Pc*, as mentioned before, was the stressor with the highest mortality).

Regarding the existence of resprout after the biotic stress condition, 12.72% of the affected plants with visible external symptoms resprouted. Regrowths were more frequent in plants subjected to double treatment (Table 2). It occurred in five of the six studied regions, except in C, where defoliation generated the highest resprout rate (6.25%). The resprout was greater in the southern regions of the Iberian Peninsula. The highest percentage of it was induced by the double treatment in S region (7.56%). M presented high percentages to defoliation and double treatment. The higher survival probabilities and regrowth rates obtained in the southern regions suggest their greater tolerance to the studied biotic stress factors.

Table 2. Probabilities of survival and resprout (%) in seedlings subjected to the following types of biotic stress: *Phytophthora cinnamomi* infection (*Pc*), mechanical defoliation (d), combined pathogen infection and mechanical defoliation (dPc) and control (c). Regions of origin (ordered from highest to lowest latitude): Picos de Europa (P), Ordesa (O), Guadarrama (G), Monfragüe (M), Cabañeros (C) and Sierra Nevada (S).

Region		Treatment			
	%	c	*Pc*	d	dPc
P	survival	80.00	52.50	62.79	61.90
	sprout	2.35	2.35	3.53	3.53
O	survival	92.86	63.04	88.64	69.77
	sprout	1.14	3.41	3.41	5.69
G	survival	100.0	50.00	90.00	84.62
	sprout	0	4.88	2.44	7.32
M	survival	95.45	91.67	85.11	87.5
	sprout	1.07	2.14	7.49	7.49
C	survival	95.0	80.0	92.5	82.5
	sprout	0.63	3.75	6.25	2.5
S	survival	92.50	88.64	86.67	83.72
	sprout	1.16	1.74	3.49	7.56
Average	survival	91.4	74.78	83.41	77.73
	sprout	1.27	2.64	4.31	4.5

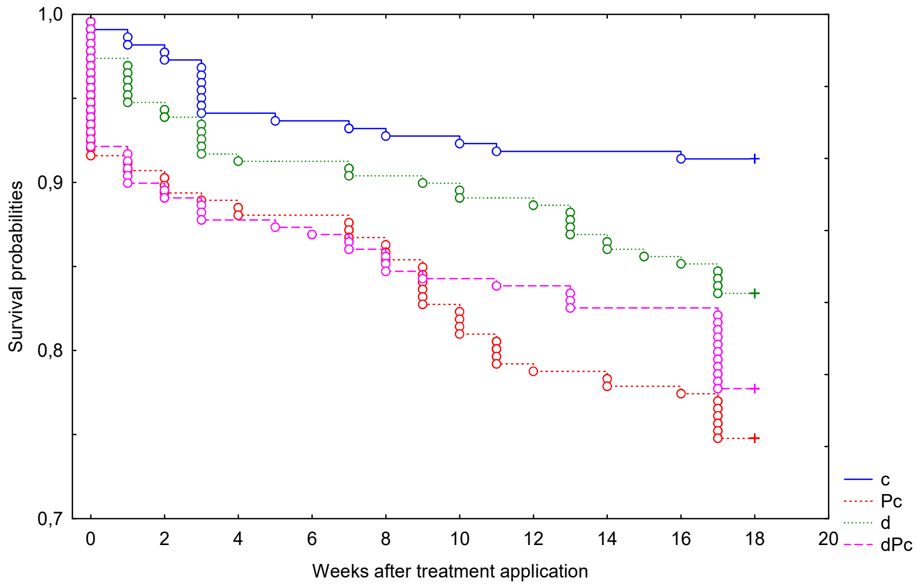


Fig. 4. Survival probabilities of *Quercus ilex* seedlings showing differences among applied treatments ($p<0.001$, $X^2 = 23.95$, df = 3, n = 905 plants). Infection with the root pathogen *Phytophthora cinnamomi* (*Pc*) was the most lethal treatment. Seedlings subjected to combined stress were also more vulnerable (dPc). Mechanical defoliation (d) produced losses but, in general, plants were able to respond with resprout. Control seedlings (c) also suffered some mortality, but within the usual rates during the first months of life.

Discussion

This study contributes to the current body of literature expanding about how plant populations from different regions differently invest in defensive traits. The individual and ecotypic variability generates differences in the response of the holm oak chemical defences against the different biotic stress factors, showing certain specificity and interactions in the production of tannins according to the stressor. This could be an adaptive strategy to the diversity of the pathogen and herbivore community present in the ecosystem. And it would be an interesting trait for the selection of better adapted to increasing biotic stressors genetic material.

Chemical defences induced by stressors

Holm oak constitutive defences varied significantly among families and regions. The region was the main source of variation of the three phenolic groups in agreement with Rodríguez-Romero *et al.* (2020) for the production of Tp and Ct. Solla *et al.* (2016) explored the geographical variation in the holm oak constitutive Tt, finding important local but not geographical differences, although their work focused on south-central Iberian Peninsula regions. On the other hand, Moreira *et al.* (2018) studied the constitutive Ct variation in 38 populations along an 18º latitudinal gradient of *Q. robur*, also obtaining significant differences. Pardo *et al.* (2018) explore the spatial variation in *Prunus lusitanica* Tp for its entire range of distribution, also finding geographic and local differences in the constitutive defences. López-Goldar *et al.* (2019), using a *Pinus pinaster* clonal collection in different countries of the Mediterranean Basin, also found geographic but not local differences within populations, by quantifying 98 plant secondary metabolites. This spatial variation could be consequence of local adaptation mechanisms promoted by host-stressors coexistence, but also of the resources availability to produce defences or other unexplored factors. The results of this work follow the same trend, although the disparity with Solla *et al.* (2016) in the Tt group shows the need to continue improving our knowledge of the constitutive defences chemical patterns in *Quercus*.

Regarding the induced defences, these showed significant differences according to the region (geographic level), family (local level) and the applied treatment (specific induction). Following the same pattern than constitutive defences, the variance of the three phenolic groups was better explained by region, although in the case of induced Ct it was similar to the variance explained by the family. Although there are some works on spatial variation in the effects of biotic stress or the production of chemical defences, few studies combine geographical variation with induction of defences by defoliation and pathogen infections. However, of those cited above, Pardo *et al.* (2018) also explored the spatial variation of Tp induced by mechanical defoliation in *Prunus lusitanica*, also finding significant local and geographical differences in induced defences. In addition, the three chemical traits evaluated in this work were highly variable within families and among regions, especially Ct. The same occurs in *Pinus pinaster* Tp from the Iberian Peninsula against the attack of the pine wilt nematode (*Bursaphelenchus xylophilus*; Zas *et al.* 2015).

As expected, the three evaluated variables showed a strong and positive correlation due to be overlapping fractions (Tp includes Tt and Tt includes Ct), as also shown by Gallardo *et al.* (2019) with holm oak seedlings from one provenance against mechanical defoliation and *Pc* infection. However, the effect of stress treatment on seedlings at the local level (among families) is better explained by Tt, while the major geographical differences in the induction of chemical defences are showed by the Ct production. Therefore, the best variable to characterize the differences in the induced defences against biotic stress are Ct at the geographical level. In fact, its use to analyze plant-herbivore interactions in the literature is common (War *et al.* 2012). The results of this work reinforce the Ct choice to analyze plant-biotic stress interactions and, above all, their spatial variation.

Specific defensive response: Trade-offs and cumulative effects.

The defensive response is triggered by biotic stress factors and shows significant differences depending on the stressor. The correlation between the three measured variables was high and positive, especially in the induction of defences by *Pc* infection. This specificity is also demonstrated by Gallardo *et al.* (2019) in holm oak seedlings subjected to the root pathogen infection and mechanical defoliation from the same provenance, identifying the *Quercus* sequences encoding enzymes for early steps of the biosynthesis of phenolic compounds, like hydrolysable tannins and Ct among others, plus genes involved in the late steps of Ct biosynthesis. Their study showed differences in response that were dependent on the stressor, especially in the Tt production for Picos de Europa region, also studied in our work. The results of this study show that this specific induction of the stressor can be extrapolated to its spatial variability analysis. Furthermore, the combined treatment of pathogen inoculation with mechanical defoliation induced the highest production of Tp and Ct in most of the studied regions. Mechanical defoliation alone caused the largest increases in Tt production. Despite several studies about plant-herbivore interactions are focused on Ct, it was observed that Tt, that is, those that include hydrolysable tannins, better characterize the induction of defences against herbivory stress in the holm oak. In fact, some specific compounds that take part of hydrolysable tannins are closely related to herbivory resistance (War *et al.* 2012, Marsh *et al.* 2020).

In this study the induced response was not always an increase in the level of chemical defences. On the contrary, in the case of Tt and especially in M and C regions, there is a down regulation between defence production and the combination of stressors. When defoliation and *Pc* infection act combined, the response capacity in Tt production decreases, which suggests that very high levels of stress could limit the resources needed to produce more defences. Mechanical defoliation induces the biosynthesis of Tt but infection by *Phytophthora* interferes with its regulation, increasing the seedlings susceptibility to biotic stress (Gallardo *et al.* 2019). This suggests that in root pathogen-affected stands, an intense defoliation event could increase the vulnerability of the holm oak, reducing its ability to restore after the attack.

Constitutive and induced defences and their consequences for plant survival

Tolerance to biotic stress, both caused by mechanical defoliation and induced by the pathogen *Pc* infection, differed significantly among Iberian provenances. In all cases, the biotic stressors reduced the survival probabilities. The most harmful was the root infection alone; even more that the combined root infection combined with defoliation. Generally, the southern regions were more tolerant to biotic stress from root and aerial damages. M and S were the most tolerant regions to the applied treatments, exceeding in all cases more than 80% survival and also high resprout rates. No other works are known about the geographical variation of chemical defences studied here and tolerance against the applied biotic stressors in holm oak. However, Camisón *et al.* (2019) profiles the defensive chemical patterns in *C. sativa* plants to *Pc*, finding more Tp and Ct in susceptible plants to the pathogen than in resistant ones. On the contrary, Cahill *et al.* (1989) showed that phenolic deposition was higher in the more resistant species than in the susceptible ones during the root infection by *Pc*. The role of tannins in plant defence has been widely studied (Peters and Constabel 2002, Roitto *et al.* 2009, Barbehenn and Constabel 2011, War *et al.* 2012). However, in some cases no effects have been found on them (Keinanen *et al.* 1999, Hikosaka *et al.* 2005). Furthermore, specific compounds within these large phenolic groups are those identified for their key role in resistance to different stressors (Feeny 1968). Focusing on the defensive response of the plant to *Phytophthora* spp, several studies cite the involvement of phenolic compounds in this interaction (Picard *et al.* 2000, Lherminier *et al.* 2003, Horta *et al.* 2010). Stong *et al.* (2013) cite this phenolic involvement with *P. ramorum* and Gallardo *et al.* (2019) do it with *Pc*. But the controversy obtained in the experiments to date show the role of tannins as defences against biotic stress have still to be clarified, and even more so its effect on pathogens. In our work, we related the tolerance variability with the induced chemical defence patterns obtained for each provenance, although there was no clear correspondence between them. A priori, the greater the induction, the more phenotypically plastic the plants will be, thus reducing the possibility that biotic stressors adapt to the induced chemical compounds (Howe and Jander 2008, Agrawal 2011). However, the most biotic stress-tolerant regions in the greenhouse were not those with the greatest induced defences. The case of M stands out, which even having the highest constitutive levels of Ct, barely underwent induction by the applied treatments and nevertheless, showed high tolerance to biotic stress. This tolerance showed a greater and positive correlation with constitutive defensive chemical levels -studied in more detail in Rodríguez-Romero *et al.* (2020)- than with those induced. This could be due to the strategy cited by Jorrín-Novo and Navarro-Cerrillo (2014) that highlights the metabolic costs (Agrawal *et al.* 2002) for the holm oak to induce defences. This induction could be avoided in biotic attack events when they are punctual. If herbivory or infection were frequent, or even permanent, as is often the case in the southern Iberian Peninsula, the differences between constitutive and induced levels could tend to be smaller. In our work, the regions with higher herbivore impact showed higher constitutive defences (see also Rodríguez-Romero *et al.* 2020), inducing fewer defences but responding with greater tolerance to biotic stressors. Defoliation as a permanent biotic stressor could have driven the high constitutive defence selection in southern regions, also facilitating a greater tolerance to other stressors such as *Pc*. In fact, our results show that the most susceptible to the root pathogen-regions were those in the north of the Peninsula.

Implications for breeding programs

Induced resistance could be exploited as an important tool for the integrated management of diseases and pests in forests with increasing biotic stressor scenarios. In addition, it would serve to predict catastrophic events or periods of high stress and to get ahead of them. This would allow the agrosystems to be strengthened before they reach severe levels of decline. However, our compression of these defensive mechanisms is still limited, so the authors highlight the need to continue working in this area.

The fact that the response is specific to the inducing stressor and that there are interactions between them complicates the tolerant and/or resistant genotype selection to pathogens. However, it constitutes a first step in the development of a holm oak decline management program that considers the current intraspecific geographical variation. This species also has a high genetic diversity and the conservation of its natural and managed forests is vital to maintain this diversity in its reservoirs, guaranteeing a varied induced response. Detailed studies of the specific induced chemical compounds by each biotic stressor would be very necessary to understand the intraspecific geographical variability in the allocation of defensive resources.

References

Agrawal AA (2011) Current trends in the evolutionary ecology of plant defence. Funct Ecol 25:420-32

Agrawal AA, Janssen A, Bruin J, Posthumus MA, Sabelis MW (2002) An ecological cost of plant defence: attractiveness of bitter cucumber plants to natural enemies of herbivores. Ecol Lett 5:377-85

Balci Y and Halmschlager E (2003) Incidence of *Phytophthora* species in oak forests in Austria and their possible involvement in oak decline. Forest Pathol 33:157–174

Baldwin IT (1990) Herbivory simulations in ecological research. Trends Ecol Evol 5:91–3.

Baptist F, Zinger L, Clement JC, Gallet C, Guillemin R, Martins JM, Sage L, Shahnavaz B, Choler Ph, Geremia R (2008) Tannin impacts on microbial diversity and the functioning of alpine soils: a multidisciplinary approach. Environ Microbiol 10:99–809

Barbehenn RV, Constabel CP (2011) Tannins in plant-herbivore interactions. Phytochemistry 72:1551-65

Barbehenn RV, Jaros A, Lee G, Mozola C, Weir Q, Salminen JP (2009) Tree resistance to *Lymantria dispar* caterpillars: importance and limitations of foliar tannin composition. Oecologia 159(4): 777-788

Boots M, Best A (2018). The evolution of constitutive and induced defences to infectious disease. Proceedings of the Royal Society B: Biological Sciences 285(1883), 20180658

Brasier CM (1992) Oak tree mortality in Iberia. Nature 360(6404), 539

Brasier CM, Robredo F, Ferraz JFP (1993) Evidence for *Phytophthora cinnamomi* involvement in Iberian oak decline. Plant Pathol 42:140–145

Brasier CM (1996) *Phytophthora cinnamomi* and oak decline in Southern Europe. Environmental constraints, including climate change. Ann For Sci 53(2-3): 347-358

Burgess TI, Scott JK, McDougall KL, Ota N, Hardy GESJ (2017). Current and projected global distribution of Phytophthora cinnamomi, one of the world's worst plant pathogens. Global Change Biology 23(4): 1661-1674

Cahill D, Legge B, Weste GM (1989) Cellular and histological changes induced by *Phytophthora cinnamomi* in a group of plant species ranging from fully susceptible to fully resistant. Phytopathology 79:417–424

Camilo-Alves CSP, da Clara MIE, Ribeiro NA (2013) Decline of Mediterranean oak trees and its association with *Phytophthora cinnamomi*: a review. Eur J For Res 132:411–432

Camisón A, Martín MA, Sánchez-Bel P, Flors V, Alcaide F, Morcuende D, Pinto G, Solla A (2019) Hormone and secondary metabolite profiling in chestnut during susceptible and resistant interactions with *Phytophthora cinnamomi*. J Plant Physiol 241:153030

Carbonero MD, Blázquez A, Fernández-Rebollo P (2004). Acorn production and defoliation as vigour signs in *Quercus* spp. Connections with edaphic conditions. In: Reunión Científica de la Sociedad Española para el Estudio de los Pastos, 44. Salamanca (España)

CICYTEX-INIAV (2020) Gestión y prevención de la enfermedad causada por *Phytophthora cinnamomi* en dehesas y montados. Instituto Nacional de Investigação Agrária e Veterinária, I.P. (INIAV, I.P.) y Centro de Investigaciones Científicas y Tecnológicas de Extremadura (CICYTEX). Mérida, Spain

Conrad AO, McPherson BA, Wood DL, Madden LV, Bonello P (2017) Constitutive phenolic biomarkers identify naïve *Quercus agrifolia* resistant to *Phytophthora ramorum*, the causal agent of sudden oak death. Tree Physiol 37(12):1686-1696

Corcobado T, Cubera E, Moreno G, Solla A (2013) *Quercus ilex* forests are influenced by annual variations in water table, soil water deficit and fine root loss caused by *Phytophthora cinnamomi*. Agric. For. Meteorol. 169:92–99

Corcobado T, Cubera E, Juarez E, Moreno G, Solla A (2014) Drought events determine performance of *Quercus ilex* seedlings and increase their susceptibility to *Phytophthora cinnamomi*. Agric For Meteorol 192-193:1–8

Corcobado T, Miranda-Torres JJ, Martín-García J, Jung T, Solla A (2017) Early survival of *Quercus ilex* subspecies from different populations after infections and co-infections by multiple *Phytophthora* species. Plant Pathol 66:792–804

Daglia M (2012) Polyphenols as antimicrobial agents. Curr. Opin. Biotechnol 23(2):174-181

Den Herder M, Moreno G, Mosquera-Losada RM, Palma JH, Sidiropoulou A, Freijanes JJS, Crous-Duran J, Paulo JA, Tomé M, Pantera A, Mantzanas K, Pachana P, Papadopoulos A, Plieninger T, Burgess PJ (2017). Current extent and stratification of agroforestry in the European Union. Agr Ecosyst Environ 241: 121-132

Díaz M, Pulido FJ (2009) 6310 Dehesas perennifolias de *Quercus* spp. En: VV.AA., Bases ecológicas preliminares para la conservación de los tipos de hábitat de interés comunitario en España. Madrid: Ministerio de Medio Ambiente, y Medio Rural y Marino. 69 p

Dicke M, Hilker M (2003) Induced plant defences: from molecular biology to evolutionary ecology. Basic Appl Ecol 4(1): 3–14

Dukes JS, Pontius J, Orwig D, Garnas JR, Rodgers VL, Brazee N, Cooke B, Theoharides KA, Stange EE, Harringto R, Ehrenfeld J, Gurevitch J, Lerdau M, Stinson K, Wick R, Ayres M (2009) Responses of insect pests, pathogens, and invasive plant species to climate change in the forests of northeastern North America: what can we predict? Can J For Res 39:231–248

Feeny PP (1968) Effect of oak leaf tannins on larval growth of the winter moth *Operophtera brumata*. J Insect Physiol 14:805-17

Fernández-Cancio Á, Manrique E, Navarro-Cerrillo R, Fernández R (2004) La Seca como problema: su evolución. Enfoque Climático. Ministerio de Medio Ambiente. Dirección General para la Biodiversidad

Gallardo A, Morcuende D, Solla A, Moreno G, Pulido FJ, Quesada A (2019) Regulation by biotic stress of tannins biosynthesis in *Quercus ilex*: crosstalk between defoliation and *Phytophthora cinnamomi* infection. Physiol Plant 165(2): 319-329

García A, Perea J, Acero R, Angón E, Toro P, Rodríguez V, Castro AGG (2010) Structural characterization of extensive farms in Andalusian dehesas. Archivos de zootecnia 59(228):577-588

Hagerman AE (1987) Radial diffusion method for determining tannin in plant extracts. J Chem Ecol 13: 437–449

Haslam E (2007) Vegetable tannins-lessons of a pythochemical lifetime. Phytochemistry 68:2713-2721

Hikosaka K, Takashima T, Kabeya D, Hirose T, Kamata N (2005) Biomass allocation and leaf chemical defence in defoliated seedlings of *Quercus serrata* with respect to carbon-nitrogen balance. Ann Bot 95:1025-32

Horta M, Caetano P, Medeira C, Maia I, Cravador A (2010) Involvement of the β-cinnamomin elicitin in infection and colonisation of cork oak roots by *Phytophthora cinnamomi*. Eur J Plant Pathol 127: 427-436

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annu Rev Plant Biol 59:41-66

Humphrey J, Swaine M (1997). Factors affecting the natural regeneration of *Quercus* in Scottish oakwoods. Insect defoliation of trees and seedlings. J Appl Ecol 34(3): 585-593

Jeffers NS, Martin JB (1986) Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Disease 70:1038-1043

Jorrín-Novo J, Navarro-Cerrillo RM (2014). Variabilidad y respuesta a distintos estreses en poblaciones de encina (*Quercus ilex* L.) en Andalucía mediante una aproximación proteómica. Ecosistemas 23(2):99-107

Jung T, Blaschke H, Neumann P (1996) Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. Eur J Forest Pathol 26:253–272

Jung T, Vettraino AM, Cech T, Vannini A (2013) The impact of invasive *Phytophthora* species in European forests 146–158 in Lamour, K. (ed.) *Phytophthora*: A global perspective. CABI international, Wallingford, UK.

Keinanen M, Julkunen-Tiitto R, Mutikainen P, Walls M, Ovaska J, Vapaavuori E (1999) Trade-offs in phenolic metabolism of silver birch: effects of fertilization, defoliation and genotype. Ecology 80:1970-86

Kraus TEC, Dahlgren EC, Zasoski RJ (2003) Tannins in nutrient dynamics of forest ecosystems - a review. Plant and Soil 256(1):41–66

Lherminier J, Benhamou N, Larrue J, Milat ML, Boudon-Padieu E, Nicole M, Blein JP (2003) Cytological characterization of elicitin-induced protection in tobacco plants infected by *Phytophthora parasitica* or phytoplasma. Phytopathology 93(10):1308-1319

López-Goldar X, Villari C, Bonello P, Borg-Karlson AK, Grivet D, Sampedro L, Zas R (2019) Genetic variation in the constitutive defensive metabolome and its inducibility are geographically structured and largely determined by demographic processes in maritime pine. J Ecol 107(5):2464-2477

Lowe S, Browne M, Boudjelas S, De Poorter M (2000) 100 of the World´s Worst Invasive Alien Species. A selection from the Global Invasive Species Database. The IUCN Invasive Species Specialist Group (ISSG). IUCN 12 pp

Makkar HPS (2003) Measurement of total phenolics and tannins using Folin-Ciocalteu method. In: Quantification of tannins in tree and shrub foliage. Springer, Dordrecht, The Netherlands, 49–51

Marsh KJ, Wallis IR, Kulheim C, Clark R, Nicolle D, Foley WJ, Salminen JP (2020) New approaches to tannin analysis of leaves can be used to explain in vitro biological activities associated with herbivore defence. New Phytol 225(1):488-498

Moctezuma C, Hammerbacher A, Heil M, Gershenzon J, Méndez-Alonzo R, Oyama K (2014) Specific polyphenols and tannins are associated with defense against insect herbivores in the tropical oak *Quercus oleoides*. J Chem Ecol 40:458–467

Moreira X, Castagneyrol B, Abdala-Roberts L, Berny-Mier y Teran JC, Timmermans BGH, Bruun HH, Covelo F, Glauser G, Rasmann S, Tack AJM (2018) Latitudinal variation in plant chemical defences drives latitudinal patterns of leaf herbivory. Ecography 41(7):1124-1134

Moreno G, Aviron S, Berg S, Crous-Duran J, Franca A, de Jalón SG, Hartel T, Mirck J, Pantera A, Palma JHN, Paulo JA, Re GA, Sanna F, Thenail C, Varga A, Viaud V, Burgess PJ (2018). Agroforestry systems of high nature and cultural value in Europe: provision of commercial goods and other ecosystem services. Agrofor syst 92(4): 877-891

Moreno G, Pulido FJ (2009) The functioning, management, and persistente of dehesas. In: Agroforestry Systems in Europe. Current Status and Future prospects. Riguero-Rodriguez A, Mosquera-Losada MR, McAdam J. (eds.). Advances in Agroforestry Series, Springer Publishers pp 127-161.

Oliva A, Lahoz E, Contillo R, Aliota G (1999) Fungistatic activity of *Ruta graveolens* extract and its allelochemicals. Chem Ecol 25(3):519-526

Olmo M, Andicoberry S, García-Moreno AM, Caño AB, Gómez-Giráldez PJ, Carbonero MD, Zamora-Rojas E, Fernández-Rebollo P, Villar R, Guerrero-Ginel JE (2017).

Producción de bellota y defoliación en el arbolado de las dehesas del proyecto Life+bioDehesa. In: 7º Congreso Forestal Español. Plasencia (España).

Pagán I, García-Arenal F (2020). Tolerance of plants to pathogens: a unifying view. Annual Review of Phytopathology 010820-012749

Pardo A, Cáceres Y, Pulido F (2018) Intraspecific variation in heritable secondary metabolites and defensive strategies in a relict tree. J Plant Ecol 11: 256–265

Pautasso M, Doring TF, Garbelotto M, Pellis L, Jeger MJ (2012) Impacts of climate change on plant diseases-opinions and trends. Eur J Plant Pathol 133:295-313

Pérez-Ramos IM (2014) El milagro de regenerar en especies mediterráneas de *Quercus* ¿Cómo serán los bosques del futuro? Ecosistemas 23(2):13-17

Pérez-Sierra A, López-García C, León M, García-Jiménez J, Abad-Campos P, Jung T (2013) Previously unrecorded low-temperature *Phytophthora* species associated with *Quercus* decline in a Mediterranean forest in eastern Spain. For Pathol 43:331–339

Peters DJ, Constabel CP (2002) Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*) Plant J 32:701-12

Picard K, Ponchet M, Blein JP, Rey P, Tirilly Y, Benhamou N (2000). Oligandrin, a proteinaceous molecule produced by the mycoparasite Pythium oligandrum induces resistance to Phytophthora parasitica infection in tomato plants. Plant Physiol 124:379-395

Plieninger T, Rolo V, Moreno G (2010). Large-scale patterns of *Quercus ilex, Quercus suber* and *Quercus pyrenaica* regeneration in Central-Western Spain. Ecosystems 13: 644-660

Porter LJ, Hirstich LN, Chan BG, 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochem 25:223-230

Pulido F, Gallardo A, Morcuende D, Moreno G, Rodríguez-Romero M, Solla A, Fernández-Rebollo P, Quesada A (2019) Determinantes de la resistencia al estrés biótico en una especie forestal modelo: la encina (*Quercus ilex*) en los Parques Nacionales. Organismo Autónomo Parques Nacionales.

Robin C, Desprez-Loustau M, Capron G, Delatour C (1998) First record of *Phytophthora cinnamomi* on cork and holm oaks in France and evidence of pathogenicity. Ann Sci For 55(8):869–883

Rodà F, Vayreda J, Ninyerola M (2009) 9340 Encinares de *Quercus ilex* y *Quercus rotundifolia*. En: VV.AA., Bases ecológicas preliminares para la conservación de los tipos de hábitat de interés comunitario en España. Madrid: Ministerio de Medio Ambiente, y Medio Rural y Marino. 94 p

Rodríguez-Romero M, Gallardo A, Pulido F (2020) Geographical and within-population variation of constitutive chemical defences in a Mediterranean oak (*Quercus ilex*). Forest Systems 29(2)

Roitto M, Rautio P, Markkola A, Julkunen-Tiitto R, Varama M, Saravesi K, Tuomi J (2009) Induced accumulation of phenolics and sawfly performance in Scots pine in response to previous defoliation. Tree Physiol 29:207-16

Sánchez ME, Caetano P, Romero MA, Navarro RM, Trapero A (2006) *Phytophthora* root rot as the main factor of oak decline in southern Spain. In: Brasier C, Jung T, Oßwald W, eds. Progress in Research on *Phytophthora* Diseases of Forest Trees. Farnham, Surrey, UK: Forest Research, 149-54

Santini A, Ghelardini L, De Pace C, Desprez-Loustau ML, Capretti P, Chandelier A, Cech T, Chira D, Diamandis S, Gaitniekis T, Hantula J, Holdenrieder O, Jankovsky L, Jung T, Jurc D, Kirisits T, Kunca A, Lygis V, Malecka M, Marcais B, Schmitz S, Schumacher J, Solheim H, Solla A, Szabó I, Tsopelas P, Vannini A, Vetrraino AM, Webber J, Woodward S, Stenlid J (2013). Biogeographical patterns and determinants of invasion by forest pathogens in Europe. New Phytol 197(1): 238-250.

Scalbert A (1991) Antimicrobial properties of tannins. Phytochemistry 30(12):3875–3883

Scanu B, Linaldeddu BT, Franceschini A, Anselmi N, Vannini A, Vettraino AM (2013) Occurrence of *Phytophthora cinnamomi* in cork oak forests in Italy. For Pathol 438(4):340–343

Schmid S, Palacio S, Hoch G (2017). Growth reduction after defoliation is independent of CO 2 supply in deciduous and evergreen young oaks. New Phytol 214(4): 1479-1490

Schultz J (1988) Plant responses to herbivores. Trends Ecol Evol 3(2):45-49

Solla A, Slobodan M, Gallardo A, Bueno A, Corcobado T, Cáceres Y, Morcuende D, Quesada A, Moreno G, Pulido FJ (2016) Genetic determination of tannins and herbivore resistance in *Quercus ilex*. Tree Genet Genom 12(6):117

Sork VL, Stowe KA, Hochwender C (1993) Evidence for local adaptation in closely adjacent subpopulations of northern red oak (*Quercus rubra* L.) expressed as resistance to leaf herbivores. American Naturalist 142:928–936

Stong RA, Kolodny E, Kelsey RG, González-Hernández MP, Vivanco JM, Manter DK (2013) Effect of plant sterols and tannins on *Phytophthora ramorum* growth and sporulation. J Chem Ecol 39(6):733-743

Tiberi R, Branco M, Bracalini M, Croci F, Panzavolta T (2016) Cork oak pests: a review of insect damage and management. Ann For Sci 73:219-232

War AR, Paulraj MG, Ahmad T, Buhroo AA, Hussain B, Ignacimuthu S, Sharma HC (2012) Mechanisms of plant defense against insect herbivores. Plant Signal Behav 7:1306-1320

Zas R, Moreira X, Ramos M, Lima MRM, Nunes da Silva M, Solla A, Vasconcelos MW, Sampedro L (2015) Intraspecific variation of anatomical and chemical defensive traits in Maritime pine *(Pinus pinaster*) as factors in susceptibility to the pinewood nematode (*Bursaphelenchus xylophilus*). Trees 29:663-673

Supplementary information

Table S1. Pearson's correlations (r values) between constitutive and induced chemical defences in *Quercus ilex* leaves (total phenols Tp, total tannins Tt and condensed tannins Ct) by regions and stressors. Regions of origin (ordered from highest to lowest latitude): Picos de Europa (P), Ordesa (O), Guadarrama (G), Monfragüe (M), Cabañeros (C) and Sierra Nevada (S). Applied treatments: inoculation with *Phytophthora cinnamomi* (*Pc*), mechanical defoliation (d) and double treatment pathogen infection/mechanical defoliation (dPc).

P	Tp by *Pc*	Ct by *Pc*	Tt by *Pc*	Tp by d	Ct by d	Tt by d	Tp by dPc	Ct by dPc	Tt by dPc
Constitutive Tp	-0.59*			-0.72*			-0.6*		
Constitutive Ct		-0.55			-0.54			-0.61*	
Constitutive Tt			-0.69*			-0,7.*			-0.61*

O	Tp by Pc	Ct by Pc	Tt by Pc	Tp by d	Ct by d	Tt by d	Tp by dPc	Ct by dPc	Tt by dPc
Constitutive Tp	-0.91*			-0.68*			-0.53		
Constitutive Ct		-0.57			-0.68*			-0.49	
Constitutive Tt			-0.84*			-0.41			-0.47

G	Tp by Pc	Ct by Pc	Tt by Pc	Tp by d	Ct by d	Tt by d	Tp by dPc	Ct by dPc	Tt by dPc
Constitutive Tp	0.22			-0.12			0.78		
Constitutive Ct		-0.24			-0.37			-0.64	
Constitutive Tt			0.79			0.32			0.51

M	Tp by Pc	Ct by Pc	Tt by Pc	Tp by d	Ct by d	Tt by d	Tp by dPc	Ct by dPc	Tt by dPc
Constitutive Tp	-0.56			-0.24			-0.73		
Constitutive Ct		-0.39			-0.69*			-0.79*	
Constitutive Tt			-0.58			0.14			-0.78*

C	Tp by Pc	Ct by Pc	Tt by Pc	Tp by d	Ct by d	Tt by d	Tp by dPc	Ct by dPc	Tt by dPc
Constitutive Tp	-0.57			-0.8*			-0.87*		
Constitutive Ct		-0.39			-0.48			-0.51	
Constitutive Tt			-0.59			-0.67*			-0.69*

S	Tp by Pc	Ct by Pc	Tt by Pc	Tp by d	Ct by d	Tt by d	Tp by dPc	Ct by dPc	Tt by dPc
Constitutive Tp	-0.8*			-0.73*			-0.68*		
Constitutive Ct		-0.62*			-0.25			-0.64*	
Constitutive Tt			-0.74*			-0.78*			-0.75*

* r-values with statistical significance: *$p < 0.05$; ns $p > 0.05$.

Table S2. Pearson's correlations (r values) among induced chemical defences, total defences and survival in *Quercus ilex* leaves (total phenols, total tannins and condensed tannins). Regions of origin (ordered from highest to lowest latitude): Picos de Europa (P), Ordesa (O), Guadarrama (G), Monfragüe (M), Cabañeros (C) and Sierra Nevada (S).

	Induced defences			Total defences (constitutive+induced)		
Region	Total phenolics	Total tannins	Condensed tannins	Total phenolics	Total tannins	Condensed tannins
P	0.24	0.09	0.28	0.29	0.12	0.39*
O	0.19	0.23	0.37*	0.27	0.28	0.43*
G	-0.28	-0.01	-0.15	-0.24	-0.01	-0.19
M	-0.12	-0.17	-0.05	-0.17	-0.21	-0.03
C	-0.09	0.32	0.1	-0.13	0.39	0.1
S	-0.21	-0.002	-0.55*	-0.29	-0.04	-0.59*

* r-values with statistical significance: *$p < 0.05$; ns $p > 0.05$

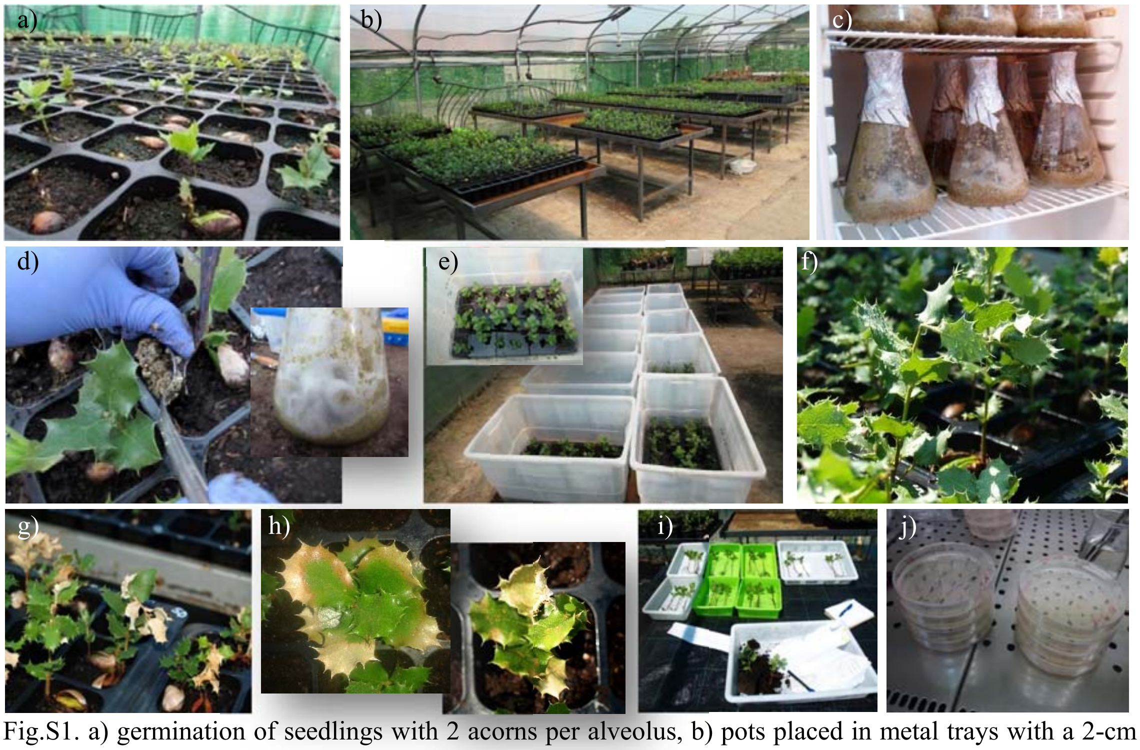
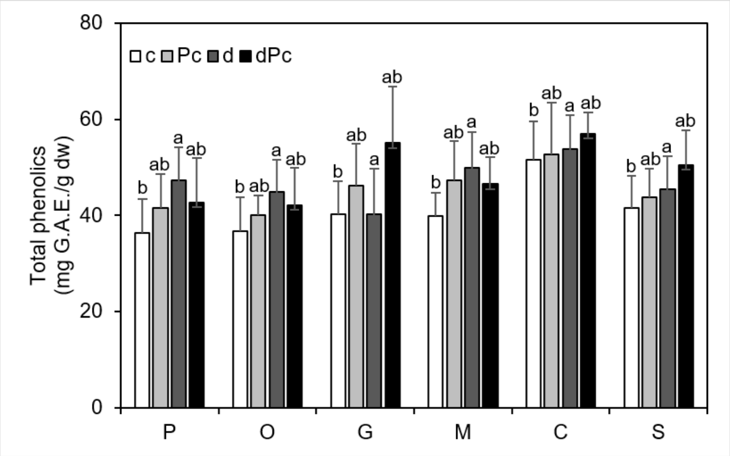


Fig.S1. a) germination of seedlings with 2 acorns per alveolus, b) pots placed in metal trays with a 2-cm deep water line to avoid water stress, c) *Phytophthora cinnamomi* inoculum growing in an incubator at 22ºC, d) inoculation with 20 cc of the mycelial culture into each cell, e) seedling flood to stimulate sporangia production and release of infective zoospores, f) mechanical wounding or mechanical defoliation of the upper third of the seedling, g) seedlings with "mechanical defoliation" treatment (d) one week later, h) inoculated seedlings (p) one week later, i) sampling for phenolic extraction, j) tests to confirm the infection in inoculated plant roots and the absence of the pathogen in the non-inoculated ones by means of seeding in selective medium NARPH.

I)



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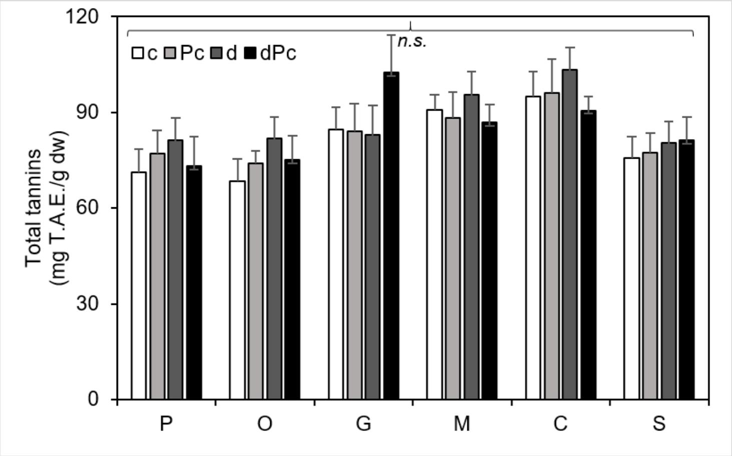

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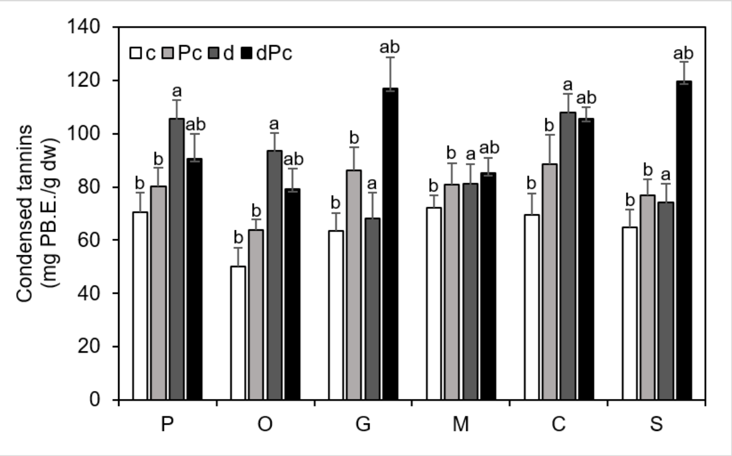

Fig.S2. Chemical analysis of phenolic compounds in *Quercus ilex* leaves after application of biotic stress treatment according to the region of origin (ordered from highest to lowest latitude): Picos de Europa (P), Ordesa (O), Guadarrama (G), Monfragüe (M), Cabañeros (C) and Sierra Nevada (S). (I) Total phenolics, (II) Total tannins, (III) Condensed tannins. Applied treatments: control treatment (c), inoculation with *Phytophthora cinnamomi* (*Pc*), mechanical defoliation (d) and double treatment pathogen infection/mechanical defoliation (dPc). Bar height represents the mean and whiskers are standard deviations. Different letters denote statistically significant differences.

CHAPTER 3

Susceptibility to *Phytophthora cinnamomi* of six holm oak (*Quercus ilex*) provenances: are results under controlled vs. natural conditions consistent?

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Submitted to Forest Systems (under revision)

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Abstract

Quercus forests have been recently affected by severe decline and mortality. This oak decline is associated with the soilborne pathogen *Phytophthora cinnamomi* (Pc). Its wide distribution and exacerbation of damage by climate change in the Mediterranean region raise the need for breeding programs based on resistant and tolerant material to the disease. This work aims to determine if holm oaks planted in four field plots with highly Pc-infected soils show differences in mortality according to their provenances after a four-year monitoring. It also evaluates whether the most tolerant provenances are those with the greatest constitutive chemical defences. In general, there was a high mortality (56.0-80.5%) but provenances with higher constitutive chemical defences better tolerate the pathogen both in greenhouse conditions and in the field. These provenances show a higher probability of survival and also a better development. In the southern provenances, some families with higher tolerance and, therefore, candidates for use in reforestation programs in areas infected by Pc, were identified.

Introduction

Plants have several mechanisms to deal with biotic and abiotic factors that cause them stress. In the current context of global change, stress is increasing and more studies are focused on the search for resistant or tolerant plant material against these stressors. The identification of tolerant species and genotypes is key in breeding programs. In addition, forest managers need this plant material to regenerate areas seriously affected by pests and diseases or with difficulties for natural regeneration, among others. The natural selection, genetic drift and local adaptation favor intraspecific diversity in tolerance to stressors (Eguiarte *et al.* 2013). Therefore, tests of geographical variation in susceptibility are very useful to find them. But stressor tolerance experiments are often carried out under very controlled conditions and even in greenhouses, therefore, very far from the natural environments affected by various combined stressors.

In this field study, the holm oak (*Quercus ilex*) has been chosen as a model species due to its wide genetic diversity and distribution. The holm oak is an evergreen oak well adapted to drought and distributed throughout the Mediterranean basin (Rodá *et al.* 2009). This is the dominant species in “dehesas”, an Iberian oak rangeland that constitutes the economic base of much of the Iberian Western rural world (Campos, 2004). In fact, they are a good example of "grazed wood pastures", common in the Mediterranean region (Den Herder *et al.* 2017), and the main agrosilvopastoral system in Europe, with more than 4.5 million hectares in the Iberian Peninsula (Moreno and Pulido 2009). But this ecosystem of high natural and sociocultural value has experienced a generalized decline in recent decades closely related to the pathogen *Phytophthora cinnamomi* (Pc; Brasier 1992, 1996, Sánchez *et al.* 2006, Corcobado *et al.* 2014). This soil-borne oomycete affects more than 5000 species in the world (Hardham and Blackman 2018) and rots the absorbent roots of the holm oak, causing their death. Previous works with *Q. ilex* and also *Q. suber* under greenhouse conditions have yielded high mortality rates but also have revealed the existence of more tolerant provenances and genotypes (Cuenca *et al.* 2018, León 2013, Rodríguez-Romero *et al.* 2020a). León (2013) and Cuenca *et al.* (2018) found a high variability in the genotype response to Pc infection. Cuenca *et al.* (2018) also demonstrated that the tolerance of holm oak to infection by Pc –and also to water stress- is genetically controlled and, therefore, susceptible to improvement. However, these results are always under controlled greenhouse conditions and with short-term experiments, so they should be treated with caution. In another previous greenhouse study of holm oaks from various Iberian provenances inoculated with Pc, significant differences were obtained in the tolerance shown according to their origin and genotype (Rodríguez-Romero *et al.* 2020a). The analysis of their phenolic defences yielded higher constitutive values in southern provenances, which were also the most tolerant to infection by the root pathogen. Under more natural conditions, Rodríguez-Molina *et al.* (2002) carried out these susceptibility tests on soils naturally infected by Pc in an environmentally controlled chamber under a 16:8 light/dark photocycle at 28–22ºC during the light-dark period. They demonstrated the high susceptibility of holm oak to the pathogen in the aforementioned conditions, independent of acorn provenance and soil origin. However, that test also addressed mortality in cork oak, which was significantly different depending on the acorn origin and soil treatment, and on their interactions. Besides this, they conclude that the susceptibility may be also related to plant age and/or growth stage. In fact, later studies carried out in the field with cork oak (*Quercus suber;* Moreira *et al.* 2018a) determine the existence of some genotypes tolerant to the disease and capable of growing in infected areas ten years after planting. However, no published results of tolerant holm oaks from different provenances to the pathogen in the field are known.

The main goal of this work is to confirm whether results on holm oak tolerance obtained in greenhouse tests according to provenances are also maintained in more natural conditions in the field. If results in both scenarios were consistent, this approach would facilitate the identification and selection of disease-tolerant plant material. To achieve it, an experiment was established in four field plots located on active decline areas with plants previously born in the greenhouse from six Iberian provenances. Subsequently, the plantation was monitored during four years, recording symptoms, mortality and growth in order to answer the following questions:

-Which provenances were the most tolerant to the disease?

-Does this tolerance correspond to that obtained in common garden conditions in a greenhouse?

-Do constitutive defences predict tolerance under field conditions?

Materials and methods

Plant material and sowing in the greenhouse

In winter 2015, holm oak acorns of seven genotypes were collected in each of the six sampled Spanish National Parks (ordered from highest to lowest latitude): Picos de Europa (P), Ordesa (O), Aigüestortes (A), Monfragüe (M), Cabañeros (C) and Sierra Nevada (S). Sowing took place under a common environment at the greenhouse on the School of Forestry of the University of Extremadura (Plasencia, UTM Zone 29N X: 748862; Y: 4435709; 395 meters above sea level). Seedlings grew in 28-well plastic trays, 450 ml in volume, filled with a soil substrate consisting of mixed peat and sand (1: 2, pH: 5.5). Pots were placed on metal trays with a 2-cm deep water line to keep the substrate at field capacity, thus avoiding water stress.

Acorn germination and seedling mortality during the first year in the greenhouse was uneven according provenance. So, the number of holm oak seedlings was different with a relative importance of: P (94 plants), O (41 plants), A (29 plants), M (116 plants), C (122 plants), S (124 plants).

Experimental field design and symptom monitoring

In March 2017, four plots were delimited on two decline areas with a negative evolution in the tree density during the last 60 years (Fig. 1). The affected areas were located on two farms in the central western Iberian Peninsula (Malpartida de Plasencia, Extremadura) and close to the greenhouses of the University of Extremadura. The chosen farms were San Esteban (SE) and Haza de la Concepción (HC) and are typical “dehesas” with a Mediterranean climate and gentle slopes (less than 7%). Within each farm, one plot was located on a slope (S) and the other on a valley (V). The plots were fenced to avoid cattle and wild fauna interference. Each plot occupied a surface of 200 m^2, leaving a symptomatic adult holm oak inside to favor the infection of new seedlings and another 100 m^2 free of vegetation for the planting of holm oaks from different provenances.

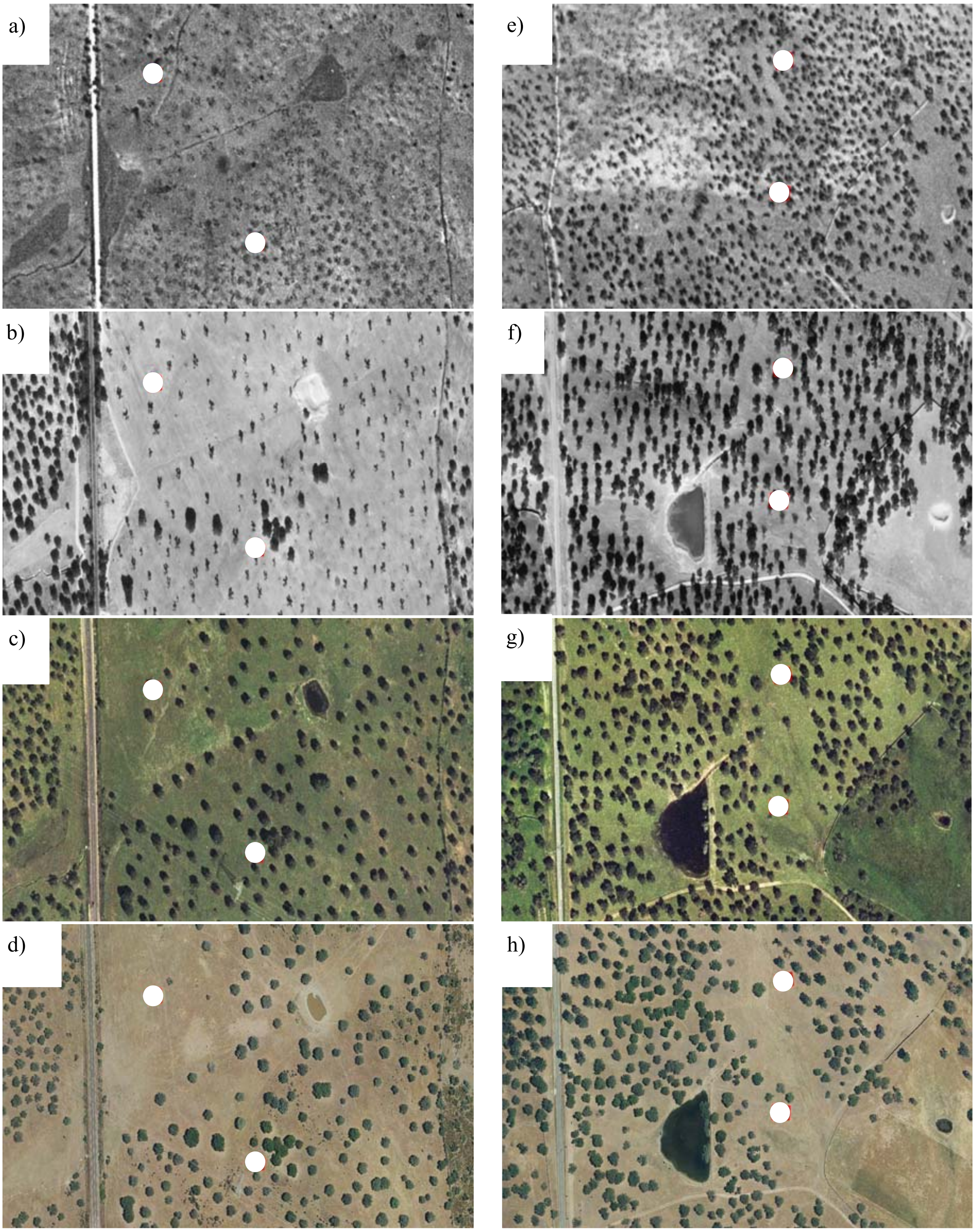

Fig. 1. Evolution of the areas affected by the oak decline in the last 60 years. a and e) Orthophotos AMS 1956-1957 over SE and HC respectively; b and f) Orthophotos Interministerial flight 1973-1986 over SE and HC respectively; c and g) PNOA 2006 Orthophotos over SE and HC respectively; d and h) PNOA 2019 orthophotos over SE and HC respectively. White dots in the orthophotos are plots within each farm. Taken from the Download Center of the National Geographic Information Center (CNIG) cartography.

The land was plowed before planting in March. Topsoil was removed to place an anti-weed mesh and thus reduce the early competition of the seedlings with grasses in the plot. Subsequently, planting holes of 30 cm in diameter and 40 cm in depth were made. The holes were spaced 60 cm and 1 meter between planting lines. Provenances and families were randomly distributed and 2-6 seedlings were used for each family. The total number of georeferenced seedlings per plot was 153 plants in SEV, 156 plants in SES, 157 plants in HCV and 155 plants in HCS. Finally, 40 cm high protective tubes with 25% shading were placed to protect the stem from rodents.

In addition to the holm oak seedlings, 10 one-year-old *Fraxinus angustifolia* seedlings and 10 one-year-old *Quercus faginea* seedlings were planted in each plot. *Q. faginea* is another Iberian oak susceptible to Pc, although more tolerant than holm oak (Moralejo *et al.* 2009, Pérez Sierra *et al.* 2013, Moreira *et al.* 2018a). *F. angustifolia* has been described as a Pc resistant species (Moralejo *et al.* 2009). Both species are more demanding in soil moisture than holm oak, so a good development of these species will indicate that water stress is not the limiting factor for the growth of holm oaks in the plots. The viability of these species will help to confirm that the main cause of holm oak mortality is the infection by Pc.

The plant development in plots was measured weekly the first year and monthly during the following three years, recording symptoms, mortality, regrowth and the possible incidence of other damages such as occasional defoliation, presence of ants and voles in some seedlings. At the end of each year, plant growth was also measured.

Pc isolation

Pc presence was confirmed in the plots before planting by isolating in roots and soils following the procedure described by Jung *et al.* (1996) and sowing the samples in selective PARPH medium (Jeffers and Martin, 1986). A molecular analysis of soil samples from the four plots also allowed the identification of other species of the same genus (*P. gonapodyides* and *P. megasperma* -A. Solla, personal communication). During the following years, twice a year, five soil and root samples were randomly selected per plot to confirm the activity of the pathogen each spring. The samples were taken in the first 20 cm of depth. The pathogen isolation was carried out following the same initial protocol and *Q. suber* leaves were used as baits. The identification of the Pc colonies in the selective medium was carried out with a microscope after 2-3 days of incubation in the dark at 25ºC. In addition, the evolution of tree condition was verified weekly the first year and monthly during the following three years.

Plot soil characterization

Soil plays a fundamental role in the disease impact. Characterizing the spatial variability in detail is key to later analyzing the results obtained in a field experiment. Before planting, soil samples were taken from the four plots for their physical-chemical description. The characteristic data of the soil for each plot were provided by the Agrarian Laboratory of the Junta de Extremadura. The main edafic components and their differences among plots are shown in Table S1 (Supplementary information). Despite certain differences among soils in the four plots, all of them presented similar and frequent basic characteristics in this type of agrosilvopastoral systems. However, the effects associated with the differences between valley and slope, and between farms were included in the statistical model for a better representation of the found variability.

Statistic analysis

To analyze the plant survival time in the field, the Kaplan-Meier estimate was used. Gehan's Wilcoxon test was used to assess differences among provenances in survival. Survival was analyzed with the "Survival Analysis" module from Statistica software. The effect of plot and provenance on mortality, symptoms, healthy development and relative height increment (difference of the final-initial plant height divided by initial height) were analyzed on a factorial design using a generalized linear mixed model (GLMM). Dependent variables were mortality (plant dead/alive), presence of symptoms (plant with symptoms/without symptoms), healthy development (plant alive without symptoms/with symptoms or dead) and height increment (cm). Data were statistically analysed using Tukey-Kramer´s test ($p<0.05$) for significant differences of means.

To analyze whether results obtained in the field are consistent with those obtained in the greenhouse test, the Pearson´s linear correlation was calculated between the variables mortality at 5 months in the field and mortality at 5 months in the greenhouse (as this period of the test greenhouse the limiting factor). Besides, the relationship between mortality at 4 years in the field and mortality in the greenhouse at the five-month duration of this test was also estimated for the same provenances. Finally, the relationship between mortality in the field at 4 years and levels of constitutive chemical defences measured in the greenhouse (data from Rodríguez-Romero *et al.* 2020) was also evaluated by Pearson's linear correlation. Statistical analyzes were performed in Statistica v10 software.

Results

Differences in susceptibility to the pathogen according to provenance

Holm oaks from all provenances and in all plots drastically reduced their survival probabilities in the Pc-naturally infected fields after four years of follow-up (p <0.001). Plant provenance had a significant effect on the mortality of the seedlings (Gehan's Wilcoxon test p <0.001, Table 1, Fig. 2 and 3). Mortality during the four years of follow-up according to provenance ranged between 56.0% and 80.5% and symptoms of Pc infection ranged between 13.8% and 34.0% in the remaining seedlings, according to provenance (Fig. 4). The relative height increase of the holm oak plants during the four years of monitoring also varied among provenances and was minimum in provenance A (0.19) and maximum in provenance C (0.58, Fig. 4). The development of these variables by plot and provenance is graphically shown in the supplementary information (Fig. S1-S4).

Table 1. Effects by GLMM of plot, provenance and their interaction on the number of dead plants, plants with symptoms and plants with healthy development (alive without symptoms), and effects on the relative increase in the plant height after four years of follow-up (difference between final and initial height divided by initial plant height. Provenances: *Quercus ilex* plants of P (Picos de Europa), O (Ordesa), A (Aigüestortes), M (Monfragüe), C (Cabañeros), and S (Sierra Nevada); F: *Fraxinus angustifolia*, Q: *Quercus faginea*.

Effect	df	Mortality	Symptoms	Healthy development	Relative height increase
Provenance	5	2.95***	2.56*	0.91	1.58*
Plot	3	25.54***	14.45***	4.51***	18.93***
Provenance x plot	15	1.24	0.91	1.02	1.17

F-values are shown along with statistical significance: **p* < 0.05; ***p* < 0.01; ****p* < 0.001; ns *p* > 0.05

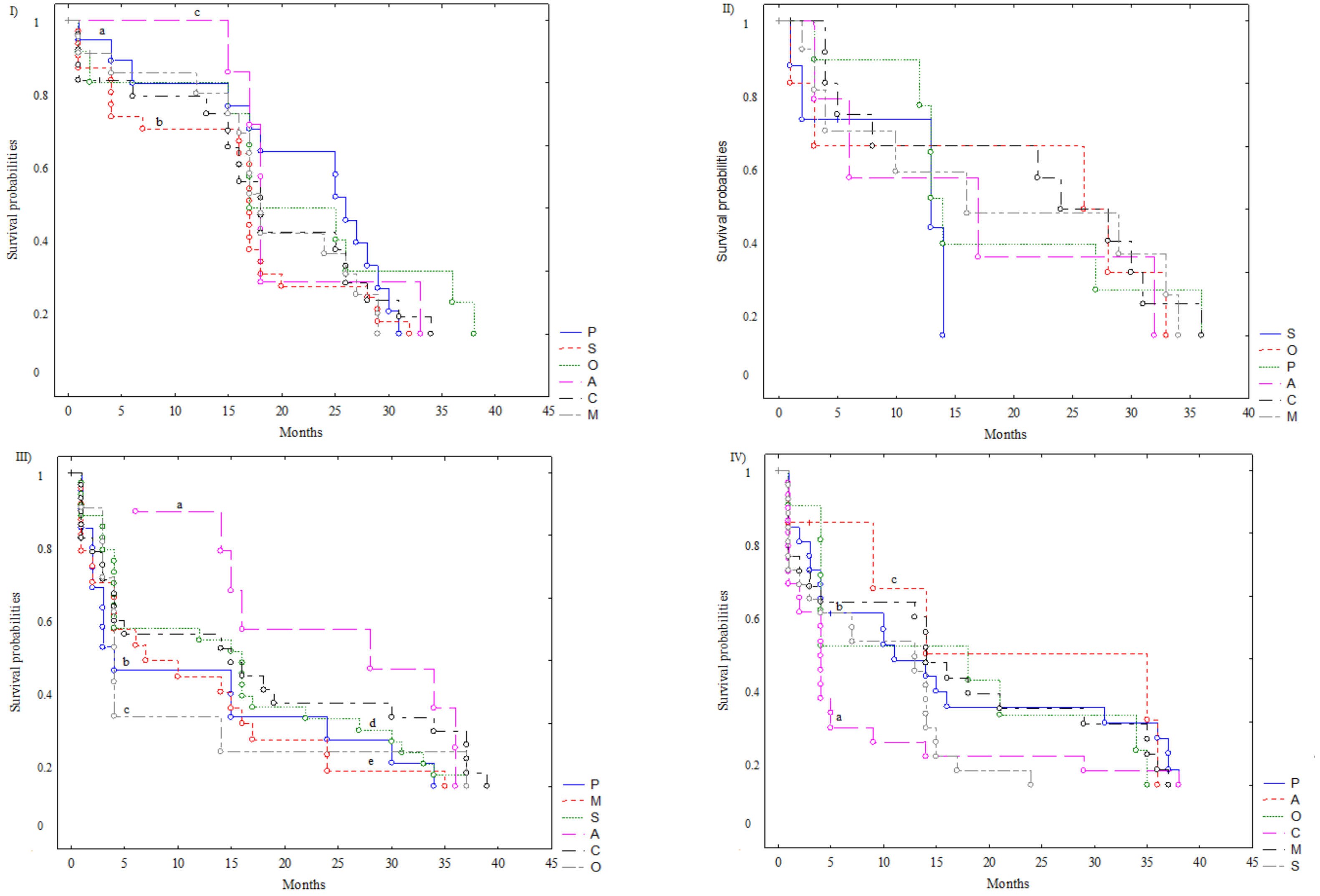

Fig. 2. Survival curve of *Quercus ilex* seedlings grown in soil naturally infected by *Phytophthora cinnamomi* according to provenance after four years of follow-up. Plots: I) SEV, II) SES, III) HCV, IV) HCS. Provenance: P (Picos de Europa), O (Ordesa), A (Aigüestortes), M (Monfragüe), C (Cabañeros), and S (Sierra Nevada). Different small letters denote statistically significant differences from Gehan's Wilcoxon test ($p < 0.05$).

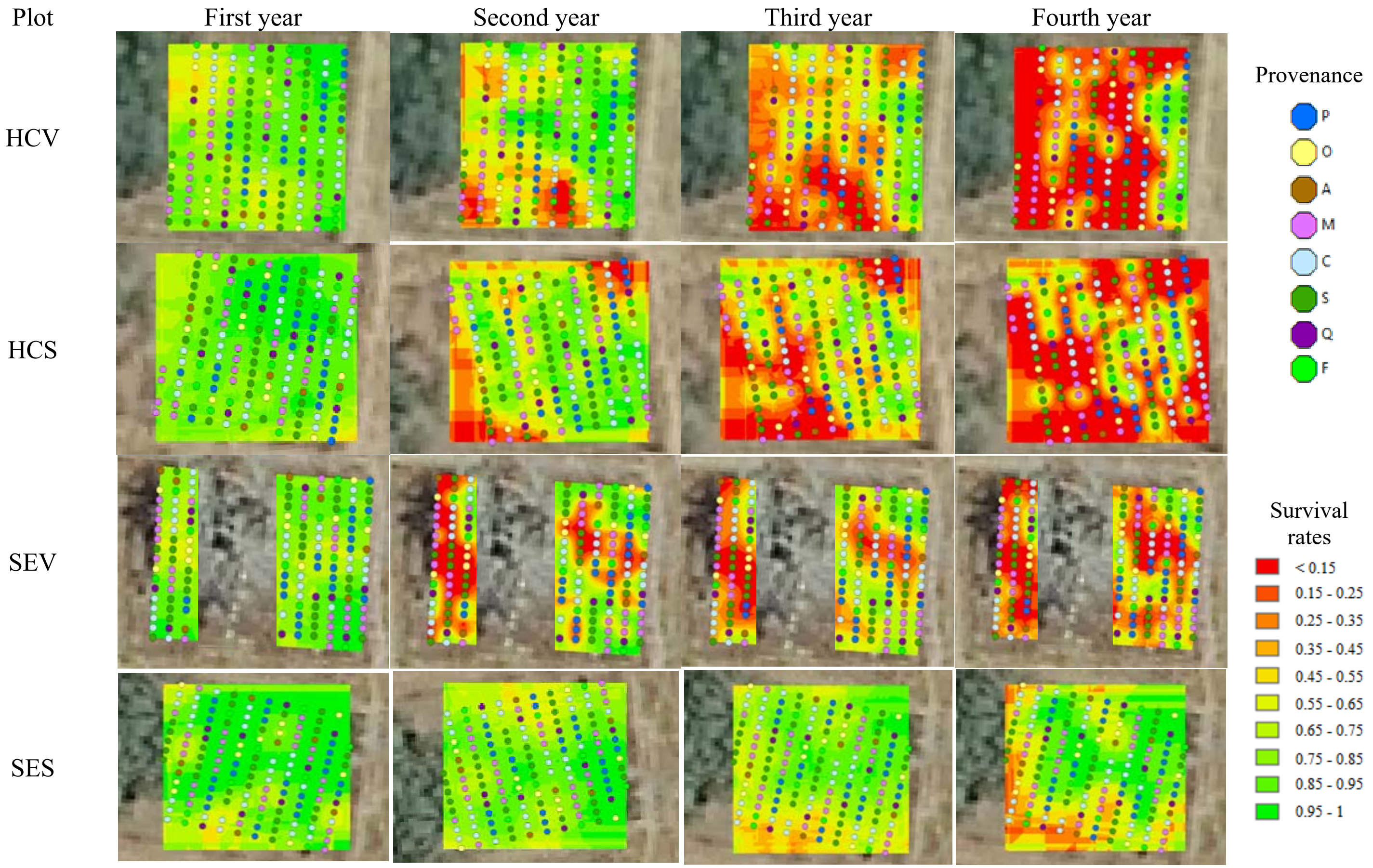

Fig. 3. Geostatistical interpolation by krigring of the survival rates during the four years of *Quercus ilex* monitoring in the field. The colored dots indicate the provenance location of the seedlings. Experimental plots: (HCV) Haza de la Concepción valley, (HCS) Haza de la Concepción slope, (SEV) San Esteban valley, (SES) San Esteban slope. Provenances/species: *Quercus ilex* plants of P (Picos de Europa), O (Ordesa), A (Aigüestortes), M (Monfragüe), C (Cabañeros), and S (Sierra Nevada); F: *Fraxinus angustifolia*, Q: *Quercus faginea*.

Control species showed very low mortality (30% in *Q. faginea*, 14% in *F. angustifolia*) and Pc symptoms (52.5% in *Q. faginea*, 0% in *F. angustifolia*). In addition, these control species showed high relative growth during the experimental period (0.8 in *Q. faginea*, 1.69 in *F. angustifolia*). These results confirm that the main cause of mortality in holm oak was Pc infection.

Tolerance, evaluated from the percentage of living plants without Pc symptoms, was higher in southern provenances (S, C and M; Tukey-Kramer's test p <0.05; Table 1 and Fig. 4, "*healthy development plants*"). Among the northern regions, P provenance showed a similar mortality than the southern regions, but a higher percentage of plants with symptoms. The regions with the lowest mortality also experienced the highest growth rates (C, M and S provenances in the south, and P provenance in the north). The provenance with the highest percentage of healthy plants was S (13.71% of living plants without infection symptoms).

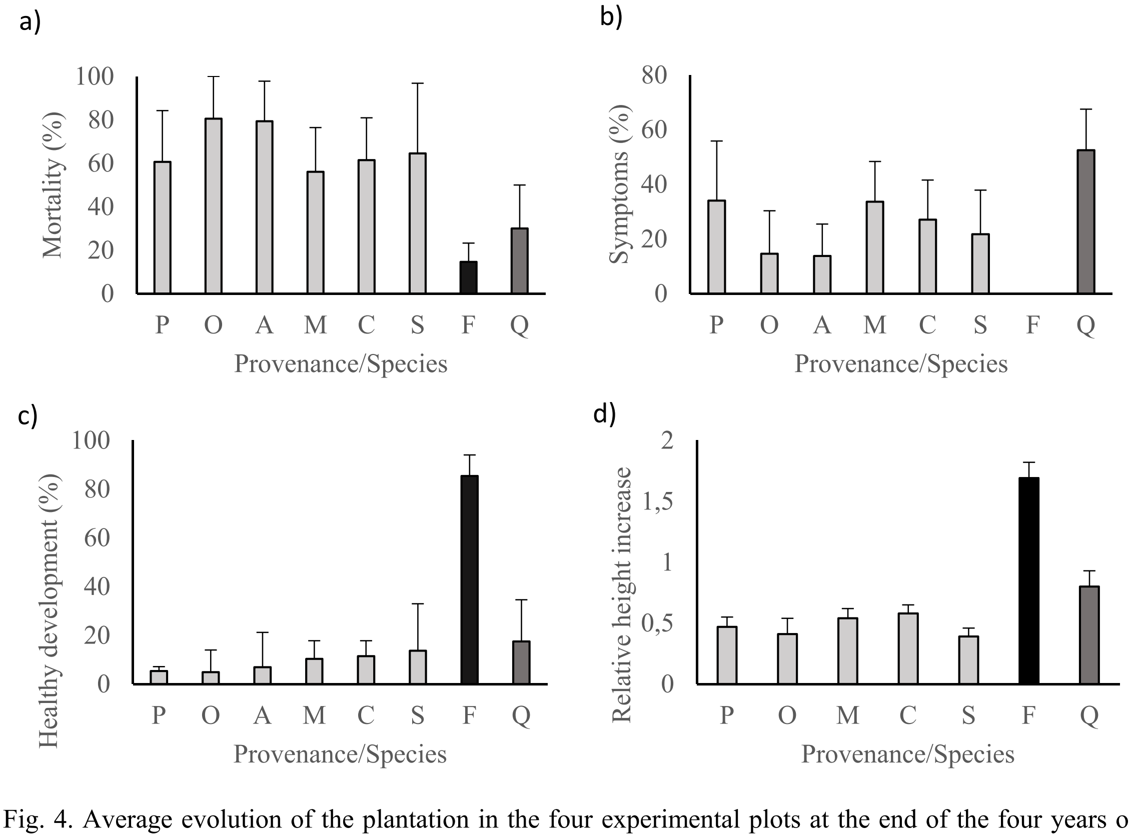

Fig. 4. Average evolution of the plantation in the four experimental plots at the end of the four years of follow-up according to provenance. Bar height represents the mean and whiskers are standard deviations. a) mortality (percentage of dead seedlings, b) percentage of symptomatic live plants, c) percentage of live and symptom-free plants with healthy development and d) relative height increase (final height minus initial divided by initial height after four years after planting). Provenances/species: *Quercus ilex* plants of P (Picos de Europa), O (Ordesa), A (Aigüestortes), M (Monfragüe), C (Cabañeros), and S (Sierra Nevada); F: *Fraxinus angustifolia*, Q: *Quercus faginea*.

The plot and its position on the slope or valley also had a significant effect on tolerance to the pathogen (Fig. S1-S4 from Supplementary Information). Mortality was higher in HC farm than in SE farm (survival 32.55% and 55.62% respectively, Tukey's test p <0.05). The position also showed a significant effect on mortality in the SE farm and mortality was higher in the valley than on the slope (survival in the valley 40.54% and 74.19% on the slope (Tukey's test p <0.05). In HC there were no significant differences according to position (Tukey's test p> 0.05). In the slope position, the highest tolerance was reached by plants from the S provenance (25.8% of the seedlings had a healthy development) and the lowest by the P provenance plants (6% of the seedlings with healthy development, alive and free of symptoms). In the valley position, the most tolerant provenance was C (9.58% of the plants showed healthy development) and the least tolerant were the O and A provenances (all their plants died in this position). The provenances most affected by the pathogen in both positions were again the northerners (P, O and A).

Growth also differed among plots (Fig. S1-S4 from Supplementary Information). The mean relative height increase of holm oaks during the four years was also higher in SE (0.55 ± 0.06) than in HC (0.30 ± 0.06) and differences between them were significant (Tukey's test p< 0.05). Relative high increase in SE was 0.15 ± 0.08 in the valley and 0.96 ± 0.08 on the slope. In HC it was 0.23 ± 0.08 in the valley and 0.39 ± 0.08 on the slope. However, only SES differed significantly from the rest (Tukey's test p< 0.05).

Consistency of tolerance to Pc under greenhouse and field conditions

From the six provenances evaluated in the field, five provenances had been previously Pc-inoculated in a greenhouse in a five-month study (Rodríguez-Romero *et al.* 2020). In the aforementioned study, mortality by Pc infection increased with latitude (N = 5 provenances, r = 0.94, p = 0.02). Provenances with the lowest mortality in the greenhouse experiment were M, C and S (located in the southern Iberian Peninsula). The increasing trend in mortality with latitude in the greenhouse experiment was maintained in the field experiment and the lowest mortality was also achieved in the southern provenances. The relationship between latitude and mortality of holm oaks from these five provenances in the naturally Pc-infected field at five months was also significant (r

= 0.91, p = 0.03). However, this mortality/latitude relationship in the field was not significant (r = 0.58, p = 0.3) four years later, probably because the northern P provenance showed mortality similar to that of southern provenances, as already highlighted above. However, living plants from this provenance showed severe infection symptoms (Fig. 4, Table S2 of supplementary information) at the end of the experiment, which would have predictably been recorded as dead plant in a longer-term test. The relationship between mortality at 5 months in the greenhouse and mortality at 5 months in the field was not significant (N = 5, r = 0.76, p = 0.14). Nor was the relationship between mortality at 5 months in the greenhouse and mortality at four years in the field (N = 5, r = 0.41, p = 0.49). However, since the P provenance had very few plants with healthy development, the mortality relationship in the greenhouse test was also analyzed with the number of dead plants and plants with severe symptoms of the disease in the field at four years of the experiment (mortality (%)+symptoms (%)), obtaining a high and significant correlation (N = 5, r = 0.88, p = 0.04; Fig. 5).

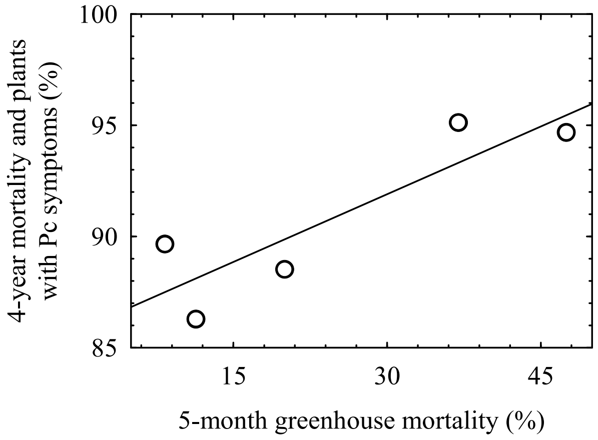

Fig. 5. High and significant Pearson´s correlation (r = 0.88, p = 0.04) between mortality of holm oaks (*Q. ilex)* from N = 5 provenances after 5 months of inoculation with *Phytophthora cinnamomi* (Pc) in the greenhouse (obtained de Rodríguez-Romero *et al.* 2020) and mortality plus plants with severe symptoms of Pc infection (% mortality + % symptoms) in naturally Pc infected soils in the field four years after planting.

Regarding chemical defence levels, from the three phenolic groups measured in the previous test (Tp total phenols, Ct condensed tannins and Tt total tannins; Rodríguez-Romero *et al.* 2020), provenances with the highest survival in the field four years later were also those with the highest constitutive phenolic defences (measured in the greenhouse test). The higher the constitutive phenolic defences, the greater the survival to the pathogen's presence both in the greenhouse and in the field (Table S2 from supplementary information). But only the constitutive Ct showed a statistically significant and strong correlation with the mortality of holm oaks registered in the naturally Pc-infected area (Pearson's correlation r = -0.99, p = 0.001; Fig. 6).

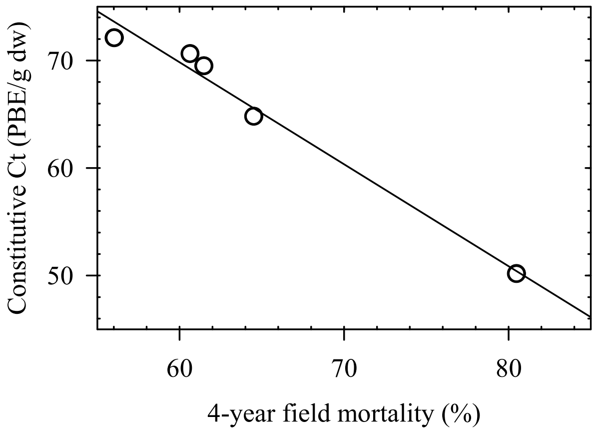

Fig. 6. High and significant Pearson´s correlation (r = -0.99, p = 0.001) between the mortality of holm oaks (*Q. ilex*) of N = 5 provenances in the field after four years of follow-up on areas affected by *Phytophthora cinnamomi* and the constitutive condensed tannins of these provenances (PBE/g.dw; obtained from Rodríguez-Romero *et al.* 2020).

Discussion

To our knowledge, this work constitutes the first field test on the susceptibility of holm oaks of different provenances in areas infected by the Pc pathogen. In addition, the four years of follow-up of its effects greatly exceeds the usual test times for susceptibility to the pathogen. Our results confirmed the high mortality of the species in the presence of Pc, but also the existence of holm oak genotypes tolerant to the disease. Survival differed significantly according to their provenances, being those from the south more tolerant. In addition, these provenances with better health status were the most tolerant in a previous greenhouse experiment (Rodríguez-Romero *et al.* 2020) and their tolerance was related to higher constitutive chemical defences.

Pc-tolerance in the field depends on the provenance

The mortality of holm oak plants was very high, according to other susceptibility tests of the same species against Pc (Robin *et al.* 1998, Serrano *et al.* 2012, Rodríguez-Molina *et al.* 2002, 2005). The high mortality of holm oak in the field during the first years after reforestation in decline areas could make regeneration very difficult in the most affected foci. However, this work shows that after four years of follow-up, some holm oaks survived, despite the Pc presence in the soil. The survival of some plants evidences Pc tolerance. These findings are in agreement with León (2013) and Cuenca *et al.* (2018), who also found tolerance in this species in some families. Furthermore, Cuenca *et al.* (2018) showed that the tolerance of Pc-infected holm oaks is genetically controlled, being susceptible to improvement. Our study in the field and longer follow-up time also showed that the plant provenance has an effect on tolerance, which facilitates the identification of the holm oak families with greatest interest for possible plant breeding programs. In general, tolerance in the southern provenances was higher than in the north, which also resulted in higher plant growth.

Although no other studies are known about the effect of geographical variability on the response of holm oak to the pathogen in the field, there is a previous study in cork oak (*Q. suber*; Moreira *et al.* 2018a). The aforementioned work also obtained variability in the response and identified some tolerant families to the disease after ten years of follow-up. This work also used *Q. faginea* in the plantation, obtaining high tolerance to the pathogen, as also occurs in our work, and proposed it as a possible rootstock of *Q. ilex* in the most affected areas. In addition, the other species used as a control of water stress, *F. angustifolia*, showed low mortality and resistance to the pathogen mentioned in other articles (Moralejo *et al.* 2009). The plant survival of these species and their different degrees of tolerance reinforce what has been described by other authors such as Jactel *et al.* (2017) about the greater resistance of mixed forests to natural disturbances that are relatively small-scale and selective in their effect. And they highlight the importance of specific functional diversity in the stand, that is, "*trees with different levels of susceptibility to different hazards should be intermixed in order to reduce the amount of exposed resources and to generate barriers against contagion*" (Jactel *et al.* 2017).

The provenances with high constitutive chemical defences are also more tolerant to the disease in the field

This analysis was performed in comparison with the previous analysis of susceptibility to the pathogen under greenhouse conditions for the same holm oak provenances. Provenances from the south of the Iberian Peninsula were more tolerant in the field. This result agrees with that obtained in the greenhouse (Rodríguez-Romero *et al.* 2020). Furthermore, in the greenhouse study a strong relationship was detected between these more tolerant provenances and the Ct constitutive levels. Our present study shows that this relationship is maintained in the field. The provenances with higher Ct constitutive levels were more tolerant to the disease both in the greenhouse and in the field. The highest levels of defences were found at low latitudes, following a classic paradigm in ecology that argues that defense production and biotic stress increase at low latitudes (Rasmann and Agrawal 2011, Pearse and Hipp 2012, Moreira *et al.* 2014, Anstett *et al.* 2016). This trend could be also linked to underlying factors associated with latitudinal variation such as climate and soil (Moreira *et al.* 2018b). However, it is noteworthy among the northern provenances that P showed similar mortality to the southern provenances, but a high percentage of severe symptoms, despite its high Ct content. On the other hand, in previous studies it was shown that these defensive traits are genetically controlled (Solla *et al.* 2016, Cuenca *et al.* 2018, Rodríguez-Romero *et al.* 2020). Therefore, the Ct would be a parameter of interest in future genetic improvement programs, but also the latitude of the genetic material sources.

The disease-tolerant families identified in this study should be followed in the long-term to test if such tolerance is maintained over time. If after a longer experiment period, tolerance continues to be kept, these families should be part of the source of genetic material in breeding programs. In addition, the existence of *Q. faginea* as a species more tolerant to the disease suggests the revision of the specific composition of oaks in these affected areas, being able to use *Q. faginea* as a substitute species in the most affected by the pathogen areas and even as rootstocks, as some studies already point out (Moreira *et al.* 2018a).

However, in a field test like this one, results should be viewed with caution, as several environmental factors also condition the plant survival. In fact, it would be very interesting in future larger field experiments to deepen the knowledge of the effects of several factors on tolerance according to provenances, such as exposure, water stress, shade and distance to the infected adult tree or the competition between oak plants and herbaceous plants during the first years in oligotrophic soils like these. Notwithstanding all these factors, the existence of tolerant holm oak families to the pathogen in some provenances suggests that the restoration of the affected by this disease-areas could be possible if tolerant or resistant plant material is used.

References

Anstett DN, Nunes KA, Baskett C, Kotanen PM (2016). Sources of controversy surrounding latitudinal patterns in herbivory and defense. Trends Ecol Evol 31: 789-802.

Brasier CM (1992). Oak tree mortality in Iberia. Nature 360, 539.

Brasier CM (1996). *Phytophthora cinnamomi* and oak decline in Southern Europe. Environmental constraints, including climate change. Ann For Sci 53: 347-358.

Campos P (2004). Towards a sustainable global economics approach for Mediterranean agroforestry systems. In: Schnabel, S., Ferreira, A. (eds.) Sustainability of agrosilvopastoral systems. Advances in GeoEcology, vol 37. Catena Verlag, Reiskirchen, Germany, pp 13–28.

Corcobado T, Cubera E, Juarez E, Moreno G, Solla A (2014). Drought events determine performance of *Quercus ilex* seedlings and increase their susceptibility to *Phytophthora cinnamomi*. Agric For Meteorol 192-193:1–8.

Cuenca B, Dorado FJ, Camisón A, Luquero L, Ocaña L, Solla A (2018). Selección de brinzales de *Quercus suber* y *Q. ilex* tolerantes a *Phytophthora cinnamomi* y al estrés hídrico. En: Libro de Resúmenes XIX Congreso de la Sociedad Española de Fitopatología, Toledo (España).

Den Herder M, Moreno G, Mosquera-Losada RM, Palma JH, Sidiropoulou A, Freijanes JJS, Crous-Duran J, Paulo JA, Tomé M, Pantera A, Mantzanas K, Pachana P, Papadopoulos A, Plieninger T, Burgess PJ (2017). Current extent and stratification of agroforestry in the European Union. Agr Ecosyst Environ 241: 121-132.

Download Center of the National Geographic Information Center (CNIG). http://centrodedescargas.cnig.es/CentroDescargas/index.jsp

Eguiarte LE, Aguirre-Liguori JA, Jardón-Barbolla L, Aguirre-Planter E, Souza V (2013). Genómica de poblaciones: nada en evolución va a tener sentido si no es a la luz de la genómica y nada en genómica tendrá sentido si no es a la luz de la evolución. Rev Esp Cienc Quim Biol 16(1).

Hardham AR, Blackman LM (2018). *Phytophthora cinnamomi*. Mol Plant Pathol. 19(2):260-285.

Jactel H, Bauhus J, Boberg J, Bonal D, Castagneyrol B, Gardiner B, González-Olabarria JR, Koricheva J, Meurisse N, Brockerhoff EG (2017). Tree Diversity Drives Forest Stand Resistance to Natural Disturbances. Current Forestry Reports 3(3): 223-243.

Jeffers NS, Martin JB (1986) Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Disease 70:1038-1043

Jung T, Blaschke H, Neumann P (1996). Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. Eur J Forest Pathol 26:253–272

León IM (2013). Selección de progenies de encina (*Quercus ilex* L. spp. ballota) y alcornoque (*Quercus suber* L.) tolerantes al patógeno *Phytophthora cinnamomi*. Universidad de Huelva, Tesis Doctoral.

Moralejo E, García-Muñoz JA, Descals E (2009). Susceptibility of Iberian tres to *Phytophthora ramorum* and *P. cinnamomi*. Plant Pathology 58(2): 271-283.

Moreira AC, Martins MS (2005). Influence of site factors on the impact of *Phytophthora cinnamomi* in cork oak stands in Portugal. Forest Pathology 35:145-162.

Moreira AC, Tapias R, Fernandes L, Rodriguez A (2018a). Field susceptibility of cork oak trees with different provenances to *Phytophthora cinnamomi*. Forest Pathology 48(5) e12461.

Moreira X, Castagneyrol B, Abdala-Roberts L, Berny-Mier y Teran JC, Timmermans BGH, Bruun HH, Covelo F, Glauser G, Rasmann S, Tack AJM (2018b). Latitudinal variation in plant chemical defences drives latitudinal patterns of leaf herbivory. Ecography 41: 1124–1134.

Moreira X, Mooney KA, Rasmann S, Petry WK, Carrillo-Gavilan A, Zas R, Sampedro L (2014). Trade-offs between constitutive and induced defences drive geographical and climatic clines in pine chemical defences. Ecol Lett 17 (5): 537– 546.

Moreno G, Pulido FJ (2009). The functioning, management, and persistente of dehesas. In: Agroforestry Systems in Europe. Current Status and Future prospects. Riguero-Rodriguez A, Mosquera-Losada MR, McAdam J. (eds.). Advances in Agroforestry Series, Springer Publishers pp 127-161.

Pearse IS, Hipp AL (2012). Global patterns of leaf defences in oak species. Evol 66: 2272– 2286.

Pérez-Sierra A, López-García C, Leon M, García-Jiménez J, Abad-Campos P, Jung T (2013). Previously unrecorded low-temperature *Phytophthora* species associated with *Quercus* decline in a Mediterranean forest in Eastern Spain. Forest Pathology, 43: 331-339.

Rasmann S, Agrawal AA (2011). Latitudinal patterns in plant defence: evolution of cardenolides, their toxicity, and induction following herbivory. Ecol Lett 14: 476– 483.

Robin CM, Desprez-Loustau GC, Delatour C (1998). First record of *Phytophthora cinnamomi* on cork and holm oaks in France and evidence of pathogenicity. Ann Sci For 55(8):869-883.

Rodá F, Vayreda J, Ninyerola M (2009). 9340 Encinares de *Quercus ilex* y *Quercus rotundifolia*. En: VV.AA., Bases ecológicas preliminares para la conservación de los tipos de hábitat de interés comunitario en España. Madrid: Ministerio de Medio Ambiente, y Medio Rural y Marino. 94 p.

Rodríguez-Molina MC, Blanco-Santos A, Palo-Núñez EJ, Torres-Vila LM, Torres-Álvarez E, Suárez de la Cámara MA (2005). Seasonal and spatial mortality patterns of holm oak seedlings in a reforested soil infected with *Phytophthora cinnamomi*. Forest Pathology 35(6): 411-422.

Rodríguez-Molina MC, Torres-Vila LM, Blanco-Santos A, Nunez EJP, Torres-Álvarez E (2002). Viability of holm and cork oak seedlings form acorns sown in soils naturally infected with *Phytophthora cinnamomi*. Forest Pathology, 32(6): 365-372.

Sánchez ME, Caetano P, Romero MA, Navarro RM, Trapero A (2006). Phytophthora root rot as the main factor of oak decline in southern Spain. In: Brasier C, Jung T, Oßwald W, eds. Progress in Research on *Phytophthora* Diseases of Forest Trees. Farnham, Surrey, UK: Forest Research, 149-54.

Serrano MS, De Vita P, Carbonero MD, Fernández F, Fernández-Rebollo PM, Sánchez E (2012). Susceptibility to *Phytophthora cinnamomi* of the commonest morphotypes of holm oak in southern Spain For Patho 42:345-347.

Solla A, Slobodan M, Gallardo A, Bueno A, Corcobado T, Cáceres Y, Morcuende D, Quesada A, Moreno G, Pulido FJ (2016). Genetic determination of tannins and herbivore resistance in *Quercus ilex*. Tree Genet Genom 28: 743–751.

Zentmyer GA (1980). *Phytophthora cinnamomi* and the diseases it causes. APS Monograph 10. Minnesota, USA: American Phytopathology Society Press. 96 pp.

Supplementary information

Table S1. Elements and soil characteristics in the four experimental plots. SEV: San Esteban Valley, SES: San Esteban Slope, HCV: Haza de la Concepción Valley, HCS: Haza de la Concepción Slope. Analysed by the Agro-food Laboratory of the Junta de Extremadura.

Determination	SEV	SES	HCV	HCS	Unit
Calcium (Ca++)	2.78	0.87	2.47	1.09	meq/100 g
Assimilable calcium	2.34	0.51	2.05	0.65	meq/100 g
Cation exchange capacity	6.75	8.90	7.59	8.46	meq/100 g
Electrical conductivity 1:5 20ºC	0.040	0.021	0.035	0.019	mmhos/cm
Assimilable phosphorus (Olsen method P)	<2.00	<2.00	3.43	<2.00	ppm
Magnesium (Mg++)	0.73	0.81	0.84	0.73	meq/100 g
Assimilable magnesium	0.57	0.65	0.68	0.57	meq/100 g
Oxidizable organic matter	1.26	1.50	2.61	1.60	%
pH value in water 1:2.5	5.40	4.80	5.20	4.80	-
Potassium (K+)	0.13	0.12	0.24	0.15	meq/100 g
Sodium (Na+)	0.20	0.13	0.10	<0.10	meq/100 g
Texture	Silty-loam	Silty-loam	Loamy	Loamy	-
Clay	10.66	9.01	8.97	8.07	%
Sand	28.42	38.54	41.85	45.43	%
Silt	60.92	52.45	49.18	46.50	%
Coarse elements	5.01	23.2	37.0	56.9	%
Total nitrogen (N Kjeldahl)	0.09	0.10	0.14	0.12	%
C/N ratio	8.11	8.70	10.9	7.75	-_
Ca/Mg ratio	3.80	1.07	2.94	1.49	_
Mg/K ratio	5.62	6.75	3.50	4.87	_
Sulphates (SO4=)	undetected	undetected	undetected	undetected	ppm

Table S2. Mortality (%) of *Quercus ilex* seedlings in the greenhouse after five months of controlled infection with *Phytophthora cinnamomi* (Pc) according to provenances (*Rodríguez-Romero *et al.* 2020), mortality (%) in the field experiment on soil naturally infected by Pc at the five months after planting and mortality at four years (end of field experiment). Means of constitutive chemical defences measured in *Q. ilex* leaves (total phenols Tp, total tannins Tt and condensed tannins Ct) from the aforementioned test carried out in the greenhouse. Average values according to provenance: plants of P (Picos de Europa), O (Ordesa), M (Monfragüe), C (Cabañeros), and S (Sierra Nevada).

Provenance	5-month greenhouse mortality (%)*	5-month field mortality (%)	4-year field mortality (%)	4-year mortality and plants with symptoms (%)	Tf*	Tt*	Ct*
P	47.5	15.96	60.64	94.68	36.44	71.35	70.64
O	36.96	17.07	80.49	95.12	36.80	68.30	50.17
M	8.33	14.52	56.03	89.65	39.97	90.72	72.11
C	20	13.11	61.47	88.52	51.67	94.83	69.50
S	11.36	12.90	64.52	86.29	41.58	75.58	64.81

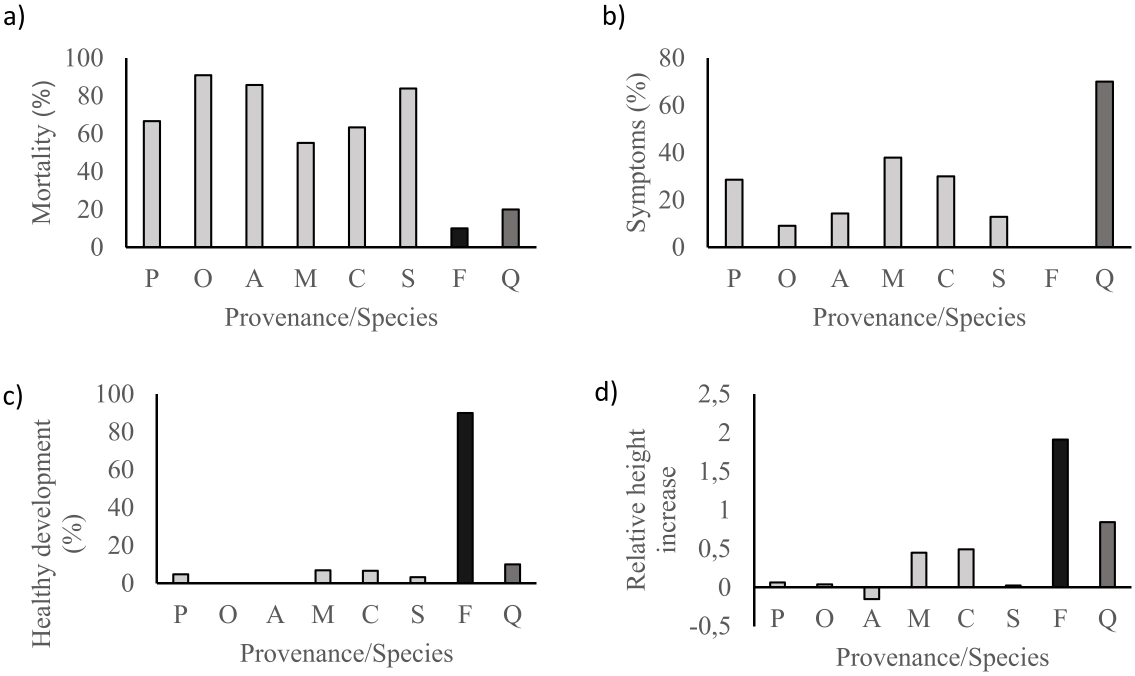

Fig. S1. Plant development in the San Esteban Valley plot (SEV) at the end of the four years of follow-up according to provenance. a) mortality (percentage of dead seedlings, b) percentage of symptomatic live plants, c) percentage of live and symptom-free plants with healthy development and d) relative height increase (final height minus initial divided by initial height after four years after planting). Provenances/species: *Quercus ilex* plants of P (Picos de Europa), O (Ordesa), A (Aigüestortes), M (Monfragüe), C (Cabañeros), and S (Sierra Nevada); F: *Fraxinus angustifolia*, Q: *Quercus faginea*.

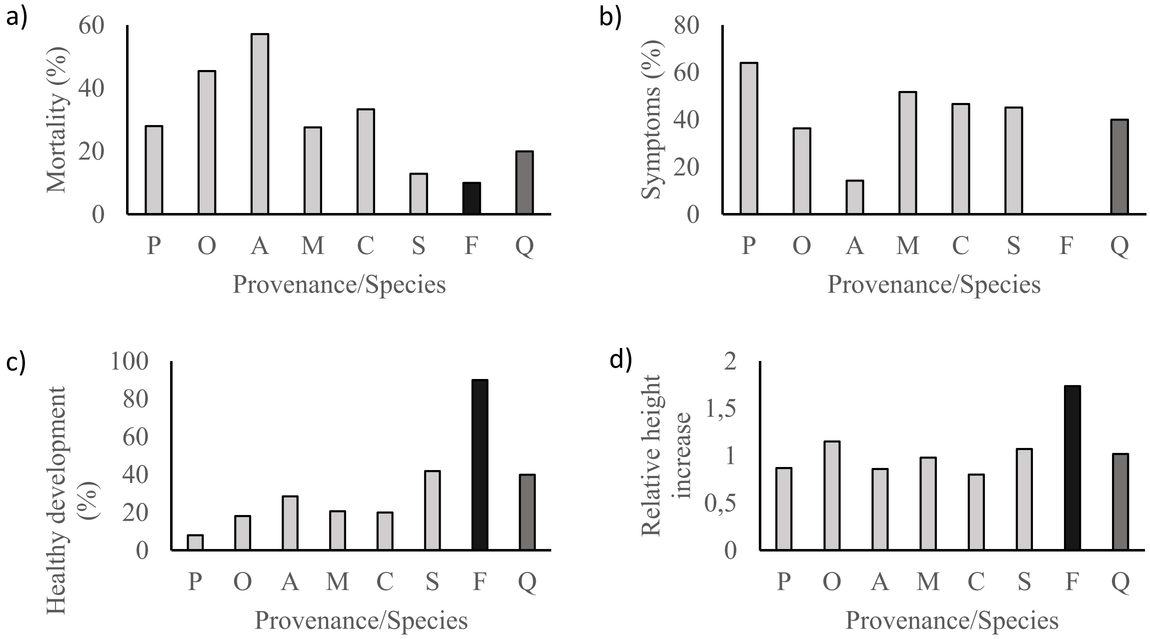

Fig. S2. Plant development in the San Esteban Slope plot (SES) at the end of the four years of follow-up according to provenance. a) mortality (percentage of dead seedlings, b) percentage of symptomatic live plants, c) percentage of live and symptom-free plants with healthy development and d) relative height increase (final height minus initial divided by initial height after four years after planting).

Provenances/species: *Quercus ilex* plants of P (Picos de Europa), O (Ordesa), A (Aigüestortes), M (Monfragüe), C (Cabañeros), and S (Sierra Nevada); F: *Fraxinus angustifolia*, Q: *Quercus faginea*.

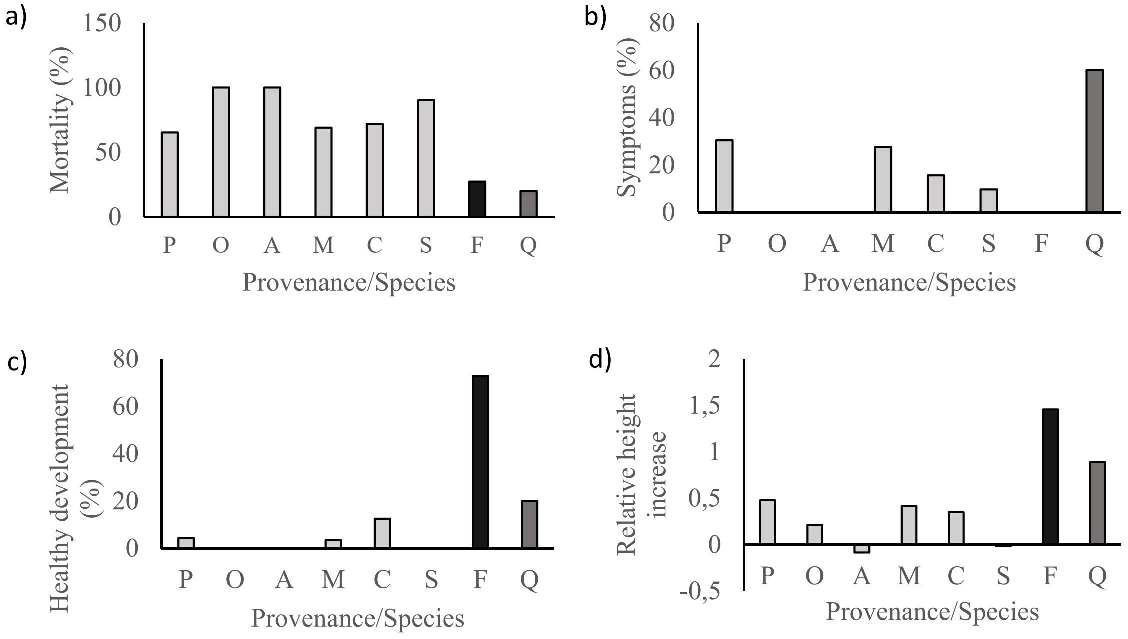

Fig. S3. Plant development in the Haza de la Concepción Valley plot (HCV) at the end of the four years of follow-up according to provenance. a) mortality (percentage of dead seedlings, b) percentage of symptomatic live plants, c) percentage of live and symptom-free plants with healthy development and d) relative height increase (final height minus initial divided by initial height after four years after planting). Provenances/species: *Quercus ilex* plants of P (Picos de Europa), O (Ordesa), A (Aigüestortes), M (Monfragüe), C (Cabañeros), and S (Sierra Nevada); F: *Fraxinus angustifolia*, Q: *Quercus faginea*.

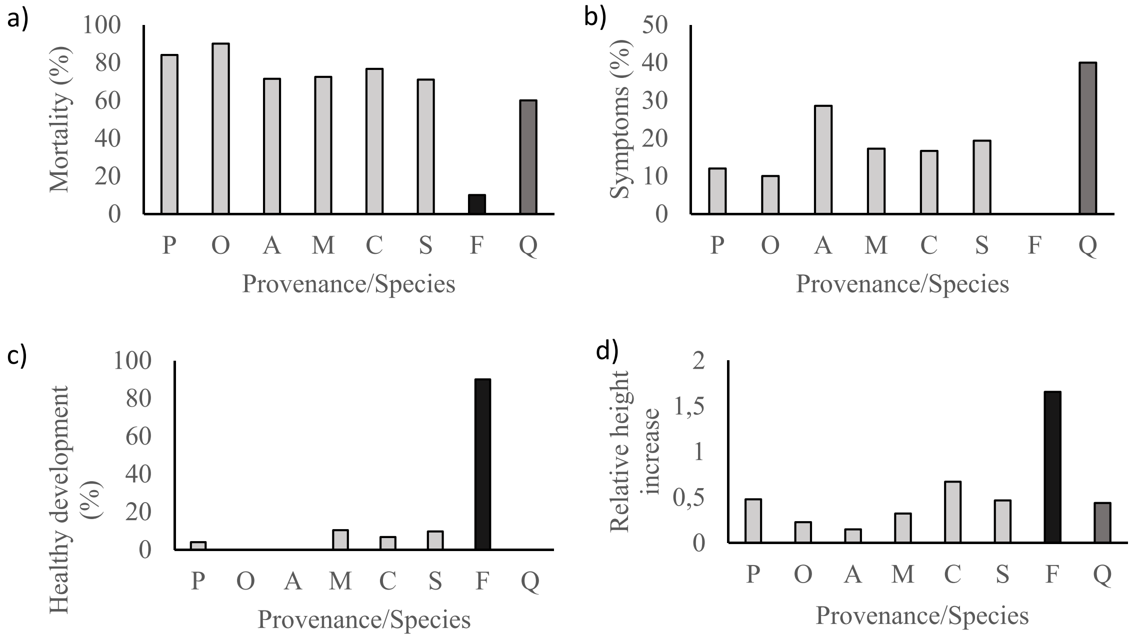

Fig. S4. Plant development in the Haza de la Concepción Slope plot (HCS) at the end of the four years of follow-up according to provenance. a) mortality (percentage of dead seedlings, b) percentage of symptomatic live plants, c) percentage of live and symptom-free plants with healthy development and d) relative height increase (final height minus initial divided by initial height after four years after planting). Provenances/species: *Quercus ilex* plants of P (Picos de Europa), O (Ordesa), A (Aigüestortes), M (Monfragüe), C (Cabañeros), and S (Sierra Nevada); F: *Fraxinus angustifolia*, Q: *Quercus faginea*.

CHAPTER 4

Allelopathic effects of three herb species on *Phytophthora cinnamomi*, a pathogen causing severe oak decline in Mediterranean wood pastures.

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Submitted to Forests (under revision)

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Abstract

The ability of three herbaceous plants (*Diplotaxis tenuifolia* (L.) DC., *Eruca vesicaria* L. and *Raphanus raphanistrum* L.) from Iberian wood pastures to reduce the *Phytophthora cinnamomi* Rands (Pc) pathogen populations through allelopathic relationships is studied. The inhibitory capacity of their aqueous root extracts (AREs) on mycelial growth and production of Pc reproductive structures is analysed *in vitro*. In addition, the effect of the herbaceous plants on *Quercu*s seedlings with those grown in Pc-infested soil is evaluated *in vivo*. Results show a strong inhibitory capacity of AREs on Pc activity *in vitro* and a protective effect of these herb species on *Quercus* plants against Pc *in vivo*. *D. tenuifolia* would be especially suited for biological control in the pathogen spread.

Key Message

The management of root allelopathic relationships in the Iberian wood pastures presents the ability of reducing the *Phytophthora cinnamomi* activity and consequently its infective capacity under field conditions.

Introduction

Plants can influence the composition of microbial communities around their roots through exudation of carbohydrates and other allelopathic compounds. Allelopathy is a naturally occurring ecological phenomenon of interference among organisms by which a plant produces one or more biochemical compounds that influence the development of other organisms (bacteria, fungi, other plants...), either negatively or positively (Molisch 1937, Rice 1984, Farooq *et al.* 2011). Some exudates present bactericidal and fungicidal activity and can affect the growth, survival and/or reproduction of various microorganisms. The allelochemicals most frequently involved in these fungicidal relationships are secondary metabolites that are not directly involved in the plant life cycle but play an important role in its defence against natural enemies. Allelochemicals are located in different parts of the plant, such as leaves, branches or roots (Ferguson *et al.* 2013). They are usually released directly into the aqueous phase of the soil, or from volatile gaseous substances in the surrounding air (Rizvi *et al.* 1992). The allelochemical release is influenced by the soil, climatic conditions and the plant itself (Singh *et al.* 2001).

Iberian agrosilvopastoral ecosystems are currently suffering an increasing decline with serious impact on oak species. The widespread *Quercus* decline is influenced by the action of biotic and abiotic stress factors. This disease is associated with different species of oomycetes and *Phytophthora cinnamomi* Rands (Pc) is the most frequently isolated from the Iberian Peninsula soils (Brasier 1992). This soil-borne pathogen affects more than 5000 trees, shrubs and herbs species in the world (Hardham and Blackman 2018) and causes root rot and death of several *Quercus* species. Its eradication from the soil in field is very complex due to the durability of its resistance structures and the easy spread by different pathways (but see Dunstan *et al*. 2010). Today, it is assumed that the control is only possible from an integrated perspective given its wide dispersion and high range of hosts. Especially susceptible are holm oak (*Q. ilex* L.) and cork oak (*Q. suber* L.), which are main tree components of Iberian wood pastures.

Among the different management tools against oak decline, biological control is presented as a still underdeveloped alternative but with certain advances in agricultural systems that could be the basis for the development of a forestry strategy (FAO, 2001).

But for this, a better knowledge of the plant-pathogen interactions and the possible allelopathies between them is essential. The plant tolerance is understood as reducing the damage caused by the pathogen infection and increasing evidence indicates that tolerance is a host defence strategy against pathogens as widespread and successful as resistance (Pagán and García-Arenal 2020). In addition to the susceptible holm oak and cork oak mentioned above, the Portuguese oak (*Q. faginea*) is also susceptible facing *P. cinnamomi*, although less than the first two (Rodríguez-Molina *et al.* 2002, Sánchez *et al.* 2005, Pérez-Sierra *et al.* 2013). Other susceptible and common species in dehesas and montados are those of *Cistus* and *Lupinus* genera (Cardillo *et al.* 2018, Moreira and Martins 2005, Serrano *et al.* 2011, Sampaio 2017). Pasture crops such as *L. luteus* are capable of increasing the density of *P. cinnamomi* viable inoculum in field while *Avena sativa* or *Triticum aestivum* crops are resistant to *P. cinnamomi* (Serrano *et al.* 2012), resulting in a potential reduction of its inoculum. However, several plant species of these agrosystems are still unknown asymptomatic hosts for pathogenic fungi (Cabeza and Bejarano 2010), and they can contribute to increase the pathogen´s population.

In addition, there are species that, instead of being susceptible to the pathogen, show resistance to it and even inhibit its infective activity due to the release of allelochemicals (Kong *et al.* 2019). In the Mediterranean flora there are various species with allelopathic effects against *P. cinnamomi* (Sampaio 2017, Ríos *et al.* 2016a 2016b, Moreira *et al.* 2018, Rodríguez-Molina *et al.* 2018). Their allelopathic property can be observed in plants used for biofumigation such as *Brassica carinata* and *B. juncea* (Ríos *et al.* 2016a 2016b, Rodríguez-Molina *et al.* 2018) and in other native flora still under study (Ríos *et al.* 2016a, Sampaio 2017, Moreira *et al.* 2018). The family to which they all belong, *Brassicaceae*, stands out for its high concentration of glucosinolates and its fungicidal effect in certain concentrations and conditions (Fenwick *et al.* 1983, Dunne 2004, Ríos *et al.* 2016a). These are sulfur compounds with proven fungicidal and biocidal capacity (Bialy *et al.* 1990, Wolf *et al.* 1984). Their defensive properties are generated by an enzymatic hydrolysis that releases volatile compounds, among which are isothiocyanates, nitriles, thiocyanates and oxazolidines, depending on the structure of the hydrolyzed glucosinolate (Fahey *et al.* 2001).

Based on the findings described by Sampaio (2017) and Moreira *et al.* (2018), three herbaceous species from the rangeland´s native flora with potential allelopathic effect facing *P. cinnamomi* were used in this study for *in vitro* and *in vivo* experiments. The work aims to answer the following questions:

1. What plant species have the greatest allelopathic ability on Pc by application of their AREs under *in vitro* conditions?

2. Do these herb species reduce the chemical defence levels of *Quercus* seedlings under *in vivo* conditions when they grow up together in Pc-infested soil?

Materials and methods

Biological material

The *Phytophthora cinnamomi* strain 5833 mating type A2 was isolated from chestnut (*Castanea sativa* Mill.) roots in central Portugal. Isolation and culture maintenance took place on V8 Juice agar medium as described by Moreira-Marcelino (2001).

Root extracts were prepared using 40-day-old plants of *Diplotaxis tenuifolia* (L.) DC., *Eruca vesicaria* (L.) Cav. and *Raphanus raphanistrum* L. A natural soil collected in Sintra was sown with seeds of these species collected in natural conditions (*E. vesicaria* and *R. raphanistrum*) and adquired in the market (*D. tenuifolia*). Plants grew under greenhouse conditions in Oeiras (INIAV) (Portugal, UTM Zone 29S X: 472148, Y: 4283072, 38 meters above sea level). After harvesting, plants were washed and the roots were frozen at -10ºC until be processed.

Acorns of *Quercus suber* (cork oak) *and Q. faginea* (Portuguese oak) were collected in in forest areas across the Concelhos of Ourique and Mafra (Portugal). These acorns were used to produce *Quercus* seedlings to assess the allelopathic effect of the herbaceous species *in vivo*.

Root extracts preparation using different methods and chemical characterization

Aqueous root extracts (AREs) were prepared by three different methods. The methods were (M1) maceration of fresh material without inactivation of enzymes (modified from Alkhail 2005), (M2) maceration of fresh material with heat inactivation of enzymes (Sampaio 2017, Moreira *et al.* 2018) and (M3) microwave dried material (modified from Hongju 1999).

AREs were prepared from 10 g of previously washed roots, with later maceration in 100 ml distilled water at room temperature in M1. All glucosinolates occur in the plant in conjunction with the hydrolytic isoenzyme myrosinase, and are located in separate cells (Hongju 1999). However, after trituration of plant tissues, they come together, so the enzyme must be inactivated to assess the glucosinolate effect. Thus, 10 g fresh weight was macerated in 100 mL distilled water at 80ºC in M2 for 10 minutes for its inactivation by heat. In the M3 method, after weighing 10 g of roots, they were microwaved at 900W for different times depending on their thickness and humidity (*R. raphanistrum* 9 minutes, *E. vesicaria* 2 minutes, *D. tenuifolia* 1 minute). Once dry, roots were crushed and macerated at room temperature with 100 mL distilled water. In all three methods, the solution was filtered and centrifuged for 10 minutes at 8000 *g* and 4°C. The supernatant was removed and filtered with Millex –GP 0.22 µm (33 mmØ) filters. AREs were frozen for conservation.

In order to know the chemical composition of AREs with the highest inhibitory ability, a general characterization was addressed. The followed procedure was the same described below with *Quercus* leaves in the *in vivo* experiment. In addition, the characterization of their volatile compounds by GC-MS was carried out at the Research Support Service of the University of Extremadura in Badajoz (Spain). Results (shown in supplementary information) were expressed as mean ± standard deviation and the differences among species (*D. tenuifolia, E. vesicaria* and *R. raphanistrum*) and treatments (field/greenhouse) were tested using a factorial ANOVA. Pairwise differences were considered as statistically significant at a value of $p<0.05$ by Tukey´s test. Statistical analyses were performed in Statistica v10 software.

In vitro *assessment of the allelopathic effect on the activity of Pc*

The AREs´inhibitory effect on *P. cinnamomi* activity was evaluated *in vitro*. The mycelial growth was measured in Petri dishes with V8 broth and ARE at 75% (v/v) as described Moreira *et al.* (2018), 12 days after their incubation at 25ºC in the dark. The dry weight mycelium (48 h at 60ºC) was recorded. Plates with V8 broth and 75% (v/v) of sterile water was used as control.

The sporangia production and the zoospores release and germination were also evaluated six days later, as well as the chlamydospores production, only for the AREs obtained by M2. The production of *P. cinnamomi* reproductive structures was obtained and analysed using non-sterile soil extract (Moreira-Marcelino 2001) with root extracts at 75% (v/v). V8-agar plugs (5) 5 mm in diameter were transferred from the edge of *P. cinnamomi* colony to the non-sterile soil extract supplemented with ARE and incubated at room temperature with indirect light. Plates were scored at six and 12 days later.

Sporangia and chlamydospores quantification was recorded as the mean number per mm^2 in the mycelium. Zoospores germination was evaluated using a zoospore suspension (100 µL) with 8.0 $x10^4$ cel /mL, plated on V8-agar 5% supplemented with ARE at 75% (v/v) and incubated at 25ºC in the dark. A treatment without ARE and supplemmented with sterile distilled water at 75% (v/v) served as a control. The colony-forming units were counted after 24 h and 48 h and the inhibition determined 48 h after plating. All trials had six repetitions per method and species. For the evaluation and taking of photographs of the different *P. cinnamomi* structures, samples were stained with lactophenol blue solution.

The percentage inhibition of *P. cinnamomi* was calculated as described by Moreira *et al.* (2018), according to the equation: Inhib (%) = 100(Control-Treatment)/Control. Data were transformed according to the equation: Inhibt = (100-Inhib (%))/100 to satisfy tests of normality. Transformed data were statistically analysed by ANOVA using the Tukey's test ($p < 0.05$) for differentiation of means. Statistical analyses were performed in Statistica v10 software.

In vivo *evaluation of the effect of allelopathic herbaceous species on* Quercus *seedlings in Pc-infested soil*

The inhibitory effect of *E. vesicaria* and *D. tenuifolia* against *P. cinnamomi* was analysed *in vivo* at INIAV greenhouses in Oeiras. Acorns of *Q. suber* and *Q. faginea* were sown in pots (10 L) with soil naturally infested with *P. cinnamomi*. The soil was collected in a decline foci located in Coruche (Portugal, soil pH: 4.9) in December. Presence of *P.*

cinnamomi in soil was confirmed by its isolation in PARPH selective medium (Jeffers and Martin 1986). Furthermore, *P. cinnamomi* inoculum was reinforced with 10 g per pot prepared according to the protocol described by Marcelino-Moreira (2001). This assay was carried out with three treatments: 1) *Quercus* species and herbaceous plants; 2) *Quercus* species and *Lupinus luteus*; 3) *Quercus* species without companion herbaceous (control). In the first treatment, 12 plants/pot of *E. vesicaria* and *D. tenuifolia* (high allelopathic effect on *P. cinnamomi*) were sown in containers with acorns of the each *Quercus* species. Ten seeds/pot of *L. luteus* (highly susceptible to *P. cinnamomi* and used as a positive control), were sown in each pot with acorns of the each *Quercus* species. There were five replicates for each species and treatment. The herbaceous plants grew together with the *Quercus* seedlings and two years later, 7-9 leaves from *Q. suber* and *Q. faginea* were collected from each pot to quantify their chemical phenolic defences in the presence of these companion herbaceous plants. It is assumed that this effect will be the combined action of herbaceous root exudates, together with possible interspecific competition relationships and even the facilitation of bacterial complexes in the soil. Thus, this *in vivo* essay attempt to be an approach to better understand the role that allelopathic relationships of herbaceous plants play on trees on Pc-infected Iberian wood pastures.

Extraction and quantification of phenolic content in Quercus *leaves*

For the determination of defensive phenolic content as described by Gallardo *et al.* (2019), *Quercus* leaves were lyophilized using a Telstar LyoQuest lyophilizer (temperature -55ºC and 0.001-0.002 mbar pressure) and ground to a fine particle size. The phenolic content was extracted from lyophilized material with 70% (v/v) aqueous methanol for 60 minutes in an ultrasonic bath at room temperature. The crude extracts were centrifuged at 8000 g for 5 minutes at 4ºC and the supernatant was collected and stored at -80ºC.

The total phenolics content (Tp) was determined by the Folin-Ciocalteu method (Singleton and Rossi 1965). Crude extracts (2 volumes) were mixed with 2 volumes of Folin-Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) and 40 volumes of 75 grams per liters of sodium carbonate. In the control tube, the extract volume was replaced by deionized water. The mixture was stirred gently and maintained in the dark and at room temperature for 60 minutes. After incubation, the absorbance was measured at 670 nm, using a UV/Vis Varian Cary-50 spectrophotometer (Palo Alto, California, USA). Gallic acid (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used as standard and results were expressed as micrograms of gallic acid equivalents (G.A.E.) per milligrams of lyophilized sample.

The total tannin content (Tt) was determined by the Folin-Denis method (Schanderl 1970). Crude extracts (2 volumes) were mixed with 2 volumes of Folin-Denis reagent (Panreac, Barcelona, Spain) and 5 volumes of 200 grams per liters of sodium carbonate. In the control tube, the extract volume was replaced by deionized water. The mixture was stirred gently and maintained in the dark and at room temperature for 30 minutes. After incubation, the absorbance was measured at 760 nm. Tannic acid (Panreac, Barcelona, Spain) was used as standard and results were expressed as micrograms of tannic acid equivalents (T.A.E.) per milligrams of lyophilized sample.

The butanol-HCl assay (Porter *et al.* 1986) was used to quantify condensed tannins (Ct) using procyanidin B2 (Sigma-Aldrich, Madrid, Spain) as a reference compound. Briefly, crude extracts were mixed with 100 volumes of n-butanol/acetone 1:1 (46% each) plus HCl (1.85%) and ferric ammonium sulphate (0.04%). In the control tube, the extract volume was replaced by methanol. Samples were heated at 70ºC. After 45 minutes of incubation, the samples were cooled and the absorbance at 550 nm was measured, with final results expressed as micrograms of procyanidin B equivalents (PB.E.) per milligrams of lyophilized sample.

Antioxidant activity (AA) and the half maximal inhibitory concentration (IC_{50}) in phenolic extracts of *Quercus* leaves were calculated to determine the root exudates ability of herbaceous species to influence the microbiota in the plant-pathogen infection. The AA was determined by the 2,2-diphenyl-1-picrylhydrazyl radical (DDPH) (Sigma-Aldrich, Madrid, Spain; Thaipong *et al.* 2006). Crude extracts (5 volumes) were mixed with 95 volumes of DPPH 3.35 mM in methanol. In the control tube, the extract volume was replaced by methanol. The mixture was stirred gently and maintained in the dark and at room temperature for 24 hours. After incubation, the absorbance was measured at 550 nm. Trolox (Sigma-Aldrich, Madrid, Spain) was used as standard and results were expressed as micromole of Trolox equivalents (T.E.) per milligrams of lyophilized sample. AA was expressed as a percentage inhibition of DPPH radical, and calculated from the equation: Scavenging activity (%) = ((Abs control – Abs sample)/Abs control) x 100. IC_{50} values were determined from the plotted graphs as scavenging activity against the concentration of the extracts. These values are defined as inhibitory concentration of the extract necessary to decrease the initial DPPH radical concentration by 50% and they are expressed in micrograms per millilitres.

Low molecular-weight phenolic compounds of the phenolics extracts from *Quercus* leaves were identified to determine which ones are involved in the *Quercus* defensive response facing Pc infection and the effect of the presence of herbaceous plants on their production. They were analysed on an Agilent 1200 liquid chromatograph instrument (Agilent Technologies, Santa Clara, CA, USA). The standard compounds used for their identification were gallic, protocatechuic, p-hydroxyphenyl, p-hydroxybenzoic, vanillic, caffeic, syringic, p-coumaric, ferulic, ellagic and salicylic acids; vescalagine, castalagine, catechin, aesculetin, epicatechin, vanillin, rutin-hydrate, myricetin, eriodictyol, quercetin, naringenin, kaempferol, syringaldehyde, and coniferyl and sinapyl aldehyde. The column used was Poroshell 120 SB-C18 (100 nm x 4.6 mm x 2.7 µm; Agilent Technologies, Santa Clara, CA, USA) and the mobile phases were water (0.1% formic acid, solvent A) and methanol (0.1% formic acid, solvent B). The gradient employed was the following: 0 min, 0% B; 1 min, 5% B; 16 min, 20% B; 30 min 50% B; 36 min, 100% B and was maintained for 5 minutes before returning to initial conditions. A flow rate of 1 mL per minute was used together with an injection volume of 0.5 µL and column temperature was fixed to 30°C. Detection was performed with a diode-array detector (255, 280, 305, 345 and 370 nm) and fluorescence detector (Ex= 275 nm, Em= 315 nm) and peak areas were used as analytical response.

Data were statistically analysed by GLM using Tukey´s test ($p<0.05$) for significant differences of means. Statistical analyses were performed in Statistica v10 software.

Results

In vitro assessment of the allelopathic effect in the activity of Phytophthora cinnamomi

The most effective method of ARE extraction was M2 (ARE extracted by heat inactivation of enzymes) due to its greater inhibition of mycelial growth and the non-viability of the reproductive structures of *P. cinnamomi* in the ARE presence (p< 0.001 Fig. 1, 2 and 3, Table 1). The species with the highest allelopathic capacity were *D. tenuifolia* and *E. vesicaria* for the same reasons (p< 0.001). There were significant differences in mycelial growth between these two species and *R. raphanistrum* (Tukey`s test p <0.001) but not between *D. tenuifolia* and *E. vesicaria* (Tukey´s test p> 0.05). The first two species caused a mycelial growth inhibition of up to 67.5% compared to the control. *R. raphanistrum* showed the lowest allelopathic activity (up to 55.73% growth inhibition).

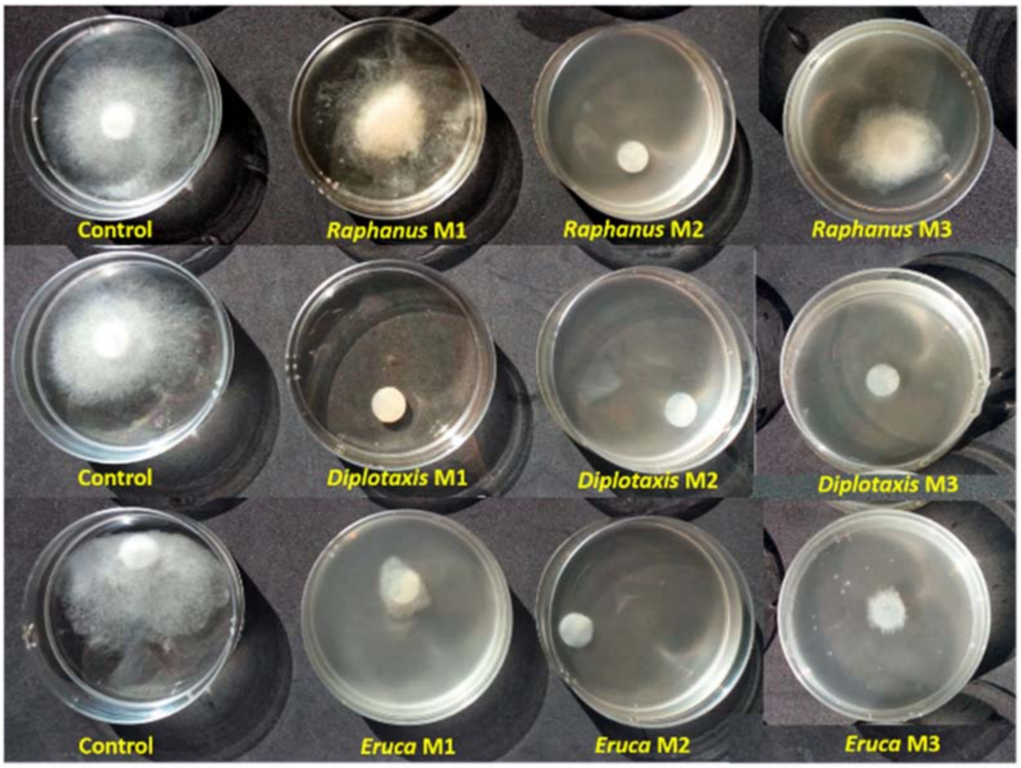

Figure 1. Inhibitory effect of different AREs extracted by three methods (M1, M2 and M3) on *Phytophthora cinnamomi* mycelial growth in V8 broth after 12 days at 25ºC in the dark. Method 2 (M2) was the most effective in all ARE, and in particular with *D. tenuifolia* ARE.

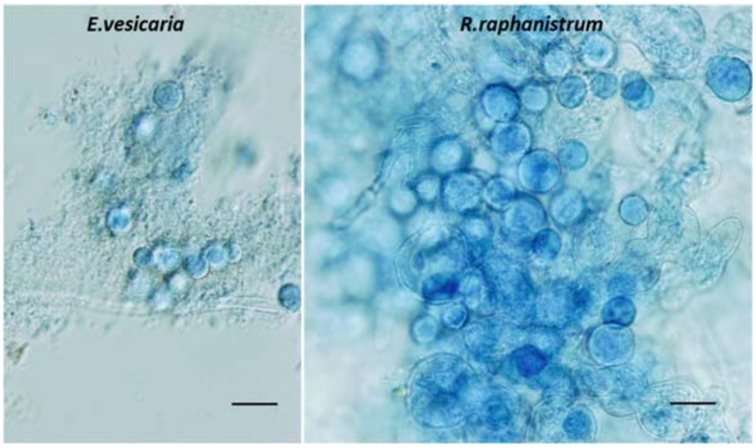

Figure 2. Zoospores encystment of *Phytophthora cinnamomi* in a non-sterile soil extract in presence of *E. vesicaria* and *R. raphanistrum* AREs. Bar 10 µm.

Table 1. Allelopathic effects of AREs on *Phytophthora cinnamomi* structures.

Allelopathic species	ARE´s pH	Method	Inhib. (%) mycelial growth	Inhib. (%) zoospore germination	Inhib. (%) sporangia after 6 days	Inhib. (%) sporangia after 12 days	Inhib. (%) chlamydospores after 12 days
Diplotaxis tenuifolia	5.38	M1	58.0				
		M2	67.5	83.7	100.0	100.0	100.0
		M3	65.3				
Eruca sativa	5.59	M1	62.4				
		M2	64.4	62.5	76.9	83.3	89.6
		M3	63.5				
Raphanus raphanistrum	4.94	M1	1.1				
		M2	55.7	37.5	30.7	33.3	44.3
		M3	19.9				

Sporangia and chlamydospore production showed reduction compared to the control with AREs application, especially with *D. tenuifolia* (100% inhibition in both, p <0.001). Significant inhibition of zoospore viability was observed on *D. tenuifolia* ARE (83.75% versus control, p <0.001). Generally, the *P. cinnamomi* mycelium in the presence of studied AREs showed lysis of the cytoplasm after six days and total destruction with *D. tenuifolia* ARE after 12 days (Figure 3). The non-viability of chlamydospores was 100% with *D. tenuifolia* ARE.

In presence of *E. vesicaria* and *R. raphanistrum* AREs, released zoospores showed a high and quick immobilization (Fig. 2) with encystment, compared to the control, in which zoospores were highly mobile for a long time. This result seems to be a good indicator of a reduction in *P. cinnamomi* activity with AREs of *E. vesicaria* and *R. raphanistrum.*

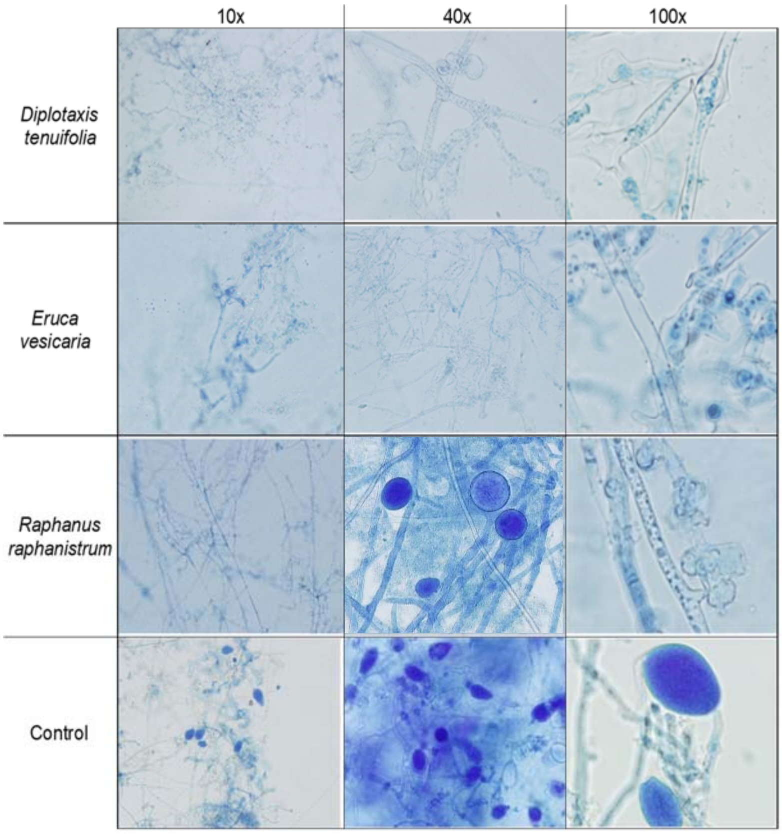

Figure 3. Mycelial growth and sporangia production. Although AREs (M2) of all species showed direct lysis of the sporangia and hyphae, the *D. tenuifolia* ARE showed the highest inhibition of *P. cinnamomi* activity (100% sporangia and chlamydospore inhibition, $p<0.001$).

In vivo *evaluation of the herbaceous species effect on* Quercus *seedlings in Pc-infested soil*

The presence of these herbaceous plants had a significant effect on the production of chemical defences on *Quercus* inoculated with *P. cinnamomi* ($p<0.05$, Table 2). The AA in *Quercus* phenolic extracts was also modified in the presence of these herb species (Table 2). The levels of chemical defences in Pc-susceptible *Quercus* species decreased especially with *D. tenuifolia* and *E. vesicaria*, without causing their death.

The Tp in *Q. faginea* leaves were higher in the control (1017.21 mg GAE/mg sample, Table S3 supplementary information) than in the herbaceous plant presence (from 797.42 to 954.79 mg GAE/mg sample, Tukey's test $p<0.05$). There were no significant differences in defence production among herbaceous plants (Tukey's test p> 0.05). In *Q. suber*, there were significant differences with the control and among herbaceous species. The highest value was reached with *L. luteus* (1566.88 mg GAE/mg sample) and the lowest with *E. vesicaria* (502.67 mg GAE/mg sample) and *D. tenuifolia* (439.59 mg GAE/mg sample).

Table 2. Effect of *Quercus* species (*Q. faginea* and *Q. suber*), herbaceous species (*D. tenuifolia, E. vesicaria* and *L. luteus*) and their interactions on *Quercus* chemical defences (total phenols Tp, total tannins Tt and condensed tannins Ct), antioxidant activity (AA and IC_{50}) and the major low molecular weight compounds in phenolic extracts (gallic acid GA, vescalagine Vesc., castalagine Cast., Catechin and ellagic acid EA).

Effect	df	Tp	Tt	Ct	AA	IC_{50}	GA	Vesc.	Cast.	Catechin	EA
Quercus species	1	0.53	309.81*	1467.94*	238.95	963.16*	10.31*	59.90*	37.76*	6730.80*	1698.40*
Herbaceous species	3	62.03*	1970.47*	433.12*	77.66*	46.09*	1.20	29.30*	77.96*	100.48*	8.09*
Quercus species*Herbaceous species	3	72.48*	12.82*	220.14*	64.03*	92.43 *	10.19*	109.00*	25.43*	93.07*	29.60*

F-values are shown along with statistical significance. *$p < 0.05$, ns $p > 0.05$.

The Tt concentration showed the same trends in both *Quercus* species, although values were significantly higher in *Q. suber* than in *Q. faginea* (Tukey's test p <0.05). The significantly higher values were reached in the control (503.09 mg TAE/mg sample in *Q. faginea*, 612.95 mg TAE/mg sample in *Q. suber*) and the lowest values with *L. luteus* (158.69 mg TAE/mg sample in *Q. faginea*, 226.31 mg TAE/mg sample in *Q. suber*). There were no significant differences between the herbaceous ones.

The Ct showed significant differences among *Quercus* and herbaceous species and were significantly higher in *Q. suber* than in *Q. faginea* (Tukey's test p <0.05). In *Q. suber*, the maximum Ct production was reached in the control (171.09 µg PBE/mg sample) and the minimum in *D. tenuifolia* (92.95 µg PBE/mg sample) and *E. vesicaria* (93.83 µg PBE/mg sample). In *Q. faginea*, the maximum Ct production was reached with *L. luteus* (104.26 µg PBE/mg sample) and the minimum one with *D. tenuifolia* (39.80 µg PBE/mg sample).

Regarding AA measured in *Quercus* leaves, both the herbaceous and *Quercus* and their interaction showed significant differences (p <0.001). AA was very high in *Quercus* grown without accompanying herbaceous plants (30.13 µmol TE/mg sample in *Q. suber*, 29.41 µmol TE/mg sample in *Q. faginea*). The highest antioxidant value was reached in the *Q. suber* with *L. luteus* (30.28 µmol TE/mg sample). The lowest antioxidant value was recorded in the *Q.* faginea with *L. luteus* (20.1 µmol TE/mg sample). Herbaceous plants with allelopathic capacity showed intermediate values in *Q. faginea* (22.74 µmol TE/mg sample in *E. vesicaria*, 23.32 µmol TE/mg sample in *D. tenuifolia*). In *Q. suber*, the minimum AA was registered with these two allelopathic species and they did not show statistically significant differences between them (26.39 µmol TE/mg sample in *E. vesicaria*, 25.72 µmol TE/mg sample in *D. tenuifolia*, Tukey test $p > 0.05$). The half maximal inhibitory concentration (IC_{50}) showed, as might be expected from its ability to measure the effectiveness of a compound's antioxidant capacity, the inverse pattern to AA. The effect of both *Quercus* and herbaceous plants showed significant differences ($p < 0.001$) in the IC_{50}. The highest values were always those of *Q. faginea*, with a maximum growing together with *L. luteus* (368.68 µg/mL) and a minimum in the control (308.36 µg/mL). In *Q. suber*, the maximum was reached with *D. tenuifolia* (286.76 µg/mL) and the minimum in the control (241.21 µg/mL). Intermediate values in *Q. faginea* were registered growing with the allelopathic *E. vesicaria* (335.93 µg/mL) and *D. tenuifolia* (312.30 µg/mL) and in *Q. suber* with *E. vesicaria* (267.33 µg/mL) and *L. luteus* (250.19 µg/mL).

From the HPLC analysis for the low molecular weight compounds in *Quercus* leaves, five of them were determinant: gallic acid, vescalagine, castalagine, catechin and ellagic acid. They showed significant differences between treatments (Tukey test $p < 0.05$, Table 2 and Table S3 from supplementary information). The major compound was vescalagine in all the samples. The minimum values were found in *Q. faginea* with *L. luteus* (4806.4 mg/L) and the maximum values in *Q. suber* with *E. vesicaria* (8341.9 mg/L). Castalagin was the second major compound, with minimum values in *Q. faginea* with *L. luteus* (413.8 mg/L) and maximum in *Q. suber* with *E. vesicaria* (663.0 mg/L). The third most abundant compound generated was catechin, with minimum values in *Q. faginea* with *D. tenuifolia* (21.9 mgL) and maximum in *Q. suber* with *E. vesicaria* (487 mg/L). Gallic acid was also lower in *Q. faginea*, but in this case with *E. vesicaria* (10.1 mg/L) and maximum once again in *Q. suber* with *E. vesicaria* (34.3 mg/L). On the contrary, ellagic acid was maximum in the control of *Q. faginea* (without herbaceous, 74.5 mg/L) and minimum in *Q. suber* with *E. vesicaria* (29.6 mgL).

Discussion

This study confirms the existence of allelopathic relationships capable of inhibiting the infective pathogen activity *in vitro* and *in vivo* conditions. From the three species studied, the ARE of *D. tenuifolia* was especially suited for its complete inhibition of the Pc sporangia production. In addition, when these herb plants grew together with *Quercus* seedlings in Pc-infested soils, the *Quercus* chemical defences lowered but did not die, which could be the result of a protective effect of the allelopathic species against infection. However, it would be necessary to explore the complex of soil microorganisms present in this allelopathic relationship for its better management before possible testing and application in the field.

*Anti-*Phytophthora *effects with AREs*

The ARE by heat inactivation of enzymes was the extraction method with the best allelopathic results. This result was expected because the method followed had been tested in Sampaio (2017) and Moreira *et al.* (2018) with conclusive results.

Regarding the species tested, the ARE of *D. tenuifolia* showed a very high effectiveness, with 67.55% inhibition of mycelial growth and 100% inhibition of sporangia production, in accordance with Moreira *et al.* (2018), although the percentage of inhibition in the aforementioned study was higher (83% for a 75% ARE concentration). Previous studies of *P. cinnamomi* inhibition with *E. vesicaria* (Ríos *et al.* 2016a, Sampaio 2017, Moreira *et al.* 2018) and *R. raphanistrum* (Sampaio 2017, Moreira *et al.* 2018) also confirm its allelopathic effect. *E. vesicaria*, whose main glucosinolates are aliphatic (glucoraphanin, glucosativin and glucoerucin) showed a high inhibition, but not the complete non-viability of its reproduction structures, according with what was discovered by Ríos *et al.* (2016a). *R. raphanistrum* showed lower inhibition values than the two previous species and very similar to that obtained by Moreira *et al.* (2018) for the mycelial growth of *P. cinnamomi*.

The total inhibition of *P. cinnamomi* sporangia production by applying the *D. tenuifolia* ARE is a very important advance demonstrated in this study. However, the *in vitro* conditions of the test must be taken into account and new experiments could be investigated under field conditions. Although *E. vesicaria* does not completely inhibit the production of sporangia, a very important reduction in the mobility of zoospores was observed when they are released in the presence of the extract. Zoospores lead to primary infections, so this reduction is key in the spread of the disease.

*Anti-*Phytophthora *herb species reduced the levels of chemical defences of* Quercus *plants* in vivo *in Pc-infested soil*

The *Quercus* phenolic defences showed significant differences depending on the companion allelopathic herbaceous species with those that grew together in *P. cinnamomi*-infested soil. The Tp and Ct were minimal with *E. vesicaria* and *D. tenuifolia* in both *Quercus*. The Ct were used in other studies as a good indicator of the Pc response (Gallardo *et al.* 2019). In this work, Ct were always higher in the susceptible *Q. suber* than in *Q. faginea*, which is more tolerant to Pc (Pérez-Sierra *et al.* 2013). With *L. luteus*, also highly susceptible to the pathogen (Serrano *et al.* 2011), both *Quercus* species increased their defensive levels against Pc, although the increase was greater in *Q. suber* (susceptible to Pc) than in *Q. faginea* (tolerant to Pc).

The lowest levels of Tp and Ct were reached when *Q. suber* grew alongside *D. tenuifolia*. There are previous studies on the increase of Tp and Ct in *Q. ilex* infected by Pc (Gallardo *et al.* 2019, Rodríguez-Romero *et al.* 2020). If we consider the chemical defence production in *Quercus* as a response to the attack they are suffering, it is expected that the *Quercus* species studied here also increase their defensive levels. However, in the presence of *E. vesicaria* and *D, tenuifolia*, aforementioned levels decreased with respect to the control (without the presence of herbaceous plants). This suggests that the allelopathic relationships established during their growth with *Quercus* in Pc-infested soil exerts a certain protective effect on *Quercus* seedlings.

It is known that interactions among plants are frequently controlled by root exudates, some of which have activity against microorganisms (Bais *et al.* 2006). In general, the high inhibitory activity of *D. tenuifolia* and *E. vesicaria* also acted *in vivo* conditions but we do not know if it was through their root exudates or if it is due to other factors involved in the joint growth of herbaceous plants with *Quercus*, such as competition between them during the first years. However, these results could be due to a decrease in the impact of Pc activity. The low infective efficacy of Pc could be explained by the release of secondary metabolites in allelopathic root exudates, or also by the facilitation of inhibitory soil bacteria against the pathogen. Notwithstanding the above, this study shows that the presence of these allelopathic herbaceous plants growing in Pc-infested soil reduced the defence level of *Q. suber*, which is susceptible to P. *cinnamomi*. Therefore, these herb species reduce the chemical defence costs against the pathogen´s attack. No previous studies are known with these species to establish comparisons, but the complex analysis of the herb-tree-pathogen interactions (Cazorla and Mercado-Blanco 2016, Shuping and Eloff 2017) should be continued to better understand the role of each component in that relationship.

As for the low molecular weight phenolic compounds, these were always minimum in *Q. faginea* and maximum in *Q. suber* (higher and lower tolerance to *P. cinnamomi*, respectively). Ockels *et al.* (2007) studied the differences in the phenolic chemical compounds of phloem tissue of *Q. agrifolia* infected with *P. ramorum*, finding a higher concentration of gallic acid. Furthermore, this compound was associated with greater tolerance to the pathogen. Del Río *et al.* (2003) also associated higher levels of catechin with greater tolerance to the genus *Phytophthora* in olive trees. Higher production of these defences would indicate the response to a higher attack caused by the infection. In the susceptible species (the cork oak) the accompanying allelopathic plant that less increases the levels of defences will be the most effective because it reduces Pc infection. This happened with *D. tenuifolia*, which confirms its inhibitory capacity against the pathogen observed *in vitro* conditions. However, ellagic acid showed the opposite pattern, and was higher in *Q. faginea* without allelopatic plants and lower in *Q. suber* with *E. vesicaria*. This compound seems to be directly related to tolerance to *Phytophthora* infection in other *Quercu*s species (Ockels *et al.* 2007, Nagle *et al.* 2011, McPherson *et al.* 2014, Conrad *et al.* 2014), although its levels in the presence of allelopathic herbaceous plants do not show a clear pattern, which suggests that there are other soil organisms (probably bacteria) also highly conditioned by plant-pathogen interaction. The authors consider the need to have a better knowledge of the surrounding microbiota relationships in order to explain its role.

Regarding AA in *Quercus* leaves, the highest one was found in *Q. suber* seedlings that grew together with *L. luteus* (both species susceptible to *P. cinnamomi*). The lowest AA was that of *Q. faginea* with *L. luteus* (*Quercus* species more tolerant to *P. cinnamomi* than *Q. suber*). Antioxidants protect biological systems against reactions or processes that can produce harmful effects in the individual. In a weakened system, as is the case of the *Q. suber* with *L. luteus* growing in a *P. cinnamomi* infested soil, it is to be expected that there would be more free radicals and therefore, a greater AA would be induced. However, in scenarios with more tolerant to Pc-species, such as *Q. faginea*, the expected AA would be lower. To strengthen the defensive response to biotic stress, seedlings induce a hypersensitive response that consists of programmed cell death to ensure the plant survival. When stress decreases, AA also drops (Irfan *et al.* 2011). In our study, when the *Quercus* species most susceptible to *P. cinnamomi* grew with *D. tenuifolia* and *E. vesicaria*, AA decreased, showing lower stress levels. In *Q. faginea*, this level also decreased in the presence of allelopathic species. Therefore, the presence of these allelopathic species generated a protective effect on *Quercus* seedlings, reducing their stress levels facing the pathogen infection. By its own definition, the higher the AA, the lower the IC_{50} and therefore, the more effective the exudate evaluated. However, both the AA and the IC_{50} in this test were calculated from the phenolic extract of *Quercus* leaves, so their values are an indirect measure of the effectiveness of the allelopathic herbs. But it would be very interesting to do a chemical characterization of their root exudates to discriminate other effects that could be involved in these plant-pathogen interactions.

The chemical composition of different plant extracts of the *Diplotaxis, Eruca* and *Raphanus* genera has been extensively studied in nutrition for its antioxidant and bactericidal properties (Nicoletti *et al.* 2007, D´Antuono *et al.* 2008, Mateu *et al.* 2018). Rarer is the chemical characterization of these species for biological control against plant pathogens. However, *Brassicaceae* family has been specially studied for its glucosinolate content for biofumigation. This is a widely used biological control tool against several pathogens. Ríos *et al.* (2016a) screened various brassicaceous plants (*D. tenuifolia* was not evaluated) to identify the most suitable and the compounds responsible for the inhibitory allelochemical activity of *P. cinnamomi*. They demonstrated the biocidal action on *P. cinnamomi* of rich in sinigrin-species, such as *B. juncea* and *B. carinata* (see also Fernández-Rebollo *et al.* 2015, Morales-Rodríguez *et al.* 2016, Ríos *et al.* 2017), while in others glucosinolates different from sinigrin (such as *E. vesicaria*) only a fungistatic effect was obtained. However, later *in vivo* tests to control the disease in cork oak with *B. carinata* pellets proved their significant effectiveness only when combined with calcium carbonate application (Rodríguez-Molina *et al*. 2018). In fact, all the authors agree on an integrated fight against the oak decline. Furthermore, the application of Brassicas uses high amounts of biomass and is not feasible in some lands due to orographic limitations. The novel approach of this work proposes the enrichment of pastures with allelopathic species against *P. cinnamomi*. The inoculum would not be eradicated from the soil, but the disease spread could be dimmed, increasing the tolerance of several species in Iberian wood pastures. The incorporation in the field of native plants capable of reducing the *P. cinnamomi* infective activity through their natural root exudates shows high potential in the possible control of the pathogen´s activity with a sustainable management in these agrosilvopastoral systems. Furthermore, the use of allelopathic plants has the advantage that they usually contain more than one antipathogenic compound, which hinders the development of resistance of pathogens (Shuping and Eloff 2017). However, it should still be deepened in several issues such as the knowledge of these relationships, their action and release in the field, the most recommended doses to avoid toxicity in other plants and whether other surrounding microorganisms are involved in them (see bacteria, for example).

References

Alkhail A (2005) Antifungal activity of some extracts against some plant pathogenic fungi. Pakistan journal of Biological Sciencies Vol 8(3): 413-417.

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. Ann. Rev. Plant. Biol. 57:233-266.

Bialy Z, Oleszek W, Lewis J, Fenwick GR (1990). Allelopathic potential of glucosinolates (mustard oil glycosides) and their degradation products against wheat. Plant and Soil 129: 277-281.

Brasier CM (1992). Oak tree mortality in Iberia. Nature, 360: 359.

Cabeza E., Bejarano J. (2010). Influencia de las cubiertas vegetales sobre la epidemiología y control de las enfermedades del olivo. En: E.J. González, R.

Ordóñez y J.A. Gil (eds.). Aspectos agronómicos y medioambientales de la agricultura de conservación: 165-175. Eumedia-Ministerio de Medio Ambientes y Medio Rural y Marino, Mundi-Prensa. Madrid.

Cardillo E, Pérez C, Rodríguez-Molina, MC (2018). El papel de las jaras en el decaimiento de encinas y alcornoques: análisis de susceptibilidad-tolerancia de *Cistus* sp a *Phytophthora cinnamomi*. En: Libro de Resúmenes XIX Congreso de la Sociedad Española de Fitopatología, Toledo (España).

Cazorla FM, Mercado-Blanco J (2016). Biological control of tree and woody plant diseases: an impossible task? BioControl 61: 233-242.

Conrad AO, Rodriguez-Saona LE, McPherson BA, Wood DL and Bonello P (2014). Identification of *Quercus agrifolia* (coast live oak) resistant to the invasive pathogen *Phytophthora ramorum* in native stands using Fourier-transform infrared (FT-IR) spectroscopy. Front. Plant Sci. 5:521.

D´Antuono LF, Elementi S, Neri R (2008). Glucosinolates in *Diplotaxis* and *Eruca* leaves: Diversity, taxonomic relations and applied aspects. Phytochemistry 69(1):187-99.

Del Río JA, Baidez AG, Botia JM, Ortuno A (2003). Enhancement of phenolic compounds in olive plants (*Olea europaea* L.) and their influence on resistance against Phytophthora sp. Food Chem. 83, 75–78.

Dunne C (2004) Control of sudden death in cultivated proteas from the South west of Western Australia. PhD Thesis. Murdock University, Australia.

Dunstan WA, Rudman T, Shearer BL, Moore NA, Paap T, Calver MC, Dell B, Hardy GESJ (2010). Containment and spot eradication of a highly destructive, invasive plant pathogen (*Phytophthora cinnamomi*) in natural ecosystems. Biological Invasions, 12, 913–925.

Fahey JW, Zalcmann AT, Talalay P (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry, 56: 5-51.

FAO (2001). Protecting plantations from pests and diseases. Report based on the work of W.M. Ciesla. Forest Plantation Thematic Papers, Working Paper 10. Forest

Resources Development Service, Forest Resources Division. FAO, Rome. (http://www.fao.org/forestry)

Farooq M, Jabran K, Cheema ZA, Wahid A, Siddique KH (2011) The role of allelopathy in agricultural pest management. Pest Management Science 67(5): 493-506

Fenwick RG, Heaney RK, Mullin WJ (1983). Glucosinolates and their breakdown products in food plants. CRC. Crit Rev Food Sci Nutr 18:123-201.

Ferguson JJ, Rathinasabapathi B, Chase CA (2013). Allelopathy: How plants suppress other plants. University of Florida, 1–5. Retrieved from http://edis.ifas.ufl.edu/hs186

Fernández-Rebollo P, Carbonero MD, Leal JR, García AM, Ríos P, Sánchez ME, Obregón S, de Haro A (2015). Producción, concentración y perfil de glucosinolatos de *Brassica carinata* y *Brassica juncea* cultivadas en la dehesa. In: Cifre J, Janer I, Gulias J, Jaume J, Medrano M (eds). Pastos y forrajes en el siglo XXI. Palma de Mallorca, SEEP, pp 193-200.

Gallardo A, Morcuende D, Solla A, Moreno G, Pulido FJ, Quesada A, 2019. Regulation by biotic stress of tannins biosynthesis in *Quercus ilex*: crosstalk between defoliation and *Phytophthora cinnamomi* infection. Physiol Plant 165: 319-329.

Hardham AR, Blackman LM (2018). *Phytophthora cinnamomi*. Mol Plant Pathol. 19(2):260-285.

Hongju H (1999) Studies for growth adaptation and identification of glucosinolates in various Chinese *Brassicas*. PhD Thesis. Herbert Utz Verlag-Wissenschaft, München, Germany.

Irfan M, Hayat S, Bashir A (2011). Regulation of antioxidant system under biotic stress in plants. In: Plant under stress. Stadium Press, New Delhi.

Jeffers S and Martin S (1986) Comparison of two media selective for *Phytophthora* and *Phythium* species. Plant Disease vol 70(11): 1038-1043.

Kong CH, Xuan TD, Khanh TD, Tran HD, Trung NT (2019). Allelochemicals and signaling chemicals in plants. Molecules 24(15): 27-37.

Mateu G, Dimitrova P, Petkova N, Ivanov I, Mihaylova D (2018). Antioxidant activity and mineral content of rocket (*Eruca sativa*) plant from Italian and Bulgarian origins. J Microbiol Biotech Food Sci 8(2): 756-759.

McPherson BA, Mori SR, Opiyo SO, Conrad AO, Wood DL, Bonello P (2014). Association between resistance to an introduced invasive pathogen and phenolic compounds that may serve as biomarkers in native oaks. Forest Ecology and Management 312: 154-160.

Molisch H (1937). Der Einfluss einer Pflanze auf die andere Allelopathie. Jena, Fischer.

Morales-Rodríguez C, Vettraino AM, Vannini A (2016). Efficacy of biofumigation with *Brassica carinata* comercial pellets (BioFence) to control vegetative and reproductive structures of *Phytophthora cinnamomi*. Plant Disease 100(2): 324-330.

Moreira A, Calha I, Passarinho JA, Sampaio A (2018). Anti-P*hytophthora* activity of root extracts from herbaceous species. Revista das Ciências Agrárias 41 (Especial): 39-47.

Moreira AC, Martins MS (2005). Influence of site factors on the impact of *Phytophthora cinnamomi* in cork oak stands in Portugal. Forest Pathology 35:145-162.

Moreira-Marcelino AC (2001). Aspectos da interacção entre *Phytophthora cinnamomi* e a doença do declínio em *Q. suber* e *Q. rotundifolia*. Dissertação para Prestação de Provas de Doutoramento, Faculdade de Engenharia de Recursos Naturais, Universidade do Algarve, Faro. pp279.

Nagle AM, McPherson BA, Wood DL, Garbelotto M, Bonello P (2011). Relationship between field resistance to *Phytophthora ramorum* and constitutive phenolic chemistry of coast live oak. Forest Pathology 41(6): 464-469.

Nicoletti R, Raimo F, Miccio G (2007). Diplotaxis tenuifolia: biology, production and properties. EJPSB. 1:36–43.

Ockels FS, Eyles A, McPherson BA, Wood DL, Bonello P (2007). Phenolic chemistry of coast live oak response to *Phytophthora ramorum* infection. J Chem Ecol 33(9):1721-32.

Pagán I, García-Arenal F (2020). Tolerance of plants to pathogens: a unifying view. Annual Review of Phytopathology 010820-012749.

Pérez-Sierra A, López-García C, Leon M, García-Jiménez J, Abad-Campos P, Jung T (2013). Previously unrecorded low-temperature *Phytophthora* species associated with *Quercus* decline in a Mediterranean forest in Eastern Spain. Forest Pathology, 43: 331-339.

Porter LJ, Hirstich LN, Chan BG, 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochem 25: 223-230.

Rice EL (1984). Allelopathy. 2nd ed., New York, Academic Press.

Ríos P, González M, Obregón S, Carbonero MD, Leal JR, Fernández P, De Haro A, Sánchez ME (2017). Brassica-based seedmeal biofumigation to control *Phytophthora cinnamomi* in the Spanish “dehesa” oak trees. Phytopathologia Mediterranea 56(3):392-399.

Ríos P, Obregón S, González M, de Haro A, Sánchez ME (2016). Screening brassicaceous plants as biofumigants for management of *Phytophthora cinnamomi* oak disease. For Path 46(6): 652-659

Ríos P, Obregón S, Haro A, Fernández-Rebollo P, Serrano M, Sánchez ME (2016). Effect of *Brassic*a biofumigant amendments on different stages of the life cycle of *Phytophthora cinnamomi*. Journal of Phytopathology 164 (9): 582-594.

Rizvi SJH, Haque H, Singh UK, Rizvi V (1992). A discipline called allelopathy. In: Rizvi SJH, Rizvi H (Eds.). Allelopathy: Basic and applied aspects. London, Chapman & Hall, p.1-10.

Rodríguez-Molina MC, Serrano-Pérez P, Santiago R, Cardillo E, Godoy B y De Santiago A (2018). Efecto de la biofumigación con pellets de *Brassica carinata* en la progresión de la enfermedad causada por *Phytophthora cinnamomi* en *Lupinus luteus*. En Libro de Resúmenes XIX Congreso de la Sociedad Española de Fitopatología, Toledo (España).

Rodríguez-Molina MC, Torres-Vila LM, Blanco-Santos A, Nunez EJP, Torres-Álvarez E (2002). Viability of holm and cork oak seedlings form acorns sown in soils naturally infected with *Phytophthora cinnamomi*. Forest Pathology, 32(6): 365-372.

Rodríguez-Romero M, Gallardo A, Pulido F (2020). Geographical and within-population variation of constitutive chemical defences in a Mediterranean oak (*Quercus ilex*). Forest Systems 29 (1): e011.

Sampaio AR (2017). Selecção de plantas com efeito alelopático para controlar *Phytophthora cinnamomi* Rands. Dissertação para obtenção do grau de Mestre em Engenharia Agronómica-Protecção de Plantas. ISA/UL, 71 pp.

Sánchez ME, Andicoberry S, Trapero A (2005). Pathogenicity of three *Phytophthora* spp. causing late seedling rot of *Quercus ilex* ssp. *ballota*. Forest Pathology, 35(2): 327-334.

Schanderl SH (1970). Method in food analysis. Academic press, New York, USA. 709 pp.

Serrano M, Fernández-Rebollo P, De Vita P, Carbonero M, Sánchez ME (2011). The role of yellow lupin (*Lupinus luteus*) in the decline affecting oak agroforestry ecosystems. Forest Pathology 4(5): 382-386.

Serrano MS, Fernández-Rebollo P, de Vita P, Sánchez ME (2012) Susceptibility of common herbaceous crops to *Phytophthora cinnamomi* and its influence on *Quercus* root rot in rangelands. European Journal of Plant Pathology 134(2): 409-414.

Shuping DSS, Eloff JN (2017). The use of plants to protect plants and food against fungal pathogens: a review. Afr J Tradit Complement Altern Med 14(4): 120-127.

Singh H, Batish D, Hohli R (2001). Allelopathy in agroecosystems: an overview. The Haworth Press, Inc, 1-42.

Singleton VL, Rossi JA (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic 16:144-158.

Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Byrne DH (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. J Food Compos Anal. 19(6-7): 669-675.

Wolf RB, Spencer GF, Kwolek WF (1984). Inhibition of velvet leaf (*Abutilon thephrasti*) germination and growth by benzyl isothiocyanate, a natural toxicant. Weed Science 32: 612–615.

Supplementary information

Chemical characterization of AREs

AREs of *D. tenuifolia*, *E. vesicaria* and *R. raphanistrum* were made from plants grown in the field (natural conditions) and others grown in the greenhouse (under controlled conditions) to identify possible differences due to the herbaceous material´s source. The AREs´pH of was always acidic and ranged from the most acidic values of *R. raphanistrum* in the field (4.94) to the closest to neutral of *R. raphanistrum* in the greenhouse (6.21). The *D. tenuifolia* pH ranged between 5.11 in the greenhouse and 5.38 in the field. The *E. vesicaria* pH did not show statistically significant differences between provenances (5.59 in the field, 5.78 in the greenhouse). There was no significant correlation between the phenolic groups evaluated and the pH of the extract (Tukey´s test p> 0.05).

The AREs´ defensive chemical profile and their AA differed significantly among herbaceous species and plant sources (p <0.001, Table S1). The Tp were lower in *D. tenuifolia* (18.44 µg GAE/mg sample in the field, 36.56 µg GAE/mg sample in the greenhouse, Table S2) and higher in R. *raphanistrum* (46.26 µg GAE/mg sample in the field, 59.18 µg GAE/mg sample in greenhouse). The *E. vesicaria* values were intermediate (39.98 µg GAE/mg sample in the field, 38.66 µg GAE/mg sample in the greenhouse) and did not show statistically significant differences (Tukey's test p >0.05).

Table S1. Effect of AREs species (*D. tenuifolia*- D, *E. vesicaria*- E and *R. raphanistrum*- R), sources (field/greenhouse) and their interactions on chemical defences (total phenols Tp, total tannins Tt and condensed tannins Ct) and antioxidant activity (AA and IC_{50}).

Effect	df	Tp	Tt	Ct	AA	IC_{50}
Source (field/greenhouse)	1	384.10*	4.49	210.76*	19.91*	749.16*
Herbaceous species (D, E, R)	2	830.08*	33.91*	886.78*	236.85*	2513.20*
Source*Species	1	132.12*	75.30*	2744.69*	12.83*	559.54*

F-values are shown along with statistical significance. **p* < 0.05, ns *p* > 0.05.

The Tt were also lower in *D. tenuifolia* (0.76 µg TAE/mg sample in the field, 4.24 µg TAE/mg sample in greenhouse, Table S2) and higher in *R. raphanistrum* from the field (8.31 µg TAE/mg sample). However, the Tt of *R. raphanistrum* grown in a greenhouse showed low values (1.69 µg TAE/mg sample). *E. vesicaria* showed intermediate values (5.42 µg TAE/mg sample in the field, 6.84 µg TAE/mg sample in greenhouse) and there were no statistically significant differences between provenances for this species (Tukey´s test $p > 0.05$).

The Ct were significantly high in *D. tenuifolia* from the field (95.66 µg PBE/mg sample) and very low in *D. tenuifolia* from the greenhouse (39.92 µg PBE/mg sample), *E. vesicaria* (37.69 µg PBE/mg sample in the field, 30.8 µg PBE/mg sample in greenhouse) and *R. raphanistrum* in the field (30.98 µg PBE/mg sample). In contrast, *R. raphanistrum* from greenhouse also showed high Ct (74.98 µg PBE/mg sample).

AA was significantly lower in *D. tenuifolia* ARE than in the other two species (0.11 µmol TE/mg sample in the field, 0.25 µmol TE/mg sample in the greenhouse, compared to 0.53 µmol TE/mg sample of *E. vesicaria* in the field and 0.5 µmol TE/mg sample in greenhouse, and compared to 0.42 µmol TE/mg sample of *R. raphanistrum* in the field and 0.48 µmol TE/mg sample in greenhouse). Among the latter, there were no significant differences (Tukey test $p > 0.05$). Therefore, the IC_{50} showed the inverse pattern, with maximum and significant values in *D. tenuifolia* in the field (24872.60 µg/mL and 12560.00 µg/mL in the greenhouse). The IC_{50} was lower in the other two species, although without significant differences between sources within them (in *E. vesicaria*, 8182.70 µg/mL in the field and 7420.00 µg/mL in the greenhouse, $p > 0.05$; in *R. raphanistrum*, 5466.04 µg/mL in the field and 5001.26 µg/mL in the greenhouse, $p > 0.05$).

Regarding the general characterization of the volatiles released by AREs, the GC/MS screening carried out by the Research Support Service of the University of Extremadura identified the presence of several compounds from the isothiocyanate family, among others: 3 -butenyl isothiocyanate, butyl isothiocyanate, n-pentyl isothiocyanate, 4-methylpentyl isothiocyanate and hexyl isothiocyanate. Only the butylisothiocyanate compound was common in the six AREs and in a higher proportion in *D. tenuifolia* than in *E. vesicaria*, and less in *R. raphanistrum*, although new tests would be necessary for its correct quantification.

Table S2. Chemical composition of aqueous root extracts according to species and source. Total phenols (Tp), total tannins (Tt), condensed tannins (Ct), antioxidant activity (AA) and the half maximal inhibitory concentration (IC_{50}). Means and standard deviation.

Species	Source	pH	Original concentration (mg/mL)	Concentration for analysis (mg/mL)	Tp (µg GAE/mg sample)	Tt (µg TAE/mg sample)	Ct (µg PBE/mg sample)	AA (µmol TE/mg sample)	IC_{50} (µg/mL)
D. tenuifolia	field	5.38	43	3.4	18.44±0.17	0.76±0.19	95.66±0.97	0.11±0.01	24872.60±894.00
D. tenuifolia	greenhouse	5.11	18.45	3.4	36.56±0.27	4.24±1.37	32.92±0.73	0.25±0.01	12560.00±795.00
E. vesicaria	field	5.59	9.95	3.4	39.98±0.57	5.42±0.00	37.69±0.81	0.53±0.01	8182.70±14.30
E. vesicaria	greenhouse	5.78	7.95	3.4	38.66±1.48	6.84±0.39	30.80±0.67	0.50±0.03	7420,00±112.10
R. raphanistrum	field	4.94	10.9	3.4	46.26±0.38	8.31±0.70	30.98±0.72	0.42±0.00	5466.04±101.19
R. raphanistrum	greenhouse	6.21	4.55	3.4	59.18±2.03	1.69±0.15	74.98±2.50	0.48±0.00	5001.26±118.57

Effects of root exudates of allelopathic herbaceous species on Quercus *chemical defences*

Table S3. Chemical composition of phenolic extracts from *Quercus* leaves grown with allelopathic root exudates and *Phytophthora cinnamomi* infection. Total phenols (Tp), total tannins (Tt), condensed tannins (Ct), antioxidant activity (AA), the half maximal inhibitory concentration (IC_{50}) and the major low molecular weight compounds in phenolic extracts (gallic acid GA, vescalagine Vesc., castalagine Cast., Catechin and ellagic acid EA). Means are shown ± standard deviation.

Quercus species	Herbaceous species	Tp (mg GAE/mg sample)	Tt (mg TAE/mg sample)	Ct (µg PBE/mg sample)	AA (µmol TE/mg sample)	IC_{50} (µg/mL)	Low-molecular weight compounds (mg/L)				
							GA	Vesc.	Cast.	Catechin	EA
Q. suber	*E. sativa*	502.7±22.7	233.7±10.5	93.8±0.9	26.3±0.7	267.3±4.4	34.2±6.3	8341.9±197.8	663.1±24.2	487±9.2	29.6±2.4
Q. suber	*D. tenuifolia*	439.5±30.8	223.0±13.6	92.9±5.9	25.7±1.2	286.7±2.8	18.2±3.5	6654.2±219.1	596.9±21.9	302.3±9.2	32.9±1.3
Q. suber	*L. luteus*	1566.8±238.3	226.3±19.5	115.0±2.0	30.2±0.5	250.1±20.3	16.5±0.8	7912.7±104.2	494.7±9.1	356.9±5.6	38.1±0.9
Q. faginea	*E. sativa*	928.2±65.8	178.4±10.8	63.1±7.8	22.7±0.9	335.9±7.7	10.1±2.1	5542.3±132.4	503.1±11.7	29.1±0.5	70.4±1.3
Q. faginea	*D. tenuifolia*	954.7±135.6	180.5±9.6	39.8±8.3	23.2±0.4	312.3±9.3	19.2±7.2	7453.7±592.8	599.2±16.5	21.9±0.5	73.1±2.7
Q. faginea	*L. luteus*	797.4±56.7	158.6±9.0	104.2±10.6	20.1±0.4	368.6±9.2	18.4±9.1	4806.4±168.9	413.8±18.4	64.7±0.8	59.7±1.3
Q. suber	control	1266.7±81.8	612.9±19.0	171.0±4.4	30.1±1.8	241.2±6.3	22.6±1.7	4925.8±373.1	607.1±44.2	433.6±30.5	33.8±3.1
Q. faginea	control	1017.1±89.4	503.0±14.0	73.2±6.1	29.4±0.3	308.3±7.1	19.3±6.9	6468.6±461.7	636.6±27.9	57.7±6.4	74.5±4.5

GENERAL DISCUSSION

GENERAL DISCUSSION

This Thesis has dealt with various aspects related to the geographical and local variation of the constitutive and induced chemical defences in the holm oak affected by biotic stressors of the root and the aerial part. The consistency of the results obtained in the greenhouse was subsequently evaluated in the field to reinforce the conclusions. This work has also covered defensive chemical aspects in two other species of Mediterranean oaks, the cork oak and the gall oak. In this case, the protective character that certain species of the Mediterranean flora with allelopathic capacity can exert on oaks stressed by the pathogen Pc was evaluated.

The three species of Mediterranean oaks are of high environmental and socio-economic importance in rural southern Europe and their conservation is highly threatened by the fast progress of oak decline. This decline is generalized, spreads worldwide and is closely related to the current increase in the pressure of biotic and abiotic stressors. All this makes it essential to increase knowledge of plant-pathogen interactions to face the new challenges in the conservation of oak stands.

In particular, this Thesis has contributed to improve the knowledge of the chemical defences involved in plant-stressor interactions, their variability as a function of provenances, populations and genotypes, and the heritability of these chemical traits. These concepts are basic in the design of a breeding and improvement program to select plant material that can be used in the restoration of the most affected areas. Likewise, the identification of protective allelopathic species of oaks against Pc infection opens a new line of biological control on a widely distributed biotic stressor whose eradication seems complex. The main results obtained from this work are discussed below in a wider context.

Geographical and local variation of the constitutive defences in *Q. ilex*

In the **first chapter**, we evaluate whether the constitutive levels of the main chemical defences in *Quercus* (total phenols and condensed tannins) vary among regions, populations and families in *Quercus ilex*. The results showed that the constitutive levels of heritable chemical defences in holm oak depend significantly on its geographical origin and family, but not on populations within regions. Previous studies in holm oak and cork oak studied their phenolic content (Faeth 1986, Dawra *et al.* 1988, Cadahía *et al.* 1993) but there are hardly any works on their geographical variation. However, a previous holm oak study estimated the concentration of total tannins in leaves and its geographic variation (Solla *et al.* 2016), although in a lower latitudinal range than that of our study. This work did not find differences among origins, but did find differences among families. Other studies such as Moreira *et al.* (2018) in a greater latitudinal gradient did find differences among the content of condensed tannins of *Q. robur* from different provenances. This disparity in the estimates of different phenolic groups evidences the need to continue studying the chemical patterns in *Quercus* and the importance of the latitudinal gradient evaluated.

Regarding the evaluated groups, total phenols and condensed tannins were positively correlated. Moreover, our results are consistent with the theory predicting that chemical defences should decrease as latitude increases (Rasmann & Agrawal 2011, Abdala-Roberts *et al.* 2016). The lowest levels of constitutive defences were found in the north of the Iberian Peninsula. However, recent studies have found a positive relationship between latitude and phenolic compounds in species such as *Q. robur* (Moreira *et al.* 2018) or *Pinus pinaster* (López-Goldar *et al.* 2019). This trend could be linked to underlying factors associated with latitudinal variation such as climate and soil (Moreira *et al.,* 2018). In our work, the type of soil described by pH also showed an apparent effect on phenolic defences. Seedlings from acidic soil regions had a higher phenolic content than those from alkaline soils. Soil pH influences the availability of nutrients for plants and, therefore, can be the cause of deficiency or toxicity (Benton 2003). Tannins can induce allelopathic responses at the ecosystem level, including toxicity against pathogens (Kraus *et al.* 2003). One of the most widespread and aggressive pathogens in recent decades is Pc, the soil oomycete that causes root rot and is identified as the main causal agent for the *Quercus* decline in the Iberian Peninsula (Brasier 1992, 1996, Sánchez *et al.* 2006, Corcobado *et al.* 2014). This pathogen grows better in acidic than basic soils (Blaker & MacDonald 1983, Dixon 1984, Duvenhage & Kotzé, 1995). In fact, calcium mineral nutrition raises soil pH and increases tolerance of *Q. ilex* to *Phytophthora* root disease (Serrano *et al.* 2013). Assuming the important role that tannins exert on soil pathogens (Scalbert 1991, Stong *et al.* 2013), plants in basic soil would not need to produce high levels of these compounds, as we see in our study, because the pathogen does not perform well at high pH. However, in acid soils, this increased production of phenolic compounds could be the defensive response to a higher pressure by the pathogen.

Heritability of defensive chemical traits

The heritability of the defensive chemical traits of *Q. ilex* was calculated in the **first chapter** from constitutive defences of the six studied provenances. The heritability obtained for total phenols and condensed tannins was moderate. In the context of tree improvement, determining the narrow-sense heritability is a useful tool to know how much of the observed variation of a certain quantitative character is due to the additive genetic component. The heritability values in our study were lower than those obtained in a previous work in the same species but for the total tannin content (Tt) with fewer families sampled (0.37 in Tp, 0.48 in Ct versus 0.83 in Tt; Solla *et al.* 2016). When the narrow-sense heritability is moderate, it is likely to allow selection (Eriksson *et al.* 2013, Alcaide *et al.* 2019, 2020) but these results must be taken with caution due to the low number of families evaluated (Jensen & Barr 1971, Visscher & Goddard 2015). However, this could enable short-term evolutionary change in response to selection and makes the selection of material from regions with higher constitutive levels meaningful in future species improvement programs.

On the other hand, genetic differentiation by quantitative traits (Q_{ST}) was moderate for Ct (0.25) and higher in Tp (0.51) in the six regions analyzed. Solla *et al.* (2016) studied the Q_{ST} in total tannins, observing a lower genetic differentiation between the *Q. ilex* regions (0.12). There is a general lack of knowledge regarding this variation in chemical defences. However, this value allows Q_{ST}-F_{ST} comparisons and thus, discrimination between natural selection and genetic drift as differentiation mechanisms in traits (Leinonen *et al.* 2013). Therefore, this study offers a first insight into the characterization of variations that *Q. ilex* secondary metabolites undergo in the Mediterranean area. It would also be very interesting to calculate the fixation index (F_{ST}) to find out if there is divergent selection in the species and thus, confirm whether the different genotypes are favored under different environmental conditions (Merilä & Crnokrak 2001).

Chemical defences induced by stressors

After analyzing the constitutive defences in holm oak, the **second chapter** of the Thesis has focused on the induction of defences by two types of biotic stressors. The damage at the root level by the soil-borne pathogen Pc and the aerial mechanical defoliation simulating the damage by herbivores under controlled greenhouse conditions contributed to a better understanding of the geographical variation in the induction of defences. The two stressors are common in oak stands that are currently suffering a serious worldwide decline. So, it is urgent to unveil their resistance mechanisms for a better control and management of oak decline.

The results of the **second chapter** showed that, as happened with the constitutive defences, the induced ones also showed a great geographical and local intraspecific variation. The plant provenance was the main source of variation of the three phenolic groups evaluated (total phenols, total tannins and condensed tannins). In addition to those cited above, other works on the geographical variation of chemical defences in forest species have found mixed results. Although there are some works on spatial variation in the effect of biotic stress or the production of chemical defences, few studies combine geographical variation with induction of defences by defoliation and pathogen infection. However, Pardo *et al.* (2018) explore the spatial variation induced in *Prunus lusitanica* Tp by mechanical defoliation for its entire range of distribution, also finding geographical and local differences. Another study on the variation of Tp induced by the attack of the pine wilt nematode (*Bursaphelenchus xylophilus*) in *Pinus pinaster* in the Iberian Peninsula also showed differences at the same spatial levels (Zas *et al.* 2015). Although to date, there are no known studies of the geographical variation of induced defences in holm oak, the results with other species suggest that this spatial variation in the defensive chemical response could be a consequence of the local adaptation mechanisms promoted by host-stressor coexistence (Valladares *et al.* 2015, VanWallendael *et al.* 2019). Additionally, they could also be due to resource availability limitations or other unexplored factors.

The three variables evaluated (Tp, Tt and Ct) showed a positive and strong correlation, as expected because they are overlapping fractions (Tp includes Tt and Tt includes Ct). This also happened in Gallardo *et al.* (2019) with holm oaks from the same origin (Picos de Europa provenance) against the two types of stress studied here (mechanical defoliation and Pc infection). The Ct content differentiated well the stress effect in the different provenances, while the effects at the local level, among individuals within the same provenance, were better explained by the Tt content. This shows that the Ct content is an adequate variable to explain the geographical variability of chemical defences induced by biotic stressors. In fact, its use to analyze plant-herbivore interactions in the literature is common (War *et al.* 2012).

In addition to local and geographical differences, the induction of chemical defences was also stressor-specific. It means the defensive response was triggered by biotic stress factors and depended on the stressor. This specificity is also demonstrated by Gallardo *et al.* (2019) in holm oak seedlings subjected to the root pathogen infection and mechanical defoliation from the same provenance. They also identified the *Quercus* sequences encoding enzymes for early steps of the biosynthesis of phenolic compounds, like hydrolysable tannins and Ct among others, plus genes involved in the late steps of Ct biosynthesis. Their study showed differences in response that were dependent on the stressor, especially in the Tt production for Picos de Europa region, also studied in our work. The consistent results of our work show that this specific induction of the stressor can be extrapolated to its spatial variability analysis. Furthermore, the combined treatment of pathogen inoculation with mechanical defoliation induced the highest production of Tp and Ct in most of the studied regions. Mechanical defoliation alone caused the largest increases in Tt production. Despite several studies about plant-herbivore interactions are focused on Ct, it was observed that Tt, that is, those that include hydrolysable tannins, better characterize the induction of defences against herbivory stress in the holm oak. In fact, some specific compounds that take part of hydrolysable tannins are closely related to herbivory resistance (War *et al.* 2012, Marsh *et al.* 2020).

From this **second chapter** it is noteworthy that the response induced by biotic stressors was not always an increase in the level of chemical defences. That is, there is an interactive effect among stressors and the existence of induction or inhibition of chemical defences depends on the provenance. In the case of Tt and especially in M and C regions, there is an inhibitory effect on the defence production due to the combination of stressors. When defoliation and Pc infection act combined, the response capacity in Tt production decreases, which suggests that very high levels of stress could limit the resources needed to produce more defences. Mechanical defoliation induces the biosynthesis of Tt but infection by *Phytophthora* interferes with its regulation, increasing the plant susceptibility to biotic stress (Gallardo *et al.* 2019). This suggests that in root pathogen-affected stands, an intense defoliation event could increase the vulnerability of the holm oak, reducing its ability to recover after the attack.

Constitutive and induced defences and their consequences for plant survival

The greenhouse tests from the **first and second chapters** and the field experiment from the **third chapter** made possible to analyze the involvement of the geographical variation of constitutive and induced chemical defences of holm oak in its survival. Tolerance to biotic stress, both caused by mechanical defoliation and induced by the pathogen Pc infection, differed significantly among Iberian provenances. In all cases, the biotic stressors reduced the survival probabilities. The most harmful was the root infection alone; even more that root infection combined with defoliation.

Generally, the southern regions were more tolerant to biotic stress from root and aerial damages in greenhouse conditions and also in the field against infection by Pc. M and S were the most tolerant regions to the applied treatments, exceeding in all cases more than 80% survival. No other works are known about the geographical variation of chemical defences studied here and tolerance against the applied biotic stressors in holm oak. However, Camisón *et al.* (2019) profiles the defensive chemical patterns in *Castanea sativa* plants to Pc, finding more Tp and Ct in susceptible plants to the pathogen than in resistant ones. On the contrary, Cahill *et al.* (1989) showed that phenolic deposition was higher in the more resistant species than in the susceptible ones during the root infection by Pc. The role of tannins in plant defence has been widely studied (Peters & Constabel 2002, Roitto *et al.* 2009, Barbehenn & Constabel 2011, War *et al.* 2012). However, in some cases no effects have been found on them (Keinanen *et al.* 1999, Hikosaka *et al.* 2005). Furthermore, specific compounds within these large phenolic groups are those identified for their key role in resistance to different stressors (Feeny 1968). Focusing on the defensive response of the plant to *Phytophthora* spp, several studies cite the involvement of phenolic compounds in this interaction (Picard *et al.* 2000, Lherminier *et al.* 2003, Horta *et al.* 2010). Stong *et al.* (2013) cite this phenolic involvement with *P. ramorum* and Gallardo *et al.* (2019) do it with Pc. But the controversy arising from experiments to date show that the role of tannins as defences against biotic stress have still to be clarified, and even more so their effect on pathogens. In our work, we related the tolerance variability with the induced chemical defence patterns obtained for each provenance, although there was no clear correspondence between them. A priori, the greater the induction, the more phenotypically plastic plants will be, thus reducing the possibility that biotic stressors adapt to the induced chemical compounds (Howe & Jander 2008, Agrawal 2011). However, the most biotic stress-tolerant regions in the greenhouse were not those with the greatest induced defences. The case of M stands out, which even having the highest Ct constitutive levels, barely underwent induction by the applied treatments and nevertheless, showed high tolerance to biotic stress. This tolerance showed a greater and positive correlation with constitutive defensive chemical levels -studied in more detail in the **first chapter**- than with those induced, that is, survival to stress is more related to constitutive defences than to induced ones. This could be due to the strategy cited by Jorrín-Novo & Navarro-Cerrillo (2014) that highlights the metabolic costs (Agrawal *et al.* 2002) for the holm oak to induce defence. This induction could be avoided in biotic attack events when they are punctual. If herbivory or infection were frequent, or even permanent, as is often the case in the southern Iberian Peninsula, the differences between constitutive and induced levels could tend to be smaller. The results of the **first chapters** showed that the southern regions, with the highest impact of herbivores, showed higher constitutive defences, inducing fewer defences but being more tolerant to biotic stressors. This result was repeated in the **third chapter**, after the field assessment on areas naturally infected by Pc. The most tolerant and best developed provenances in these areas after four years of follow-up were the southern ones. Defoliation as a permanent biotic stressor could have driven the high constitutive defence selection in southern regions, also facilitating a greater tolerance to other stressors such as Pc. In fact, our results show that the most susceptible to the root pathogen-regions were those in the north of the Iberian Peninsula.

Applicability for selection programs

The constitutive chemical patterns of defence in holm oak showed high geographical and local variability. The lowest defensive phenolic concentrations were found in the northern regions and on alkaline soils, while the highest constitutive values were found in the south and on acid soils (higher Tp in Cabañeros, higher Ct in Monfragüe provenance). Likewise, the heritability of the evaluated characters was moderate but enough to show genetic control and opportunities for selection. The high variability of defensive patterns may be a source of useful ecotypes in areas under high levels of biotic stress and, especially, in forests affected by the infection with pathogens of the genus *Phytophthora*, which are known to suffer from tannin inhibition (Stong *et al.* 2013). This suppressive ability of tannins is a key factor, since it is expected that plants with elevated tannin levels will develop better under unfavourable environmental conditions. Southern provenances on acidic soils appear to be candidates for the selection to restore oak decline areas, although in a widely distributed species such as this one, other factors must be taken into account. That is, good candidates due to their high defensive levels may not be adapted suitably to other types of soil, climatic conditions or phenological conditions, among others. Confirmatory studies are needed in order to establish a sound basis for future selection.

Additionally, the induced resistance could be exploited as an important tool for the integrated management of diseases and pests in forests with increasing biotic stressor scenarios. It would also serve to predict catastrophic events or high stress periods. This would allow the agrosystems to be strengthened before they reach severe levels of decline. However, our compression of these defensive mechanisms is still limited and the results of this Thesis show that the tolerance of the holm oak to these two types of applied biotic stressors is more related to the constitutive defences than to the induced ones. This facilitates the selection of plant material. But on the other hand, the fact that the response is stressor-specific and that there are interactions between them complicates the tolerant and/or resistant genotype selection. However, it constitutes a first step in the development of a holm oak decline management program considering the current intraspecific geographical variation. This species also has a high genetic diversity and the conservation of its natural and managed forests is vital to maintain this diversity in its reservoirs, guaranteeing a varied induced response.

These findings on the biotic stressor effects on the chemical defences and survival of holm oak may facilitate the development of genetic material selection programs in the integrated control of the widespread *Quercus* decline. However, for the identification of material, tests under more natural conditions are essential. The **third chapter** of the Thesis has focused on evaluating whether the tolerance results obtained in the greenhouse were also maintained in the field. To our knowledge, this work constitutes the first field test on the susceptibility of holm oaks of different provenances in Pc-infected areas. In addition, the four years of follow-up of its effects greatly exceeds the usual test times for susceptibility to the pathogen. Our results confirmed the high mortality of the species in the presence of Pc, but also the existence of holm oak genotypes tolerant to the disease. These findings are in agreement with León (2013) and Cuenca *et al. (*2018), who also found tolerance in this species in some families under greenhouse conditions. Furthermore, Cuenca *et al.* (2018) showed that the tolerance of Pc-infected holm oaks is genetically controlled, being susceptible to improvement. Our study in the field and longer follow-up time also showed that the plant provenance has an effect on tolerance, which facilitates the identification of the holm oak families with greatest interest for possible plant breeding programs. The southern provenances were the most tolerant to Pc in the field, as occurred in the greenhouse, and their tolerance was related to higher constitutive chemical defences. The positive and strong correlation between tolerance to disease and content of constitutive condensed tannins is very remarkable.

Although no other studies are known about the effect of geographical variation on the response of holm oak to the pathogen in the field, there is a previous study in cork oak (Moreira *et al.* 2018a). The aforementioned work also obtained variability in the response and identified some tolerant families to the disease after ten years of follow-up. This work also used *Q. faginea* in the plantation, obtaining high tolerance to the pathogen, as also occurs in our work, and proposed it as a possible rootstock of *Q. ilex* in the most affected areas. In addition, the other species used as a control of water stress, *F. angustifolia*, showed low mortality and resistance to the pathogen mentioned in other articles (Moralejo *et al.* 2009). The plant survival of these species and their different degrees of tolerance reinforce what has been described by other authors such as Jactel *et al.* (2017) about the greater resistance of mixed forests to natural disturbances that are relatively small-scale and selective in their effect.

The disease-tolerant families identified in our study should be followed in the long-term to test if such tolerance is maintained over time. If after a longer experiment period, tolerance continues to be kept, these families should be part of the source of genetic material in breeding programs. However, in a field test, results should be viewed with caution, as several environmental factors also condition the plant survival. But it would be very interesting in future larger field experiments to deepen the knowledge of the effects of several factors on tolerance according to provenances (exposure, water stress, shade and distance to the infected adult tree or the competition between oak plants and herbaceous plants during the first years in oligotrophic soils).

Anti-Phytophthora effects with AREs

In addition to defensive response of the holm oak to biotic stress, the role that other species of the Mediterranean flora could have on the stress generated by the pathogen Pc in two other species of Mediterranean oaks, the cork oak and the gall oak, was analyzed. The **fourth chapter** of the Thesis focused first on a screening of the allelopathic effect of various species. Selected some herbaceous plants from the Brasicaceae family as interesting for their allelopathic capacity, this was tested under laboratory conditions. The most efficient method for obtaining the AREs was the followed by Sampaio (2017) and Moreira *et al.* (2018) with conclusive results. Regarding the species tested, the ARE of *D. tenuifolia* was especially suited for its complete inhibition of the Pc sporangia production. It also reduced mycelial growth, although to a lesser extent than in a previous trial (83% for to 75% ARE concentration; Moreira *et al.* 2018). Previous studies of Pc inhibition with *E. vesicaria* (Ríos *et al.* 2016a, Sampaio 2017, Moreira *et al.* 2018) and *R. raphanistrum* (Sampaio 2017, Moreira *et al.* 2018) also confirm its allelopathic effect. *E. vesicaria*, whose main glucosinolates are aliphatic (glucoraphanin, glucosativin and glucoerucin) showed a high inhibition, but not the complete non-viability of its reproduction structures, according with what was discovered by Ríos *et al.* (2016a). Although *E. vesicaria* does not completely inhibit the production of sporangia, a very important reduction in the mobility of zoospores was observed when they are released in the presence of the extract. Zoospores lead to primary infections, so this reduction is key in the spread of the disease. *R. raphanistrum* showed lower inhibition values than the two previous species and very similar to that obtained by Moreira *et al.* (2018) for the mycelial growth of Pc.

Anti-*Phytophthora* herb species and chemical defences of *Quercus* plants *in vivo* in Pc-infested soil

The possible protective effect that these herb plants could contribute to Pc-stressed oaks was estimated *in vivo*, by means of a greenhouse test with naturally Pc-infected soil. The oak phenolic defences showed significant differences depending on the companion allelopathic herb species with those that grew together in Pc-infested soil. When these herb plants grew together with oak seedlings in Pc-infested soils, the *Quercu*s chemical defences lowered but plants did not die, which could be the result of a protective effect of the allelopathic species against infection. The Tp and Ct were minimal with *E. vesicaria* and *D. tenuifolia* in both *Quercus*. If we take the condensed tannin content as a reference, Ct were always higher in the susceptible *Q. suber* than in *Q.* faginea, which is more tolerant to Pc (Pérez-Sierra *et al.* 2013). With *L. luteus*, also highly susceptible to the pathogen (Serrano *et al.* 2011), both oak species increased their defensive levels against Pc, although the increase was greater in *Q. suber* (susceptible to Pc) than in *Q. faginea* (tolerant to Pc). If, as we saw in previous chapters, *Quercus* Ct increases in response to the attack they are suffering, it is expected that the oak species studied here also increase their defensive levels. However, in the presence of *E. vesicaria* and *D. tenuifolia*, aforementioned levels decreased with respect to the control (without the presence of herbaceous plants). This suggests that the allelopathic relationships established during their growth with *Quercus* in Pc-infested soil exerts a certain protective effect on *Quercus* seedlings.

Plant interactions are controlled by root exudates, which sometimes have an allelopathic effect against various microorganisms (Bais *et al.* 2006). The allelopathic capacity of *D. tenuifolia* and *E. vesicaria* was observed *in vivo* conditions, but we do not know if it was through their root exudates or if it is due to other factors involved in the joint growth of herbaceous plants with *Quercus*, such as competition between them during the first years. However, these results could be due to a decrease in the impact of Pc activity. The low infective efficacy of Pc could be explained by the release of secondary metabolites in allelopathic root exudates, or also by the facilitation of inhibitory soil bacteria against the pathogen. Even with everything, it was found that the defence levels in the cork oak, more susceptible to Pc than the gall oak, decreased in the presence of the exudates of the two herbaceous plants evaluated. Therefore, these herb species reduce the chemical defence costs against the pathogen's attack. No previous studies are known with these species to establish comparisons, but the complex analysis of the herb-tree-pathogen interactions (Cazorla & Mercado-Blanco 2016, Shuping & Eloff 2017) should be continued to better understand the role of each component in that relationship.

As for the low molecular weight phenolic compounds, these were always minimum in *Q. faginea* and maximum in *Q. suber* (higher and lower tolerance to Pc, respectively). Several studies relate some of the compounds studied with a greater tolerance to the genus *Phytophthora* (Ockels *et al.* 2007, Del Río *et al.* 2003, Nagle *et al.* 2011, McPherson *et al.* 2014, Conrad *et al.* 2014). However, the levels of some compounds did not show a clear pattern, which suggests that there are other soil organisms (probably bacteria) also highly conditioned by plant-pathogen interaction.

Regarding the antioxidant activity in oak leaves, the highest one was found in *Q. suber* seedlings that grew together with *L. luteus* (both species susceptible to Pc). The lowest AA was that of *Q. faginea* with *L. luteus* (*Quercus* species more tolerant to Pc than *Q. suber*). Antioxidants protect biological systems against reactions or processes that can produce harmful effects in the individual. In a weakened system, as is the case of the *Q. suber* with *L. luteus* growing in a Pc-infested soil, it is to be expected that there would be more free radicals and therefore, a greater AA would be induced. However, in scenarios with more tolerant to Pc-species, such as *Q. faginea*, the expected AA would be lower. To strengthen the defensive response to biotic stress, seedlings induce a hypersensitive response that consists of programmed cell death to ensure the plant survival. When stress decreases, AA also drops (Irfan *et al.* 2011). In our study, when the *Quercus* species most susceptible to Pc grew with *D. tenuifolia* and *E. vesicaria*, AA decreased, showing lower stress levels. In Q. *faginea*, this level also decreased in the presence of allelopathic species. Therefore, the presence of these allelopathic species generated a protective effect on *Quercus* seedlings, reducing their stress levels facing the pathogen infection.

Brassicaceae family has been specially studied for its glucosinolate content for biofumigation. This is a widely used biological control tool against several pathogens. Ríos *et al.* (2016a) screened various brassicaceous plants (*D. tenuifolia* was not evaluated) to identify the most suitable and the compounds responsible for the inhibitory allelochemical activity of Pc. They demonstrated the biocidal action on Pc of rich in sinigrin-species, such as *B. juncea* and *B. carinata* (see also Fernández-Rebollo *et al.* 2015, Morales-Rodríguez *et al.* 2016, Ríos *et al.* 2017), while with others glucosinolates different from sinigrin (such as *E. vesicaria*) only a fungistatic effect was obtained. However, its effectiveness in the field has only been proven with infected cork oaks when combined with calcium carbonate applications (Rodríguez-Molina *et al.* 2018). In fact, integrated control is becoming more and more meaningful as the only way to manage the oak decline. The novel approach of this work proposes the enrichment of pastures with allelopathic species against Pc. The inoculum would not be eradicated from the soil, but the disease spread could be dimmed, increasing the tolerance of several species in Iberian wood pastures. The incorporation in the field of native plants capable of reducing the Pc infective activity through their natural root exudates shows high potential in the possible control of the pathogen's activity with a sustainable management in these agrosilvopastoral systems. However, it should still be deepened in several issues such as the knowledge of these relationships, their action and release in the field, the most recommended doses to avoid toxicity in other plants and whether other surrounding microorganisms are involved in them (see bacteria, for example).

Synthesis and future prospects

To sum up, this Thesis offered new insights on the defensive chemical response of three Mediterranean *Quercus* species to biotic stress. In the first place, it was shown that there is a diversity in the constitutive chemical defences of the oak that depends on the origin and the genotype. Generally, higher levels of constitutive defences were observed at lower latitudes and on acidic rather than basic soils. Furthermore, total phenols and condensed tannins showed a positive correlation. The narrow-sense heritability of these chemical traits was moderate.

This study contributes to the current body of literature expanding about how plant populations from different regions differently invest in defensive traits. The individual and ecotypic variability generates differences in the response of the holm oak chemical defences against the different biotic stress factors, showing certain specificity and interactions in the production of tannins according to the stressor. This could be an adaptive strategy to the diversity of the pathogen and herbivore community present in the ecosystem. And it would be an interesting trait for the selection of better adapted to increasing biotic stressors genetic material. Detailed studies of the specific induced chemical compounds by each biotic stressor would be very necessary to understand the intraspecific geographical variability in the allocation of defensive resources.

To our knowledge, this work includes the first field test on the susceptibility of holm oaks of different provenances in naturally Pc-infected areas. In addition, the four years of follow-up of its effects greatly exceeds the usual test time for susceptibility to the pathogen. Results confirmed the high mortality of the species in the Pc presence, but also the existence of holm oak genotypes tolerant to the disease. Survival differed significantly according to their provenances, being those from the south more tolerant. In addition, these provenances with better health status were the most tolerant in the greenhouse experiment and their tolerance was related to higher constitutive chemical defences. Lastly, the existence of tolerant holm oak families to the pathogen in some provenances suggests that the restoration of the affected areas could be possible if tolerant or resistant plant material is used.

Finally, this Thesis confirms the existence of allelopathic relationships capable of inhibiting the infective pathogen activity *in vitro* and *in vivo* conditions. From the three species studied, the ARE of *D. tenuifolia* was especially suited for its complete inhibition of the Pc sporangia production. In addition, when these herb plants grew together with *Que*rcus seedlings in Pc-infested soils, the *Quercus* chemical defences lowered but they did not die, which could be the result of a protective effect of the allelopathic species against infection. However, it would be necessary to explore the complex of soil microorganisms present in this allelopathic relationship for its better management before possible testing and application in the field.

References

Abdala-Roberts L, Moreira X, Rasmann S, Parra-Tabla V, Mooney KA (2016). Test of biotic and abiotic correlates of latitudinal variation in defenses in the perennial herb *Ruellia nudiflora*. J Ecol 104: 580-590.

Agrawal AA (2011). Current trends in the evolutionary ecology of plant defence. Funct Ecol 25:420-32.

Agrawal AA, Janssen A, Bruin J, Posthumus MA, Sabelis MW (2002). An ecological cost of plant defence: attractiveness of bitter cucumber plants to natural enemies of herbivores. Ecol Lett 5:377-85.

Alcaide F, Solla A, Cherubini M, Mattioni C, Cuenca B, Camisón Á, Martín MA (2020). Adaptive evolution of chestnut forests to the impact of ink disease in Spain. Jnl of Sytematics Evolution.

Alcaide F, Solla A, Mattioni C, Castellana S, Martín MA (2019). Adaptive diversity and drought tolerance in *Castanea sativa* assessed through EST-SSR genic markers. Forestry 92 (3): 287-296.

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. Ann. Rev. Plant. Biol. 57:233-266.

Barbehenn RV, Jaros A, Lee G, Mozola C, Weir Q, Salminen JP (2009). Tree resistance to *Lymantria dispar* caterpillars: importance and limitations of foliar tannin composition. Oecologia. 159: 777-788.

Benton JJ (2003). Agronomic handbook: Management of crops, soils and their fertility. CRC press LLC. USA.

Blaker NS & MacDonald JD (1983). Influence of container médium pH on sporangium formation, zoospore reléase and infection of rhododendron by *Phytophthora cinnamomi*. Plant Disease 67: 259-263.

Brasier CM (1992). Oak tree mortality in Iberia. Nature 360, 539.

Brasier CM (1996). *Phytophthora cinnamomi* and oak decline in Southern Europe. Environmental constraints, including climate change. Ann For Sci 53: 347-358.

Cadahía E, Conde E, Cañellas I, García-Vallejo MC (1993). Variación del contenido de taninos en las bellotas de *Quercus suber* L. y *Quercus ilex* L. durante el proceso de maduración. Congreso Forestal Español, Lourizán. Tomo 11. 485.

Cahill D, Legge B, Weste GM (1989). Cellular and histological changes induced by *Phytophthora cinnamomi* in a group of plant species ranging from fully susceptible to fully resistant. Phytopathology 79:417–424.

Camisón A, Martín MA, Sánchez-Bel P, Flors V, Alcaide F, Morcuende D, Pinto G, Solla A (2019). Hormone and secondary metabolite profiling in chestnut during susceptible and resistant interactions with *Phytophthora cinnamomi*. J Plant Physiol 241:153030

Cazorla FM & Mercado-Blanco J (2016). Biological control of tree and woody plant diseases: an impossible task? BioControl 61: 233-242.

Conrad AO, McPherson BA, Wood DL, Madden LV, Bonello P (2017). Constitutive phenolic biomarkers identify naïve *Quercus agrifolia* resistant to *Phytophthora ramorum*, the causal agent of sudden oak death. Tree Physiol 26: 1–11.

Corcobado T, Cubera E, Juarez E, Moreno G, Solla A (2014). Drought events determine performance of *Quercus ilex* seedlings and increase their susceptibility to *Phytophthora cinnamomi*. Agric For Meteorol 192-193:1–8.

Cuenca B, Dorado FJ, Camisón A, Luquero L, Ocaña L, Solla A (2018). Selección de brinzales de *Quercus suber* y *Q. ilex* tolerantes a *Phytophthora cinnamomi* y al estrés hídrico. En: Libro de Resúmenes XIX Congreso de la Sociedad Española de Fitopatología, Toledo (España).

Dawra RK, Makkar HPS, Singh B (1988). Total phenolics, condensed tannins and protein-precipitable phenolics in young and mature leaves of oak species. J. Agric. Food Chem. 36: 953-957.

Del Río JA, Baidez AG, Botia JM, Ortuno A (2003). Enhancement of phenolic compounds in olive plants (*Olea europaea* L.) and their influence on resistance against Phytophthora sp. Food Chem. 83, 75–78.

Dixon G (1984). Plan pathogens and their control in horticulture. Horticultural crops.

Duvenhage JA & Kotzé JM (1991). The influence of calcium on saprophytic growth and pathogenicity of *Phytophthora cinnamomi* and on resistance of avocado to root rot. South African Avocado Growers'Association Yearbook 14: 13-14.

Eriksson G, Ekberg I, Clapham D (2013). Genetics applied to forestry. Department of Plant Biology and Forest Genetics, SLU. Uppsala, Sweden.

Faeth SH (1986). Indirect interactions between temporally separated hervibores mediated by the host plant. Ecology 67: 479-454.

Feeny PP (1968). Effect of oak leaf tannins on larval growth of the winter moth *Operophtera brumata*. J Insect Physiol 14:805-17

Fernández-Rebollo P, Carbonero MD, Leal JR, García AM, Ríos P, Sánchez ME, Obregón S & de Haro A (2015). Producción, concentración y perfil de glucosinolatos de *Brassica carinata* y *Brassica juncea* cultivadas en la dehesa. In: Cifre J, Janer I, Gulias J, Jaume J, Medrano M (eds). Pastos y forrajes en el siglo XXI. Palma de Mallorca, SEEP, pp 193-200.

Gallardo A, Morcuende D, Solla A, Moreno G, Pulido FJ, Quesada A (2019). Regulation by biotic stress of tannins biosynthesis in *Quercus ilex*: crosstalk between defoliation and *Phytophthora cinnamomi* infection. Physiol Plant 165: 319-329.

Hikosaka K, Takashima T, Kabeya D, Hirose T, Kamata N (2005). Biomass allocation and leaf chemical defence in defoliated seedlings of *Quercus serrata* with respect to carbon-nitrogen balance. Ann Bot 95:1025-32.

Horta M, Caetano P, Medeira C, Maia I, Cravador A (2010). Involvement of the β-cinnamomin elicitin in infection and colonisation of cork oak roots by *Phytophthora cinnamomi*. Eur J Plant Pathol 127: 427-436.

Howe GA & Jander G (2008). Plant immunity to insect herbivores. Annu Rev Plant Biol 59:41-66.

Irfan M, Hayat S, Bashir A (2011). Regulation of antioxidant system under biotic stress in plants. In: Plant under stress. Stadium Press, New Delhi.

Jactel H, Bauhus J, Boberg J, Bonal D, Castagneyrol B, Gardiner B, González-Olabarria JR, Koricheva J, Meurisse N, Brockerhoff EG (2017). Tree diversity drives forest stand resistance to natural disturbances. Current Forestry Reports 3(3): 223-243.

Jensen EL, Barr GR (1971). Standard errors of heritability estimates calculated from variance component analysis of a two-way classification. J. Anim. Sci 32 (6): 1069-1077.

Jorrín-Novo J, Navarro-Cerrillo RM (2014). Variabilidad y respuesta a distintos estreses en poblaciones de encina (*Quercus ilex* L.) en Andalucía mediante una aproximación proteómica. Ecosistemas 23(2):99-107.

Keinanen M, Julkunen-Tiitto R, Mutikainen P, Walls M, Ovaska J, Vapaavuori E (1999). Trade-offs in phenolic metabolism of silver birch: effects of fertilization, defoliation and genotype. Ecology 80:1970-86 .

Kraus TEC, Dahlgren EC, Zasoski RJ (2003). Tannins in nutrient dynamics of forest ecosystems - a review. Plant and Soil 256: 41–66.

Leinonen T, McCairns RJS, O´Hara RB, Merilä J (2013). Q_{ST}–F_{ST} comparisons: evolutionary and ecological insights from genomic hetero-geneity. Nat Rev Genet 14:179–190.

León IM (2013). Selección de progenies de encina (*Quercus ilex* L. spp. ballota) y alcornoque (*Quercus suber* L.) tolerantes al patógeno *Phytophthora cinnamomi*. Universidad de Huelva, Tesis Doctoral.

Lherminier J, Benhamou N, Larrue J, Milat ML, Boudon-Padieu E, Nicole M, Blein JP (2003). Cytological characterization of elicitin-induced protection in tobacco plants infected by *Phytophthora parasitica* or phytoplasma. Phytopathology 93(10):1308-1319.

López-Goldar X, Villari C, Bonello P, Borg-Karlson AK, Grivet D, Sampedro L, Zas R (2019). Genetic variation in the constitutive defensive metabolome and its inducibility are geographically structured and largely determined by demographic processes in maritime pine. J. Ecol. 107, 2464–2477.

Marsh KJ, Wallis IR, Kulheim C, Clark R, Nicolle D, Foley WJ, Salminen JP (2020). New approaches to tannin analysis of leaves can be used to explain in vitro biological activities associated with herbivore defence. New Phytol 225(1):488-498.

McPherson BA, Mori SR, Opiyo SO, Conrad AO, Wood DL, Bonello P (2014). Association between resistance to an introduced invasive pathogen and phenolic compounds that may serve as biomarkers in native oaks. Forest Ecology and Management 312: 154-160.

Merilä J & Crnokrak P (2001). Comparison of genetic differentiation at marker loci and quantitative traits. J. Evol. Biol 14: 892-903.

Moralejo E, García-Muñoz JA, Descals E (2009). Susceptibility of Iberian trees to *Phytophthora ramorum* and *P. cinnamomi*. Plant Pathology 58(2): 271-283.

Morales-Rodríguez C, Vettraino AM, Vannini A (2016). Efficacy of biofumigation with *Brassica carinata* comercial pellets (BioFence) to control vegetative and reproductive structures of *Phytophthora cinnamomi*. Plant Disease 100(2): 324-330.

Moreira X, Castagneyrol B, Abdala-Roberts L, Berny-Mier y Teran JC, Timmermans BGH, Bruun HH, Covelo F, Glauser G, Rasmann S, Tack AJM (2018a). Latitudinal variation in plant chemical defences drives latitudinal patterns of leaf herbivory. Ecography 41 1124–1134.

Moreira AC, Tapias R, Fernandes L, Rodriguez A (2018b). Field susceptibility of cork oak trees with different provenances to *Phytophthora cinnamomi*. Forest Pathology 48(5) e12461.

Moreira A, Calha I, Passarinho JA, Sampaio A (2018c). Anti-P*hytophthora* activity of root extracts from herbaceous species. Revista das Ciências Agrárias 41 (Especial): 39-47.

Nagle AM, McPherson BA, Wood DL, Garbelotto M, Bonello P (2011). Relationship between field resistance to *Phytophthora ramorum* and constitutive phenolic chemistry of coast live oak. Forest Pathology 41(6): 464-469.

Ockels FS, Eyles A, McPherson BA, Wood DL, Bonello P (2007). Phenolic chemistry of coast live oak response to *Phytophthora ramorum* infection. J Chem Ecol 33(9):1721-32.

Pardo A, Cáceres Y, Pulido F (2018). Intraspecific variation in heritable secondary metabolites and defensive strategies in a relict tree. J Plant Ecol 11: 256–265.

Pérez-Sierra A, López-García C, León M, García-Jiménez J, Abad-Campos P, Jung T (2013). Previously unrecorded low-temperature *Phytophthora* species associated with *Quercus* decline in a Mediterranean forest in eastern Spain. For Pathol 43:331–339.

Peters DJ & Constabel CP (2002). Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*) Plant J 32:701-12.

Picard K, Ponchet M, Blein JP, Rey P, Tirilly Y, Benhamou N (2000). Oligandrin, a proteinaceous molecule produced by the mycoparasite Pythium oligandrum induces resistance to Phytophthora parasitica infection in tomato plants. Plant Physiol 124:379-395.

Rasmann S & Agrawal AA (2011). Latitudinal patterns in plant defence: evolution of cardenolides, their toxicity, and induction following herbivory. Ecol Lett 14: 476– 483.

Ríos P, Obregón S, González M, de Haro A, Sánchez ME (2016a). Screening brassicaceous plants as biofumigants for management of *Phytophthora cinnamomi* oak disease. For Path 46(6): 652-659.

Ríos P, González M, Obregón S, Carbonero MD, Leal JR, Fernández P, De Haro A, Sánchez ME (2017). Brassica-based seedmeal biofumigation to control *Phytophthora cinnamomi* in the Spanish “dehesa” oak trees. Phytopathologia Mediterranea 56(3):392-399.

Rodríguez-Molina MC, Serrano-Pérez P, Santiago R, Cardillo E, Godoy B, De Santiago A (2018). Efecto de la biofumigación con pellets de *Brassica carinata* en la progresión de la enfermedad causada por *Phytophthora cinnamomi* en *Lupinus luteus*. En Libro de Resúmenes XIX Congreso de la Sociedad Española de Fitopatología, Toledo (España).

Roitto M, Rautio P, Markkola A, Julkunen-Tiitto R, Varama M, Saravesi K, Tuomi J (2009). Induced accumulation of phenolics and sawfly performance in Scots pine in response to previous defoliation. Tree Physiol 29:207-16.

Sampaio AR (2017). Selecção de plantas com efeito alelopático para controlar *Phytophthora cinnamomi* Rands. Dissertação para obtenção do grau de Mestre em Engenharia Agronómica-Protecção de Plantas. ISA/UL, 71 pp.

Sánchez ME, Caetano P, Romero MA, Navarro RM, Trapero A (2006). *Phytophthora* root rot as the main factor of oak decline in southern Spain. In: Brasier C, Jung T, Oßwald W, eds. Progress in Research on Phytophthora Diseases of Forest Trees. Farnham, Surrey, UK: Forest Research, 149-54.

Scalbert A (1991). Antimicrobial properties of tannins. Phytochemistry 30: 3875–3883.

Serrano M, Fernández-Rebollo P, De Vita P, Carbonero M, Sánchez ME (2011). The role of yellow lupin (*Lupinus luteus*) in the decline affecting oak agroforestry ecosystems. Forest Pathology 4(5): 382-386.

Serrano MS, Fernández-Rebollo P, De Vita P, Sánchez ME (2013). Calcium mineral nutrition increases the tolerance of *Quercus ilex* to *Phytophthora* root disease affecting oak rangeland ecosystems in Spain. Agroforestry Systems, 87(1): 173-179.

Shuping DSS, Eloff JN (2017). The use of plants to protect plants and food against fungal pathogens: a review. Afr J Tradit Complement Altern Med 14(4): 120-127.

Solla A, Slobodan M, Gallardo A, Bueno A, Corcobado T, Cáceres Y, Morcuende D, Quesada A, Moreno G, Pulido FJ (2016). Genetic determination of tannins and herbivore resistance in *Quercus ilex*. Tree Genet Genom 28: 743–751.

Stong RA, Kolodny E, Kelsey RG, González-Hernández MP, Vivanco JM, Manter DK (2013). Effect of plant sterols and tannins on *Phytophthora ramorum* growth and sporulation. J Chem Ecol 39(6):733-743.

Valladares F, Bastias CC, Godoy O, Granda E and Escudero A (2015). Species coexistence in a changing world. Front. Plant Sci. 6:866.

VanWallendael A, Soltani A, Emery NC, Peixoto MM, Olsen J, Lowry DB (2019). A Molecular View of Plant Local Adaptation: Incorporating Stress-Response Networks. Annu. Rev. Plant Biol. 70 (1): 559-583.

Visscher PM & Goddard ME (2015). A general unified framework to assess the sampling variance of heritability estimates using pedigree or marker-based relationships. Genetics 199: 223-232.

War AR, Paulraj MG, Ahmad T, Buhroo AA, Hussain B, Ignacimuthu S, Sharma HC (2012). Mechanisms of plant defense against insect herbivores. Plant Signal Behav 7:1306-1320.

Zas R, Moreira X, Ramos M, Lima MRM, Nunes da Silva M, Solla A, Vasconcelos MW, Sampedro L (2015). Intraspecific variation of anatomical and chemical defensive traits in Maritime pine *(Pinus pinaster*) as factors in susceptibility to the pinewood nematode (*Bursaphelenchus xylophilus*). Trees 29:663-673.

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

1. Geographical variability is a significant source of intraspecific variation of constitutive and induced defences in holm oak. Its constitutive phenolic defences vary geographically and locally. They increase with decreasing latitude and tend to be higher in acidic soils than in alkaline ones.

2. Defensive chemical traits have moderate heritability and could allow genetic selection, although these results should be taken with caution given the low number of genotypes evaluated.

3. Survival to biotic stress is more related to constitutive defences than to those induced by the plant. This principle was demonstrated both in greenhouse conditions and in the field. Plants from provenances with higher levels of constitutive condensed tannins were more tolerant.

4. The major induction was expected to obtain a priori greater plant survival. However, the most tolerant provenances were those with higher constitutive levels of defences and not those with the highest induction. Costs of induction may be so high that it does not ensure plant survival, and yet those provenances with lower induced but high constitutive levels tolerate the biotic stressor.

5. The induced chemical response is specific to the biotic stressor that causes it. The induced response does not always increase the defences. Total tannins decrease when Pc infection is combined with mechanical defoliation in some provenances. These reveal that oak stands affected by Pc will be more vulnerable to other stressful events, as demonstrated in this case with herbivory.

6. Provenance and families within provenances were identified with tolerance to the pathogen Pc in the field after four years of follow-up. This test should be kept in time to confirm this capacity for future breeding programs.

7. The allelopathic capacity of *D. tenuifolia* and *E. vesicaria* on the mycelial growth and production of sporangia in Pc was demonstrated under laboratory conditions

and was very effective. These two allelopathic herbaceous plants against Pc also demonstrated their effectiveness *in vivo* under greenhouse conditions. When they were grown together with gall oak and cork oak plants in Pc-infected soils, their presence reduced the oak's chemical defences, but oak plants did not die after two years of testing. This result suggests a protective effect of allelopathic herbaceous plants against infection in oak trees.

8. The geographical and local variation on the chemical defence production in holm oak is an interesting source for the identification of resistant and tolerant plant material to increasing biotic stress. The survival of some holm oaks in areas severely affected by Pc demonstrates this. Furthermore, the identification of allelopathic Mediterranean plants capable of reducing Pc virulence and protecting cork oaks and gall oaks from stress offers alternative tools for the oak stands conservation.