Forest Systems
31 (2), e011, 10 pages (2022)
eISSN: 2171-9845
https://doi.org/10.5424/fs/2022312-17977
INIA-CSIC

RESEARCH ARTICLE

OPEN ACCESS

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

Manuela Rodríguez-Romero[1,2], Enrique Cardillo[1], Ramón Santiago[1] and Fernando Pulido[2]

[1]*Centro de Investigaciones Científicas y Tecnológicas de Extremadura (CICYTEX), Junta de Extremadura. Pol. Ind. El Prado, C/Pamplona s/n, 06800 Mérida, Spain.* [2]*Instituto de Investigación de la Dehesa (INDEHESA), Universidad de Extremadura. Av. Virgen del Puerto s/n, 10600 Plasencia, Spain.*

Abstract

Aim of study: Quercus forests are being affected by severe decline and mortality. The oak decline is associated with the soilborne pathogen *Phytophthora cinnamomi* (Pc), among others. This work aims to determine if *Quercus ilex* growing in Pc-infested soils show mortality differences according to their provenance in the field. It also evaluates whether the most tolerant provenances are those with the greatest constitutive chemical defences.

Area of study: Acorns from six Spanish National Parks with natural presence of *Q. ilex* were collected for sowing in the greenhouse and later be planted on soils naturally infested by Pc in the surroundings of Plasencia, western Spain.

Materials and methods: Seedlings were planted in four field plots located in two areas with oak decline: 153, 156, 157 and 155 plants in plot I, II, III and IV, respectively. The presence and infection of Pc was confirmed before their installation and during the experiment. Symptoms, regrowth, mortality and development was recorded for four years.

Main results: There was a high mortality (56.0-80.5%) with differences among provenances. The most tolerant provenances in the field coincided with those identified under greenhouse conditions in a previous test. Provenances with higher constitutive condensed tannins better tolerate the pathogen under both conditions. In the southern provenances, some families with higher tolerance and, therefore, candidates for use in reforestation programs in areas infected by Pc, were identified.

Research highlights: The restoration of Pc-affected areas would be possible through the use of *Q. ilex* plant material with high constitutive defences, more tolerant to the pathogen.

Additional key words: biotic stress; condensed tannins; field susceptibility; geographical variability; tolerance; total phenolics

Abbreviations used: A (Aigüestortes National Park); C (Cabañeros National Park); Ct (Condensed tannins); HC (Haza de la Concepción Farm); M (Monfragüe National Park); O (Ordesa National Park); P (Picos de Europa National Park); Pc *(Phytopthora cinnamomi)*; S (Sierra Nevada National Park); SE (San Esteban Farm); Slo (Slope); Tp (Total phenols); Tt (Total tannins); Val (Valley).

Authors' contributions: Analysed the data and wrote the paper: MRR and FP. All authors conceived, designed and performed the experiments; contributed reagents/materials/analysis tools; read and approved the final manuscript.

Citation: Rodríguez-Romero, M; Cardillo, E; Santiago, R; Pulido, F (2022). Susceptibility to *Phytophthora cinnamomi* of six holm oak *(Quercus ilex)* provenances: are results under controlled *vs.* natural conditions consistent? Forest Systems, Volume 31, Issue 2, e011. https://doi.org/10.5424/fs/2022312-17977

Supplementary material (Tables S1-S2; Figs. S1-S4) accompanies the paper on SJAR's website.

Received: 19 Jan 2021. **Accepted:** 13 Jun 2022.

Funding agencies/institutions	Project / Grant
Spanish Ministry of Agriculture, Food and Environment	Project 956 (TANOAK) "Determinants of biotic stress resistance in a model forest species: A new adaptive management tool in National Parks"
Spanish National Institute for Agriculture and Food Research and Technology (INIA)/CICYTEX	Grant FPI-INIA to Manuela Rodríguez-Romero

Competing interests: The authors have declared that no competing interests exist.

Correspondence should be addressed to Manuela Rodríguez-Romero: manuela.rodriguez@juntaex.es

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 (Rodá *et al.,* 2009). The holm oak is an evergreen oak well adapted to drought and distributed throughout the Mediterranean basin. 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 & Pulido, 2009). But this ecosystem of high natural and sociocultural value has experienced a generalized decline in the last decades due to the oomycete *Phytophthora cinnamomi* (Pc) (Brasier, 1992, 1996; Sánchez *et al.,* 2006; Corcobado *et al.,* 2014). This soil-borne pathogen known from more than 5000 species in the world (Hardham & Blackman, 2018) causes root rots on holm oak tree, resulting in tree death, although there are recent studies showing that the diversity of *Phytophthora* species linked to oak decline is higher than previously thought (Corcobado *et al.,* 2017; Mora- Sala *et al.,* 2019; Ruiz-Gómez *et al.,* 2019). Previous works with *Q. ilex* and also *Quercus suber* under greenhouse conditions have yielded high mortality rates but also have revealed the existence of more tolerant provenances and genotypes (León, 2013; Cuenca *et al.,* 2018; Moreira AC *et al.,* 2018; Rodríguez-Romero *et al.,* 2022). 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 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.,* 2022). The analysis of their phenolic defences yielded higher constitutive values (those that are relatively stable or whose variation in the plant depends on internal factors) 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 *Q. suber*, which was significantly different depending on the acorn origin and soil treatment, and on their interactions. Besides this, they concluded 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 (Moreira AC *et al.,* 2018) determined 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 for four years, recording symptoms, mortality and growth in order to answer the following questions: (i) which provenance is the most tolerant to the *P. cinnamomi* oak decline? (ii) does this tolerance correspond to that obtained in common garden conditions in a greenhouse? and (iii) do constitutive defences predict tolerance under field conditions?

Material and methods

Plant material and sowing in the greenhouse

In winter 2015, holm oak acorns of seven genotypes (28 acorns/genotype) were collected in each of the six sampled Spanish National Parks (ordered from highest to lowest latitude): Picos de Europa (P) (UTM 30 X: 357338, Y: 4784442); Ordesa (O) (UTM 31 X: 257277, Y: 4728450); Aigüestortes (A) (UTM 31 X:331157, Y: 4713657); Monfragüe (M) (UTM 30 X: 247904, Y: 4410162); Cabañeros (C; UTM 30 X: 374709, Y: 4357164) and Sierra Nevada (S) (UTM 30 X: 478055, Y: 4112818). 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 to provenance. So, the number of holm oak seedlings at the end of the first year that could be planted in the field 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) which are typical "dehesas" with a Mediterranean climate and gentle slopes (< 7%). Within each farm, one plot was located on a slope (Slo) and the other on a valley (Val). The plots were fenced to avoid cattle and wild fauna interference. Each plot occupied a surface of 200 m^2, leaving a symptomatic (chlorotic, wilted or dead foliage) 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.

The land was plowed before planting in March. Topsoil was removed to place an anti-weed mesh and thus reduce the early competition of a year-old 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 (4 families/provenance). The total number of georeferenced seedlings per plot was 153 plants in SEVal, 156 plants in SESlo, 157 plants in HCVal and 155 plants in HCSlo. Finally, 40 cm high protective tubes with 25% shading were placed to protect the stem from rodents.

In addition to the holm oak seedlings, one-year-old *Fraxinus angustifolia* seedlings and one-year-old *Quercus faginea* seedlings were planted in each plot. *Quercus faginea* is another Iberian oak susceptible to Pc, although more tolerant than holm oak (Moralejo *et al.,* 2009; Pérez Sierra *et al.,* 2013; Moreira AC *et al.,* 2018). *Fraxinus*

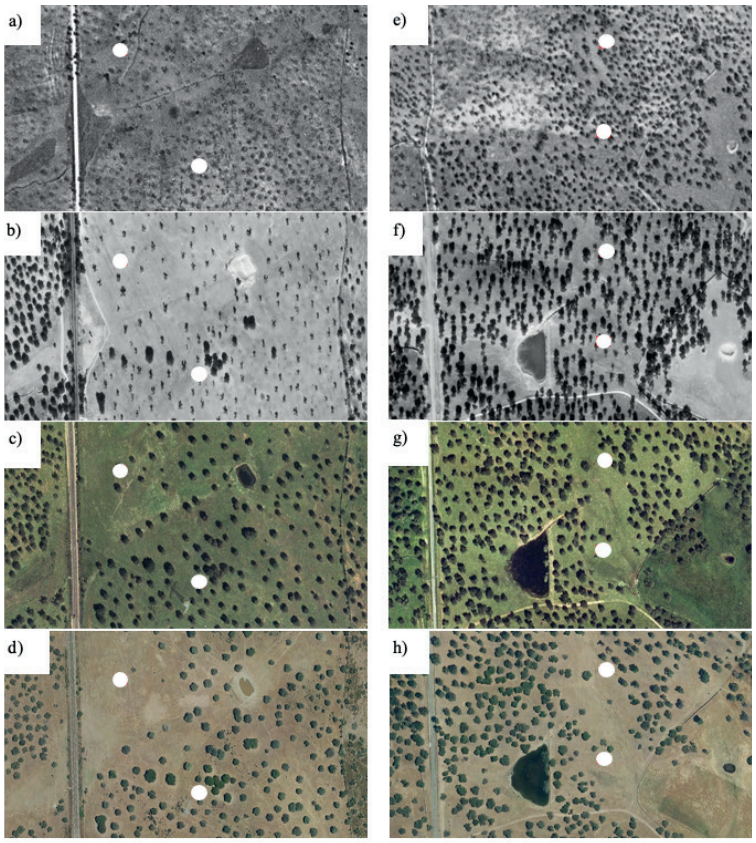

Figure 1. Evolution of the areas affected by the oak decline in the last 60 years. Orthophotos AMS 1956-1957 (a and e), Interministerial flight 1973-1986 (b and f), PNOA 2006 (c and g), and PNOA 2019 (d and h) over SE and HC, respectively. White dots in the orthophotos are plots within each farm. Taken from the Download Center of the National Geographic Information Center (http://centrodedescargas.cnig.es/CentroDescargas/index.jsp).

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 would had not indicated that water stress was the limiting factor for holm oaks growth 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 the plots was measured weekly the first year and monthly during the following three years, recording at each visit the number of plants with symptoms (chlorotic, wilted or dead foliage), the number of dead plants or with regrowth and some observations as the possible incidence of other damages (occasional defoliation, presence of ants and voles in some seedlings). At the end of each year, the growth of the plants was also measured to estimate annual increments.

Pc isolation

Pc presence was confirmed in all plots and under all provenances before planting by isolating the pathogen from roots and soils following the procedure described by Jung *et al.* (1996) and sowing these samples in selective PARPH medium (Jeffers & Martin, 1986). A molecular analysis of soil samples from the four plots also allowed the identification of other species of the same genus, as *Phytophthora gonapodyides* (Corcobado *et al.,* 2010) among others. However, Corcobado *et al.* (2017) show that Pc is the most virulent *Phytophthora* species under Mediterranean conditions exposed also to summer drought, so it was selected only this species for the detection and annual confirmation of its presence during the follow-up of the test. During the following years, twice a year, five soil and root samples from *Q. ilex* seedlings were randomly selected per plot to confirm the activity of the pathogen each spring. The process of isolation of the pathogen was also performed on roots and soil of control species. All 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. Root and soil samples were plated on PARPH selective medium, at a rate of 3 Petri dishes for each soil and root sample collected in each plot (30 Petri dishes/plot twice a year). 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 [suppl]. 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.

Statistical 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 were 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 (being the term of the greenhouse test the limiting period). 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.,* 2022) was also evaluated by Pearson's linear correlation. Statistical analyses were performed in Statistica v10 software.

Results

Statistical analysis

Holm oaks from all provenances and in all plots drastically reduced their survival probabilities in the Pc infected

Table 1. GLMM to detect the effects of plot, provenance and their interaction on the number of dead plants, plants with symptoms, 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).

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: $p > 0.05$.

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 Fig. 3). Mortality associated with Pc infection 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 in holm oak (Fig. 4). The relative height increases 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 material (Figs. S1-S4 [suppl]).

Control species showed very low mortality and Pc symptoms in *F. angustifolia* (14% and 0% respectively) but *Q. faginea* was relatively susceptibility to Pc (30% and 52.5% respectively). 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, especially for *F. angustifolia*, 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).

The plot and its position on the slope or valley also had a significant effect on tolerance to the pathogen (Figs. S1-

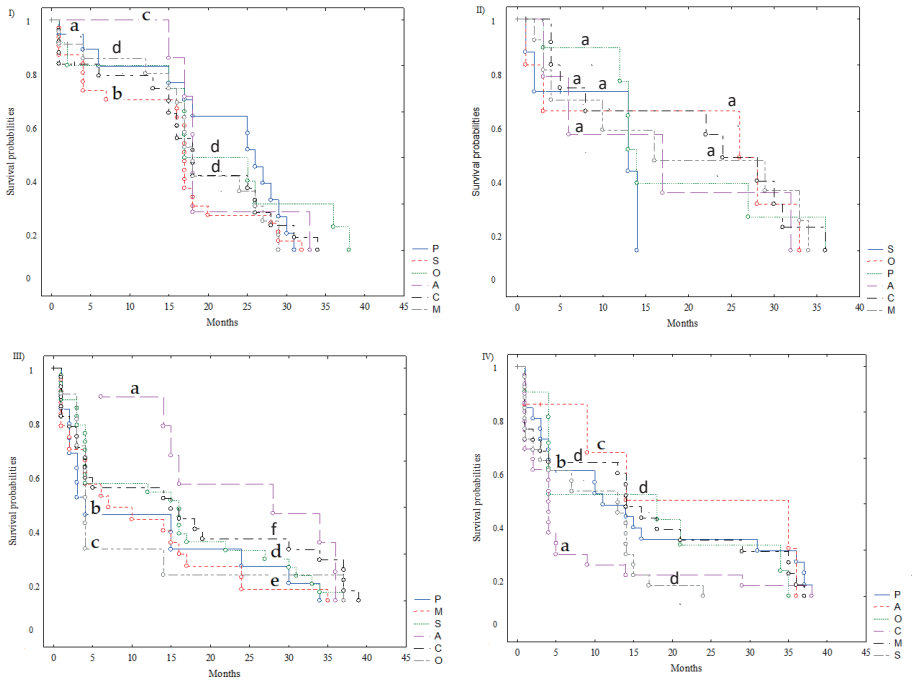

Figure 2. Kaplan-Meier survival curves of *Quercus ilex* seedlings grown in soil naturally infected by *Phytophthora cinnamomi* according to provenance after four years of follow-up (time in months). Plots: I) SEVal, II) SESlo, III) HCVal, IV) HCSlo. Provenance: P (Picos de Europa), O (Ordesa), A (Aigüestortes), M (Monfragüe), C (Cabañeros), and S (Sierra Nevada). Different letters denote statistically significant differences from Gehan's Wilcoxon test ($p< 0.05$).

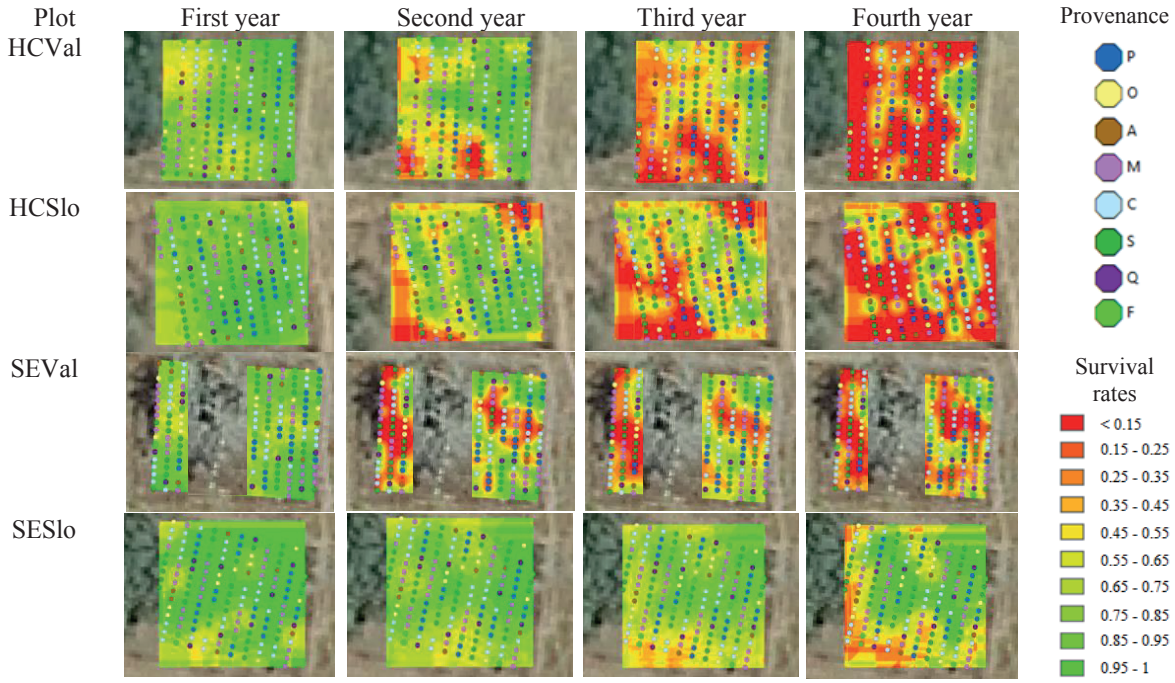

Figure 3. Survival rates during the four years of *Quercus ilex* monitoring in the field. The colored dots indicate the provenance location of the seedlings. Experimental plots: HCVal, Haza de la Concepción valley; HCSlo, Haza de la Concepción slope; SEVal, San Esteban valley; SESlo, San Esteban slope. 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.*

S4 [suppl]). 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 (Figs. S1-S4 [suppl]). 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 0.2 ± 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 5-month study (Rodríguez-Romero *et al.,* 2022). 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 [suppl] 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

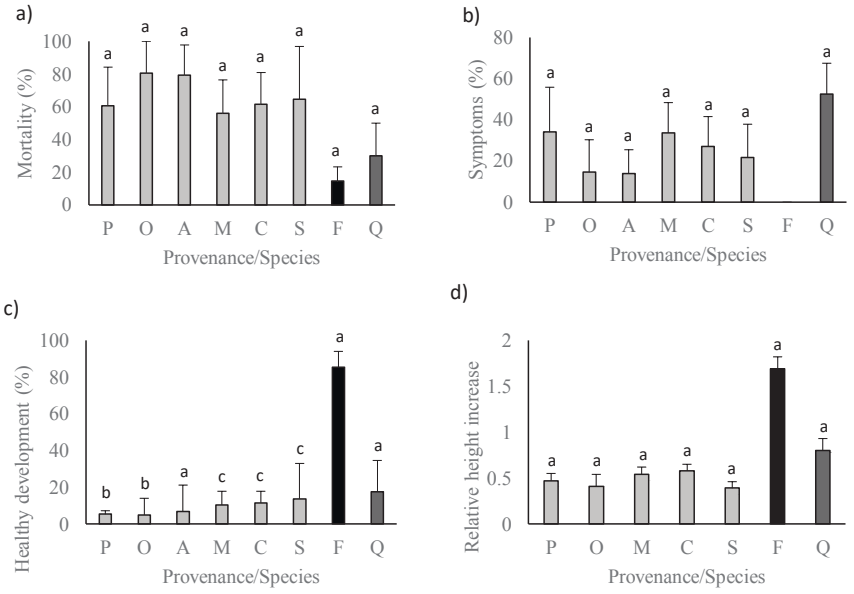

Figure 4. Average evolution of the plantation in the four experimental plots at the end of the four years of follow-up according to provenance. Mean and standard deviations are represented: a) mortality (percentage of dead seedlings), b) percentage of symptomatic live plants, c) percentage of live and 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.* Different letters denote statistically significant differences.

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).

Regarding chemical defence levels, from the three phenolic groups measured in the previous test (Tp, total phenols; Ct, condensed tannins; and Tt, total tannins), 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

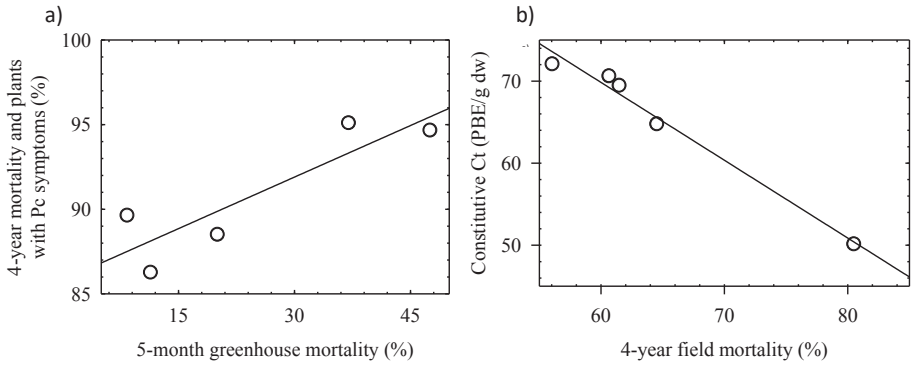

Figure 5. a) Pearson´s correlation between five provenances holm oak mortality plus plants with severe symptoms of *Phytophthora cinnamomi* (Pc) infection in naturally Pc infected soils after four years planting and mortality after 5 months of inoculation with Pc in the greenhouse (r = 0.88, p = 0.04), and b) Pearson´s correlation between mortality after four years on areas affected by Pc and the constitutive condensed tannins of these provenances (PBE/g dw) measured in greenhouse (r = -0.99, p = 0.001). Greenhouse data are available at Rodríguez-Romero *et al.* (2022).

survival to the pathogen's presence both in the greenhouse and in the field (Table S2 [suppl]). 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).

Discussion

To our knowledge, this work constitutes the first field test on the susceptibility of holm oaks of different provenances in oak decline affected 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.,* 2022) and their tolerance was related to higher constitutive chemical defences.

Pc-tolerance in the field depends on the provenances

The mortality of holm oak plants was very high, according to other susceptibility tests of the same species against Pc (Robin *et al.,* 1998; Rodríguez-Molina *et al.,* 2002, 2005; Serrano *et al.,* 2012). 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 (Moreira AC *et al.,* 2018). 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 works (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.,* 2022). 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 & Agrawal, 2011; Pearse & 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 X *et al.,* 2018). 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.,* 2022). 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 areas by the pathogen and even as rootstocks, as some studies already point out (Moreira AC *et al.,* 2018).

However, in a field test like this one, results should be viewed with caution, as several environmental factors also condition the plant survival, and among them, the combined action of several species of *Phytophthora* present in the oak decline foci such as this study area (Corcobado *et al.,* 2014, 2017; Ruiz-Gómez *et al.,* 2018; San-Eufrasio *et al.,* 2021). 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 diseased areas could be possible if tolerant or resistant plant material is used.

Acknowledgments

Authors would like to thank the Cork, Wood and Charcoal Institute (ICMC, Mérida) staff for the technical assistance received and especially Luis Sánchez (in memoriam). Likewise, we thank the Agri-food Laboratory of the Junta de Extremadura for the soil analyses and the Forest Nurseries of the Junta de Extremadura for the transfer of *Fraxinus angustifolia* and *Quercus faginea* seedlings.

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. https://doi.org/10.1016/j.tree.2016.07.011

Brasier CM, 1992. Oak tree mortality in Iberia. Nature 360: 539. https://doi.org/10.1038/360539a0

Brasier CM, 1996. *Phytophthora cinnamomi* and oak decline in Southern Europe. Environmental constraints, including climate change. Ann For Sci 53: 347-358. https://doi.org/10.1051/forest:19960217

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

Corcobado T, Cubera E, Pérez-Sierra A, Jung T, Solla A, 2010. First report of *Phytophthora gonapodyides* involved in the decline of *Quercus ilex* in xeric conditions in Spain. New Dis Rep 22: 33. https://doi.org/10.5197/j.2044-0588.2010.022.033

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. https://doi.org/10.1016/j.agrformet.2014.02.007

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. https://doi.org/10.1111/ppa.12627

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. Proc XIX Congreso Sociedad Española de Fitopatología, Toledo (Spain).

Den Herder M, Moreno G, Mosquera-Losada RM, Palma JH, Sidiropoulou A, Freijanes JJS *et al.,* 2017. Current extent and stratification of agroforestry in the European Union. Agr Ecosyst Environ 241: 121-132. https://doi.org/10.1016/j.agee.2017.03.005

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): 42-56. https://doi.org/10.1016/S1405-888X(13)72077-1

Hardham AR, Blackman LM, 2018. *Phytophthora cinnamomi*. Mol Plant Pathol 19(2): 260-285. https://doi.org/10.1111/mpp.12568

Jactel H, Bauhus J, Boberg J, Bonal D, Castagneyrol B, Gardiner B *et al.,* 2017. Tree diversity drives forest stand resistance to natural disturbances. Curr Forest Rep 3(3): 223-243. https://doi.org/10.1007/s40725-017-0064-1

Jeffers NS, Martin JB, 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Dis 70: 1038-1043. https://doi.org/10.1094/PD-70-1038

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. https://doi.org/10.1111/j.1439-0329.1996.tb00846.x

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.

Mora-Sala B, Gramaje D, Abad-Campos P, Berbegal M. 2019. Diversity of *Phytophthora* species associated with *Quercus ilex* L. in three Spanish regions evaluated by NGS. Forests 10(11): 979. https://doi.org/10.3390/f10110979

Moralejo E, García-Muñoz JA, Descals E, 2009. Susceptibility of Iberian trees to *Phytophthora ramorum* and *P. cinnamomi*. Plant Pathol 58(2): 271-283. https://doi.org/10.1111/j.1365-3059.2008.01956.x

Moreira AC, Tapias R, Fernandes L, Rodriguez A, 2018. Field susceptibility of cork oak trees with different provenances to *Phytophthora cinnamomi*. Forest Pathol 48(5): e12461. https://doi.org/10.1111/efp.12461

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. https://doi.org/10.1111/ele.12253

Moreira X, Castagneyrol B, Abdala-Roberts L, Berny-Mier y Teran JC, Timmermans BGH, Bruun HH *et al.,* 2018. Latitudinal variation in plant chemical defences drives latitudinal patterns of leaf herbivory. Ecography 41: 1124-1134. https://doi.org/10.1111/ecog.03326

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 *et al.* (eds.). Advances in Agroforestry Series, Springer, pp: 127-161. https://doi.org/10.1007/978-1-4020-8272-6_7

Pearse IS, Hipp AL, 2012. Global patterns of leaf defences in oak species. Evol 66: 2272-2286. https://doi.org/10.1111/j.1558-5646.2012.01591.x

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 Pathol 43: 331-339. https://doi.org/10.1111/efp.12037

Rasmann S, Agrawal AA, 2011. Latitudinal patterns in plant defence: evolution of cardenolides, their toxicity, and induction following herbivory. Ecol Lett 14: 476-483. https://doi.org/10.1111/j.1461-0248.2011.01609.x

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. https://doi.org/10.1051/forest:19980801

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

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 Pathol 32(6): 365-372. https://doi.org/10.1046/j.1439-0329.2002.00297.x

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 Pathol 35(6): 411-422. https://doi.org/10.1111/j.1439-0329.2005.00423.x

Rodríguez-Romero M, Gallardo A, Pérez A, Pulido FJ, 2022. Interactive effects of biotic stressors and provenance on chemical defence induction by holm oak (*Quercus ilex*). Trees 36: 227-240. https://doi.org/10.1007/s00468-021-02201-z

Ruiz-Gómez F, Pérez-De-Luque A, Sánchez-Cuesta R, Quero J, Cerrillo RN, 2018. Differences in the response to acute drought and *Phytophthora cinnamomi* Rands infection in *Quercus ilex* L. seedlings. Forests 9: 634. https://doi.org/10.3390/f9100634

Ruiz Gómez FJ, Navarro-Cerrillo RM, Pérez-de-Luque A *et al.,* 2019. Assessment of functional and structural changes of soil fungal and oomycete communities in holm oak declined dehesas through metabarcoding analysis. Sci Rep 9: 5315. https://doi.org/10.1038/s41598-019-41804-y

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: Progress in research on *Phytophthora* diseases of forest trees; Brasier C *et al.* (eds.). Farnham, Surrey, UK: Forest Research, pp: 149-154.

San-Eufrasio B, Castillejo MÁ, Labella-Ortega M, Ruiz-Gómez FJ, Navarro-Cerrillo RM, Tienda-Parrilla M *et al.,* 2021. Effect and response of *Quercus ilex* subsp. *ballota* [Desf.] Samp. seedlings from three contrasting Andalusian populations to individual and combined *Phytophthora cinnamomi* and drought stresses. Front Plant Sci 12: 722802. https://doi.org/10.3389/fpls.2021.722802

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 Pathol 42: 345-347. https://doi.org/10.1111/j.1439-0329.2011.00758.x

Solla A, Slobodan M, Gallardo A, Bueno A, Corcobado T, Cáceres Y *et al.,* 2016. Genetic determination of tannins and herbivore resistance in *Quercus ilex*. Tree Genet Genom 28: 743-751. https://doi.org/10.1007/s11295-016-1069-9