



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

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Abstract

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.

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. A better understanding of its resistance mechanisms is urgently required to enable a better control of its decline. In this work, the ability of holm oaks from six Iberian provenances to respond to multiple biotic damage is studied through an analysis of their induced chemical defence patterns. Using 2016 seedlings established in a common garden trial (6 regions × 12 families/region × 7 seedlings/family × 4 treatments), biotic damage was induced at the root level (by infection with the widespread pathogen *Phytophthora cinnamomi*) and at the above-ground 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 present 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 amongst stressors whose sign (induction/inhibition) depends on the provenance. These findings on biotic stressor effects on the chemical defences and survival of holm oak can contribute to the development of genetic material selection programs in the integrated control of the widespread decline of *Quercus*.

Keywords Combined biotic stress · Geographical variability · Herbivory · Induced chemical defences · Oak decline · *Phytophthora cinnamomi*

Introduction

Plants have different strategies to deal with external biotic attacks. One of them is the production of constitutive and induced chemical compounds, secondary metabolites with a key role in their defence against biotic stress. In fact, phenolic compounds such as tannins range from 5 to 10% dry weight of tree leaves (Barbehenn and Constabel 2011). The constitutive resistance levels correspond to defensive traits that are relatively stable or whose variation depends on internal factors (Schultz 1988). They are genetically controlled and typically show extensive variation amongst genotypes and provenances (Dicke and Hilker 2003; Solla et al. 2016; Gallardo et al. 2019). The induced chemical defences have their origin in phenotypic plasticity that allows individuals to modify their phenotype in response to changes in their environment. Therefore, a large number of biotic factors can alter the constitutive chemical characteristics of plants

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in response to attack (Dicke and Hilker 2003). Constitutive defence prevents infection in the first place, whilst induced defence typically shortens the infectious period (Boots and Best 2018).

The main chemical defences in *Quercus* are phenolic compounds. 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 (Mandal et al. 2010; Castillo-Reyes et al. 2015; Matei et al. 2020) and have a high antimicrobial activity (Scalbert 1991; Daglia 2012).

Oak forests are suffering a worldwide decline related to the rise in average temperatures and the invasion of highly aggressive alien pathogens (Dukes et al. 2009, Santini et al. 2013, Pérez-Ramos 2014). Several *Phytophthora* species are associated with oak decline all over the world (Jung et al. 1996; Balci and Halmschlagler 2003; Pérez-Sierra et al. 2013), and the Mediterranean Basin is not exempt from these pathogens. 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 favoured by global warming, spreading towards the poles (Burgess et al. 2017). Of the many pathogen infections, *Pc* is the primary causal agent of the decline of *Quercus* 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 (Lowe et al. 2000) and its mitigation is a high-priority task that requires the isolation of affected areas to avoid the spread of the pathogen, measures to ensure healthy plant vigour and, in the long term, the selection and implementation of resistant plants (Camilo-Alves et al. 2013). The action of this pathogen, together with the lack of tree regeneration, are the main threats to *Quercus* conservation in the Mediterranean Basin (Moreno and Pulido 2009; Plieninger et al. 2010; Jorrín-Novo and Navarro-Cerrillo 2014).

In the Iberian Peninsula, and mostly within its south-western area, some *Quercus* species such as holm oak (*Q. ilex*) and cork oak (*Q. suber*) form agrosystems known as “dehesas” in Spain and “montados” in Portugal. They are good examples of “grazed wood pastures”, common in the Mediterranean region (Den Herder et al. 2017). These socio-ecological systems are considered by the European Union to be of high natural and cultural value (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 holm oak growth (Schmid et al. 2017) and compromises its regeneration (Carbonero et al. 2004; Olmo et al. 2017). In recent decades, intense defoliation has been coupled with the spread of *Pc* and it is expected that the combination of several biotic stress factors will further weaken the already aged forests (Plieninger et al. 2010).

To date, the study of oak decline has not considered the differences amongst individuals and regions in terms of susceptibility to biotic stressors. The holm oak is the most abundant *Quercus* species in the western Mediterranean area. This is because of its ecological amplitude, growing both in siliceous and calcareous soils and enduring long periods of summer drought. Whilst some studies have been published on geographic variability in the induction of certain defensive chemical traits in holm oak against biotic attack (Solla et al. 2016; Pulido et al. 2019), this is the first study to consider the combined attack at root and aerial levels and their possible interactions. Studies that focus on the effects of combined multiple biotic stress on tree chemical defences and their geographic variability is still scarce. However, previous works on other species, such as that of Pardo et al. (2018) on the chemical defences induced by defoliation in *Prunus lusitanica*, found significant geographical differences in their chemical response. Phenolics are a chemical group widely studied in response to biotic stress in other forest species. Condensed tannins in particular, commonly included as phenols, have demonstrated their induced defensive specificity (Osier and Lindroth 2001; War et al. 2012; Gallardo et al. 2019). Kim et al. (2012) studied phenolic profile in leaves of five different *Quercus* species, proving the presence of many chemical constituents. Brossa et al. (2009) established that major constituents in *Q. ilex* leaves are flavanols and flavonols, although proanthocyanidins and many soluble tannins had a relevant presence in all leaf samples. This work focuses on the evaluation of total phenols, total tannins (included within total phenols) and condensed tannins (included within total tannins) induced by biotic stress to determine the possible intraspecific differences in resistance traits of *Q. ilex*. Resistance traits are those whose aim is to minimise damage whereas tolerance traits are those whose aim is to reduce the impact of damage on plant fitness. Since they prevent the entrance and inhibit the development of the pathogen, phenolic chemical defences are considered resistance traits. Monitoring the survival of plants affected by biotic stressors, as a proxy of plant fitness, can be used to assess their tolerance to damage, especially when the relation between survival and damage is examined. The tolerance to biotic stress or “the ability of the host to reduce

the stress factor damage” (Pagán and García-Arenal 2020) will depend, amongst other factors, on the genes involved in the production of these chemical defences (Gallardo et al. 2019). The existence is, therefore, expected 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, facilitates a priori 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, knowledge about 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. The purpose of the trial was 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?

Based on the current knowledge about the induced response of different biotic stressors in other tree species, the applied treatments were expected to induce a defensive chemical response in the holm oak seedlings which will be specific to the biotic stressor and will have decisive effects on 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. Within each region, acorns were collected from 12 randomly selected mother trees (hereinafter referred to as “families”) to study the chemical defences of their seedlings (half-siblings). 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 latitude, 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 29 N X: 748,862; Y: 4,435,709; 395 m above sea level). Collected acorns were sown in December 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). To ensure the successful germination of the seedlings, two acorns were sown per alveolus, subsequently selecting the first to emerge (Fig. S1 from supplementary information).

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

Application of treatments and symptom assessment

Once a minimum development had been 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 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 each year to confirm its virulence and kept cold at 10 °C. For this test, it was reactivated 2 weeks prior to its use in the inoculum to cause infective lesions in the root system of 1-month-old *Q. ilex* and *Castanea sativa* seedlings.

The inoculum was prepared in Erlenmeyer flasks following the procedure described by Jung et al. (1996) with plugs of the UEX 1 strain in V8 multivitamin juice broth, whole oat grains and fine vermiculite, and incubated for 5 weeks at 22 °C (Fig. S1 supplementary information). Plant infection

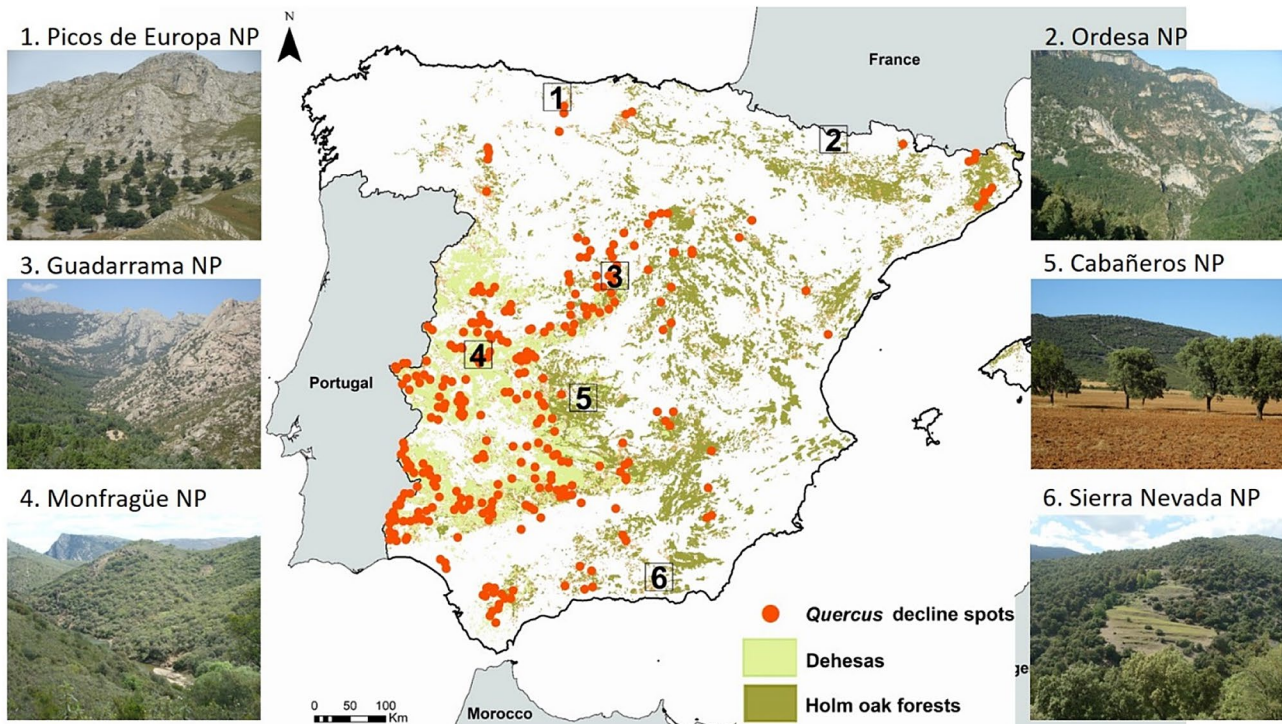


Fig. 1 Distribution range of dehesas (light green area), holm oak forests (dark green area) and location of the studied regions in Spain (numbered black squares) according to Díaz and Pulido (2009) and Rodà et al. (2009): 1. Picos de Europa, 2. Ordesa, 3. Guadarrama, 4. Monfragüe, 5. Cabañeros and 6. 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 presence of *Phytophthora cinnamomi* (CICYTEX-INIAV 2020)

was facilitated by inoculating the soil with 20 cc of the mycelial culture into each cell. After inoculation, the seedlings were lightly watered and flooded the following day in chlorine-free water to stimulate sporangia production and the release of infective zoospores.

The simulation of the mechanical damage that an herbivore could cause on the seedling was made through mechanical defoliation 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 control of 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 analysing 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 analysing interactions between two types of biotic damage. In the double treatment, both biotic stressors were applied at the same time. The development of external symptoms (chlorotic, wilted or dead foliage) and the

evolution of mortality and resprouting were recorded daily for each plant during 4 months.

Sampling and secondary metabolite quantification

Three weeks after the application of treatments, when symptoms were evident and mortality had begun to increase, sampling was performed for the quantification of secondary metabolites. Three individual plants per family (half-siblings) 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 -80°C . To confirm Koch's postulates, from the fine roots of plants infected with *Pc*, the pathogen was successfully re-isolated in selective NARPH (nystatin–ampicillin–rifampicin–pentachloronitrobenzene–hymexazol) corn meal agar medium, a modified PARPH medium (Jeffers and Martin 1986). As expected, *Pc* was not found in control treatment or mechanical defoliation seedlings. The time lag after treatment application was established based on tests of pathogenicity, resistance to simulated herbivory and assessment of the induced systemic response of the plants (Kúć 2001; Sánchez et al. 2004; Izaguirre et al. 2007).

As previous studies on the chemical characterisation of defensive compounds in holm oak (Pulido et al. 2019) reported phenolic content in leaves to be more stable throughout the year than in stems, roots and acorns, where the content varies depending on the season, for the purposes of this study, it was decided to employ leaf phenol extraction. Leaf samples were freeze-dried (Telstar LyoQuest lyophilizer, temperature $-55\text{ }^{\circ}\text{C}$ and 0.001–0.002 mbar pressure) and ground to a fine particle size. Once the milling had been completed, tissue samples were stored at $-80\text{ }^{\circ}\text{C}$ until analysis. The phenolic content of the plants was extracted from lyophilized material with 70% (v/v) aqueous methanol for 60 min in an ultrasonic bath at room temperature, as described by Gallardo et al. (2019). The crude extracts were centrifuged at 10,000 rpm for 5 min at $4\text{ }^{\circ}\text{C}$ and the supernatant was collected and stored at $-80\text{ }^{\circ}\text{C}$.

The total phenolic content (Tp) of the extract was determined by the Folin–Ciocalteu method (Makkar, 2003). For this, 0.2 ml of the 20-fold diluted extract was mixed with 1 ml of 10% Folin–Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) and 0.8 ml of 7.5% (w/v) sodium carbonate. In the control tube, the extract volume was replaced with methanol. The mixture was stirred gently and maintained in the dark and at room temperature for 45 min. 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 acetic acid, 60 μM ascorbic acid and 0.1% BSA. Uniform wells were made on plates containing the same volume of solidified agarose and constant volumes of samples were loaded and incubated at $30\text{ }^{\circ}\text{C}$ 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 with methanol. Samples were heated at 70° . After 45 min 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, family (nested in region) and treatments on secondary metabolite patterns were analysed through a general linear mixed model (GLMM). Region, family (nested in region) and treatment were used as fixed factors given the relatively low number of sampled regions and families and their non-random selection within the natural range of distribution of the species. However, to calculate the percentage of variance explained by each factor, these factors were then considered randomised and estimated using the restricted maximum likelihood (REML) method. The Tp, Tt and Ct contents were used as dependent variables. Germination time and seedling size (measured from their weight during the harvesting of samples) were included in the model as continuous covariates to control their potential disturbing factor. Interactions between treatment and region were also included in the model. To test for inducibility upon mechanical damage and inoculation with *Pc*, differences between family means of constitutive defences (control treatment) and those induced by each treatment were calculated. For the analysis of the response induced by the treatments, the application of two treatments (*Pc* and *d*), each with two levels (0/1), and their interactions (for control and combined treatments) were considered. To check normality of the residuals of the model, the data were analysed using the Kolmogorov–Smirnov test, whilst homoscedasticity was checked using Levene’s test.

The heritability of chemical traits (\hat{h}^2) was based on the variance components of the model. The narrow-sense heritability 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 generalised selfing rate of 1–3% 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 ($\frac{1}{4}\text{VA}$). The following equation was used:

$$\hat{h}^2 = \text{VA}/\text{VP} = \frac{(\sigma^2f(\text{gen})) \times (1/r)}{(\sigma^2f(\text{gen})) + (\sigma^2r) + (\sigma^2e)},$$

where $\sigma^2f(\text{gen})$ is the variance component amongst families (genotypes), σ^2r the amongst-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):

$$Q_{ST} = \frac{(\sigma^2_r)}{(2 \times \sigma^2_{f(\text{gen})}) + (\sigma^2_r)}$$

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 amongst treatments in survival. Survival was analysed with the “Survival Analysis” module from the Statistica v10 software. The effect of region, family (nested in region), treatments and their interactions on the tolerance and resprouting of the seedlings was also analysed through a GLMM, using these as fixed factors and survival as the binomial dependent variable. The relationship between chemical-dependent variables and tolerance was calculated at regional level using Pearson’s linear correlation. Statistical analyses were performed in the Statistica v10 software.

Results

Chemical defence induction by treatments

The Folin–Ciocalteu analysis method yielded Tp concentrations in *Q. ilex* leaves of between 36.44 and 56.98 mg G.A.E./g dw. Tt concentrations fluctuated more and were between 68.3 and 103.36 mg T.A.E./g dw, whilst Ct was the variable with the greatest range of variation, with values between 50.17 and 119.74 mg P.B.E./g dw. Significant differences amongst 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 significant differences ($p < 0.001$) in the treatment/region interaction thus indicating geographical variation of the response to treatments.

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 specific response to the stressor varied geographically and locally (Table 1). 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 neutralised the production of defences in some regions, being intermediate between that induced by the pathogen and that induced by mechanical defoliation. In other regions, the double treatment 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 the region, and the sign of the interaction (neutralisation or enhancement) varied geographically.

The constitutive defences (control treatment, $N = 36$ seedlings per region) showed statistically significant correlations with those induced by the different treatments only in some cases ($N = 36$ seedlings per region and treatment; Table S1 from supplementary information). Correlation varied according to the region, the induction treatment and the phenolic group (Fig. 3). The significant correlations were always negative. The regions with the highest significant and negative correlation between constitutive and induced defences were P and S.

Heritability of defensive chemical traits

Defences showed significant narrow-sense heritability across regions (\hat{h}^2) for Tp (0.43 ± 0.09), Tt (0.18 ± 0.05) and Ct (0.5 ± 0.26). The quantitative genetic differentiation (Q_{ST}) was 0.2, 0.44 and 0.07, respectively (Table 2).

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

Effect	df	Total phenolics	Total tannins	Condensed tannins	Survival	Resprout
Region	5	25.30***	31.36***	6.09***	35.87***	0.21
Family (region)	62	2.66***	1.90***	2.47***	30.72***	6.44
<i>P. cinnamomi</i> inoculation (Pc)	1	9.63**	1.20	16.37***	22.74***	2.55
Mechanical defoliation (d)	1	44.95***	9.54**	79.18***	0.17	11.89***
Pc × d	1	6.80**	8.09**	1.99	12.97***	0.3
Region × d	5	0.34	0.58	2.19*	2.27*	0.35
Region × Pc	5	1.73	1.93	5.69***	6.79***	0.36
Region × Pc × d	5	3.09**	2.25*	4.31***	5.04***	0.19

Treatments: inoculation with *Phytophthora cinnamomi* (Pc) and mechanical defoliation (d)

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

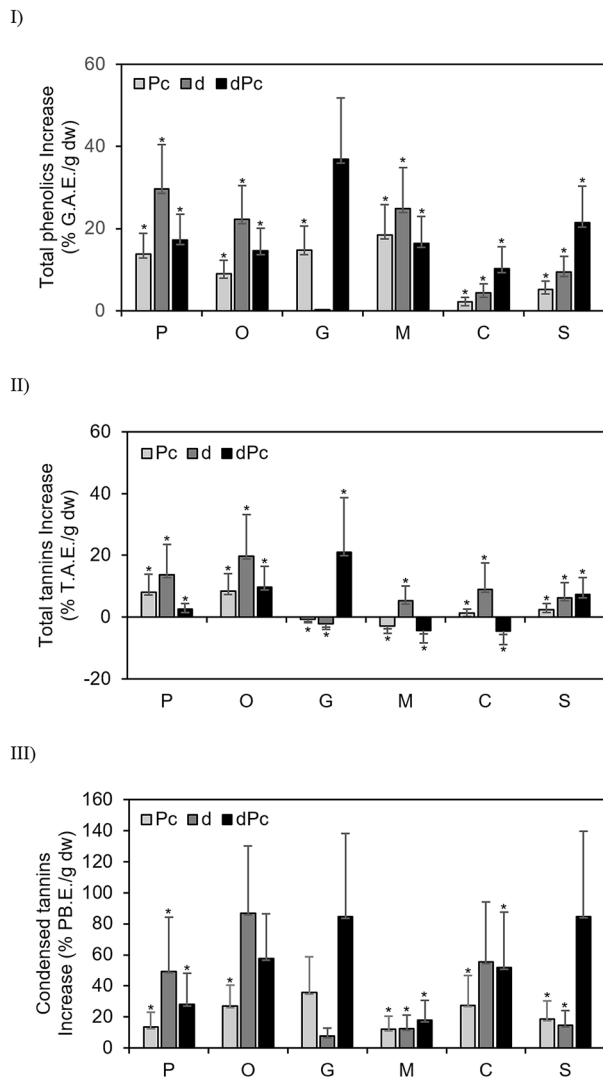


Fig. 2 Phenolic compounds induction in leaves of *Quercus ilex* (percentage increase compared to the control treatment) after biotic stress according to the region of origin (ordered from highest to lowest latitude): Picos de Europa (P), Ordesa (O), Guadarrama (G), Monfragüe (M), Cabañeros (C) and Sierra 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. *Indicates statistically significant differences between constitutive (control treatment) and induced defences (GLMM results)

Induction and survival

The Pc inoculation and Pc × d interaction treatments had a statistically significant effect on survival ($p < 0.001$, Table 1 and Table S2 from supplementary information), but defoliation did not. Survival was dependent on region and family

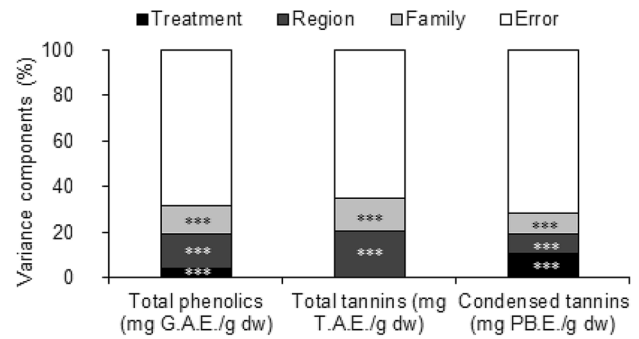


Fig. 3 Components of the variance of total phenols, total tannins and condensed tannins amongst treatments, regions and families, expressed as percentages of the total variance. The error value corresponds to the differences amongst individuals within each family. The statistical significance of the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

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

Statistic	Total phenolics	Total tannins	Condensed tannins
$\sigma^2 f(\text{gen})$	15.35	20.74	79.87
$\sigma^2 r$	29.02	121.1	41.97
$\sigma^2 e$	88.9	273.94	473.97
\hat{h}^2	0.43 ± 0.09	0.18 ± 0.05	0.5 ± 0.26
Q_{ST}	0.20	0.44	0.07

($p < 0.001$). In addition, there were significant differences in region survival to each stressor, and therefore, variation in tolerance (especially in the region × Pc treatment interaction and in the region × Pc × d interaction (Table 1, $p < 0.001$).

Induced defence levels did not show a significant correlation with survival ($p > 0.05$, Table S2 from supplementary information). Only in the O and S regions was there a significant correlation between Ct production and survival ($r = 0.37$ in O, $r = -0.55$ in S, $p < 0.05$). The absolute defence values (sum of constitutive and induced) also showed no statistically 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 3, Fig. 4). Infection with the root pathogen was the most damaging (74.78% survival versus 91.4% of the control). Pc

Table 3 Probabilities of survival and resprouting (%) 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)

Region	%	Treatment			
		c	<i>Pc</i>	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

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)

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 when facing stressors than northern regions, in particular the M and S regions (survival probabilities greater than 80%). C showed

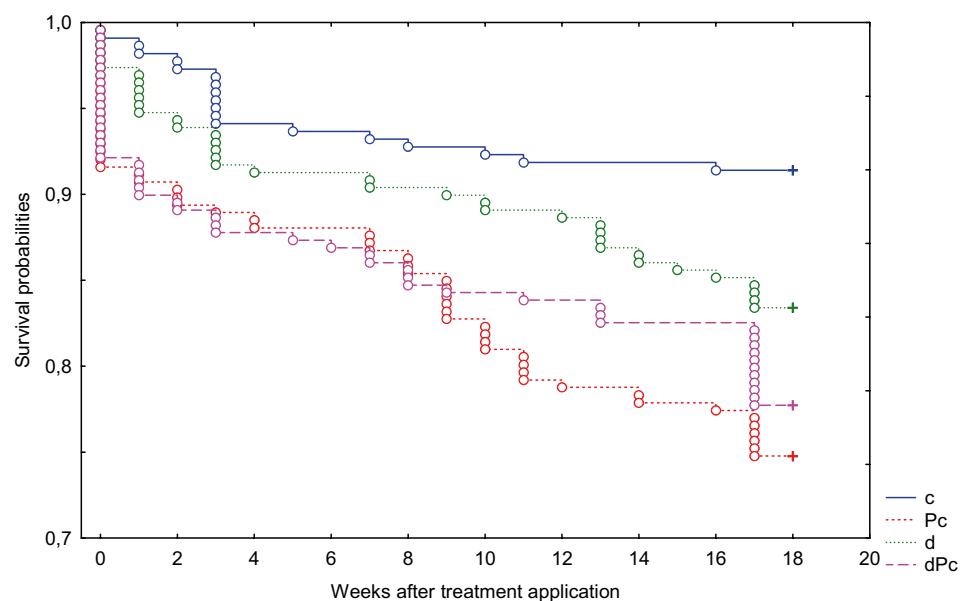
high tolerance to defoliation but not so much to *Pc* infection and double treatment. It was observed that mortality due to defoliation increased when combined with *Pc* infection, that is *Pc* increases the susceptibility of seedlings that also suffer herbivory. However, defoliation combined with root infection caused lower mortality than when *Pc* acted alone (*Pc*, as mentioned before, was the stressor with the highest mortality).

As for resprouting after the biotic stress condition, only the effect of the defoliation treatment was significant ($p < 0.001$, Table 1). Of the affected plants with visible external symptoms, 12.72% resprouted. Regrowths were more frequent in plants subjected to double treatment (Table 3). This was the case in five of the six studied regions, with the exception being C where defoliation generated the highest resprout rate (6.25%). Resprouting was greater in the southern regions of the Iberian Peninsula. The highest resprout percentage was induced by the double treatment in the S region (7.56%). M presented high resprout percentages for both defoliation and the double treatment. The higher survival probabilities and regrowth rates obtained in the southern regions suggest their greater tolerance to the studied biotic stress factors.

Discussion

This study contributes to the current body of literature by expanding on how plant populations from different regions invest differently in their defensive traits. Individual and ecotypic variability generate variations in the response of the holm oak's chemical defences against different biotic stress factors, showing a certain specificity and interactions

Fig. 4 Survival probabilities of *Quercus ilex* seedlings showing differences amongst 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 resprouting. Control seedlings (c) also suffered some mortality, but within the usual rates during the first months of life



in the production of tannins depending on the stressor. This could be an adaptive strategy to the diversity of the pathogen and herbivore community present in the ecosystem.

Chemical defences induced by stressors

The constitutive defences of holm oak varied significantly amongst families and regions. Region was the main source of variation of the three phenolic groups, which is in agreement with Rodríguez-Romero et al. (2020) for the production of Tp and Ct. Solla et al. (2016) explored geographical variation in holm oak constitutive Tt, finding important local but not geographical differences, although their work focussed on south-central Iberian Peninsula regions. For their part, Moreira et al. (2018) studied the constitutive Ct variation in 38 populations along an 18° latitudinal gradient of *Q. robur*, also obtaining statistically significant differences. Pardo et al. (2018) explored 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 when quantifying 98 plant secondary metabolites. This spatial variation could be due to local adaptation mechanisms promoted by the coexistence of host stressors, as well as the availability of resources 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 knowledge of the chemical patterns of the constitutive defences in *Quercus*.

Regarding the induced defences, these showed significant differences according to region (geographic level), family (local level) and applied treatment (specific induction). Following the same pattern as in constitutive defences, the variance of the three phenolic groups was best explained by region, although in the case of induced Ct it was similar to the variance explained by family. Although some studies have been published on spatial variation in the effects of biotic stress or the production of chemical defences, only a few studies have combined geographical variation with induction of defences by defoliation and pathogen infections. However, of those cited earlier, Pardo et al. (2018) also explored the spatial variation of Tp induced by mechanical defoliation in *Prunus lusitanica*, finding significant local and geographical differences in induced defences. Osier and Lindroth (2001) also found in relation to aspen phytochemistry that artificial defoliation induced genotype-dependent Ct. In addition, the three chemical traits evaluated in this work were highly variable within families and amongst regions, especially Ct. A similar finding was reported in *Pinus pinaster* Tp from the Iberian Peninsula against the attack of

pine wilt nematode (*Bursaphelenchus xylophilus*; Zas et al. 2015).

As expected, because they are overlapping groups, the three evaluated variables showed a strong and positive correlation (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. Major geographical differences in the induction of chemical defences were also shown in Ct production. The best variable for the characterisation of differences in induced defences against biotic stress can therefore be considered to be Ct at the geographical level because the effect of the factors evaluated in the assays was statistically significant both when evaluated independently and in their interactions. In fact, its use to analyse plant–herbivore interactions in the literature is common (War et al. 2012). The results of this work reinforce the choice of Ct to analyse 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 also high and positive, especially in the induction of defences by *Pc* infection. This specificity was also demonstrated by Gallardo et al. (2019) in holm oak seedlings from the same provenance subjected to root pathogen infection and mechanical defoliation, identifying the *Quercus* sequences encoding enzymes for early steps of the biosynthesis of phenolic compounds, like hydrolysable tannins and Ct amongst 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 Tt production for the Picos de Europa region, also studied in our work. The results of the present 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. Whilst several studies on plant–herbivore interactions have focussed on Ct, it was observed in the present study that Tt, that is those that include hydrolysable tannins, better characterise the induction of defences against herbivory stress in the holm oak. In fact, some specific compounds that form 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 the M and C regions,

the double treatment results in an inhibition, as opposed to an induction, of defences. When defoliation and *Pc* infection are 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 susceptibility of seedlings 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. However, the method to determine Tt in this experiment could be interfering in the anomaly performance, since the measurement of BSA halos could incur greater precision errors when halos are small.

Heritability of constitutive chemical defences

The heritability of Tp, Tt and Ct in *Q. ilex* leaves was moderate. 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 obtained in our study is likely to allow selection (Eriksson et al. 2013; Alcaide et al. 2019). Nevertheless, these results must be taken with caution due to the low number of genotypes evaluated (Jensen and Barr 1971; Visscher and Goddard 2015). Although there are hardly any heritability works on these chemical traits in holm oak, Tt heritability values were lower than those obtained in a previous study (Solla et al. 2016). However, Tp and Ct did obtain heritability of interest for the selection of material in future species improvement programs.

Genetic differentiation by quantitative traits (Q_{ST}) was higher in Tt (0.44) than in a previous work (0.12; Solla et al. 2016). Although little is known about Q_{ST} in holm oak chemical defences, $Q_{ST}-F_{ST}$ comparisons would facilitate discrimination between natural selection and genetic drift as differentiation mechanisms in traits (Leinonen et al. 2013). Future analyses with a greater number of sampled families and an estimation of the fixation index (F_{ST}) would be key to detect if there is divergent selection (Merilä and Crnokrak 2001).

Constitutive and induced defences and their consequences for plant survival

Tolerance to biotic stress, both caused by mechanical defoliation and induced by *Pc* infection, differed significantly amongst Iberian provenances. In all cases, the biotic stressors reduced the survival probabilities. The most harmful was root infection alone, even more than the combination of root infection and defoliation.

Generally, the southern regions were more tolerant to biotic stress from root and aerial damage. M and S were the most tolerant regions to the applied treatments, exceeding in all cases more than 80% survival rates as well as having high resprout rates. As far as the authors of the present study are aware, no other works have been published on the geographical variation of the chemical defences studied here and the tolerance against the applied biotic stressors in holm oak. However, Camisón et al. (2019) profile the defensive chemical patterns in *C. sativa* plants to *Pc*, finding more Tp and Ct in plants susceptible to the pathogen than in resistant ones. In contrast, Cahill et al. (1989) showed that phenolic deposition was higher in the more resistant species than in the susceptible ones during root infection by *Pc*. A similar result was reported by Ullah et al (2017) in the antifungal activity of Ct against *Melampsora larici-populina* in *Populus nigra*. 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 (Keinanen et al. 1999; Hikosaka et al. 2005). Furthermore, specific compounds in these large phenolic groups have been identified for their key role in resistance to different stressors (Feeny 1968). Focussing on the defensive response of the plant to *Phytophthora* spp, several studies have cited the involvement of phenolic compounds in this interaction (Picard et al. 2000; Lherminier et al. 2003; Horta et al. 2010). Stong et al. (2013) reported this phenolic involvement with *P. ramorum* and Gallardo et al. (2019) with *Pc*. However, given the contrasting results obtained in the experiments that have been conducted to date, the role of tannins in a plant's defence against biotic stress still has 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 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 despite 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 that were 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 sporadic. 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 (Perea et al. 2014) 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 regions most susceptible to the root pathogen 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, as our understanding of these defensive mechanisms is still limited, the authors underline 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 selection of genotypes tolerant and/or resistant to pathogens. However, it constitutes a first step in the development of a holm oak decline management program that considers current intraspecific geographical variations. 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 chemical compounds induced by each biotic stressor are required to understand intraspecific geographical variability in the allocation of defensive resources.

Author contribution statement Conceived, designed and performed the experiments: MR, AG, AP and FP. Analysed the data: MR and FP. Contributed reagents/materials/analysis tools: MR, AG and FP. Wrote the paper: MR and FP. All the authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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