

## Article

# Allelopathic Effects of Three Herb Species on *Phytophthora cinnamomi*, a Pathogen Causing Severe Oak Decline in Mediterranean Wood Pastures

Manuela Rodríguez-Romero <sup>1,2,\*</sup>, Belén Godoy-Cancho <sup>1</sup>, Isabel M. Calha <sup>3</sup>, José António Passarinho <sup>3</sup> and Ana Cristina Moreira <sup>3</sup>

<sup>1</sup> Instituto del Corcho, la Madera y el Carbón Vegetal (ICMC)/Centro de Investigaciones Científicas y Tecnológicas de Extremadura (CICYTEX), Junta de Extremadura. Pol. Ind. El Prado, C/Pamplona s/n, 06800 Mérida, Spain; belen.godoy@juntaex.es

<sup>2</sup> Instituto de Investigación de la Dehesa (INDEHESA), Universidad de Extremadura (UEX), Av. Virgen del Puerto s/n, 10600 Plasencia, Spain

<sup>3</sup> Instituto Nacional de Investigação Agrária e Veterinária (INIAV, IP), Quinta do Marquês, 2784-505 Oeiras, Portugal; isabel.calha@iniav.pt (I.M.C.); jose.passarinho@iniav.pt (J.A.P.); cristina.moreira@iniav.pt (A.C.M.)

\* Correspondence: manuela.rodriguez@juntaex.es

**Citation:** Rodríguez-Romero, M.; Godoy-Cancho, B.; Calha, I.M.; Passarinho, J.A.; Moreira, A.C. Allelopathic Effects of Three Herb Species on *Phytophthora cinnamomi*, a Pathogen Causing Severe Oak Decline in Mediterranean Wood Pastures. *Forests* **2021**, *12*, 285. <https://doi.org/10.3390/f12030285>

Academic Editor: Fred O. Asiegbu

Received: 10 December 2020

Accepted: 25 February 2021

Published: 2 March 2021

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

**Abstract:** The ability of three herbaceous plants (*Diplotaxis tenuifolia* (L.) DC., *Eruca vesicaria* L. and *Raphanus raphanistrum* L.) from Iberian wood pastures to reduce *Phytophthora cinnamomi* Rands pathogen populations through allelopathic relationships is studied. The inhibitory capacity of their aqueous root extracts (AREs) on mycelial growth and production of *P. cinnamomi* reproductive structures is analysed *in vitro*. In addition, *Quercus* seedlings were grown in infested by *P. cinnamomi*-soils and with the presence or absence of allelopathic and susceptible herb species to the pathogen to assess the defensive chemical response of *Quercus* seedlings through their leaf phenolic compounds. Results show a strong inhibitory capacity of AREs on *P. cinnamomi* activity *in vitro* and a protective effect of these herb species on *Quercus* plants against *P. cinnamomi* *in vivo*. *D. tenuifolia* would be especially suited for biological control in the pathogen suppression.

**Keywords:** allelopathy; biocontrol; dehesa and montado's herb species; *Phytophthora cinnamomi*; *Quercus* decline

## 1. Introduction

Plants can influence the composition of microbial communities around their roots through exudation of carbohydrates and other allelopathic compounds. Allelopathy is a naturally occurring ecological phenomenon of interference among organisms by which one of them produces one or more biochemical compounds that influence the growth and development of others (bacteria, fungi, plants...), either negatively or positively [1–3]. Some exudates present bactericidal and fungicidal activity and can affect the growth, survival and/or reproduction of various microorganisms. The allelochemicals most frequently involved in these fungicidal relationships are secondary metabolites that are not directly involved in the plant life cycle but play an important role in its defence against natural enemies. Allelochemicals are located in different parts of the plant, such as leaves, branches or roots [4]. They are usually released directly into the aqueous phase of the soil, or from volatile gaseous substances in the surrounding air [5]. The allelochemical release is influenced by the soil, climatic conditions and the plant itself [6].

Iberian agrosilvopastoral ecosystems are currently suffering an increasing decline with serious impact on oak species. The widespread *Quercus* decline is influenced by the action of biotic and abiotic stress factors. This disease is associated with different species

of oomycetes and *Phytophthora cinnamomi* Rands is the most frequently isolated from the Iberian Peninsula soils [7]. This soil-borne pathogen affects more than 5000 trees, shrubs and herbs species in the world [8] and causes root rot and death of several *Quercus* species. Its eradication from the soil in field is very complex due to the durability of its resistance structures and the easy spread by different pathways [9]. Nowadays, the control is understood only from an integrated perspective, given its wide dispersion and the large host range. Especially susceptible are holm oak (*Q. ilex* L.) and cork oak (*Q. suber* L.), which are main tree components of Iberian wood pastures. The Portuguese oak (*Q. faginea*) is also susceptible to *P. cinnamomi*, although less than the first two [10–12]. The main chemical defences in *Quercus* are phenolic compounds and their induction before the attack of biotic stressors has been studied before [13,14], so they can be used to evaluate the damage caused in plants. However, allelopathic phenomena in the co-occurring plants could mitigate the stress caused in *Quercus* species [15]. The chemical defence levels in *Quercus* leaves would be a measure of the strength of the host's response to the pathogen.

Among the different management tools against oak decline, biological control is presented as a still underdeveloped alternative but with certain advances in agricultural systems that could be the basis for the development of a forestry strategy [16]. There are species that show resistance to it and even inhibit its infective activity due to the release of allelochemicals [17]. In the Mediterranean flora there are various species with allelopathic effects against *P. cinnamomi* [18–22]. The allelopathic property can be observed in plants used for biofumigation such as *Brassica carinata* and *B. juncea* [19,20,22] and in other native flora still under study [18,19,21]. The family to which they all belong, *Brassicaceae*, stands out for its high concentration of glucosinolates, which show fungicidal effect in certain concentrations and conditions [19,23,24]. These are sulfur compounds with proven fungicidal and biocidal capacity [25,26]. Their defensive properties are generated by an enzymatic hydrolysis that releases volatile compounds, among which are isothiocyanates, nitriles, thiocyanates and oxazolidines, depending on the structure of the original glucosinolate [27].

Based on the findings described by Sampaio [18] and Moreira et al. [21], three herbaceous species from the Mediterranean native flora with potential allelopathic effect facing *P. cinnamomi* were used in this study for in vitro and in vivo experiments.

The work aims to answer the following questions:

- (1) What plant species have the greatest allelopathic ability on *P. cinnamomi* by application of their AREs under in vitro conditions?
- (2) Do these herb species reduce the need to invest in chemical defences of *Quercus* seedlings under in vivo conditions when they grow up together in *P. cinnamomi*-infested soil?

## 2. Materials and Methods

### 2.1. Biological Material

The *Phytophthora cinnamomi* strain 5833 mating type A2 was isolated from chestnut (*Castanea sativa* Mill.) roots in central Portugal. Isolation and culture maintenance took place on V8 Juice agar medium as described by Moreira-Marcelino [28].

Root extracts were prepared using 40-day-old plants of *Diplotaxis tenuifolia* (L.) DC., *Eruca vesicaria* (L.) Cav. and *Raphanus raphanistrum* L. A natural soil collected in Sintra, Lisbon, was sown with seeds of these species collected in natural conditions (*E. vesicaria* and *R. raphanistrum*) and acquired in the market (*D. tenuifolia*). Plants grew under greenhouse conditions without fertilization and watered twice a week in Oeiras (INIAV) (Portugal, UTM Zone 29S X: 472148, Y: 4283072, 38 m above sea level). At harvest, the roots were washed to remove the soil and later they were cut to be frozen at  $-10^{\circ}$  until processed. The upper part of the plants was discarded.

Acorns of *Quercus suber* (cork oak) and *Q. faginea* (Portuguese oak) were collected in forest areas across the Counties of Ourique and Mafra (Portugal). These acorns were used

to produce *Quercus* seedlings to assess the allelopathic effect of the herbaceous species *in vivo*.

### 2.2. Root Extracts Preparation Using Different Methods and Chemical Characterization

All glucosinolates occur in the plant in conjunction with the hydrolytic isoenzyme myrosinase, and are located in separate cells [29]. However, after trituration of plant tissues, they come together, so the enzyme must be inactivated to assess the glucosinolate effect. In attempt to inactivate the myrosinase, aqueous root extracts (AREs) were prepared by three different methods. The methods were (M1) maceration of fresh material without inactivation of enzymes (modified from Alkhail [30]), (M2) maceration of fresh material with heat inactivation of enzymes [14,18] and (M3) microwave dried material (modified from Hongju [29]).

AREs were prepared from 10 g of previously washed roots, with later maceration in 100 mL distilled water at room temperature in M1. In M2, 10 g fresh weight was macerated in 100 mL distilled water at 80 °C for 10 min for its inactivation by heat. In the M3 method, after weighing 10 g of roots, they were microwaved at 900W for different times depending on their thickness and humidity (*R. raphanistrum* 9 min, *E. vesicaria* 2 min, *D. tenuifolia* 1 min). Once dry, roots were crushed and macerated at room temperature with 100 mL distilled water. In all three methods, the solution was filtered and centrifuged for 10 min at 8000× *g* and 4 °C. The supernatant was removed and filtered with Millex –GP 0.22 µm (33 mmØ) filters (Merck Millipore Ltd., Carrigtwohill, Cork, IRL). AREs were frozen at –10 °C for conservation.

### 2.3. In Vitro Assessment of the Allelopathic Effect on the Activity of *P. cinnamomi*

The AREs' inhibitory effect on *P. cinnamomi* activity was evaluated *in vitro*. The mycelial growth was measured in Petri dishes with V8 broth and ARE at 75% (*v/v*) as described Moreira et al. [21], 12 days after their incubation at 25 °C in the dark. Then, the mycelium was harvested from the broth, filtered, washed and dried in an oven for 48 h at 60 °C. The dry weight mycelium was recorded. Plates with V8 broth and 75% (*v/v*) of sterile water was used as control.

The effect of ARE obtained by M2 was evaluated in the sporangia production, zoospore release and germination and in the production of chlamydospores. The production of *P. cinnamomi* reproductive structures was obtained and analysed using non-sterile soil extract [28] with root extracts at 75% (*v/v*). V8-agar plugs (5) 5 mm in diameter were transferred from the edge of *P. cinnamomi* colony to the non-sterile soil extract supplemented with ARE and incubated at room temperature with indirect light. Plates were scored at six and 12 days later. Zoospores germination was evaluated using a zoospore suspension (100 µL) with  $8.0 \times 10^4$  cel/mL, plated on V8-agar 5% supplemented with ARE at 75% (*v/v*) in sterile distilled water and incubated at 25 °C in the dark. A treatment without ARE and supplemented with sterile distilled water at 75% (*v/v*) served as a control. The colony-forming units were counted after 24 h and 48 h and the inhibition determined 48 h after plating. Sporangia and chlamydospores quantification was recorded as the mean number per mm<sup>2</sup> at four equidistant spots on the mycelium at 10×, 40× and 100× under a microscope. All trials had six repetitions per method and species. For the evaluation and taking of photographs of the different *P. cinnamomi* structures, samples were stained with lactophenol blue solution.

The percentage inhibition of *P. cinnamomi* was calculated as described by Moreira et al. [21], according to the equation: Inhibition (%) = 100 (Control-Treatment)/Control. Data were transformed according to the equation: Inhibition<sub>transformed</sub> = (100-Inhibition (%))/100 to satisfy tests of normality. Transformed data were statistically analysed by ANOVA using the Tukey's test ( $p < 0.05$ ) for differentiation of means. Statistical analyses were performed using the Statistica v10 software (StatSoft, Inc., Tulsa, OK, USA).

#### 2.4. In Vivo Evaluation of the Effect of Allelopathic Herbaceous Species on *Quercus* Seedlings in *P. cinnamomi*-Infested Soil

The inhibitory effect of *E. vesicaria* and *D. tenuifolia* against *P. cinnamomi* was tested in vivo at INIAV greenhouses in Oeiras. Acorns of *Q. suber* and *Q. faginea* were sown in pots (10 L; two acorns per pot, without mixing *Quercus* species in each pot) with soil naturally infested with *P. cinnamomi*. The soil was collected in December in a cork oak woodland located in Biscainho, Coruche (Portugal, UTM Zone 29S X: 532757, Y: 4306056, 71 m above sea level; soil pH: 4.9). In this area, several foci were detected in 2016, showing trees with symptoms of decline infected by *P. cinnamomi*. The soil was collected from an area where the trees died. Presence of *P. cinnamomi* in soil was confirmed by its isolation using cork oak young leaves as baits and plated in PARPH selective agar medium [31]. Furthermore, *P. cinnamomi* inoculum was reinforced with 10 g per pot of inoculum composed by millet seeds (*Panicum milliaceum* L.) colonized during three weeks by an equal mixture of the three isolates *P. cinnamomi* 1538, 1539 and 5833, incubated at 25 °C in dark, according to the protocol described by Moreira-Marcelino [28]. The control experiment was prepared in the same way, but using sterilized seeds of *P. milliaceum*. This assay was carried out with three treatments: (1) *Quercus* species and *E. vesicaria* and *D. tenuifolia*; (2) *Quercus* species and *Lupinus luteus*; (3) only *Quercus* species (control). In the first treatment, 12 plants/pot of *E. vesicaria* and *D. tenuifolia* (high allelopathic effect on *P. cinnamomi*) were sown in containers with acorns of the *Quercus* species. Ten seeds/pot of *L. luteus* (highly susceptible to *P. cinnamomi* and used as a positive control), were sown in each pot with acorns of the *Quercus* species. There were five replicates for each species and treatment. After sowing, the three treatments were grown for two years before leaf harvest. Then, 7–9 leaves from each *Quercus* seedling were collected per pot to quantify their chemical phenolic defences [14,15]. It is assumed that this effect will be the combined action of herbaceous root exudates, together with possible interspecific competition relationships and even the facilitation of bacterial complexes in the soil. Thus, this in vivo essay attempts to be an approach to better understand the role that allelopathic relationships of herbaceous plants play on trees on *P. cinnamomi*-infested Iberian wood pastures.

#### Extraction and Quantification of Phenolic Content in *Quercus* Leaves

For the determination of defensive phenolic content as described by Gallardo et al. [14], *Quercus* leaves were lyophilized using a Telstar LyoQuest lyophilizer (temperature -55 °C and 0.001–0.002 mbar pressure; Telstar, Terrassa, Spain) and ground to a fine particle size. The phenolic content was extracted from lyophilized material with 70% (*v/v*) aqueous methanol for 60 min in an ultrasonic bath at room temperature. The crude extracts were centrifuged at 8000× *g* for 5 min at 4 °C and the supernatant was collected and stored at -80 °C.

The total phenolics content (TPC) was determined by the Folin-Ciocalteu method [32]. Crude extracts (2 volumes) were mixed with 2 volumes of Folin-Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) and 40 volumes of 75 g per liters of sodium carbonate. In the control tube, the extract volume was replaced by deionized water. The mixture was stirred gently and maintained in the dark and at room temperature for 60 min. After incubation, the absorbance was measured at 670 nm, using a UV/Vis Varian Cary-50 spectrophotometer (Palo Alto, CA, USA). Gallic acid (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used as standard and results were expressed as micrograms of gallic acid equivalents (G.A.E.) per milligrams of lyophilized sample.

The total tannin content (TTC) was determined by the Folin-Denis method [33]. Crude extracts (2 volumes) were mixed with 2 volumes of Folin-Denis reagent (Panreac, Barcelona, Spain) and 5 volumes of 200 g per liters of sodium carbonate. In the control tube, the extract volume was replaced by deionized water. The mixture was stirred gently and maintained in the dark and at room temperature for 30 min. After incubation, the absorbance was measured at 760 nm. Tannic acid (Panreac, Barcelona, Spain) was used as

standard and results were expressed as micrograms of tannic acid equivalents (T.A.E.) per milligrams of lyophilized sample.

The butanol-HCl assay [34] was used to quantify condensed tannins (CTC) using procyanidin B2 (Sigma-Aldrich, Madrid, Spain) as a reference compound. Briefly, crude extracts were mixed with 100 volumes of n-butanol/acetone 1:1 (46% each) plus HCl (1.85%) and ferric ammonium sulphate (0.04%). In the control tube, the extract volume was replaced by methanol. Samples were heated at 70 °C. After 45 min of incubation, the samples were cooled and the absorbance at 550 nm was measured, with final results expressed as micrograms of procyanidin B equivalents (PB.E.) per milligrams of lyophilized sample.

Antioxidant activity (AA) and the half maximal inhibitory concentration (IC<sub>50</sub>) in phenolic extracts of *Quercus* leaves were calculated to determine the root exudates ability of herbaceous species to influence the microbiota in the plant-pathogen infection. The AA was determined by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (Sigma-Aldrich, Madrid, Spain; [35]). Crude extracts (5 volumes) were mixed with 95 volumes of DPPH 3.35 mM in methanol. In the control tube, the extract volume was replaced by methanol. The mixture was stirred gently and maintained in the dark and at room temperature for 24 h. After incubation, the absorbance was measured at 550 nm. Trolox (Sigma-Aldrich, Madrid, Spain) was used as standard and results were expressed as micromole of Trolox equivalents (T.E.) per milligrams of lyophilized sample. AA was expressed as a percentage inhibition of DPPH radical, and calculated from the equation: Scavenging activity (%) = ((Abs control – Abs sample)/Abs control) × 100. IC<sub>50</sub> values were determined from the plotted graphs as scavenging activity against the concentration of the extracts. These values are defined as inhibitory concentration of the extract necessary to decrease the initial DPPH radical concentration by 50% and they are expressed in micrograms per milliliters.

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

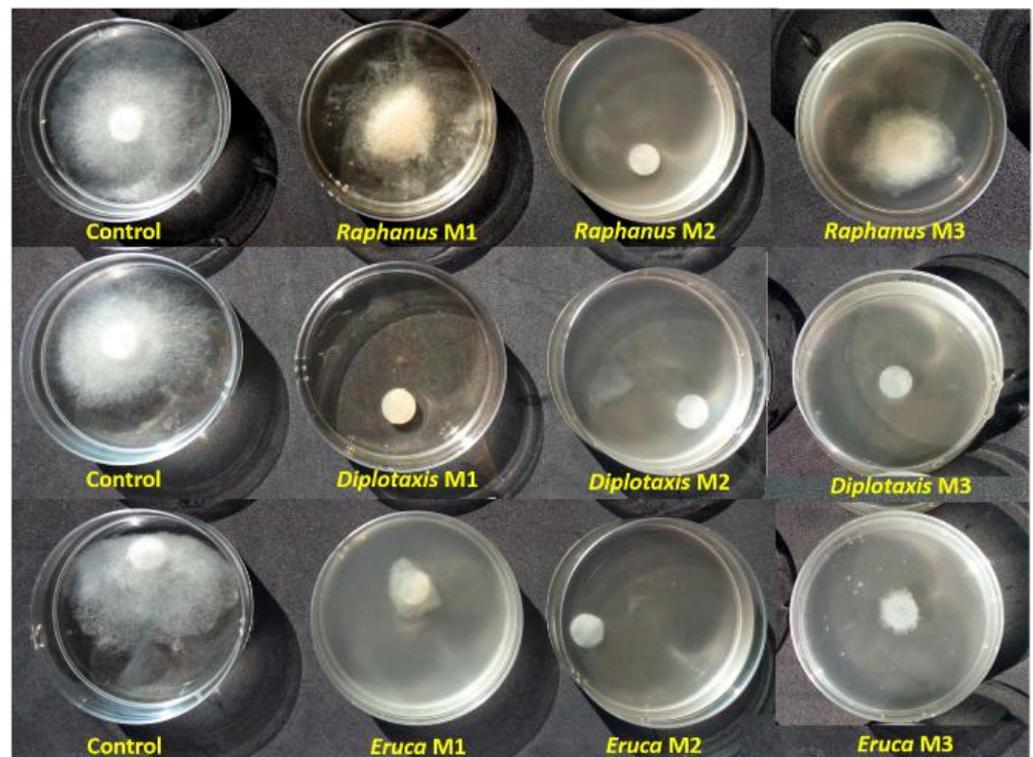
The effect of *Quercus* species (*Q. faginea* and *Q. suber*), herbaceous species (*D. tenuifolia*, *E. vesicaria* and *L. luteus*) and their interactions on *Quercus* chemical defences (total phenols TPC, total tannins TTC and condensed tannins CTC), antioxidant activity (AA and IC<sub>50</sub>) and the major low molecular weight compounds in phenolic extracts (gallic acid GA, vescalagine Vesc., castalagine Cast., Catechin and ellagic acid EA) were analysed through a general linear model (GLM) using Tukey's test ( $p < 0.05$ ) for significant differences of means. *Quercus* and herb species were used as random factors and the TPC, TTC, CTC, AA, IC<sub>50</sub> and the major low molecular weight compounds were used as dependent variables. Data were analysed to check normality (by Kolmogorov-Smirnov test) and homoscedasticity (through Levene's test). Interactions between *Quercus* and herb species

were also included in the model. Statistical analyses were performed using the Statistica v10 software.

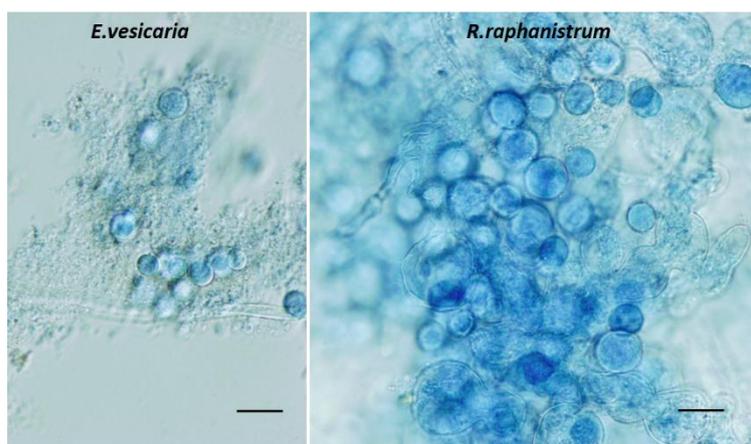
### 3. Results

#### 3.1. In Vitro Assessment of the Allelopathic Effect in the Activity of *Phytophthora cinnamomi*

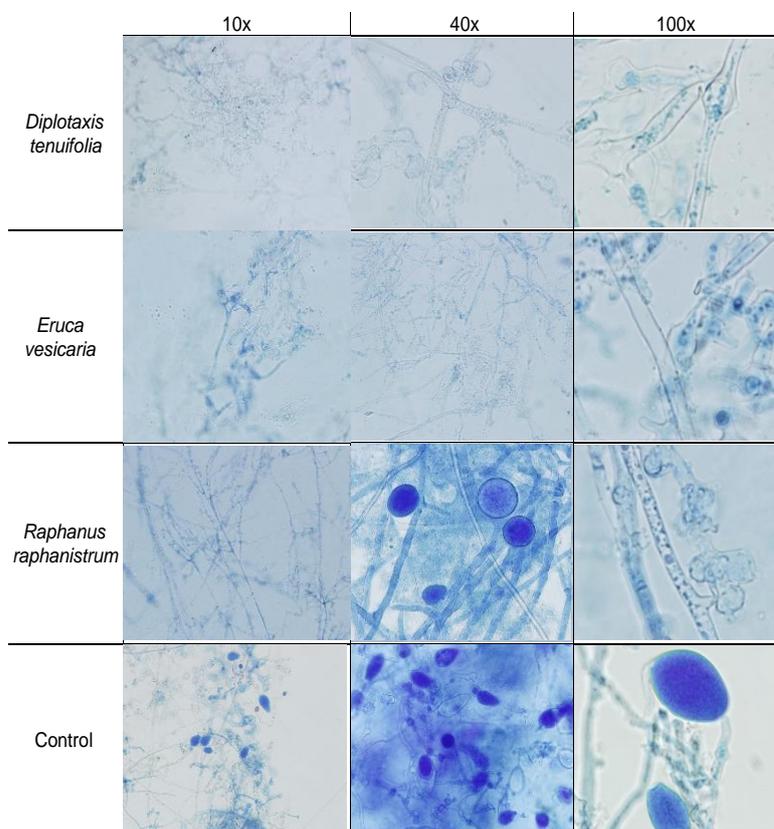
The most effective method of ARE extraction was M2 (ARE extracted by heat inactivation of enzymes) due to its greater inhibition of mycelial growth ( $p < 0.001$  Figures 1–3, Table 1). The species with the highest allelopathic capacity were *D. tenuifolia* and *E. vesicaria* for their greater inhibition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



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



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



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

**Table 1.** Allelopathic effects of AREs on *Phytophthora cinnamomi* structures (percentages are the mean and standard errors of six replicates per method and species).

Allelopathic Species	<i>Diplotaxis tenuifolia</i>			<i>Eruca sativa</i>			<i>Raphanus raphanistrum</i>			
	Method	M1	M2	M3	M1	M2	M3	M1	M2	M3
ARE's pH		5.38			5.59			4.94		
Inhib. (%) mycelial growth		58.0 ± 0.5	67.5 ± 0.2	65.3 ± 0.3	62.4 ± 0.2	64.4 ± 0.2	63.5 ± 0.01	1.1 ± 0.01	55.7 ± 0.01	19.9 ± 0.1
Inhib. (%) zoospore germination		83.7 ± 0.6			62.5 ± 0.3			37.5 ± 1.2		
Inhib. (%) sporangia after 6 days		100			76.9 ± 0.5			30.7 ± 2.5		
Inhib. (%) sporangia after 12 days		100			83.3 ± 0.3			33.3 ± 1.2		
Inhib. (%) chlamydo-spores after 12 days		100			89.6 ± 0.4			44.3 ± 2.7		

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

In presence of *E. vesicaria* and *R. raphanistrum* AREs, released zoospores showed a high and quick immobilization (Figure 2) with encystment, compared to the control, in which zoospores were highly mobile for a long time. This result seems to be a good indicator of a reduction in *P. cinnamomi* activity with AREs of *E. vesicaria* and *R. raphanistrum*. Zoospore encystment in the presence of *D. tenuifolia* could not be reported because ARE from that species completely inhibited sporangia production.

### 3.2. In Vivo Evaluation of the Herbaceous Species Effect on *Quercus* Seedlings in *P. cinnamomi*-Infested Soil

The presence of these herbaceous plants had a significant effect on the production of chemical defences on *Quercus* grown in *P. cinnamomi*-infested soil ( $p < 0.05$ , Table 2). The AA in *Quercus* phenolic extracts was also modified in the presence of these herb species (Table 2). The levels of chemical defences in *P. cinnamomi*-susceptible *Quercus* species decreased especially with *D. tenuifolia* and *E. vesicaria*, and the *Quercus* plants remained alive (Figure 4, Table S3 Supplementary Materials).

Overall, in the three major phenolic groups (total phenol content TPC, total tannin content TTC and condensed tannin content CTC) and for the *Quercus* species tested (3 phenolic groups × 2 *Quercus* species), 3/6 were in accordance with predictions (suppressed chemical response when grown with the two allelopathic species and elevated response when grown with *L. luteus* or no companion plant [control]); 1/6 showed no clear pattern; and 2/6 showed suppression of chemical response with all three species of companion plants.

From the HPLC analysis for the low molecular weight compounds in *Quercus* leaves, five of them were determinant: gallic acid, vesicalagine, castalagine, catechin and ellagic acid. These compounds showed significant differences between treatments (Tukey test  $p < 0.05$ , Table 2 and Table S3 from Supplementary Materials). In these five low molecular weight phenolic compounds assessed for the two *Quercus* species (10 combinations), 1/10 was in accordance with predictions (suppressed chemical response when grown with the two allelopathic species and elevated response when grown with *L. luteus* or no companion plant [control]); 6/10 showed no clear pattern; 2/10 showed suppression of chemical

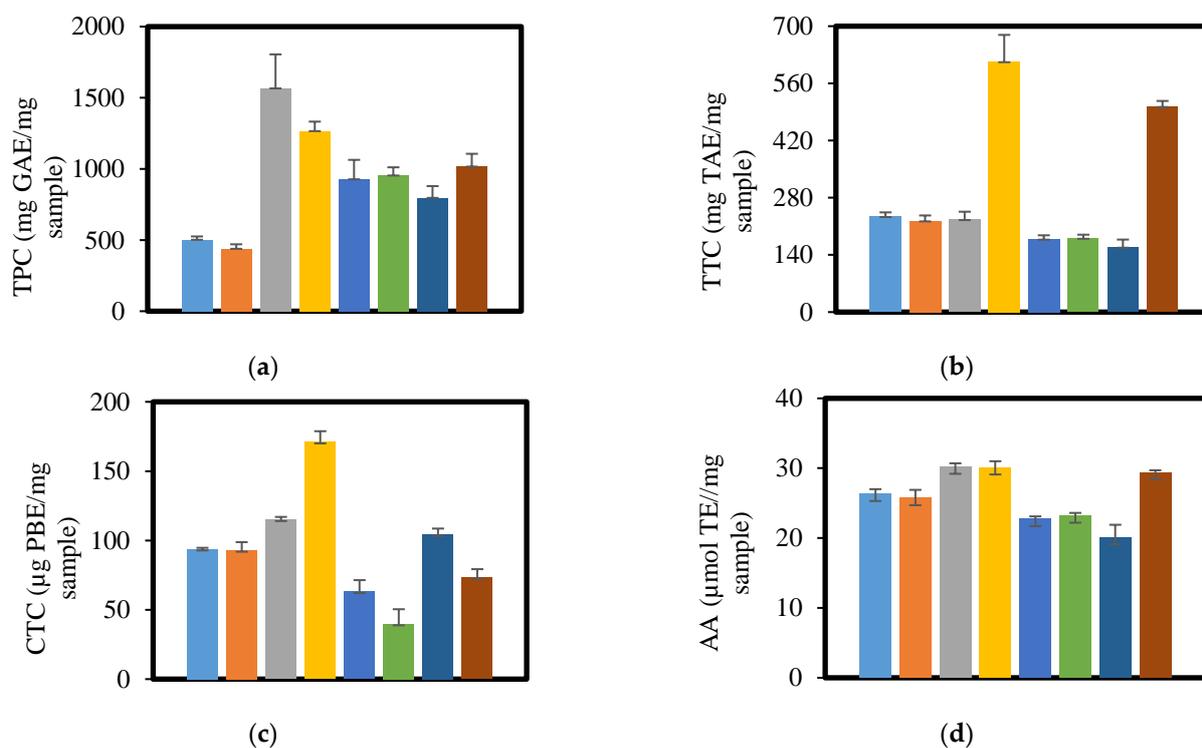
response with all three species of companion plants; and 1/10 showed enhance chemical response with all three species of companion plants.

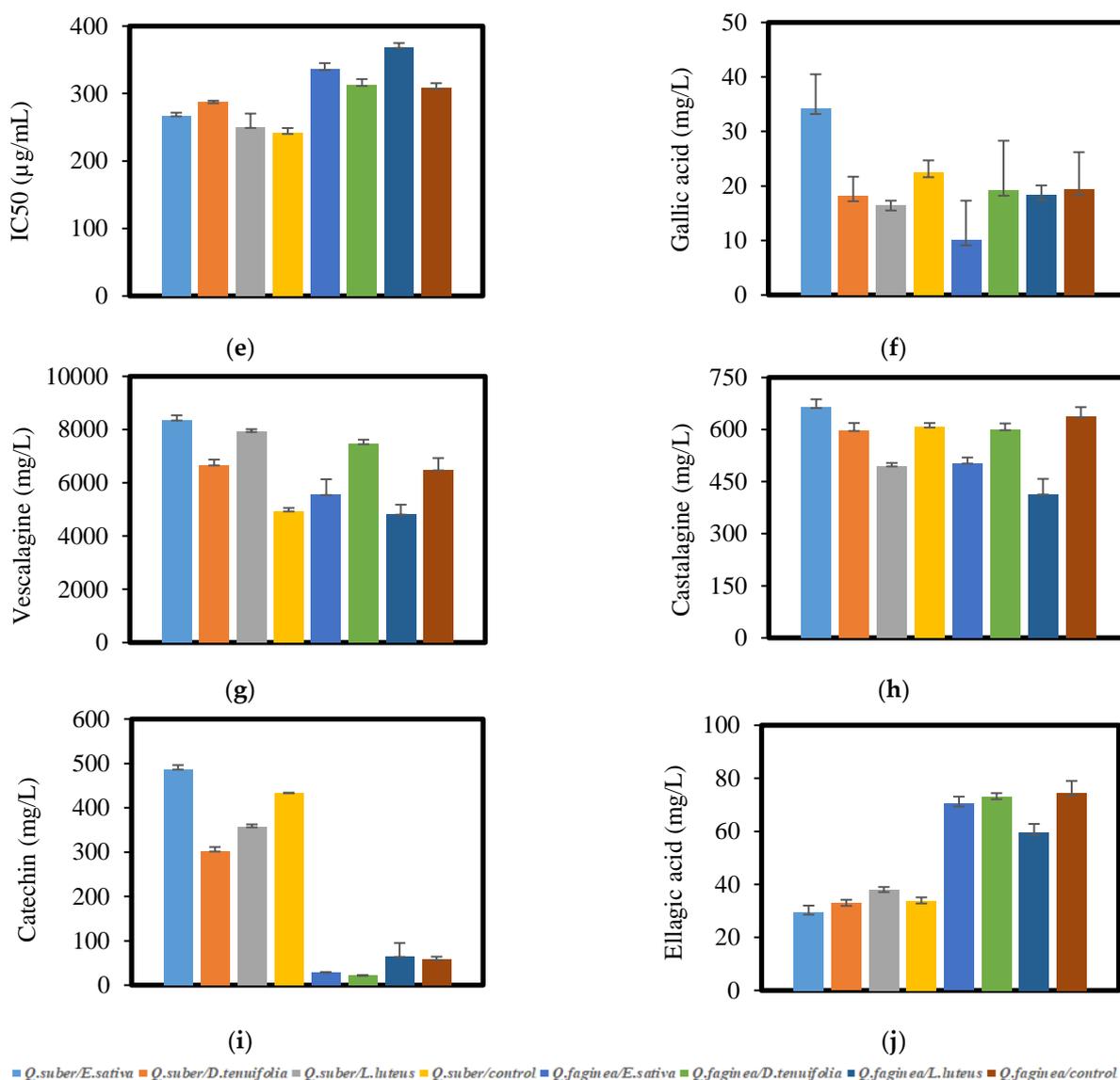
Regarding antioxidant activity (AA) measured in *Quercus* leaves, both the herbaceous and *Quercus* and their interaction showed significant differences ( $p < 0.001$ ). AA was very high in *Q. suber* with *L. luteus*, high in the control, intermediate in *Quercus* grown with the allelopathic herbaceous and very low in the *Q. faginea* with *L. luteus*. The half maximal inhibitory concentration (IC<sub>50</sub>) showed, as might be expected from its ability to measure the effectiveness of a compound's antioxidant capacity, the inverse pattern to AA. The effect of both *Quercus* and herbaceous plants showed significant differences ( $p < 0.001$ ) in the IC<sub>50</sub> (Figure 4, Table S3 Supplementary Materials).

**Table 2.** Effect of *Quercus* species (*Q. faginea* and *Q. suber*), herbaceous species (*D. tenuifolia*, *E. vesicaria* and *L. luteus*) and their interactions on *Quercus* chemical defences (total phenols TPC, total tannins TTC and condensed tannins CTC), antioxidant activity (AA and IC<sub>50</sub>) and the major low molecular weight compounds in phenolic extracts (gallic acid GA, vescalagine Vesc., castalagine Cast., Catechin and ellagic acid EA).

	<i>Quercus</i> Species	Herbaceous Species	<i>Quercus</i> Species * Herbaceous Species
df	1	3	3
TPC	0.53	62.03 *	72.48 *
TTC	309.81 *	1970.47 *	12.82 *
CTC	1467.94 *	433.12 *	220.14 *
AA	238.95 *	77.66 *	64.03 *
IC <sub>50</sub>	963.16 *	46.09 *	92.43
GA	10.31 *	1.20	10.19 *
Vesc.	59.90 *	29.30 *	109.00 *
Cast.	37.76 *	77.96 *	25.43 *
Catechin	6730.80 *	100.48 *	93.07 *
EA	1698.40 *	8.09 *	29.60 *

F-values are shown along with statistical significance \*  $p < 0.05$ .





**Figure 4.** Chemical composition of phenolic extracts from *Quercus* leaves grown with allelopathic root exudates and *Phytophthora cinnamomi* infection. (a) Total phenols (TPC), (b) total tannins (TTC), (c) condensed tannins (CTC), (d) antioxidant activity (AA), (e) the half maximal inhibitory concentration (IC<sub>50</sub>) and the major low molecular weight compounds in phenolic extracts (f) gallic acid, (g) vescalagine, (h) castalagine, (i) catechin and (j) ellagic acid. Means are shown  $\pm$  standard deviation.

## 4. Discussion

### 4.1. Anti-Phytophthora Effects with AREs

AREs prepared by method M2 (maceration in hot water to inactivate myrosinase) showed the strongest activity against *P. cinnamomi*. This result was expected because the method followed had been tested in Sampaio [18] and Moreira et al. [21] with conclusive results.

Regarding the species tested, the ARE of *D. tenuifolia* showed a very high effectiveness, with 67.55% inhibition of mycelial growth and 100% inhibition of sporangia production, in accordance with Moreira et al. [21], although the percentage of inhibition in the aforementioned study was higher (83% for a 75% ARE concentration). Previous studies of *P. cinnamomi* inhibition by *E. vesicaria* [18,19,21] and *R. raphanistrum* [18,21] also confirm their allelopathic effect. *E. vesicaria*, whose main glucosinolates are aliphatic (glucoraphanin, glucosativin and glucoerucin) showed a high inhibition, but not the complete non-viability of its reproduction structures, according with what was discovered by Ríos

et al. [19]. *R. raphanistrum* showed lower inhibition values than the two previous species and very similar to that obtained by Moreira et al. [21] for the mycelial growth of *P. cinnamomi*.

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

#### 4.2. In vivo Evaluation of the Herbaceous Species Effect on *Quercus* Seedlings in *P. cinnamomi*-Infested Soil

The *Quercus* phenolic defences showed significant differences depending on the companion allelopathic herbaceous species with those that grew together in *P. cinnamomi*-infested soil. The allelopathic effect associated with the lowering of chemical defences in *Quercus* suggests a suppression of *P. cinnamomi* levels in the soil. Furthermore, the increase in *Quercus* chemical defences grown with a *P. cinnamomi* highly susceptible species (*L. luteus*) may be due to the stimulation of *P. cinnamomi* levels in the soil.

The lowest TPC and CTC values were reached with *E. vesicaria* and *D. tenuifolia* in both *Quercus*. The CTC were found used in other studies as a good indicator of the *P. cinnamomi* response [14]. In this work, CTC were always higher in *Q. suber* than in *Q. faginea*, which is more tolerant to *P. cinnamomi* [14]. With *L. luteus*, also highly susceptible to the pathogen [36], both *Quercus* species increased their defensive levels against *P. cinnamomi*, although the increase was greater in *Q. suber* (susceptible to *P. cinnamomi*) than in *Q. faginea* (tolerant to *P. cinnamomi*).

There are previous studies on the increase of TPC and CTC in *Q. ilex* infected by *P. cinnamomi* [14,37]. If we consider the chemical defence production in *Quercus* as a response to the attack they are suffering, it is expected that the *Quercus* species studied here also increase their defensive levels. However, in the presence of *E. vesicaria* and *D. tenuifolia*, aforementioned levels decreased with respect to the control (without the presence of herbaceous plants). This suggests that the allelopathic relationship of the herb species with the pathogen reduces the inoculum levels of *P. cinnamomi* also in vivo conditions and therefore, the magnitude of the defensive response in *Quercus* is reduced.

It is known that interactions among plants are frequently controlled by root exudates, some of which have activity against microorganisms [38]. In general, the high inhibitory activity of *D. tenuifolia* and *E. vesicaria* also acted in vivo conditions but we do not know if it was through their root exudates or if it is due to other factors involved in the joint growth of herbaceous plants with *Quercus*, such as competition between them during the first years. However, a diminished chemical response in *Quercus* was suggestive of reduced levels of *P. cinnamomi*. This could be explained by the release of secondary metabolites in allelopathic root exudates, or also by the facilitation of inhibitory soil bacteria against the pathogen. In addition, this study shows that the presence of these allelopathic herbaceous plants growing in *P. cinnamomi*-infested soil reduced the defence level of *Q. suber*, which is susceptible to *P. cinnamomi*. Therefore, these herb species reduce the chemical defence costs in TPC and CTC production against the pathogen's attack. No previous studies are known with these species to establish comparisons, but the complex analysis of the herb-tree-pathogen interactions [39,40] should be continued to better understand the role of each component in that relationship.

As for the low molecular weight phenolic compounds, these were always minimum in *Q. faginea* and maximum in *Q. suber* (except ellagic acid). Ockels et al. [41] studied the differences in the phenolic chemical compounds of phloem tissue of *Q. agrifolia* infected with *P. ramorum* and tested gallic acid in vitro bioassays, finding a strong dose-dependent inhibitory effects against *P. ramorum* and *P. cinnamomi*. Del Río et al. [42] also associated

higher levels of catechin with greater tolerance to the genus *Phytophthora* in olive trees. Higher production of these defences would indicate the response to a higher attack caused by the infection. In the susceptible species (the cork oak) the accompanying allelopathic plant that less increases the levels of defences will be the most effective because it reduces *P. cinnamomi* infection. This happened with *D. tenuifolia*, which confirms its inhibitory capacity against the pathogen observed in vitro conditions. However, ellagic acid showed the opposite pattern, and was higher in *Q. faginea* without allelopathic plants and lower in *Q. suber* with *E. vesicaria*. This compound seems to be directly related to tolerance to *Phytophthora* infection in other *Quercus* species [41,43–45], although its levels in the presence of allelopathic herbaceous plants do not show a clear pattern, which suggests that there are other soil organisms (probably bacteria) also highly conditioned by plant-pathogen interaction. The authors consider the need to have a better knowledge of the surrounding microbiota relationships in order to explain its role.

Regarding AA in *Quercus* leaves, the highest one was found in *Q. suber* seedlings that grew together with *L. luteus* (both species susceptible to *P. cinnamomi*). The lowest AA was that of *Q. faginea* with *L. luteus* (*Quercus* species more tolerant to *P. cinnamomi* than *Q. suber*). Antioxidants protect biological systems against reactions or processes that can produce harmful effects in the individual. In a weakened system, as is the case of the *Q. suber* with *L. luteus* growing in a *P. cinnamomi* infested soil, it is to be expected that there would be more free radicals and therefore, a greater AA would be induced. However, in scenarios with more tolerant to *P. cinnamomi*-species, such as *Q. faginea*, the expected AA would be lower. To strengthen the defensive response to biotic stress, seedlings induce a hypersensitive response that consists of programmed cell death to ensure the plant survival. When stress decreases, AA also drops [46]. In our study, when the *Quercus* species most susceptible to *P. cinnamomi* grew with *D. tenuifolia* and *E. vesicaria*, AA decreased, showing lower stress levels. In *Q. faginea*, this level also decreased in the presence of allelopathic species. Therefore, the presence of these allelopathic species generated a protective effect on *Quercus* seedlings, reducing their stress levels facing the pathogen infection. By its own definition, the higher the AA, the lower the IC<sub>50</sub> and therefore, the more effective the exudate evaluated. However, both the AA and the IC<sub>50</sub> in this test were calculated from the phenolic extract of *Quercus* leaves, so their values are an indirect measure of the effectiveness of the allelopathic herbs. But it would be very interesting to do a chemical characterization of their root exudates to discriminate other effects that could be involved in these plant-pathogen interactions.

The chemical composition of different plant extracts of the *Diplotaxis*, *Eruca* and *Raphanus* genera has been extensively studied in nutrition for its antioxidant and bactericidal properties [47–49]. Rarer is the chemical characterization of these species for biological control against plant pathogens. However, *Brassicaceae* family has been specially studied for its glucosinolate content for biofumigation. This is a widely used biological control tool against several pathogens. Ríos et al. [19] screened various brassicaceous plants (*D. tenuifolia* was not evaluated) to identify the most suitable and the compounds responsible for the inhibitory allelochemical activity of *P. cinnamomi*. They demonstrated the biocidal action on *P. cinnamomi* of rich in sinigrin-species, such as *B. juncea* and *B. carinata* (see also [50–52]), while in others glucosinolates different from sinigrin (such as *E. vesicaria*) only a fungistatic effect was obtained. However, later in vivo tests to control the disease in cork oak with *B. carinata* pellets proved their significant effectiveness only when combined with calcium carbonate application [22]. In fact, all the authors agree on an integrated fight against the oak decline. Furthermore, the application of *Brassicaceae* uses high amounts of biomass and is not feasible in some lands due to orographic limitations. The novel approach of this work proposes the enrichment of pastures with allelopathic species against *P. cinnamomi*. The inoculum would not be eradicated from the soil, but the disease spread could be dimmed, increasing the tolerance of several species in Iberian wood pastures. The incorporation in the field of native plants capable of reducing the *P. cinnamomi* infec-

tive activity through their natural root exudates shows high potential in the possible control of the pathogenic activity with a sustainable management in these agrosilvopastoral systems. Furthermore, the use of allelopathic plants has the advantage that they usually contain more than one antipathogenic compound, which hinders the development of resistance of pathogens [40]. However, it should still be deepened in several issues such as the knowledge of these relationships, their action and release in the field, the most recommended doses to avoid toxicity in other plants and whether other surrounding microorganisms such as bacteria are involved in them.

## 5. Conclusions

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

**Supplementary Materials:** The following are available online at [www.mdpi.com/1999-4907/12/3/285/s1](http://www.mdpi.com/1999-4907/12/3/285/s1): Table S1. Effect of AREs species (*D. tenuifolia*- D, *E. vesicaria*- E and *R. raphanistrum*- R), sources (field/greenhouse) and their interactions on chemical defences (total phenols Tp, total tannins Tt and condensed tannins Ct) and antioxidant activity (AA and IC<sub>50</sub>). Table S2. Chemical composition of aqueous root extracts according to species and source. Total phenols (Tp), total tannins (Tt), condensed tannins (Ct), antioxidant activity (AA) and the half maximal inhibitory concentration (IC<sub>50</sub>). Means and standard deviation. Table S3. Chemical composition of phenolic extracts from *Quercus* leaves grown with allelopathic root exudates and *Phytophthora cinnamomi* infection. Total phenols (Tp), total tannins (Tt), condensed tannins (Ct), antioxidant activity (AA), the half maximal inhibitory concentration (IC<sub>50</sub>) and the major low molecular weight compounds in phenolic extracts (gallic acid GA, vescalagine Vesc., castalagine Cast., Catechin and ellagic acid EA). Means are shown ± standard deviation.

**Author Contributions:** Conceived, designed and performed the experiments: M.R.-R., B.G.-C., I.M.C., J.A.P. and A.C.M. Analysed the data: M.R.-R., B.G.-C. and A.C.M. Contributed reagents/materials/analysis tools and wrote the paper: all authors. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work has been partially funded by the “Cross-border cooperation project for the integral assessment of dehesa and montado PRODEHESA-MONTADO”, a project financed by the European Regional Development Fund (ERDF) through the INTERREG V-A Spain-Portugal Program (POCTEP) 2014-2020; and by the Spanish National Institute for Agriculture and Food Research and Technology (INIA)/CICYTEX funds through the grant FPI-INIA to Manuela Rodríguez.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

**Acknowledgments:** Authors would like to thank the ICMC, INIAV and the staff support from the Project “Declínio do Montado no Alentejo” (PDR2020-101-031496), and specially Fernando Pulido, from the UEX, for the technical assistance received and for the critical review of this article.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

- Molisch, H. Der Einfluss einer Pflanze auf die andere Allelopathie. *Nature* **1937**, *141*, doi:10.1038/141493a0.
- Rice, E.L. *Allelopathy*, 2nd ed.; Academic Press: New York, NY, USA, 1984.
- Farooq, M.; Jabran, K.; Cheema, Z.A.; Wahid, A.; Siddique, K.H. The role of allelopathy in agricultural pest management. *Pest Manag. Sci.* **2011**, *67*, 493–506.
- Ferguson, J.J.; Rathinasabapathi, B.; Chase, C.A. *Allelopathy: How Plants Suppress Other Plants*; University of Florida: Gainesville, FL, USA, 2013; pp. 1–5. Available online: <http://edis.ifas.ufl.edu/hs186> (accessed on 25 February 2021).
- Rizvi, S.J.H.; Haque, H.; Singh, U.K.; Rizvi, V. A discipline called allelopathy. In *Allelopathy: Basic and Applied Aspects*; Rizvi, S.J.H., Rizvi, H., Eds.; Chapman & Hall: London, UK, 1992; pp.1–10.
- Singh, H.; Batish, D.; Hohli, R. *Allelopathy in Agroecosystems: An Overview*; The Haworth Press, Inc.: Pennsylvania, PA, USA, 2001; pp. 1–42.
- Brasier, C.M. Oak tree mortality in Iberia. *Nature* **1992**, *360*, 359.
- Hardham, A.R.; Blackman, L.M. *Phytophthora cinnamomi*. *Mol. Plant Pathol.* **2018**, *19*, 260–285.
- Dunstan, W.A.; Rudman, T.; Shearer, B.L.; Moore, N.A.; Paap, T.; Calver, M.C.; Dell, B.; Hardy, G.E.S.J. Containment and spot eradication of a highly destructive, invasive plant pathogen (*Phytophthora cinnamomi*) in natural ecosystems. *Biological Invasions* **2010**, *12*, 913–925.
- Rodríguez-Molina, M.C.; Torres-Vila, L.M.; Blanco-Santos, A.; Nunez, E.J.P.; Torres-Álvarez, E. Viability of holm and cork oak seedlings from acorns sown in soils naturally infected with *Phytophthora cinnamomi*. *For. Pathol.* **2002**, *32*, 365–372.
- Sánchez, M.E.; Andicoberry, S.; Trapero, A. Pathogenicity of three *Phytophthora* spp. causing late seedling rot of *Quercus ilex* ssp. *ballota*. *For. Pathol.* **2005**, *35*, 327–334.
- Pérez-Sierra, A.; López-García, C.; Leon, M.; García-Jiménez, J.; Abad-Campos, P.; Jung, T. Previously unrecorded low-temperature *Phytophthora* species associated with *Quercus* decline in a Mediterranean forest in Eastern Spain. *For. Pathol.* **2013**, *43*, 331–339.
- Solla, A.; Milanovic, S.; Gallardo, A.; Bueno, A.; Corcobado, T.; Cáceres, Y.; Morcuende, D.; Quesada, A.; Moreno, G.; Pulido, F.J. Genetic determination of tannins and herbivore resistance in *Quercus ilex*. *Tree Genet. Genomes* **2016**, *12*, 117.
- Gallardo, A.; Morcuende, D.; Solla, A.; Moreno, G.; Pulido, F.J.; Quesada, A. Regulation by biotic stress of tannins biosynthesis in *Quercus ilex*: Crosstalk between defoliation and *Phytophthora cinnamomi* infection. *Physiol. Plant* **2019**, *165*, 319–329.
- John, J.; Sarada, S. Role of phenolics in allelopathic interactions. *Allelopath. J.* **2012**, *29*, 215–230.
- FAO. *Protecting Plantations from Pests and Diseases*; Report based on the work of W.M. Ciesla, Forest Plantation Thematic Papers, Working Paper 10; Forest Resources Development Service, Forest Resources Division, FAO: Rome, Italy, 2001. Available online: <http://www.fao.org/forestry> (accessed on 25 February 2021).
- Kong, C.H.; Xuan, T.D.; Khanh, T.D.; Tran, H.D.; Trung, N.T. Allelochemicals and signaling chemicals in plants. *Molecules* **2019**, *24*, 27–37.
- Sampaio, A.R. *Seleção de plantas com efeito alelopático para controlar Phytophthora cinnamomi Rands*. Dissertação para obtenção do grau de Mestre em Engenharia Agronómica-Proteção de Plantas; School of Agriculture, University of Lisbon: Lisbon, Portugal, 2017; p. 71.
- Ríos, P.; Obregón, S.; González, M.; de Haro, A.; Sánchez, M.E. Screening brassicaceous plants as biofumigants for management of *Phytophthora cinnamomi* oak disease. *For. Path.* **2016**, *46*, 652–659.
- Ríos, P.; Obregón, S.; Haro, A.; Fernández-Rebollo, P.; Serrano, M.; Sánchez, M.E. Effect of *Brassica* biofumigant amendments on different stages of the life cycle of *Phytophthora cinnamomi*. *J. Phytopathol.* **2016**, *164*, 582–594.
- Moreira, A.; Calha, I.; Passarinho, J.; Sampaio, A. Anti-*Phytophthora* activity of root extracts from herbaceous species. *Revista das Ciências Agrárias* **2018**, *41*, 39–47.
- Rodríguez-Molina, M.C.; Serrano-Pérez, P.; Santiago, R.; Cardillo, E.; Godoy, B.; De Santiago, A. Efecto de la biofumigación con pellets de *Brassica carinata* en la progresión de la enfermedad causada por *Phytophthora cinnamomi* en *Lupinus luteus*. In *Libro de Resúmenes XIX Congreso de la Sociedad Española de Fitopatología*; SEF: Toledo, Spain, 2018; p. 124.
- Fenwick, R.G.; Heaney, R.K.; Mullin, W.J. Glucosinolates and their breakdown products in food plants. *CRC Crit. Rev. Food Sci. Nutr.* **1983**, *18*, 123–201.
- Dunne, C. Control of sudden death in cultivated proteas from the South west of Western Australia. Ph.D. Thesis, Murdoch University, Perth, Australia, 2004.
- Bialy, Z.; Oleszek, W.; Lewis, J.; Fenwick, G.R. Allelopathic potential of glucosinolates (mustard oil glycosides) and their degradation products against wheat. *Plant Soil* **1990**, *129*, 277–281.
- Wolf, R.B.; Spencer, G.F.; Kwolek, W.F. Inhibition of velvet leaf (*Abutilon theophrasti*) germination and growth by benzyl isothiocyanate, a natural toxicant. *Weed Sci.* **1984**, *32*, 612–615.
- Fahey, J.W.; Zalcmann, A.T.; Talalay, P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* **2001**, *56*, 5–51.
- Moreira-Marcelino, A.C. *Aspectos da interação entre Phytophthora cinnamomi e a doença do declínio em Q. suber e Q. rotundifolia*; Dissertação para Prestação de Provas de Doutorado, Faculdade de Engenharia de Recursos Naturais, Universidade do Algarve: Faro, Portugal, 2001; p. 279.
- Hongju, H. Studies for growth adaptation and identification of glucosinolates in various Chinese *Brassicacae*. Ph.D. Thesis, Herbert Utz Verlag-Wissenschaft, München, Germany, 1999.

30. Alkhail, A. Antifungal activity of some extracts against some plant pathogenic fungi. *Pak. J. Biol. Sci.* **2005**, *8*, 413–417.
31. Jeffers, S.; Martin, S. Comparison of two media selective for *Phytophthora* and *Phythium* species. *Plant Dis.* **1986**, *70*, 1038–1043.
32. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdc-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.
33. Schanderl, S.H. *Method in Food Analysis*; Academic Press: New York, NY, USA, 1970, 709 p.
34. Porter, L.J.; Hirstich, L.N.; Chan, B.G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **1986**, *25*, 223–230.
35. Thaipong, K.; Boonprakob, U.; Crosby, K.; Cisneros-Zevallos, L.; Byrne, D.H. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J. Food Compos. Anal.* **2006**, *19*, 669–675.
36. Serrano, M.; Fernández-Rebollo, P.; De Vita, P.; Carbonero, M.; Sánchez, M.E. The role of yellow lupin (*Lupinus luteus*) in the decline affecting oak agroforestry ecosystems. *For. Pathol.* **2011**, *4*, 382–386.
37. Rodríguez-Romero, M.; Gallardo, A.; Pulido, F. Geographical and within-population variation of constitutive chemical defences in a Mediterranean oak (*Quercus ilex*). *For. Syst.* **2020**, *29*, e011.
38. Bais, H.P.; Weir, T.L.; Perry, L.G.; Gilroy, S.; Vivanco, J.M. The role of root exudates in rhizosphere interactions with plants and other organisms. *Ann. Rev. Plant Biol.* **2006**, *57*, 233–266.
39. Cazorla, F.M.; Mercado-Blanco, J. Biological control of tree and woody plant diseases: An impossible task? *BioControl* **2016**, *61*, 233–242.
40. Shuping, D.S.S.; Eloff, J.N. The use of plants to protect plants and food against fungal pathogens: A review. *Afr. J. Tradit. Complement. Altern. Med.* **2017**, *14*, 120–127.
41. Ockels, F.S.; Eyles, A.; McPherson, B.A.; Wood, D.L.; Bonello, P. Phenolic chemistry of coast live oak response to *Phytophthora ramorum* infection. *J. Chem. Ecol.* **2007**, *33*, 1721–32.
42. Del Río, J.A.; Baidez, A.G.; Botia, J.M.; Ortuno, A. Enhancement of phenolic compounds in olive plants (*Olea europaea* L.) and their influence on resistance against *Phytophthora* sp. *Food Chem.* **2003**, *83*, 75–78.
43. Conrad, A.O.; Rodríguez-Saona, L.E.; McPherson, B.A.; Wood, D.L.; Bonello, P. Identification of *Quercus agrifolia* (coast live oak) resistant to the invasive pathogen *Phytophthora ramorum* in native stands using Fourier-transform infrared (FT-IR) spectroscopy. *Front. Plant Sci.* **2014**, *5*, 521.
44. Nagle, A.M.; McPherson, B.A.; Wood, D.L.; Garbelotto, M.; Bonello, P. Relationship between field resistance to *Phytophthora ramorum* and constitutive phenolic chemistry of coast live oak. *For. Pathol.* **2011**, *41*, 464–469.
45. McPherson, B.A.; Mori, S.R.; Opiyo, S.O.; Conrad, A.O.; Wood, D.L.; Bonello, P. Association between resistance to an introduced invasive pathogen and phenolic compounds that may serve as biomarkers in native oaks. *For. Ecol. Manag.* **2014**, *312*, 154–160.
46. Irfan, M.; Hayat, S.; Bashir, A. Regulation of antioxidant system under biotic stress in plants. In *Plant Under Stress*; Stadium Press: New Delhi, India, 2011.
47. Mateu, G.; Dimitrova, P.; Petkova, N.; Ivanov, I.; Mihaylova, D. Antioxidant activity and mineral content of rocket (*Eruca sativa*) plant from Italian and Bulgarian origins. *J. Microbiol. Biotech. Food Sci.* **2018**, *8*, 756–759.
48. Nicoletti, R.; Raimo, F.; Miccio, G. *Diplotaxis tenuifolia*: Biology, production and properties. *EJPSB* **2007**, *1*, 36–43.
49. D’Antuono, L.F.; Elementi, S.; Neri, R. Glucosinolates in *Diplotaxis* and *Eruca* leaves: Diversity, taxonomic relations and applied aspects. *Phytochemistry* **2008**, *69*, 187–99.
50. Ríos, P.; González, M.; Obregón, S.; Carbonero, M.D.; Leal, J.R.; Fernández, P.; De Haro, A.; Sánchez, M.E. Brassica-based seed-meal biofumigation to control *Phytophthora cinnamomi* in the Spanish “dehesa” oak trees. *Phytopathologia Mediterranea* **2017**, *56*, 392–399.
51. Fernández-Rebollo, P.; Carbonero, M.D.; Leal, J.R.; García, A.M.; Ríos, P.; Sánchez, M.E.; Obregón, S.; de Haro, A. Producción, concentración y perfil de glucosinolatos de *Brassica carinata* y *Brassica juncea* cultivadas en la dehesa. In *Pastos y Forrajes en el Siglo XXI*; Cifre, J., Janer, I., Gulias, J., Jaume, J., Medrano, M., Eds.; SEEP: Palma de Mallorca, Spain, 2015; pp. 193–200.
52. Morales-Rodríguez, C.; Vettraino, A.M.; Vannini, A. Efficacy of biofumigation with *Brassica carinata* comercial pellets (BioFence) to control vegetative and reproductive structures of *Phytophthora cinnamomi*. *Plant Dis.* **2016**, *100*, 324–330.