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Coexistent Mediterranean woody species as a driving factor of *Phytophthora cinnamomi* infectivity and survival

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Abstract

The long-term conservation of Mediterranean mixed oak forests is seriously threatened by the massive mortality of *Quercus suber* caused by the exotic pathogen *Phytophthora cinnamomi*. This species frequently grows in mixed forests under natural conditions, but nothing is known about how its level of disease might be altered by the diversity and identity of coexisting neighbours varying in susceptibility to the exotic pathogen. Here we analysed the individual and combined effects of *Q. suber* and the main coexisting tree species (*Quercus canariensis* and *Olea europaea* subsp. *europaea* var. *sylvestris*) in mixed forests of southern Spain on the production of infective and survival spores of *P. cinnamomi*. Through in vitro experiments, it was demonstrated that mixtures of *Q. suber* and *Q. canariensis* highly stimulated the production of *P. cinnamomi* zoospores in comparison with both species in monocultures. *Olea europaea* did not stimulate zoospore production. Under controlled conditions, the initial and final densities of inoculum in soil planted with monocultures of *O. europaea* and *Q. canariensis* did not differ. However, inoculum densities significantly decreased along the experiment in *Q. suber* mixtures with *O. europaea* and *Q. canariensis*. *Phytophthora cinnamomi* was able to infect and cause root rot symptoms on all tree species, including *O. europaea* var. *sylvestris*. We concluded that mixed stands of *Q. suber* and *Q. canariensis* are able to stimulate *P. cinnamomi* infectivity and survival much more than monospecific stands, and consequently under favourable conditions for root disease development, the coexistence of *Q. suber* and *Q. canariensis* might exacerbate Mediterranean forests decline. This study also constitutes the first report of *O. europaea* var. *sylvestris* as host and inductor of *P. cinnamomi* sporulation under controlled conditions.

KEYWORDS

Olea europaea subsp. *europaea* var. *sylvestris*, *Phytophthora* interactions, *Quercus canariensis*, *Quercus suber*, soil pathogens

1 | INTRODUCTION

Exotic pathogen invasions are among the main causes of biodiversity impoverishment worldwide (Desprez-Loustau et al., 2007; Fisher et al., 2012; Garbelotto & Pautasso, 2012; Santini & Battisti, 2019). Several of these pathogens are well-known for their severe economic and ecological impacts, as for instance *Cryphonectria parasitica* (Murrill) Barr, the causal agent of the chestnut blight which nearly wiped out *Castanea* spp. in Europe, North America, Asia and Africa (Rigling & Prospero, 2018; Robin & Heiniger, 2001), or the Dutch elm disease caused by *Ophiostoma ulmi* (Buism.) and *O. novo-ulmi* (Brasier) which nearly destroyed the elm trees from Europe,

Asia and North America during the last century (Brasier, 1991; Santini & Battisti, 2019).

Mediterranean ecosystems are especially vulnerable to invasions by forest pathogens (Garbelotto, 2008; Garbelotto & Pautasso, 2012). In this way, the invasion of the soilborne pathogen *Phytophthora cinnamomi* Rands is threatening the persistence of oak forests and open-woodland ecosystems in the Mediterranean Basin (Brasier, 1996; Frisullo et al., 2018; Romero et al., 2007; Sánchez, Caetano, Ferraz, & Trapero, 2002; Scanu et al., 2013), as well as the native Banksia forests in Australia (Shearer, Crane, Cochrane, & Dunne, 2013) and the endemic oak forests in the Western United States (Garbelotto, Hüberli, & Shaw, 2006). *P. cinnamomi* is one of the most devastating plant pathogens on earth, listed among the 100 World's Worst Invasive Alien Species (Lowe, Browne, Boudjelas, & De Poorter, 2000). This soilborne oomycete causes collar and root rot on a broad range of susceptible plants (Erwin & Ribeiro, 1996; Hardham, 2005).

Numerous studies have focused on determining individual plant reactions against *P. cinnamomi*, from herbaceous species (Kueh et al., 2012; Serrano, Ferández-Rebollo, De Vita, & Sánchez, 2012) to woody plants such as *Quercus* spp. (Robin, Desprez-Loustau, Capron, & Delatour, 1998; Serrano et al., 2012) or *Eucalyptus* spp. (Shearer & Dillon, 1995; Simamora, Stukely, Barber, Hardy, & Burgess, 2017). However, species vulnerable to *P. cinnamomi* frequently grow in mixtures under natural conditions. Therefore, it is plausible to expect that the dynamics of the disease on a specific target species might be altered by the diversity and identity of their neighbours, and more specifically by their susceptibility, tolerance or resistance to the exotic pathogen. For instance, Jayasekera et al. (2017) reported the presence of selfed oospores of *P. cinnamomi* in roots of *Lupinus angustifolius* L. under the influence of *Acacia pulchella* R.Br., an Australian endemic plant resistant to this pathogen (Cahill, Legge, Grant, & Weste, 1989), while none were produced when *Acacia* was absent (Jayasekera, McComb, Shearer, & Hardy, 2007). The coexistence of both species, which are common in Australian jarrah forests, modified *P. cinnamomi* heterothallism. In a similar way, *Lupinus luteus* L., a common legume planted in the oak rangeland ecosystems of the Iberian Peninsula, significantly stimulates the viability of *P. cinnamomi* chlamydospores and zoospore production, increasing oak root infection and disease development (Serrano, Ferández-Rebollo, et al., 2012). Despite the relevance of understanding disease dynamics caused by *P. cinnamomi* in diverse plant communities, to our knowledge there are no studies that have explored the impacts of different tree species mixtures on the infectivity and survival of the pathogen.

In this study, we aim to explore how the life cycle of *P. cinnamomi* might be affected by the composition of the tree community in mixed Mediterranean forests dominated by *Quercus suber* L. We focused our study in forests of the southern Iberian Peninsula, where *Q. suber* is seriously affected by *P. cinnamomi* root disease (Romero et al., 2007). In the wetter areas, *Q. suber* coexist with the deciduous and shade-tolerant *Quercus canariensis* Willd., whereas in the drier areas *Q. suber* coexist with the evergreen and shade-intolerant *Olea europaea* subsp. *europaea* var. *sylvestris* L. (Gomez-Aparicio et al., 2012). Whereas *Q. suber* has been described as highly susceptible to the pathogen (Brasier, 1992; Robin et al., 1998; Robin, Capron, & Desprez-Loustau, 2001), *Q. canariensis* is considered moderately susceptible (Moralejo, García-Muñoz, & Descals, 2009), while no evidences of *P. cinnamomi* infection have been found for *O. europaea* by log inoculations (Moralejo et al., 2009). Specifically, we aimed to determine: (a) the individual effects of *Q. suber*, *Q. canariensis* and *O. europaea* subsp. *europaea* var. *sylvestris* on *P. cinnamomi* zoospore production; (b) the interactive effects of coexistent species (*Q. suber*—*Q. canariensis* and *Q. suber*—*O. europaea* subsp. *europaea* var. *sylvestris*) on *P. cinnamomi* infectivity ability and (c) the viable inoculum dynamics of *P. cinnamomi* in soil colonised by roots of each tree species in monoculture or in mixtures. We hypothesized that (a) there would be a significant variation in the magnitude of zoospore stimulation depending on the host susceptibility to *P. cinnamomi*; (b) mixtures of *Q. suber* with less susceptible or resistant hosts (like *Q. canariensis* and *O. europaea*, respectively) would stimulate zoospore production to a lower extent than *Q. suber* monocultures; (c) the dynamics of viable inoculum of *P. cinnamomi* in the soil will vary depending on host susceptibility. It is expected that the density of viable chlamydospores in soil planted with susceptible hosts would rapidly decrease because of spore germination to infect roots, to later increase as a result of infected root tissue degradation. However, a slight reduction without further increase on viable inoculum would be expected in soil planted with resistant hosts and without plants and (d) the dynamics of viable inoculum in soil planted with species mixtures would be led by the most susceptible host, showing a similar dynamics than the most susceptible species in monoculture.

2 | MATERIALS AND METHODS

2.1 | Plant material

The experiments were conducted with seedlings of three tree species: *Q. suber* (Qs), *Q. canariensis* (Qc) and *O. europaea* subsp. *europaea* var. *sylvestris* (Oe). *Quercus* seedlings were obtained from acorns collected in Los Alcornocales Natural Park (Cádiz, southern Spain) in December 2017. Acorns were stored in humid substrate at 4°C until sowing. Before sowing, healthy acorns of the two *Quercus* species were selected by flotation (Gribko & Jones, 1995) and surface-sterilised with a 10% sodium hypochlorite solution. *O. europaea* var. *sylvestris* seedlings were obtained from commercial seeds from Semillas Silvestres S.L. To improve germination, seeds of *O. europaea* var. *sylvestris* were subjected to mechanical scarification and soaked in water for 2 days previous to sowing (Costa-Pérez & Sánchez-Landa, 2001). Acorns and *O. europaea* var. *sylvestris* seeds were potted in quick-pot

containers (trays of 27 cm × 53 cm × 15 cm, with 32 containers of 300 cm^3 each) filled with wet vermiculite. Trays were incubated in the greenhouse and watered as required from December 2017 to February 2018 for in vitro experiments and to June 2018 for *in planta* experiments.

2.2 | Oomycete material

In all the experiments, an isolate of *P. cinnamomi* with A2 mating type (PE90) obtained from roots of Holm oak was used (Sánchez, Andicoberry, & Trapero, 2005). It was stored in paraffin-tubes at the mycological collection of the Agronomy Department of the University of Córdoba (Spain). Before starting the experiments, the isolate was passaged through Granny Smith apples to ensure it had not lost its pathogenicity because of long-term storage (Erwin & Ribeiro, 1996), and then re-isolated on NARPH (Corn Meal Agar amended with nystatin, ampicillin, rifampicin, ntachloronitrobenzene, and hymexazol) (Hüberli, Tommerup, & Hardy, 2000) for use in later inoculations.

2.3 | Effect of tree species on *P. cinnamomi* zoospore production

In vitro experiments were conducted to determine the influence of the three tree species to stimulate sporangia germination and zoospore release of *P. cinnamomi*, following the methodology described by Serrano, Ferández-Rebollo, et al. (2012). Six millimetre diameter agar plugs from the margin of *P. cinnamomi* colonies growing in CA medium (carrot 20%-Agar, Dhingra & Sinclair, 1995) for 3 days at 22°C, were plated in the center of sterilised glass beakers (330 ml) (one plug per beaker) containing 35 ml of PA medium (Pea 20%-Agar, Trione, 1974). The beakers were incubated for 4 days at 22°C in the dark. At that time, 125 ml of sterilised mineral saline solution (MSS, Chen & Zentmyer, 1970) were poured into each beaker to stimulate sporangial production. Two 2-months-old seedlings of the different tree species were held with 40-mm-thick sterilised polyurethane plugs acting as lids for the beakers for immersing of the root into the saline solution. Each beaker contained seedlings of *Q. suber* (Qs), *Q. canariensis* (Qc) or *O. europaea* (Oe) added in monoculture (Qs-Qs, Oe-Oe and Qc-Qc) or in mixed pairs (Qs-Oe and Qs-Qc). The combination Oe-Qc was not included since these two species do not usually coexist under natural conditions. Two seedlings of *O. europaea* were included per each seedling of *Q. suber* or *Q. canariensis* because of its smaller root system. Three beakers were prepared per tree species combination, whereas three additional beakers without plants were used as controls. There were in total six plant treatments (three monocultures, two mixtures and a control) and 18 beakers. All beakers were incubated in the dark at 22°C for 55 hr to sporangia germination and zoospore release (Serrano, Ferández-Rebollo, et al., 2012). After the incubation period, three 10-ml aliquots (subsamples) were taken out from each beaker and strongly shaken to promote zoospore encystment. The production of zoospores was counted in a Neubauer chamber (0.1 µl of MSS solution). Three counts were performed per aliquot, which made a total of nine counts per beaker.

2.4 | Effect of tree species on the density of viable *P. cinnamomi* chlamydospores and root rot development

The influence of the tree species on the number of viable *P. cinnamomi* chlamydospores in the substrate was evaluated following the methodology described by Serrano, Ferández-Rebollo, et al. (2012). Firstly, substrate (peat, AgroPicazo) was infested by adding an aqueous suspension of chlamydospores of *P. cinnamomi* (2.9×10^4 chlamydospores × mL^{-1}), which corresponded to around 200 viable chlamydospores × g^{-1} of dry soil (Serrano, Ríos, González, & Sánchez, 2015). One week after peat infestation, 6-months old seedlings of *Q. suber* (Qs), *Q. canariensis* (Qc) and *O. europaea* var. *sylvestris* (Oe) were planted in pots containing 2 L of infested peat. In each pot, two seedlings were planted in pairs both in monoculture (Qs-Qs, Oe-Oe and Qc-Qc) and in mixtures (Qs-Oe and Qs-Qc). To balance root volume among species, three *O. europaea* var. *sylvestris* seedlings were potted per each *Q. suber* and *Q. canariensis* seedling. Four replicates (pots) were prepared per tree species combination, plus four additional pots containing infested peat without plants acting as controls, making a total of six plant treatments (three monocultures, two mixtures and a control). All pots were placed in plastic trays without drainage (57 cm × 41 cm × 9 cm) to avoid cross contamination. These pots were incubated in a greenhouse with 12 h light/day at 18–24°C. One week after plants were potted in infested peat, every pot including the controls was partially filled with tap water as described in Serrano, Ferández-Rebollo, et al. (2012), maintaining substrate flooding for 2 days per week until the end of the experiment, 11 weeks later. The saturated water content (θ_s) was calculated as the average difference between the weight at the time of watering (maximum value) and the weight obtained just before the next watering (minimum value) for each pot, expressed as percentage.

At weeks 4 and 12, one peat sample (50 ml vol.) was taken from the rhizosphere of each pot using a sterile tube. Peat samples were air dried separately at room temperature, sieved (2 mm pore size) and processed as described in Serrano, Ferández-Rebollo, et al. (2012). Ten g of homogenised dry substrate was suspended in 100 ml sterilised water-agar (0.2%) and shaken. One millilitre aliquots were taken from the substrate-water-agar mix and plated onto Petri dished containing NARPH *Phytophthora* selective medium (Hüberli et al., 2000), using a sterile glass spreader to distribute the material over the agar surface. For each substrate sample, a total of

20 Petri dishes were prepared. Dishes were incubated at 22°C in the dark for 24 hr, and then the agar surface was washed with sterile water, removing the soil-water-agar mix. Dishes were incubated at 22°C in the dark for another 48 hr and the growing colonies morphologically identified as *P. cinnamomi* were counted. Inoculum density was expressed as colony forming units per g of dry soil (cfu × g^{-1}).

At the end of the experiment, severity of root symptoms was assessed for each plant on a 0–4 scale according to the percentage of root necrosis (0 = 0%, 1 = 1–33%, 2 = 34–66%, 3 = more than 67% and 4 = dead tissue; Romero et al., 2007). In addition, root segments from each woody species were washed and plated on NARPH medium for *P. cinnamomi* re-isolation (Romero et al., 2007; Serrano, Ferández-Rebollo, et al., 2012). A subsample of the infected roots of the three tree species was also used to make histologically thin sections. Root tissue material was fixed in a 4% EM grade glutaraldehyde solution and afterward included into resin Spurr (EMS) according to the protocols for dehydration and inclusion followed in the Microscopy Service of the University of Seville, where the histological sections were made. Samples were sectioned with a Leica Ultramicrotome UC7, obtaining 450-nm thick longitudinal sections of the roots in a longitudinal position which were stained in a 1% toluidine blue solution. Root sections were observed under a compound microscope (Nikon Eclipse 80i, ×100) to check for the existence of *P. cinnamomi* infective and survival structures.

2.5 | Data analysis

Zoospore production converted to (number of zoospore $ml^{-1})^{1/2}$ (Steel & Torrie, 1985) did not meet the assumptions of normality and heteroscedasticity of parametric statistical analyses, and therefore was analysed using the non-parametric Kruskal–Wallis test, with plant treatment as factor.

Chlamydospore density converted to (cfu × $g^{-1})^{1/2}$ (Steel & Torrie, 1985) was analysed using a two-way ANOVA with plant treatment, sampling date and their interaction as factors. If a significant interaction was found, the inoculum density in the soil planted with the different species combinations were separately compared with the control soil without plants. Additionally, differences in chlamydospore density among the six plant treatments at the end of the experiment were analysed by a one-way ANOVA. The severity of root symptoms at the end of the experiment was analysed separately for each tree species using one-way ANOVAs, including plant treatment as factor with three levels for *Q. suber* (Qs-Qs, Qs-Oe and Qs-Qc) and two levels for *O. europaea* (Oe-Oe, Qs-Oe) and *Q. canariensis* (Qc-Qc, Qs-Qc). When ANOVA revealed significant differences, mean values were compared among them by Fisher's LSD tests (Steel & Torrie, 1985) for α = .05. Statistix software was used for statistical analyses (Analytical Software, Tallahassee, FL).

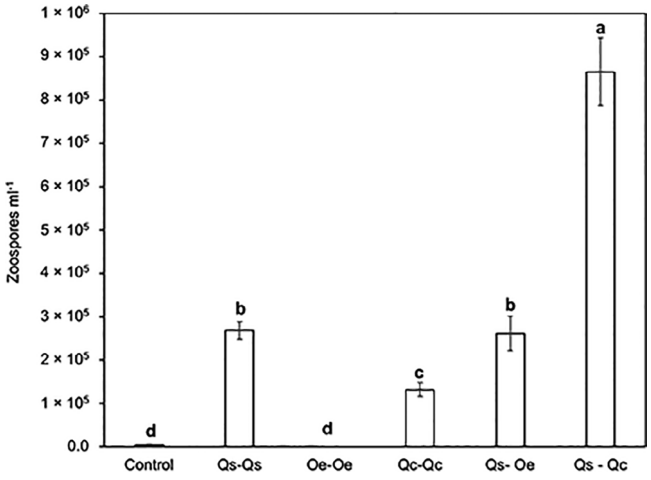

FIGURE 1 Average values and standard errors of indirect sporangial germination (zoospore production) of *Phytophthora cinnamomi* induced by woody plant combinations tested and control without plants. Bars with different letters significantly differ according to Fisher's LSD test for $p < .05$

3 | RESULTS

3.1 | Effect of tree species on *P. cinnamomi* zoospore production

We found significant differences among plant treatments in zoospore production ($p < .0001$, Kruskal–Wallis tests). When grown in monoculture, the two *Quercus* species, and particularly *Q. suber*, significantly stimulated the production of *P. cinnamomi* zoospores more than the control without plants. Monocultures of *O. europaea* did not differ from controls in zoospore production (Figure 1). *Q. suber* combined with *O. europaea* reached a similar production of zoospores (~2.6×10^5) to monoculture. However, the combination of *Q. suber* and *Q. canariensis* achieved the maximum spores' stimulation (~8.6×10^5), over three times higher than in *Q. suber* monocultures.

3.2 | Effect of tree species on the density of viable inoculum of *P. cinnamomi*

The average maximum and minimum soil water content (θ_s) during each flooding period was 100–73%.

There were significant differences among plant treatments ($DF = 5$, $F = 7.36$, $p < .0001$), sampling dates ($DF = 2$, $F = 57.61$ $p < .0001$), and their interaction ($DF = 10$, $F = 7.55$, $p < .0001$) on the density of viable *P. cinnamomi* chlamydospores. The density of inoculum in control substrate without plants showed a significant decrease at Week 4, maintaining a constant inoculum level until the end of the experiment (Figure 2). Viable inoculum density also decreased from the start of the experiment to Week 4 in the three tree monocultures.

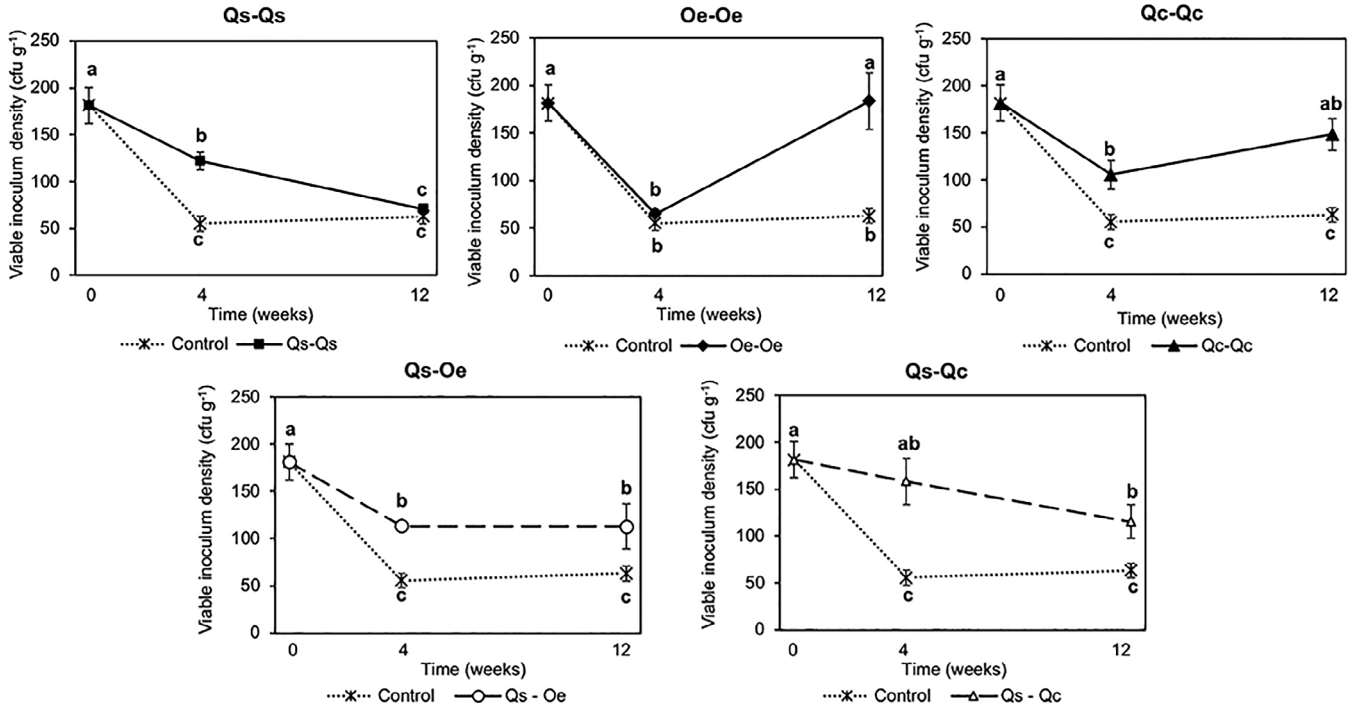

FIGURE 2 Average values and standard errors of viable colony forming units of *Phytophthora cinnamomi* per g of dry soil (cfu × g^{-1}) in the infested soil planted with the different plant combinations and the control without plant over 12 weeks. For each graph, the inoculum density in the soil planted with the different species combinations were compared with the control soil without plants, and values with distinct letters significantly differed according to Fisher's LSD test for $p < .05$. (Oe, *Olea europaea subsp euopaea* var. *sylvestris*; Qc, *Quercus canariensis*; Qs, *Quercus suber*)

However, the magnitude of the decrease was higher in the *O. europaea* monoculture (reaching levels similar to control) than in the two *Quercus* monocultures. At Week 12, the density of viable inoculum continued decreasing in *Q. suber* monocultures, reaching densities which did not differ to the control substrate. On the contrary, viable chlamydospores densities in substrate planted with monocultures of *Q. canariensis* and *O. europaea* increased at Week 12, reaching values similar to those at the beginning of the experiment and significantly higher than in control substrate (Figure 2). When in mixtures, chlamydospore density decreased through the course of the experiment, particularly for the *Q. suber-O. europaea* combination, having in all cases higher inoculum density than control substrates (Figure 2).

At the end of the experiment, there were significant differences among plant treatments in chlamydospore density ($DF = 5$, $F = 6.55$, $p = .0012$). The viable inoculum density was significantly higher in peat planted with monocultures of *O. europaea* (183.5 ± 29.5) and *Q. canariensis* (148.2 ± 17.1) than with monocultures of *Q. suber* (63.0 ± 7.7), which did not differ from control substrates without plants. Substrates planted with mixtures of *Q. suber-O. europaea* and *Q. suber-Q. canariensis* had intermediate density values (113.2 ± 24.1 and 115.2 ± 18.1, respectively) that differed significantly only from the largest and lowest densities found in *O. europaea* monocultures and control substrates, respectively.

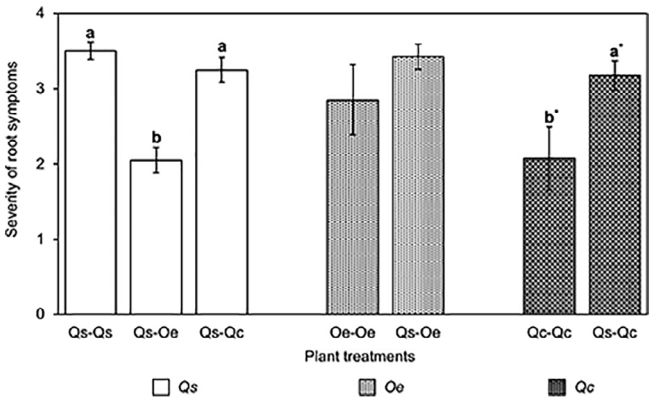

FIGURE 3 Average values and standard error of the severity of root symptoms for each plant species at the end of the experiment. For each set of treatments with a specific species of interest, bars with different letter significantly differ according to Fisher's LSD test for $p < .05$. *For *Q. canariensis* $p = .0538$

3.3 | Root rot development on tree species

All species exhibited symptoms of root rot consisting of necrosis and absence of feeder roots. For each species, there were differences among treatments in the severity of root symptoms showed by *Q. suber* ($DF = 1$, $F = 26.38$, $p = .0002$) and *Q. canariensis*

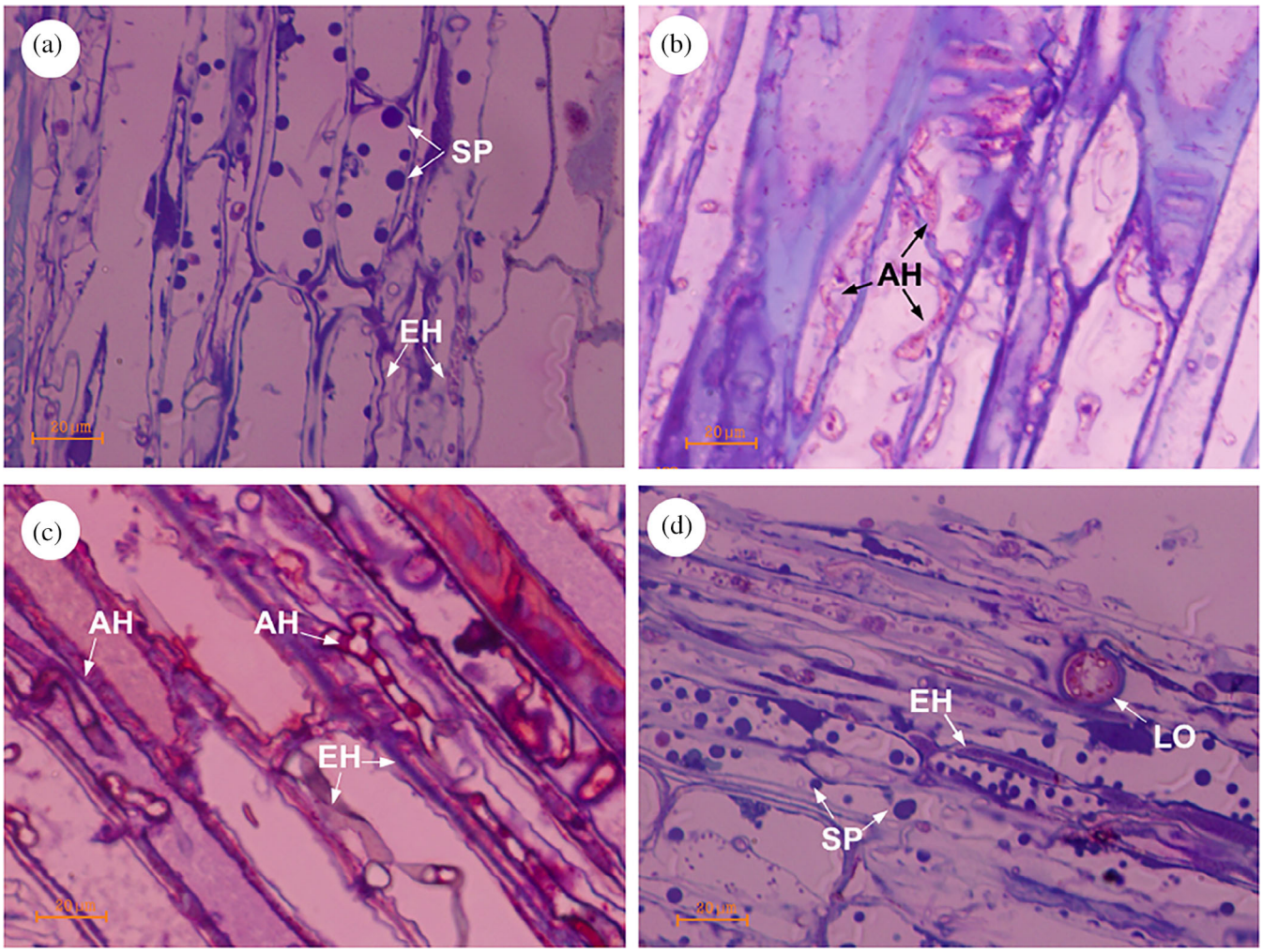

FIGURE 4 Longitudinal 450-nm thick sections of *Olea europaea* subsp. *europaea* var. *sylvestris*, *Quercus canariensis* and *Quercus suber* roots infected by *Phytophthora cinnamomi* and stained with 1% Toluidine Blue. (a) *O. europaea* subsp. *europaea* var. *sylvestris* root with the cellular structure quite intact, but with a high colonisation of *P. cinnamomi* and abundance of thick-walled spherical propagules (SP) and high development of intercellular hyphae (EH); (b) *Q. canariensis* root with cellular structure intact and abundance of intracellular hyphae (AH) colonisation; (c) *Q. suber* roots appeared also intact with inter- and intracellular hyphae colonisation; (d) spherical propagules like-oospores (LO) inside *O. europaea* subsp. *europaea* var. *sylvestris* root

(*DF* = 1, *F* = 5.73, *p* = .0538), but not in *O. europaea*. *Q. suber* showed significantly more severe symptoms in monoculture or mixed with *Q. canariensis* than when grown in mixtures with *O. europaea* (Figure 3). *Quercus canariensis* showed higher severity of roots symptoms when grown in mixtures with *Q. suber* than in monocultures (*p* = .0538 Figure 3). *P. cinnamomi* was always re-isolated from necrotic roots from each plant species. The percentages of re-isolation were 30.2 ± 10.4 for *Q. suber*, 52.3 ± 4.7 for *Q. canariensis* and 69.0 ± 2.3 for *O. europaea*, which reached the highest percentages of re-isolation.

In the histological sections, the cellular structure of *O. europaea*, *Q. canariensis* and *Q. suber* roots appeared quite intact, showing a high degree of colonisation by coralloid-type hyphae of *P. cinnamomi* (Figure 4). In root fragments of *O. europaea*, a large number of thick-walled spherical propagules were observed (Figure 4a). Additionally, bigger globose structures with smooth double wall like-oospores were observed sporadically in *O. europaea* (Figure 4d) and *Q. canariensis* roots, but never in *Q. suber* roots.

4 | DISCUSSION

4.1 | Influence of tree species on *P. cinnamomi* zoospore production

The release of zoospores from the sporangia and the ability of these zoospores to move towards favourable infection sites led by chemical and electrical gradients are key factors for *P. cinnamomi* disease dissemination and establishment (Hardham, 2005; Hickman, 1970). Differences found among tree species in the ability to stimulate *P. cinnamomi* sporulation were consistent with their differential susceptibility to the pathogen, corroborating our first hypothesis.

Q. suber, which is the most susceptible species tested in this study (Moralejo et al., 2009; Robin et al., 1998, 2001), led the highest zoospore production, significantly differing from both *Q. canariensis* and *O. europaea*. In fact, the last species did not differ from the control treatment without plants. Unfortunately, our experimental design did not allow us to discriminate whether the low number of zoospores found in the control treatment without plants and in substrates with *O. europaea* monocultures was because of a reduction in sporangia production, or on the contrary, it was consequence of a decrease in sporangia germination. Other studies have also reported zoospores of *Phytophthora* spp., including *P. cinnamomi*, to be quickly attracted by susceptible plant roots, while rarely attacked non-hosts roots (Hickman, 1970; van West, Appiah, & Gow, 2003; Zentmyer, 1961). Our results are in agreement with Serrano, Ferández-Rebollo, et al. (2012) who demonstrated that the susceptible species *Lupinus luteus* highly stimulated the production of *P. cinnamomi* zoospore in comparison with other resistant (wheat, oat) and tolerant species (vetch). In the same way, Zhang et al. (2019) found that root exudates of *Glycine max*, a susceptible host of *P. sojae*, attracted more zoospores of this pathogen and encouraged zoospore encystment than root exudates of resistant and non-hosts of the pathogen.

Our results provide novel evidence showing that tree species mixtures can affect disease dynamics in a very different way than species monocultures. We expected mixtures of a susceptible tree species (*Q. suber*) with less (*Q. canariensis*) or non-susceptible species (*O. europaea*) to stimulate production of *P. cinnamomi* zoospores to a lower extent than highly susceptible monocultures. However, and contrary to our second hypothesis, we found the maximum production of infective spores in mixtures of *Q. suber* and *Q. canariensis*. *Phytophthora* zoospore density is highly dependent on plant combination (Raftoyannis & Dick, 2006), however, several mechanisms have been described to explain zoospore accumulation in rhizosphere. Several studies concluded that zoospores of most *Phytophthora* species show attraction to a wide range of chemicals exudates such as vitamins, phenolic compounds, growth regulators, a variety of sugars, organic acids and amino acids produced by hosts (Hickman, 1970; Hosseini et al., 2014; Khew & Zentmyer, 1973; Malajczuk & McComb, 1977) and even non-hosts plants (Malajczuk & McComb, 1977). The interactive effects among species found in this study might be mediated by synergistic effects of their root exudates. Thus, in a previous study, Hickman (1970) found *P. cinnamomi* to be strongly stimulated by the glutamic and aspartic acid present in hosts and non-hosts root, however, the presence of an inhibitor in the non-hosts plants cancelled their effect. In this way, citric acid was able to inhibit the stimulatory effect of susceptible host root exudates on *P. sojae* (Zhang et al., 2019). Variation in amino acid composition and concentration led changes in *Phytophthora* spp. response (Hickman, 1970). However, other study concluded that the interaction *Phytophthora*–plant is mediated by electrochemical signals, whereby zoospores are attracted by the ionic gradient associated to the electrical field established in the rhizosphere (van West et al., 2002). Unfortunately, little is known about the composition of the root exudates and electrotactic in the Mediterranean tree species of study, either in monoculture or in mixtures. Nevertheless, the results obtained in this study suggest a higher inoculum potential, and consequently a greater dissemination and establishment capacity of *P. cinnamomi*, in mixed forests where the two *Quercus* species coexist than in mixed *Q. suber*–*O. europaea* forests or in *Q. suber* monospecific stands. Understanding why certain tree species combinations can be particularly conducive to disease dynamics should be considered a relevant research line for the future that might help to predict disease dynamics under natural conditions.

4.2 | Effect of tree species on the density of viable inoculum of *P. cinnamomi* and root rot disease development

We found that the viable inoculum dynamics of *P. cinnamomi* varied depending on host susceptibility, in agreement with our third hypothesis. However, our results do not totally support results from previous studies regarding inter-specific differences in their susceptibility degree to *P. cinnamomi*. Thus, whereas the severity of symptoms registered in *Q. suber* and *Q. canariensis* when grown in monocultures were in concordance with the results previously shown by Moralejo et al. (2009), *P. cinnamomi* was also able to infect the feeder roots and cause severe disease in 6-months old seedlings of *O. europaea* var. *sylvestris*, a species previously described as resistant to the pathogen (Moralejo et al., 2009). Moreover, *Olea europaea* was able to stimulate *P. cinnamomi* aggressiveness, despite the fact that its roots did not encourage zoospore production. Among the causes associated to the different responses found for this tree species against *P. cinnamomi* might be the use of distinct inoculation methods, different varieties (i.e., the commercial vs. wild variety), or different plant ages. In this sense, other studies have also found susceptibility to *Phytophthora* species to vary with plant ontogeny, mortality rates declining with plant age (Simamora et al., 2017). Further research is needed to confirm if *O. europaea* var. *sylvestris* susceptibility to *P. cinnamomi* may be associated with plant-age or not.

According to our third hypothesis, it was expected a sharp decrease in inoculum density in substrate likely because of the rapid germination of chlamydospores to produce sporangia and zoospores to infect susceptible plants and increase pathogen populations (Weste & Ruppin, 1977). However, in our study the decrease of *P. cinnamomi* population showed different magnitudes depending on the tree species colonising the substrate. In this way, the density of chlamydospores slightly decreased in peat planted with *Q. suber* as its roots were infected and necrosed. However, the less lignification observed in *O. europaea* roots in comparison with both *Quercus* species roots, specially *Q. suber*, might induce a rapid root tissue degradation explaining the significant increment of viable inoculum detected in substrate planted with this species at the end of the experiment. Consequently, as Hwang and Ko (1978) reported, chlamydospores produced inside roots are released to the substrate acting as inoculum.

Species mixtures in Mediterranean forests led to changes in *P. cinnamomi* behavior. In contrast with our fourth hypothesis, the

presence of *Q. canariensis* and particularly *O. europaea* mixed with *Q. suber* altered the expected dynamics of *P. cinnamomi* inoculum. According to our results, *P. cinnamomi* could more easily infect and degrade feeder roots of *O. europaea* than of *Q. suber* when both species coexisted. Consequently, *P. cinnamomi* viable inoculum increased in substrate faster in *Q. suber*–*O. europaea* mixtures than when *Q. suber* grew alone. These results supported the significantly higher severity of root rot symptoms registered for *O. europaea* than for *Q. suber*, although this *Quercus* species is considered as highly susceptible to *P. cinnamomi* (Moralejo et al., 2009; Robin et al., 1998, 2001). In contrast, in mixtures *Q. suber*–*Q. canariensis*, the severity of the root disease was similarly high for both species. This may be because of the synergistic effect of both *Quercus* species, which largely stimulated the production of infective zoospores. Probably as a result of this strong stimulation, after a slightly decrease of *P. cinnamomi* inoculum density in substrate, the combined effect of the root exudates of *Q. suber* and *Q. canariensis* sustained the inoculum density over the experiment. In this substrate the densities reached were significantly higher than in the control substrate without plants and in substrate planted with *Q. suber* as monoculture. These results supported the high influence of *Q. canariensis* on *P. cinnamomi* epidemiology, even although it had been described as less susceptible to *P. cinnamomi* than others *Quercus* spp., including *Q. suber* (Moralejo et al., 2009).

An extensive *P. cinnamomi* colonisation was detected in feeder roots of the three woody species, including *O. europaea*. In addition, spherical propagules were abundantly found in seedling roots of this species, but never in *Quercus* spp. roots. Previous studies found *P. cinnamomi* chlamydospores in roots of susceptible hosts (Crone, McComb, O'Brien, & Hardy, 2013; Jung, Colquhoun, & Hardy, 2013), including *Q. suber* (Ruiz-Gómez, Sánchez-Cuesta, Navarro-Cerrillo, & Pérez-de-Luque, 2012) and *Q. ilex* (Redondo et al., 2015), but never in species reported to be resistant such as *Acacia pulchella*, *Zea mays* L., *Triticum aestivum* L. and *Avena sativa* L. (Cahill et al., 1989; McCarren, McComb, Shearer, & Hardy, 2005; Serrano, Ferández-Rebollo, et al., 2012). The results obtained in this study suggested that these propagules found in *O. europaea* roots might be associated with the significant increment of *P. cinnamomi* inoculum density detected in soil planted with *O. europaea* seedlings. Based on the relevance of the results presented here, it is evident that more research is needed to verify the role of *O. europaea* roots on *P. cinnamomi* inoculum production.

Under the studied conditions, putative oospores (sexual resistant spores) were sporadically observed in roots of *Q. canariensis* and *O. europaea* var. *sylvestris*. Previous studies conducted in Australia found abundant selfed oospores formed in feeder roots of herbaceous annual species (Crone et al., 2013; Jung et al., 2013), as well as woody species such as *Banksia grandis* Willd. and *Eucalyptus marginata* Donn ex Sm. (Jung et al., 2013). The production of oospores has severe consequences for *P. cinnamomi* long-term survival, since oospores are considered much more resistant than chlamydospores (Jung et al., 2013). Although more research is needed to confirm *P. cinnamomi* behavior, according to our study *P. cinnamomi* might act as facultative homothallic in roots of *Q. canariensis* and *O. europaea* var. *sylvestris*. These findings might question *P. cinnamomi* life cycle described in the Iberian Peninsula, as well as the viability of the control methods currently developed.

Overall, the results obtained also suggest that mixed stands of *Q. suber* and *Q. canariensis* are able to stimulate *P. cinnamomi* infectivity and survival much more than monospecific stands, causing a severity of root rot on *Q. canariensis* significantly higher than when both *Quercus* species grew separately. Nevertheless, *Q. suber* showed a high severity of root rot symptoms regardless of the plant combination (monoculture or coexisting with *Q. canariensis*). Our results therefore suggest that, under favourable conditions for root disease development (periodical waterlogged soil and warm temperatures), the coexistence of *Q. suber* and *Q. canariensis* might extremely exacerbate Mediterranean forests' decline. This study also constitutes the first report of *O. europaea* subsp. *europaea* var. *sylvestris* as host and inductor of *P. cinnamomi* sporulation under controlled conditions. However, further investigations are required to determine the consequences of this study's findings under field conditions.

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