# Response of *Quercus ilex* seedlings to *Phytophthora* spp. root infection in a soil infestation test

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**Summary**

*Phytophthora* species are the main agents associated with oak (*Quercus* spp.) decline, together with the changing environmental conditions and the intensive land use. The aim of this study was to evaluate the susceptibility of *Quercus ilex* to the inoculation with eight *Phytophthora* species. Seven to eight months old *Q. ilex* seedlings grown from acorns, obtained from two Spanish origins, were inoculated with *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. megasperma*, *P. nicotianae*, *P. plurivora*, *P. psychrophila* and *P. quercina*. All *Phytophthora* inoculated seedlings showed decline and symptoms including small dark necrotic root lesions, root cankers, and loss of fine roots and tap root. The most aggressive species were *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. plurivora* and *P. psychrophila* followed by*P. megasperma*., while *Phytophthora quercina* and *P. nicotianae* were the less aggressive species. Results obtained confirm that these *Phytophthora* species could constituted a threat to *Q. ilex* ecosystems and the implications are further discussed.

**Keywords:** *Quercus* spp, Pathogenicity, Inoculation, Holm oak

## Introduction

The genus *Quercus*, comprises over 450 species distributed mainly throughout the Northern hemisphere (Xia et al. 2014). Over 70 species are known to be present in Spain and approximately 20 % are native (Sánchez de Lorenzo-Cáceres 2001). The evergreen holm oak (*Quercus ilex* L.) is the dominant tree in Spanish woodlands covering an area of 2.8 M ha (15.3 % of the forested area) (MAGRAMA 2014). It can also be found in 2.4 M ha of oak rangelands mixed with cork oak (*Quercus suber* L.) and "quejigo" oak [*Quercus faginea* (Cout) Camus)] (MAGRAMA 2014).

Since the last century, European oak forests are suffering a decline (Brasier 1996; Jung et al. 1996, 2000). The increase of pathogens threatening *Quercus*, along with the changing environmental conditions and the intensive land use, has resulted in a serious complex syndrome that is diminishing *Quercus* woodlands (Brasier 1992ab, 1996, 2008; Brasier et al. 1993b; Jung et al. 1996, 1999, 2000; Moreira and Martins 2005; Camilo-Alves et al. 2013). Amongst others, pathogens contributing to oak decline include: *Biscogniauxia mediterranea* (de Not.) Kuntze, *Botryosphaeria stevensii* Shoem., *Lembosia quercina* (Ellis & G. Martin) Tracy & Earle, *Pesotum piceae* J.L. Crane & Schokn., *Phomopsis quercina* (Sacc.) Höhn. ex Died., *Phytophthora* spp., *Pythium sterilum* Belbahri & Lefort and *Pythium spiculum* B. Paul (Brasier 1996; Jung et al. 1996, 2000; Gallego et al. 1999; Luque et al. 2000; Rizzo et al. 2002; Romero et al. 2007, Jiménez et al. 2008). Climate change, leading to an increase in mean temperatures, together with more frequent droughts followed by flooding episodes, are some of the abiotic factors causing weakening of the trees (Brasier 1992b, 1996; Sánchez et al. 2002; Corcobado et al. 2013). Once the tree health balance is disturbed, biotic damaging agents such as *Phytophthora* species can lead to the decline (Brasier et al. 1993b; Brasier 1996; Hansen and Delatour 1999; Jung et al. 2000; Sánchez et al. 2006; Camilo-Alves et al. 2013; Corcobado et al. 2013). The vigour of the tree is also affected by changes in the microbial composition of the rhizosphere. Jönsson (2006) suggested that the presence of microorganisms in the soil and mycorrhizal colonization made oak less susceptible to *Phytophthora* spp. infection. Lower ectomycorrhizal root colonization and diversity have been observed in *Phytophthora*-infected oak stands (Corcobado et al., 2014). Hence, disturbances in *Quercus* forests cause shifts in mycorrhizal soil communities, which in the presence of pathogens such as *Phytophora* spp., contribute to the decline (Corcobado et al., 2014).

The genus *Phytophthora* includes some of the most devastating plant pathogens comprising more than 150 species with different host ranges (Hardham and Blackman 2010; Scibetta et al. 2012; Thines 2013; Jung

et al. 2016; Panabières et al. 2016). It is present in natural and anthropogenic ecosystems causing large environmental and economic losses (Erwin and Ribeiro 1996; Kroon et al. 2012; Jung et al. 2016). Numerous surveys conducted in Europe reported *Phytophthora* as the main damaging agent associated with oak decline. In Spain and Portugal, the invasive pathogen *P. cinnamomi* Rands was established as the causal agent of decline of *Q. ilex* and *Q suber* in the Iberian Peninsula, although it is not the only *Phytophthora* species involved (Brasier 1992ab, 1996; Brasier et al. 1993b; Tuset et al. 1996; Gallego et al. 1999; Sánchez et al. 2002, 2003, 2006; Moreira and Martins 2005; Navarro et al. 2004; Corcobado et al. 2010; Pérez-Sierra et al. 2013). This situation is similar in other oak woodlands and maquis from Mediterranean regions in France, Italy and Turkey, where oaks are also affected by other *Phytophthora* species such as *P. cactorum* (Lebert & Cohn) J. Schröt., *P. cambivora* (Petri) Buisman, *P. citricola* complex, *P. cryptogea* Pethybr. & Laff., *P. gonapodyides* H.E. Petersen, *P. megasperma* Drechsler, *P. psychrophila* T. Jung & E.M. Hansen, *P. quercina* T. Jung and *P. syringae* (Kleb.) Kleb. (Brasier 1996; Robin et al. 1998; Hansen and Delatour 1999; Vettraino et al. 2002; Balci and Halmschlager 2003a; Linaldeddu et al. 2014; Scanu et al. 2015).

Several studies have been carried out to determine the susceptibility of oak (e.g. *Q. robur* and *Q. suber*) to different *Phytophthora* spp. *Phytophthora cinnamomi*, *P. cryptogea*, *P. drechsleri* Tucker, *P. gonapodyides*, *P. megasperma*, *P. psychrophila*, *P. quercina* and *P. syringae* have been inoculated on *Q. ilex*, in which lesions on the roots of young seedlings were observed (Tuset et al. 1996; Robin et al. 1998, 2001; Gallego et al. 1999; Maurel et al. 2001; Rodríguez-Molina et al. 2002; Sánchez et al. 2002, 2005; Pérez-Sierra et al. 2013; Linaldeddu et al. 2014; Martín-García et al. 2015). Considering the importance of *Q. ilex* as the most representative tree in the Spanish forest ecosystems and the lack of information regarding the role of some *Phytophthora* spp. in its decline, the aim of this study was to investigate the response of *Q. ilex* seedlings to the inoculation with eight different *Phytophthora* species using a soil infestation method.

## Material and Methods

Plant material

Seven to eight months old *Q. ilex* subsp. *ballota* seedlings grown from acorns were used. Acorns were selected from two different origins; an oak rangeland (a silvopasture farming system) located in Cáceres in Extremadura region in western Spain (39º58´N, 6º5´W; mean T = 16.5 ºC; annual P = 803 mm), and from La Yesa, a Mediterranean mixed forest stand, in which holm oaks are grown competing with other tree species in Comunidad Valenciana in eastern Spain. In both cases acorns were collected from vigorous trees. The acorns from La Yesa were provided by the Forest Research Centre CIEF (Centro para la Investigación y Experimentación Forestal, Valencia). Acorns from both origins were surface sterilized and pre-germinated in trays with thermo-sterilized sand incubated at 20 ºC under 12 h photoperiod. Once the roots emerged, pre-germinated acorns were transplanted to Quick pot PE trays (52 × 29 upper surface and 19 cm high). Each cell contained approximately 1,700 ml in volume of vermiculite-sand-peat substrate mixture (1:1:1, v/v/v) previously autoclaved three times. To avoid root disturbance during inoculation, two cavities were made in the substrate before sowing by placing 2 sterile 15 ml tubes 6 cm apart. One pre-germinated acorn per cell was planted between the tubes and plants maintained in the greenhouse at 20-25 ºC and watered every two weeks.

*Phytophthora* isolates

*Phytophthora* isolates used in the pathogenicity tests were selected from the *Phytophthora* collection maintained in soil solution extract and oatmeal agar tubes at the Instituto Agroforestal Mediterráneo (IAM-UPV, Valencia, Spain). All were isolated from *Q. ilex* during previous surveys of forest ecosystems and nurseries. Eight *Phytophthora* species were selected: *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. megasperma*, *P. nicotianae*, *P. plurivora*, *P. psychrophila* and *P. quercina* (Table1).

Soil infestation pathogenicity test

The potting mix consisted of vermiculite-sand-peat (1:1:1, v/v/v), oat grains (20 $cm^3$) and V8 broth (200 mL/L V8 juice, 800 mL/L demineralized water and 3 g/L $CaCo_3$). The mixture was autoclaved 3 times and then inoculated with the selected *Phytophthora* species isolates previously grown on V8 media (V8A). The inoculated media were incubated for 6 weeks in the dark at room temperature (Pérez-Sierra et al. 2013). After this time, the inoculum mixture was rinsed with demineralized water before inoculations.

105 Seedlings were selected for the test based on morphological homogeneity and healthy appearance. For
106 inoculation, 20 ml of inoculum mixture per 1 L potting medium was added to the cavities previously made
107 in each cell where the seedling was grown. Negative control plants (henceforth called uninoculated plants)
108 were inoculated with non-infested mixture and the experiment was repeated twice. In total, 24 seedlings
109 were inoculated per *Phytophthora* spp. and control. Each 12 seedlings repetition contained 9 seedlings from
110 Cáceres and 3 from La Yesa. For inoculations with *P. megasperma*, only 15 seedlings from La Yesa were
111 included (7 and 8 seedlings in each repetition). All seedlings were watered the day before the inoculation.
112 Immediately after inoculation, the seedlings were flooded for 48 h and the flooding was repeated every two
113 weeks to stimulate formation of zoosporangia, as previously described (Pérez-Sierra et al. 2013). The
114 experiment was harvested 6 months after inoculation.

115 Seedlings were uprooted and the root system was washed carefully under running water to remove the
116 substrate. Reisolations from all seedlings were performed by plating symptomatic fine root fragments in
117 CMA-PARPBH (Jeffers and Aldwinckle 1987) and baiting the substrate with Granny Smith apples (Erwin
118 and Ribeiro 1996) to confirm Koch´s postulates.

119 Seedling analysis

120 Seedlings were evaluated immediately after inoculation and every two weeks thereafter in order to
121 determine aerial condition. Number of leaves on each seedling and above-ground symptoms were evaluated
122 using a visual scale, where 0 = symptoms-free plant, 1 = limited foliar chlorosis and necrosis, 2 = wilting,
123 dieback, defoliation,and 3 = dead plant (Jönsson et al. 2003).

124 To assess root condition of inoculated seedlings two different approaches were used at the end of the
125 experiment. First, symptom severity was assessed, using a visual descriptive scale from 1 to 4 (1 = root loss
126 from 0-25 %, 2 = root loss from 26-50 %, 3 = root loss from 51-75 %, 4 = root loss from 76-100 %) (Pérez-
127 Sierra et al. 2013). Symptom severity was calculated using the McKinney Index (MI; McKinney 1923)
128 based on the scale given above, and a Kruskal-Wallis test applied to the data to compare between
129 *Phytophthora* species. For the second approach, the dry weight of the root biomass was measured. The
130 aerial tissues were separated from the root system by cutting at the root collar, placed into paper bags, and
131 dried for 5 days in an oven at 35 ºC. The dry weights of the aerial tissues and root system were recorded.
132 An analysis of variance (ANOVA) was performed for the factors treatment, origin and the interaction

133 treatment x origin. Mean values were compared using the Student's least significant difference test at the
134 95 % confidence level. Correlation between the different parameters were determined by calculating
135 Pearson´s coefficients (r). All analyses were performed using the package SPSS 16.0 (SPSS Inc., Chicago
136 IL).

137 An ANOVA was performed to determine differences in mean number of final leaves, length of the stem,
138 weight of fine roots (Ø<2mm), weight of main roots (Ø>2mm), weight of the complete root system, weight
139 of the aerial tissues and survival days obtained from the different *Phytophthora* treatments and the acorn
140 origin. Pearson´s coefficients were calculated to determine correlations between the measured parameters.
141 Finally, survival time of the seedlings was also assessed using the Kaplan-Meier estimate, a product-limit
142 estimate:

143 $$S(t) = \prod_{j=1}^{k} \cdot \left(\frac{nj-dj}{nj}\right)$$

144 where $n_j$ = number of seedlings alive before the time $t_{(j)}$ and $d_{(j)}$ = number of dead seedlings at time $t_{(j)}$ for
145 $t_{(k)} \leq t \leq t_{(k+1)}$. This non-parametric analysis was carried out using the same software and the log-Rank test
146 (Collett 2003) was used to compare the survival curves of the seedlings inoculated with the different
147 *Phytophthora* species.

148

149 **Results**

150 All *Q. ilex* seedlings inoculated with the different *Phytophthora* isolates showed root symptoms (small dark
151 necrotic lesions, root cankers, loss of fine roots, tap root rot), as well as aerial symptoms (decline, chlorosis,
152 wilting, dieback, defoliation, slow growth rate, leaf spots). Reisolations from symptomatic roots confirmed
153 Koch´s postulates. In contrast, control treatment seedlings showed non-specific symptoms in the root
154 system and the aerial tissues, which were not associated with positive reisolations of *Phytophthora* and new
155 healthy rootlets were growing in almost all control seedlings at the end of the experiments.

156 ANOVA showed significant differences among the treatments (*Phytophthora* species inoculated) and the
157 uninoculated control plants in number of leaves, weight of fine roots, symptom severity , MI and survival
158 time with a confidence level of 99 % (Table 2). These parameters were not significantly different, based on

159 the origin of the acorns. The interaction treatment x origin showed non-significant effect for these
160 parameters. The analysis of MI showed *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. plurivora* and *P.*
161 *psychrophila* were aggressive species causing severe symptoms ranging from 95.8 % to 98.8 %. The lowest
162 MI corresponded to *P. nicotianae* (83.3 %) which was significantly higher than the uninoculated plants
163 (Table 2). Seedlings inoculated with *P. nicotianae*, *P. quercina* and the uninoculated plants had
164 significantly higher fine root weights compared with the other treatments (*P*-value > 0.05) (Table 2). The
165 *P. gonapodyides* treatment caused the lowest survival time of the seedlings, followed by *P. cinnamomi*.
166 These seedlings showed non-significant differences among them in terms of survival but they showed
167 differences compared with *P. psychrophila, P. megasperma, P. quercina, P. nicotianae* and uninoculated
168 controls (*P*-value < 0.05) (Table 2).

169 ANOVA also showed significant differences among the treatments and the uninoculated plants in length of
170 the stem, weight of the complete root system, weight of the main roots and weight of the aerial tissues. For
171 these parameters, the factor acorn origin was also significant (*P*-value < 0.05). Due to this finding, these
172 parameters were examined separately by origin (Table 3). The interaction treatment x origin showed non-
173 significant effect for these parameters. For seedlings from Cáceres, plants inoculated with *P. psychrophila*,
174 *P. cinnamomi, P. gonapodyides* and *P. cryptogea* showed significant lower stem length (*P*-value < 0.05).
175 For seedlings from La Yesa, plants inoculated with *P. megasperma* showed lower stem length compared
176 with plants inoculated with *P. cryptogea*, *P. quercina* and uninoculated controls.

177 Regarding the weight of the complete root system and the main roots, *P. cryptogea, P. psychrophila*, *P.*
178 *plurivora, P. gonapodyides* and *P. cinnamomi* were the most aggressive species for both acorn origins. In
179 seedlings grown from La Yesa acorns, the most aggressive species were also *P. megasperma* and *P.*
180 *nicotianae*. Regardless of the origin of the acorns, seedlings inoculated with *P. quercina* did not differ
181 significantly in weight of the complete root system from the uninoculated controls.

182 The survival curves (Fig. 1) agreed with the results obtained from the Kruskal-Wallis analysis.
183 *Phytophthora megasperma, P. nicotianae, P. quercina,* and the uninoculated plants showed highest survival
184 at the end of the experiment, ranging from 62.5 to 91.7 %. *Phytophthora cinnamomi, P. cryptogea, P.*
185 *gonapodyides, P. plurivora* and *P. psychrophila* were more aggressive causing lower survival of plants at
186 the end of the experiment: 4.2 %, 4.2 %, 8.3 %, 20.8 % and 45.5 %, respectively.

187 Pearson´s analysis of the global data set, showed that the correlation between the different parameters
188 studied in the experiment (Table 4) was significant (*P*-value < 0.001). The coefficients between MI and the
189 other parameters examined were negative and particularly strong between MI and the different weights (W)
190 of the root system (W main roots r = -0.7591; W fine roots r = -0.7994) and the aerial tissues (r = -0.7066).
191 The correlation between the weights of the aerial tissues and the root system of the seedlings was positive,
192 also showing a strong relationship among these parameters (W main roots r = 0.8048; W fine roots r =
193 0.7407). Finally, there was a positive correlation between the total weight of the seedling (aerial tissues and
194 root systems) and survival time (W aerial tissues r = 0.4265; W main roots r = 0.4051; W fine roots r =
195 0.3391) (Table 4).

196

197 **Discussion**

198 All *Phytophthora* isolates inoculated on *Q. ilex* were pathogenic. The most aggressive species were *P.*
199 *cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. plurivora* and *P. psychrophila*, followed by *P. megasperma*,
200 while *P. quercina* and *P. nicotianae* were the least aggressive species, with plants inoculated with *P.*
201 *quercina* having the longest survival rates. For seedligs grown from Cáceres acorns, *P. nicotianae* was the
202 least aggressive species, while seedlings grown from La Yesa acorns the least aggressive was *P. quercina*.

203 Results observed in seedlings inoculated with *P. cinnamomi* were in agreement with several studies and
204 field observations, which demonstrated the devastating action of this wide-host range pathogen in the
205 Iberian Peninsula (Brasier 1992a, 1992b, 1996; Brasier et al. 1993b; Robin et al. 1998; Tuset et al. 1996;
206 Gallego et al. 1999; Luque et al. 2000, 2002; Sánchez et al. 2002, 2003, 2005, 2006; Moreira and Martins
207 2005; Navarro et al. 2004; Camilo-Alves et al. 2013, Hernández-Lambraño et al. 2018; Sena et al. 2018).
208 The *P. cinnamomi* pathogenicity test showed high mortality rates that could be associated with a rapid root
209 rot affecting not only the feeder roots but also the tap root. Loss of fine roots, cankers and dieback of the
210 tap root with necrotic lesions were observed in inoculated seedlings as previously described for *P.*
211 *cinnamomi* infection (Brasier et al. 1993b; Robin et al. 2001; Sánchez et al. 2005; Redondo et al. 2015).
212 *Phytophthora cinnamomi* is well adapted to the Spanish environmental and edaphic conditions causing oak
213 decline with the exception of the eastern limestone Mediterranean area, which constrains its development
214 due to the high calcium content soils (Schmitthenner and Canaday 1983; Ríos et al. 2016).

*Phytophthora gonapodyides* was considered a ubiquitous and opportunistic or weak pathogen (Brasier 1993a, Hansen and Delatour 1999). However, Jung et al. (1996) reported that *P. gonapodyides* produced a wilting toxin able to cause root rot and stem lesions on *Q. robur* seedlings. In 2010, *P. gonapodyides* was reported as *Q. ilex* pathogen (Corcobado et al. 2010). Subsequent studies showed the aggressiveness of this species in holm oak (Pérez-Sierra et al. 2013; Corcobado et al. 2017). Corcobado et al. (2017) observed the highest necrosis lengths in the roots and high mortality rates in seedlings infected with *P. gonapodyides* compared with seedlings infected with *P. quercina*. Our results agree with these findings since seedlings inoculated with *P. gonapodyides* caused the most rapid mortality, high MI, limited aerial tissue development and a significant reduction in the root system. *Phytophthora gonapodyides* could be considered along with *P. cinnamomi* in the category of main biotic threats to holm oak seedlings in Spanish forests as from our results it has similar behaviour as *P. cinnamomi*.

Regarding seedlings inoculated with *P. cryptogea*, results obtained agree with pathogenicity studies carried out with Spanish *Q. ilex* material by Sánchez et al. (2005). *Phytophthora cryptogea* zoospores attack the feeder roots, and the pathogen progresses through the root system reaching the main root and causing dieback with necrotic lesions and small cankers. As the root system diminishes rapidly, the aerial tissues do not develop correctly, leading to high mortality rates. *Phytophthora cryptogea* has been reported in several Mediterranean ecosystems associated with oak decline (Vettraino et al. 2002; Balci and Halmschlager 2003a; Sánchez et al. 2005; Pérez-Sierra et al. 2013; Linaldeddu et al. 2014; Scanu et al. 2015, Mora-Sala et al. unpublished data). The versatility of *P. cryptogea* and the ability to persist in water bodies, soil or plant tissue until favourable conditions appear, might allow it to establish and to develop throughout the oak forest ecosystems in Spain, then becoming then a dangerous pathogen.

*Phytophthora nicotianae* is a polyphagous, broad-range pathogen responsible for major economic losses in agricultural and ornamental sectors worldwide (Erwin and Ribeiro 1996; Álvarez et al. 2007; Panabières et al. 2016). It has been reported among the main *Phytophthora* species present in the nursery industry especially in Mediterranean regions threatening afforestation of *Quercus* stands (Moralejo et al. 2009; Pérez-Sierra et al. 2012; Pérez-Sierra and Jung 2013; Jung et al. 2016; Panabières et al. 2016). Climate change and global trade are driving *P. nicotianae* to an advantageous position over other *Phytophthora* species as its high optimum temperature, longevity, dispersal capacity and hybridisation capacity enable it to adapt to the changing worldwide climate scenarios (Panabières et al. 2016). This report is the first time

that *P. nicotianae* was tested on *Q. ilex* and the results demonstrated that this host is susceptible to the pathogen, despite *P. nicotianae* being less aggressive than the other *Phytophthora* species tested. La Yesa seedlings were more susceptible to *P. nicotianae* than Cáceres seedlings, possibly due to the quality of the acorns, as the management of the oaks in the two origins differ. While in Cáceres, the holm oak is the only tree species in this agricultural scenario, in La Yesa, oaks are part of a Mediterranean mixed forest. In Cáceres, oaks are maintained for the production of acorns to feed the livestock, which generally produces bigger acorns.

*Phytophthora plurivora* is a well-known aggressive oak pathogen (Jung et al. 1996, 2000; Hansen and Delatour 1999; Vettraino et al. 2002; Balci and Halmschlager 2003a, 2003b; Mrázková et al. 2013; Jung and Burgess 2009; Jankowiak et al. 2014), but our study represents the first soil infestation test conducted on holm oak with this species. *Q. ilex* seedlings inoculated with *P. plurivora* showed absence of fine roots, necrotic lesions, open cankers, dieback of the whole root system and collar rot. In some cases, no tap root was present. These symptoms agree with those reported in other woody hosts leading to a high mortality rate, high MI and low root and aerial tissues weight (Jung and Burgess 2009). As homothallic species, *P. plurivora* is a broad host range pathogen having high environmental versatility (Jung and Burgess 2009). It could be considered as a potentially easy spreading species in Spanish natural ecosystems. Indeed, it has already been detected in different areas of Spain (Català et al. 2017; Mora-Sala et al. unpublished data).

*Phytophthora psychrophila* was firstly recovered from soil from *Q. robur*, *Q. petraea* and *Q. ilex* in Bavaria and Southern France (Jung et al. 2002). In 2013, *P. psychrophila* was reported in Comunidad Valenciana (eastern Spain) causing *Q. ilex* and *Q. faginea* dieback in a Mediterranean oak forest (Pérez-Sierra et al. 2013) and it has also been detected in Spanish oak stands (Català et al. 2017; Mora-Sala et al. unpublished data). In the present study, *P. psychrophila* behaved as an aggressive pathogen, which caused dieback of the root system, mainly the fine roots, showed necrotic lesions and open cankers. The results observed agreed with a previous pathogenicity test performed on *Q. ilex* (Pérez-Sierra et al. 2013) and the symptoms obtained were similar to those observed by Jung et al. (2002) in a soil infestation test conducted on *Q. robur* seedlings.

*Phytophthora quercina* is a proven pathogen of oak, widespread in oak-dominated ecosystems (Hansen and Delatour 1999; Jung et al. 1999; Vettraino et al. 2002; Balci and Hamschlager 2003a, 2003b; Pérez-Sierra et al. 2013; Català et al. 2017; Mora-Sala et al. unpublished data). *Phytophthora quercina* is considered a

fine root nibbler, which causes major loses of fine roots weakening the tree progressively but effectively (Jung et al. 1999; Jönsson et al. 2003, Corcobado et al., 2017). Tsao (1990) stated that a tree can have substantial loss of fine roots without showing above-ground symptoms. Our results concur with this description, showing a pathogenic behaviour rotting feeder roots and causing small necrotic lesions and cankers. In this context, it can be hypothesized that a decrease in survival rate of inoculated seedlings would have occurred if the test lasted longer. As observed with *P. nicotianae*, La Yesa seedlings resulted to be more susceptible to the pathogen than Cáceres seedlings.

*Phytophthora megasperma* is considered an opportunistic pathogen and has been isolated and detected in declining oak forests (Hansen and Delatour 1999; Jung et al. 2000; Vettraino et al. 2002; Pérez-Sierra et al. 2013; Mora-Sala et al. unpublished data). The study shows a reduction of the root system and a limited development of the aerial tissues and survival rates were lower than when the other *Phytophthora* species were inoculated. *Phytophthora megasperma* behaves in a similar way to *P. quercina* and this result is similar to the one obtained previously by Pérez-Sierra et al. (2013).

In the present study different parameters were evaluated to assess the pathogenicity of *Phytophthora* species on *Q. ilex*, and it is remarkable that most of these parameters agree in the results. Both Spanish acorn origins tested behaved the same way in terms of MI, fine root rot, defoliation and survival rates. Nevertheless, the seedlings from both acorn origins diverge in terms of weight of the aerial tissues, stem length and weight of the main roots. The results obtained showed that the McKinney index or the survival function were suitable to assess *Phytophthora* pathogenicity tests.

This pathogenicity test demonstrates that *Q. ilex* was susceptible to a range of *Phytophthora* species, apart from *P. cinnamomi*. The *Phytophthora* species tested are well known nursery pathogens affecting a broad range of host plants including woody hosts, such as *Quercus* species (Jung et al. 2016). The present and previous studies demonstrated that several *Phytophthora* species constituted a threat to *Quercus* ecosystems. The relevance of this group of plant pathogens and the increasing number of hosts that are emerging in different scenarios highlights the need for improving the control of plant material. In this context, the nursery industry and international plant trade should implement effective phytosanitary measures to avoid *Phytophthora* dispersal to naïve natural ecosystems and geographical areas where the pathogen is not present.

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**Compliance with ethical standards.** The authors declare that ethical standards have been followed and that no human participants or animals were involved in this research.

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**Table 1.** *Phytophthora* isolates used in the pathogenicity test.

| ***Phytophthora* spp.** | **Code** | **Host** |
|---|---|---|
| *P. cinnamomi* | Ps 1630 | *Q. ilex* (roots) |
| *P. cryptogea* | Ps 962 | *Q. ilex* (roots) |
| *P. gonapodyides* | Ps 789 | *Quercus* sp. |
| *P. megasperma* | Ps 1619 | *Q. ilex* (soil) |
| *P. nicotianae* | Ps 956 | *Q. ilex*(roots) |
| *P. plurivora* | Ps 932 | *Q. ilex* |
| *P. psychrophila* | Ps 1030 | *Quercus* sp. |
| *P. quercina* | Ps 982 | *Q. ilex* (soil) |

**Table 2.** Kruskal-Wallis and one-way ANOVA for non-significant parameters according to the origin of the inoculated material. Results of the parameters analysed in the pathogenicity test coming from Cáceres and La Yesa acorn origins.

| ***Phytophthora* spp.** | **No. leaves** | **W fine roots (mg)** | **MI** | **Survival time (days)** |
|---|---|---|---|---|
| *P. cinnamomi* | 6.5 ± 0.95 **ab** | 63 ± 19.59 **a** | 95 ± 2.46 **d** | 92.1 ± 6.10 **ab** |
| *P. cryptogea* | 8.9 ± 0.99 **abc** | 41 ± 14.51 **a** | 96 ± 1.72 **d** | 98.5 ± 7.31 **b** |
| *P. gonapodyides* | 6.8 ± 1.08 **ab** | 45 ± 25.22 **a** | 97 ± 1.44 **d** | 78.8 ± 6.82 **a** |
| *P. megasperma* | 4.6 ± 1.33 **a** | 32 ± 10.84 **a** | 95 ± 2.67 **cd** | 136.5 ± 11.50 **cd** |
| *P. nicotianae* | 15.5 ± 1.91 **d** | 230 ± 49.13 **b** | 83 ± 4.43 **b** | 147.5 ± 5.67 **d** |
| *P. plurivora* | 10.2 ± 2.05 **bc** | 41 ± 19.47 **a** | 96 ± 2.29 **d** | 101.4 ± 8.23 **b** |
| *P. psychrophila* | 6.8 ± 1.08 **ab** | 35 ± 9.79 **a** | 98 ± 1.14 **d** | 122.4 ± 9.03 **c** |
| *P. quercina* | 13.5 ± 2.11 **cd** | 185 ± 33.24 **b** | 84 ± 3.30 **bc** | 139.3 ± 7.85 **cd** |
| Negative control | 16.7 ± 2.70 **d** | 245 ± 77.30 **b** | 72 ± 6.72 **a** | 156.3 ± 1.28 **d** |

All *P*-values are significant at $P < 0.01$.

Values with the same letter for each column do not differ significantly according Fisher´s LSD test ($P = 0.05$)

W = weight; MI = McKinney Index

**Table 3.** Kruskal-Wallis one-way ANOVA. Results of the parameters obtained in the pathogenicity test of the different *Phytophthora* species inoculated on *Quercus ilex* seedlings from Cáceres.

| **Species** | **L stem (cm)** | **W** aerial tissues **(mg)** | **W complete root system (mg)** | **W main roots (mg)** |
|---|---|---|---|---|
| *P. cinnamomi* | 9.6 ± 1.19 **a** | 393 ± 79.59 **a** | 711 ± 172.58 **a** | 640 ± 156.68 **a** |
| *P. cryptogea* | 11.14 ± 0.88 **a** | 561 ± 69.96 **ab** | 421 ± 76.80 **a** | 401 ± 61.30 **a** |
| *P. gonapodyides* | 9.61 ± 0.60 **a** | 486 ± 93.75 **ab** | 642 ± 183.31 **a** | 602 ± 158.34 **a** |
| *P. nicotianae* | 18.06 ± 1.80 **c** | 1827 ± 292.60 **c** | 898 ± 257.27 **b** | 1627 ± 214.94 **b** |
| *P. plurivora* | 11.39 ± 1.17 **ab** | 607 ± 106.44 **ab** | 618 ± 186.71 **a** | 566 ± 161.82 **a** |
| *P. psychrophila* | 8.25 ± 0.66 **a** | 425 ± 83.75 **ab** | 590 ± 90.93 **a** | 553 ± 87.04 **a** |
| *P. quercina* | 11.08 ± 1.03 **b** | 876 ± 151.35 **b** | 1520 ± 221.89 **b** | 1339 ± 192.15 **b** |
| Negative control | 14.6 ± 1.46 **b** | 1372 ± 262.39 **c** | 1925 ± 449.74 **b** | 1642 ± 356.46 **b** |

All *P*-values are significant at $P < 0.01$.

Values with the same letter for each column do not differ significantly according Fisher´s LSD test ($P = 0.05$)

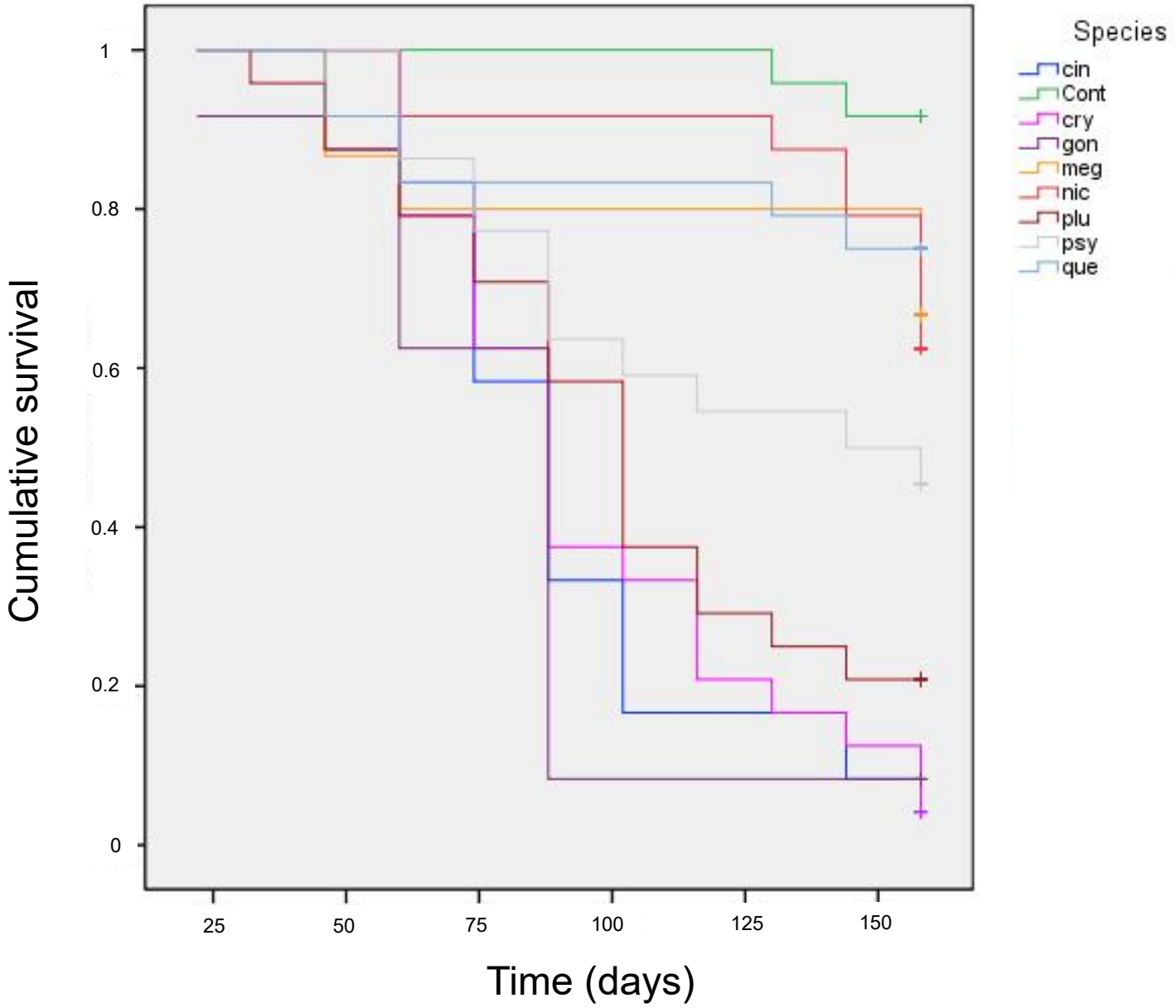
L = length; W = weight

**Table 4.** Pearson´s correlation coefficient (r) between the different parameters studied in the *Phytophthora* spp. pathogenicity test on *Quercus ilex* seedlings.

| | **L** | **No. leaves** | **W**aerial tissues | **Wmain roots** | **Wfine roots** | **MI** | **Survival time** |
|---|---|---|---|---|---|---|---|
| **L** | | | | | | | |
| **No. leaves** | 0.5886 | | | | | | |
| **Waerial part** | 0.8310 | 0.6356 | | | | | |
| **Wmain roots** | 0.6519 | 0.5122 | 0.8048 | | | | |
| **Wfine roots** | 0.5847 | 0.5376 | 0.7407 | 0.8137 | | | |
| **MI** | -0.6200 | -0.5084 | -0.7066 | -0.7591 | -0.7994 | | |
| **Survival time** | 0.3301 | 0.4698 | 0.4265 | 0.4051 | 0.3391 | -0.3547 | |

All correlations are significant. *P*-values < 0.001

L = length; W = weight; MI = McKinney Index


Cumulative survival
1
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100
125
150
Time (days)
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