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The Use of qPCR Reveals a High Frequency of *Phytophthora quercina* in Two Spanish Holm Oak Areas

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Abstract: The struggling Spanish holm oak woodland situation associated with *Phytophthora* root rot has been studied for a long time. *Phytophthora cinnamomi* is considered the main, but not the only species responsible for the decline scenario. This study verifies the presence and/or detection of *Phytophthora* species in two holm oak areas of Spain (southwestern "dehesas" and northeastern woodland) using different isolation and detection approaches. Direct isolation and baiting methods in declining and non-declining holm oak trees revealed *Phytophthora cambivora*, *Phytophthora cinnamomi*, *Phytophthora gonapodyides*, *Phytophthora megasperma*, and *Phytophthora pseudocryptogea* in the dehesas, while in the northeastern woodland, no *Phytophthora* spp. were recovered. Statistical analyses indicated that there was not a significant relationship between the *Phytophthora* spp. isolation frequency and the disease expression of the holm oak stands in the dehesas. *Phytophthora quercina* and *P. cinnamomi* TaqMan real-time PCR probes showed that both *P. cinnamomi* and *P. quercina* are involved in the holm oak decline in Spain, but *P. quercina* was detected in a higher frequency than *P. cinnamomi* in both studied areas. Thus, this study demonstrates that molecular approaches complement direct isolation techniques in natural and seminatural ecosystem surveys to determine the presence and distribution of *Phytophthora* spp. This is the first report of *P. pseudocryptogea* in Europe and its role in the holm oak decline should be further studied.

Keywords: *Quercus ilex* L.; *Phytophthora cinnamomi*; *Phytophthora quercina*; *Phytophthora pseudocryptogea*; qPCR

1. Introduction

Holm oak (*Quercus ilex* L.) grows spontaneously throughout the Mediterranean basin, from the Iberian Peninsula to Turkey in the North and from Morocco to Tunisia in the South, having its optimum growing conditions in the Western Mediterranean regions [1]. Holm oak is a low nutrient demanding species, which prefers dry soils situated in Spain from sea level up to 2000 m high, although the most dense holm oak forests' altitude ranges from 200 to 800 m. This species is well adapted to Mediterranean xeric conditions, with an early active taproot development and little branching at the expense of shoot development [2].

In Spain, holm oak is the most abundant evergreen *Fagaceae* tree species, covering almost all Spanish provinces except the Canary Islands and Galicia regions, where it is scarce [1]. About 2.8 M ha of the Spanish forestry surface are holm oak woodlands and 2.4 M ha are oak rangelands (henceforth called dehesas) (which consist mainly of holm oaks mixed with cork oaks (*Quercus suber* L.), and even a deciduous oak (*Quercus faginea* Lam.)) [3].

Holm oak constitutes a fundamental pillar of the Spanish dehesa, an agro–silvo–pastoral system, benefiting from the use of its fruit mainly for livestock during the autumn season and the grass growing

underneath the canopy for grazing. Its wood it is also a valuable asset. In addition, it hosts migrant birds from Central and Northern Europe during the winter season. This complex system is suffering a significant decline due to biotic and abiotic factors [4,5]. The Spanish dehesas' decline associated with *Phytophthora* root rot has been studied since the end of the 20th century [6–11]. *Phytophthora cinnamomi* Rands. is considered the main pathogen responsible for the decline of this ecosystem [4,7–10,12,13], but it is not the only *Phytophthora* species infecting holm oaks [14–16].

On the other hand, Spanish natural oak woodlands are also undergoing this decline caused by *Phytophthora* spp. [17,18]. Several studies across Europe demonstrate the association of declining oak woodlands with *Phytophthora quercina* T. Jung, among other species, causing root infections [19–24]. In addition, abiotic factors, such as increasing temperatures and water stress, are being enhanced by climate changing conditions, which have a negative impact on the tree health status, weakening the stands and making holm oaks more susceptible to *Phytophthora* and *Pythium* infection [9,24–27]. Moreover, in view of the lack of regeneration of the stands, reforestations and afforestations are conducted with nursery material, with the consequent risk of introducing alien *Phytophthora* species to natural ecosystems [14,28–31].

Some *Phytophthora* species infect plants without causing external symptoms and this plant material is transported worldwide, allowing pathogens to be disseminated without generating any alert at the inspection points [30,32]. Denman et al. [33] reported that leaves from holm oak and rhododendron saplings remained asymptomatic when they were infected with *Phytophthora ramorum* Werres, De Cock, and Man in't Veld and *Phytophthora kernoviae* Brasier, Beales, and S.A. Kirk, two invasive species affecting ornamental and natural ecosystems. In 2006, imported ornamental *Grevillea* plants, which were asymptomatic, were found to be infected with *Phytophthora niederhauserii* Z.G. Abad and J.A. Abad [30]. Thus, visual screening for monitoring *Phytophthora* without complementary tests is not an appropriate management tool. The direct isolation of *Phytophthora* species on semiselective media from affected tissue or baiting techniques do not always generate quick and sensitive results, making it difficult to accurately monitor forest areas [5,20]. Economic and environmental losses caused by *Phytophthora* worldwide [31,34,35] require the use of all available techniques to detect and identify invasive species as quickly as possible. Combining direct isolation and baiting techniques with molecular tools, such as quantitative real-time PCR, increases the specificity, reproducibility, and sensitivity of the assessments, adding efficiency and accuracy to the diagnosis, an essential part of forest management strategies.

The aim of this study was to verify the presence of *Phytophthora* species in the holm oak rhizosphere in southwestern Spanish dehesas and in a northeastern Spanish holm oak woodland. In addition, the association between the *Phytophthora* species and the symptomatology of the holm oaks was studied in the dehesas by taking samples from declining and non-declining stands. For this purpose, different *Phytophthora* spp. isolation and detection approaches were performed: Direct isolation on semiselective media and apple and soil baiting using leaf material. Moreover, as *P. cinnamomi* and *P. quercina* are considered among the main pathogens associated with holm oak decline, their presence and relative abundance were studied in the samples using specific TaqMan real-time PCR probes.

2. Materials and Methods

2.1. Study Sites and Sampling

Studies were conducted in autumn 2012 and 2013 at 10 and 15 mature dehesas, respectively, located in the Extremadura region (southwestern Spain) (Table 1). This region has siliceous soils with *Pyro bourgaeanae-Querceto rotundifoliae sigmetum* vegetation series, and calcareous soils with *Paeonio coriaceae-Querceto rotundifoliae sigmetum* vegetation series, within an altitude ranging from 300 to 600 m [36]. At each site, two different areas were studied: A declining area where three symptomatic trees were randomly selected and a non-declining area with three randomly selected asymptomatic trees. Trees severely affected by aerial pathogens or insect pests were discarded. In the 2012 survey,

one soil sample including fine roots from the rhizosphere around the base of each tree was collected (60 samples in total) by making three 20–30 cm deep holes at approximately 1 m distance from the trunk and bulked, obtaining a representative 0.5 kg sample, as described by Pérez-Sierra et al. [18] (Table 1). In the 2013 survey, sites 1 to 5 were sampled as described above, but in the remaining ten sites, two pooled samples per site were collected (Table 1). In sites 6 to 15 from 2013, one pooled sample from 3 declining trees and one pooled sample from 3 non-declining trees were collected at approximately 1 m distance from the trunk of each tree at 20–30 cm depth. Fifty samples in total were collected in 2013.

Table 1. Description of the survey conducted in 2012 and 2013 in the dehesas of the Extremadura region and in 2013 in the oak woodland of Montseny Biosphere Reserve.

2012 Dehesas			
Site	**Number of Samples**	***X* Coordinate**	***Y* Coordinate**
1	6	748324.99	4428259.51
2	6	248632.54	4460613.6
3	6	752500	4418487
4	6	694464.02	4431470.91
5	6	752500	4418487
6	6	750948.57	4437972.39
7	6	742685	4456109
8	6	753940.25	4450439.88
9	6	248428	4459568
10	6	749280	4457282
2013 Dehesas			
Site	**Number of Samples**	***X* Coordinate**	***Y* Coordinate**
1	6	748324.99	4428259.51
2	6	248632.54	4460613.6
3	6	752500	4418487
4	6	694464.02	4431470.91
5	6	761398,91	4425067.28
6	2	750948.57	4437972.39
7	2	742685	4456109
8	2	753940.25	4450439.88
9	2	248428	4459568
10	2	749580	4457274
11	2	279799	4430500
12	2	285614.18	4435261.32
13	2	281973	4432507
14	2	724766.4	4438845.56
15	2	246007.77	4396525.56
2013 Montseny Biosphere Reserve (Oak Woodland)			
Site	**Number of Samples**	***X* Coordinate**	***Y* Coordinate**
MS 2	1	450134	4625428
MS 6	1	458610	4621206
MS 12	1	457172	4620252
MS 13	1	457197	4620078
MS 14	1	455346	4619895
MS 16	1	454763	4621083
MS 18	1	455161	4621632
MS 22	1	455266	4618911
MS 23	1	454086	4619117
MS 24	1	453979	4619403
MS 25	1	452961	4620152
MS 26	1	452734	4619947
MS 27	1	451398	4622040
MS 28	1	450537	4622715
MS 29	1	449829	4622703

As differences in the *Phytophthora* spp. have been identified in eastern Spanish holm oak surveys [18], a study area located in northeastern Spain was included in the study. Montseny mountains is a 31,063 ha area located in Catalonia that since 1978 has been a biosphere reserve. It is made up of primarily siliceous rocks, with limestone rocks located on the western slopes of the mountains [37,38]. *Quercus ilex* is located in the lower altitudes among a *Quercetum-ilicis-galloprovinciale* vegetation series [38,39]. Fifteen holm oak declining stands showing defoliation, dead branches, and dieback symptoms and whose altitude ranged from 293 to 868 m were sampled as described above for 2012 just after a precipitation period in autumn 2013 (Table 1).

All the samples from the different surveys were transported to the laboratory, where roots were separated from soil for processing and soil was conserved at 5 °C until processing.

2.2. *Phytophthora spp.* Isolation

Roots from each sample were carefully washed under tap water and blotted on filter paper and direct isolation was performed on CMA-PARPB, as described by Jeffers and Martin [40], with and without the addition of hymexazol. Green apple baits were used for soil isolation. Granny Smith apples were surface disinfested with 95% ethanol. Four perpendicular 1 cm^2 holes were cut, filled with soil and remains of fine roots, and moistened with sterile water. These filled holes were sealed with tape and incubated in covered trays at 20 °C. The apples were examined daily until lesions developed. Small tissue fragments from the edge of the lesions were plated on CMA-PARPB with and without hymexazol and incubated at 20 °C in the dark. Oomycete-like colonies grown both from root and soil samples were transferred to potato dextrose agar (PDA) (Biokar-Diagnostics, Beauvais, France) and incubated at 20 °C in the dark for 7 days for further identification. Pure cultures of all putative *Phytophthora* isolates were obtained by transferring single hyphal tips to PDA plates.

Additionally, in the 2013 surveys, soils were also baited using leaflets of *Camellia* sp., *Rhododendron* sp., and *Viburnum* sp., following the methods described by Jung et al. [41,42]. Isolations were made using CMA-PARBPH as the selective agar medium [40] and processed as described above.

2.3. *Culture DNA Extraction, Sequencing, and Statistical Analyses*

DNA was extracted from pure cultures of putative *Phytophthora* grown on PDA by scraping the mycelium and grinding to a fine powder under liquid nitrogen, using the commercial kit EZNA Plant Miniprep Kit (Omega Bio-Tek, Doraville, GA, USA) following the manufacturer's instructions. Ribosomal DNA ITS amplifications were carried out using the universal primers ITS6 and ITS4 [43,44]. The PCR reaction final volume was 25 µL: PCR buffer 1×, 2.5 mM $MgCl_2$, 200 µM each dNTP, 0.4 µM of each primer, 1 U of DNA Taq polymerase (Dominion MBL, Córdoba, Spain), and 1 µL of template DNA. All PCR reactions were performed in a PTC 200 thermocycler (MJ Research Inc., Waltham, MA, USA) with the following parameters: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; and 72 °C for 10 min. Amplified products were sequenced at Macrogen Europe (Amsterdam, The Netherlands). The isolates were identified to the species level by conducting Basic Local Alignment Search Tool (BLAST) and comparing with the sequence data on international collection databases (*Phytophthora* Database, https://www.phytophthoradb.org and GenBank, https://www.ncbi.nlm.nih.gov/genbank/).

The total number of *Phytophthora* spp. isolates (*Phytophthora* pool) obtained and the number of isolates from each *Phytophthora* species were converted into frequencies relative to the total number of *Phytophthora* isolates recovered in the dehesas surveys. An analysis of variance (ANOVA) was performed with the 2012 and 2013 dehesas' data using a general linear model (GLM) in SAS version 9.0 (SAS Institute, Cary, NC, USA), in order to study the relationship between the frequency and diversity of *Phytophthora* spp. and the symptomatology shown by the trees in the dehesas. Mean values were compared using the Fischer's least significant difference (LSD) procedure at p-value = 0.05.

2.4. Environmental Samples: DNA Extraction and P. cinnamomi and P. quercina qPCRs

Roots and soil from both types of holm oak stands were tested with specific TaqMan probes for the main two oak *Phytophthora* pathogens, *P. cinnamomi* and *P. quercina* [16,45]. Each soil sample was passed through a 2 mm sieve to remove the organic matter and gravel. Once it was homogenized, 50–80 g per sample was lyophilized overnight and pulverized using FRITSCH Variable Speed Rotor Mill-PULVERISETTE 14 (ROSH, Oberstein, Germany). DNA was extracted in duplicate with the Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. The root samples were first ground using a mortar and pestle under liquid nitrogen and then extraction was performed from 60 to 80 mg using the Power Plant Pro DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA).

Real-time PCR was performed on a Rotor-Gene Q 5plex HRM (QIAGEN, Hilden, Germany) and data were analyzed with the Software Version 2.0.2. (QIAGEN) following the MIQE guidelines [46]. The primers quercina_F (GGTCTTGTCTGGCGTATGG), quercina_R (AGCTACTTGTTCAGACCGAAG), and the hydrolysis probe (6-FAM/GCTGTAAAA/ZEN/GGCGG CGGCTGTTGC/IaBlk-FQ/) designed by Català et al. [16] were used to detect *P. quercina* in DNA from all the soil and root samples collected in the study. In addition, *P. cinnamomi* was also tested with the primers P cin FF (CAATTAGTTGGGGGCCTGCT), P cin RF (GCAGCAGCAGCCGTCG), and the P cin hydrolysis probe (TTGACATCGACAGCCGCCGC) [45]. The qPCRs were performed in a total volume of 25 µL using Premix Ex TAQ (Probe qPCR; Takara Biotechnology (Dalian), Co., Ltd., China). Reactions consisted of 12.5 µL Premix Ex Taq (2×), 2.5 µL of primers–probe mix (500 nM of each primer and 250 nM probe), 1 µL of BSA (5 mg/mL) and 2 µL of template DNA. Two-step PCR was performed with the following cycling conditions: 95 °C for 1 min; 45 cycles of 95 °C for 5 s and 60 °C for 45 s for *P. quercina*, while for *P. cinnamomi*, 45 cycles of 95 °C for 5 s and 60 °C for 60 s. Two replicates were performed alongside standard dilution curves of *P. quercina* (isolate Ps-982 from Mediterranean Agroforestry Institute–UPV collection) and *P. cinnamomi* (isolate Ps-727 from Mediterranean Agroforestry Institute–UPV collection). Probe sensitivity was tested with serial dilution of each DNA ranging from 0.2 ng/µL to 2 fg/µL for *P. quercina* DNA; 2 ng/µL *P. cinnamomi* DNA (2 ng/µL) was serially diluted (1:10, $1{:}10^2$, $1{:}10^3$, $1{:}10^4$, $1{:}10^5$, $2{:}10^6$). Negative samples were diluted and tested again to avoid false negatives.

3. Results

3.1. Phytophthora spp. Isolation

In the 2012 survey, *Phytophthora* spp. were detected in three dehesas, which represented 30% of the sampled sites. Three isolates of *Phytophthora* were recovered through the apple baiting method, one of each of: *Phytophthora cambivora* (Petri) Buisman (from a non-declining site), *P. cinnamomi*, and *Phytophthora gonapodyides* (H.E. Petersen) Buisman (from declining sites) (Table 2).

In 2013, the dehesas were surveyed and the soils baited in addition to the other methods already described for 2012. *Phytophthora* spp. were recovered in the 2013 dehesas survey from 21 holm oak samples, which represented 42% of the total samples, with 20% from declining samples and the remaining 22% from non-declining samples. A total of 165 Oomycetes isolates were obtained in 2013: 59 *Phytophthora* spp. isolates (clustered into four species) and 107 *Pythium* spp. isolates. In 2013, 39% of the *Phytophthora* spp. isolates were recovered from declining sites, and 61% were recovered from non-declining sites (Table 3). Regarding the isolation method, 13.4% of the *Phytophthora* spp. isolates were isolated directly from roots, 5.1% from apple baits, and 81.3% from leaf baits. As for the diversity of species obtained in 2013, *P. cinnamomi*, *P. gonapodyides*, *Phytophthora megasperma* Drechsler and *Phytophthora pseudocryptogea* Safaiefarahani, Mostowfizadeh, G.E. Hardy, and T.I. Burgess were isolated (Table 3). The range of abundance according to isolation was 39% *P. cinnamomi*, 35.6% *P. gonapodyides*, 20.3% *P. megasperma*, and 5.1% *P. pseudocryptogea*.

Table 2. Number of isolates of *Phytophthora* spp. obtained from *Quercus ilex* roots and soil in the 2012 survey in the dehesas of the Extremadura region according to the symptomatology of the sampled trees and results of the TaqMan real-time PCR assays. Results obtained from samples in each site are grouped according to whether samples were from declining or non-declining trees.

Site	Symptomatology	Isolates			qPCR			
					CIN		QUE	
		CAM	CIN	GON	Roots	Soil *	Roots	Soil *
1	d	0	0	1 [a]	ndt	0/3	ndt	3/3
1	nd	0	0	0	ndt	2/3	ndt	3/3
2	d	0	0	0	ndt	0/3	ndt	1/3
2	nd	0	0	0	ndt	0/3	ndt	1/3
3	d	0	0	0	ndt	0/3	ndt	2/3
3	nd	0	0	0	ndt	1/3	ndt	0/3
4	d	0	0	0	ndt	0/3	ndt	1/3
4	nd	0	0	0	ndt	0/3	ndt	3/3
5	d	0	0	0	ndt	0/3	ndt	2/3
5	nd	0	0	0	ndt	1/3	ndt	1/3
6	d	0	0	0	ndt	1/3	ndt	3/3
6	nd	0	0	0	ndt	0/3	ndt	2/3
7	d	0	0	0	ndt	0/3	ndt	2/3
7	nd	0	0	0	ndt	0/3	ndt	3/3
8	d	0	0	0	ndt	2/3	ndt	2/3
8	nd	0	0	0	ndt	0/3	ndt	3/3
9	d	0	0	0	ndt	2/3	ndt	0/3
9	nd	1 [a]	0	0	ndt	1/3	ndt	2/3
10	d	0	1 [a]	0	ndt	1/3	ndt	3/3
10	nd	0	0	0	ndt	0/3	ndt	3/3

d = declining; nd = non-declining; CAM = *Phytophthora cambivora*; CIN = *Phytophthora cinnamomi*; GON = *Phytophthora gonapodyides*; QUE = *Phytophthora quercina*; [a] = isolated from soil with apple baiting; ndt = not determined; * = number of positive samples detected out of the total number of samples.

Table 3. Number of isolates of *Phytophthora* spp. obtained from *Q. ilex* roots and soil in the 2013 survey in the dehesas of the Extremadura region according to whether samples were from declining or non-declining trees and results of the TaqMan real-time PCR assays. PCR results obtained from samples in sites 1 to 5 are grouped according to whether samples were from declining or non-declining trees.

Site	Symptomatology	Isolates				qPCR			
						CIN		QUE	
		CIN	GON	PSC	MEG	Roots *	Soil *	Roots *	Soil *
1	d	0	1 [b]	0	0	0/3	2/3	2/3	3/3
1	nd	4 [r,b]	8 [r,b]	0	4 [b]	2/3	2/3	3/3	3/3
2	d	0	0	0	0	0/3	1/3	2/3	1/3
2	nd	0	3 [b]	0	3 [b]	1/3	2/3	1/3	3/3
3	d	4 [b]	0	0	0	3/3	2/3	0/3	0/3
3	nd	0	0	0	0	2/3	1/3	1/3	1/3
4	d	0	0	0	2 [b]	0/3	2/3	3/3	3/3
4	nd	0	3 [b]	0	0	0/3	1/3	2/3	3/3
5	d	3 [b]	0	0	0	2/3	0/3	1/3	2/3
5	nd	2 [b]	6 [b]	0	0	1/3	1/3	3/3	3/3
6	d	0	0	0	0	−	−	+	−
6	nd	0	0	0	0	−	−	−	+
7	d	0	0	0	0	+	+	+	+
7	nd	0	0	0	0	−	−	+	+
8	d	0	0	0	0	+	−	−	+
8	nd	0	0	0	0	+	−	+	+

Table 3. *Cont.*

Site	Symptomatology	Isolates				qPCR			
						CIN		QUE	
		CIN	GON	PSC	MEG	Roots *	Soil *	Roots *	Soil *
9	d	0	0	0	0	−	−	−	+
9	nd	0	0	0	1 [b]	+	+	+	−
10	d	3 [r,b]	0	0	0	+	+	+	−
10	nd	0	0	0	0	−	+	−	−
11	d	1 [b]	0	0	0	−	+	+	−
11	nd	0	0	0	0	−	−	+	+
12	d	0	0	0	0	+	−	−	+
12	nd	0	0	0	0	+	−	−	+
13	d	0	0	0	0	+	+	−	+
13	nd	0	0	0	0	−	−	+	−
14	d	0	0	0	1 [b]	−	−	−	−
14	nd	0	0	0	0	−	−	+	+
15	d	5 [a,b]	0	2 [b]	0	+	+	+	+
15	nd	1 [r]	0	1 [a]	0	+	−	−	+

d = declining; nd = non-declining; CIN = *P. cinnamomi*; GON = *P. gonapodyides*; MEG = *Phytophthora megasperma*; PSC = *Phytophthora pseudocryptogea*; QUE = *P. quercina*; [r] = isolated from roots; [a] = isolated from soil with apple baiting; [b] = isolated from baiting soil with leaves; * = number of positive detected samples out of the total number of samples; + = positive; − = negative.

Twenty-three isolates of *P. cinnamomi* were isolated from eight samples in the dehesas in 2013 (Table 3); percentages from declining and non-declining samples are shown in Figure 1. Twenty-one cultures of *P. gonapodyides* were isolated from five samples, with most of the samples from non-declining sites (Table 3, Figure 1). Twelve *P. megasperma* isolates were isolated from five samples (Table 3), and most of these samples were from non-declining trees (Figure 1). Three *P. pseudocryptogea* cultures were isolated from two samples (Table 3, Figure 1).

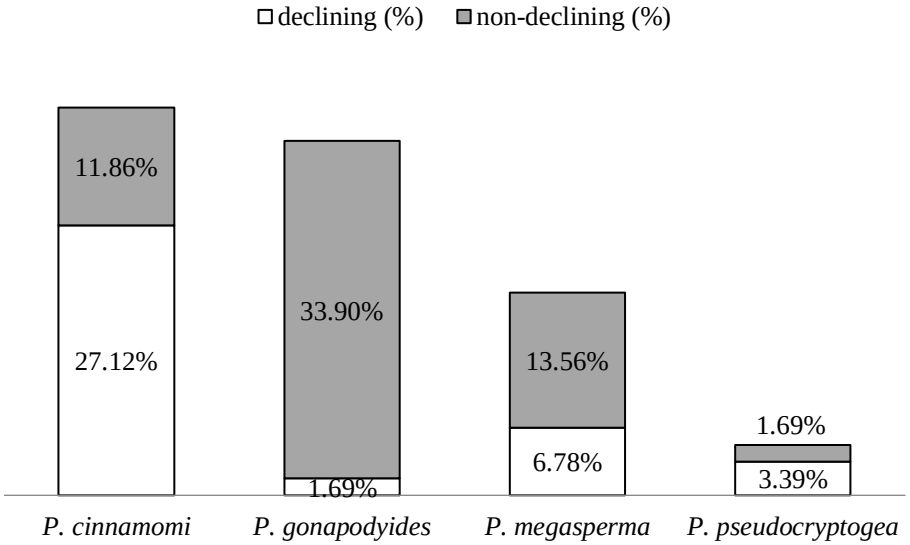

Figure 1. Percentage of each *Phytophthora* species cultures isolated in the dehesas 2013 survey according to whether the holm oaks were declining or non-declining.

The statistical analysis showed that the factors' symptomatology (p-value = 0.3626) and dehesa (p-value = 0.3087) were not significant for the frequency of the different *Phytophthora* species present in the dehesas in 2013. Considering the different species isolated separately, only the presence of *P. gonapodyides* was significantly higher in non-declining samples (p-value = 0.0366). The presence of either one species or another was not significantly associated with the dehesa factor (*P. cinnamomi*

p-value = 0.2277, *P. gonapodyides* *p*-value = 0.9176 and *P. megasperma* *p*-value = 0.7029). *P. pseudocryptogea* was only isolated in one dehesa, but from both declining and non-declining sites.

No *Phytophthora* isolates were recovered from the 15 samples of the Montseny Biosphere Reserve by direct isolation on semiselective media from affected tissues and/or the baiting.

3.2. Environmental Samples: Hydrolysis Probes—*P. cinnamomi* and *P. quercina* qPCRs

The *Phytophthora quercina* standard curve plot showed that the correlation between the Cq-value and the DNA concentration was high (r^2 = 0.99966), with an efficiency of 0.90389. For *P. quercina*, the limit of detection (LOD) was established at a DNA concentration of 2 fg/µL.

Phytophthora quercina was detected in all the surveyed dehesas in 2012 (65.1% of the samples). Of these, 31.8% came from declining holm oak trees and 33.3% from non-declining trees (Table 2). In 2013, *P. quercina* was detected in all the surveyed dehesas (79.6% of the samples) (Table 3). A total of 66.7% of the soil samples were positive for *P. quercina*: 27.8% were from declining soil samples, while 38.9% were from non-declining soil samples. A total of 55.6% of the root samples were positive for *P. quercina*: 24.1% were from declining holm oak fine roots, while 31.5% were from non-declining holm oak roots. In the survey conducted in Montseny Biosphere Reserve in 2013, 66.7% of the samples were positive for *P. quercina*, of which 40% were from root samples and 53.3% from soil samples (Table 4).

Table 4. Results of the TaqMan real-time PCR assays obtained from *Q. ilex* roots and soil in the 2013 survey in the oak woodland of Montseny Biosphere Reserve.

Site	qPCR			
	CIN		QUE	
	Roots	Soil	Roots	Soil
MS 2	−	+	+	+
MS 6	+	+	−	−
MS 12	+	+	−	−
MS 13	−	−	+	+
MS 14	−	−	−	+
MS 16	−	−	−	+
MS 18	−	−	−	−
MS 22	−	−	+	+
MS 23	+	−	+	+
MS 24	+	+	+	−
MS 25	−	−	−	−
MS 26	+	+	+	−
MS 27	−	−	−	+
MS 28	+	−	−	−
MS 29	+	−	−	+

CIN = *P. cinnamomi*. QUE = *P. quercina*. + = positive detection; − = negative detection.

The *Phytophthora cinnamomi* standard curve revealed a high correlation between the Cq-value and the DNA concentration (r^2 = 0.99731), with a reaction efficiency of 0.92014. The LOD was established at 4 fg/µL.

Phytophthora cinnamomi was detected in seven out of the ten surveyed dehesas in 2012 (19.7% of the samples) (Table 2). A total of 12.1% of the soil detections came from declining holm oak trees and 7.6% from non-declining. In 2013, *P. cinnamomi* was detected in 11 dehesas (57.4% of the samples) (Table 3). A total of 38.9% of the soil samples were positive for *P. cinnamomi*; 22.2% were from declining trees, while 16.7% were from non-declining trees. A total of 33.9% of the root samples were positive for *P. cinnamomi*, with 22.2% from declining oak fine roots and 14.8% from non-declining oak roots. In Montseny Biosphere Reserve, 53.3% of the samples were positive for *P. cinnamomi*, of which 46.7% were from root samples and 33.3% from soil samples (Table 4).

4. Discussion

This study provides evidence that molecular approaches complement direct isolation methods of *Phytophthora* species from fine roots from holm oak in natural (Montseny Biosphere Reserve) and seminatural (dehesas) ecosystems, confirming that it is not only *P. cinnamomi* that is involved in the holm oak decline in Spain, but *P. quercina* is also present. Moreover, this is the first report of *P. pseudocryptogea* in Europe.

Regarding traditional isolation methods, an increase in *Phytophthora* isolation was observed in the dehesas in 2013 compared with the sampling conducted in the dehesas in 2012. This was probably not only due to the implementation of the leaf baiting technique, but also because in 2013, isolation of *Phytophthora* spp. from fine roots was more successful. This could be explained by the fact that under favorable environmental conditions, *Phytophthora* spp. infected the tree root systems and rotted fine roots containing the pathogens detached from the plant, so the pathogens can establish again in the soil [47]. *Phytophthora* spp. dispersion requires warm temperatures and free water to produce infective zoospores; if not, they remain as resistant structures in the soil [34]. Furthermore, the efficiency of *Phytophthora* isolation techniques can be compromised by the climatic conditions suffered during the period previous to the survey and by the presence of other microorganisms [8,34]. In fact, the dehesa regions where the surveys were conducted received less precipitation in 2012 than in 2013 [48]. Thus, according to this, the environmental conditions for *Phytophthora* spp. isolation were more favorable in 2013 than in 2012 in the southwestern Spanish dehesas, as they were recovered in 2013 from fresh lesions [49]. Another possible explanation for the low efficiency of *Phytophthora* recovery in Montseny Biosphere Reserve is the presence of other fast-growing species in the samples, such as *Pythium* spp., making the isolation difficult. *Pythium* spp. were recorded in very low numbers in dehesas in 2012 (explained by the absence of favorable environmental conditions), but their presence was very relevant in the 2013 dehesas and in the Montseny Biosphere Reserve surveys. The genus *Pythium* is present in almost all soils and, as the isolation medium used for *Phytophthora* isolation is semiselective [34,40], *Pythium* spp. were also isolated with a high frequency in our study and were able to mask *Phytophthora* spp. presence.

Oak decline, associated with abiotic and biotic factors, has been occurring across Europe during the past decades [4,11,22,28,42,50]. Among the several *Phytophthora* species that have been associated with this decline, *P. cinnamomi* has been considered the main biotic factor responsible for oak mortality in Spain since the 1990s [8,9,51]. In 2013, *P. cinnamomi* was the most frequent species isolated in the infested dehesas, as was expected according to previous studies [7,52]. In addition to *P. cinnamomi*, Corcobado et al. [15] reported that *P. gonapodyides* was also involved in oak decline in this region. In our surveys, *P. gonapodyides, P. megasperma*, and *P. pseudocryptogea* were recovered at low frequencies, and these species may play an important role as causal agents of the disease, as reported in other studies, where they were also recovered at low frequencies [14,25,28,52]. There is no statistical evidence to support a differential distribution of *Phytophthora* species among the dehesas in 2013. Moreover, statistical results indicated that there was not a significant relationship between the *Phytophthora* spp. isolation frequency and the symptomatology of the holm oak stands. Our results showed a higher percentage of *Phytophthora* spp. recovery in 2013 from non-declining sites than from declining sites. *Phytophthora cinnamomi*, which was found in six dehesas, either from declining or from non-declining trees, is a primary root pathogen of woody species, considered a hemibiotrophic organism with life strategies which can change from biotrophic to necrotrophic, according to the environmental conditions [53–55]. This species is also present in plant reservoirs and, depending on its behavior, will determine if the plant remains asymptomatic or not [54–57]. Furthermore, *P. cinnamomi* is highly aggressive to holm oaks, as demonstrated previously [11,12,25,58–60]. Tsao [61] stated that a certain percentage of lost roots is required for symptoms to emerge and our results in the 2013 survey provide evidence that a tree symptomatology is not always an indication about the conditions of its root system. Statistical analyses showed that *P. gonapodyides* is more frequent in non-declining stands, in agreement with the results of Vettraino et al. [28], while the other species found did not show any statistical

pattern. *Phytophthora gonapodyides* is known to attack the small or fine feeder roots [62] and to produce a wilting toxin [41]. Nevertheless, Brasier et al. [62] stated that *P. gonapodyides* is often in balance with the unstressed oak root system, but this can change under stress conditions, contributing to a rapid decline. Hansen et al. [63] suggested that some *Phytophthora* spp. from clade 6 ecologically related to *Phytophthora chlamydospora* could cause limited root damage with no above ground disease symptoms contributing to the oak decline. *Phytophthora megasperma* isolated in the present study had been previously associated with oak decline [28,42]. Although it is considered a pathogen of herbaceous plants and agricultural trees, it can become a serious problem when the oak balance is broken due to other factors, such as droughts or waterlogging [34]. A similar behavior has been indicated for other *Phytophthora* spp. such as *P. gonapodyides* [10]. *Phytophthora megasperma*, *P. quercina*, *P. psychrophila*, *Phytophthora drechsleri*, and *Phytophthora syringae* have also been associated with oak decline [18,19,25], although these species were not found in our samples. Nevertheless, *P. pseudocryptogea* in the present study was isolated for the first time in Europe and from a holm oak-rangeland in Spain. This species was described by Safaiefarahani et al., who re-evaluated the *P. cryptogea* complex [64]. Although it was isolated from three soil samples in the present study, from three soil samples, the role of *P. pseudocryptogea* in holm oak decline remains unknown. The pathogenicity of this species in holm oak should be further studied.

The results obtained with the *P. quercina* probe are relevant, since it has always been thought that the holm oak decline in acidic soils in Spain is caused primarily by *P. cinnamomi. Phytophthora cinnamomi* was present in a high number of samples in both study locations, as was expected, but surprisingly it was not the most frequent species detected. *Phytophthora quercina* was shown as the most frequent species in this study, and the number of positive samples was higher in both studied areas. Molecular diagnoses provide faster and more sensitive detection of *Phytophthora* spp. [16,45,65–72].

5. Conclusions

Different *Phytophthora* species were detected and identified in the study areas, regardless of whether they cause symptoms of decline or not. Further research is needed to clarify the effect of these pathogens in combination and abiotic factors in the oak stands. The implementation of the different direct and baiting isolation techniques for the isolation of *Phytophthora* spp., along with the available molecular detection techniques, allows a better diagnosis and understanding of the role of *Phytophthora* spp. in the holm oak forest areas.

Author Contributions: B.M.-S. conducted field sampling, performed experimental work and data analysis in lab experiments, discussed the results, and wrote the paper. M.B. participated in the data analyses, discussed the results, and revised the manuscript. P.A.-C. designed the study and revised the manuscript.

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