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Susceptibility of Iberian trees to *Phytophthora ramorum* and *P. cinnamomi*

E. Moralejo*, J. A. García-Muñoz and E. Descals

Institut Mediterrani d'Estudis Avançats, IMEDEA (CSIC-UIB). Miquel Marquès 21, 07190 Esporles, Balearic Islands, Spain

The capacity of *Phytophthora ramorum* to colonize the inner bark of 18 native and two exotic tree species from the Iberian Peninsula was tested. Living logs were wound-inoculated in a growth chamber with three isolates belonging to the EU1 and two to the NA1 clonal lineages of *P. ramorum*. Most of the *Quercus* species ranked as highly susceptible in experiments carried out in summer, with mean lesion areas over 100 cm^2 in *Q. pubescens*, *Q. pyrenaica*, *Q. faginea* and *Q. suber* and as large as 273 cm^2 in *Q. canariensis*, *ca*. 40 days after inoculation. *Quercus ilex* ranked as moderately susceptible to *P. ramorum*, forming lesions up to 133 cm^2 (average 17·2 cm^2). *Pinus halepensis* and *P. pinea* were highly susceptible, exhibiting long, narrow lesions; but three other pine species, *P. pinaster*, *P. nigra* and *P. sylvestris*, were resistant to slightly susceptible. No significant difference in aggressiveness was found between the isolates of *P. ramorum*. In addition, there was evidence of genetic variation in susceptibility within host populations, and of significant seasonal variation in host susceptibility in some *Quercus* species. The results suggest a high risk of some Iberian oaks to *P. ramorum*, especially in forest ecosystems in southwestern Spain, where relict populations of *Q. canariensis* grow amongst susceptible understory species such as *Rhododendron ponticum* and *Viburnum tinus*. One isolate of *P. cinnamomi* used as positive control in all the inoculations was also highly aggressive to Iberian oaks and *Eucalyptus dalrympleana*.

Keywords: invasive plant pathogen, Mediterranean forests, *Phytophthora hedraiandra*, *Phytophthora polonica*, *Phytophthora syringae*, sudden oak death

Introduction

The Iberian Peninsula (Spain and Portugal) is one of the main centres of diversity of the genus *Quercus* in Eurasia, with more than nine native species forming extensive forests and woodlands (Ruiz de la Torre, 2002). Since the early 1980s, extensive areas of holm oak (*Quercus ilex*) and cork oak (*Q. suber*) woodlands in the southwest of the Iberian Peninsula have been severely damaged by *Phytophthora cinnamomi*, an alien and invasive pathogen infecting roots of many woody species (Brasier *et al*., 1993; Brasier, 1996; Moreira & Martins, 2005). The disease is characterized by a severe decline and occasional sudden death of the oak trees (Sánchez *et al*., 2002).

Since 1995, a progressive decline and sudden death of coast live oaks (*Q. agrifolia*), California black oaks (*Q. kelloggii*) and tan oaks (*Lithocarpus densiflorus*), superficially resembling that of the Iberian oaks, has occurred in coastal areas of central California. In 2000, the causal agent of sudden oak death (SOD) in California was demonstrated to be *Phytophthora ramorum* (Rizzo *et al*., 2002), a newly described species which had been previously isolated from infected rhododendron and viburnum ornamentals in European nurseries (Werres *et al*., 2001).

Phytophthora ramorum is a non root-invading pathogen and grows at lower temperatures than *P. cinnamomi*. Unlike the evergreen oak disease of Iberia, SOD is characterized by the presence of above-ground infections on the trunk caused by aerially dispersed propagules, often leading to bleeding cankers (Davidson *et al*., 2005). Both oak diseases are caused by exotic Phytophthoras invading natural ecosystems mostly in Mediterranean-type climates. However, *P. cinnamomi* is a relatively old invader, already having a heavy impact on some woodlands in three of the five Mediterranean biomes of the world (Weste & Marks, 1987; Sánchez *et al*., 2002), whereas the risk posed by *P. ramorum* in other areas outside California and Oregon is a controversial issue.

To date, *P. ramorum* is an internationally regulated pathogen subject to quarantine, with negative effects on the ornamental plant trade. Although all *P. ramorum* isolates are considered conspecific, the American and European populations probably have a different provenance. They differ in their mating type, genetic profiles (AFLPs and SNPs), phenotypes and adaptive trajectories, reflecting at least two independent introduction

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*E-mail: vieaemr@uib.es

events (Brasier *et al*., 2006a; Ivors *et al*., 2006). More than 90 plant species have been reported so far as natural hosts (COMTF, http://nature.berkeley.edu/comtf/html/host), and the number of potential hosts determined in the laboratory, either for Europe or other geographic areas, is rapidly increasing (> 100; RAPRA Project, http://rapra.csl.gov.uk/). Through the ornamental trade, *P. ramorum* has spread into nurseries across Europe including the Mediterranean area (Moralejo & Werres, 2002). This increases the risk of transfer to natural or semi-natural ecosystems, as has already occurred locally in southwest England and the Netherlands (Brasier *et al*., 2004; Brasier, 2008).

Southern Europe, including the Mediterranean basin, is considered a risk area for the establishment of *P. ramorum* on natural vegetation (Brasier *et al*., 2004; Brasier & Jung, 2006; Moralejo *et al*., 2006a). This concern is supported by preliminary *in vitro* studies on the susceptibility of holm oak forest species (Moralejo *et al*., 2006a,b), by recent findings of natural infections on native Mediterranean species planted or naturalized in the UK, e.g. *Rhododendron ponticum*, *Viburnum tinus* and *Q. ilex* (Denman *et al*., 2005a,b) and by taxonomic affinities between constituents of the Californian and Mediterranean ecosystems. Furthermore, recent ecological niche models, based on SOD distribution in California, predict moderate to high risks of establishment of *P. ramorum* in Spain and Portugal (Kluza *et al*., 2007). These are consistent with previous risk maps for Europe built with the software CLIMEX, indicating high similarity indexes between SOD areas in California and those in northwest Spain and Portugal (R. Baker, CSL, UK, personal communication). Although these models are excellent prospective tools for pest risk assessment, they lack essential information of the pathogen-host(s)-environment disease triangle. A more realistic estimation of the risk of disease establishment can only be attained if data on the geographical distribution of the hosts are integrated into the model. Advanced prediction models should also include the risk of tree mortality if the disease becomes established, given that this phase of *P. ramorum* epidemiology has significant ecological and economic implications.

Brasier & Kirk (2001) developed an inoculation method on detached living logs to estimate the ability of *Phytophthora* pathogens to colonize the cambial zone of major tree species in the UK. In the case of *P. ramorum*, it has proved useful to predict the inner bark (phloem) susceptibility of tree species to infection in recent field outbreaks in the UK (Brasier *et al*., 2004, 2006b). Similar log tests performed by Hansen *et al*. (2005) have confirmed already known susceptibilities of forest species from the western coast of the USA, such as tan oak, and they have also detected other potential hosts. Using the log inoculation method, the capacity of *P. ramorum* and other *Phytophthora* species, including *P. cinnamomi*, to invade stems of major Mediterranean tree species from the Iberian Peninsula and the Balearic Islands has been assessed, in an attempt to evaluate the risk posed to their forest habitats.

Materials and methods

Isolates

Five isolates of *P. ramorum* were used in all the experiments, three from the European lineage UE1: P1376 (obtained from *Viburnum tinus* cv. Eve Price), P1577 (from *Rhododendron catawbiensis*) and P1578 (from *Rhododendron grandiflora*); and two from the North American clonal lineage NA1: P1403 (*Vaccinium ovatum*; Oregon, USA) and P1579 (*Quercus agrifolia*, California, USA). For comparative purposes for assessing the aggressiveness of *P. ramorum*, one isolate of *P. cinnamomi*, a well-known pathogen of roots of oak trees, and *P. syringae*, an *a priori* less aggressive pathogen to trees, were initially included in the experiment. However, at the onset of the experiments the presence of the newly described species *P. hedraiandra* was discovered in nurseries in Spain (de Cock & Lévesque, 2004; Moralejo *et al*., 2007) and the new species *P. polonica* was also described (Belbahri *et al*., 2006). *Phytophthora hedraiandra* is usually found in ornamental nurseries in Spain infecting rhododendron and viburnum (Moralejo *et al*., 2007); hence, there is an increasing interest in knowing its potential host range. Therefore, single isolates of *P. hedraiandra* (P3842) and *P. polonica* (CBS119650) substituted *P. syringae* in the inoculations as positive controls. Stock cultures were maintained on carrot agar (CA) (Brasier, 1967) at 20°C without illumination. Isolates were grown on CA in 90 mm diameter Petri dishes for 5–10 days at 20°C under continuous white light.

Plant material

Twenty tree species were selected for the experiments, ranging from hygrophilous to drought-tolerant. Together they represent more than 80% of the Mediterranean and sub-Mediterranean forest surface of the Iberian Peninsula. Temperate tree species of northern Spain, such as *Fagus sylvatica*, *Quercus robur* and *Castanea sativa*, were excluded as they have been studied by others in the UK. The list of tree species and sampling sites are provided in Table 1. Most of the tree species were felled in summer since previous inoculation tests carried out by others suggested a higher susceptibility to *P. ramorum* in that season. But due to lack of space in the quarantine chamber and to limited availability of plant material in summer, some tree species were felled in winter. If species inoculated in winter showed occasionally severe lesions, the inoculations were repeated in the following summer (e.g. *Quercus* species).

In the field, previously marked healthy trees, about 20–30 cm diameter at breast height, were felled at ground level by the Forest Services of the regional governments of the Balearic Islands and Catalonia. Single logs 1–1·2 m long × 15–25 cm diameter were sampled from each felled tree. The bottom and top ends of the logs were identified and immediately sealed with a grafting synthetic resin (Arbokol, Kollant Ltd.) to retard drying. The logs were

Table 1 Tree species used in the inoculation experiments, with habitats and sources

Species	Family	Common name	Life form	Forest type[a]	Locality[b]
Acer monspessulanum	Aceraceae	Montpellier maple	Deciduous tree	sMed	Prades, Catalonia
Arbutus unedo	Ericaceae	Strawberry tree	Evergreen shrub	Med	Genova, Mallorca
Celtis australis	Cannabaceae	European nettle tree	Deciduous tree	Med	Binissalem, Mallorca
Corylus avellana	Betulaceae	Common hazel	Deciduous tree	sMed-temp	Prades, Catalonia
Cupressus sempervirens	Cupressaceae	Cypress	Evergreen conifer	Exotic (Med)	Esporles, Mallorca
Eucalyptus dalrympleana	Myrtaceae	White mountain gum	Evergreen tree	Exotic	Hostalrich, Catalonia
Fraxinus angustifolia	Oleaceae	Narrow-leaf ash	Deciduous tree	Med-sMed	Hostalrich, Catalonia
Ilex aquifolium	Aquifoliaceae	European holly	Evergreen shrub	sMed-temp	Prades, Catalonia
Olea europaea	Oleaceae	Olive	Evergreen tree	Med	Porreres, Mallorca
Pinus halepensis	Pinaceae	Aleppo pine	Evergreen conifer	Med	Bunyola, Mallorca
Pinus nigra	Pinaceae	Black pine	Evergreen conifer	Med-sMed	Prades, Catalonia
Pinus pinaster	Pinaceae	Maritime pine	Evergreen conifer	Med-sMed	Prades, Catalonia
Pinus pinea	Pinaceae	Stone pine	Evergreen conifer	Med	Arbucies, Catalonia
Pinus sylvestris	Pinaceae	Scots pine	Evergreen conifer	sMed-temp	Prades, Catalonia
Quercus canariensis	Fagaceae	Algerian oak	Semi-deciduous tree	Med-sMed	Hostalrich, Catalonia
Quercus faginea	Fagaceae	Portuguese oak	Semi-deciduous tree	Med-sMed	Arbucies, Catalonia
Quercus ilex	Fagaceae	Holm oak	Evergreen tree	Med	Valldemossa, Mallorca
Quercus pubescens	Fagaceae	Downy oak	Semi-deciduous tree	sMed	Prades, Catalonia
Quercus pyrenaica	Fagaceae	Pyrenean oak	Semi-deciduous tree	sMed	Prades, Catalonia
Quercus suber	Fagaceae	Cork oak	Evergreen tree	Med	Arbucies, Catalonia

[a] Med = Mediterranean; sMed = subMediterranean; temp = temperate.

[b] All trees were collected from Mallorca (Balearic Islands) and Catalonia (NE Spain).

inoculated in the quarantine chamber 24–48 h after cutting. Inoculations were performed in seven independent experiments from June 2004 to June 2006 (Table 2).

Log inoculation technique and lesion measurement

The protocol of Brasier & Kirk (2001) was followed with minor modifications. A 6 mm diameter hole was made with a flamed cork borer through the bark down to the wood and the bark plug lifted. A 6 mm diameter plug from the margin of a colony growing actively on CA was taken with the same cork borer, inserted in the hole with the aerial mycelium facing the wood. A drop of sterile distilled water was added and the bark plug replaced. Moist cotton wool was placed over this and covered with a piece of aluminium foil secured with strips of PVC tape. Eight inoculation points were arranged on the log along three transverse lanes separated by *ca.* 25 cm and each inoculation point on the lane by *ca.* 10 cm (three points at each extreme and two points in the central lane). To accommodate genetic heterogeneity within a host population, a randomized block design was used in which the inoculation points were the experimental units grouped into blocks (individual logs). Treatments (pathogen isolates/ species and controls) were randomly applied to different experimental units within the block. Thus, five of the eight points were inoculated with the *P. ramorum* isolates, one with *P. cinnamomi* and one with the negative control in all trunks and tree species, whilst the vacant point was respectively inoculated with *P. syringae*, *P. hedraindra* or *P. polonica* in different experiments. Repetition of the block inoculations, with mostly eight logs per tree species, provided the replications for the treatments. Negative controls (one per log) consisted of plain CA plugs applied as above. Each of the eight logs per tree species was coded with a capital letter (A–H) and the position of each isolate or control on the log annotated.

Inoculated logs were stood upright, sealed with double polyethylene film to ensure suitable humidity and incubated at 20 ± 1°C in the same quarantine chamber. After *ca.* 40 days, the outer bark was carefully removed with hammer and chisel and the outline of any necrotic lesions on the inner bark marked with a pen and traced on transparent paper. The resulting images were scanned. The lesion area was calculated with the Olympus DP12 Software version 3·2. To confirm the presence of live mycelium three pieces of tissue from the wood/bark interface were taken from the border and around the point of inoculation of each lesion and transferred to PARP selective medium (CA amended with 5 μg mL^{-1} of pimaricin, 100 μg mL^{-1} of ampicillin, 25 μg mL^{-1} of rifampicin and 100 μg mL^{-1} of pentachloronitrobenzene). Isolates were identified to species on colony pattern and morphology of sporangia, chlamydospores and oogonia. At the end of the experiments, the bark debris was bagged and autoclaved. The logs were smeared with a 5% bleach solution, air-dried and burnt.

Data analysis

Data were analysed using the Statistica 6·0. software package (Statsoft Inc., 2001). Differences in lesion areas for each isolate per tree species were analysed with a general linear model (GLM), treating logs as a random blocking factor, isolates as a fixed factor and lesion areas as the dependent variable. To meet ANOVA assumptions of

Table 2 Susceptibility rank of Iberian trees to *Phytophthora ramorum* and analysis of variance for bark lesions of each tree species after inoculating with seven *Phytophthora* species/isolates

		All Phytophthoras[a] ANOVA[c]			
Species	*P. ramorum* lesion[b] (range) (cm^2)	df	MS	*F*	Date
Highly susceptible to *Pr*					
Quercus suber	> 300 (13·8–793)				Jun 2006
Isolates		6	0·12	0·80	
Block		1	0·93	6·22*	
Quercus canariensis	273·2 (1·8–708·6)				Jun 2006
Isolates		6	2·07	3·7**	
Block		7	1·21	2·16	
Quercus faginea	210·1 (1·7–562·4)				Jun 2006
Isolates		6	1·03	3·56***	
Block		5	5·04	17·38***	
Quercus pyrenaica	146·2 (3·2–372·8)				Jun 2005
Isolates		6	0·44	4·41**	
Block		7	2·55	25·43***	
Quercus pubescens	109·6 (1·9–416·6)				Jun 2005
Isolates		6	1·72	5·98***	
Block		7	0·87	3·0**	
Pinus halepensis	51·1 (1·7–230·6)				Jun 2004
Isolates		6	1·14	3·92**	
Block		7	0·54	1·84	
Quercus canariensis	48·1 (1·4–245·3)				Dec 2005
Isolates		6	1·03	6·5***	
Block		7	1·92	12·14***	
Eucalyptus dalrympleana	43·0 (1·1–304·1)				Jun 2006
Isolates		6	0·78	1·74	
Block		5	0·71	1·57	
Pinus pinea	21·3 (1·1–96·7)				Jun 2006
Isolates		6	0·11	0·67	
Block		5	1·49	9·32***	
Moderately susceptible					
Quercus ilex	17·2 (0·7–133·2)				Jun 2004
Isolates		6	0·35	1·15	
Block		6	0·88	2·86*	
Arbutus unedo	14·8 (3·2–39·2)				Dec 2004
Isolates		6	0·43	14·72***	
Block		7	0·75	25·71***	
Quercus faginea	11·3 (1·3–66·2)				Dec 2005
Isolates		6	0·43	3·39**	
Block		7	1·31	10·29***	
Slightly susceptible					
Ilex aquifolium	9·2 (1·0–63·4)				Jul 2005
Isolates		6	0·03	0·28	
Block		7	0·50	5·19***	
Acer monspessulanum	8·7 (1·0–47·4)				Jul 2005
Isolates		6	0·04	0·28	
Block		7	0·4	2·90*	
Quercus suber	7·3 (1·4–42·9)				Dec 2005
Isolates		6	0·24	5·93***	
Block		6	0·46	11·35***	
Corylus avellana	6·8 (1·0–40·8)				Jul 2005
Isolates		6	0·11	1·28	
Block		7	0·62	7·19***	
Quercus ilex	5·4 (0·7–66·1)				Dec 2004
Isolates		6	0·33	3·87**	
Block		7	0·30	3·52**	
Pinus sylvestris	4·3 (1·3–17·3)				Jul 2005
Isolates		6	0·13	1·85	
Block		6	0·16	2·31	

Table 2 *Continued*

Species	*P. ramorum* lesion[b] (range) (cm^2)	All Phytophthoras[a] ANOVA[c] df	MS	*F*	Date
Pinus pinaster	4·1 (1·0–15·4)				Jul 2005
Isolates		6	0·15	2·71*	
Block		7	0·23	4·16**	
Resistant					
Pinus nigra	2·8 (0·7–12·7)				Jun 2005
Isolates		6	0·30	3·50**	
Block		6	0·05	0·59	
Celtis australis	2·1 (1·3–3·0)				Mar 2006
Isolates		6	0·00	0·47	
Block		2	0·05	9·91**	
Olea europaea	1·5 (0·8–2·9)				Dec 2004
Isolates		6	0·03	1·64	
Block		7	0·03	2·01	
Cupressus sempervirens	1·5 (0·7–2·4)				Mar 2006
Isolates		6	0·03	1·29	
Block		4	0·03	1·17	
Fraxinus angustifolia	1·2 (0·8–1·9)				Dec 2005
Isolates		6	0·01	1·02	
Block		7	0·01	1·11	

[a] Five isolates of *P. ramorum* and one isolate each of *P. cinnamomi*, *P. hedraiandra*, *P. syringae* and *P. polonica* were used across experiments.

[b] Mean lesion area and range are the pooled results of five isolates of *P. ramorum.*

[c] There were eight treatments (*Phytophthora* species/isolates) per log (blocking factor) and tree species. Negative controls were excluded from the analysis. Data were $\log_{10} + 0{\cdot}5$ transformed before analyses. Levels of significance: *($P < 0{\cdot}05$); **($P < 0{\cdot}01$); ***($P < 0{\cdot}001$).

homogeneity of variance, data sets were transformed [$\log_{10} + 0{\cdot}5$] and negative controls were excluded from the analysis. The effect of season on host susceptibility to *P. ramorum* was also examined following previous reports of seasonal variation in host susceptibility to *Phytophthora* (Brasier & Kirk, 2001; Luque *et al*., 2002; Hansen *et al*., 2005). For this purpose, trunks of *Quercus ilex*, *Q. suber*, *Q. faginea* and *Q. canariensis* were inoculated both in winter and summer. To reduce variation due to different genetic backgrounds, logs were taken from the same location (i.e. tree population). Seasonal effects on oak susceptibility were analysed using a two-way ANOVA with season and tree species as fixed factors and lesion sizes as the dependent variable. An independent *t*-test was performed for each of the four tree species to compare the effect of summer vs. winter inoculations on lesion development. Differences in aggressiveness between *P. ramorum* isolates across experiments were analysed with GLM, treating tree species as a blocking factor and lesion size as the dependent variable.

Results

Susceptibility categories

Lesion sizes caused by *Phytophthora* isolates on the tree trunks ranged from 0·71 to 793 cm^2 across the experiments (Table 2). To rank data into meaningful susceptibility categories, experimental observations ($n = 1319$) were subject to an exploratory analysis. A bar chart showing the frequency distributions of the mean lesion sizes of each of the isolate-host combinations ($n = 192$ including controls) resulted in a marked L-shaped pattern (lognormal distribution), with two major leap rungs at *ca*. 10 and 20 cm^2 (Fig. 1). This pattern largely deviated from the normal distribution to be expected if continuous rates of lesion expansion were scaled across the selected trees. Both larger leap rungs were chosen as thresholds separating relatively susceptible categories. Accordingly, trees were considered (i) resistant to an isolate/species as long as the mean lesion-sizes of replicates did not differ from those in negative controls (usually < 3 cm^2); (ii) slightly susceptible when lesion sizes were significantly larger than in negative controls but below 10 cm^2; (iii) moderately susceptible between 10 and 20 cm^2; and (iv) highly susceptible above 20 cm^2.

Isolation percentages of the *Phytophthora* species from necrotic lesions were usually high (> 85%), and, in general, they were higher in summer than in winter. No *Phytophthora* species was isolated from the negative controls. It was noticed that colonies formed from tissue derived from small lesion areas were appressed and compact (i.e. inhibited), while those from large lesions were usually more diffuse and grew faster.

Susceptibility of *Quercus* species

The outer bark normally did not show any symptoms except on cork oak, which occasionally produced tarry exudates as a response to *P. ramorum* in the winter

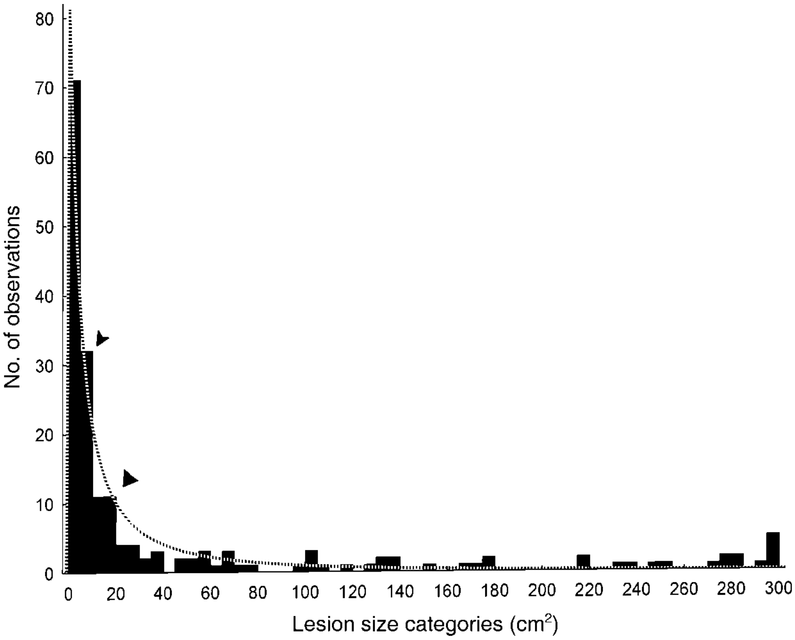

Figure 1 Log-normal distribution of frequencies of mean lesion areas (cm^2) formed by all *Phytophthora* isolate-host combinations, including negative controls ($n = 192$). The two arrowheads indicate main columns separating relative susceptibility categories. Resistant (lesions < 3 cm^2); slightly susceptible (lesions 3–10 cm^2); moderately susceptible (lesions 10–20 cm^2); and highly susceptible (lesions > 20 cm^2). Kolmogorov-Smirnov goodness of fit test: $d = 0{\cdot}09$, $P < 0{\cdot}10$; $\chi = 34{\cdot}75$, $P < 0{\cdot}01$.

inoculation. After removing the periderm, the inner bark of trunks of all inoculated oaks generally exhibited a distinctive brownish necrotic lesion, sometimes with a marked dark borderline. Six out of eight trunks of *Q. suber* inoculated in summer formed overlapping lesions. This made it difficult to distinguish between lesion areas caused by each of the isolates and controls. A closer examination of these trunks was made by removing the necrotic phloem 3–7 mm deep into the wood in order to expose the borders of the discoloured areas. This usually revealed that isolates produced large lesions, whereas controls remained around the point of inoculation. In most cases, the lesion areas on the wood next to the cambium were estimated to exceed 300 cm^2.

Phytophthora ramorum

Five out of six *Quercus* species ranked in the highly susceptible (lesions > 20 cm^2) category when inoculated with *P. ramorum* (Fig. 2). The pooled mean lesion areas of the five *P. ramorum* isolates on *Q. canariensis*, *Q. faginea*, *Q. pyrenaica*, *Q. pubescens* and *Q. suber* were greater than 100 cm^2 in trials carried out in summer (Fig. 2, Table 2). Single lesions were as large as 793 cm^2, as for example those produced by P1579 on *Q. suber*; 709 cm^2 by P1403 on *Q. canariensis*; 562 cm^2 by P1376 on *Q. faginea*; 416 cm^2 by P1403 on *Q. pubescens*; and 373 cm^2 by P1577 on *Q. pyrenaica* in trials carried out in summer. *Quercus ilex* ranked in the moderately susceptible category (mean lesion area = 17·2 cm^2), although lesion sizes up to 133 cm^2 were observed in the summer trial (Table 2).

Holm oak (*Q. ilex*), cork oak (*Q. suber*) and Portuguese oak (*Q. faginea*) were on average slightly susceptible in the winter trials, although some inoculations attained the highly susceptible category (Fig. 2). This suggests a critical threshold in the host-pathogen interactions (*cf.* summer trials), similar to that reported by Brasier & Kirk (2001) for *Alnus*. Cork oak exhibited irregularly rounded red-brown lesions with a dark outer zone line, usually giving rise to tarry exudates strikingly similar to those observed on *Q. agrifolia* in California (Rizzo *et al.*, 2002), whereas the other *Quercus* species usually exhibited dry, diamond-shaped lesions. Secondary necrotic lesion islands in the phloem, separated by healthy tissue from the main lesion, were also frequently observed on *Quercus suber* and sporadically in other *Quercus* species. These islands of discoloured phloem often coalesced with the main lesion beneath the surface along the sapwood, as reported by Brown & Brasier (2007) and Parke *et al.* (2007).

Other Phytophthoras

The *P. cinnamomi* isolate caused significantly smaller lesions than those of *P. ramorum* on *Q. canariensis* (127·8 vs. 273·2 cm) and on *Q. faginea* (100·7 vs. 210·1 cm^2) in the summer inoculations, but were comparable to or larger than those of *P. ramorum* on *Q. pubescens* (133·5 vs. 109·6) and *Q. pyrenaica* (239·8 vs. 146·2) (Fig. 2). The newly described species *P. hedraiandra* formed generally smaller lesions than *P. ramorum* on all *Quercus* spp. tested. Nonetheless, in the summer trials, it still developed mean lesion areas of 170·9 and 101·3 cm^2 on *Q. canariensis* and *Q. faginea*, respectively (Fig. 2).

Susceptibility of pines

Lesions formed by *P. ramorum* on Aleppo pine (*P. halepensis*) and stone pine (*P. pinea*) were long and narrow, often exceeding 50 cm in length. Both pine species ranked in the highly susceptible category (Fig. 3).

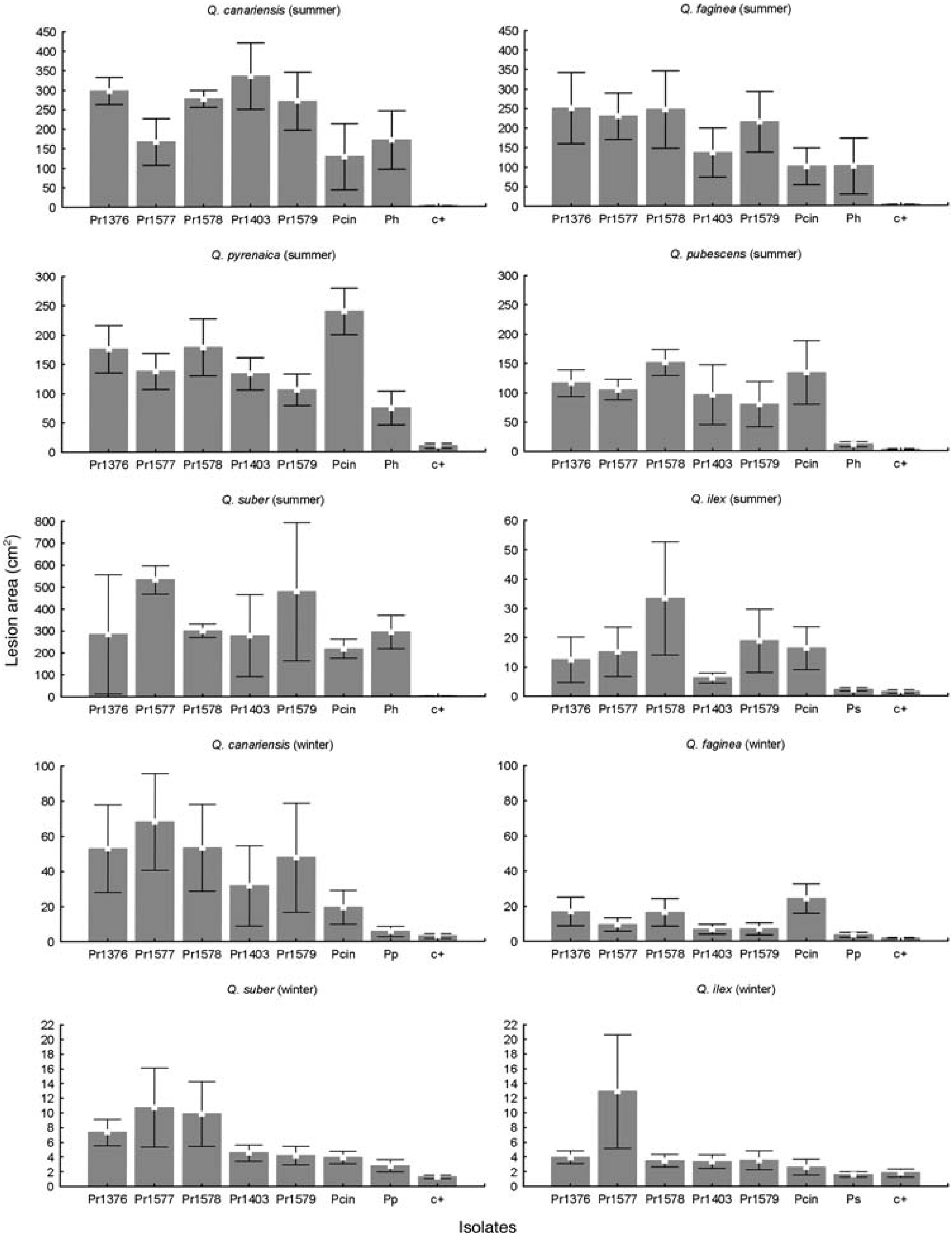

Figure 2 Mean (± SE) lesion area formed on six Iberian *Quercus* species 40 days after inoculation with several *Phytophthora* species. Inoculation trials were carried out during summer and winter. Trees inoculated in summer were highly susceptible (lesions > 20 cm^2) to *P. ramorum*, except *Q. ilex* which was moderately susceptible (lesions 10–20 cm^2). Pr = *P. ramorum*; Pcin = *P. cinnamomi*; Ph = *P. hedraiandra*; Pp = *P. polonica*; Ps = *P. syringae*; and c+ = control.

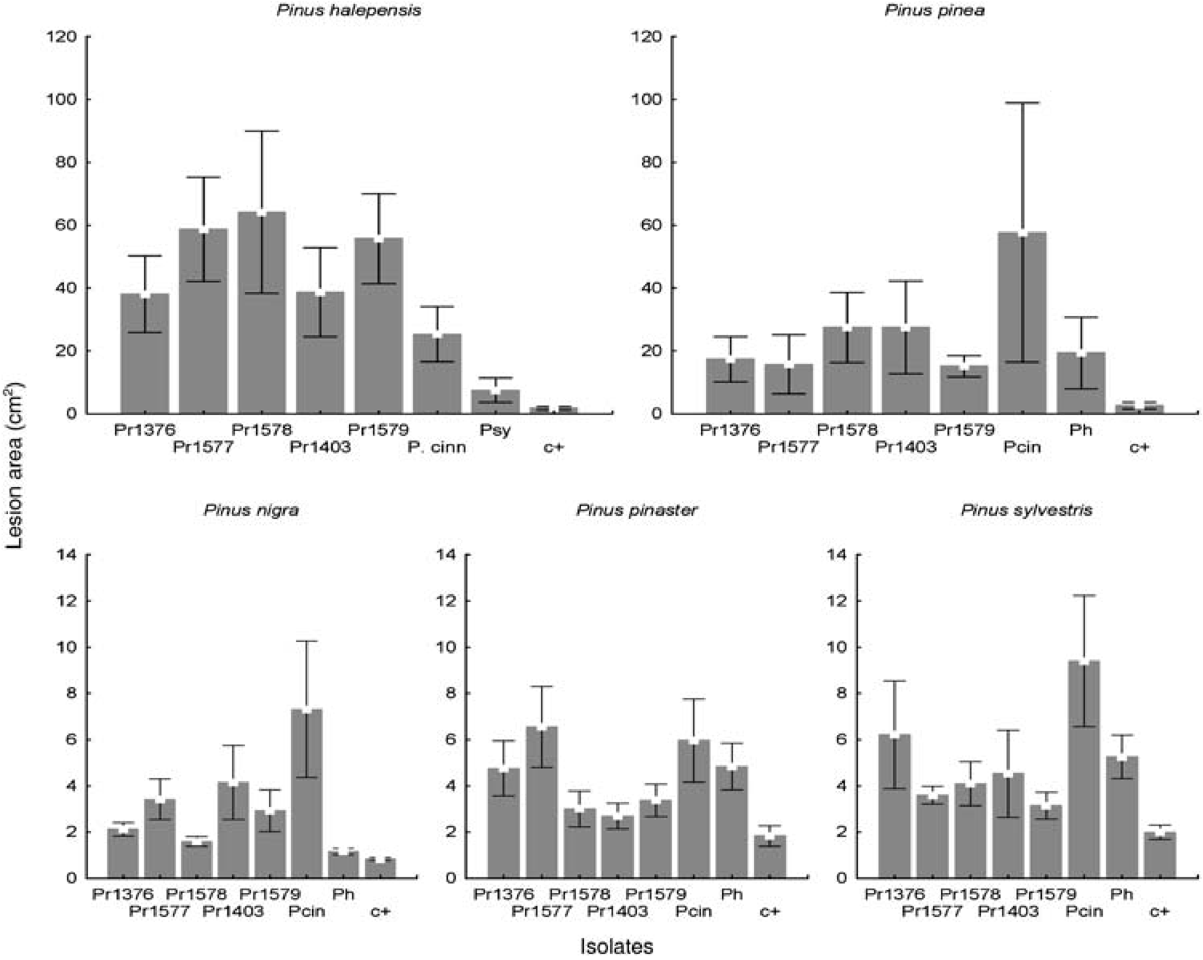

Figure 3 Mean (± SE) lesion sizes formed by *Phytophthora* isolates on pines (*Pinus* spp.) from Iberia. Different scalings were used to show lesion sizes of highly susceptible trees *P. halepensis* and *P. pinea* (lesions > 20 cm^2) and resistant (lesions < 3 cm^2) to slightly susceptible (lesions 3–10 cm^2) trees *P. nigra*, *P. pinaster* and *P. sylvestris* to *P. ramorum*. Pr = *P. ramorum* isolates; Pcin = *P. cinnamomi*; Ph = *P. hedraiandra*; Psy = *P. syringae*; and c+ = control.

Of the other three pines, *P. pinaster* and *P. nigra* were consistently resistant and *P. sylvestris* slightly susceptible to *P. ramorum* (Fig. 3). *Phytophthora cinnamomi* formed larger lesions on *P. pinea* than the other *Phytophthora* species, although they were not statistically significant (Table 2), and *P. hedraiandra* was as pathogenic as *P. ramorum* to *P. pinea*, *P. pinaster* and *P. sylvestris* (Fig. 3). In all species inoculated, necrosis of the inner bark was brownish. This made it easy to distinguish lesions from the surrounding healthy, pale, cream-coloured tissue. Discolouration decreased into the wood.

Susceptibility of other trees

The exotic tree *Eucalyptus dalrympleana*, used in forest plantations in northeastern Spain, was highly susceptible to *P. ramorum* and *P. cinnamomi* and moderately susceptible to *P. hedraiandra* (Fig. 4). *Phytophthora cinnamomi* was the most aggressive, producing lesions as large as 717·6 cm^2 (data not shown). The strawberry tree (*Arbutus unedo*) was highly susceptible to *P. syringae* (av. 45·5 cm^2) and moderately so to *P. ramorum* (av. 14·8 cm^2) and *P. cinnamomi* (av. 11·6 cm^2) (Fig. 4). Lesion sizes caused by *P. ramorum* isolates on the strawberry tree harvested and inoculated in winter were consistent (Fig. 4). A heterogeneous group of tree species formed by holly (*Ilex aquifolium*), hazel (*Corylus avellana*) and Montpellier maple (*Acer monspessulanum*) fell in the slightly susceptible category with respect to *P. ramorum* (Fig. 4). All of these, however, showed large variation in lesion area development; in some instances, single lesions achieved the highly susceptible category. *Olea europea*, *Fraxinus angustifolia*, *Celtis australis* and *Cupressus sempervirens* were consistently resistant to the development of inner bark lesions by all the *Phytophthora* species and isolates tested.

Seasonal and within-host population variation in lesion development

There were no statistical differences in aggressiveness to Iberian trees between *P. ramorum* isolates ($F_{4,797} = 1{\cdot}83$;

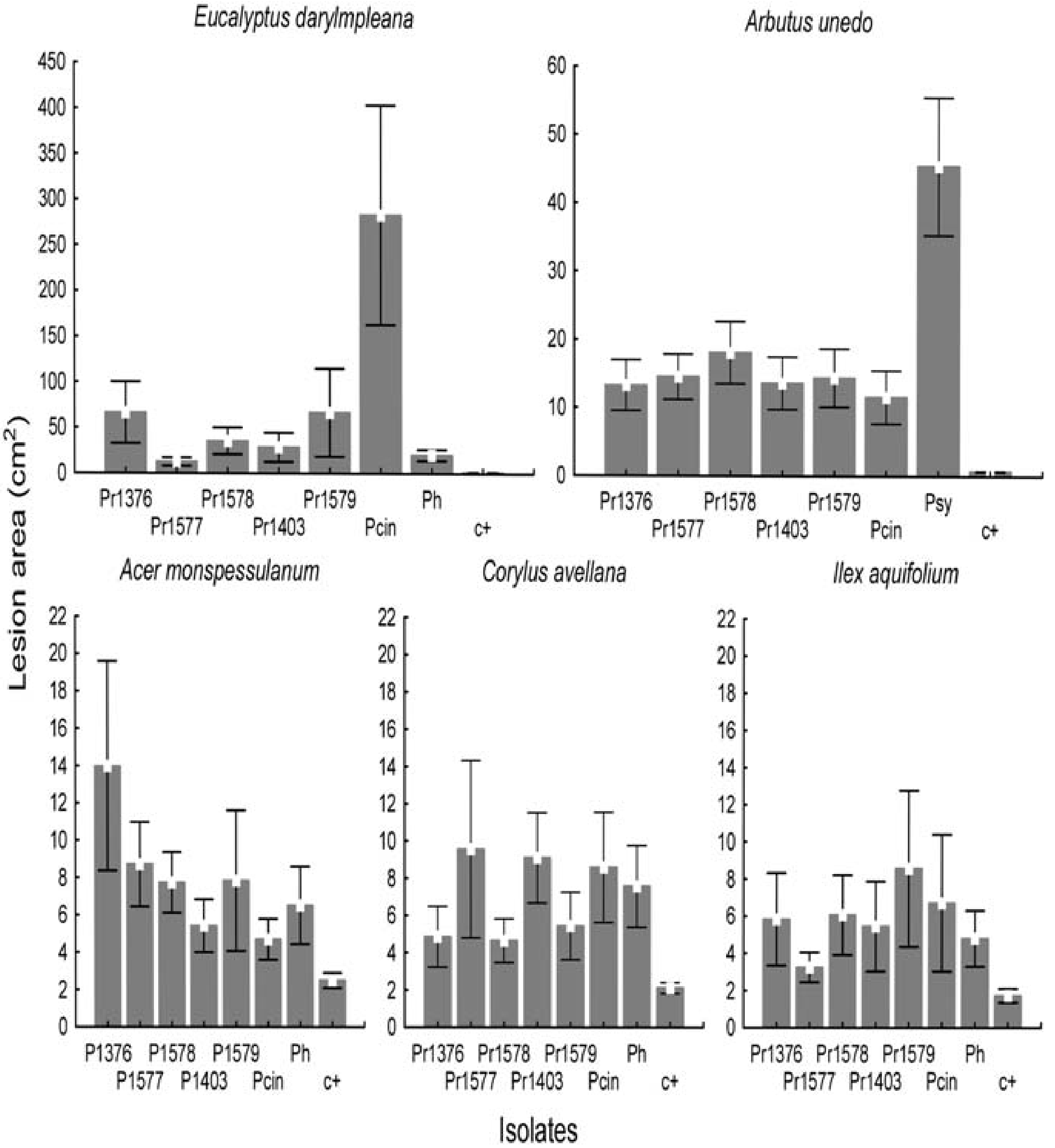

Figure 4 A heterogeneous group of Iberian and exotic tree species showing diverse lesion area (mean ± SE) responses to *Phytophthora ramorum* and other *Phytophthora* species 40 days after inoculation. Species highly to moderately susceptible (top) and slightly susceptible (bottom) to *Phytophthora* species. Strawberry tree (*Arbutus unedo*) logs were harvested and inoculated in winter; all the other tree species were harvested and inoculated in summer. Pr = *P. ramorum*; Pcin = *P. cinnamomi*; Ph = *P. hedraiandra*; Ps = *P. syringae*; and c+ = control.

$P = 0{\cdot}12$). However, the three European isolates produced on average slightly larger lesions (7·8%) than the two American isolates throughout the experiments. Of the top 24 mean lesion-sizes over 100 cm^2 on *Quercus* spp., 12 were due to the EU1 clonal lineage and six to the NA1 lineage; four corresponded to *P. cinnamomi* and two to *P. hedraiandra*.

There were highly significant ($F_{1,256} = 144{\cdot}60$; $P < 0{\cdot}001$) differences between the winter and summer trials in lesion areas caused by *P. ramorum* on four of the *Quercus* species tested. Trees inoculated in summer generally formed lesions several times larger than those obtained in winter (Fig. 5). However, in the winter trials, large lesions (> 50 cm^2) occasionally developed on individual logs. For example, P1577 produced a lesion of 66·1 cm^2 on *Q. ilex*, and P1376 of 66·2 cm^2 on *Q. faginea*, while all isolates produced a lesion larger than 191 cm^2 on *Q. canariensis* on at least one occasion. Overall, significant differences in lesion development were found within tree species that were slightly to highly susceptible to *P. ramorum* (Table 2).

Discussion

This study suggests that the development of large canker lesions on oaks similar to those caused by *P. ramorum* in California might occur locally in some forests and

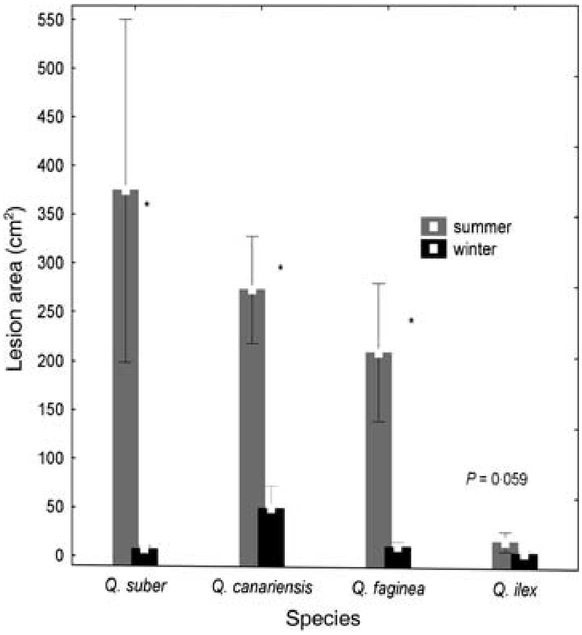

Figure 5 Seasonal variation in susceptibility of Iberian oaks to *Phytophthora ramorum*. Lesion areas (mean ± SE) developed on four Iberian oak species by *P. ramorum* in summer and winter. Data correspond to five isolates which were pooled for the analysis. Asterisks indicate significant differences in lesion sizes between the summer and winter inoculation for each oak species using an independent *t*-test ($P < 0·05$).

woodlands of Iberia if the pathogen became established in the canopy or surrounding vegetation. Previous research had focused on assessing foliage susceptibility of woody plants associated with holm oak forests as an indicator of the risk of *P. ramorum* establishment in this predominant type of vegetation in the Mediterranean (Moralejo *et al*., 2006a), but no attempts to date had been made to estimate the risk of tree mortality. The log inoculation method used in this study does not elucidate the infection process but provides a good estimate of tree response to colonization of the pathogen through the cambial zone (Brasier & Kirk, 2001). In natural situations, however, zoospores or sporangia instead of mycelia are believed to be responsible for infections, and must gain entrance through the outer bark. This could be an important barrier to infection, providing a potential for field resistance in some otherwise susceptible hosts.

The high level of aggressiveness exhibited by *P. ramorum* to the inner bark of Iberian oaks was somewhat unexpected. Differences in susceptibility to *P. ramorum* between oak species were suggested by Rizzo *et al.* (2002) to have a phylogenetic base after noting that red oaks (subsection *Erytrobalanus*: *Q. agrifolia*, *Q. kelloggii*, *Q. palustris*, *Q. rubra*) were highly susceptible in the field or in inoculation tests, whereas several American oaks belonging to the white section (*Lepidobalanus*) were unaffected. Conversely, the inoculation tests on Iberian oaks in the present study, which included species within subsection *Lepidobalanus* (*Q. canariensis*, *Q. pyrenaica*, *Q. faginea* and *Q pubescens*), the *Cerris* group (*Q. suber*) and the *Sclerophyllodrys* group (*Q. ilex*), revealed that oaks not belonging to the red section can also be highly susceptible to *P. ramorum*. Tooley & Kyde (2007) reached the same conclusion, showing that stems of two white oak saplings from eastern USA, *Q. lobata* and *Q. prinus*, were highly susceptible in laboratory inoculations. These results therefore preclude the use of phylogenetic signals for predicting the tree host range of *P. ramorum*.

In the present tests carried out here, three common lesion shapes, diamond-shaped, fusiform and rounded, could be identified in the inner bark. They reflect unequal lateral and longitudinal mycelial growth rates along the cambial zone. The first two shapes were normally observed on oak and pine, respectively, whereas rounded irregular lesions were only found on cork oak. In epidemiological terms, wider lesions, such as those formed on oaks, suggest a higher risk for stem girdling, and hence tree mortality, to occur. By contrast, large lesions that remain narrow, such as those developed on Aleppo and maritime pine, are likely to be indicative of progressive, sublethal lesions.

The present study reveals that the living inner bark (phloem) of six *Quercus* species in Iberia is highly to moderately susceptible to *P. ramorum*: lesion sizes were > 100 cm^2 in 32·8% ($n = 345$) of the overall inoculations. The range of lesion sizes measured was similar, or even much larger, on *Q. canariensis* (mean lesion range = 29·4–335·0 cm^2) than on tan oak (*Lithocarpus densiflorus*) (16·8–232·6 cm^2) (Hansen *et al.*, 2005) and than that reported by Brasier *et al.* (2006b) for European beech (*Fagus sylvatica*) (245 cm^2) in log inoculations carried out in the UK. Because lesion sizes on Iberian oaks are comparable to those of tan oak and European beech, two of the most susceptible hosts to *P. ramorum* in the field, it is thought that lethal bark cankers may develop on Iberian oaks if trunks become infected.

Among the susceptible oaks, *Q. ilex*, *Q. suber*, *Q. canariensis* and *Q. pubescens*, and to a lesser extent *Q. faginea* and *Q. pyrenaica*, thrive in areas of Spain and Portugal where climatic conditions might be conducive to disease (Kluza *et al.*, 2007). Cork oak and *Q. canariensis*, in particular, commonly live on siliceous substrates in subhumid to humid habitats near coastal areas, where environmental conditions are similar to those encountered in the geographical range of *Q. agrifolia* in California. At the local scale, the results highlight the risk posed especially by forest ecosystems in southern Spain (e.g. Los Alcornocales Natural Park) where relict populations of *Q. canariensis* surrounded by cork oak woodlands exist alongside *Rhododendron ponticum*, *Viburnum tinus* and other potentially susceptible host species in the understorey.

Due to the dominance of holm oak in forests and woodlands across the Mediterranean basin, it is important to determine its degree of susceptibility to *P. ramorum*. In the present inoculation tests, holm oak was on average moderately susceptible to *P. ramorum*, although large lesions > 50 cm^2 were observed sporadically on some trunks. Conversely, Brasier *et al.* (2002) reported that logs of holm oak originating from the UK were highly susceptible when inoculated with isolates belonging to the NA1 and EU1 lineages of *P. ramorum*. Moreover, Denman *et al.* (2005a,b) reported foliage and shoot infections on holm oak in recent outbreaks in the UK. Understorey plants forming part of holm and cork oak forests may also favour *P. ramorum* establishment. Leaves and fruits of *Arbutus unedo*, *Viburnum tinus*, *Rhamnus alaternus* and other shrubs were demonstrated by Moralejo *et al.* (2006a,b) to be highly susceptible to zoospore infection, and some of them could sustain sporulation. It is noteworthy that holm oak stands could carry both foliage and stem infections, and hence potentially drive *P. ramorum* epidemics on their own, as occurs with tan oaks in California and Oregon.

Transitions from Mediterranean to sub-Mediterranean and temperate forest domains are not linear, exhibiting complex patterns associated with topography, substrate and microclimate. They often show mixed overlaps and species substitutions, especially in the north and northeast of Iberia (Ruiz de la Torre, 2002). Due to the ecological amplitude exhibited by *Q. ilex*, in its northern range in temperate and sub-Mediterranean zones it frequently forms mosaics of mixed forests with beech (*Fagus sylvatica*), sweet chestnut (*Castanea sativa*), *Q. pubescens* and *Q. pyrenaica* (Ruiz de la Torre, 2002). In these mixed hardwood forests, infected *Q. ilex* foliage could act as a source of inoculum, leading to trunk infections on chestnut and beech for example, chestnut being moderately and beech highly susceptible to *P. ramorum* (Brasier & Jung, 2006). As in the area affected by SOD in California, a diversity of forest and vegetational types occurs in Catalonia (NE Spain) forming intricate mosaics along short transects (30 kms) from the coast to the nearby mountain ranges. For example, in the Montseny Park near the coast, mixed populations can be found of *Q. ilex*, *Q. suber*, *Q. canariensis*, *Q. pubescens*, *Q. faginea*, *C. sativa* and *F. sylvatica*. In coastal areas in NE Spain and the Balearic Islands, holm oak also often forms part of a succession of mixed evergreen forests with *Pinus halepensis* and/or *P. pinea* under xeric to mesic conditions. However, although the inner bark of both pine species is highly susceptible, it is less likely that severe mortality could occur because these species only exhibited non-girdling lesions in the present tests and because the climate might be less conducive to disease establishment.

In the inoculations, a number of tree species, including hazel, holly and Montpellier maple, were on average slightly susceptible to *P. ramorum* but showed marked variation in lesion development. Except for hazel, these species are usually found relatively scattered in mixed hardwood forests in sub-Mediterranean and temperate areas. In epidemiological terms, therefore, the risk may not be significant. On the other hand, three pine species forming extensive forests and plantations in Spain and Portugal, *P. pinaster*, *P. nigra* and *P. sylvestris*, were resistant or slightly susceptible to *P. ramorum* in the inoculations. Similar results were obtained by Brasier *et al.* (2002) for *P. nigra* and *P. sylvestris* in the UK. Therefore, it is thought that *P. ramorum* will probably not threaten these forest types and plantations. Of major concern is the possibility of *Eucalyptus* plantations becoming infected, as they are usually in areas where very favourable conditions for *P. ramorum* establishment are found, e.g. in the northwest and north of Spain, as well as in Portugal. However, as stated before, caution should be taken when extrapolating species susceptibility to the whole genus. The inoculation tests were made on *E. dalrympleana*, which is not as widely planted as *E. globulus* or *E. camaldulensis*.

Phytophthora cinnamomi is well-known as an aggressive root pathogen of oak and *Eucalyptus* species. The present study reveals its high capacity for also invading the inner bark of these trees, although this does not necessarily correlate with root susceptibility. At present, it causes decline and death of evergreen oaks in southern and central Iberia, but there seems to be a potential for *P. cinnamomi* to spread northward by infecting semi-deciduous oaks and *Eucalyptus* plantations as a result of global climatic change (Brasier, 1996). Hence it is another factor of concern, as it could lead to a regression of sub-Mediterranean forests. Despite being less aggressive than *P. ramorum* and *P. cinnamomi*, the newly described species *P. hedraiandra* also shows some trends for becoming an invader. It is currently known to infect *Rhododendron* and *Viburnum* in nurseries and, like *P. ramorum*, is being subject to global spread via the plant trade (Man in't Veld *et al.*, 2007; Moralejo *et al.*, 2007). In addition to *P. ramorum*, both *P. hedraiandra* and *P. kernoviae* may likewise be a threat to plant communities formed by *Rhododendron ponticum*-*Quercus canariensis*-*Viburnum tinus* in southern Spain.

With most hosts, susceptibility to *P. ramorum* appears to vary according to the organ and tissue attacked and to the season of the year (Hansen *et al.*, 2005; Denman *et al.*, 2006). Indeed, in the present study, the effect of season was very marked with the four *Quercus* species tested. This was not related to temperature fluctuations, since all the experiments were performed at 20°C. A similar behaviour has been reported by Hansen *et al.* (2005) on trunks of tan oak and by Brasier & Kirk (2001) for alder and several other tree species within the Fagaceae. The cause of this remains poorly understood. Seasonal variation in host susceptibility could be partially explained if phenolic or similar compounds are stored in the cambial zone in winter and mobilized to other organs during the growing season (Ockels *et al.*, 2007). Accordingly, critical thresholds in lesion development might reflect synergies between the concentrations of different inhibitors and their effect on fungal morphogenesis, especially mycelial branching patterns. More difficult to explain is the observed variation in the sizes of individual lesions within a host. Some variation could be attributed to experimental error due to differences in inoculation depth. However, Parke *et al.* (2007) recently found no differences in inner bark lesion sizes on excised logs of tan oaks, irrespective of whether the inner bark, the cambium or the sapwood was inoculated. A second possibility is that the concentrations of inhibitors are not homogeneously spread along the cambial zone. However, if this were the case, lesion contours would have been shaped by concentration gradients, a feature that has not been seen. The observations of large differences in lesion sizes between isolates within individual trunks might suggest a capacity of some isolates to overcome or suppress the effect of inhibitors at critical thresholds. This could explain why this phenomenon happened more often in hosts falling into the slightly and moderately susceptible categories. This hypothesis is more likely, taking into account the strong selection pressures that inhibitors might exert on fungal morphogenesis.

In the current tests, lesion sizes caused by different *Phytophthora* species varied widely (1–792 cm^2) amongst *Quercus* and other tree species. Even within highly susceptible *Quercus* species, some individual stems of *Q. pyrenaica* and *Q. faginea* apparently had complete resistance to infection. A similar within-population variation in lesion development has been reported for *Q. robur*/*P. ramorum* (Brasier & Jung, 2006), for *Castanea sativa*/*P. cambivora* (Robin *et al*., 2006) and for *Alnus glutinosa*/*P. alni* (Brasier & Kirk, 2001). In California, Dodd *et al*. (2005) studied differences in susceptibility to *P. ramorum* within and between *Q. agrifolia* populations by inoculating shoots. They found a higher variation in susceptibility and genetic diversity within populations than among *Q. agrifolia* populations. This is consistent with previous studies on the genetic structure of the populations of *Quercus* species in Spain and Europe based on AFLP and other genetic profiles, where a higher genetic heterogeneity was found within than between populations (Coelho *et al*., 2006). It is reasonable to assume, therefore, that similar responses to infection (i.e. lesion size ranges) would have been obtained if trees had been sampled from any other area of the geographical range of the species, as they are unlikely to have been previously exposed to the pathogen; hence, their populations would not have been genetically structured by it. These general observations further strengthen the validity of the results and conclusions of the inoculation tests.

Although the above study was carried out on trees of Iberian sources, some of the conclusions can be extended to forests and woodlands in other areas of the western Mediterranean, including North Africa, southern France and Italy. To date there are no reports of field infections in the Mediterranean area and this could lead to a serious underestimation of the threat posed by *P. ramorum* to Mediterranean forests and, in consequence, a temptation to relax current plant health measures. It should be emphasised that many western Mediterranean ecosystems whose plant members are potentially susceptible to *P. ramorum* have not yet been exposed to the pathogen. If introduced, its ability to invade each of these habitats will depend on complex interactions between the pathogen, the host and the environment, which have still either only been partially investigated, or not investigated at all. Until these ecosystems are shown to be resistant then, on the basis of the information provided here, it is believed that the precautionary principle should prevail.

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