J. Phytopathology **156**, 577–587 (2008)

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Strawberry Tree Blight in Spain, a New Disease Caused by various *Phytophthora* Species

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Received September 12, 2007; accepted November 20, 2007

Keywords: Mediterranean plants, ornamental plant pathogens, ITS sequence, sporulation, *Arbutus unedo*

Abstract

During surveys for *Phytophthora ramorum* in garden centres in Majorca, Spain, 31 isolates of *Phytophthora* were recovered from potted strawberry trees (*Arbutus unedo*) showing leaf and twig blights. Many isolates of *Phytophthora syringae* and *Phytophthora citrophthora* as well as single isolates of *P. ramorum, Phytophthora tropicalis* and *Phytophthora nicotianae* were identified on morphological features and on the sequences of the internal transcribed spacer regions from ribosomal DNA genes. *Phytophthora syringae* was collected most frequently in late autumn and winter, whereas *P. citrophthora* was dominant during late summer and autumn. *In vitro* pathogenicity of *P. syringae* and *P. citrophthora* was compared with that of *P. ramorum* by inoculating intact detached leaves of *A. unedo* with zoospores and twigs with mycelial plugs. In addition, *in vitro* sporangial production was examined on inoculated excised leaves and on agar plugs at 12, 15 and 20°C. *Phytophthora citrophthora* produced the largest lesions both on leaves and on twigs at all temperatures. *Phytophthora ramorum* formed lesions comparable in size to those of *P. syringae*, but it significantly produced more sporangia on excised leaves and agar plugs. In a log inoculation assay, *P. syringae* caused large lesions in the inner bark, whereas those of *P. ramorum* were moderate. Strawberry tree blight has not yet been observed in natural ecosystems in the western Mediterranean areas. Possible biological and environmental limitations hindering disease spread in the wild are discussed.

Introduction

The strawberry tree (*Arbutus unedo* L., Ericaceae) is an evergreen shrub 3–10 m tall, native to the Mediterranean and western Europe, and a major component of the understorey of the holm oak (*Quercus ilex* L.) woodlands and of the 'maquis' vegetation. It is being increasingly planted in gardens worldwide and sought after for its lustrous leaves, conspicuous persistent red fruits and tolerance to calcareous soils and drought. Due to its relatively recent exploitation as an ornamental crop, natural planting stocks that differ little genetically from wild strawberry trees are propagated in nurseries. Like other Ericaceae (e.g. *Rhododendron*, *Pieris*, *Vaccinium*, etc.) used in gardening, *A. unedo* is also susceptible to many fungal pathogens causing leaf spots, e.g. *Septoria unedinis* Roberge, *Cercospora molleriana* Winter and *Pestalotiopsis maculans* (Corda) Nag Raj, and twig diebacks, e.g. *Botryosphaeria sensu lato*, (Muntañola-Cvetkovic et al., 2001; Trapero et al., 2003).

Most ericaceous plants grown in nurseries, particularly rhododendrons, are susceptible to root and crown rots by soil-borne species of *Phytophthora* such as *Phytophthora cinnamomi* Rands (Benson and Cochran, 1980; Erwin and Ribeiro, 1996; Krebs and Wilson, 2002). In addition, the aerial organ parts of many ericaceous ornamental plants are also frequently attacked by *Phytophthora* spp. For example, Gerlach et al. (1976) reported a leaf and shoot tip blight on *Pieris japonica* caused by *Phytophthora citrophthora* (R. E. Smith and E. H. Smith) Leonian. Similar symptoms are found on rhododendron, but are caused by a range of phytophthoras including *P. citricola* Sawada, *Phytophthora syringae* Klebahn and several other species, many of which frequently occur in nurseries (Benson and Jones, 1980; Linderman, 1986; Backhaus, 1994). Leaf and twig blight on rhododendron caused by the recently described species *Phytophthora ramorum* Werres, de Cock and Man in't Veld is of major international concern because the same pathogen also causes sudden oak death in the coastal forests of California and south-west Oregon of the USA (Werres et al., 2001; Rizzo et al., 2002). In these

forests, *P. ramorum* causes a disease damaging plants is at the community level. General symptoms include bleeding trunk cankers that are often lethal in several species of the Fagaceae as well as shoot dieback and leaf and twig blights on many native understorey plants including madrone: *Arbutus menziessii* (Rizzo et al., 2002; Maloney et al., 2004). Furthermore, *P. ramorum* already infects an increasing number of ornamental plants in nurseries, many in the Ericaceae, and could affect many others (Tooley et al., 2004). Through the international trade of ornamental plants, *P. ramorum* has spread widely in nurseries across Europe (e.g. Moralejo and Werres, 2002; Inman et al., 2003; Lane et al., 2003; Pintos Varela et al., 2003) and recently within the USA (Hansen et al., 2003). Recognition of all potential ornamental hosts of *P. ramorum* is needed because these might be a means of worldwide dispersal of the pathogen to natural ecosystems. If all potential hosts of dangerous pathogens are known and subjected to plant Health Regulation, the risk of spreading them to the natural ecosystems will be reduced.

In February 2002, *P. ramorum* was detected on rhododendron for the first time in two garden centres in Majorca, Balearic Islands, Spain (Moralejo and Werres, 2002). In preliminary pathogenicity tests on excised leaves of several Mediterranean shrubs, it was shown that *A. unedo* was highly susceptible to *P. ramorum* (Moralejo and Hernández, 2002). It was therefore included as a potential host to be surveyed in garden centres and in the wild. During surveys for *P. ramorum* over the next 3 years, isolates of several species of *Phytophthora* were recovered from a previously unreported disease, leaf and twig blight, commonly affecting potted strawberry trees (*A. unedo*) in Spain. To implement an effective disease management strategy the pathogen have to be identified and information abort the epidemiology of disease obtained. Therefore, the objectives of this research were: (i) to determine the aetiology of the new disease in Spain, named here strawberry tree blight, (ii) to characterize the pathogenicity of the isolates and (iii) to examine the *in vitro* sporulation potential of the species recovered.

Materials and Methods

Isolation

Surveys for *Phytophthora* on strawberry trees were conducted in several garden centres on the Mediterranean island of Majorca (Spain) between 2002 and 2005. Isolates were collected from potted plants showing leaf and twig blights and occasionally stem cankers. Samples of infected leaves and twigs were placed in unused labelled polyethylene bags. In the laboratory, parts of the plant diseased with material were cut out, washed in tap water, dipped in 70% ethanol for 30 s, rinsed in sterile deionized water and later blotted dry on filter paper. From the leading edge of the necrotic lesions, pieces of ca. 5×5 mm of tissue were plated onto P_5ARP, a medium semiselective for oomycetes, consisting of cornmeal agar (CMA; Sigma Chemical Company, St. Louis, MO, USA) amended with 5 μg/ml of pimaricin, 100 μg/ml of ampicillin, 25 μg/ml of rifampicin and 100 μg/ml of pentachloronitrobenzene (Erwin and Ribeiro, 1996). The plates were incubated at 20°C in the dark for 72 h. Pure cultures were obtained by transferring single hyphal tips from the edge of the colonies to Petri dishes with CMA. Stock cultures were maintained at 12°C in the dark and the CMA slants were covered with sterile paraffin oil. Representative isolates of each taxon have been deposited at CBS (Centraalbureau voor Schimmelcultures) as *P. syringae* P11013 = CBS119645, P10413 = CBS119646, P4942 = CBS119647, P11313 = CBS119648; P. citrophthora P4142 = CBS119651. P3742 = CBS-119652. P3212 = CBS119653; *P. tropicalis* P11515 = CBS119654 and *P. nicotianae* P11615 = CBS119655.

Morphology and physiology

Isolates were grown on CMA, carrot agar: CA (Brasier, 1967), malt extract agar: MEA (Sigma), potato dextrose agar: PDA (Sigma) and unclarified V8 juice agar: 2 g $CaCO_3$, 200 ml of V8A (Campbell juice) and 15 g agar in 800 ml distilled water (Erwin and Ribeiro, 1996), at 20°C in the dark, and the colony morphology examined at 7 days. Radial growth rates (mm/day) at 20°C were determined for two replicates of each isolate on both CA and V8A. Two perpendicular radii were measured daily over a 4-day period and the average radial growth calculated.

The presence of sporangia, hyphal swellings and chlamydospores on all agar media was checked for 2 weeks. To induce the formation of sporangia, three 12-mm-diam. disks were taken from the edge of a 7-day-old colony (on CA), and placed in a 60-mm-diam. Petri dish flooded with 10 ml of soil extract solution (50 g of filtered oak forest soil in 1000 ml of distilled water, autoclaved at 121°C for 15 min). The dishes were kept for 48–72 h at 20°C under white light (12 h photoperiod). The shape, type of papillae and caducity of sporangia were recorded using a compound microscope. The presence of hyphal swellings on mycelium in the soil solution was examined. About 25 asexual structures (sporangia and chlamydo spores) chosen at random were measured for each isolate using a calibrated eyepiece on a compound microscope (× 400).

The formation of gametangia on CA was examined after 10 and 30 days at 20°C in the dark. Heterothallism was investigated for non-self-fertile isolates by pairing them with known A1 and A2 mating type isolates of *Phytophthora nicotianae* Breda de Haan on CA at 20°C in darkness, (Brasier, 1972). Sexual structures were measured as explained above. Leaves infected by *P. syringae* often showed gametangia embedded in the lesion when observed at ×50 magnification with a Leitz wild dissecting microscope. Further observations were made after clearing the tissue with 70% ethanol at 50°C for one hour followed by a bath in 1% hypochloride for 5 min.

ITS sequencing and molecular phylogeny

Mycelial DNA was extracted from pure cultures grown in sterile pea broth (Kroon et al., 2004) and checked for quality as previously described (Belbahri et al., 2006a,b). Amplification of the internal transcribed spacer (ITS) regions of the ribosomal DNA were conducted using the previously described universal primers ITS4 and ITS6 that target conserved regions in the 18S and 28S rDNA genes (White et al., 1990; Cooke et al., 2000). PCR product purification and DNA sequencing were performed according to Belbahri et al. (2006a,b). The sequences obtained were registered in GenBank (Table 1). These sequences together with those of phytophthora species included in Cooke et al. (2000) were used to build a molecular phylogeny (Belbahri et al., 2006a,b). Sequences were aligned manually using Seaview (Galtier et al., 1996). The maximum likelihood (ML) trees were produced using the PHYML software program (Guindon and Gascuel, 2003) available from the (http://atgc.lirm.fr/phyml/) website with the Hasegawa, Kishino and Yano model allowing transitions and transversions to have potentially different rates and the General Time Reversible model allowing all rates to be different. For the correction of the among-site rate variations, the proportion of invariable sites (I) and the 'a' parameter of g distribution (G), with eight rate categories, were estimated by the program and taken into account in all analyses. Non-parametric ML bootstraps (with 100 replicates) were calculated using PHYML and Bayesian inferences were obtained with MRBAYES v.3.0 (Huelsenbeck and Ronquist, 2001), using the same models of DNA evolution as for the ML analyses. The program was run for 2 000 000 generations, sampled every 100 generations, with four simultaneous chains. The trees sampled before the chains became stationary were discarded. Neighbour-joining plot and Treeview were used to view ML and Bayesian trees respectively.

Table 1
Isolates of *Phytophthora* collected from diseased *Arbutus unedo* in garden centres of Majorca (Spain)

Species	Isolate	Symptoms	Year	GenBank accession number
P. citrophthora	P2912	Twig blight	2002	AY946249
P. citrophthora	P3212	Twig blight	2002	AY961609
P. citrophthora	P3742	Twig blight	2002	AY881004
P. citrophthora	P4142	Leaf blight	2002	DQ648129
P. nicotianae	P11613	Leaf blight	2005	DQ357827
P. syringae	P2612	Twig blight	2002	AY946262
P. syringae	P2712	Twig blight	2002	AY946251
P. syringae	P2812	Twig blight	2002	AY946250
P. syringae	P3312	Twig blight	2002	AY881003
P. syringae	P4542	Leaf blight	2002	DQ648128
P. syringae	P4642	Leaf blight	2002	AY946258
P. syringae	P4842	Leaf blight	2002	AY946256
P. syringae	P4942	Leaf blight	2002	AY946255
P. syringae	P7042	Leaf blight	2003	AY881006
P. syringae	P7514	Leaf blight	2004	AY946254
P. syringae	P10113	Leaf blight	2004	EF050524
P. syringae	P10213	Leaf blight	2004	DQ648138
P. syringae	P10313	Leaf blight	2004	N/T
P. syringae	P10413	Leaf blight	2004	DQ648136
P. syringae	P10513	Leaf blight	2004	EF050525
P. syringae	P10613	Leaf blight	2004	AY961606
P. syringae	P10713	Leaf blight	2004	DQ648142
P. syringae	P10813	Leaf blight	2004	DQ648130
P. syringae	P10913	Leaf blight	2004	AY946245
P. syringae	P11013	Leaf blight	2004	AY881007
P. syringae	P11113	Leaf blight	2004	AY946246
P. syringae	P11213	Leaf blight	2004	AY946247
P. syringae	P11313	Leaf blight	2004	AY946248
P. syringae	P11413	Leaf blight	2004	DQ648149
P. ramorum	P62	Leaf blight	2002	N/T
P. tropicalis	P11515	Twig blight	2005	DQ357829

N/T: not tested.

Pathogenicity tests

Pathogenicity was assessed *in vitro* on excised leaves and twigs of *A. unedo*. Six isolates, three putatively identified as *P. syringae*, two as *P. citrophthora* and one as *P. ramorum*, were used in the main inoculation tests carried out in 2004. The first two taxa represented the most common species encountered in the survey. *Phytophthora tropicalis* Aragaki and Uchida and *P. nicotianae* were found at the end of the survey; therefore, pathogenicity of these two species was tested separately on detached leaves.

Young twig cuttings ca. 20 cm long selected randomly were collected from at least 10 different strawberry tree individuals in a holm oak (*Q. ilex*) woodland near the town of Esporles in Majorca in May 2004. The basal leaves of the cuttings were removed and saved for the detached-leaf assay. All plant material was rinsed in running tap water and dried in a laminar flow hood. On each twig, a 5-mm-long flap of the outer bark was lifted ca. 10 cm below the shoot tip with a sterile razor blade. A ca. 3×3 mm agar block was taken from the edge of a 7-day-old colony on CA and placed under the flap with the mycelium facing the stem, covered with a piece of autoclaved water-soaked cotton and wrapped with Parafilm. Three replicate twigs were inoculated with each isolate, and placed in 250-ml flasks containing 200 ml of autoclaved deionized water. For controls, three twigs were inoculated with blocks of plain CA and treated as described above. The twigs were sprayed with deionized water, covered with clean transparent polyethylene bags and held at 20°C under white light (12 h photoperiod). After 48 h, the bag was removed and the length of the discoloured tissue measured 10 days after the inoculation. A small piece of discoloured tissue was placed on P_5ARP and kept at 20°C for 5 days for re-isolation of the pathogen.

For the inoculation on detached leaves, a zoospore suspension of each isolate was prepared. For sporangial production, three 12-mm-diam. disks from each colony were removed using a cork borer from a 7-day-old colony grown on CA; placed in a 6-cm-diam. Petri dish with 10 ml of soil extract solution, and incubated at 20°C under white light for 3 days. Zoospore release was induced by chilling the plates at 7°C for 45 min, and then exposing them to room temperatures for 30 min. Based on haemocytometer counts the

suspension was adjusted to 2–4 × 10^4 zoospores/ml. Sets of five leaves of *A. unedo* were placed with the adaxial side down on a metal grid in moist chambers consisting of transparent plastic boxes lined on the bottom with sterile paper towels soaked in water. The abaxial sides of four of the five leaves were inoculated by placing a single 100 μl drop of zoospore suspension near the centre of the midrib. The remaining leaf was used as a control by placing a drop of sterilized deionized water. The boxes were held at 20°C under cool white light from 30-W fluorescent tubes suspended 30 cm above the chambers (12 h photoperiod). Seven days after inoculation, the leaves were scanned and the area of the lesions calculated with the Olympus DP12 Soft version 3.2. (Olympus America Inc., Scientific Equipment Group, Melville, NY).

Isolate P10413, representing the most common phenotype of *P. syringae*, five isolates of *P. ramorum* and a single isolate of *P. cinnamomi* were used to determine their capacity for colonizing the inner bark of strawberry tree logs in the winter of 2004. Eight logs (10–20 cm diam. × 1.2 m long) were wound inoculated with 7-mm-diam. mycelial plugs at eight equidistant points (Brasier and Kirk, 2001). The trunks were sealed with two polyethylene films to ensure suitable humidity and incubated at 20°C in a quarantine chamber. After 40 days, the outer bark was shaved, the outline of the necrotic lesion traced on transparent paper and the image scanned. The lesion area was calculated as above. Differences in lesion size among isolates were analysed using a LSD test in one-way analysis of variance (ANOVA) (STATISTICA 6.1; Statsoft, Tulsa, OK, USA).

Sporangial production

The effect of temperature on sporangial production on detached leaves, on leaf disks and on agar cultures was assessed for the same isolates used in the leaf and twig pathogenicity tests. The temperatures selected were 20°C for sporulation on detached leaves, 12°C on leaf disks, and 15 and 20°C on agar colony plugs. For sporangial formation on detached leaves, we used the same inoculated leaves as in the detached-leaf experiment explained in the above section. On day 6 a single 100 μl drop of sterile distilled water was placed on the original inoculation point and leaves were incubated further. Four inoculated-leaf replicates were used for each isolate. On day 7, the water droplet was then suctioned off the leaf with a micropipette and transferred to a 1.5 ml centrifuge microtube. The leaf surface below the water drop (including all the necrotic parts resulting from the infection) was then gently scraped with a flamed scalpel to dislodge sporangia. A 200 μl drop of sterile distilled water was placed on the leaf lesion, the scrapings suspended in the drop and this again suctioned and added to a 1.5-ml centrifuge microtube.

Mycelial plugs taken with a 12-mm-diam. core borer from the edge of a 5-day-old colony were placed on multidish wells (Nunc, Roskilde, Denmark). Four mycelial plugs were used for each isolate as replicates. Disks of 12-mm diam. from the edge of 7-day-old leaf lesions were placed on multidish wells. The plates with 24 microwells, where each row was occupied by one isolate, were incubated under continuous light on a chilling-hot plate for 72 h, at 15 and 20°C for the mycelial plugs, and at 12°C for leaf disks. A drop of cotton blue in lactic acid was subsequently added to each well and the number of sporangia counted. For this, the contents of the microplates were carefully introduced into a 1.5 ml centrifuge microtube, vortexed at high speed for 30 s, and five 10-μl aliquots of the suspension pipetted onto a microscope slide. Sporangia in each drop were counted and the means calculated. Differences in mean numbers of sporangia among isolates at each temperature were analysed with Fisher's LSD test of a one-way ANOVA design using the GLM (General Linear Model), with isolates as categorical predictors. Sporulation data were $\log_{10}$ transformed to meet the ANOVA assumption of homogeneity of variance (Levene's test).

Results

Disease symptoms

Three major types of symptoms on strawberry trees were observed during the survey: leaf and twig blights and, to a lesser extent, stem cankers. Natural leaf infections usually began on the petioles or at the leaf margins as small, water-soaked, black or brownish necroses showing a diffuse front. Lesions often advanced first along the midrib and petiole, progressing then downwards into the twig. Subsequently the upper leaves sometimes wilted and were quickly desiccated, mostly remaining attached to the stem and appearing as dieback. In addition, necroses were commonly observed on twig tips of saplings. Strawberry tree blights were mostly observed during the rainy season from October to April, and could be differentiated from those of leaf and twig necroses caused by *Botryosphaeria sensu lato* because they were not sunken nor surrounded by a marked purple halo.

Morphological identification

Thirty-one isolates of *Phytophthora* were recovered from strawberry tree plants exhibiting the above described symptoms in five garden centres (Table 1). Most of them were first assigned to two phenotypic groups, i.e. *P. syringae* like and *P. citrophthora* like, based on colony patterns, radial growth rates, sporangial shapes and sexual behaviour (Waterhouse and Waterston, 1964a and Waterhouse and Waterston, 1964b; Waterhouse, 1970). The remaining three isolates were putatively identified as *P. ramorum*, *P. nicotianae* and *P. tropicalis*. A brief morphological description of each is given below:

Phytophthora syringae. This was the most common species of *Phytophthora* recorded (77.4%). Twenty-two *P. syringae*-like isolates were similar in their radial growth rate and colony morphology. They were characterized by having slow radial growth rate (< 2.7 mm/day at 20°C on all agar media), petaloid to

chrysanthemum colony pattern on CA and V8A, and very slow growth on PDA. Sporangia were absent on all media, except a few on CMA in P4942, but they were readily produced in soil–water extract solution. Sporangia were mainly semipapillate and some non-papillate, predominantly non-caducous, 49.3 ± 9.4 × 32.6 ± 5.0 μm (±SD), ovoid to obpiriform, sometimes irregularly shaped and borne on long sporangiophores or lax sympodia (Fig. 1a and b). Zoospores were released through an exit pore averaging 8.5 μm in width. Catenulate hyphal swellings were formed in water by most isolates, but no chlamydospores were seen in any of the media. Gametangia in culture were sparse probably due to long *in vitro* storage. Conversely, sexual organs formed abundantly on the host tissue (leaf) when incubated at 12°C for 2 weeks in darkness. In agar, gametangia were distributed patchily, and exhibited oogonia with smooth walls, 30.8 ± 4.2 μm in diam., and paragynous antheridia, usually with the stalks tangled below the oogonia. Oospores were globose to subglobose, 27.2 ± 3.0 μm in diam. and mostly plerotic (Fig. 1c).

Phytophthora citrophthora like: This was the second most common *Phytophthora* recovered (12.9%). Isolates were mainly collected from larger stem lesions during the summer and autumn. Unlike that of *P. syringae*, isolates of *P. citrophthora* were rather heterogeneous in morphological and physiological traits. Colonies were fast growing, with mean radial growth rates on CA and CMA from 10 to 13 mm/day at 20°C, unpatterned and submerged in CMA, radiate to striate on CA, stellate to petaloid and fluffy on MEA, stellate on V8A, and stellate to petaloid on PDA. Sporangia were formed on agar only by isolates P3742

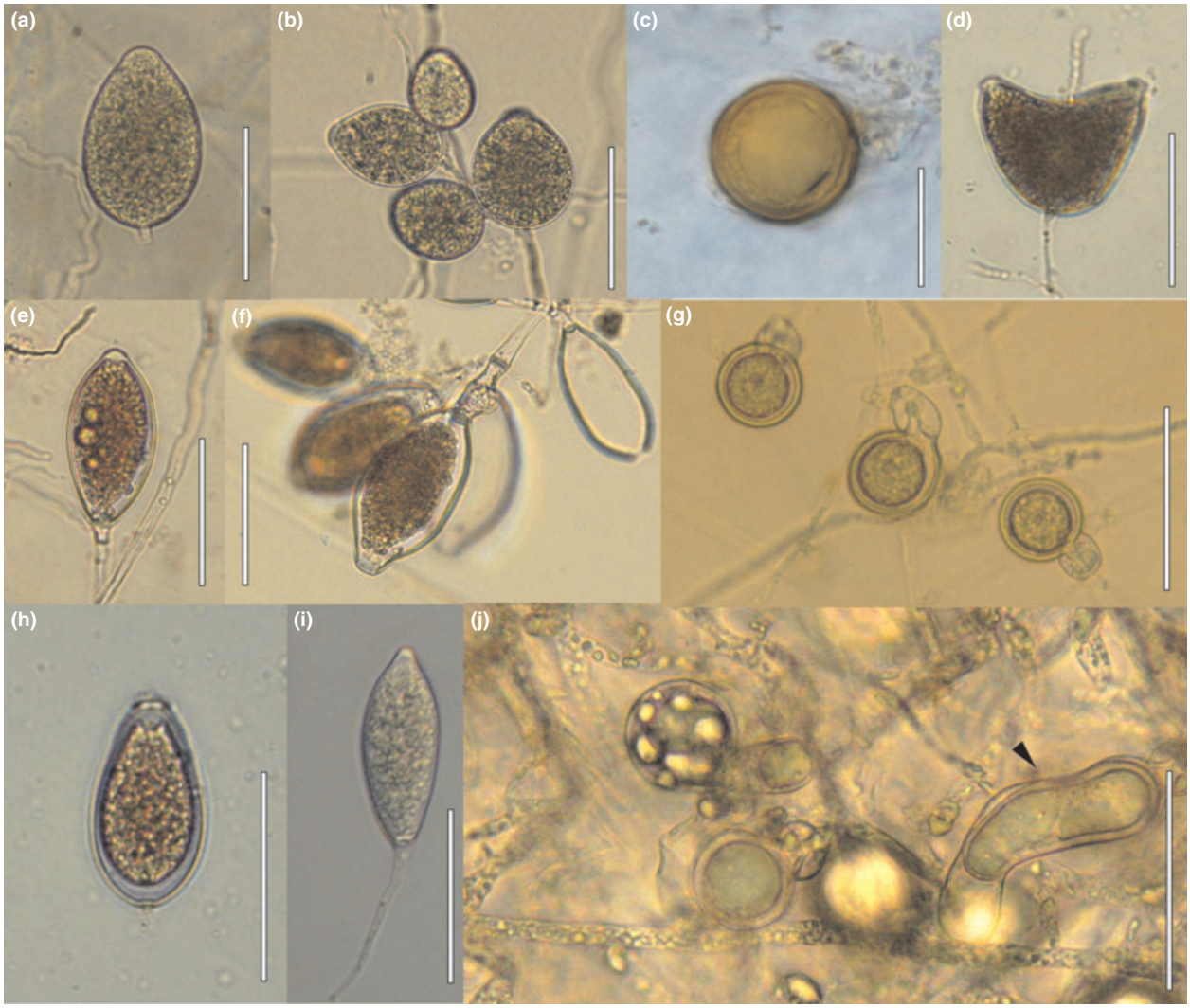

Fig. 1 Morphological features of four species of *Phytophthora* recovered from diseased shoots of *Arbutus unedo*. (a) A semipapillate ovoid sporangium of *P. syringae*; (b) a rare case of sporangia of *P. syringae* borne on a compact sympodium; (c) mature oogonium and tangled antheridium of *P. syringae*; (d) bi-papillate sporangium of *P. citrophthora*; (e) ellipsoid sporangium of *P. citrophthora*; (f) umbellate sporangiophores of *P. citrophthora*; (g) oospores, oogonia and amphigynous antheridia produced in the colony side of *P. citrophthora* in an interspecific pairing between *P. citrophthora* and *P. nicotianae*; (h) characteristic deciduous sporangium of *P. ramorum*; (i) naviculate sporangium of *P. tropicalis* with a long pedicel; (j) oogonia formed in pairings between *P. tropicalis* and *P. nicotianae* showing distorted shapes (arrow). Bars = 50 μm except for (c) which is 20 μm

and P4142 but readily in water soil extract by all them. Sporangia were mostly non-caducous, papillate, ovoid to obpiriform, some subglobose or less frequently ellipsoid, 53.1 ± 7.3 × 35.5 ± 3.5 μm, some bearing two apices (Fig. 1d). Isolate P3742 varied greatly in shape, being predominantly limoniform and with the base tapering (Fig. 1e). In many cases, sporangia were arranged in close umbels (Fig. 1f). A few small chlamydospores were produced only by isolate P3212. No gametangia were found in pure cultures. Oospores were sparsely produced by P3212 and P4142, and more abundantly by P3742 in pairings with a *P. nicotianae* A1 mating type isolate (Fig. 1g). On *P. citrophthora* colony and bordering the overlapping zone, oogonia were spherical, mean diam. 24.6 ± 2.2 μm, ranging from 21.1 to 28.1 μm. Oospores were aplerotic, mean diam. 19.3 μm and thin walled. Antheridia were amphigynous, doliiform and 12.3 × 12.4 μm. However gametangia may show dimensions corresponding to both parents and are therefore not necessarily those of the type description of *P. citrophthora*.

Phytophthora ramorum: A single isolate was recovered from a leaf lesion in 2002. Despite an intense survey on the same set of potted *A. unedo* plants, there were no more findings. In culture, *P. ramorum* was easily distinguished from the other isolates by its unique combination of slow growth rate and formation of large chlamydospores on all agar media, along with caducous sporangia arranged in clusters with a sessile or short pedicel. Sporangia measured 42.2 ± 7.8 × 24.8 ± 4.7 μm (Fig. 1h). Isolate P62 formed few gametangia when paired with an isolate of *Phytophthora cambivora* belonging to the A2 mating type (Kaminski and Werres, personal communication), confirming that the isolate was of the A1 mating type. The colony patterns and growth rates were also characteristic of the European subpopulation of *P. ramorum*. Further molecular tests to determine the lineage, as recently described by Ivors et al. (2006) were not carried out.

Phytophthora nicotianae. A single isolate of the A2 mating type was obtained from a leaf lesion. The radial growth rate was 7 mm/day on CA and below 2 mm/day on MEA and PDA. Hyphal branches were short, and the mycelium bushy to coralloid on PDA. The colony pattern was characteristically stoloniferous, with undefined margins on CMA, MEA and PDA, and submerged and unpatterned on CA. *Phytophthora nicotianae* was readily identified by its non-caducous, papillate, globose to obpyriform sporangia, and on the abundant chlamydospores and hyphal swellings on CA. When the isolate was mated with a known A1 mating type of *P. nicotianae*, it produced abundant oospores with amphigynous antheridia.

Phytophthora tropicalis. The single isolate obtained exhibited a relatively high radial growth rate (10.5 mm/day) on CA at 20°C. The colony on CA was stellate to slightly petaloid and powdery on the margins; slow growing, flattened and slightly stellate on PDA; and stellate to petaloid and appressed on MEA. Sporangia were abundant, formed on umbellate sporangiophores, caducous, bearing very long pedicels (mostly twice the sporangial length), papillate or semipapillate, averaging 35.5 × 21.4 μm (23.8–51.3 × 16.5–25.8 μm), mostly obovoid to limoniform or elongated ellipsoidal, often with a tapered base, length : breadth ratio was ca. 1.8 (Fig. 1i). Some sporangia were curved or 'mouse shaped'. No chlamydospores were observed. Gametangia were abundant 5 days after pairing with an A2 isolate of *P. nicotianae*, confirming that our isolate was A1. Oogoniatormed on the colony side of *P. tropicalis* were spherical to globose, and frequently with elongated and distorted shapes (Fig. 1j).

Identification by ITS sequencing and phylogenetic position

The morphological identification of isolates of the five *Phytophthora* species was confirmed by comparing their ITS sequences with those of well-known species published in GenBank. The amplification of the ITS produced a fragment of 813 bp in all isolates of *P. syringae*. Sequence alignments showed 100% identity, except for isolates P3312 and P11213, which differed from the others by 2 and 6 bp respectively. Both isolates however clustered together with the other *P. syringae* isolates in the phylogenetic analysis. Amplification of the ITS produced an 873 bp fragment in all isolates of *P. citrophthora*. Isolates P2912 and P4142 shared identical ITS sequences, but differed from P3742 and P3212 in 1 and 2 bp respectively (Fig. 2). The ITS sequence of *P. tropicalis* was 779 bp long and differed at least in 3 bp from the sequences of those of *P. tropicalis* in GenBank. The phylogenetic position of the five *Phytophthora* species based on the ITS sequences mostly conformed to those observed in the tree topologies of Cooke et al. (2000) and Kroon et al. (2004) (Fig. 2).

Pathogenicity

All isolates tested for pathogenicity produced lesions either on detached leaves or on excised stems. In both experiments, controls remained healthy. All isolates were successfully recovered from inoculated hosts by plating pieces of tissue ca. 5 × 5 mm from the margins of the necrotic lesion onto PARP selective medium, thus meeting Koch's postulates. No *Phytophthora* was recovered from the controls.

Lesions on leaves were conspicuous for all isolates 6 days after zoospore inoculation. The symptoms developed were similar to those observed in natural infections. Two to 3 days after the inoculation, the first signs of necrosis appeared at the point of inoculation. The lesions spread faster along the midrib in both directions, on many occasions eventually reaching the petiole. There were significant differences in lesion area between species ($F = 18.4$, $P < 0.001$) in the leaf inoculations (Fig. 3). The two *P. citrophthora* isolates were significantly ($P < 0.01$) more aggressive than the others, with the mean lesion area ca. 10 cm^2, whereas no significant differences in pathogenicity were observed among the *P. syringae* isolates and *P. ramorum* with

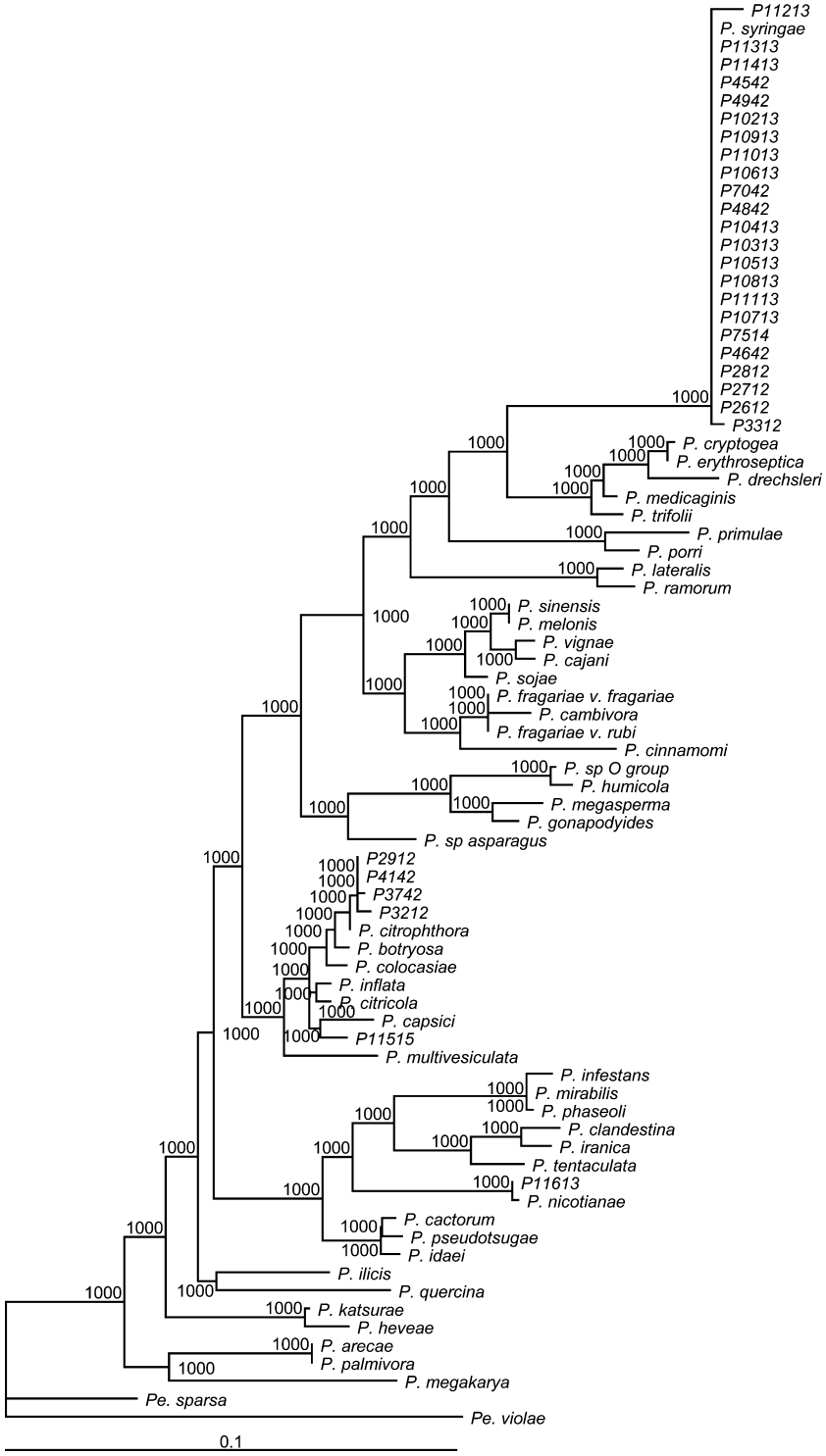

Fig. 2 Neighbour-joining tree obtained from the sequence data of the internal transcribed spacer (ITS) regions of the rDNA of isolates of *Phytophthora* spp. collected from *Arbutus unedo* and reported in GenBank using the maximum likelihood method with the general time reversible model (GTR) + G + I. The numbers at the nodes are non-parametric bootstrap values higher than 80%. Lengths of branches are proportional to the number of substitutions per site as indicated by the scale

the exception of isolate P4942 which formed a larger lesion than *P. ramorum* (Fig. 3).

In the twig inoculations there were significant ($F = 18.4$, $P < 0.001$) differences in lesion length among species (see Fig. 4). *Phytophthora citrophthora* caused the most extensive lesions, which agrees with observations on natural infections. The two *P. citrophthora* isolates were significantly ($P < 0.01$) more aggressive than the other isolates, with the mean lesion length ranging from 85 to 90 mm (Fig. 4). The *P. ramorum* and *P. syringae* isolates (Fig. 4) showed no significant ($P = 0.67$) differences in pathogenicity. The most pathogenic isolates on leaves (i.e. *P. citrophthora*) also affected twigs most severely.

In the log inoculation trial, *P. syringae* significantly formed the largest inner bark necrotic lesions, with a mean area of 45.5 cm^2, ranging from 20.2 to 101.5 cm^2. The other five *P. ramorum* isolates were

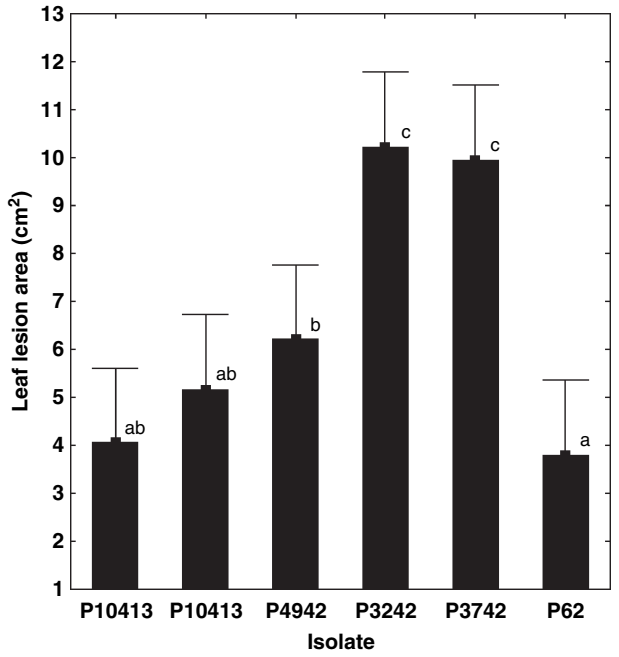

Fig. 3 Differences in aggressiveness between isolates of *Phytophthora* spp. on excised leaves of *Arbutus unedo*. Intact leaves were inoculated with a zoospore suspension (10^4 zoosp/ml) and the lesion area measured 7 days after inoculation. Values in each column followed by the same letter are not significantly different at P = 0.05, Fisher's protected least significant difference test. Error bars denote 95% confidence intervals. n = 4

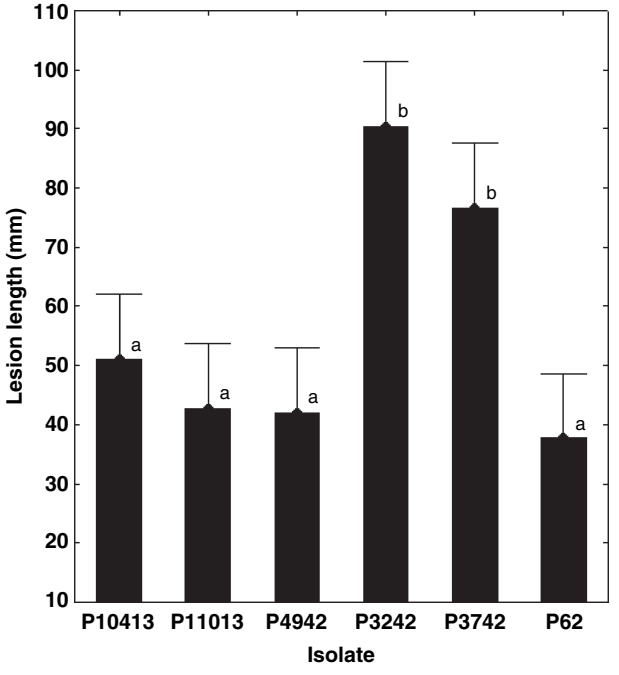

Fig. 4 Mean lesion length on twigs of *Arbutus unedo* 10 days after wound inoculation with several *Phytophthora* spp. Values in each column followed by the same letter are not significantly different at P = 0.05, Fisher's protected least significant difference test. Error bars denote 95% confidence intervals. n = 3

moderately aggressive to the trunks, with mean area lesions between 13.2 and 18.7 cm^2 (ranging from 3.2 to 39.3 cm^2). The mean lesion area of *P. cinnamomi* was 11.6 cm^2 (ranging from 2.5 to 27.7 cm^2). The mean necrotic area of the controls was below 0.71 cm^2. The isolates were recovered from all the inoculated points except in the controls.

Sporangial production

All isolates formed sporangia on lesions in the detached-leaf inoculations (Table 2). *Phytophthora ramorum* significantly formed the highest number of sporangia at 20°C ($P < 0.001$), with up to 843 per leaf, and sporangial numbers were always ca. twice those of *P. syringae* (highest average 317 sporangia). The latent period, i.e. the time from infection to sporangial formation, was <96 h for *P. ramorum*. Numerous chlamydospores were formed by the latter.

On agar plugs at either 15 or 20°C, again there were significant ($P < 0.01$) differences in sporulation between isolates (Table 2). *Phytophthora ramorum* formed the highest number of sporangia at both temperatures and significantly ($P < 0.01$) more than the other isolates, except for P3742. The average number of sporangia formed by *P. ramorum* was 5447. In a factorial ANOVA analysis to evaluate the temperature effect, there was no significant difference in *P. ramorum* sporulation on agar plugs at 15 and 20°C.

On infected disks at 12°C, there was an important decrease in sporulation (sporangial formation) of all *P. syringae* isolates when compared with those on agar plugs. There was also a significant ($P < 0.005$) difference in sporulation among isolates. *Phytophthora ramorum* sporulation decreased if compared with the agar plug inoculation, but a considerable number of sporangia were still formed. *Phytophthora citrophthora* produced a statistically comparable number to that of

Table 2
Mean number of sporangia formed on detached leaves of *Arbutus unedo*, infected leaf disks of *A. unedo* and 12-mm-diam. agar plugs at different temperatures by representative isolates of *Phytophthora* spp. recovered from *A. unedo* in Spain

		No. of sporangia			
Species	Isolates	Detached leaves (20°C)[z]	Mycelium plugs (20°C)[z]	Mycelium plugs (15°C)[z]	Leaf disks (12°C)[z]
P. syringae	P10413	66^{a}	537^{ab}	1122^{ab}	3^{a}
	P11013	288^{b}	525^{ab}	398^{abc}	7^{a}
	P4942	195^{b}	339^{a}	331^{ac}	4^{a}
P. citrophthora	P3212	72^{ab}	617^{ab}	138^{c}	174^{b}
	P3732	141^{ab}	1072^{b}	1514^{bd}	145^{b}
P. ramorum[x]	P62	813^{c}	4074^{c}	6310^{d}	692^{b}

[z]Values in each column followed by the same letter are not significantly different. The number of sporangia was Fisher's protected least significant difference test (P = 0.05).
[x]P62 belongs to the European subpopulation of *P. ramorum* (A1 mating type).

P. ramorum. Of the *P. syringae* isolates, only P4942 formed a few sporangia.

Discussion

Our study provides clues for the understanding of the aetiology of strawberry tree (*A. unedo*) blight by identifying the causal agents and comparing their aggressiveness and capacity to sustain sporangia on the host. Morphological identification of isolates to species level is well supported by a phylogenetic analysis based on ITS sequences (Fig. 2).To our knowledge, these are the first records of *P. syringae*, *P. citrophthora*, *P. tropicalis*, *P. nicotianae* and *P. ramorum* on strawberry trees in garden centres and nurseries of Spain. All species had earlier been reported infecting ericaceous ornamentals in Europe and the USA (e.g. Gerlach et al., 1976; Benson and Jones, 1980; Erwin and Ribeiro, 1996; Werres et al., 2001; Hong et al., 2006), but only *P. cactorum* (Stuntz and Seliskar, 1943) and *P. ramorum* (Maloney et al., 2004) had been reported infecting the closely related species *Arbutus menziesii* in the Pacific coast of the USA.

Disease incidence seems to differ markedly between the species of *Phytophthora* involved. Although the source of the primary inoculum in nurseries has not yet been investigated, the frequency of isolation of *P. syringae* and diversity where infected individuals are found suggests to that it is well established on strawberry trees of the main garden centre suppliers across the west Mediterranean. The incidence of blight caused by *P. syringae* seems to be seasonal, favoured by prolonged periods of cool (10–15°C), rainy weather during late autumn and winter, as most findings in garden centres occurred during this period. However, the optimal temperatures for *in vitro* mycelial growth and sporulation were somewhat higher in culture. This slight discrepancy could be explained because *P. syringae* infections are heightened when plants are dormant in winter, as suggested by Bostock and Doster (1985) for infections of *P. syringae* in almond trees in orchards in California.

Phytophthora citrophthora isolates exhibit high aggressiveness, forming largest lesions on stems and leaves in either the field or the inoculation tests. Unlike *P. syringae*, it has rarely been isolated from natural ecosystems in Europe and hence seems to be mostly confined to human-managed environments, especially citrus orchards (Cohen et al., 2003) and ornamental nurseries (Erwin and Ribeiro, 1996). Infections in nurseries may start at the onset of the rainy season in late summer, or earlier when overhead irrigation is practised. Its incidence seems to be directly related to the total rainfall and to the duration of mild to warm temperatures through autumn under Mediterranean conditions, as occurs in the epidemiology of 'orange brown rot' in Spain (Tuset, 1988). In our study, a high phenotypic variation was found among isolates of *P. citrophthora*, which is genetically correlated with small differences in the rDNA ITS nucleotide sequences. Cohen et al. (2003) found high genetic and pathogenicity variations between isolates of *P. citrophthora* collected from citrus groves in the Mediterranean island of Corsica, France. The ITS sequence of our *P. citrophthora* isolates matched that of the main cluster (G1) of isolates collected from *citrus* orchards in Corsica (Cohen et al., 2003). However, unlike the Corsican isolates, not all the *P. citrophthora* isolates from Majorca were sterile; some formed a substantial number of gametangia when paired with the A1 isolate of *P. nicotianae*.

The single findings of *P. ramorum*, *P. tropicalis* and *P. nicotianae* on leaves of *A. unedo* suggest an almost 'universal' susceptibility of this shrub towards *Phytophthora*, as seems to happen with rhododendron (Brasier et al., 2004). The *P. tropicalis*-like isolate agreed with the previous morphological description of the few findings in Europe in being of the A1 mating type (Gerlach and Schubert, 2001), but differed in the position of 3 bp in respect to the ITS sequence of other isolates of *P. tropicalis* published in GenBank, as well as in the formation of irregularly shaped oogonia. Therefore, its taxonomic status within the *P. capsici*/P. tropicalis complex has to be further examined. Despite an intense survey of *P. ramorum*, it was recovered only once from *A. unedo* in the same garden center where it had been previously isolated from rhododendron plants 5 to 7 m apart. We hence believe it is possibly a case of *in situ* infection.

Temperatures below 18°C at the start of the rainy season in autumn hamper the development of epidemics by tropical or subtropical *Phytophthora* spp. such as *P. nicotianae*, *P. citrophthora* and maybe *P. tropicalis* on native hosts in Mediterranean climates. In contrast, *P. ramorum* and *P. syringae* seem to be better adapted to the Mediterranean climate by being able to sporulate and infect at lower temperatures. While *P. syringae* is highly aggressive in nurseries either on leaves, twigs or trunks, we have never seen leaf infections on wild strawberry trees in the Balearic Islands or in Catalonia (Moralejo, unpublished data). This failure of infection outdoors could be due to *P. syringae* having been recently introduced in this area, or to ecological and physiological constraints hindering its spread. The first supposition is unlikely as citrus species, reported as hosts (Tuset, 1988), have long been planted in the Mediterranean basin. The second assumption could be related to its low sporangial production and persistence. The combination of both factors would have a negative effect on the basic reproductive number R_0, i.e. the average number of new lesions produced by a lesion during its period of infectiousness in the early stages of the epidemic (Segarra et al., 2001) in a random low-density plant distribution, as happens for *A. unedo* in nature. The case of *P. ramorum* could be quite different, however. It is capable of infecting all aboveground organs including fruits in all stages of maturity (Moralejo et al., 2006) and of sporulating profusely on them. The results of our inoculation assays reveal no significant differences with *P. syringae* in aggressiveness to leaves or twigs. In contrast, sporangial density was much higher

both *in planta* and *in vitro*, and the latent period comparatively shorter. In addition, *P. ramorum* is a multiple-host pathogen producing deciduous sporangia, which are easily dispersed from leaf to leaf by rain splash, reaching higher altitudes in the canopies. This suggests a higher potential for inoculum production and infection rates within the same weather parameters favouring *P. syringae* epidemics. In addition, management practices in nurseries such as overhead sprinkling, high-density planting and shading create the ideal environment for rapid disease propagation. Together with *A. menziesii* and *A. unedo*, *A. canariensis* has also shown high susceptibility to *P. ramorum* in artificial inoculations (Moralejo et al., unpublished data). There have been a few recent interceptions of *A. unedo* plants infected by *P. ramorum* in Spain (Defra interception charts: http://www.defra.gov.uk/planth/interc/18dec04.pdf). Taking into account all the above, we conclude that *P. ramorum* might become an important pathogen of *A. unedo* if it is introduced in the nurseries where this is propagated, and it might also spread to the understories of the Mediterranean holm oak forest (Moralejo et al., 2006).

Acknowledgements

We are grateful to the 'Conselleria de Medi Ambient del Govern Balear' for funding the survey of *Phytophthora ramorum* in the Balearic Islands and to Joan Coll and Diego Olmo and the 'Conselleria d'Agricultura del Govern Balear'. Part of this research was also funded by the EU Project RAPRA.

Supporting Information

The following supporting information is available for this article:

Figure S5. Necrotic lesions formed by *phytophthora* spp. on *Arbutus unedo*. A. on excised leaves inoculated with zoospores after seven days. B. on twigs, 10 days after wound-inoculation.

Additional Supporting Information may be found in the online version of the article.

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