Phytophthora iranica, a New Root Pathogen of Myrtle from Italy

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ABSTRACT

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A homothallic *Phytophthora* sp. producing persistent and markedly papillate sporangia was isolated, together with *P. nicotianae*, from the rotted roots of seedlings of myrtle (*Myrtus communis*), grown in pots in a commercial nursery in Sardinia, Italy. The homothallic species isolated from myrtle was identified as *P. iranica* on the basis of morphological and cultural characteristics; it differed from other known *Phytophthora* spp. belonging to Waterhouse's group I in its higher cardinal growth temperatures. In addition, the isolate of *P. iranica* from myrtle was distinguished from *P. cactorum* by the electrophoretic protein pattern and the serological reaction of mycelial extracts, from *P. clandestina* by its simple antheridia and the absence of a prominent basal plug in the sporangia, and from both *P. cactorum* and *P. clandestina* by its persistent sporangia. In pathogenicity tests, *P. iranica* proved to be nonpathogenic to wound-inoculated potato tubers, less pathogenic than *P. nicotianae* to apple fruit, and only weakly pathogenic on tomato seedlings or the stem and roots of myrtle seedlings. This is the only record of *P. iranica* outside Iran.

During the early summer of 1990, a severe decline of pot-grown seedlings of myrtle (*Myrtus communis* L.) was observed in a nursery of S.A.F./E.N.C.C. in Sardinia, Italy. The decline was associated with root and crown rot, and *Phytophthora nicotianae* Breda de Haan was consistently isolated from rotted tissues and confirmed as the primary etiologic agent by fulfilling Koch's postulates (1). Isolations from rotted roots on a selective antibiotic medium amended with hymexazol (10) also yielded a few cultures of a *Phytophthora* species with papillate sporangia but distinct from *P. nicotianae*. The isolations were tentatively identified as *P. iranica* Ershad, a homothallic *Phytophthora* sp. reported in Iran (6,7) and as yet never recorded elsewhere.

This study was undertaken to confirm the identity of the homothallic isolates recovered from myrtle roots and to determine their pathogenicity.

MATERIALS and METHODS

Isolation. Rotted roots of wilted myrtle seedlings were washed free of soil in tap water and blotted dry on filter paper. Root segments, 2–4 mm long, were plated on BNPRAH selective medium (10), incubated at 24 C in the dark, and examined daily over 10 days for the development of *Phytophthora* spp. Emerging hyphae of *Phytophthora* were subcultured on Difco potato-dextrose agar (PDA) and V8 juice agar (V8A) (14).

Identification. The isolates were identified on the basis of colony morphology, mycelial characteristics and growth rate, cardinal growth temperatures, and morphology and dimensions of both sporangia and gametangia.

The growth rate and the cardinal temperatures were determined by transferring 5-mm-diameter mycelial plugs to PDA in petri dishes and incubating the dishes in the dark at temperatures from 5 to 40 C, with 5 C increments, and then at 27, 28, and 37 C. Radial growth of the colonies was measured daily for up to 9 days. The test was repeated three times, with six replicate dishes each. Analysis of variance of the data was performed, and Fisher's test was used to separate treatment means (16).

Sporangia were produced both on V8A in petri dishes in the dark at 24 C and in saline salt solution, according to the method reported by Chen and Zentmyer (4). Gametangia were produced by hyphal tip isolates in single cultures on V8A in the dark at 24 C. Specimens for the measurements of both sporangia and gametangia were mounted in distilled water on glass slides. Each set of data was compiled on the basis of 100 measurements.

Electrophoresis of mycelial proteins. The following fungal isolates from the collection of the Institute of Plant Pathology of the University of Catania were tested: four isolates from myrtle roots provisionally referred to as *P. iranica*, an isolate of *P. cactorum* (Lebert & Cohn) J. Schrö. from *Fragaria* × *ananassa* Duchesne (strawberry), an isolate of *P. capsici* Leonian from *Capsicum annuum* L. (pepper), an isolate of *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian from *Citrus sinensis* (L.) Osbeck (orange), and isolates of *P. nicotianae* from myrtle, citrus soil, *Forsythia* × *intermedia* Zab. (golden bells), and *Simmondsia chinensis* (Link) C.K. Schneid. (jojoba).

Mycelial extracts were obtained from 9-day-old stationary cultures grown on carrot broth (14) at 24 C in the dark. Mycelium was harvested by filtration into muslin and washed three times with sterile distilled water.

The proteins were extracted by grinding blotted-dry mycelium with a pestle in a mortar containing quartz sand in 0.2 phosphate buffer, pH 7.0, with 0.001 M EDTA (1 ml of buffer per 1 g of mycelium). The mycelial fragments were removed by centrifugation at 40,000 *g* for 40 min. The soluble proteins of the supernatant were precipitated with 70% ammonium sulfate and then recovered by centrifugation at 9,800 *g* for 25 min. The resulting pellet was dissolved in the sample buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, and 0.002% bromophenol blue). All the above steps were conducted at 4 C.

The proteins extracted were analyzed on 7.5% polyacrylamide gels in a nondissociating buffer system (5,13). Each sample, containing 14 μg of proteins, as determined by the Bradford method (2), was pipetted into the stacking gel (3.5% polyacrylamide), using a Protean-Dual apparatus (Bio-Rad Laboratories, Richmond, CA). The electrophoresis was carried out at 4 C. The gels were stained with silver stain (11). The values of the similitude index (SI) among the electrophoretic protein profiles were calculated as previously described (3).

Serology. Serological relationships between *P. iranica* from myrtle roots and other *Phytophthora* species were examined by enzyme-linked immunosorbent assay (ELISA). The *Phytophthora* isolates tested by ELISA were the same as those used in polyacrylamide gel electrophoresis. A commercial kit (Phytophthora E Kit, Agri-Diagnostics Associates, Cinnamison, NJ) was used in a standard ELISA precoated multiwell

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format. Mycelial extracts were prepared from 8-day-old cultures grown on PDA in the dark at 24 C. Abrasive pads supplied with the kit were used to grind up aerial mycelium, and then the mycelium was washed from the pads on a vortex mixer in test tubes containing 4 ml of the extraction buffer supplied in the kit. Each extract (100 μl) was pipetted into the wells of a microtiter plate. The plate was incubated for 10 min at room temperature (22–24 C) with continuous shaking, then was washed five times with the wash solution supplied with the kit. The enzyme-conjugated antibody (100 μl) was added to each well, and the plate was incubated for 10 min and washed again thoroughly. Substrate (100 μl) was pipetted into each well, and after 10 min of incubation, 50 μl of stop solution was added. The absorbance values were determined at 405 nm with a Titertek Multiscan 3100 ELISA plate reader (Flow Laboratories, McLean, VA).

The mycelial extracts were plated in duplicate wells, and the test was repeated three times. Negative controls (supplied in the kit) were included in each multiwell plate.

Pathogenicity tests. The following isolates were used in the pathogenicity tests: an isolate of *P. cactorum* from *Malus* × *domestica* Borkh. (apple); one of *P. cryptogea* Pethybr. & Lafferty from *Pistacia lentiscus* L. (lentisk, or mastic tree); one tentatively classified as *P. iranica* and one of *P. nicotianae*, both recovered originally from rotted roots of myrtle; and isolate IMI 180615 of *P. cryptogea* from the culture collection of the International Mycological Institute, Kew, England. Four different methods were used in the pathogenicity tests:

1) Fruit of apple cv. Golden Delicious and tubers of potato (*Solanum tuberosum* L.) cv. Spunta were surface-sterilized with 1% NaOCl for 2 min, rinsed in sterile distilled water, and inoculated with 5-mm-diameter plugs taken from actively growing colonies on PDA. Six fruit and six tubers per isolate were inoculated by inserting the mycelium plug in a hole made with a 5-mm-diameter cork borer, then sealing the hole with cellophane tape. Control plants were punctured and inoculated with PDA plugs. The fruit and tubers were incubated in the dark at 24 C for 4 days. The effects of inoculation on fruits were evaluated, 4 days after inoculation, by measuring the diameter of the brown lesions that appeared on the surface of the peel. Potato tubers were sectioned longitudinally 6 days after inoculation, and the rotted area of the section was measured with an automatic area meter to determine the size of the lesion. All the isolates were compared for pathogenicity. The experiment was repeated three times.

2) Pot-grown 30-day-old seedlings of tomato (*Lycopersicon esculentum* Mill.) cvs. Petomech and Marmande were inoculated on the stem, 2–5 cm above the soil surface, by inserting a 2-mm-diameter plug, taken from the edge of cultures actively growing on PDA, into a longitudinal incision made with a sterile scalpel. The wound was sealed with adhesive tape. Control plants were inoculated with plugs of PDA only. The seedlings were kept in a growth chamber at 24 C, under cool-white fluorescent light, with a 16-hr photoperiod. Symptoms were observed 7 days after the inoculation. Five seedlings of each cultivar were inoculated with each fungal isolate, and the test was repeated twice.

3) Pot-grown 3-yr-old seedlings of myrtle were inoculated in the stem with a 3-mm-diameter mycelial disk cut from the edge of a colony actively growing on PDA at 24 C in the dark. A cut was made with a sterile scalpel in the bark of the stem of each plant, 0.5 cm above the soil line. A disk was placed into each cut under the bark, and the cut was sealed with a strip of Parafilm. Agar disks from uninoculated PDA were inserted into the stems of control plants. One isolate of *P. iranica* and one of *P. nicotianae*, both recovered originally from myrtle roots, were used. Each treatment was replicated five times on single plants. After inoculation, plants were kept in a growth chamber at 24 C, under cool-white fluorescent light, with a 16-hr photoperiod. After 14 wk, disease severity was evaluated on a scale of 1–3, with 0 = no necrosis and wound completely healed, 1 = necrosis not extending beyond the wound margin, 2 = necrotic lesion up to 1.0 cm long, and 3 = necrotic lesion girdling the stem.

4) Pot-grown 1-yr-old seedlings of myrtle were inoculated in the root with the same *P. iranica* and *P. nicotianae* isolates used for inoculating stems. The seedlings were transplanted into 400-ml plastic pots containing a mixture of steam-pasteurized sandy loam field soil and vermiculite (1:2, v/v) and rice inoculum at the rate of 2% (w/v). The inoculum was prepared according to a

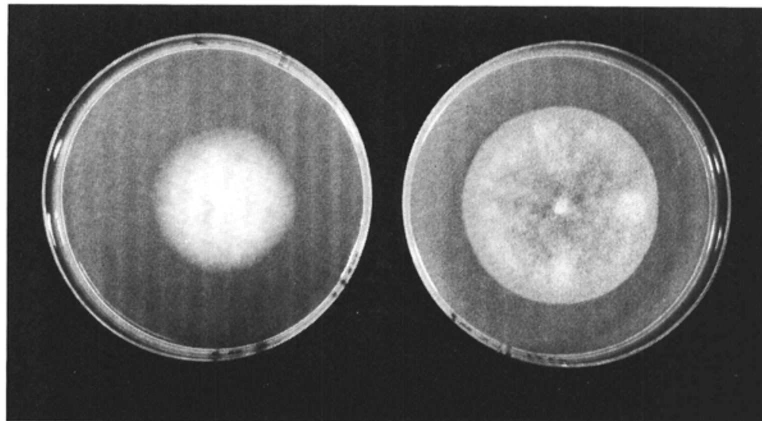

Fig. 1. Cultures of *Phytophthora iranica* from myrtle on (left) potato-dextrose agar and (right) V8 juice agar after 6 days of growth at 24 C in the dark.

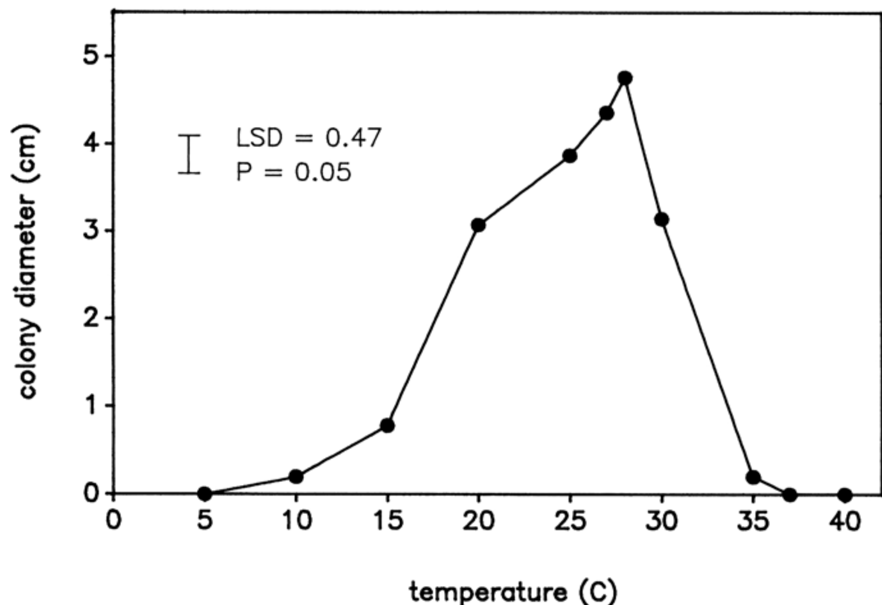

Fig. 2. Effect of temperature on the radial growth of cultures of *Phytophthora iranica* from myrtle on potato-dextrose agar after 6 days of incubation in the dark.

Fig. 3. (A, B, and C) Distinctly papillate sporangia stained with cotton blue in lactophenol, with (A) an inconspicuous basal plug at the insertion of the sporangiophore, and **(D, E, F, and G)** sexual structures of *Phytophthora iranica* from myrtle mounted in distilled water showing paragynous attachment of antheridia to the base of oogonia. Scale bar = 10 μm.

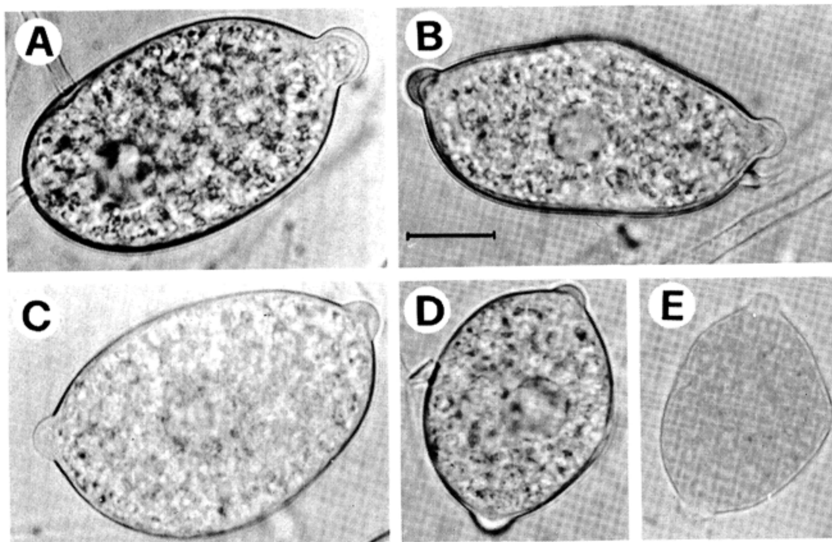

Fig. 4. (A) Monopapillate and **(B–E)** pluripapillate sporangia of *Phythophthora iranica* from myrtle in distilled water. Scale bar = 10 μm.

previously tested method (9), with the following slight modifications: 150 cm^3 of rice was thoroughly moistened with 200 ml of V8 juice broth in a 500-ml Erlenmeyer flask, autoclaved twice, and seeded with 20 ml of a mycelial suspension obtained by flooding an 8-day-old colony, grown on PDA, with sterile distilled water, then scraping the surface with a sterile scalpel. After inoculation, the flasks were incubated in the dark at 24 C for 3 wk. Control seedlings were transplanted in the same soil mixture prepared with uninoculated rice. The seedlings were kept in a growth chamber as previously described. Three weeks after transplanting, and at 2-wk intervals thereafter, the pots were flooded for 48 hr by plugging the drain hole of the pot and adding water until 5–10 mm of free water was collected above the soil surface. After 14 wk, disease severity was evaluated according to a scale of 1–5 based on a visual estimate of the percentage of the root mass rotted and the severity of symptoms on the aboveground part of the plant, with 0 = roots healthy, 1 = up to 25% rotted roots, 2 = 26–50% rotted roots, 3 = 51–75% rotted roots, 4 = severe root and crown rot and leaf yellowing, and 5 = severe root rot resulting in wilting of the plant. Each treatment was replicated five times on single plants, and the experiment was repeated twice.

Phytophthora spp. were reisolated from infected tissues of artificially inoculated plants on BNPRAH medium.

RESULTS

Identification. The isolates of *P. iranica* grew slowly (radial growth rate, 7.5 mm per day on PDA at 28 C), producing uniform colonies with dense mycelium on both PDA and V8A (Fig. 1). The cardinal temperatures for radial growth on PDA were 10 C minimum, 27–28 C optimum, and 35 C maximum (Fig. 2).

The hyphae produced on V8A measured 3.5–5.5 μm in diameter. Intercalary and terminal chlamydospores were observed occasionally. Sporangiophores were simple and sympodially branching.

Sporangia were produced more abundantly on aerial mycelium; they were terminal and less frequently intercalary, occasionally with lateral attachment, externally proliferating, persistent, with an inconspicuous basal plug, distinctly papillate, and often with two or three papillae (Figs. 3 and 4). The sporangia were spherical, ovoid, or ellipsoid to obpyriform, and pluripapillate sporangia were often distorted (Fig. 4). The sporangia formed on V8A at 25 C were 14–46 μm (mean 32.7 μm) long, including the papilla, and 11–37.5 μm (mean 26.0 μm) wide; the sporangia formed in salt solution were 35–59 μm (mean 46 μm) long and 27.5–42 μm (mean 35.5 μm) wide. The length-to-width ratio averaged 1.25 (range 1–1.5) on V8A and 1.30 (range 1–1.5) in salt solution.

Gametangia were produced in single culture (Fig. 3) and differentiated within 2 wk at 25 C on V8A. The oogonia measured 22–39 μm (mean 30 μm) in diameter on V8A and 26–35 μm (mean 30 μm) in salt solution. The antheridia were simple, always paragynous, with a spherical, ellipsoid, or ovoid shape, and averaged 9.8 μm in diameter (range 8.0–14 μm); the attachment of the antheridium was commonly near the oogonial stalk, both on solid and in liquid media.

The oospores were single, spherical, aplerotic (sometimes markedly so), smooth, and frequently thick walled (up to 2.5 μm). The thickness of the oospore wall appeared to be influenced by the medium; it averaged 1.1 μm in salt solution and 1.8 μm on V8A. Diameter of the oospores ranged between 15 and 23 μm (mean 21 μm) on V8A and between 22 and 30 μm (mean 25 μm) in salt solution.

Electrophoretic and serological tests. The four isolates identified as *P. iranica* on the basis of morphological characteristics showed identical protein profiles, confirming that they are conspecific. Similarly, the isolates of *P. nicotianae* from different hosts showed very similar electrophoretic protein patterns (SI values between the isolates of *P. nicotianae* were always <1.5).

The five species of *Phytophthora* tested showed different electrophoretic patterns of total mycelial proteins (Fig. 5). SI values obtained from the comparison between the electrophoretic profiles of *P. iranica* isolates and the profiles of isolates of other species were always >2.5, indicating that the isolates were taxonomically distinct.

When assayed by ELISA, the mycelial extracts of *P. iranica* isolates produced a less intense reaction than the extracts of the other species of *Phytophthora*.

Pathogenicity tests. All the isolates of *Phytophthora* infected the apple fruit (Fig. 6), but there was a marked difference in virulence. The isolates of *P. nicotianae* and *P. cactorum* were more virulent than the isolates of *P. iranica* and *P. cryptogea* (Table 1). All the isolates except *P. iranica* were pathogenic on potato tubers. *P. cactorum* was only weakly virulent; it produced a white mycelium in the cavity of the inoculation wound and rotted only the tissues lining the cavity. *P. nicotianae* and *P. cryptogea* isolate IMI 180615 induced a discoloration of infected tissues, which turned brown after the tubers were sectioned. The tubers inoculated with the isolate of *P. cryptogea* from lentisk showed an extensive pinkish discoloration of the internal tissues, whereas the tubers inoculated with *P. iranica*, as well as the control tubers, appeared sound (Fig. 6).

On tomato seedlings wound-inoculated with *P. cactorum, P. cryptogea*, or *P. nicotianae*, a brown, sunken lesion girdled the stem and caused collapse of the seedlings 7 days after inoculation. The isolates of *P. iranica* induced only a slightly brown discoloration of the stem tissues lining the incision made to inoculate the seedlings.

On myrtle seedlings stem-inoculated with *P. iranica* or *P. nicotianae*, the mean disease severity ratings were 1.2 and 2.4, respectively, compared with a disease rating of 0.0 on the control seedlings. The differences between the means were significant ($P \leq 0.01$). The species inoculated were reisolated from the lesions, whereas no *Phytophthora* sp. was recovered from the control seedlings.

Both *P. nicotianae* and *P. iranica* incited root decay on myrtle seedlings transplanted into artificially infested soil, but they differed noticeably in virulence. The mean disease severity ratings were 1.0 in seedlings inoculated with *P. iranica* and 3.8 (ranging from 2 to 5) in seedlings inoculated with *P. nicotianae*. The differences between the means were significant ($P \leq 0.01$). The control seedlings showed no symptoms of root decay. The species of *Phytophthora* inoculated was reisolated from rotted roots in each treatment. No *Phytophthora* species was recovered from the roots of the control seedlings.

DISCUSSION

The homothallic species of *Phytophthora* producing papillate sporangia and paragynous antheridia are placed in Waterhouse's group I, which originally comprised only *P. cactorum* (18). Subsequently, three more species—*P. clandestina* Taylor, Pascoe, & Greenhalgh, *P. iranica*, and *P. pseudotsugae* P.B. Hamm & E.M. Hans.—were included in the group (12,15).

The homothallic *Phytophthora* sp. recovered from myrtle roots can be readily separated from *P. cactorum* by its noncaducous sporangia and oospores, which vary a great deal in size. It also differs from *P. cactorum* in the slower growth, the colony pattern, the higher cardinal growth temperatures, and the relatively sparse production and slow differentiation of gametangia on agar media. The abundance and the early production of gametangia in culture could be supporting characters to separate *P. cactorum* from other *Phytophthora* spp. (19).

The results of the electrophoresis of mycelial proteins indicate that the homothallic *Phytophthora* sp. isolated from myrtle is distinct from *P. cactorum* and from other papillate species of Waterhouse's group II. Mycelium of *P. iranica* did not react as strongly as other papillate species with the ELISA test kit, con-

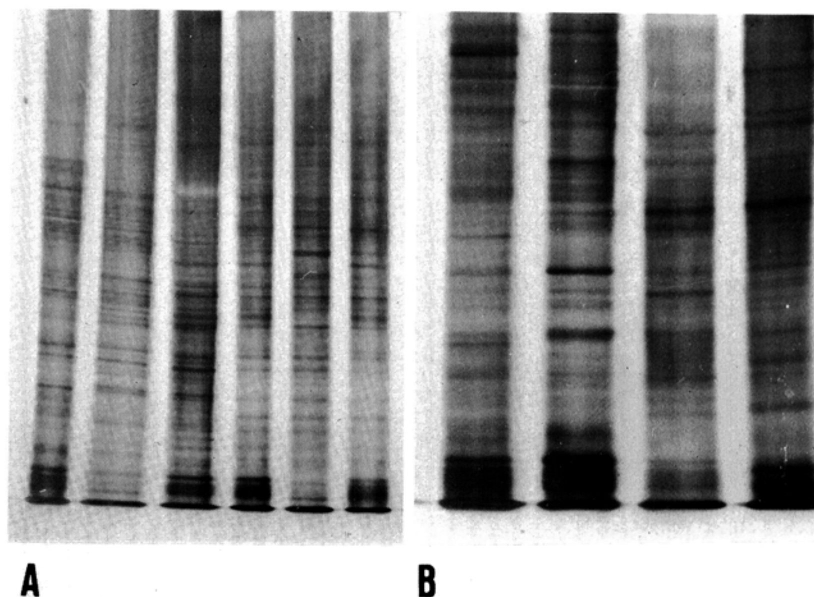

Fig. 5. (A) Electrophoretic protein patterns shown by isolates of *Phytophthora* spp. in polyacrylamide slab gel: (left to right) *P. iranica* from myrtle, *P. citrophthora* from orange, *P. cactorum* from strawberry, *P. nicotianae* from golden bells, *P. nicotianae* from myrtle, and *P. nicotianae* from jojoba. **(B)** A detail showing major differences between species: (left to right) *P. iranica, P. citrophthora, P. cactorum*, and *P. nicotianae* from golden bells.

firming that substantial differences exist between the protein pattern of *P. iranica* and those of the other species of *Phytophthora* tested.

The species found on myrtle, although showing some characteristics in common with both species, differs from *P. clandestina* and *P. pseudotsugae* in its higher cardinal temperatures for growth. Moreover, *P. clandestina* was reported to form caducous sporangia, with a conspicuous basal plug, and complex, frequently amphigynous antheridia (17), whereas the *Phytophthora* sp. recovered from myrtle produced only simple paragynous antheridia and persistent sporangia, with an inconspicuous basal plug. Finally, both *P. clandestina* and *P. pseudotsugae* are reported to be host-restricted (8,17).

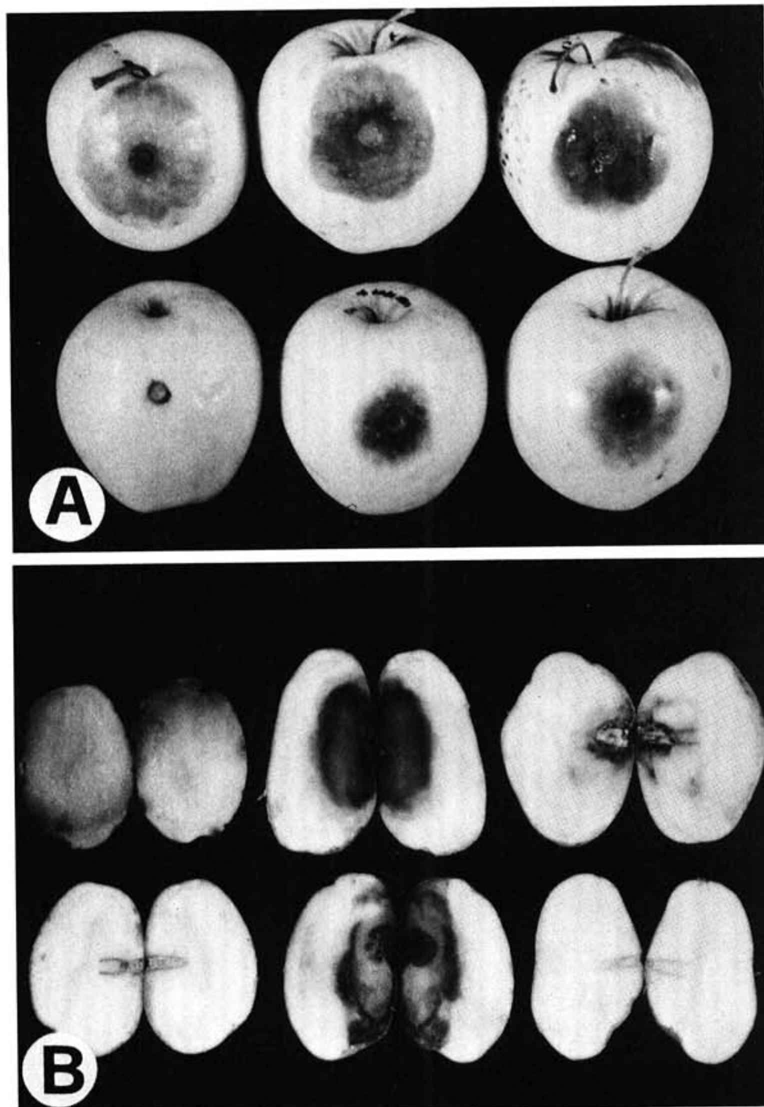

Fig. 6. (A) Results of pathogenicity tests of isolates of *Phytophthora* spp. on apple fruit, 4 days after inoculation: (top row, left to right) *P. cactorum* from apple, *P. cryptogea* from lentisk, *P. nicotianae* from myrtle; (bottom row, left to right) control, *P. cryptogea* isolate IMI 180615, *P. iranica* from myrtle. **(B)** Sections of potato tubers wound-inoculated with isolates of *Phytophthora* spp., 6 days after inoculation: (top row, left to right) *P. cryptogea* from lentisk, *P. cryptogea* isolate IMI 180615, *P. cactorum* from apple; (bottom row, left to right) *P. iranica* from myrtle, *P. nicotianae* from myrtle, control.

Table 1. Pathogenicity tests of *Phytophthora* spp. on apple fruit and potato tubers

		Lesion size[z] (cm²)	
Species	**Host**	**Apple fruit**	**Potato tubers**
P. cactorum	Apple	16.0 a	0.0 c
P. cryptogea	Lentisk	10.2 c	21.9 a
Isolate IMI 180615	Tomato	3.7 e	11.3 b
P. iranica	Myrtle	7.4 d	0.0 c
P. nicotianae	Myrtle	12.4 b	9.8 b
Control	...	0.0 f	0.0 c

[z] Means of six replicate values. Values followed by the same letter do not differ significantly according to Duncan's multiple range test ($P = 0.01$).

The homothallic *Phytophthora* sp. recovered from myrtle roots showed a close similarity in its morphological and cultural characteristics to *P. iranica*; it conformed to all the characteristics used by Stamps et al (15) to identify this species except it occasionally produced sporangia with lateral attachments, a characteristic not reported for *P. iranica*. Slight differences were observed in the mean size of sporangia and in the sexual structures between the *Phytophthora* sp. recovered from myrtle and the original description of *P. iranica* (6). These differences could be attributed, however, to the cultural conditions used and are within the range of intraspecific variability. As these differences do not warrant the separation of a new taxon, we have chosen to refer to the homothallic isolates of *Phytophthora* recovered from myrtle as *P. iranica*.

P. iranica was reported by Ershad (6) to be nonpathogenic on apple fruit and pathogenic on potato tubers, whereas in our study, the strain of *P. iranica* recovered from myrtle was moderately pathogenic on apple fruit but did not infect potato tubers. In accordance with Ershad's report (6), the strain of *P. iranica* from myrtle also proved to be a weak pathogen. Moreover, it appeared less polyphagous than other more common species of *Phytophthora*, such as *P. cactorum*, *P. cryptogea*, and *P. nicotianae*. Unlike *P. nicotianae*, *P. iranica* did not induce lethal effects on artificially inoculated myrtle seedlings. This suggests that the latter species was an opportunistic pathogen on myrtle roots and was not capable of causing severe disease in the absence of damage from other causes. Conversely, the results of pathogenicity tests confirm *P. nicotianae* as the primary etiologic agent of the decline of myrtle seedlings observed in Sardinia (1).

Ershad's specific description of *P. iranica* (6) was based on a single isolate recovered from roots of *Solanum melongena* L. (eggplant) in Iran. The present finding of *P. iranica* on myrtle, the only one following Ershard's report, broadens the range of known host plants of this *Phytophthora* sp. and consolidates the taxonomic status of *P. iranica* as a species in Waterhouse's group I.

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