Forsythia: A New Host of *Phytophthora nicotianae* in Italy

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ABSTRACT

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During the summer of 1990, seedlings of forsythia (*Forsythia viridissima*), grown in pots in a production nursery in Campania (Italy), showed symptoms of decline associated with root and crown rot. *Phytophthora nicotianae* of A_2 mating type was isolated from decayed tissues. Identification of the isolate was based on both morphological and physiological characters, and on the electrophoretic pattern of total native mycelial proteins. *P. nicotianae* was confirmed as the causal agent of this decline by fulfilling Koch's postulates. Seedlings of forsythia inoculated with the *P. nicotianae* isolate developed symptoms identical to those observed in natural infections. The species inoculated was reisolated from the basal stem and roots of symptomatic seedlings. This is the first report of *P. nicotianae* as a pathogen of forsythia.

Forsythias (Oleaceae), also known as golden bells, are commonly used as ornamental shrubs in gardens, primarily because of their bright flowers. *Forsythia viridissima* Lindl., *F. suspensa* (Thunb.) Vahl, *F. giraldiana* Ling., *F. ovata* Nakai, and *F. europea* Degen & Baldacci, with their varieties and hybrids, such as *Forsythia* × *intermedia* Zab., are the most widely grown species.

During the summer of 1990, numerous 1- to 3-yr-old seedlings of *F. viridissima,* grown in pots with overhead sprinkler irrigation in a production nursery of S.A.F./E.N.C.C. in Campania (Italy) showed decline symptoms, including leaf chlorosis, lack of shoot growth, wilting, leaf scorch, and eventual plant collapse. Dying seedlings often appeared in groups that received excess irrigation water because they were in areas where the sprinklers overlapped. This syndrome was associated with root and collar rot. A *Phytophthora* sp. with papillate sporangia was consistently isolated from necrotic root and basal stem tissues.

The objectives of this study were to identify the species of *Phytophthora* associated with declining forsythia seedlings and to evaluate its pathogenicity to forsythia.

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MATERIALS AND METHODS

Isolation. Isolations were made from basal stem and roots of forsythia seedlings which showed decline symptoms. Roots were washed free of soil in tap water and blotted dry on filter paper. Small pieces of stem bark and root segments (1–2 mm) were placed in petri dishes containing the BNPRAH selective medium for the isolation of *Phytophthora* (15). Hymexazol was added at a concentration of 25 mg/L of medium. Petri dishes were incubated in the dark at 22 C, and emerging colonies of *Phytophthora* were subcultured on Difco potato-dextrose agar (PDA). The same selective medium was used for reisolations from infected roots and basal stem of artificially infected seedlings in pathogenicity tests.

Fungal isolates. A single hyphal isolate (C_{501}) from forsythia and the following isolates of *Phytophthora nicotianae* Breda de Haan (syn. *P. parasitica* Dastur), identified and characterized in previous studies, were used: a single zoospore isolate ($C_{88\text{-}1}$) from jojoba (*Simmondsia chinensis* (Link) C.K. Schneid.) (3), of A_2 mating type; a mass isolate (C_{92}) from kentia (*Howeia forsteriana*) (C. Moore & F.v. Muell.) Becc.) (3), of A_1 mating type; two mass isolates (C_{105} and C_{106}) from laurel (*Laurus nobilis* L.), both of A_2 mating type; and a single hyphal isolate (C_{301}) from myrtle (*Myrtus communis* L.) (1), of A_2 mating type. The identification numbers of the isolates are those of the culture collection at the Institute of Plant Pathology, University of Catania, Italy.

Morphological and physiological studies. Cardinal temperatures for vegetative growth of isolate C_{501} from forsythia, and of isolates C_{105} and C_{106}, both from laurel, were determined by transferring 5-mm-diameter mycelial plugs to PDA in petri dishes and incubating the dishes in the dark at temperatures between 5 and 40 C. The diameters of the colonies were measured after 6 days of incubation. The test was repeated twice, with six replicate dishes each. Analysis of variance of the data was performed to differentiate treatment means.

Sporangia and chlamydospores of isolate C_{501} were produced on both V8 juice agar medium (V8A) (18) and mineral salt solution, using the procedure described by Chen and Zentmyer (4). Measurements were taken on 150 sporangia, mounted in water, per type of medium. To determine the size and shape of sporangia, 12-day-old colonies grown on V8A at 24 C in the dark were used. The isolates were paired with isolates of opposite mating types for production of gametangia and oospores. Paired cultures were grown in petri dishes on V8A in the dark at 24 C. Measurements were taken on 60 oogonia and oospores mounted in water.

Polyacrylamide gel electrophoresis. Mycelial proteins were extracted using a previously described procedure (3) with slight modifications. Mycelium from 8-day-old cultures grown on carrot broth (18) and incubated in the dark at 24 C was harvested by filtration into muslin and washed with sterile distilled water. Mycelium was blotted dry and ground with a mortar and pestle with quartz sand in 0.1 M phosphate buffer, pH 7.0, with 0.001 M EDTA (1 ml of buffer per gram 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% $(NH_4)_2SO_4$ 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% bromphenol blue). All steps were conducted at 4 C.

The proteins extracted were analyzed on 7.5% polyacrylamide gels in a discontinuous nondissociating buffer system (6,17). Each sample, containing approximately 200 μg of proteins as determined by both the method of Lowry et al (13) and Bradford's method (2), was pipetted onto the stacking gel (5% polyacrylamide). An electrophoresis apparatus (Protean-Dual, Bio-Rad Laboratories, Richmond, CA) was used. The electrophoresis was conducted at 4 C. The gels were stained with Coomassie Blue (7). Each experiment was repeated three times.

Pathogenicity tests. Pot-grown 3-yr-old seedlings of forsythia (*F. viridissima*) were transplanted into 25-cm-diameter plastic pots (one seedling per pot) filled with a mixture of steam-pasteurized sandy loam field soil and vermiculite (1:2 v/v), and rice-inoculum (14) at the rate of 2% (w/v) of the mixture. The inoculum was prepared by growing the *Phytophthora* isolate from forsythia in 250-ml Erlenmeyer flasks containing 80 g of rice moistened with 50 ml of V8 juice broth. After inoculation, flasks were incubated in the dark at 24 C until the mycelium had colonized the entire substrate. The inoculum, obtained as described, was rinsed with sterile distilled water over cheesecloth in a Büchner funnel and then mixed with the potting medium. Seedlings used as controls were transplanted into pots containing the same soil mixture prepared with uninoculated rice. After transplanting, seedlings were kept in a screenhouse for 3 mo, and the pots were flooded every 2 wk by immersing them in water for 48 hr. Control seedlings received the same treatment. Three months after transplanting, the roots of the seedlings were washed free of soil and examined for disease. Disease severity was evaluated according to a scale 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 plants. Each treatment was replicated six times on single plants; and the experiment was repeated three times, in June, July, and September 1991.

The same procedure with slight modifications was used to inoculate 1-yr-old forsythia seedlings. Seedlings were transplanted into a 50 × 50 × 15 cm garden frames containing infested soil mixture prepared according to the procedure described above. Seedlings used as controls were transplanted into frames containing soil mixture prepared with uninoculated rice. Ten seedlings were transplanted into each frame. The seedlings were irrigated only when necessary, thus avoiding waterlogging the soil. Three months after transplanting, the roots of the seedlings were examined for disease. Disease severity was evaluated according to the same rating scale used for 3-yr-old seedlings. The test was repeated three times, in June, July, and September 1991, each with four replicate frames. Fungal isolates obtained from infected roots and basal stem of artificially inoculated plants were identical to isolate C_{501}.

In addition to forsythia seedlings, tomato (*Lycopersicon esculentum* Mill. 'Marmande') and tobacco (*Nicotiana tabacum* L. 'Kentucky 15' and 'White Burley') were included in a host-range susceptibility study. The isolates C_{105}, C_{301}, and C_{501} were used in these tests. Pot-grown 60-day-old seedlings of tomato and tobacco were inoculated on the stem, 4 cm above the soil surface, by inserting a 3-mm-diameter plug taken from the edge of actively growing cultures on PDA into a longitudinal incision made with a sterile scalpel, which was then sealed with adhesive tape. The seedlings were incubated in a growth chamber at 24 C under cool-white fluorescent light, with a 16-hr photoperiod. Agar plugs with no fungal mycelium were placed into the stem incision of seedlings used as controls. The test was repeated three times, with five seedlings of each cultivar per fungal isolate. Equal numbers of seedlings were used as controls.

RESULTS

Identification. The isolate from forsythia formed uniform colonies on PDA, with sparse aerial hyphae and undefined margins. The colony morphology was similar to that of colonies formed by isolates of *P. nicotianae* from other hosts (Fig. 1). The isolate from forsythia grew faster at temperatures between 15 and 35 C than did isolates C_{105} and C_{301}, from laurel and myrtle, respectively (Fig. 2). The isolate from forsythia did not grow at 5 and 40 C, and grew very slowly at 37 C. Optimal growth was observed at 27–28 C.

Markedly papillate sporangia, occasionally with two apexes, subspherical, or less frequently, broadly ovoid or obpyriform in shape, were produced on V8A. Sporangia were frequently caducous with a very short pedicel (mean length less than 5 μm) (Fig. 3). The medium used for sporangium production affected the degree of caducity. The percentages of detached pedicellate sporangia were 7.4 and 43% on V8A and mineral salt solution, respectively. The sporangia produced on V8A were 29–57 μm long (mean 44.2 μm), including the papilla, and 25–44 μm wide (mean 33.8 μm). The

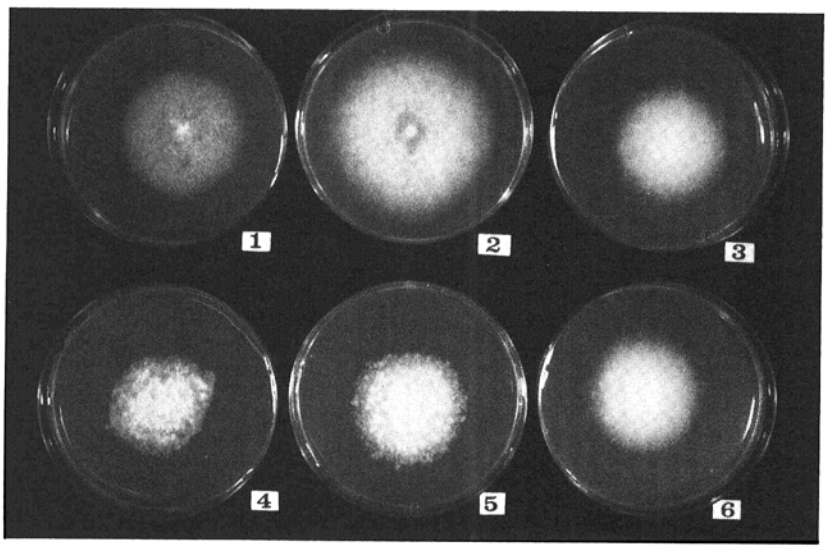

Fig. 1. Colony morphology of isolates of *Phytophthora nicotianae* grown for 8 days at 24 C on potato-dextrose agar. Isolates are (1) C_{301} from myrtle, (2) C_{501} from forsythia, (3) C_{105} from laurel, (4) C_{92} from kentia, (5) $C_{88\text{-}1}$ from jojoba, (6) and C_{106} from laurel.

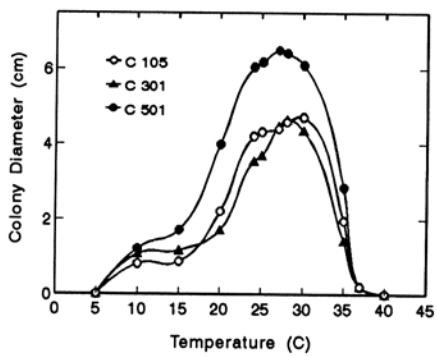

Fig. 2. Effect of temperature on growth of mycelium of isolates C_{105}, C_{301}, and C_{501} of *Phytophthora nicotianae*. Colony diameter was measured 6 days after the isolates were grown on potato-dextrose agar in the dark.

dimensions of sporangia formed in mineral salt solution were 25–72 μm long (mean 50.9 μm) and 18–52 μm wide (mean 39.4 μm). The mean length-to-width ratio of sporangia was 1.3 ± 0.3 (fiducial limits at $P = 0.01$) on both media. On V8A, the isolate differentiated chlamydospores readily and formed hyphal swellings, more or less spherical, with radiating hyphae.

The isolates tested formed sexual reproductive structures only when paired with the opposite mating type (Table 1). The isolate from forsythia produced gametangia and oospores when mated with the isolate of *P. nicotianae* of A_1 mating type. On the contrary, it did not form sexual structures when paired with isolates of the A_2 mating type, proving itself to be the A_2 type. The antheridia were always amphigenous (Fig. 3). The oogonia measured 25–37 μm in diameter (mean 30.7 μm), and their stalks were often excentric. The oospores were either plerotic or slightly aplerotic, with diameters ranging between 21 and 34 μm (mean and modal values 28.3 and 29 μm, respectively).

All isolates examined, including the isolate from forsythia, showed identical electrophoretic banding patterns of native mycelial proteins (Fig. 4).

Pathogenicity tests. Seedlings of forsythia transplanted into artificially infested soil developed leaf chlorosis and wilting associated with root and basal stem rot. On 1-yr-old seedlings transplanted in June, July, and August, the mean disease severity ratings were 1.6, 1.2, and 0.6, respectively, compared with a disease rating of 0.0 on the control seedlings.

Flooding of the soil caused extensive decay of the root system on 3-yr-old seedlings transplanted into artificially infested soil. The mean disease ratings on 3-yr-old seedlings transplanted in June, July, and September were 3.5, 3.0, and 3.3, respectively. Seedlings used as controls showed no symptoms, except a slight necrosis of rootlets, probably as a consequence of flooding.

No *Phytophthora* sp. was recovered from necrotic rootlets of seedlings used as controls. *P. nicotianae,* on the contrary, was consistently reisolated from rotted tissues of basal stem and roots of seedlings transplanted into artificially infested soil.

Tomato seedlings inoculated on the stem with the isolate C_{501} developed a brown, sunken lesion that girdled the stem and caused the collapse of the seedlings 2 days after the inoculation. The other two isolates proved less virulent on tomato, inducing only a brown, sunken lesion extending longitudinally on the stem. The length of the stem lesions 7 days after inoculation averaged 1.2 and 3.5 cm for the seedlings inoculated with the isolates C_{301} and C_{105}, respectively.

All three isolates proved to be very weakly virulent on tobacco. On seedlings of both tobacco cultivars, they induced only a slightly brown discoloration of the stem tissues lining the incision made to inoculate the seedlings.

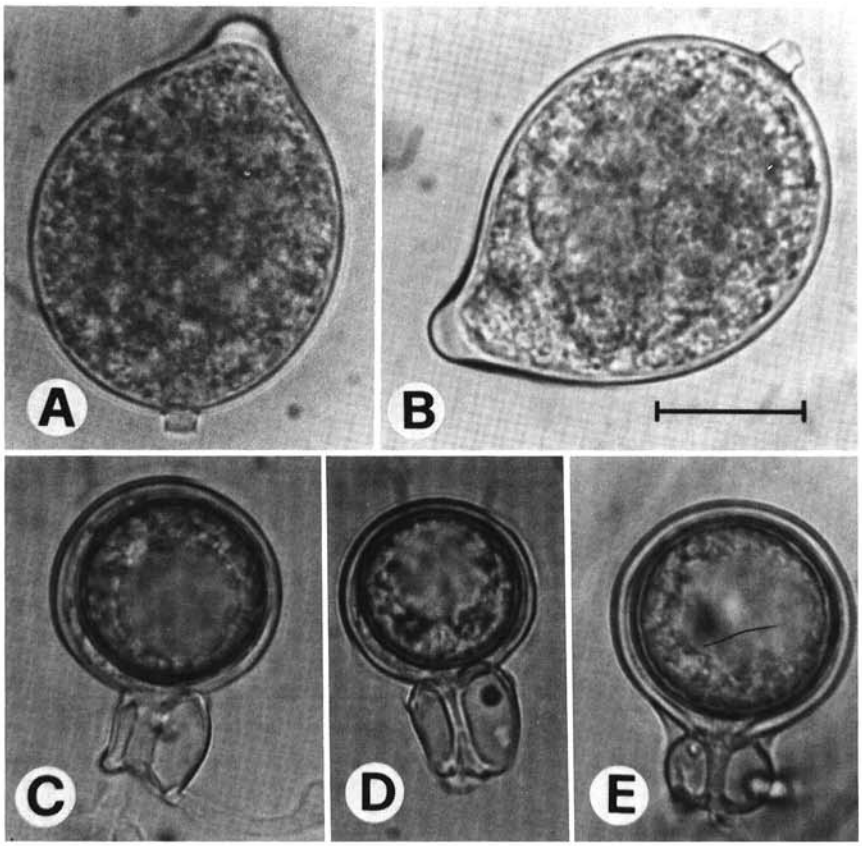

Fig. 3. (A) and **(B)** pedicellate sporangia produced by the isolate C_{501} from forsythia on V8 juice agar. **(C), (D),** and **(E)** sexual structures differentiated by paired cultures of isolates $C_{501} \times C_{88\text{-}1}$. Bar = 20 μm.

Table 1. Determination of the mating types of *Phytophthora nicotianae* isolates from several hosts[a]

Isolate	Host	Mating type of isolates				
		$C_{88\text{-}1}(A_2)$	$C_{92}(A_1)$	$C_{105}(A_2)$	$C_{106}(A_2)$	$C_{301}(A_2)$
C_{501}	Forsythia	−[b]	+[b]	−	−	−
C_{301}	Myrtle	−	+	−	−	...
C_{106}	Laurel	−	+	−	...	...
C_{105}	Laurel	−	+	...	...	...
C_{92}	Kentia	+	...	...	...	...

[a] Cultures were paired on V8A.
[b] + = Production of sexual structures; − = sexual structures not produced.

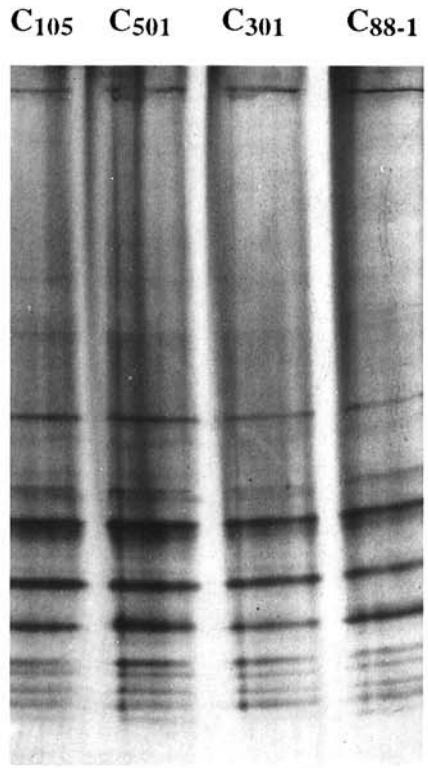

Fig. 4. Electrophoretic patterns of total mycelium proteins shown by isolates of *Phytophthora nicotianae* in 7.5% polyacrylamide slab gel, in a native system. Isolates were obtained from laurel (C_{105}), forsythia (C_{501}), myrtle (C_{301}), and jojoba ($C_{88\text{-}1}$).

DISCUSSION

The isolate of *Phytophthora* obtained from forsythia was identified as *P. nicotianae* on the basis of both the morphological and cultural characteristics (19) and the electrophoretic banding pattern of mycelial proteins. With the electrophoretic pattern it was possible to identify this isolate unequivocally in spite of its unusual morphological and cultural characters, such as fast radial growth rate and high degree of sporangial caducity. The results of this study confirmed the diagnostic value of the electrophoretic pattern of total native mycelial proteins in identification of *P. nicotianae* isolates, and were consistent with those obtained in previous studies (3,16) showing that protein patterns of *P. nicotianae* isolates, of A_1 and A_2 types, obtained from different hosts, were either identical or very similar to each other.

Stamps et al, in their taxonomic key (19), following Waterhouse classification criterion (22,24,25), differentiated two varieties of *P. nicotianae*: *P. n. nicotianae* and *P. n. parasitica.* This distinction was based primarily on morphological characters (23). The separation of *P. nicotianae* into varieties, however, has been a controversial aspect of the taxonomy of *Phytophthora* (8,9,12,20). Very recently, extensive evidence has been provided indicating that this grouping of *P. nicotianae* is questionable, as it is based on unreliable characters (10). The isolate recovered from forsythia was thus referred to simply as *P. nicotianae,* although it showed features which fit the variety *P. n. nicotianae*, such as sizes of oospores and oogonia, the production of caducous sporangia, and hyphal swellings with radiating hyphae, as well as oogonia with an excentric stalk.

P. nicotianae occurs widely in warm temperate regions and has a very broad range of known hosts, including members of the family Oleaceae (5,11,21). This is, however, the first record of *P. nicotianae* on forsythia. *P. cinnamomi* (26) was the only species of *Phytophthora* reported previously on forsythia.

Koch's postulates were completed by reproducing symptoms in artificially inoculated forsythia seedlings identical to those observed in the nursery. Symptoms were more severe on seedlings that were flooded after being transplanted into artificially infested soil mixture than on nonflooded seedlings, indicating that waterlogging was conducive to disease development. The overhead sprinkler irrigation system used in the nursery apparently provided conditions which favored the outbreak of root and stem rot of forsythia seedlings: flooding of pot soil and dispersal of *P. nicotianae* propagules.

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