Phytophthora and *Pythium* species Associated with Crown Rot in New York Apple Orchards

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ABSTRACT

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During the summers of 1978 and 1979, isolations were made from 23 apple trees showing typical crown rot symptoms in 10 western New York orchards. Two species of *Phytophthora* and one of *Pythium* plus other unidentified isolates of *Phytophthora* and *Pythium* were recovered on a pimaricin-vancomycin-PCNB medium. The most frequently isolated species was *Phytophthora megasperma*, which was recovered from eight trees. *Phytophthora cactorum*, generally regarded as the causal organism, was recovered from three trees. Other pythiaceous fungi, including *Pythium irregulare*, two unidentified isolates of *Phytophthora*, and five unidentified isolates of *Pythium*, were each recovered from only one tree. Relative pathogenicity of these isolates was determined in vitro by using an excised twig assay and in vivo by using seedlings grown in artificially infested soil. All species were pathogenic to some extent, but *Ph. cactorum* isolates were most pathogenic in both assays. All tested isolates of *Ph. megasperma* were consistently pathogenic, implicating this species for the first time in crown rot of apple trees in New York. *Ph. megasperma* isolates, like those of *Ph. cactorum*, exhibited varying degrees of virulence to specific apple cultivars.

Additional key words: collar rot, *Malus pumila*.

Crown rot and collar rot of apple trees are caused by the same *Phytophthora* species and the difference is only in the initial site of infection. Crown rot is typified by invasion of the root-crown tissues of an apple tree (ie, where the major roots emerge from the lower trunk) with pathogenesis extending distally along the primary roots. The collar rot infection court is at the soil line on the tree trunk; the fungus can move both laterally and longitudinally and can eventually girdle the tree by forming a necrotic "collar" around the trunk (14,28). A distinct margin between healthy and infected tissues is usually evident in both cases. Baines (2) first showed that *Phytophthora cactorum* (Lebert and Cohn) Schroeter caused collar rot symptoms on apple trees in an Indiana orchard, and soon thereafter, Welsh (33) showed that the same pathogen was involved in a crown rot disorder of apple trees in British Columbia. *Ph. cactorum* has since been reported from many apple-growing areas worldwide (25). This is not, however, the only *Phytophthora* species associated with this disease syndrome. *Ph. syringae* (23,26,27), *Ph. megasperma* (17,22), *Ph. cambivora* (20,29), *Ph. drechsleri* (17), and an unidentified species of *Phytophthora* (20) have also been found associated with apple trees exhibiting typical crown rot-collar rot symptoms. In addition, *Pythium ultimum* has been identified as a collar rot pathogen (4), and other *Pythium* species have been suggested as pathogens of apple roots (21,24).

In New York, where crown rot is the symptom commonly observed, *Ph. cactorum* had been the only species implicated in the disease prior to this work (1). Because of the increasing evidence from several apple-growing regions of the possible involvement of other *Phytophthora* and *Pythium* species, this study was undertaken to determine whether such species were associated with the crown rot problem in western New York apple orchards and to determine their relative pathogenicity. The preliminary findings of this study were reported earlier (7).

MATERIALS AND METHODS

Root and crown tissue samples were collected from apple trees (*Malus pumila* Miller) exhibiting typical crown rot symptoms (Fig. 1; and 15,33) in orchards in the western New York apple-growing counties of Wayne, Monroe, and Orleans during the summers of 1978 and 1979. Orchards planted to apple cultivars on MM.106 rootstocks were of principal concern because this rootstock has been reported to be susceptible to crown rot under field conditions (13,15) and it is widely planted.

Samples were kept cool and moist until isolation procedures began (usually the following day). In the laboratory, root-crown pieces were thoroughly rinsed to remove all soil debris and were washed under running tap water for 2–24 hr. Samples were blotted dry, rinsed in sterile water, and blotted dry again. Small segments

Fig. 1. Crown rot symptoms on MM.106 apple rootstock. Infection originated in the root-crown zone (arrows) and extended distally along the primary roots. *Phytophthora megasperma* was recovered from this tree.

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of periderm, ~5 mm^3, were cut from the margin area between necrotic and healthy-appearing tissue and were pressed into a modified pimaricin-vancomycin-PCNB (PVP) selective cornmeal agar medium (30): 17 g of Difco cornmeal agar (CMA) and 940 ml of distilled water amended with 10 mg pimaricin (0.4 ml Pimafucin®, Aldrich Chemical Co., Milwaukee, WI 53233), 300 mg vancomycin hydrochloride (Eli Lilly Co., Indianapolis, IN 46200), and 50 mg PCNB (66.7 mg Terraclor® 75 WP, Olin Corp., Little Rock, AR 72200), each in 20 ml of sterile distilled water. Isolation plates were incubated in the dark at 25 C for up to 7 days and were examined daily. Hyphal tips growing into the selective medium were transferred to fresh PVP and incubated again in the dark at 25 C. Isolates continuing to grow on PVP and free of contamination were transferred to CMA slant tubes and then stored in polyethylene bags at 4 C.

Both oospores and zoosporangia were required for identification of these isolates. To induce oospore formation, isolates were plated on 0.5% hemp seed agar (HSA), which was prepared by autoclaving 5 g of cracked hemp seed wrapped in cheesecloth with 17 g of Difco-Bacto agar in 1 L of distilled water and then removing the seed bag (W. L. Bruckart III, *personal communication*). Plates were incubated in the dark at 25 C and oospores usually formed in 2–3 wk. Clear V-8 juice agar containing β-sitosterol and $CaCl_2$ (19) was as effective as HSA in producing oospores. The protocol described by Mircetich and Matheron (19) was adapted for sporangia production. Sporangia were produced on V-8 juice agar (V8A) plugs of a test isolate flooded with unsterile soil extract solution 18–48 hr after incubation on the laboratory bench (~21–24 C).

Pathogenicity of pythiaceous fungi first was determined by an excised-twig assay (8) on dormant McIntosh and Empire apple twigs. Cultures for this assay were grown for 7 days at 25 C on pimaricin-amended cornmeal agar plus agar (PCMAA) in Pyrex® storage jars. Twigs were then inserted vertically, distal end up, into the agar just within the colony periphery, and the jars were kept for another 7 days at 25 C. Net necrosis length (total length of necrosis minus the depth of agar in the jar) (NNL) was used to measure pathogenicity.

Pathogenicity was also determined by using a seedling assay based on the procedures of Mircetich and Matheron (19). Inocula were prepared by growing pythiaceous isolates on a sterile mixture of 200 cc of fine-textured vermiculite saturated with 100 ml of V-8 juice broth (V8B) in a 500-ml Erlenmeyer flask at 25 C for 7 wk in the dark. Five- to 7-wk-old Grimes Golden apple seedlings were transplanted into 946-ml (1-qt) plastic containers (with a drain hole drilled at the base) containing 750 cc of autoclaved potting mixture of soil, sand, and peat moss (1:1:1, v/v) infested with 10–15 cc of vermiculite-V8B inoculum. One seedling was planted in each container, and there were six plants per treatment. Controls were transplanted into soil to which uninfested vermiculite-V8B had been added. All plants were maintained in a growth chamber (24 C day, 18 C night, uncontrolled RH, and a 16-hr photoperiod at 12.6 klux) for 8 wk. During this period plants were flooded three times at 2-wk intervals, (beginning 2 wk after transplanting) for 48 hr each time (19). Flooding was achieved by plugging the drain hole in each container with a rubber stopper and adding water until 10–20 mm of standing water was on the soil surface. Containers were allowed to dry down after each flooding before normal watering was resumed. Plants were fertilized with water-soluble 20-20-20 (N-P-K) fertilizer every other week and were sprayed with a benomyl suspension four times during the 8-wk period to control powdery mildew (caused by *Podosphaera leucotricha*). Pathogenicity of a species was determined and relative virulence of an isolate within a species was assessed based on four parameters: number of seedlings dead or dying, root dry weight, shoot dry weight, and shoot height.

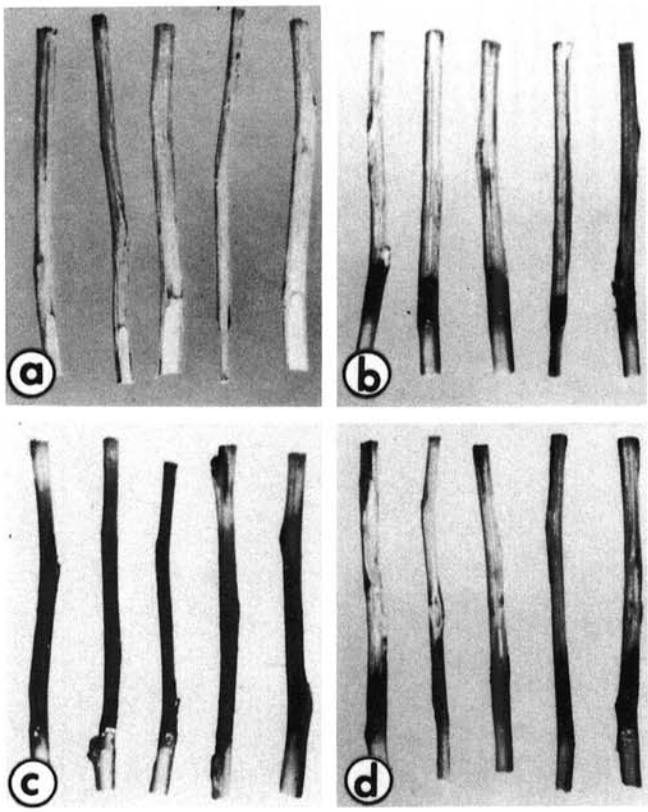

Fig. 2. Excised dormant apple twigs 7 days after inoculation with pythiaceous fungi associated with crown rot of orchard apple trees. Periderms have been removed to show necrosis. a, Check, uninoculated; b, c, and d, inoculated with: *Phytophthora megasperma* isolate 029, *Ph. cactorum* isolate 007, and *Pythium* species I isolate 005, respectively.

RESULTS

Phytophthora and *Pythium* species were recovered from 14 of 23 symptomatic apple trees sampled from 10 orchards during the summers of 1978 and 1979. *Phytophthora* species were isolated more frequently than were *Pythium* species (12 trees in five orchards and five trees in four orchards, respectively). *Ph. megasperma* Drechsler was most frequently isolated (eight trees in four orchards) followed by *Ph. cactorum* (three trees in two orchards). Two unidentified *Phytophthora* isolates, *Phytophthora* species I and *Phytophthora* species II, were each recovered from only one tree in separate orchards. *Pythium irregulare* Buisman and five unidentified *Pythium* species isolates, I–V, were each recovered from only a single tree. Although two species were occasionally recovered from the same tree, there was no consistency in the occurrence of different species together.

All measurements of reproductive structures for isolates of both *Phytophthora* and *Pythium* were an average of 25–50 observations. *Phytophthora* species were identified by using Waterhouse's key (31) and genus description (32). *Ph. cactorum* was easily recognized by its distinctly papillate sporangia (41 × 30 μm and terminal on sympodially branched sporangiophores) and its paragynously fertilized oogonia (32 μm, each containing a single oospore 26 μm in diameter). Both sexual and asexual structures of *Ph. cactorum* were plentiful on solid media (eg, CMA). Sporangia of *Ph. megasperma*, varying greatly in both size and shape, were produced only in soil extract solution. Sporangia measured 61 × 38 μm, proliferated internally on unbranched sporangiophores, lacked obvious papillae, and possessed broad exit pores. The antheridia of this species were most often paragynous, but occasionally were amphigynous. Isolates of *Ph. megasperma* fell into two distinct, nonverlapping groups based on sizes of oogonia and oospores. Oogonia and oospores averaged 37 and 32 μm, respectively, for 24 small-spored isolates and averaged 54 and 47 μm, respectively, for 11 large-spored isolates. These two groups are consistent with those described by Waterhouse (31,32) for *Ph. megasperma* var. *sojae* (oogonia less than 45 μm) and *Ph. megasperma* var. *megasperma* (oogonia greater than 45 μm). However, in light of Kuan and Erwin's (11) findings that *Ph. megasperma* exhibits a continuous

range in oogonium size from 30 to 58 μm, we have designated all *Ph. megasperma*-like isolates recovered from apple root-crowns as only *Ph. megasperma*. The identities of *Ph. megasperma* isolates were confirmed by O. K. Ribeiro (Department of Plant Pathology, University of California, Riverside 92521). *Phytophthora* species I produced nonpapillate sporangia (43 × 27 μm) abundantly in soil extract and infrequently on solid media. These obpyriform-to-ovoid structures proliferated internally on unbranched sporangiophores and had broad exit pores. No oospores could be produced when paired with the appropriate A^1 and A^2 mating types (O. K. Ribeiro, *personal communication*), but large spherical hyphal swellings occurring singly, in chains, or in aggregates were numerous on solid media. The only distinct morphological structures of *Phytophthora* species II were the ovoid sporangia (55 × 37 μm) produced in soil extract solution and not on solid media, which appeared to have small exit pores and proliferated internally on unbranched sporangiophores. This isolate also failed to produce oospores when crossed (O. K. Ribeiro, *personal communication*). *Py. irregulare* was identified according to the description in Middleton's monograph (16), and its identity was confirmed by R. D. Lumsden (USDA Soilborne Disease Laboratory, Beltsville, MD 20705). This fungus had both terminal and intercalary spherical sporangia (24 μm on HSA) and irregularly spherical, terminal, or intercalary oogonia (21 μm) fertilized by one or two antheridia. Oospores were aplerotic and averaged 18 μm. *Pythium* species I–V were morphologically similar. They were characterized by spherical sporangia, typically terminal, with average diameters ranging from 29 to 33 μm (produced in soil extract solution). Empty sporangia in soil extract solution exhibited the "delicate emission tubes" characteristic of the genus *Pythium* (16). None of these isolates produced oospores, either by the methods of Lumsden et al (12) or those of Mircetich and Fogle (18).

An American Type Culture Collection isolate of *Ph. cactorum* (ATCC #16695) was used in both pathogenicity studies, and *Ph. megasperma* isolate 112, isolated from apple orchard soil, was used in the seedling assay for pathogenicity. In the excised-twig assay, *Ph. cactorum* isolates were significantly more pathogenic on both cultivars than were the other isolates tested (Fig. 2. and Table 1). In this test, isolates of *Ph. cactorum* also differed signficantly ($P = 0.05$) in virulence. The isolates of other *Phytophthora* and *Pythium* species exhibited various degrees of virulence resulting in bark necrosis (NNL) ranging from 5.3 to 17.1 mm on Empire twigs and from 1.2 to 11.8 mm on McIntosh twigs (Table 1). Virulences of the unidentified *Pythium* species isolates tended to fall in the upper end of these ranges, those of *Ph. megasperma* isolates tended to fall in the lower end, and those of *Phytophthora* species I, *Phytophthora* species II, and *Py. irregulare* were interspersed within these two groups. *Ph. megasperma* isolates differed in virulence to Empire and McIntosh twigs. The controls had no necrosis. Necrosis was consistently greater on Empire twigs than on McIntosh twigs, which indicated a difference in the susceptibility of these two cultivars to the tested pathogens.

Individual isolates had varying effects on growth in the seedling assay for pathogenicity (Fig. 3 and Table 2). *Ph. cactorum* caused the greatest mortality in Grimes Golden seedlings. *Phytophthora* species I was the only other pathogen that caused substantial seedling mortality. All other species, however, did have adverse effects on seedling growth (Table 2). *Ph. cactorum* isolates consistently caused the most severe damage followed in order by *Pythium* species I–V and *Ph. megasperma* isolates. *Phytophthora* species I, *Phytophthora* species II, and *Py. irregulare* were interspersed among these three groups. In addition, *Ph. megasperma* isolates differed significantly ($P = 0.05$) in virulence to Grimes Golden apple seedlings. Only one isolate of this species caused no significant growth reduction compared to the control.

DISCUSSION

The data presented in this paper strongly suggest that several pythiaceous fungi, in addition to *Ph. cactorum*, are a potential threat to apple trees in western New York. The frequent isolation and pathogenicity of *Ph. megasperma* implicates this species in the crown rot syndrome for the first time in this area. There is also evidence that suggests the possible pathogenic involvement of other *Phytophthora* species. The role of these pathogens in crown and root diseases of apple trees in New York is not fully understood, but it may be analogous to the situation described by Kouyeas (10) for crown rot of stone fruit trees: "It seems probable that in Europe stone fruit apoplexy is due to species of *Phytophthora* in many more cases than hitherto realized."

TABLE 1. Pathogenicities of *Phytophthora* (*Ph.*) and *Pythium* (*Py.*) isolates on excised dormant twigs of two apple cultivars

Isolate no.	Species	Empire twigs		McIntosh twigs	
		Necrosis[x] (mm)	LSD mean comparison[y]	Necrosis[x] (mm)	LSD mean comparison[y]
097	*Ph. cactorum*	51.1[z]	a	41.5	b
042	*Ph. cactorum*	47.9[z]	ab	47.5	a
007	*Ph. cactorum*	45.7[z]	bc	43.5	b
16695	*Ph. cactorum*	41.3[z]	c	42.2	b
044	*Py.* sp. IV	17.1	d	11.8	c
005	*Py.* sp. I	16.5	de	4.0	fgh
047	*Py.* sp. V	15.7	def	7.6	de
013	*Py.* sp. II	14.0	defg	5.7	def
041	*Py.* sp. III	13.9	defg	7.4	de
095	*Py. irregulare*	13.4	defgh	2.3	gh
070	*Ph. megasperma*	12.9[z]	defgh	3.3	fgh
081	*Ph. megasperma*	11.7	efghi	2.5	gh
011	*Ph. megasperma*	11.5	efghi	3.5	fgh
029	*Ph. megasperma*	10.7	fghi	2.9	fgh
001	*Ph.* sp. I	10.1	ghij	8.1	d
075	*Ph. megasperma*	8.8	ghij	1.2	h
088	*Ph. megasperma*	8.2	hij	2.8	fgh
054	*Ph. megasperma*	8.1	hij	1.9	h
055	*Ph. megasperma*	8.1	hij	4.9	efg
082	*Ph.* sp. II	7.2	ij	2.4	gh
012	*Ph. megasperma*	5.3	j	2.1	gh
...	Control	no necrosis		no necrosis	
		LSD = 5.3 mm		LSD = 3.0 mm	

[x] Mean net necrosis length (total length of necrosis − depth of agar) on 15 replicate twigs determined by using an excised-twig assay (8).
[y] Means were compared using a protected LSD test (5) with $P = 0.05$. Numbers followed by the same letter are not significantly different.
[z] Necrosis had moved the entire length of at least one twig in the treatment.

The recent involvement of *Phytophthora* species other than *Ph. cactorum* or occasionally *Ph. syringae* in this disease is probably ascribable to a better understanding of *Phytophthora* biology, to improved techniques for working with this group of fungi (particularly the development of pimaricin-vancomycin-PCNB selective medium [6,30]), and to closer scrutiny of the problem. *Ph. cactorum* readily produces both sporangia and oospores on commonly used agar media (eg, CMA) whereas other *Phytophthora* species often require an aquatic environment to produce sporangia and pairing with compatible mating types to produce oospores (31). Consequently, *Ph. cactorum* may have been the only species *identified* in association with the crown rot-collar rot syndrome, especially if this was the pathogen investigators were looking for. In addition, popular clonal rootstocks, preferred for their dwarfing ability and genetic consistency, may be more susceptible to the various *Phytophthora* species than were the seedling rootstocks used in the past. The possibility that apple growers may be bringing these pathogens into their orchards from the nursery (9) needs to be considered in New York.

Pythium species, especially *Py. sylvaticum*, were initially reported as apple root pathogens by Mulder (21) and have been recently discussed at length by Sewell (24). The isolation of *Pythium* species from apple root crown tissues and the pathogenicity of these isolates to apple twigs and seedlings are consistent with these reports and suggest that these fungi may be significant in below-ground disorders of apple trees. Whether they play a specific role in the crown rot-collar rot syndrome in New York needs further clarification.

We have demonstrated here that *Ph. megasperma* isolates differ significantly in virulence to the three apple cultivars used in this investigation. This characteristic has already been demonstrated for *Ph. cactorum* (1,3). Therefore, as with *Ph. cactorum* (1,3,28), the most virulent isolates of *Ph. megasperma* should be used in screening apple rootstock material for crown rot resistance.

Ph. cactorum was consistently the most virulent among the species tested in our pathogenicity assays. It has been suggested that *Ph. cactorum* is more virulent than *Ph. syringae* (26) and *Ph. megasperma* (22). This greater virulence of *Ph. cactorum* to apple makes it a much more obvious problem, but not necessarily a more important one. As Mulder (21) stated, ". . . hardly anything is known about chronic latent root diseases which only take a certain toll of the plant as a whole without leading to clear symptoms." The

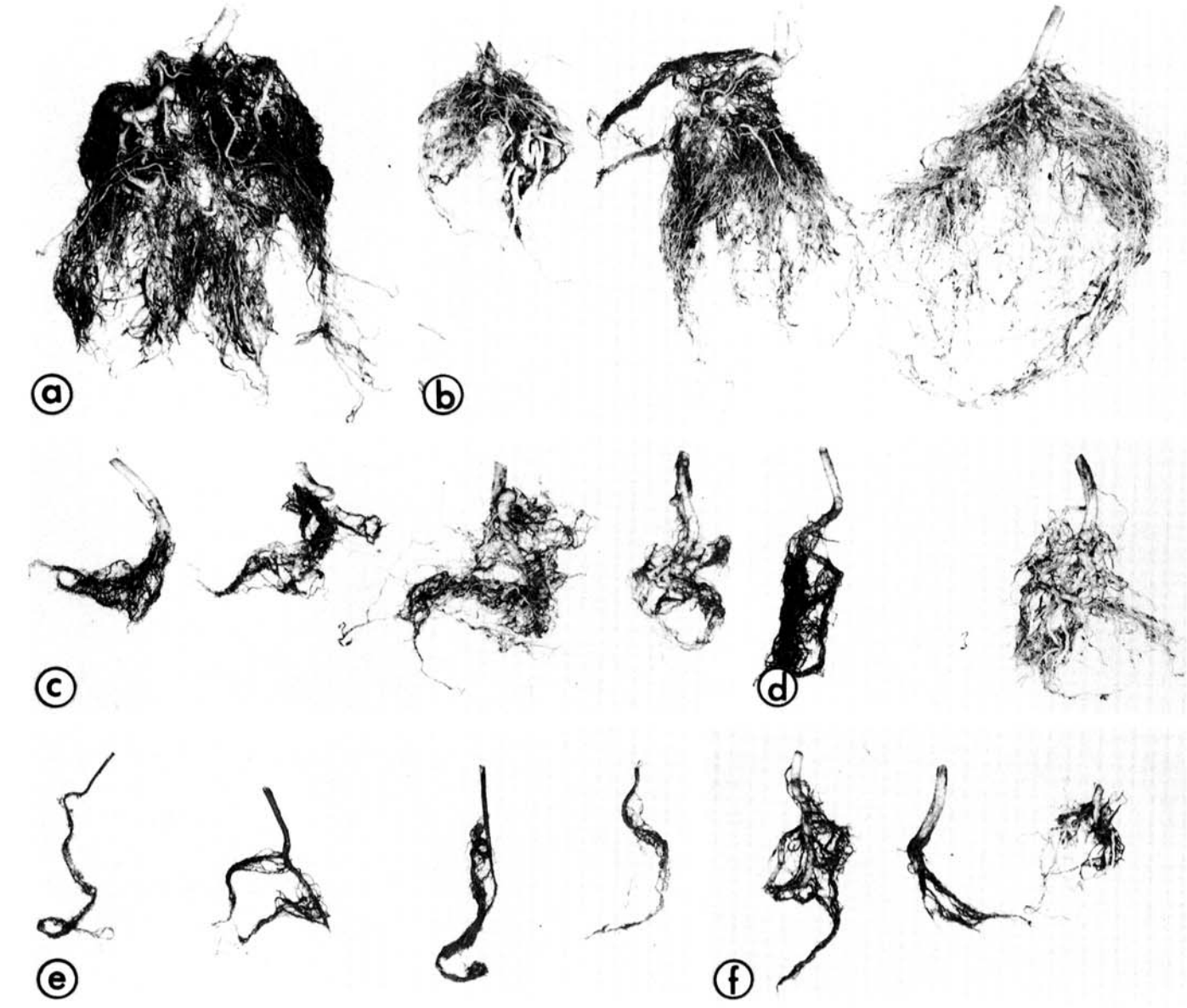

Fig. 3. Representative root systems of Grimes Golden apple seedlings that were grown for 8 wk in soil artificially infested with: a, control, uninfested; b, *Phytophthora megasperma* isolates (left to right) 011, 012, and 029; c, *Ph. megasperma* isolates 075, 081, 088, and 112; d, *Phytophthora* species. I and II isolates 001, and 082, respectively; e, *Ph. cactortum* isolates 007, 042, 097, and 16695; f, *Pythium* species. I–III isolates 005, 013, and 041, respectively.

TABLE 2. Effects of *Phytophthora* (*Ph.*) and *Pythium* (*Py.*) isolates on Grimes Golden apple seedlings growing in artificially infested soil

Isolate no.	Species	No. seedlings dead or dying[x]	Root dry weight (mg)	LSD mean comparison[y]	Shoot dry weight (mg)	LSD mean comparison[y]	Shoot height (mm)	LSD mean comparison[y]
007	*Ph. cactorum*	6	28	a	448	a	73	a
042	*Ph. cactorum*	5	60	ab	838	abc	103	ab
16695	*Ph. cactorum*	4	75	ab	668	ab	92	a
047	*Py.* sp. V	2	103	ab	782	abc	92	a
001	*Ph.* sp. I	3	125	ab	1,092	abcd	128	ab
097	*Ph. cactorum*	5	133	ab	947	abcd	113	ab
075	*Ph. megasperma*	1	142	abc	878	abc	118	ab
081	*Ph. megasperma*	0	148	abc	1,153	abcd	127	ab
013	*Py.* sp. II	2	152	abc	1,032	abcd	123	ab
041	*Py.* sp. III	1	175	abc	988	abcd	127	ab
044	*Py.* sp. IV	2	210	abcd	1,392	abcde	158	abc
005	*Py.* sp. I	1	217	abcde	1,068	abcd	123	ab
112	*Ph. megasperma*	1	252	abcdef	139	abcd	135	ab
055	*Ph. megasperma*	2	278	bcdefg	1,282[z]	abcd	148	ab
070	*Ph. megasperma*	2	298	bcdefgh	1,558	bcdef	180	bc
088	*Ph. megasperma*	1	383	cdefgh	1,788[z]	cdef	180	bc
082	*Ph.* sp. II	1	450	defgh	1,960	defg	182	bc
054	*Ph. megasperma*	0	463	efgh	2,515	fgh	273	de
095	*Py. irregulare*	0	475	fgh	1,957	defg	185	bc
011	*Ph. megasperma*	0	512	ghi	2,413[z]	efgh	238	cd
012	*Ph. megasperma*	0	527	hi	2,820[z]	ghi	240	cd
029	*Ph. megasperma*	0	737	ij	3,237[z]	hi	307	de
···	Control	0	893	j	3,787	i	348	e
			LSD = 248 mg		LSD = 1,023 mg		LSD = 86 mm	

[x]Number of seedlings dead or dying out of a possible six after 8 wk in a growth chamber.
[y]Means were compared using a protected LSD test (5) with $P = 0.05$. Numbers followed by the same letter are not significantly different.
[z]Treatments from which at least two leaves were removed during the course of this experiment because of powdery mildew (caused by *Podosphaera leucotricha*).

damaging role of sublethal pythiaceous fungi to apple trees and their interaction with the environmental stresses caused by extremes of temperature and soil moisture need further investigation.

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