Phytophthora Species Associated with Crown and Root Rot of Apple in Chile

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ABSTRACT

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Phytophthora cactorum, *P. cryptogea*, *P. gonapodyides*, and *P. megasperma* were isolated from necrotic root and crown tissues or the rhizospheres of apple trees exhibiting typical symptoms of *Phytophthora* root and crown rot in the Central Valley of Chile. Representative isolates of all four species were pathogenic on a variety of apple rootstocks and scions in trials conducted on excised shoots and 1-year-old MM.106 rootstock grown for 4 months in infested potting medium. *P. cactorum* was the most frequently isolated species and the most virulent in pot tests, although a significant *Phytophthora* sp.-apple genotype interaction was observed. This is the first report of any species other than *P. cactorum* causing root and crown rot of apple trees in Chile.

Additional keywords: apple rootstocks, *Malus*

Over 30,000 ha of apple (*Malus × domestica* Borkh) are cultivated in Chile, mainly on seedling rootstocks that are generally considered less susceptible to *Phytophthora* spp. than many clonal apple rootstocks (12). However, susceptible clonal rootstocks (e.g., MM.106) are becoming prevalent among new apple plantings in the country, thereby increasing the probability of outbreaks of *Phytophthora* crown and root rot. Risk of the disease is greatest where the susceptible rootstocks are used on *Phytophthora* spp.-infested soils that are subject to prolonged periods of water saturation. Wet conditions favor infection of deciduous fruit trees by the pathogens (3,27,31,32).

Several *Phytophthora* spp. can attack the crown and the roots of apple (6,12). *P. cactorum* (Lebert & Cohn) J. Schroet. has been the species most frequently associated with crown and root rot of apple throughout much of the world, but additional pathogens include *P. cambivora* (Petri) Buisman, *P. citricola* Sawada, *P. cryptogea* Pethybr. & Lafferty, *P. drechsleri* Tucker, *P. megasperma* Drechs., *P. parasitica* Dastur, *P. syringae* (Kleb.) Kleb., and several unidentified *Phytophthora* spp. (1,6,8,9, 11–13,21,23,28). Additionally, *P. gonapodyides* has been isolated from the soil

of apple orchards in England (2,6). Although some of these species are known to affect other fruit crops in Chile (14–18,20,22,28,29), the species of *Phytophthora* causing crown and root rot of apple in the country remain unknown. Consequently, the objectives of this study were to identify and evaluate the pathogenicity of the species of *Phytophthora* associated with crown and root rot of apple in Chile.

MATERIALS AND METHODS

Isolation. Samples of necrotic root or crown tissues were removed from diseased apples grafted on either MM.106 or apple seedling rootstocks in 18 commercial orchards in the Central Valley of Chile (34 to 37° latitude south) during 1995 and 1996. At least 10 pieces (5 to 10 mm in length) of crown or root tissues, selected at the margins of canker lesions, were placed in 1.7 % corn meal agar (CMA, Difco Laboratories) amended with ACMA (per liter: ampicillin, 150 mg; pimarin, 10 mg; rifampicin, 16 mg; pentachloronitrobenzene [PCNB], 100 mg; benomyl, 10 mg; and hymexazol, 30 mg). Cultures were incubated for 5 to 7 days at 20°C in darkness. Emerging *Phytophthora* colonies were initially identified by gross colony morphology and the presence of coenocytic hyphae with wide branching angles. Representative isolates were subcultured on CMA (6,16).

Soil samples were collected around the roots of diseased apple trees, 10 to 15 cm beneath the soil surface. Two 200-ml subsamples of the soil from each tree were transferred to a plastic container and flooded with distilled water to a depth of 30 to 35 mm. Then, one apple fruit, previ-

ously punctured three times 1 cm deep with a hydrodermic needle, was submerged in each container and incubated at 20 to 23°C for 4 to 7 days. Fruit that developed round, firm, water-soaked lesions were surface disinfested in 70% ethanol for 1 min, then the skin was removed and four to five pieces of tissue from the margin of each lesion were transferred to ACMA. The culture plates were incubated and observed for emerging colonies that were subcultured as described above.

Identification of *Phytophthora* spp.

Phytophthora spp. were identified according to the keys of Waterhouse (26) and Stamps et al. (24). Colony morphologies were determined after 5 days on CMA and V8 juice agar at 20 to 23°C. The ability to grow at 5, 30, and 35°C was determined after 5 days on CMA. Sporangia were produced by growing each isolate in carrot juice broth for 48 h at 20 to 23°C, rinsing in sterile distilled water, treating them for 3 to 5 min with cool saline solution containing (per liter) 2.36 g of Ca(NO₃)₂, 0.5 g of KNO₃, 1 g of MgSO₄ · 7H₂O, and 1 ml of chelated iron solution (ethylenediaminetetra-acetic acid, 13 g /liter; KOH, 7.5 g/liter, FeSO₄ · 7H₂O, 24.9 g/liter), after which they were incubated in 1.5% (wt/vol) nonsterile soil extract solution for 48 h at 20 to 23°C under continuous light (6,16). Gametangia were produced either in single culture or in pairings with *P. cinnamomi* isolates of known mating type, and observed for 20 to 30 days of incubation at 20°C in darkness on unclarified V8 juice agar amended with βV-8 A (per liter: β-sitosterol, 30 mg dissolved in 5 ml of heated ethanol; tryptophan, 20 mg; and thiamine-HCl, 1 mg).

Two isolates (M95-122.9 and M95-93.5), tentatively identified as *P. gonapodyides*, were further compared with isolates of *P. cryptogea* (K15-2 and NY413) and *P. drechsleri* Tucker (V20) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of soluble mycelial proteins. Soluble proteins were extracted from 2 to 3 g of mycelium of each isolate, obtained after growth in carrot broth for 72 h at 20°C. The mycelium was washed twice with sterile distilled water and ground in liquid nitrogen with a mortar and pestle, after which protein profiles were produced using previously described techniques (17).

Pathogenicity tests. Representative isolates of *Phytophthora* spp. isolated from affected apple trees and from soil samples

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were tested for pathogenicity in excised, 1-year-old shoots of apple rootstocks or cultivars, always collected during dormancy, as described previously (10,25). In the first experiment, shoots from apple rootstocks M.9, M.25, MM.106, and MM.111 and cv. Granny Smith were used. In the second experiment, shoots from apple cvs. Braeburn, Fuji, Royal Gala, and Granny Smith were used. For each isolate of *Phytophthora*, five shoots 5 to 6 mm in diameter and 15 cm long were buried 2 to 3 cm deep in a mixture containing one volume of inoculum (15-day-old carrot broth-vermiculite cultures) per three volumes of sterile vermiculite and incubated for 14 days at 20°C. Shoots of each rootstock or cultivars buried in sterile vermiculite were left as controls. The length of the canker was measured and data were analyzed for variance following a completely randomized factorial design. Orthogonal comparisons

were performed between Granny Smith and other apple cultivars or clonal rootstocks.

In the third experiment, four isolates of *P. cactorum*, two of *P. cryptogea*, two of *P. gonapodyides*, and one of *P. megasperma* were tested for pathogenicity on 1-year-old plants of apple rootstock MM.106. Each of the rootstock plants was transplanted to a 7-liter pot containing either an infested soil mixture (1:1 mixture of organic soil:sand) or the same soil amended so that 3% of the final volume was a 14-day-old inoculum mixture (40:56:4, vol/vol, carrot broth:vermiculite:oat medium at 23°C). Plants were incubated for 4 months in a screenhouse, and were kept constantly wet by placing them in trays filled with 2 to 3 cm of water. Additionally, pots were subjected to six flooding periods by immersing them in water for 48 h. An equal number of both flooded and unflooded plants were grown

in noninoculated soil mixture as controls. At the conclusion of the experiment, the roots were washed free of the soil mix and pathogenicity was evaluated on the basis of crown rot incidence, root rot severity (visually estimated on a 1-to-5 scale, where 1, 2, 3 and 4 were ≥ 75 , 50 to 75, 25 to 50, and $\leq 25\%$ rotted roots, respectively, and 5 = 100% healthy roots), and root weight. Results were subjected to analysis of variance according to a completely randomized design replicated five times, and means separated according to the Waller-Duncan *k*-ratio *t* test.

To confirm the association of the appropriate *Phytophthora* spp. and symptoms, isolations were performed on ACMA at the end of each pathogenicity test from samples taken from diseased plant materials and from control plants.

Detection of *Phytophthora* spp. in the irrigation water. The presence of *Phytophthora* spp. in the water used to surface irrigate two apple orchards with history of *Phytophthora* crown rot was tested serologically twice during the growing season (October to January) using an enzyme-linked immunosorbent assay (ELISA) kit for the detection of *Phytophthora* spp. (Sigma-Aldrich, St. Louis) as previously described (19). Samples consisted of 100 ml of water collected near the beginning and end of the irrigation furrows (i.e., both before and after the water had passed through three different rows containing diseased trees from which *P. cactorum* had been isolated previously). Following reaction of the samples with kit reagents, optical density (OD) was measured in an ELISA Microplate Reader EL307C, (Biotek Instruments) at $\lambda = 405$ nm. Only those samples with an $OD_{\text{sample}}/OD_{\text{negative check}} > 1.1$ were considered positive for the presence of *Phytophthora* spp.

RESULTS

Isolation and identification. *Phytophthora* spp. were isolated from 2 out of 37 (5.4%) symptomatic apple seedling rootstocks and from 13 out of 53 (24.5%) symptomatic clonal rootstocks, mainly MM.106. Similarly, *Phytophthora* spp. were recovered from 32 out of 85 (42.5%) soil samples taken from the root zone of symptomatic apple trees.

The isolates identified as *P. cactorum*, *P. cryptogea*, *P. megasperma* and *P. gonapodyides*. *P. cactorum*, and *P. cryptogea* were recovered from diseased tissue and soil samples, whereas *P. megasperma* and *P. gonapodyides* were isolated only from soil samples.

Isolates identified as *P. gonapodyides* produced rosette colonies on CMA and on V8 juice agar. The isolates of this species made poor growth at 4°C, optimum growth at 25 to 30°C; near-optimal growth at 33°C; and poor to no growth at 36°C. Sporangia were typical for those of Waterhouse Group VI (ovoid, nonpapillate,

Table 1. Similarity coefficients from comparisons of isolates of *Phytophthora gonapodyides*, *P. cryptogea*, and *P. drechsleri* based upon electrophoretic banding patterns of total soluble mycelial proteins²

Trial, no.	Species	Isolate	Isolates of <i>Phytophthora</i> spp.				
			1	2	3	4	5
Trial 1							
1	<i>P. gonapodyides</i>	M95 122.9	100	85	50	31	38
2	<i>P. gonapodyides</i>	M95 93.5	...	100	53	40	41
3	<i>P. cryptogea</i>	K15-2	...	...	100	80	50
4	<i>P. drechsleri</i>	V20	...	...	...	100	53
5	<i>P. cryptogea</i>	NY413	...	...	...	...	100
Trial 2							
1	<i>P. gonapodyides</i>	M122.9	100	83	23	48	30
2	<i>P. gonapodyides</i>	M93.5	...	100	49	44	39
3	<i>P. cryptogea</i>	K15-2	...	...	100	82	48
4	<i>P. drechsleri</i>	V20	...	...	...	100	56
5	<i>P. cryptogea</i>	NY413	...	...	...	...	100

² Similarity coefficient for each pair of compared isolates was calculated as (number of bands in common)/(total number of bands for the two isolates) $\times$ 200.

Table 2. Canker development on excised, 1-year-old shoots of apple rootstocks and cultivars inoculated with four species of *Phytophthora* isolated from apple orchards in Chile^x

Trial, apple ^z	Canker length (mm) obtained after inoculation with ^y			
	<i>P. cactorum</i>	<i>P. cryptogea</i>	<i>P. gonapodyides</i>	<i>P. megasperma</i>
Trial 1				
M.9	55.1 $\pm$ 3.8	73.6 $\pm$ 3.0	50.3 $\pm$ 2.3	69.2 $\pm$ 2.8
M.25	54.2 $\pm$ 1.4	66.4 $\pm$ 2.7	48.0 $\pm$ 0.8	68.0 $\pm$ 2.2
MM.106	54.3 $\pm$ 3.0	32.0 $\pm$ 5.6	55.8 $\pm$ 2.8	57.4 $\pm$ 7.4
MM.111	59.3 $\pm$ 3.8	46.6 $\pm$ 12.1	31.5 $\pm$ 5.6	32.8 $\pm$ 3.8
Granny Smith	17.5 $\pm$ 2.5	22.0 $\pm$ 6.7	38.9 $\pm$ 1.9	37.8 $\pm$ 6.5
Granny Smith vs. others				
Mean square	58.45	42.64	2.25	14.52
<i>Pr</i> > <i>F</i>	0.0001	0.0001	0.0030	0.0001
Trial 2				
Braeburn	46.3 $\pm$ 9.5	46.4 $\pm$ 4.3	40.4 $\pm$ 6.9	28.0 $\pm$ 3.1
Fuji	28.5 $\pm$ 3.0	50.4 $\pm$ 11.2	45.8 $\pm$ 0.8	47.2 $\pm$ 5.8
Royal Gala	41.6 $\pm$ 5.7	37.0 $\pm$ 3.5	31.7 $\pm$ 2.8	38.2 $\pm$ 6.5
Granny Smith	17.8 $\pm$ 5.3	22.4 $\pm$ 6.4	22.4 $\pm$ 5.6	37.8 $\pm$ 6.5
Granny Smith vs. others				
Mean square	16.54	18.48	10.71	0.00
<i>Pr</i> > <i>F</i>	0.0001	0.0001	0.0001	1.00

^x Analysis of variance for a 4 $\times$ 5 and 4 $\times$ 4 interaction *Phytophthora* spp.–apple rootstock or cultivar for trials 1 and 2, respectively, was significant (*P* < 0.05) for the effect of *Phytophthora* spp., apple rootstock, or cultivar, and for the interaction *Phytophthora* spp.–apple rootstock or cultivar.

^y Means of five replicates and $\pm$ standard deviation obtained after 16 days at 20°C in an infested vermiculite substrate. Canker lesions were not developed on noninoculated controls.

^z Apple rootstock or cultivar.

borne singly on the sporangiophores, and proliferating).

The SDS-PAGE protein profiles of two isolates identified as *P. gonapodyides* were distinguishable from the protein profiles obtained for two isolates identified as *P. cryptogea* (between-species similarly coefficient [SC] values below 53%; Table 1). Compared with *P. gonapodyides*, the *P. drechsleri* isolate had SC values of 31 to 48%. SC values between *P. cryptogea* and *P. drechsleri* isolates exceeded 80% only when isolate K15-2 of *P. cryptogea* was used but varied from 53 to 56 when isolate NY413 of *P. cryptogea* was used. The SC values between the two isolates of *P. cryptogea* varied from 48 to 50% (Table 1).

Pathogenicity tests. Reddish to brown canker lesions, from 17.5 to 73.6 mm in length, were produced by all isolates of *P. cactorum*, *P. cryptogea*, *P. gonapodyides*, and *P. megasperma* on excised 1-year-old shoots of apple rootstocks M.9, M.25, MM.106, and MM.111 and apple cvs. Braeburn, Fuji, Royal Gala, and Granny Smith. Control shoots remained healthy except for a slight, superficial oxidation developed on the cut surface. Canker length caused by *P. cactorum*, *P. cryptogea*, *P. gonapodyides*, and *P. megasperma* varied from 17.5 to 59.3, 22.4 to 73.6, 22.4 to 55.8, and 28.0 to 69.2 mm, respectively. Canker length varied significantly ($P < 0.01$) with *Phytophthora* spp. and apple genotype used, and the *Phytophthora* spp.–apple genotype interactions were also highly significant ($P < 0.01$). For instance, *P. cactorum* produced a longer canker on shoots of MM.111 than on those of other rootstocks, whereas *P. cryptogea*, *P. gonapodyides*, and *P. megasperma* produced the longer canker on shoots of M.9, MM.106, and M.9, respectively. Granny Smith shoots was significantly less suscep-

tible than those on all rootstocks or other cultivars (Table 2).

All *Phytophthora* isolates were pathogenic 1-year-old plants of apple rootstock MM.106 causing root rot, canker lesions at the crown of most plants, and significantly ($P < 0.05$) reducing root weight (Table 3). The root weight reduction relative to the flooded but noninoculated control plants varied among the species of *Phytophthora* from 7.3 to 34.9%. Isolates of *P. cactorum* were the most virulent, with root weight reductions from 27.8 to 34.9%, whereas isolates of *P. cryptogea* were the least virulent, with root weight reductions of 7.3 and 12.2% (Fig. 1). Canker lesions varied significantly ($P < 0.05$) from 23 to 40 mm in length on inoculated plants and were not observed on control plants (Table 3).

The appropriate *Phytophthora* sp. was successfully isolated from the roots of diseased plants exposed to each isolate.

Detection of *Phytophthora* in the irrigation water. Only 1 of 16 (6.3%) water samples taken at the beginning of the irrigation furrows before the irrigation water passed by a diseased apple tree were positive for detecting a *Phytophthora* sp. In contrast, 10 of 32 (31.3%) samples taken adjacent to the affected trees and 14 of 16 (87.5%) samples collected at the end of the irrigation furrows after the water passed through the affected region were positive for a *Phytophthora* sp.

DISCUSSION

Although *Phytophthora* root and crown rot has long been recognized as an important disease of apples in Chile, *P. cactorum* is the only pathogen previously reported as a causal agent in this country (14,22). However, in addition to *P. cactorum*, we also isolated *P. cryptogea*, *P. gonapodyides*, and *P. megasperma* from infected apple trees, the soil surrounding them, or

both, and the pathogenicity of representative isolates of the four species was confirmed in tests on excised shoots (10,25) and 1-year-old potted trees of apple rootstocks MM.106. Whereas *P. cactorum* has a wide host range, including several deciduous fruits crops (6), it has been reported as a pathogen only on apple and almond (14) in Chile. In contrast, *P. cryptogea* has been recovered extensively from a variety of fruit crops in the Central Valley of Chile, including kiwifruit (15,17), grapevine (20), raspberry (29,30), and some stone fruits (Wilcox and Latorre, unpublished). In addition to this report on apples, *P. megasperma* also has been reported as a pathogen of raspberry in Chile (29,30).

It is likely that isolation frequencies obtained in this study understate the true incidence of infected samples, because some *Phytophthora* spp. are sensitive to the fungicides used in the isolation medium (i.e., benomyl, hymexazol, and pimaricin; 6). However, the absence of these compounds made isolation of *Phytophthora* spp. almost impossible, due to the rapid overgrowth of *Pythium* spp. and *Fusarium* spp.

P. gonapodyides is a heterothallic species assigned to the Group VI of Waterhouse (26). It has been previously isolated from the tissue and soil samples from the root zone of a variety of woody hosts in Europe and North America (2,6). In 1993, Brasier et al. (2) reassigned pathogenic isolates recovered from Douglas fir trees, which previously had been assigned to *P. drechsleri*, to this taxon. Although *P. gonapodyides* is morphologically similar to both *P. drechsleri* and *P. cryptogea*, isolates that we identified as *P. gonapodyides* were distinguished by their characteristic colony morphology (rosette to chrysanthemum pattern), mycelial growth charac-

Table 3. Pathogenicity and relative virulence of four *Phytophthora* spp. recovered from apple orchards determined on 1-year-old plants of apple rootstock MM.106^x

Inoculated with	Isolate	Crown rot incidence ^y	Root rot ^z	Root fresh weight (g)	Canker length (mm)
<i>P. cactorum</i>	M95-18	5/5	2.2 bcd	34.2 d	40 b
<i>P. cactorum</i>	M95-123-7	5/5	2.4 bcd	31.3 d	24 ab
<i>P. cactorum</i>	M95-143-2	5/5	1.4 d	30.9 d	26 ab
<i>P. cactorum</i>	M95-145-2	5/5	2.2 bcd	33.7 d	34 b
<i>P. cryptogea</i>	M95-68-15	4/5	3.0 bc	45.5 ab	30 ab
<i>P. cryptogea</i>	M95-136-5	5/5	3.2 ab	44.3 abc	40 b
<i>P. gonapodyides</i>	M95-93-5	4/5	3.2 ab	38.5 bcd	23 ab
<i>P. gonapodyides</i>	M95-122-9	3/5	2.2 bcd	35.6 cd	25 ab
<i>P. megasperma</i>	M95-68-6	4/5	2.0 cd	34.4 d	31 ab
Uninoculated					
Flooded	...	0/5	4.4 a	47.4 a	0.0 a
Unflooded	...	0/5	...	...	...

^x Plants were grown for 4 months (August–December) in artificially infested soil mix under screen-house conditions. Plants were constantly maintained on trays with 2 to 3 cm of water and were flooded every 2 weeks for a 48-h period by immersing pods in water. Means of five replicate plants per treatments. Values in each column followed by a common letter are not significantly different ($P < 0.05$) according to the Waller-Duncan k -ratio t test.

^y Number of plant with canker at the crown/total number of plants tested.

^z Root mass rotted based on a visual estimation where 1, 2, 3, and 4 = ≥ 75 , 50 to 75, 25 to 50, and $\leq 25\%$ rotted roots, respectively, and 5 = 100% healthy roots.

Fig. 1. Relative root weight reduction of apple rootstock MM.106 caused by *Phytophthora cactorum*, *P. megasperma*, *P. gonapodyides*, and *P. cryptogea*. Means followed by the same letter are not significantly different according to Waller-Duncan k -ratio t test ($P < 0.05$).

teristics (relatively slow growing, maximum temperature <35°C), and protein profiles that were clearly distinct from those of isolates identified as *P. drechsleri* and *P. cryptogea*. Thus, although our study involved only a limited number of isolates, we believe that these data support the concept of maintaining the three *Phytophthora* spp. as separate taxa (2,6,7,31). Two isolates from this study identified as *P. gonapodyides* have been deposited in the collection of the International Mycological Institute (IMI 368574 and 368577), as has an isolate of *P. megasperma* (IMI 368579).

Although all four *Phytophthora* spp. were pathogenic in our tests, *P. cactorum* was both the most-frequently isolated species and the most virulent specie on MM.106 rootstock, findings which are consistent with reports from several other parts of the world (e.g., 1,6,12,14,21,23). However, consistent with other reports (4,5,27) we also found a significant *Phytophthora* sp.-apple genotype interaction, reaffirming that caution must be employed when drawing general conclusions about the susceptibility of a given rootstock or cultivar to *Phytophthora* root, crown, or collar rot. Nevertheless, it appears that, under current conditions in Chile, disease control decisions, including rootstock choice, should be influenced more strongly by their effects on disease caused by *P. cactorum* than that caused by other *Phytophthora* spp.

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