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Characterization of *Phytophthora citrophthora* and *P. inundata* associated to foot and root rot of citrus trees in Chile

Alejandra Vial, Bernardo A. Latorre[1] and Juan Ortúzar
Facultad de Agronomía e Ingeniería Forestal,
Pontificia Universidad Católica de Chile, Casilla 306-22, Santiago, Chile.

Abstract

A. Vial, B.A. Latorre and J. Ortúzar. Characterization of *Phytophthora citrophthora* and *P. inundata* associated to foot and root rot of citrus trees in Chile. 2006. Cien. Inv. Agr. 33(3):205-216. Citrus production has increased considerably in Chile to about 17300 ha mainly planted with lemon, mandarin, and sweet orange. Although irrigation managements have improved and tolerant rootstocks have been commonly used, *Phytophthora* foot rot (gummosis) and root rot occur frequently. Symptoms included slow decline, moderate leaf chlorosis, reduced growth, lack of tree vigor and dieback. These symptoms were associated to extensive canker lesions and gummosis at the base of the trunk, and root rot, often extending from the main roots into the feeder roots. The frequent outbreaks of *Phytophthora* diseases in Chilean citrus groves motivated this survey to characterize the species of *Phytophthora* associated to foot and root rot. A total of 27 pathogenic isolates of *Phytophthora* were obtained in 2003 and 2004. Morphologically, 20 isolates were identified as *P. citrophthora*; however, they were distinctively divided into sterile and fertile isolates. The latter were morphologically similar to *P. citrophthora sensu lato* but, they induced oospore production when pairing against *P. cinnamomi* A2. These fertile isolates exhibited a 98% match with several *P. citrophthora* strains and were considerably less aggressive than sterile isolates. *Phytophthora inundata*, a relatively new described species, was identified on the basis of analysis of the ITS regions of rDNA. Isolates of *P. inundata* were weakly pathogenic on citrus and, possibly were found as secondary pathogen without importance on foot and root rot development on citrus trees. Therefore, *P. citrophthora* was the cause of foot and root rot found on Chilean citrus groves, planted in temperate and relative dry weather conditions. The isolation and pathogenicity of *P. citrophthora* on *Citrus macrophylla* and Carrizo citrange, considered resistant rootstocks, could be suggesting that these isolates have overcome resistant genes associated with *Phytophthora* resistant citrus rootstocks.

Key words: Citrus diseases, gummosis, oomycetes, *Phytophthora inundata, P. cryptogea* taxonomy, rootstocks.

Introduction

In recent years, citrus production has expanded considerably in Chile to about 17.300 ha, mainly planted with lemon *(Citrus limon* (L.) Burm.), mandarin (*C. reticulata* Blanco) and sweet orange (*C. sinensis* (L.) Osbeck). Although irrigation managements have improved and the use of tolerant rootstocks has increased, *Phytophthora* foot rot and root rot occur frequently. The aerial symptoms were characterized by slow decline, moderate leaf chlorosis, reduced growth, lack of tree vigor and dieback. These symptoms were associated with extensive canker lesions and gummosis at the base of the trunk, and root rot, extending from the main roots into the feeder roots (Timmer and Menge, 1993; Timmer *et al*., 2003).

Several species of *Phytophthora* have been associated to this syndrome but, *P. citrophthora* (Smith and Smith) Leonian and *P. parasitica*

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[1] Corresponding author: blatorre@uc.cl

Dastur (sin. *P. nicotianae* Breda de Haan) have been identified as the most destructive specie causing foot rot (gummosis) and root rot, often considered as two different diseases affecting citrus production worldwide (Klotz, 1973; Martins *et al*., 2001; Siviero *et al*., 2002; Timmer and Menge, 1993; Timmer *et al*., 2003; Vernière *et al*., 2004; Zitko *et al*., 1991).

Rootstocks, resistant to *P. citrophthora* and *P. parasitic*a, have been obtained and extensively use in citrus production. Resistance appears to be specie dependant without correlation between root rot and foot rot resistance. Eventually, resistant genes can be overcome (Afek *et al*., 1990; Agostini *et al*., 1991; Feld *et al*., 1990; Ferguson *et al*., 1990 ; Graham, 1990; Matheron and Matejka, 1992, 1993; Matheron *et al*., 1998; Vernière *et al*., 2004).

In Chile, *P. citrophthora,* and *P. parasitica* were reported on lemon and sweet orange associated to foot rot and, *P. citrophthora* to citrus brown rot on lemon fruits (Besoain *et al*., 1998; Mujica and Vergara, 1980). However, a complete characterization of the *Phytophthora* species involved in these syndromes, and their relative importance and distribution remain unknown in Chile. The frequent outbreaks of *Phytophthora* diseases in Chilean citrus groves motivated this survey with the aim to characterize the species of *Phytophthora* associated to foot and root rot and to evaluate their aggressiveness on the main rootstocks at present used in citrus production in Chile. A brief summary has been reported (Vial and Latorre, 2004).

Materials and methods

Isolation. Disease samples of infected roots and crown tissues were obtained from three to four trees in each of ten citrus groves distributed in a 500 km axis from north to south (Ovalle, to Requinoa, Chile). Each sample was thoroughly washed in tap water to dislodge the soil adhered to the rots and crown tissues. Small fragments (5 to 10 mm long) were obtained. Each segment was surface disinfected for 1 to 2 s on 75% ethanol, blotted on paper towel, and immediately placed on Petri plates containing 17 $g \cdot L^{-1}$Difco corn meal agar (CMA) amended per liter with 150 mg of ampicillin (Laboratorio Chile, S.A. Santiago, Chile), 10 mg of pyramicine (Delvocid, Gistbrocades, Holland), 16 mg of rifampicin (Rifaldin, Lepetit), 20 mg of benomyl (Benlate, E. I. du Pont de Nemours and Co., Wilmington, DE), 100 mg of pentachloronitrobencene (Brassicol, Hoescht) and 30 mg of hymexazol (Tachigaren, Sankio, Japan) (ACMA). Cultures were incubated in darkness at 20ºC for 7 days. Mycelial growth was examined under light microscope for the presence of a hyaline, coenocytic mycelium, branching at right angles (Erwin and Ribeiro, 1996). Hyphal tips from emerging *Phytophthora* colonies were subcultured on ACMA.

Sporangial morphology. Sporangia were produced after growing each isolate in carrot juice broth media (CJB, 500 g of fresh carrots were boiled per liter of distilled water for 10 to 15 min) for 48 to 72 h at room temperature (20 to 22ºC) under continuous fluorescent light. The mycelium obtained was washed in sterile distilled water (SDW) and treated with cool saline salt solution (2.36 g $Ca(NO_3)$, 0.5 g KNO_3, 1 g $MgSO_4 \cdot 7H_2O$, 1 mL of the following solution: EDTA (13 $mg \cdot mL^{-1}$), KOH (7.5 $mg \cdot mL^{-1}$) and, $FeSO_4$ (12.45 $mg \cdot mL^{-1}$)), washed again in tap water and then incubated in 2% (w/v) non-sterile soil extract solution for 2 to 4 days under continuous fluorescent light at room temperature (Ribeiro, 1978). Sporangia were characterized under light microscope for shape, size, presence or absence of papilla, proliferations and branching of the sporangiophores.

Gametangial morphology. Gametangia production was studied on clarified V8 juice agar (1000 mL of Campbell V-8 juice centrifuged with 11 g $CaCO_3$ for 20 min at 4000 rpm; the supernatant was diluted 1:4 with distilled water) amended per liter, after autoclaving, with: 30 mg ß- sitosterol, 20 mg tryptophan, 100 mg $CaCl \cdot 2H_20$, and 1 mg thiamine-HCl" (AV8) (Erwin and Ribeiro, 1996). Each isolate was plated in triplicate as single culture or in pairing with *P. cinnamomi* A1 or A2 mating types. Cultures were incubated for 20 to 30 days at 20ºC in complete darkness before to determine oospore production by direct observation under light microscope.

Molecular characterization
Isolates M-1, M-21 and M-58 were selected for molecular identification using the ITS sequence analysis of rDNA. These analyses were performed by CABI Bioscience (Wallingfor, England, UK).

Effect of temperature. The effect of 5, 10, 20, 30 and 35°C on mycelial growth was determined. Each isolate was plated in triplicate on ACMA and incubated for 5 days before to determine colony diameter.

Pathogenicity tests. Twenty seven isolates of *Phytophthora* spp. were initially tested for pathogenicity on fruits of lemon cv. Fino 49. Mature fruits, yellow and uniform in size were selected, washed in running tap water and surface disinfected for 1 to 2 min in 75% ethanol. Fruits were inoculated with a mycelial plug (5 mm in diameter) that was placed in a hole aseptically made with a cork borer. An equal number of injured, but noninoculated fruits, served as control. The diameter of the necrotic lesion developed was determined after 7 days of incubation in humid chamber at 20ºC.

Fifteen isolates, pathogenic on lemon fruits, were selected and tested on one-yr-old twigs of *C. macrophylla* (Afek *et al*., 1990). Twigs were surface disinfected in 75% ethanol for 1 to 2 min and inoculated with mycelial plug (5 mm long) that was inserted aseptically underneath the bark. The inoculation site was wrapped with Parafilm to avoid a rapid dehydration. An equal number of twigs injured but noninoculated were leaf as control. The length of the necrotic lesion developed was determined after 10 to 15 days at 20ºC in humid chambers.

The same 15 isolates were tested on seedlings of *C. macrophylla* and Carrizo citrange (*C. sinensis* x *Poncirus trifoliate*) growing in tubs (15x7x7 cm). Seedlings were inoculated with a mycelial suspension prepared from mycelia growing on CJB for 5 days at 20ºC under continuous light, that was macerated in a blender and adjusted to 1-2x10^5 propagules·mL^{-1}. Seedlings were equally reinoculated after 30 days. Seedlings were subjected to four floodings every 14 days each for 48 h. An equal number of seedlings of each rootstock, injured but noninoculated and none flooding, served as controls. Root fresh weight, root dry weight (24 h at 60ºC) and root rot was determined after 90 days at 20 to 24ºC. Root rot was visually estimated for each seedling on a 1 to 10 scale (1 < 10%, and 10 > 90% of the root mass), according to the percentage of root mass rotten.

The relative susceptibility of *C. macrophylla*, Carrizo citrange and Rouge lemon (*C. jambhiri* Lush.) to selected isolates of *P. citrophthora and P. inundata* was studied under screenhouse conditions. One-yr-old plants were grown in 25 liter containers with 15 liter of a nonesterilized soil mixture. Each plant was inoculated with 100 mL of a mycelial suspension (5-7x10^5 propagules·mL^{-1}) prepared as described above, that was delivered in each of four 30-cm-holes made with a sterile knife around the trunk. An equal number of noninoculated but, injured plants were left as controls. Plants were subjected to six flooding episodes, each for 48 h every 14 days starting 24 h after the inoculation. After flooding plants were drained and returned to normal irrigation regime. Root fresh weight, root dried weight and root rot was determined 180 days after inoculation.

The same rootstocks were trunk inoculated using mycelium plugs from two selected isolates of *P. citrophthora* and one from *P. inundata* that was inserted underneath the bark on trunk approximately 5 cm above soil level. The inoculation site was immediately wrapped with Parafilm to avoid a rapid dehydration. The length of the necrotic lesion developed and the presence or absence of gum exudation were determined after 30 days of the inoculation.

At the end of each pathogenicity test, small segments of disease tissues taken from inoculated plants and non inoculated controls were plated on ACMA for reisolation of the pathogen.

Statistical analyses. The effect of temperature on mycelial growth was adjusted to a polynomial model where x = incubation temperature and y = colony diameter.

All percent values were transformed to [arsin (sqrt (x)] before analysis. Results from

pathogenicity tests on lemon fruits and twigs were analyzed for variance according to a complete randomized design with four and or five replicates, each of one fruit or one twig as experimental unit. Means were separated according to Waller Duncan k ratio t tests using the Multstat program (Shane *et al*., 1990).

Results obtained on seedlings inoculations were studied for variance according to a complete randomized design with a 3x2 factorial arrangement, three *Phytophthora* sp., and two rootstocks, with four replicates each of one seedling. Means were separated according to Tukey tests using SigmaStat (Systat Software, Inc., USA).

The rootstock susceptibility was designed as a complete randomized design with 3x3 factorial arrangements of treatments, three *Phytophthora* sp., and three rootstocks, with five replicates each of one plant. Means were separated according to Tukey tests using SigmaStat.

Results

Isolation and identification. Twenty seven isolates of *Phytophthora* spp. were obtained in eight of ten citrus groves sampled in 2003 and 2004 (Table 1). On the basis of colony morphology, eleven sterile isolates were identified as *P. citrophthora* (sterile group) (Newhook, *et al*., 1978; Stamps, *et al*., 1990; Waterhouse, 1963). Nine unidentified isolates were classified in Group II and seven in Group VI of Waterhouse (Newhook, *et al*., 1978; Waterhouse, 1963). Based on polymerase chain reaction (PCR), using internal transcribed

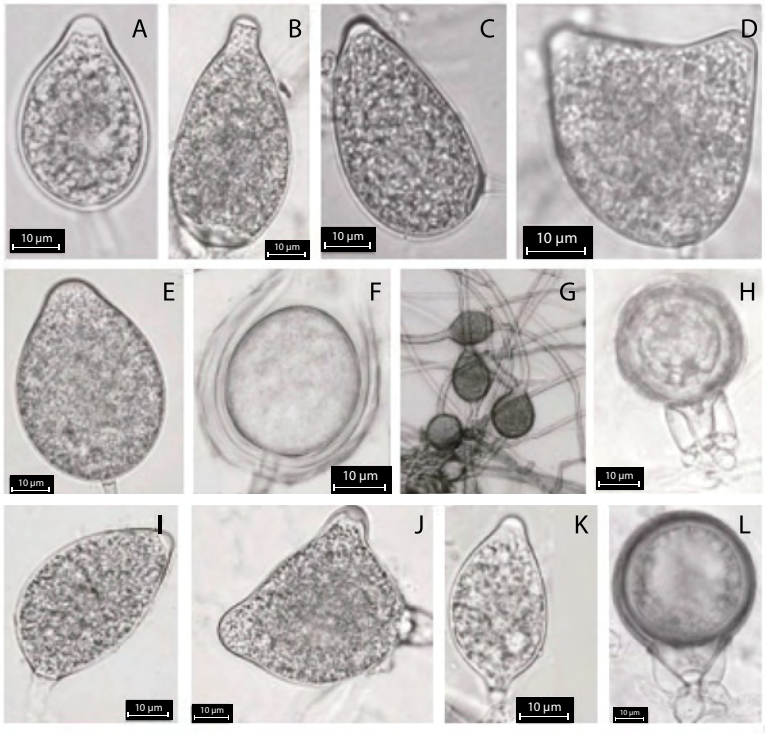

Figure 1. *Phytophthora* species isolated from citrus groves in Chile. Sporangia produced by sterile isolates of *P. citrophthora* (A-D). *Phytophthora inundata* (E-H), ovoid non papillate sporangium (E), internal proliferation of the conidiophore (F), spherical hyphal swellings (G), oospore and amphigynous antherium (H). Reproductive structure produced by fertile isolates of *P. citrophthora* (I-L), sporangia (I-K) and oospore and amphigynous antheridia (L).

Figura 1. *Especies de* Phytophthora *aisladas de huertos cítricos en Chile. Esporangios producidos por aislamientos estériles de* P. citrophthora *(A-D).* Phytophthora inundata (E-H), *esprangios ovoides no papilados (E), proliferación interna del conidióforo (F), protuberancias esféricas del micelio (G), oosporas y anteridios amfígenos (H). Estructura reproductiva de aislamientos fértiles de* P. citrophthora (*I-L), esporangio (I-K) y oosporas con anteridio amfígeno (L).*

Table 1. Isolates of *Phytophthora* spp. recovered from citrus rootstocks in 2003 and 2004 in Chile.
Cuadro 1. *Aislamientos de* Phytophthora spp. *obtenidos desde portainjertos para cítricos en 2003-2004 en Chile.*

Host[1]			*Phytophthora*	
Species	Rootstock	Locality	Species	Isolates
Lemon	Macrophylla	Quillota	*P. citrophthora*	M-1[2]
Lemon	Macrophylla	Quillota	*P. citrophthora*	M-2
Lemon	Macrophylla	Chiñihue	*P. citrophthora*	M-51
Lemon	Macrophylla	Chiñihue	*P. citrophthora*	M-52
Lemon	Macrophylla	Chiñihue	*P. citrophthora*	M-53
Lemon	Macrophylla	Chiñihue	*P. citrophthora*	M-54
Lemon	Sour orange	El Monte	*P. citrophthora*	NA-10
Lemon	Sour orange	El Monte	*P. citrophthora*	NA-16
Lemon	Sour orange	El Monte	*P. citrophthora*	NA-17
Lemon	Sour orange	El Monte	*P. citrophthora*	NA-22
Lemon	Sour orange	El Monte	*P. citrophthora*	NA-26
Lemon	Sour orange	El Monte	*P. inundata*	NA-18
Lemon	Sour orange	El Monte	*P. inundata*	NA-19
Lemon	Sour orange	El Monte	*P. inundata*	NA-20
Lemon	Sour orange	El Monte	*P. inundata*	NA-21[2]
Lemon	Sour orange	El Monte	*P. inundata*	NA-23
Mandarin	Carrizo citrange	Requinoa	*P. inundata*	C-29
Mandarin	Carrizo citrange	Requinoa	*P. inundata*	C-30
Mandarin	Carrizo citrange	Ovalle	*P. citrophthora*[3]	C-46
Mandarin	Carrizo citrange	Requinoa	*P. citrophthora*[3]	C-31
Mandarin	Carrizo citrange	Requinoa	*P. citrophthora*[3]	C-32
Sweet orange	Carrizo citrange	Mallarauco	*P. citrophthora*[3]	C-56
Sweet orange	Carrizo citrange	Mallarauco	*P. citrophthora*[3]	C-57
Sweet orange	Carrizo citrange	Mallarauco	*P. citrophthora*[3]	C-58[2]
Sweet orange	Swingle citrumelo	Nogales	*P. citrophthora*[3]	Ci-36
Sweet orange	Swingle citrumelo	Nogales	*P. citrophthora*[3]	Ci-40
Sweet orange	Swingle citrumelo	Nogales	*P. citrophthora*[3]	Ci-41

[1]Lemon = *Citrus limon*; Mandarin = *C. reticulata*; Sweet orange = *C. sinensis*; Macrophylla = *C. macrophylla;* Sour orange = *C. aurantium;* Carrizo citrange = *C. sinensis* x *Poncirus trifoliata*; Swingle citrumelo = *C. paradisi* x *P. trifoliata*.
[2]Isolates identified by CABI Bioscience (Wallingfor, England, UK) based on molecular characterization. M1 = IMI 392460, M-21 = IMI 392461, M-52 = 392462.
[3]Fertile isolates of *P. citrophthora*.
[1]*Limón* = Citrus limon*; Mandarín* = C. reticulata; *Naranjo dulce* = C. sinensis*; Macrophylla* = C. macrophylla*; Naranjo agrio* = C. aurantium*; Carrizo citrange* = C. sinensis x Poncirus trifoliata*; Swingle citrumelo* = C. paradisi x P. trifoliata.
[2]*Aislamientos identificados molecularmente por CABI Bioscience (Wallingfor, England, UK). M1 = IMI 392460, M-21 = IMI 392461, M-52 = 392462*.
[3]*Aislamiento fértil de* P. citrophthora.

spacer (ITS) of the rADN, a selective sterile isolates (M-1) and one fertile isolate (M-58) were confirmed by CABI BioSciences as *P. citrophthora* (IMI 392460 and IMI 392462, respectively). Similarly, a selective isolate (M-21), tentatively classified within Waterhouse's Group VI was identified as *P. inundata* Brasier, Sánchez-Hernández and Kirk (IMI 392461).

*S*terile *P. citrophthora* was obtained from lemon grafted on *C. macrophylla*, and sour orange. It produced white rosette, and slightly cottony colonies, on ACMA. Sporangia were papillated, persistent, ovoid, obpyriform, limoniform or distorted, having with one or two apex, of 46.5±8.6 x 29.9±4.3 μm and 1.6±0.3 length: breath ratio (Figure 1). Oospores and mycelial growth at 35ºC was not obtained (Figure 2). Chlamidospores and mycelial swellings were not produced.

Fertile *P. citrophthora* was obtained from mandarin and sweet orange grafted on Carrizo citrange, and from sweet orange grafted on Swingle citrumelo. They developed white, petaloid and slightly cottony colonies on ACMA. Sporangia were persistent, papillated to semipapillated, ovoid, obpyriform or distorted, with one or two apexes, of 53.5±12.7 x 31.9±5.8 μm and 1.7± 0.4 length:breath ratio (Figure 1).

Aplerotic oospores were produced only when pairing against *P. cinnamomi* mating type A2 (Figure 1). Hyphal swelling, chlamidospores, sporangial proliferations, and mycelial growth at 35ºC were not obtained (Figure 2).

Isolates identified as *P. inundata* were recovered from lemon grafted sour orange and mandarin grafted on Carrizo citrange. They developed white stellate slightly cottony colonies on ACMA, produced round mycelial swellings, none papillated, persistent, ovoid to obpyriform sporangia of 55.2±15.8 x 38.6±11.0 μm and 1.5±0.3 length:breath ratio. Internal and external sporangial proliferations were obtained. Sporangiophores were single or sympodial. Oogonia were smooth and aplerotic oospores were produced when pairing against *P. cinnamomi* mating type A2. Antheria were always amphigynous (Figure 1). These isolates exhibited no mycelial growth at 35ºC with optima between 25 and 30ºC (Figure 2).

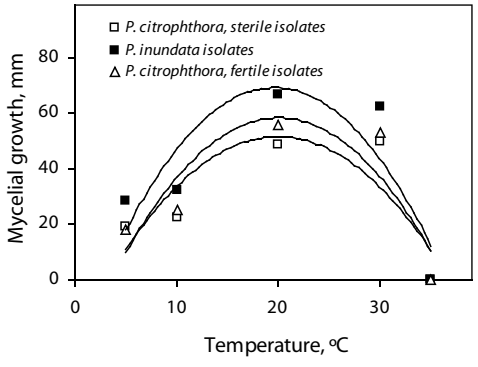

Figure 2. Growth temperature curves obtained with sterile and fertile isolates of *Phytophthora citrophthora* and isolates of *P. inundata* obtained from citrus trees in Chile. Polynomial equations for *P. citrophthora* were $y = -0.18x^2 + 7.23x - 20.50$ ($R^2 = 0.68$) and $y = -0.22x^2 + 8.58x - 27.49$ ($R^2 = 0.75$) for sterile and fertile isolates, respectively. Polynomial equation for isolates of *P. inundata* was $y = -0.24x^2 + 9.44x - 23.20$ ($R^2 = 0.72$).
Figura 2. *Curvas de respuesta del crecimiento del micelio a la temperatura obtenidas con aislamientos fértiles y estériles de* Phytophthora citrophthora *y aislamientos de* P. inundata *obtenidos de cítricos en Chile. Ecuaciones polinómicas obtenidas para* P. citrophthora *fueron iguales a* $y = -0{,}18x^2 + 7{,}23x - 20{,}50$ ($R^2 = 0{,}68$) *e* $y = -0{,}22x^2 + 8{,}58x - 27{,}49$ ($R^2 = 0{,}75$) *para aislamientos fértiles y estériles, respectivamente. Relación polinómica obtenida para aislamientos de* P. inundata *fueron iguales a* $y = -0{,}24x^2 + 9{,}44x - 23{,}20$ ($R^2 = 0{,}72$).

Pathogenicity tests

Lemon. Twenty isolates (100%) identified as sterile and fertile of *P. citrophthora* induced a brown to reddish necrotic lesion on lemon fruits that were respectively 70.4±17.1 and 65.9±22.5 in diameter while only three of seven isolates (43%) of *P. inundata* produced very small brown necrotic lesions of 1.7±1.6 mm after 7 days of incubation (Figure 3). All isolates of *P. citrophthora* were significantly ($p = 0.05$) more virulent than isolates of *P. inundata*. Reisolations made from disease tissue taken from inoculated lemon fruits were successful.

Twigs. Eight isolates of sterile *P. citrophthora* were significantly ($p = 0.05$) more virulent than five fertile isolates of *P. citrophthora* developing brown to reddish canker lesions of 30.1±4.6 mm and 18.8±2.6 mm in length on one-yr-old twigs of *C. macrophylla*. Isolates of *P. inundata* were none pathogenic (Figure 3). The reisolation of *P. citrophthora* from canker lesions was successful only from disease twigs.

Seedlings. A partial root rot was obtained regardless of the *Phytophthora* isolate used and *Phytophthora spp*. were reisolated from rotten root tissues. Root rot percentages varied significantly between the specie of *Phytophthora* (S) ($p < 0.001$), the rootstocks (R) ($p = 0.004$), and the SxR interaction was also significant ($p = 0.05$). Independently of the rootstocks, *P. citrophthora* was significantly more aggressive than *P. inundata*. *Citrus macrophylla* was significant more resistant than Carrizo citrange when seedlings were inoculated with sterile isolates of *P. citrophthora*. Similarly, significant ($p = 0.05$) root weight reductions were least pronounced on *C. macrophylla*, 13.5 and 15.3% for fresh and dry root weight loss, respectively, and Carrizo citrange with reductions exceeding 30.3% (Table 2).

Rootstocks. Trunk inoculations induced brown to reddish canker lesions and gum exudation only when rootstocks were inoculated with the sterile isolate of *P. citrophthora*. The effect of the rootstock, *Phytophthora* species and the SxR interaction were highly significant ($p < 0.001$) (Table 3). Based on these results, Carrizo citrange was the most resistant rootstock followed by *C. macrophylla* and Rough lemon.

Table 2. Pathogenicity of *Phytophthora citrophthora, P. inundata* and *Phytophthora sp*. determined on seedlings of *Citrus macrophylla* and Carrizo citrange (*Citrus sinensis* x *Poncirus trifoliata*).
Cuadro 2. *Patogenicidad de* Phytophthora citrophthora, P. inundata *y* Phytophthora sp. *determinada en plántulas de* Citrus macrophylla *y Carrizo citrange* (Citrus sinensis x Poncirus trifoliata).

Rootstocks (R)	*P. citrophthora* (I)[1]			Mean
	Sterile group (n= 8)	Fertile group (n=5)	*P. inundata* (n=2)	
Root rot (%)[2]				
C. macrophylla	43.8 ± 2.7 aA[3]	49.5 ± 1.0aA[3]	20.0 ± 10.8 aB[3]	
Carrizo citrange	63.8 ± 2.3 bA	52.5 ± 5.0a A	23.8 ± 7.5 aB	
	df	*p*	*F*	
Interaction R x I	2	0.050	3.561	
Root fresh weight (%)[2]				
C. macrophylla	17.1 ± 11.4	14.8 ± 9.2	8.5 ± 10.3	13.5 a[3]
Carrizo citrange	32.1 ± 3.3	36.2 ± 7.5	22.0 ± 9.8	30.3 b
Mean	24.6 A[3]	25.8 A	15.3 A	
	df	*p*	*F*	
Interaction R x I	2	0.773	0.261	
Root dry weight (%)[2]				
C. macrophylla	22.0 ± 22.8	19.2 ± 22.6	4.5 ± 9.1	15.3 a[3]
Carrizo citrange	36.1 ± 12.0	40.8 ± 16.3	29.7 ± 11.4	35.5 b
Mean	29.0 A	30.0 A	17.1 A	
	df	*p*	*F*	
Interaction R x I	2	0.910	0.171	

[1]Fertile and sterile conformed two groups of *P. citrophthora* isolates that induced and do not induced oospore production when pairing against *P. cinnamomi* A2, respectively.
[2]Data were expressed as percent root weight reduction relative to untreated checks.
[3]Means followed by the same lower and capital letter within columns and files, respectively, were not significantly different according to Tukey's test (p =0.05). Percentages were transformed to [arsin (sqrt (x)] before analysis but untransformed data are presented.
[1]Fértil y estéril conforman dos grupos de aislamientos de P. citrophthora *que inducen o no inducen formación de oosporas al aparearlos con* P. cinnamomi *A2, respectivamente.*
[2]Datos expresados como porcentaje de reducción del peso radical en relación con los testigos sin tratar.
[3] Promedios seguidos por letras minúsculas o mayúsculas en cada columna o fila, respectivamente no fueron significativamente diferentes según la prueba de Tukey (p = 0.05). Los porcentajes fueron transformados a arcoseno (√x) previo al análisis, pero se presentan valores sin transformar.

Sterile *P. citrophthora* was the only specie pathogenic after trunk inoculations.

The sterile and fertile isolates of *P. citrophthora* and *P. inundata* induced root rot and a considerably root mass reduction on inoculated plants after 180 days of the inoculation that appeared to be specie and rootstock dependant (Table 3). Root rot significantly (p = 0.003) varied with the specie of *Phytophthora* (S), and rootstocks (R), and the interaction SxR was also a significant (p = 0.005). All isolates of *P. citrophthora* were equally aggressive on *C. macrophylla,* and no significant differences were obtained on Carrizo citrange. However on Rough lemon, root rot was significantly more pronounced with sterile (78%) than fertile isolates (16%) of *P. citrophthora* (Table 3).

Only the rootstocks had a significant (p <0.001) effect on root weight reduction, being *C. macrophylla* significantly less affected than Carrizo citrange and Rough lemon (Table 3).

Reisolations made from disease tissue taken from inoculated rootstock were always successful.

Discussion

The objective of these studies was to characterize and identify the species of *Phytophthora* associated to foot rot and root rot, two different diseases frequently found on citrus groves in Chile. To fulfill this objective, twenty seven isolates of *Phytophthora* were obtained in a survey performed in 2003 and 2004.

Table 3. Pathogenicity of *Phytophthora citrophthora* and *P. inundata* to *Citrus macrophylla*, Carrizo citrange (*Citrus sinensis x Poncirus trifoliate*) and Rough lemon (*C. jambhiri*), commonly used as citrus rootstocks in Chile.
Cuadro 3. *Patogenicidad de* Phytophthora citrophthora *and* P. inundata *determinada en* Citrus macrophylla, *Carrizo citrange (*Citrus sinensis x Poncirus trifoliate*) y limón rugoso (*C. jambhiri*), comúnmente empleados como portainjertos* en Chile.

Phytophthora species	Rootstocks			Mean
	C. macrophylla	Carrizo citrange	Rouge lemon	
		Trunk inoculations		
Canker length (mm)				
P. citrophthora[2]				
Sterile group	14.6 ± 10.6 aA[3]	5.0 ± 5.0 aB[3]	39.4 ± 11.1aC[3]	
Fertile group	0.0 ± 0.0 bA	0.0 ± 0.0 aA	0.0 ± 0.0 bA	
P. inundata	0.0 ± 0.0 bA	0.0 ± 0.0 aA	0.0 ± 0.0 bA	
	df	*p*		
Species (S)	2	<0.001		
Rootstocks (R)	2	<0.001		
Interaction S x R	4	<0.001		
Residual	36			
		Root inoculations		
Root fresh weight (%)[1]				
P. citrophthora[2]				
Sterile group	9.6 ± 13.2	27.2 ± 20.7	53.2 ± 35.8	38.0 a[3]
Fertile group	14.6 ± 19.9	22.1 ± 23.7	20.5 ± 10.5	30.0 a
P. inundata	10.2 ± 14.7	16.5 ± 11.6	40.3 ± 27.1	31.6 a
Mean	11.5 A[3]	28.3 B[3]	48.2 B[3]	
	df	*p*		
Species (S)	2	0.241		
Rootstocks (R)	2	<0.001		
Interaction S x R	4	0.172		
Residual	36			
Root dry weight (%)[1]				
P. citrophthora[2]				
Sterile group	11.4 ± 16.2	40.9 ± 18.3	70.7 ± 5.4	41.0 a[3]
Fertile group	19.0 ± 16.9	32.0 ± 19.3	37.1 ± 16.7	32.0 ab
P. inundata	13.5 ± 17.8	24.0 ± 6.9	33.7 ± 33.1	33.2 b
Mean	14.6 A	32.3 B	47.2 B	
	df	*p*		
Species (S)	2	0.130		
Rootstocks (R)	2	<0.001		
Interaction S x R	4	0.183		
Residual	36			
Root rot (%)[1]				
P. citrophthora[2]				
Sterile group	20.0 ± 7.1 aB[3]	16.0 ± 13.4 aA	78.0 ± 13.0 aC	
Fertile group	14.0 ± 15.2bA	24.0 ± 15.2 a A	16.0 ± 8.9 bA	
P. inundata	8.0 ± 4.5 aA	16.0 ± 11.4 aA	24.0 ± 33.6 bA	
	df	*p*		
Species (S)	2	0.003		
Rootstocks (R)	2	0.003		
Interaction S x R	4	0.005		
Residual	36			

[1]Data were expressed as percent root weight reduction relative to untreated checks.
[2]Fertile and sterile conformed two groups of *P. citrophthora* isolates that induced and do not induced oospore production when pairing against *P. cinnamomi* A2, respectively.
[3]Means followed by the same lower and capital setter within columns and files, respectively were not significantly different according to Tukey's test (p =0.05). Percentages were transformed to [arsin (sqrt (x)] before analysis but untransformed data are presented.
[1]Datos expresados como porcentaje de reducción del peso radical en relación con los testigos sin tratar.
[2]Fértil y estéril conforman dos grupos de aislamientos de P. citrophthora *que inducen o no inducen formación de oosporas al aparearlos con* P. cinnamomi *A2, respectivamente.*
[3]Promedios seguidos por letras minúsculas o mayúsculas en cada columna o fila, respectivamente no fueron significativamente diferentes según la prueba de Tukey (p = 0,05). Los porcentajes fueron transformados a arcoseno (√x) previo al análisis, pero se presentan valores sin transformar.

Morphologically, a sterile group of isolates was unambiguously identified as *P. citrophthora* (Erwin and Ribeiro, 1996; Newhook, *et al*., 1978; Stamps *et al*., 1990; Waterhouse, 1963). In general, sex organs have been excluded in the description of this species (Erwin,and Ribeiro,1996; Newhook, *et al*., 1978; Stamps et al., 1990). However, another group of fertile isolates, belonging to Waterhouse's Group II (Newhook, *et al*., 1978; Waterhouse, 1963), were also ascribed to *P. citrophthora* on the basis of sequence analysis of the ITS region of rDNA (Cooke *et al*., 2000). These isolates were morphologically close to *P. citrophthora* but, they induced oospore production when pairing with *P. cinnamomi* A2. Nevertheless, they exhibited a 98% match with several *P. citrophthora* strains. Evidences of fertile strains among *P. citrophthora* have been previously reported but, oospore production in citrus trees in nature is unknown (Erwin and Ribeiro, 1996; Cohen, *et al*., 2003; Latorre *et al*., 1991; Mchau and Coffey, 1994).

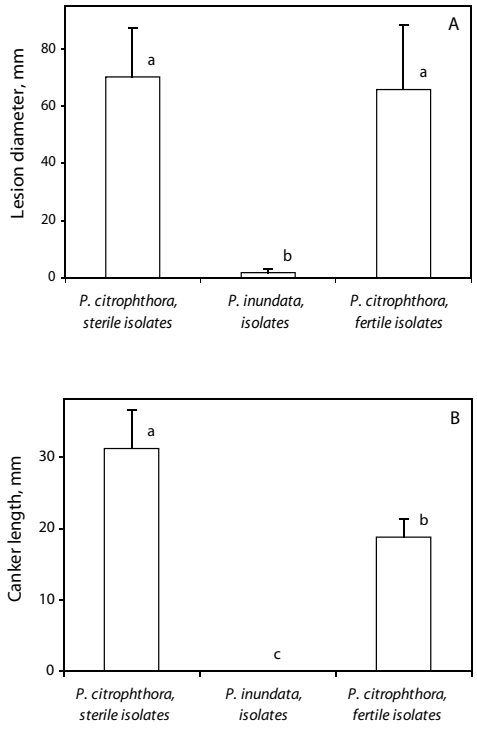

Figure 3. Pathogenicity tests preformed with sterile and fertile isolates of *Phytophthora citrophthora* and *P. inundata* obtained form citrus groves in Chile. A. Pathogenicity on fruits of lemon cv. Fino 49. B. Pathogenicity on one-yr-old twigs of *Citrus macrophylla*. Values are means four replications of eight and six sterile and fertile isolates of *P. citrophthora*, respectively, and two isolates of *P. inundata*. Means followed by the same letter were not statistically different according to Waller Duncan k ratio t tests (p = 0.05). Bars = standard deviation.

Figura 3. *Pruebas de patogenicidad realizadas con aislamientos estériles y fértiles de* Phytophthora citrophthora *y aislamientos de* P. inundata, *obtenidos en huertos cítricos en Chile. A. Petogenicidad en limón cv. Fino 49. B. Patogencidad en ramillas de un año de edad de* Citrus macrophylla. *Cada punto es el promedio de cuatro repeticiones de ocho y seis aislamientos estériles y fértiles de* P. citrophthora, *respectivamente, y dos aislamientos de* P. inundata. *Promedios seguidos por una misma letra no fueron estadísticamente diferentes de acuerdo con la prueba de Waller Duncan k ratio t tests (p = 0,05). Barras = desviación estándar.*

A group of unidentified isolates that were tentatively identified as *P. cryptogea* (Vial and Latorre, 2004) were finally ascribed to *P. inundata* within ITS Clade 6 (Brasier *et al*., 2003). This was based on the results of the analysis of the ITS regions of rDNA that revealed 99% similitude with references isolates of *P. inundata* and were distinctively different from *P. gonapodyides*, *P. drechsleri* and *P. cryptogea*, including Chilean isolates Ph3 (IMI 393160) and Ph5 of *P. cryptogea,* recovered from petunia (Ampuero and Latorre personal communication). In contrast to the original description, the Chilean isolates of *P. inundata* produced round hyphal swellings on liquid media, and mycelial growth was not obtained at 35ºC (Brasier *et al*., 2003).

Phytophthora inundata has been reported in a relatively wide range of host plants in several countries, especially prevalent in areas of flooding soils. However, to our knowledge this the first report of the presence of *P. inundata* on lemon and mandarin grafted, respectively on sour orange and Carrizo citrange, in South America. Previously, it was found on *Vitis vinifera* in Chile (isolates V94-1 and V94-49) (Brasier *et al*., 2003).

According to the results obtained, *P. citrophthora* was the most frequent species found on citrus groves in Chile. In contrast, there was any evidence for the presence of *P. parasitica*, reported previously from citrus trees in Chile (Mujica and Vergara, 1980).

The Chilean isolates of *P. citrophthora* induced brown rot symptoms on lemon fruits and they induced both foot rot and root rot, which are

often recognized as two separate *Phytophthora* diseases of citrus trees (Matheron *et al*., 1998; Timmer and Menge, 1993; Timmer *et al*., 2003). However, it was interesting that sterile isolates of *P. citrophthora* were more aggressive than the fertile isolates. This was particularly evident by the inability of fertile isolates to infect the bark tissue of a resistant rootstock (*C. macrophylla*), moderately resistant rootstock (Carrizo citrange), and a very susceptible rootstock (Rough lemon) (Erwin and Ribeiro, 1996; Matheron *et al*., 1998). Similarly, these isolates appeared to be equally pathogenic on lemon fruits but, significantly less aggressive than sterile isolates on one-yr-old twigs of *C. macrophylla,* suggesting a poor ability to cause foot rot and gummosis (Afek *et al*.,1990).

All isolates identified as *P. inundata* were less aggressive than *P. citrophthora* without any ability to infect neither the bark of *C. macrophylla*, *Carrizo citrange* and Rough lemon or one-yr-old twigs of *C. macrophylla*. Therefore, it was very possible that *P. inundata* was associated as a secondary root rot pathogen having little or no importance on foot and root rot development on citrus groves.

Therefore, *P. citrophthora* was the cause of foot and root rot found on Chilean citrus groves, planted in semiarid climate conditions. Two distinctively different strains of *P. citrophthora*, sterile and fertile strains, were identified. However, sterile strains were considerably more aggressive than fertile strains. The epidemiological significance of these findings remains to be determined.

Previously, *P. citrophthora* was recovered from other fruit crops in Chile but, to our knowledge it is the first report demonstrating Kock postulates on citrus crops and the first report of this species on mandarin in Chile (Besoain *et al*., 1998; Latorre and Muñoz, 1993; Latorre *et al*., 1991; Mujica and Vergara, 1980). The isolation and pathogenicity of *P. citrophthora* on *C. macrophylla* and Carrizo citrange, considered resistant rootstocks, could suggest that these isolates have overcome rootstock resistance. Nevertheless, this hypothesis remains to be probed.

Resumen

La producción de cítricos ha aumentado considerablemente en Chile, con alrededor de 17300 ha, principalmente destinadas a la producción de limón, mandarín y naranjo. La pudrición del pie (gomosis) y la pudrición de las raíces ocurren frecuentemente. Esto a pesar que el manejo del agua de riego ha mejorado y se han introducido portainjertos tolerantes. Los síntomas de esta enfermedad consisten en un lento y progresivo decaimiento, clorosis foliar moderada, menor crecimiento, escaso vigor y muerte parcial de ramas y ramillas. Estos síntomas están asociados con la presencia de cancros y gomosis en la base del tronco y podredumbre radical, que compromete raíces primarias y secundarias. La ocurrencia relativamente frecuente de focos de esta enfermedad en huertos cítricos en la zona central de Chile, motivó esta investigación que tuvo el propósito de caracterizar las especies de Phytophthora asociadas a este síndrome. Se obtuvo 27 aislamientos de *Phytophthora* entre 2003 y 2004. Morfológicamente, 20 aislamientos se identificaron como *P. citrophthora* entre los cuales se identificaron aislamientos estériles y fértiles. Los aislamientos fértiles fueron morfológicamente indiferenciables de *P. citrophthora sensu lato* pero, desarrollaron oosporas al aparearlos con *P. cinnamom*i A2 y presentaron 98% de similitud al compararlos molecularmente con *P. citrophthora*. Los aislamientos fértiles fueron relativamente menos agresivos que los estériles. *Phytophthora inundata*, corresponde a una especie recientemente descrita en el mundo, identificada sobre la base de la caracterización de la región ITS del rADN. Los aislamientos de *P. inundata* fueron débilmente patogénicos en cítricos y posiblemente tienen secundaria importancia en el desarrollo de esta enfermedad. Por lo tanto, según los resultados obtenidos en esta investigación *P. citrophthora* es la causa de la pudrición del pie y de las raíces en huertos cítricos chilenos, generalmente plantados en zonas templadas y relativamente secas. El aislamiento y patogenicidad de *P. citrophthora* obtenida desde *Citrus macrophylla* y Carrizo citrange, considerados como portainjertos resistentes, sugiere una posible pérdida de resistencia de estos portainjertos.

Palabras clave: Cítricos, enfermedades, gomosis, oomycetes, *Phytophthora inundata, P. cryptogea,* portainjertos, taxonomía.

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