Comparative Use of Soil Infested with Chlamydospores to Screen for Relative Susceptibility to Phytophthora Foot Rot in Citrus Cultivars

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ABSTRACT

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Soil-agar blocks infested with chlamydospores of *Phytophthora parasitica* were an effective inoculum source for screening citrus rootstocks for relative susceptibility to foot rot. Mean percentage of stem girdling, lesion area, and relative lesion area were similar on stems inoculated with soil-agar blocks containing seven to 100 chlamydospores or inoculated with 1,000 zoospores. Foot rot severity ratings were significantly higher when inoculum consisted of 0.5-cm-diameter mycelial agar disks compared with chlamydospores or zoospores. Commercial rootstocks known to be moderately resistant to or with field tolerance to foot rot were rated as susceptible when mycelial agar disks were used as an inoculum source. The use of quantifiable inoculum sources such as chlamydospores or zoospores may allow evaluation of rootstocks with intermediate levels of resistance to foot rot.

Foot rot caused by *Phytophthora* spp. is a serious disease of citrus worldwide, primarily affecting nursery and young orchard trees (3,19). Foot rot management strategies rely primarily on resistant rootstocks (7–10) and, to a lesser extent, on cultural practices that minimize exposure of susceptible scion tissues to the fungus (3) and applications of systemic fungicides to the foliage, trunk, or soil (2,12,14).

The commercial use of a rootstock is contingent on its tolerance of several virus and nematode diseases, degree of cold hardiness, and horticultural field performance, in addition to its resistance to foot rot (7,8,10). The screening program of citrus hybrids for rootstock material at the U.S. Department of Agriculture (USDA) in Orlando, FL, evaluates resistance to *P. parasitica* Dastur as one of the initial screening procedures (7). This method allows large numbers of selections to be screened as young seedlings. Evaluations for blight, tristeza tolerance, cold hardiness, and horticultural performance require plant material in grafted combinations. Since 1960, more than 5,000 citrus hybrids have been screened in the USDA rootstock program in Florida. Approximately 95–98% of these selections were eliminated in the initial screening process because they were rated as more susceptible than Carrizo citrange (*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.), a rootstock with a moderate level of resistance to Phytophthora foot rot.

Citrus tristeza virus, citrus blight (unknown etiology), and diseases vectored by citrus nematodes (*Tylenchulus semipenetrans* Cobb) and burrowing nematodes (*Radopholus citrophilus* Huettel) are more serious diseases that require almost exclusive reliance on host resistance for disease management (7,10). Thus, selection of rootstocks that possess an intermediate level of resistance below that of Carrizo would be desirable if these selections have tolerance to other diseases and/or exceptional horticultural characteristics.

Screening techniques for resistance to *Phytophthora* spp. use high zoospore concentrations for root rot inoculations and mycelial agar disks for foot rot inoculations (1,4,5). The inoculum density of agar disks, however, is not quantifiable and is probably higher than that present under field conditions. Thus, only rootstocks with a high level of resistance to *Phytophthora* are selected, whereas those that possess an intermediate level of resistance may be eliminated before other characteristics can be evaluated.

Chlamydospores serve as the primary overwintering structures of this pathogen and can infect citrus directly with germination hyphae or indirectly by production of sporangia that release motile zoospores (15). Thus, this natural source of inoculum was investigated for screening for resistance to *P. parasitica*.

These experiments were conducted to: 1) demonstrate that chlamydospores are an effective and quantifiable inoculum for causing foot rot; 2) compare effects of mycelial agar disk, zoospore, and chlamydospore-inoculum sources on lesion development in rootstocks that vary in susceptibility to *P. parasitica*; and 3) screen selected common and newer rootstocks for resistance with the three inoculum sources.

MATERIALS AND METHODS

Plant propagation and cultivar selection. Plants were grown in a soilless medium in the greenhouse at soil temperatures ranging from 22 to 28 C and ambient temperatures from 24 to 32 C. Six separate experiments were conducted. Plants in experiments 1–3 were approximately 2 yr old, whereas those in experiments 4–6 were 6–8 mo old.

P. trifoliata 'Large Flower' and sweet orange (*C. sinensis* 'Thomson navel') were used in experiments 1 and 2, and Carrizo, *C. macrophylla* P. J. Wester, and sour orange (*C. aurantium* L.) were used in experiment 3. Carrizo, *C. macrophylla*, sour orange, rough lemon (*C. limon* (L.) N. L. Burm. '86-138'), and sweet orange cv. Ridge Pineapple were used in experiment 4. Experiment 5 used Carrizo; sour orange; rough lemon cv. 86-138; mandarin orange (*C. reticulata* Blanco 'Cleopatra'); and sweet orange cvs. Bedmar Vernia, Koethen, Madam Vinous, Natal, Ridge Pineapple, Ruffert, Sanguine Grosse Ronde, Sweet Seedling, Thomson navel, and Valencia. Carrizo, sour orange, mandarin orange cvs. Cleopatra and Sun Chu Sha Ka, rough lemon cvs. 8166 and Vangasay, and *P. trifoliata* hybrids Swingle (*P. trifoliata* × *C. paradisi* Macfady.) and HRS-812 (*P. trifoliata* × *C. reticulata*) were used in experiment 6.

Inoculum culture and preparation. Two pathogenic isolates of *P. parasitica* mating type A2 were used in all studies. Isolate 1275 was obtained from the

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USDA, Agricultural Research Service, Horticultural Research Laboratory collection in Orlando, FL, and isolate 201 was obtained from a foot rot lesion on a 3-yr-old rough lemon growing in St. Cloud, FL.

Mycelial agar disk inocula were obtained from the edge of 7- to 10-day-old *P. parasitica* cultures grown on carrot agar (5% finely grated carrots, 1.8% Bacto agar, w/w) in the dark at 25 C. Disks were cut from the carrot agar with a No. 3 (0.5-cm-diameter) cork borer.

Zoospores were produced by the method of Henderson et al (6). Zoospore densities were estimated with a hemacytometer and the suspensions were diluted in deionized water to obtain the inoculum level desired in each experiment. Inoculum suspensions were serially plated on a selective medium (PARPH) (containing 10 mg of pimaricin, 250 mg of ampicillin, 10 mg of rifampicin, 100 mg of pentachloronitrobenzene, 50 mg of hymexazol, and 17 g of Difco cornmeal agar in 1 L of deionized water) to determine viable propagule densities (17,18).

Chlamydospores of *P. parasitica*, produced in vitro by the method of Tsao (16), were extracted from mycelial mats by comminuting mycelia for 2 min in an Omni-mixer (Omni International, Waterbury, CT), followed by manual maceration in a tissue grinder and sonication for 2 min. The suspension was added to citrus soil that had been oven-dried at 70 C for 48 hr and manually incorporated to evenly distribute the inocula. Infested soil was air-dried to 4% soil moisture and stored in the dark at 25 C. The infested soil was plated on PARPH selective medium 72 hr before each experiment to quantify propagule density. All fungal colonies that were observed growing 24–36 hr after plating originated from chlamydospores when viewed under a dissecting microscope at ×40. Weighed quantities of chlamydospore-infested (CI) soil containing the desired inoculum dose were evenly spread in a 95 × 30 × 3 mm clear rectangular plastic dish and infused with 2% cornmeal agar cooled to 43 C. The solidified soil agar was cut into 1.0 × 0.5 cm blocks in experiments 1 and 2 and 0.5 × 0.5 cm blocks in experiments 3–6. Randomly selected soil-agar blocks were ground up and plated on PARPH selective medium to determine mean propagule density per block. Controls consisted of agar blocks prepared with sterilized soil.

Inoculation procedures. In experiments 1 and 2, plant material consisted of Thomson navel orange scion/*P. trifoliata* rootstocks grown in 7.5-L pots. Stems of the scion and rootstock were mechanically wounded 2–3 cm above the budunion and soil level, respectively, by cutting the bark with a scalpel to the secondary xylem wood to make a 0.5 × 1.5 cm long inverted "V". Soil-agar blocks were placed between the cut bark and wood tissue, the stem was wrapped with premoistened absorbent cotton, and the cotton was wrapped with Parafilm (American Can Co., Greenwich, CT) to secure it to the stem and maintain a moist environment. The inoculation site was covered by enclosing the scion and rootstock stems with a 1-L plastic cup and filling it with wet vermiculite.

In experiments 3–6, unbudded plant material was used. Inverted "V" cuts measuring 0.7 cm long on each side were made on opposite sides of the stem at the soil line and a 0.5 × 0.5 cm soil-agar block or mycelial agar disk was inserted in the wounded area. The inoculation sites were covered with steam-pasteurized soil.

Zoospore inoculations in experiments 1–4 were accomplished by pipetting 2 ml of the zoospore suspension into the premoistened cotton after the cotton was wrapped around the wounded stem. The wet cotton was then wrapped with Parafilm. Zoospore inoculation was modified in experiments 5 and 6. A precut waxed medicine cup was wrapped around the wounded stem area in the shape of a funnel and secured and sealed from leaks with Parafilm. One milliliter of zoospore suspension was pipetted into the funnel and incubated for 1 hr before the funnel was wrapped with premoistened cotton. The inoculation site was covered with steam-pasteurized citrus soil and watered daily.

Data collection and statistical analysis. The extent of lesion development on stems was visually rated 8 wk after inoculation. In experiments 1 and 2, ratings were based on a scale of 1–5 as described by Grimm and Hutchison (5). Lesion ratings in other experiments were based on a pretransformed rating scale of 0–5 (Table 1) (11). Lesion-rating values were assigned based on the percentage of girdling of a 2-cm length of stem, amount of callus tissue, and vertical extension of lesions. In experiments 3–6, lesion area (millimeters squared) was estimated with a LI-3100A area meter (LI-COR, Inc., Lincoln, NE) or by measuring lesion extension and width. Relative lesion area was calculated as the ratio of lesion area divided by the stem area of the 2-cm stem section to remove the effects of differential growth rates and vigor between rootstocks (13).

Plants were arranged in randomized complete blocks, except in experiments 5 and 6 where plants were completely randomized. There were five replicates per inoculation method for each species or cultivar tested, except in experiment 4 where there were four to six replicates depending on availability of plant material. Data were subjected to analysis of variance. Controls were removed before data analysis. Pretransformed rating values are presented as weighted, mean-percentage stem girdling obtained from angular transformation tables of percentages to degrees (11). Differences among the three levels of CI soil in experiments 1 and 2 and rootstocks in experiments 5 and 6 were determined with Waller-Duncan's k-ratio t test. In experiments 3–6, inoculation treatment main effect sum of squares were partitioned into single degree of freedom components to determine differences between agar disk, zoospore, and CI soil inoculation methods by orthogonal contrasts.

RESULTS

Chlamydospore-infested soil-agar blocks were effective inocula in causing foot rot lesions on susceptible Thompson navel scion (Table 2). Stem lesion ratings were increased significantly with each increase in CI soil propagule density of one, nine, and 32 colonies per soil-agar block. Foot rot lesions did not develop on the resistant *P. trifoliata* rootstock regardless of propagule density in CI soil. *P. parasitica* was reisolated from lesioned tissue plated on PARPH selective medium.

Foot rot lesion development was significantly greater on rootstooks inoculated with mycelial agar disks, compared with zoospore suspensions and CI soil-agar blocks, and significantly greater when plants were inoculated with CI soil-agar blocks compared with zoospore suspensions (Table 3). In experiments 5 and 6, mean lesion area on plants inoculated with mycelial agar disks ranged from 3.0 to 3.3 times greater than plants inoculated with the highest densities of

Table 1. Comparison of disease ratings scales for *Phytophtora parasitica* stem lesions on citrus

Lesion rating	Area with lesions (%)[y]	
	Grimm and Hutchison scale (5)	Pretransformed scale
NA/0[z]	NA	0
1	0	10
2	<25	35
3	25–50	65
4	50–90	90
5	100	100

[y] Weighted mean percentage area with lesions area calculated by multiplying untransformed mean lesion rating by 18 degrees and converting the angular transformation of degrees to percentages (11).

[z] NA = Not applicable and refers to Grimm and Hutchison scale; 0 refers to pretransformed scale.

zoospores and chlamydospores. Similar results were obtained in experiments 3 and 4.

The modified method of zoospore inoculation used in experiments 5 and 6 allowed a more sensitive determination of inoculum density effects on lesion development. Infections were erratic, and there were no significant differences in lesion development on plants inoculated in experiments 3 and 4 with varying large quantities of zoospores; consequently, data are not presented. Plants inoculated via the modified method with 1,000 zoospores had significantly greater lesion development ($P = 0.05$) compared with plants inoculated with 100 zoospores. On the other hand, lesion development on plants inoculated with chlamydospores was not affected by inoculum densities ranging from 14 to 312 propagules. Lesion size (lesion area, relative lesion area) was similar on plants inoculated with 1,000 zoospores or 14–200 chlamydospores (Table 3).

Commercial and newly released rootstocks and several sweet orange cultivars were screened for resistance to *P. parasitica* with the three inoculum sources and rated susceptible to resistant based on weighted mean percentage stem girdling (Table 4). Susceptibility ratings of sweet orange cultivars screened in experiment 5 have been published (13). Rootstock by inoculum source interactions were significant because of the use of susceptible sweet orange cultivars and resistant Carrizo and Swingle rootstocks, plus the differential response of the screened material to the three inoculum sources.

Assignment of resistance in many rootstocks to *P. parasitica* was based on ratings from plants inoculated with zoospores and chlamydospores because all rootstocks would have been rated susceptible based on mycelial agar disk inoculations (Table 4). For example, the resistant rootstock Swingle had 97% stem girdling on plants inoculated with mycelial agar disks, whereas zoospore and chlamydospore inoculations produced only 29 and 16% stem girdling, respectively. The three *C. limon* cultivars, 86-138, 8166, and Vancasay, averaged 100% stem girdling with mycelial agar disk inoculations, but only 75 and 54% stem girdling with zoospore and chlamydospore inoculations. Cultivar 86-138 was rated more susceptible than 8166 and Vancasay because of significantly greater stem girdling on plants inoculated with zoospores and chlamydospores.

DISCUSSION

We compared the use of soil infested with varying densities of chlamydospores of *P. parasitica* with zoospore suspensions and mycelial agar disks to screen citrus germ plasm for resistance to foot rot. To facilitate inoculation of stem wounds and quantification of chlamydospores, the infested soil inoculum was prepared as a solidified soil-agar matrix. The inoculum potential can be quantified more accurately with chlamydospores than with traditional inoculum sources (zoospore suspensions or mycelial agar disks). Also, large quantities of genetically uniform inocula can be prepared and stored for an extended time with little or no change in pathogenicity.

The use of CI soil was an effective and consistent inoculum source in screening citrus rootstocks for resistance to Phytophthora foot rot. The extent of lesion development, however, generally did not depend on the range of chlamydospore inoculum densities used under these experimental conditions. Significant inoculum density effects were obtained with the modified zoospore inoculation method. Future screening methods should consider inoculum source and density when assessing susceptibility of rootstocks with unknown reaction to *Phytophthora*.

The use of mycelial agar disks for greenhouse screening of foot rot resistance in young plant material is a severe test because of high inoculum density. Commercial rootstocks that have field tolerance of foot rot were rated susceptible when young plants were inoculated with mycelial agar disks but not with CI soil or zoospores. For example, rough lemon has been shown to be highly variable in its resistance to foot rot (21). In our study, both the Florida and South African selections were rated susceptible when mycelial agar disks were the inoculum source. Some selections of rough lemon, including the South African selection 8166, had previously been reported resistant to foot rot (21). Resistance was observed in cv. 8166 when CI soil and zoospores were the inoculum sources.

The susceptibility ratings of commercial rootstocks were in general accordance with field performance of these rootstocks. Although Carrizo and sour orange had extensive lesion development when mycelial agar blocks were the inoculum source, Carrizo consistently had less lesion development than sour orange in our screening experiments.

Table 2. Effect of three inoculum densities of chlamydospore-infested (CI) soil on foot rot stem lesion ratings of *Citrus sinensis* 'Thompson navel' and *Poncirus trifoliata* 'Large Flower'

Treatment (g of CI soil)	Mean inoculum density (colonies)	Stem lesion rating[y]	
		C. sinensis	*P. trifoliata*
0.01	1	1.05 a[z]	1.00 a
0.10	9	1.90 b	1.15 a
0.50	32	2.60 c	1.05 a

[y] Based on the Grimm and Hutchison scale (5). Means of 10 plants from experiments 1 and 2 combined.

[z] Means within columns not significantly different according to Waller-Duncan's k-ratio t test ($P = 0.05$).

Table 3. Effect of *Phytophthora parasitica* inoculum source on weighted mean percentage stem girdling, lesion area, and relative lesion area on citrus[v]

Treatment[w]	Experiment 5				Experiment 6			
	Mean inoculum density	Stem girdling[x]	Lesion area (mm^2)	Relative lesion area[y]	Mean inoculum density	Stem girdling[x]	Lesion area (mm^2)	Relative lesion area[y]
Agar disk	0.5 cm	86	703	1.904	0.5 cm	100	453	1.732
Zoospore 1×	100/ml	32	129	0.373	100/ml	11	2	0.007
Zoospore 10×	1,000/ml	71	237	0.708	1,000/ml	69	139	0.527
CI soil 1×	20	78	307	0.830	14	42	106	0.396
CI soil 5×	100	80	261	0.763	NT[z]	NT	NT	NT
CI soil 10×	200	86	297	0.837	140	73	137	0.512

[v] Values are means of all rootstocks used in each experiment (experiment 5, n = 70; experiment 6, n = 40).

[w] Orthogonal contrasts: Agar disk vs. zoospore and CI soil; zoospore vs. CI soil significant throughout at $P = 0.001$.

[x] Weighted mean percentage calculated by multiplying mean stem lesion ratings by 18 and converting the angular transformation of degrees to percentages.

[y] Lesion area divided by area of a 2-cm section of stem.

[z] No treatment.

Table 4. Effect of *Phytophthora parasitica* inoculum source on mean percentage stem girdling of citrus cultivars with varying levels of resistance to Phytophthora foot rot[w]

Citrus cultivar	Mean percentage stem girdling[x]			
	Inoculum source			
	Mycelial agar	Zoospore	Chlamydospore	Rating[y]
C. reticulata				
Cleopatra	100 a[z]	63 c	99 a	MS
Sun Chu Sha Ka	100 a	32 d	50 cd	MR
C. limon				
86-138	100 a	97 ab	71 bc	S
8166	100 a	71 c	38 de	MR
Vancasay	98 a	56 cd	53 cd	MR
P. trifoliata hybrids				
HRS-812	100 a	68 c	35 de	MR
Swingle	97 b	29 d	16 e	R
Carrizo	100 a	79 bc	53 cd	MR
C. aurantium				
sour orange	100 a	98 a	89 ab	MS

[w] Experiment 6 only. Values are means of five replicates.
[x] Calculated by multiplying mean stem lesion ratings by 18 and converting the angular transformation of degrees to percentages.
[y] S = Susceptibile, MS = moderately susceptible, MR = moderately resistant, and R = resistant.
[z] Means within columns followed by the same letter not significantly different according to Waller-Duncan's k-ratio t test ($P = 0.05$).

These are widely used rootstocks with field tolerance of Phytophthora foot rot (3,4,20). In California, foot rot lesions on sweet orange scions budded on Carrizo rootstocks have not been observed to progress through the rootstock (J. Menge, *personal communication*), whereas in Texas, lesions occur on the sour orange rootstock and advance to the scion under conditions favorable for disease development (G. Smith, *personal observation*).

The usefulness of greenhouse tests with 6- to 8-mo-old seedlings to evaluate susceptibility to foot rot has been questioned because of the similar rankings of susceptible and resistant rootstocks (20). In those experiments, zoospore concentrations were substantially higher than in our experiments, ranging from 25 to 50×10^3 zoospores per plant in watertight collars. These high zoospore densities could account for the failure to discriminate between resistant and susceptible cultivars.

Phytophthora resistance in citrus is controlled by multiple genes with complex dominance (7). The moderate to high levels of resistance in Swingle, Carrizo, and HRS-812 have been inherited from *P. trifoliata*, a major germ plasm source for resistance to *Phytophthora*. Other factors contribute to resistance or tolerance of foot rot (7,13,19,20). Rootstock vigor and root and callus tissue formation appear to be important factors in the foot rot tolerance of certain rootstocks, particularly rough lemon. The extent of bark and periderm formation may influence the formation of growth cracks in the bark that expose cambial parenchymatous tissue to natural infection. Our results demonstrate that inoculum density may affect the susceptibility rating of a rootstock.

The success of screening experiments depends on frequent watering and covering the wounded stem with soil to prevent the drying of inoculated and infected tissues and to maintain conditions favorable for disease development. The use of watertight funnels around the stem for zoospore inoculations was essential in our tests to consistently obtain lesions. The modified zoospore inoculation method used in experiments 5 and 6 allowed submersion of wounded tissue in zoospore-infested water for 1 hr. This resulted in greater lesion development with much lower inoculum densities compared with the previous method used in experiments 3 and 4, where larger numbers of zoospores (5,000–20,000) were pipetted onto premoistened cotton. The screening of young plants in greenhouses will continue to be the primary method to evaluate resistance to foot rot until assays based on tissue or cellular reaction to pathogen toxins or elicitors are developed or phytoalexin-type compounds produced in resistant reactions are identified.

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