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GROWTH COMPARISON OF CITRUS ROOTSTOCKS AFTER ARTIFICIAL INFECTION WITH *PHYTOPHTHORA*

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Abstract. ***Phytophthora* is one of the most economically important soil-borne pathogens affecting Florida's citrus industry. The most effective control in the field is based on the use of tolerant rootstocks in connection with sound management strategies. This study describes the rapid greenhouse evaluation of seven rootstocks for tolerance to *Phytophthora* root diseases. Seedlings of the rootstocks Sun Chu Sha, Cleopatra mandarin, US-897, Sour orange, 'Pineapple' sweet orange, Carrizo citrange, and Swingle citrumelo were inoculated with citrus roots derived from a field site known to be heavily infested with *Phytophthora* and maintained in tubs containing a commercially available potting mix. The experiment was terminated after four weeks and shoot length, shoot mass, and root mass were determined. Infection with *Phytophthora* was quantified using enzyme-linked immunosorbent assays (ELISA), and species were identified by restriction fragment length polymorphism analysis in combination with polymerase chain reaction (PCR). Shoot length, root mass and shoot mass were significantly reduced in Carrizo, 'Pineapple', and Swingle, exhibiting overall reductions of 44%, 48%, and 50%, respectively, whereas total**

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reductions observed for Sun Chu Sha and Cleopatra were less than 25%. Values for Sour Orange and US-897 ranged in between the two groups. Results obtained in this study are in accordance with results obtained from field trials and provide valuable information to aid in the development of new citrus rootstocks that are tolerant to *Phytophthora*.

Phytophthora species cause some of the most serious soilborne diseases of numerous economically important crops and woody plants worldwide (Erwin and Ribeiro, 1996). Although commonly referred to as fungi, *Phytophthora* and other oomycetes such as *Pythium* differ from true fungi in various morphological, biochemical and molecular characteristics and are more closely related to the heterokont algae (Cavalier-Smith, 1986; Erwin and Ribeiro, 1996).

The most important species posing a severe threat to Florida's citrus industry are *Phytophthora citrophthora* Smith & Smith, *Phytophthora nicotianae* Breda de Haan (syn. *P. parasitica* Dastur) and *Phytophthora palmivora* Butler. *P. citrophthora* mainly causes brown rot of fruits and outbreaks are usually limited to conditions with intermittent periods of prolonged rainfall (Graham et al., 1998). *P. nicotianae* has long been regarded the major agent causing foot rot and root rot in citrus orchards and nurseries and rootstocks such as Swingle citrumelo, and the trifoliate orange (*Poncirus trifoliata* (L.) Raf.) were generally considered as tolerant towards this pathogen. In contrast, rootstocks such as Cleopatra mandarin and Sour orange that do not have trifoliate orange parentage, exhibited a higher susceptibility to *P. nicotianae*. It is now known that *P. palmivora* is a far more aggressive and competitive pathogen of citrus roots and fruit than *P. nicotianae* (Graham et al., 1998; Zitko and Timmer, 1994). Rootstocks previously considered tolerant to *Phytophthora* diseases have now proven to be highly susceptible in the presence of this pathogen (Graham et al., 2003). Some rootstocks susceptible to *P. nicotianae* on the other hand display a greater resistance towards *P. palmivora*.

While *Phytophthora* diseases affecting above-ground plant parts like leaves, stems or fruit are easily detected and diagnosed, root diseases occurring below-ground are less visible and may not be detected until many years after infection (Tsao, 1990). The most effective disease control in the field is based on the use of a tolerant rootstock in combination with proper management. Methods for the rapid testing of promising rootstock candidates for resistance to *Phytophthora* and other diseases have already been employed and are continuously refined at the U.S. Horticultural Research Laboratory (Bowman and Garnsey, 2001; Bowman et al., 2001, 2002). The present study describes the greenhouse evaluation of seven citrus rootstocks in response to *Phytophthora* root infection after artificial inoculation of soil with infected root material derived from citrus trees growing at a local field site. Different growth parameters were measured and infection rate was determined with immunological methods. Differences between rootstocks are discussed and results evaluated in regard to the present knowledge of rootstock performance.

Materials and Methods

For inoculated treatments, citrus roots from Swingle citrumelo (*Citrus paradisi* × *Poncirus trifoliata*) rootstocks were collected from the soil under the canopy of trees, located at the Florida Research Center, Vero Beach (Indian River County), at a site determined by survey to be heavily infested with *P. palmivora* and *P. nicotianae* (J. Graham, University of Florida, personal communication). Roots were washed to remove soil and insects and kept moist until use. For uninoculated treatments, citrus roots derived from healthy Swingle citrumelo rootstocks growing under controlled conditions in the USHRL greenhouses were collected, washed and kept moist until use.

A commercially available potting mix composed of steam-sterilized peat/perlite/vermiculite (Pro-Mix BX, Premier Horticulture, Inc., Red Hill, Pa.) was filled into 63 cm × 40 cm × 22 cm drained plastic tubs to a depth of 10 cm. A mixture of 360 g of roots, collected from the field or from the greenhouse as described above and ranging from fibrous roots to roots with a diameter of 1 cm, was layered on top of the soil and covered with potting mix to a final depth of 18 cm. Thirteen week-old seedlings of seven rootstock genotypes (Table 1) were planted into tubs, with three randomized replicates for each rootstock in each tub. Three tubs were prepared for each treatment, resulting in a total of nine replicates for each rootstock for each of the two treatments. Tubs were placed into non-drained 75 cm × 50 cm × 15 cm plastic trays and were arranged in a completely randomized manner on the greenhouse benches at the USHRL laboratory. Plants were grown under natural light conditions with a maximum photosynthetic photon flux (PPF) of 1200-1300 µmol s^{-1} m^{-2}. Soil was kept saturated by maintaining the water at a level of one inch above the bottom of the tub. Plants were irrigated by addition of a water-soluble fertilizer mix, 20N-10P-20K (Peters Professional, The Scotts Company, Marysville, Ohio) once every week, applied with a proportioner at a rate of 500 mg $liter^{-1}$ N. Before each fertilizer application, water was allowed to drain from the tubs. Four weeks after initiating the experiment, length of shoots above soil level was determined and plants were extracted from the soil. Roots were washed thoroughly with tap water to remove adhering soil particles and blotted dry. Fresh weight of shoots and roots was noted and roots were stored at -80 °C until used for DNA extraction and enzyme-linked immunosorbent assays (ELISA).

Roots from each plant were ground in liquid nitrogen with a mortar and pestle. To identify *Phytophthora* species in infected roots, DNA was extracted from 100 mg of ground-up tissue using the Plant DNeasy Mini Kit (Quiagen, Valencia, Calif.) according to the manufacturer's instructions. Root material was combined from all three plants of each rootstock within each tub, resulting in one composite sample of each rootstock per tub for DNA extraction. Species identification was performed using polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP) of ribosomal DNA genes as described by Cooke and Duncan (1997), Cooke et al. (2000) and Grote et al. (2002).

For the quantification of *Phytophthora* infection with ELISA a multiwell-test system (Agdia Incorporated, Elkhart,

Table 1. Plant material used in this study.

Rootstock	Parentage
Swingle citrumelo	*Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.
Carrizo citrange	*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata*
US-897	*Citrus reticulata* 'Cleopatra' × *Poncirus trifoliata*
Pineapple sweet orange	*Citrus sinensis*
Sour orange	*Citrus aurantium* (L.)
Cleopatra mandarin	*Citrus reticulata* Blanco
Sun Chu Cha	*Citrus reticulata*

Ind.) using a polyclonal anti-*Phytophthora* antibody and a monoclonal alkaline phosphatase-conjugated secondary antibody was used. For each reaction, 1 mL of extraction buffer was added to 100 mg of ground-up root tissue. Each plant was analyzed individually. Samples were mixed vigorously, incubated at room temperature for five minutes and centrifuged in an Eppendorf microcentrifuge 5417 C at 20800 g_n for five minutes. One hundred microliters of supernatant was used for each well; all samples were tested in duplicate. Positive controls were prepared as described above using freeze-dried mycelia of *P. palmivora* (isolate P99-59-1) and included in each assay. Linearity of the test system and detection threshold was determined using tenfold serial dilutions of extracts prepared from freeze-dried fungal tissue of the same isolate. All assays were performed according to the manufacturer's instructions. Absorbance values were measured at 405 nm using a Spectra Max Pro 190 Microplate Spectrophotometer (Molecular Devices Corporation, Sunnyvale, Calif.) in combination with the software Soft Max Pro, Version 2.6.

All data were tested by analysis of variance using Statistica version 6.0 (StatSoft, Tulsa, Okla.). Students-Newman-Keuls (SNK) test was used for mean comparison when the F-test was significant at $P < 0.05$.

Results and Discussion

Four weeks after initiating the experiment shoot length of uninfected plants ranged from 26 cm to 48 cm, while shoot length of citrus seedlings growing in tubs inoculated with *Phytophthora* was significantly reduced by an average of 33% (Table 2). Percent reduction of shoot growth varied significantly between rootstocks and was most pronounced in Swingle and 'Pineapple', with reductions exceeding 40% (Fig. 1A). Reduction of shoot length was smallest in Sun Chu Sha and Cleopatra with 21% and 27%, respectively. Similar results were obtained when comparing the fresh weight of shoots of healthy and infected plants. The weight of shoots of healthy plants ranged from 5.4 g for Sun Chu Sha to almost 13 g for 'Pineapple' with an average of 8.5 g (Table 2). Shoot weight of infected plants was only 5.2 g on average and thus reduced by 39%. Reduction of shoot weight was significantly different between rootstocks (Fig. 1B), with smallest reductions of 25% and 27% observed in Sun Chu Sha and Cleopatra, respectively, and largest reductions of 46% and 50% observed in 'Pineapple' and Swingle, respectively. Other than the visible reduction in shoot growth no symptoms of decline were observed in any of the infected plants.

The fresh weight of roots of healthy plants ranged from 3 g to 6 g with an average of 4.5 g as opposed to infected plants which had an average root mass of only 2.5 g (Table 2). Percent reduction of root weight overall was 44%, with the rootstocks forming two significantly different groups (Fig. 1C). Root mass was reduced by more than 56% in 'Pineapple', Carrizo and Swingle, whereas percent reductions observed for US-897, Sour orange, Cleopatra and Sun Chu Sha ranged from 21% to 36%.

Molecular tools have shown to be very accurate and highly sensitive in identifying and detecting *Phytophthora* and other pathogenic oomycetes (Cohen et al., 2003; Cooke et al., 2000; Kageyama et al., 2003) and are now frequently used as an alternative to traditional detection methods based on baiting and selective agar plating. According to results obtained with polymerase chain reaction and analysis of restriction fragment patterns, roots of all diseased plants in this study were predominantly infected with *Phytophthora palmivora*. *P. nicotianae* was detected in less than half of the samples from infected plants. No *Phytophthora* was detected in any of the samples derived from healthy roots.

Based on the results obtained from different growth parameters of seedlings above- and below-ground, Sun Chu Sha and Cleopatra mandarin exhibited the best performance and may be categorized as tolerant or possibly resistant to *Phytophthora palmivora*. The high variability of Cleopatra seedlings in regard to root loss may be attributed to the simultaneous, but moderate infection of roots with *P. nicotianae* detected by PCR analysis. As has been shown by Graham et al. (2003) and other studies performed in this laboratory (data not shown) Cleopatra is notably more susceptible to this species when compared with *P. palmivora*. Performance of 'Pineapple' sweet orange, Carrizo citrange and Swingle citrumelo was significantly inferior regarding all growth parameters analyzed in this study. Consequently, these rootstocks must be classified as susceptible to *P. palmivora*. Rootstocks US-897 and Sour orange may be regarded as moderately tolerant, since growth reductions ranked in between the two groups. These results are mostly in agreement with field observations and results from testing with *Phytophthora* spp. in combination with heavy flatwood soils (Bowman et al., 2002, 2003).

The superior performance of Sun Chu Sha, Cleopatra, Sour orange and US-897 under the conditions used in this study is further expressed by their ability to maintain a functional equilibrium between above-ground and below-ground biomass as expressed by their root to shoot values (Table 3). When log-transformed, a strong linear relationship exists be-

Table 2. Mean length of shoots, mean fresh weight of shoots and mean fresh weight of roots of uninfected and infected plants four weeks after inoculation with *Phytophthora*. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$.

	Shoot length (cm)			Shoot weight (g)			Root weight (g)		
Rootstock	Not infected	Infected	*P*	Not infected	Infected	*P*	Not infected	Infected	*P*
Sun Chu Cha	25.83	20.50	*	5.37	4.00	*	3.27	2.79	ns
Cleopatra mandarin	33.78	24.50	***	6.47	4.72	**	3.21	2.54	ns
US-897	47.72	31.94	***	7.45	4.69	***	2.95	2.01	***
Sour orange	30.17	21.17	***	10.75	6.89	***	5.71	3.63	**
Pineapple sweet orange	40.89	24.22	***	12.59	6.77	***	5.96	2.62	***
Carrizo citrange	39.11	25.00	***	7.48	4.74	***	4.70	1.98	***
Swingle citrumelo	38.33	22.94	***	9.44	4.75	***	5.54	2.16	***
Mean	36.55	24.33	***	8.51	5.22	***	4.48	2.53	***

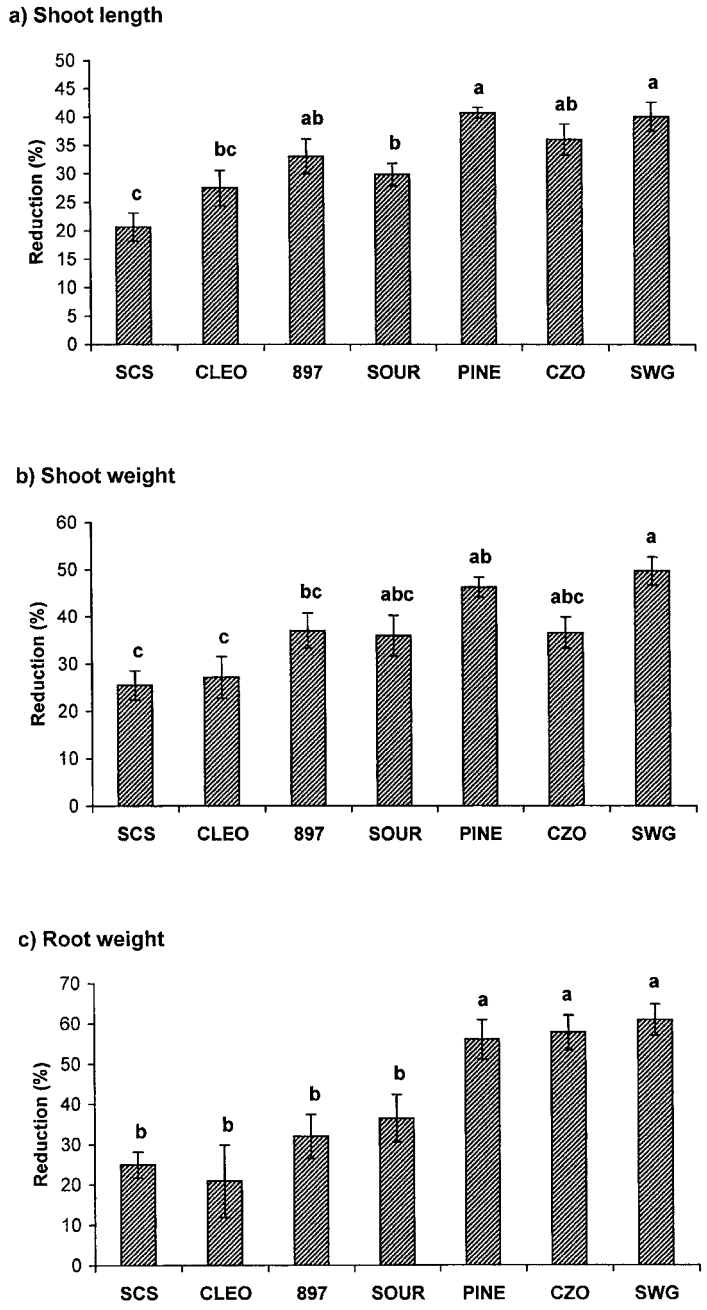

Fig. 1. Percent reduction of a) shoot length, b) shoot weight and c) root weight of infected plants four weeks after inoculation with *Phytophthora*. SCS, Sun Chu Sha; CLEO, Cleopatra mandarin; 897, US-897; SOUR, Sour orange; PINE, 'Pineapple' sweet orange; CZO, Carrizo citrange; SWG, Swingle citrumelo. Vertical bars represent standard errors. Different letters indicate significant differences between means according to the Student-Newman-Keuls test for $P < 0.05$.

tween root mass and shoot mass and changes only occur in response to drastic changes in development or environmental conditions (Poorter and Nagel, 2000).While root to shoot values were not significantly altered after infection in Sun Chu Sha, Cleopatra, Sour orange and US-897, values significantly decreased in 'Pineapple', Carrizo and Swingle. Higher tolerance of plants towards the pathogen may thus be correlated with an allocation of resources from above-ground to below-ground, necessary for defense and the preservation of an intact root system.

Table 3. Root to shoot values of uninfected and infected plants four weeks after inoculation with *Phytophthora*. R, fresh weight of roots; S, fresh weight of shoots. ***, $P < 0.001$; **, $P < 0.01$; ns, $P > 0.05$.

	Root to shoot value (logR/logS)		
Rootstock	Not infected	Infected	*P*
Sun Chu Sha	0.69	0.74	ns
Cleopatra mandarin	0.63	0.55	ns
US-897	0.53	0.42	ns
Sour orange	0.72	0.65	ns
Pineapple sweet orange	0.70	0.48	**
Carrizo citrange	0.76	0.42	***
Swingle citrumelo	0.76	0.46	***
Mean	0.68	0.53	***

In order to quantify activity of *Phytophthora* in infected roots, enzyme-linked immunosorbent assays (ELISA) were performed for each individual plant. The linearity of the system was confirmed by comparing known amounts of the pathogen with absorbance values at 405 nm and determined to fall within values of 0.2 to 1.8, equivalent to 1 ng to 100 ng of freeze-dried fungal tissue. Average ELISA values obtained from roots of non inoculated, healthy plants were zero, as opposed to 0.91 in infected plants. Absorbance values varied significantly between the rootstocks (Fig. 2) with lowest values from 0.46 to 0.78 observed in roots of Sun Chu Sha, Sour orange and Cleopatra mandarin. Highest values between 0.91 and 1.21 were obtained for 'Pineapple', Swingle, US-897 and Carrizo. Assuming that absorbance values are positively correlated with the degree of infection, as has been shown by Miller et al. (1997) and Timmer et al. (1993), infection levels of roots from the latter are thus up to three times higher than those of roots from Sun Chu Sha. These results support the main findings obtained from growth characteristics described above, but are unexpected in regard to US-897. Despite the only moderate root loss observed in plants from this rootstock, infection levels estimated with ELISA were very high and in the same range than those of the susceptible rootstocks Swingle and Carrizo. According to Graham (1995) tolerance of a rootstock is not always correlated with lower levels of absorbance values in the initial stage of infection. Also, immunological methods detect pathogens even when they are

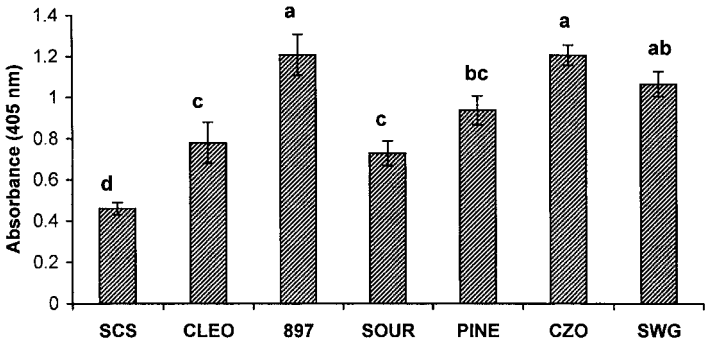

Fig. 2. Quantification of *Phytophthora* in roots of infected plants with enzyme-linked immunosorbent assays (ELISA) four weeks after inoculation. Mean values are expressed as absorbance at 405 nm. SCS, Sun Chu Sha; CLEO, Cleopatra mandarin; 897, US-897; SOUR, Sour orange; PINE, 'Pineapple' sweet orange; CZO, Carrizo citrange; SWG, Swingle citrumelo. Vertical bars represent standard errors. Different letters indicate significant differences between means according to the Student-Newman-Keuls test for $P < 0.05$.

not viable (Miller et al., 1997) and may not reflect an active infection of the plant tissue. High absorbance values obtained for roots from US-897 may thus be the result of an initially high infection rate, overcome by the release of antifungal or other defense-related substances at a later stage of infection.

Graham (1990, 1995) defined tolerance of a rootstock as the condition in which plants are infected but show a greater capacity of regenerating roots or limit spread of the infection by other means. Previous experiments performed in this laboratory (data not shown) demonstrated that the growth rate of roots varies between rootstocks but is not higher in the better performing rootstocks Sun Chu Sha and Cleopatra under the present conditions. These results, together with results from growth data and ELISA, indicate that Sun Chu Sha and Cleopatra may achieve their tolerance towards *P. palmivora* by limiting the spread of infection structurally or biochemically, rather than by regenerating roots.

In summary, results obtained in this study identify Swingle citrumelo, Carrizo citrange, and 'Pineapple' sweet orange as susceptible, US-897 and Sour orange as moderately tolerant, and Sun Chu Sha and Cleopatra mandarin as tolerant towards root infection caused by *Phytophthora palmivora* and are in agreement with field observations and previous greenhouse studies. In dual inoculations of citrus with *P. palmivora* and *P. nicotianae*, *P. palmivora* appeared to be the dominant pathogen. Growth data obtained from studies performed in the greenhouse thus provide a valuable source of information to aid in the development of new citrus rootstocks with tolerance to *Phytophthora* before establishing long-term trials in the field.

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Literature Cited

Bowman, K. D. and S. M. Garnsey. 2001. A comparison of five sour orange rootstocks and their response to citrus tristeza virus. Proc. Fla. State Hort. Soc. 114:73-77.

Bowman, K. D., J. P. Shapiro, and S. L. Lapointe. 2001. Sources of resistance to Diaprepes weevil in subfamily Aurantiodeae, Rutacea. HortScience 36:332-336.

Bowman, K. D., J. P. Albano, and J. H. Graham. 2002. Greenhouse testing of rootstocks for resistance to *Phytophthora* species in flatwoods soil. Proc. Fla. State Hort. Soc. 115:10-13.

Bowman, K. D., J. H. Graham, and R. C. Adair, Jr. 2003. Young tree growth in a flatwoods rootstock trial with Diaprepes weevil and *Phytophthora* diseases. Proc. Fla. State Hort. Soc. 116:249-251.

Cavalier-Smith, T. A. 1986. The Kingdom Chromista: Origin and systematics, p. 309-347. In: F. E. Round and D. J. Chapman (eds.), Progress on Phycological Research.Vol. 4. Biopress, Bristol.

Cohen, S., V. Allasia, P. Venard, S. Notter, C. Vernière, and F. Panabières. 2003. Intraspecific variation in *Phytophthora citrophthora* from citrus trees in eastern Corsica. Europ. J. Plant Pathol. 109:791-805.

Cooke, D. E. L. and J. M. Duncan. 1997. Phylogenetic analysis of *Phytophthora* species based on the ITS1 and ITS2 sequences of ribosomal DNA. Mycol. Res. 101:667-677.

Cooke, D. E. L., A. Drenth, J. M. Duncan, G. Wagels, and C. M. Brasier. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. Fungal Genetics and Biology 30:17-23.

Erwin, D. C. and O. K. Ribeiro. 1996. *Phytophthora* Diseases Worldwide. APS Press, Amer. Phytopathol. Soc., St. Paul.

Graham, J. T. 1990. Evaluation of tolerance of citrus rootstocks to *Phytophthora* root rot in chlamydospore-infested soil. Plant Dis. 74:743-746.

Graham, J. H. 1995. Root regeneration and tolerance of citrus rootstocks to root rot caused by *Phytophthora nicotianae*. Phytopathology 85:111-117.

Graham, J. H., L. W Timmer, D. L. Drouillard, and T. L. Peever. 1998. Characterization of *Phytophthora* spp., causing outbreaks of citrus brown rot in Florida. Phytopathology 88:724-729.

Graham, J. H., D. B. Bright, and C. W. McCoy. 2003. *Phytophthora-Diaprepes* weevil complex: *Phytophthora* spp. relationship with citrus rootstocks. Plant Dis. 87:85-90.

Grote, D., A. Olmos, A. Kofoet, J. J. Tuset, E. Bertolini, and M. Cambra. 2002. Specific and sensitive detection of *Phytophthora nicotianae* by simple and nested-PCR. Europ. J. Plant Pathol. 108:197-207.

Kageyama, K., M. Suzuki, A. Priyatmojo, Y. Oto, K. Ishiguro, H. Suga, T. Aoyagi, and H. Fukui. 2003. Characterization and identification of asexual strains of *Pythium* associated with root rot of rose in Japan. J. Phytopathol. 151:485-491.

Miller, S. A., L. V. Madden, and A. F. Schmitthenner. 1997. Distribution of *Phytophthora* spp. in field soils determined by immunoassays. Phytopathology 87:101-107.

Poorter, H. and O. Nagel. 2000. The role of biomass allocation in the growth response of plants to different levels of light, CO_2, nutrients and water: a quantitative review. Austral. J. Plant Physiol. 27:595-607.

Timmer, L. W., J. A. Menge, S. E. Zitko, E. Pond, S. A. Miller, and E. L. V. Johnson.1993. Comparison of ELISA techniques and standard isolation methods for *Phytophthora* detection in citrus orchards in Florida and California. Plant Dis. 77:791-796.

Tsao, P. H. 1990. Why many *Phytophthora* root rots and crown rots of tree and horticultural crops remain undetected. Bul. Oepp/Eppo Bul. 20:11-17.

Zitko, S. E. and L. W. Timmer. 1994. Competitive parasitic abilities of *Phytophthora parasitica* and *P. palmivora* on fibrous roots of citrus. Phytopathology 84:1000-1004.