Comparison of Seasonal Infection of Citrus Roots by *Phytophthora citrophthora* and *P. nicotianae* var. *parasitica*

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ABSTRACT

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Excised feeder roots from mature citrus trees located in climatically different regions were infected with zoospores of *Phytophthora citrophthora* and *P. nicotianae* var. *parasitica* at different times of the year under identical laboratory conditions. Zoospores encysted on and caused infection in roots from all locations year round. Both pathogens had the most encysted zoospores on roots from November to January and the least from March to May. Infection by *P. nicotianae* var. *parasitica* was consistently higher than *P. citrophthora* in the excised summer roots (May to September) and lower in January 1998 and November 1997. Infection by both species of *Phytophthora* dropped to a minimum in March when carbohydrate levels in roots were lowest. Temperature was not solely responsible for determining seasonal fluctuations of *P. nicotianae* var. *parasitica* and *P. citrophthora*. Root carbohydrate content also could not be correlated with seasonal infection. Other physiological or microbial factors associated with citrus roots may cause seasonal *Phytophthora* infection fluctuations. Infection of citrus seedling roots by *P. citrophthora* at 16°C reduced root starch content but not glucose compared to *P. nicotianae* var. *parasitica*, whereas infection at 30°C by *P. nicotianae* var. *parasitica* lowered root glucose but not starch content compared to *P. citrophthora*.

Phytophthora nicotianae Breda de Haan var. *parasitica* Dastur and *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian are serious fungus-like pathogens of citrus and account for millions of dollars in crop losses annually (41). In regions with a Mediterranean climate, such as southern California, *P. nicotianae* var. *parasitica* infection can be found in citrus groves in the summer and *P. citrophthora* in the winter (22,27,29,37). Several explanations have been put forward to explain this phenomenon, the most obvious of which is temperature. *Phytophthora nicotianae* var. *parasitica* was dormant in southern California from November to June when soil temperatures drop below 12°C, and citrus roots cease to grow (24,25). The temperature range for growth of *P. nicotianae* var. *parasitica* is 8 to 36°C, with the optimum approximately 31°C (12). So it would seem that soil temperatures that are too cold for the pathogen to grow most likely cause lack of infection by *P. nicotianae* var. *parasitica* in the winter. The temperature range for growth of *P. citrophthora* is 6 to 32°C, with the optimum approximately 26°C. Summer soil temperatures at

the rooting depth of citrus in southern California range from 17 to 34°C (Fig. 1) (4,40). However, in many areas of southern California, summer soil temperatures never exceed that at which *P. citrophthora* can grow. Furthermore, summer soil temperatures decrease with depth so the pathogen should be able to survive a few centimeters deeper in the soil. Thus, we hypothesize that changes in soil temperature do not explain the lack of infection by *P. citrophthora* in the summer.

Another possible explanation for the seasonality of *P. citrophthora* is the seasonal carbon partitioning in citrus roots. In the winter, citrus roots are high in starch and low in sugars and in the spring, starch levels drop as they are metabolized to fuel the shoot flush (15,36). During the summer, sugar levels in the roots increase and as tree growth decreases in the fall, excess photosynthate is again stored as starch (9,16). A review of the literature on *Phytophthora* metabolism revealed discrepancies in the ability of *P. nicotianae* var. *parasitica* to produce amylase and metabolize starch. Oliveira (31) and Timmer et al. (38) believed that the organism could not use starch as a carbon source. Ho and Foster (19) also implied that *P. nicotianae* var. *parasitica* has only limited ability to hydrolyze starch, which varied between isolates. On the other hand, Mehrotra (28) determined that *P. nicotianae* var. *parasitica* could metabolize starch. After testing 50 carbon sources, including nine sugars, alcohols and amino acids, Wills (42) found

that the best carbon source for *P. nicotianae* var. *parasitica* was starch or dextrin (a starch oligomer). Hendrix and Apple (17) determined that *P. nicotianae* var. *parasitica* grew better on vegetable oils, which like starch are also composed of carbon chains, than it did on glucose. In 1966, Hendrix et al. (18) found that growth of *P. nicotianae* var. *parasitica* on glucose could be increased by the addition of mineral oil, also a carbon chain molecule like starch. If *P. nicotianae* var. *parasitica* is unable to metabolize starch, this together with low temperatures would prevent the fungus from infecting citrus roots in the winter when stored starch levels are high.

P. citrophthora, on the other hand, grows more profusely on starches than sugars (35), therefore it was hypothesized to infect citrus roots mainly in the winter. To further complicate the issue, in his study of amylase activity of *Phytophthora* spp., Oliveira (31) found Brazilian isolates of *P. citrophthora* growing on cacao (*Theobroma cacao* L.) to be amylase negative. It is now believed that these isolates were, in fact, not *P. citrophthora*, but a new species of *Phytophthora* (32). Dimitman and Zentmyer (6) also noticed nutritional differences between the two species of *Phytophthora*. They found significant differences in the abilities of *P. citrophthora* and *P. nicotianae* var. *parasitica* to metabolize amino acid-carbohydrate combinations. *P. citrophthora* grew well in a chemically defined media containing maltose or the starch oligomer dextrose plus the amino acid L-asparagine, whereas *P. nicotianae* var. *parasitica* only produced one-seventh as much mycelial growth, by weight, in this media. *P. nicotianae* var. *parasitica* grew better in media containing sugar monomers such as glucose or fructose plus glutamine or glutamic acid, whereas *P. citrophthora* grew very sparsely in this medium.

The objective of the first part of this study was to determine if there is a correlation between carbon partitioning in citrus roots and *Phytophthora* infection. To minimize the effect of temperature on *Phytophthora* activity, root samples were removed from the field and immediately infected under uniform, controlled conditions in the laboratory.

The objective of the second part of this study was to search for differences in carbon metabolism between the two species of *Phytophthora* by analyzing the starch

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and sugar levels of infected citrus seedling roots, grown under different light and temperature conditions.

MATERIALS AND METHODS

Attraction and encystment of zoospores to excised citrus roots. To eliminate the possibility of variation between isolates, a combination of zoospores from four isolates each of *P. citrophthora* and *P. nicotianae* var. *parasitica* collected from widely dispersed citrus groves was used. The isolates from the warmest climates were M325 (*P. nicotianae* var. *parasitica*) and M329 (*P. citrophthora*) from Yuma, AZ and supplied by M. E. Matheron, Uni-

versity of Arizona, Yuma Agricultural Center. The authors collected the remaining isolates. The *P. citrophthora* isolates were M331 from Riverside, CA, M330 from Ventura, CA, and M332 from Visalia, Tulare County, CA. The *P. nicotianae* var. *parasitica* isolates were M328 from Fresno, CA, M327 from Riverside, CA, and M326 from Pauma Valley, San Diego County, CA. All isolates were obtained from a single zoospore to ensure pure cultures.

Zoospore preparation was started 10 days before root samples were collected from the field. Three 5-mm-diameter agar plugs from a 4- to 5-day-old culture of

each isolate grown on CV8 agar (30), were placed in two 100 × 15 mm petri dishes. The four single-spore isolates described above were used to reduce differences between isolates within each species. Agar plugs were covered with 20 ml of 0.50-strength CV8 broth and incubated at 24°C in the dark for 1 week. The nutrient broth was decanted, and the mycelium was rinsed three times with sterile water to ensure removal of all the nutrients. The mycelium was covered with 20 ml of soil extract (1%) and placed under fluorescent grow lights for 3 to 4 days to induce sporangia formation. Soil extract was prepared by placing 10 g of soil from a citrus grove in 1 liter of deionized water. The mixture was shaken to disperse the soil and left overnight. The next day, the mixture was vacuum filtered through Whatman No. 40 filter paper. This extract was stored in a refrigerator until used.

At least 1 h before the zoospores were needed, the soil extract was decanted and replaced with tap water, and then the agar plugs bearing mycelium and sporangia were refrigerated at 4°C for 30 min. Zoospores were released from sporangia after the cultures had been removed from the refrigerator and allowed to warm up to room temperature (24°C) on the lab bench.

A similar procedure to that outlined above was followed for the preparation of fluorescent zoospores with the following differences. Mycelium was grown in a 60 × 15 mm petri dish containing 5 ml of 0.50-strength CV8 broth. On the day the roots were to be inoculated, at least 4 h before the sporangia were scheduled to be refrigerated, 100 ppm of fluorescent brightener "Calcofluor White M2R New" (disodium salt of 4,4'-bis[4-anilino-6-diethylamino-*s*-triazin-2-ylamino]-2,2'-stilbene-disulfonic acid, (Sigma-Aldrich, St Louis, MO) was added to each petri dish (39). The dishes were incubated in the dark at room temperature until refrigeration prior to zoospore release.

Roots were collected six times a year from four citrus groves that had been carefully selected to represent maximum differences in climate and to be free from natural *Phytophthora* infection. The four locations were: the University of Arizona Agricultural Center at Yuma Mesa, Yuma; Pauma Valley in San Diego County, CA; Agricultural Experiment Station, University of California, Riverside; and Orange Cove, northeast of Visalia, in Tulare County, CA. All trees were approximately 15-year-old sweet orange, (*C. sinensis* (L.) Osbeck, cv. Valencia) scions on Troyer Citrange rootstock, (*C. sinensis* × trifoliata orange, *Poncirus trifoliata* (L.) Raf.). Seven trees were selected from each grove for root sampling. Approximately 0.50 kg of soil containing a clump of feeder roots was removed with a spade from the northeast side of each tree just under the drip line from a soil depth of 10 to 15 cm. Care

Fig. 1. Attachment and encystment of fluorescent zoospores of *Phytophthora* to fine citrus roots collected from widely dispersed locations at six different times a year. **A**, *P. citrophthora* zoospores, **B**, *P. nicotianae* var. *parasitica* zoospores, and **C**, comparison of *P. citrophthora* and *P. nicotianae* var. *parasitica* zoospores using combined data from the different locations. Error bars represent standard error of the mean.

was taken to minimize damage to the roots during harvesting. Samples were placed in sealed plastic bags to ensure that desiccation of the roots did not occur and were transported back to the laboratory in ice chests to maintain a constant temperature. The roots were inoculated within 1 day of harvest. This procedure was repeated at 2-month intervals from November 1996 to September 1998.

To compare infection by the two species of *Phytophthora*, four sets each of 0.5 g of carefully washed feeder roots of uniform diameter, from five trees at each location, were placed in petri dishes (100 × 15 mm) containing 20 ml of deionized water. One set was inoculated with *P. nicotianae* var. *parasitica* zoospores and the second with *P. citrophthora* zoospores at a rate of 2,000 zoospores per ml. A control set of roots was not inoculated to confirm there was no background *Phytophthora* infection in the sampled citrus groves. Roots were incubated on the lab bench at 24°C under fluorescent lights for 11 h per day for 2 days to allow root infection to take place before being dried and frozen for the *Phytophthora* infection assay. The fourth set of feeder roots was frozen for carbohydrate analysis.

Zoospore attraction to and encystment on the citrus roots described above was also determined. Two sets of six root tips, 1 cm long, from the above five trees from each location, were selected using a dissecting microscope at ×10 magnification to ensure that the two sets were of similar appearance and undamaged. These were placed in small petri dishes (35 × 10 mm) containing 3 ml of deionized water. Each petri dish was inoculated with fluorescent zoospores of *P. citrophthora* or *P. nicotianae* var. *parasitica* at a concentration of 2,000 zoospores per ml. Two hours after inoculation, the infested root tips were refrigerated to halt further pathogen growth. The encysted fluorescent zoospores were counted using an ultra violet light source at a magnification of ×125. Counting was restricted to a 2-mm region of the root, 20 µm behind the tip. The average number of encysted zoospores per treatment was calculated.

Determination of *Phytophthora* mycelium in excised citrus feeder roots. The 0.5 g of frozen, inoculated root samples from the five trees at each location described above, were immersed in liquid nitrogen and ground to a fine powder with a pestle and mortar. The quantity of *Phytophthora* mycelium per root sample was assayed using a *Phytophthora* Detection Kit (Agri-Screen, Neogen Corporation, Lansing, MI). The kit uses an enzyme-linked immunosorbent assay (ELISA) for cell wall components of *Phytophthora* mycelium to detect *Phytophthora* infection (38). One hundred microliters of the samples, plus the positive control provided with the kit, were loaded in duplicate in the

wells of the microtiter plates supplied with the kit, as well as a standard solution containing a known volume of *Phytophthora* mycelium. After removing the sample plus extraction buffer from the microtiter plate, 100 µl of skim milk was added to each well to block any empty sites not occupied by *Phytophthora*. The skim milk was prepared by mixing powdered nonfat dry milk (Carnation, Glendale, CA) with water (1:4, vol/vol). After adding the substrate to the microtiter plate, the plate was vortexed only until some of the wells had become bright green, and then the stop solution was added. If the reaction was allowed to proceed for too long, the wells became too dark to be read accurately, and the measurements of *Phytophthora* mycelium were no longer quantitative. The plate was read immediately on a Biorad Microplate Reader (Model 3550-UV, Hercules, CA) at λ405 nm.

The positive control supplied with the *Phytophthora* detection kit was calibrated to express micrograms of mycelium. Mycelium from four isolates (to minimize any effect caused by differences between isolates) of *P. citrophthora* and *P. nicotianae* var. *parasitica* was grown in 0.50-strength CV8 broth for 2 weeks. The inoculation plug of agar was then removed, and the mycelium was air-dried. Two different techniques for preparing the standards were tested. In the first, 0.025-g aliquots of dried mycelium from each species was boiled for 5 min in 1 ml of extraction buffer (Neogen Corporation). In the second, 0.025 g of dry mycelium was ground in liquid nitrogen before adding 1 ml of extraction buffer. Both extraction methods gave similar results. Weighed samples of ground mycelium from different isolates of the two pathogens were placed in 2 ml of extraction buffer for 30 min. The buffer was then decanted and serially diluted with more extraction buffer. Aliquots (100 µl) of the different dilutions of both species of *Phytophthora* were compared to the positive control supplied with the kit. This enabled an absolute value in micrograms of mycelium to be assigned to the results of the *Phytophthora* infection assay and the calculation of weight of mycelium per gram of root sample.

Extraction of soluble sugars from citrus roots. The fourth set of 0.50 g of citrus feeder roots from each of the five trees from all four locations, which had been previously frozen awaiting carbohydrate analysis, was ground to a fine powder in liquid nitrogen. The soluble carbohydrates were extracted for glucose analysis (26), and an aliquot of the resulting solution decolorized by passing through 1 ml of charcoal powder in a serum filter to remove color pigments, which would interfere with the spectrophotometer readings.

Analysis of carbohydrate content of excised citrus roots. The glucose content was analyzed using hexokinase from a

commercially available kit, (Glucose (HK) 20, Sigma-Aldrich). The hexokinase was dissolved in 50 ml of double-distilled water; otherwise the manufacturer's recommendations were followed. A series of 1.0 mM glucose standards, plus 20 µl of each root sample, were loaded per well in triplicate in a microtiter plate followed by 100 µl of the hexokinase solution. The loaded plate was placed on a shaker table at 100 rpm for 30 min and the absorbance read with a Biorad Microplate Reader (Model 3550-UV) at λ340 nm. Hexokinase phosphorylates the glucose, which then reacts with NADP⁺ to give D-gluconate-6-P plus NADPH and H⁺. The NADPH is optically opaque at λ340 nm, and the concentration can be determined quantitatively with a photospectrometer and the glucose concentrations thereby calculated (5,23). The weight of glucose per gram of root tissue was calculated.

A second aliquot of the soluble carbohydrate extracted as described above was used for the analysis of fructose and fructose containing sugars such as sucrose (21). Water-soluble sugars such as fructose react with resorcinol (1,3-benzenediol C₆H₆O₂) under acid conditions to give a red color that can be measured spectrophotometrically at λ520 nm (34). The resorcinol solution was prepared by dissolving 0.1 g of resorcinol (Sigma-Aldrich) in 60 ml of 95% ethanol in a 100-ml volumetric flask adding 20 ml concentrated HCl (36%), and adding double-distilled water to reach a volume of 100 ml when the solution had cooled to room temperature. Adding 2 ml of the resorcinol mixture to 100 µl of the samples extracted from the citrus roots and comparing them with a series of 20 mM sucrose standard solutions determined the sucrose content of the citrus roots.

The root tissue remaining after the soluble sugars had been removed was resuspended in 1.0 ml 2N KOH to extract the remaining starch as described by Robbins and Pharr (33). The starch was digested with amyloglucosidase and the amount of glucose released was determined as described above.

Greenhouse assay of effects of *P. nicotianae* var. *parasitica* and *P. citrophthora* infestation on carbohydrate content of citrus seedling roots. To investigate changes in carbohydrate content of citrus roots caused by *Phytophthora* infection, seedlings were grown under different greenhouse and growth chamber conditions. Troyer citrange (*Poncirus trifoliata* (L.) Raf. × *Citrus sinensis* (L.) Osbeck) citrus seedlings were planted in 10 × 10 × 10-cm fiber pots in a Vista coarse sandy loam from a citrus nursery in Fallbrook, CA. The soil was infested with *P. citrophthora* or *P. nicotianae* var. *parasitica*, which had been growing on sterilized millet moistened with 0.50-strength CV8 broth for 3 weeks (14,30), at a rate of 1% (wt) or with uninfested millet. The

experiment was established in a randomized block design in three locations to simulate different seasons. Spring and autumn conditions were simulated in a greenhouse with daily temperatures fluctuating from 19 to 32°C and daytime light intensity averaging 630- $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Winter conditions were simulated in a growth chamber maintained at 15°C with 10 h of simulated daylight at an intensity of 350- $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 14 h of darkness. The third environment, simulating summer conditions, was a growth chamber set at 30°C, with 14 h of simulated daylight at an intensity of 940- $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 10 h of darkness. All plants were watered with dilute (14%) Hoagland's solution (20). There were 10 replicates per treatment in each environment, and the experiment was performed three times.

Five of the seedlings were harvested after 6 weeks and the remainder after 12 weeks. The soil was carefully washed off the roots, which were visually assessed for root damage by three independent observers. Shoots and roots were weighed, and the roots carefully divided into two equal

fractions. One fraction was used for glucose, sucrose, and starch analysis as described above. The other fraction was used to estimate *Phytophthora* infection using the ELISA plates supplied with the Agri-Screen Detection Kit (method described above). Results of the three experiments were combined for data analysis.

Where appropriate, data were analyzed by analysis of variance using PROC GLM procedure (SAS Institute, Cary, NC). If $P > F$ was less than 0.01, treatment means were separated with Waller-Duncan multiple range test at $P = 0.05$ for each dependent variable.

RESULTS

Attraction and encystment of zoospores to excised citrus roots. There was more variation in attachment and encystment of *P. nicotianae* var. *parasitica* zoospores than *P. citrophthora* zoospores to roots from the different locations especially in the late autumn and winter months (Fig. 1A and 1B). In November 1996, the average number of *P. nicotianae* var. *parasitica* encysted zoospores observed per root

tip ranged from 37 on Yuma roots to 71 on Orange Cove roots and in January 1997, from 30 on Yuma roots to 50 on Orange Cove roots. Yuma and Orange Cove are the locations furthest apart geographically and have the largest differences in minimum winter soil temperatures (Fig. 2). In January 1998, the numbers of *P. nicotianae* var. *parasitica* encysted zoospores ranged from 57 on Orange Cove roots to 24 on Yuma roots and in May 1998, from 27 on Orange Cove roots to 8.1 on Yuma roots. At other times of the year the differences observed in the numbers of encysted zoospores between the locations for *P. nicotianae* var. *parasitica* were minimal, for example in March 1997 the numbers ranged from 3.8 on Orange Cove roots to 5.7 on Riverside roots.

The variation in the number of encysted zoospores of *P. citrophthora* from the four locations tend to be less than the seasonal variation. In November 1998, numbers ranged from 58 at Yuma, and 51 in Pauma Valley in November 1996, to 32 at Orange Cove and 30 in Pauma Valley in November 1998, whereas only seven encysted zoospores were observed on roots from Yuma and eight from Riverside in May 1997, and five from Pauma Valley and six from Orange Cove in July 1998.

The attachment and encystment of *P. nicotianae* var. *parasitica* and *P. citrophthora* were compared using the combined data from the four locations for each organism. For both pathogens, significantly more zoospores were observed attached to roots collected in November and January than to roots collected during the rest of the year according to a Waller Duncan separation of means with $P \leq 0.05$, and for *P. nicotianae* var. *parasitica* the number of zoospores attached to the March roots were significantly lower than in the summer (May, July, and September) (Fig. 1C). Overall, seasonal attachment and encystment of zoospores observed for the two *Phytophthora* species were similar, higher in late autumn and winter, dropping sharply in March and remaining low during the summer.

Determination of *Phytophthora* mycelium in excised citrus feeder roots. When excised citrus feeder roots from four widely different locations were inoculated with *Phytophthora* zoospores under uniform temperature conditions in the laboratory, the roots became infected by *P. citrophthora* and *P. nicotianae* var. *parasitica* throughout the year (Fig. 3). The amount of mycelium of *P. citrophthora* found in the roots from Pauma Valley was lower than that from any of the other locations in 10 of 12 sampling periods, fluctuating from 135.3 ng in November 1997 to a low of 22.7 ng in May 1997, with an average value of 71.0 ng (Fig. 3A). The average weight of *P. citrophthora* mycelium from the other locations varied from 103.0 ng from Riverside to 110.3 ng from Orange

Fig. 2. Soil temperatures at a depth of 15 cm in typical citrus-growing regions of southern California and Arizona.

Cove, with all locations following approximately the same seasonal trend. If the Pauma Valley samples are excluded, as the soil in this area is naturally suppressive to *P. citrophthora* (7), the largest variation in mycelial weight among the four locations occurred in May 1998, when Orange Cove roots contained 165.4 ng of mycelium, and Riverside roots contained 58.6 ng. In September 1998, Yuma roots contained the highest weight of mycelium (190.1 ng), and Orange Cove roots contained the least (84.6 ng). Differences were also large in September 1997, but this time the highest weight was in the Riverside roots, with 192.9 ng, and the lowest was in Yuma, with 89.3 ng. These results indicate that there are no consistent overall differences in the susceptibility to infection amongst the roots from the four locations.

The largest difference among the locations was in May 1998, with 242.7 ng of mycelium in the roots from Yuma and only 94.2 ng in the roots from Orange Cove (Fig. 3B). In November 1997, the differences were again large, with 208.3 ng of mycelium in the roots from Yuma and 92.5 ng from Orange Cove. However, when the average weight of *P. nicotianae* var. *parasitica* mycelium for the 2 years is considered, the lowest value of 92.6 ng came from Riverside, and the highest value of 136.3 ng came from Yuma. Again there were no clear differences among the four locations and the results from the four locations tend to follow the same seasonal trend.

The greatest difference between the two species in mycelial weight in the roots occurred in January 1998, with 162.7 ng of *P. nicotianae* var. *parasitica* and 65.7 ng of *P. citrophthora*; 167.7 ng of *P. nicotianae* var. *parasitica* and 89.1 ng of *P. citrophthora* in May 1998; 153.2 ng of *P. nicotianae* var. *parasitica* and 82.5 ng of *P. citrophthora* in July 1998; and 185.1 ng of *P. citrophthora* and 95.5 ng of *P. nicotianae* var. *parasitica* in January 1998. These numbers indicate (Fig. 3C), that there was more *P. nicotianae* var. *parasitica* than *P. citrophthora* mycelium infecting the late spring and summer roots.

Analysis of carbohydrate content of excised citrus roots. There were no consistent seasonal or locational differences in the glucose content of the roots, other than locations which had high levels of glucose in the winter of 1996 to 1997 had comparatively lower glucose levels in 1997 to 1998 and vice versa (Fig. 4A). In November 1996, roots from Pauma Valley and Yuma contained 1.49 and 1.08 mg of glucose per g of root tissue, respectively, whereas in November 1997, roots from these two locations contained 0.49 and 0.07 mg of glucose, respectively. Conversely, roots from Riverside and Orange Cove contained 0.27 and 0.10 mg of glucose, respectively, in January 1997 and 1.29 and 1.06 mg in January 1998.

The content of water-soluble sugars such as sucrose in the roots was higher for all locations in 1998 than 1997, except for Yuma (Fig. 4B). There were no consistent seasonal or location related trends for the sucrose content of the roots. Values ranged from as much as 6.38 μ g and 6.24 μ g per g of root tissue from Orange Cove roots in January and March of 1998, respectively, to as low as 0.75 μ g and 0.50 μ g in roots from Pauma Valley and Riverside, respectively, in March 1997.

The starch content of the roots is shown in Fig. 4C. The starch content of the roots from Yuma followed the pattern of high values in the winter, which decreased in the spring as the stored starch reserves

were metabolized to fuel the shoot growth (11,16). In January 1997, roots collected from Yuma contained 2.44 μ g of glucose equivalent per gram tissue and 3.52 μ g in January 1998, compared to 0.06 μ g in March 1997 and 0.37 μ g in March 1998. The effect can be seen to a lesser degree for roots from the other locations in March 1998, with values ranging from 2.17 μ g of glucose equivalent per gram of tissue from Orange Cove to 1.25 μ g from Pauma Valley in January, dropping to 0.73 μ g from Pauma Valley and 0.57 μ g from Orange Cove. This trend was not followed in 1997 when the roots from all locations except Yuma had their maximum starch content in March, with roots from Riverside reaching

Fig. 3. Infection by *Phytophthora* in the laboratory of excised fine citrus root collected from widely dispersed locations at different times of the year. A, *P. citrophthora*, B, *P. nicotianae* var. *parasitica*, and C, comparison of infection by the two species using combined data from all locations. Error bars represent standard error of the mean.

9.54 μg of glucose equivalent per gram of root tissue. There was again a trend for those groves that had high starch in March 1997 (Riverside, Orange Cove, and Pauma Valley) to have lower levels of starch in March 1998, except for Yuma, which had low starch content in January 1997 and higher content in January 1998. While summer maximum soil temperatures separated as expected, with Yuma having the warmest followed by Orange Cove, Riverside, and Pauma Valley, the winter maximum soil temperatures from all four sites were similar. The minimum soil temperatures from the three northern locations were similar throughout the 2-year period, while those from the Yuma site were again higher except for January 1998, indicating that soil temperature was not the cause of fluctuations in root starch content.

Greenhouse assay of effects of *P. nicotianae* var. *parasitica* and *P. citrophthora* infestation on carbohydrate content of citrus seedling roots. At 16°C, visual inspection of the roots revealed that seedlings infected with *P. citrophthora* had

fewer healthy roots than those infected with *P. nicotianae* var. *parasitica*. Sixteen percent of roots infected with *P. citrophthora* appeared healthy 6 weeks after infestation and 6% after 12 weeks, compared to 63% after 6 weeks and 64% 12 weeks after infestation with *P. nicotianae* var. *parasitica* (Table 1). At 24°C, only roots infected with *P. citrophthora* showed a significant decline in health, with 43% healthy 6 weeks after infestation and 67% healthy 12 weeks after infestation, while over 97% of the *P. nicotianae* var. *parasitica* infected roots and the control roots remained healthy. At 30°C after 6 weeks, 67% of *P. citrophthora* infected roots and 85% of *P. nicotianae* var. *parasitica* infected roots remained healthy but after 12 weeks, 90% of the *P. citrophthora* infected roots compared to 62% of the *P. nicotianae* var. *parasitica* roots remained healthy.

At all three temperatures tested, infected citrus seedling roots contained significantly more *Phytophthora* mycelium than control roots (Table 1). At 16°C, 6 weeks after infestation there were no significant

differences between treatments, but after 12 weeks, the *P. citrophthora* infected seedling roots contained 161 ng of mycelium while the *P. nicotianae* var. *parasitica* roots contained only 97 ng of mycelium. At 24°C, seedling roots contained 148 ng of *P. citrophthora* mycelium 6 weeks after infestation and 161 ng 12 weeks after infestation, compared with 67 ng and 113 ng of *P. nicotianae* var. *parasitica* mycelium. At 30°C, no significant differences in the mycelial weight between the two pathogens were found in the seedling roots.

Phytophthora infestation caused a decline in seedling root and shoot weight. At 16°C, roots of seedlings infested with *P. citrophthora* or *P. nicotianae* var. *parasitica* or control roots weighed 0.66, 0.91, or 1.06 g, respectively, 6 weeks after infestation. Twelve weeks after infestation, seedling roots weighed on average 0.70, 1.49, or 2.53 g, respectively. At 24°C, the roots of seedlings infected with *P. citrophthora* weighed significantly less compared to those infected with *P. nicotianae* var. *parasitica* and control roots. At 12 weeks after infestation, *P. citrophthora* infected roots still weighed less than roots from the two other treatments, but the difference was no longer statistically significant. At 30°C, both 6 and 12 weeks after infestation, roots of seedlings infected with *P. nicotianae* var. *parasitica* weighed significantly less than those from the other two treatments.

The only significant differences in seedling shoot weight occurred at 24°C, 6 weeks after infestation when shoots from seedlings infected with *P. citrophthora* weighed an average of 0.49 g, compared to values of 1.07 and 1.13 g for the *P. nicotianae* var. *parasitica* and control shoots, respectively.

Phytophthora infestation also affected the carbohydrate content of the citrus seedling roots. Glucose content of infected seedling roots declined compared to the control in all treatments except at 30°C 12 weeks after infestation by *P. citrophthora*. The maximum decrease occurred at 16°C 12 weeks after infestation, with over 90% reduction in glucose content. The smallest decrease occurred in *P. citrophthora* infected seedlings at 30°C 12 weeks after infestation where seedling roots contained 0.975 μg of glucose, compared to 1.014 μg in the control and 0.244 μg in the *P. nicotianae* var. *parasitica* infected roots.

The changes in the sucrose content were complex. In some cases, such as at 16°C, *P. citrophthora* infected roots had over 33 g of sucrose per g of root tissue while control and *P. nicotianae* var. *parasitica* infected roots contained approximately 15 μg (Table 1). At 30°C after 6 weeks, *P. citrophthora* and *P. nicotianae* var. *parasitica* infected roots again contained more sucrose than control roots, 15.79 μg and 12.63 μg , respectively, compared to only 7.23 μg in control roots. Twelve weeks

Fig. 4. Carbohydrate content of fine citrus roots collected from four widely dispersed locations at different times of the year. **A**, glucose content, **B**, sucrose content, and **C**, starch content (measured as glucose equivalent). Error bars represent the standard error of the mean.

after infestation at 30°C there were no statistically significant differences in sucrose content. At 24°C 6 weeks after infestation, the sucrose content of roots infected with either pathogen was less than that of control roots. Twelve weeks after infestation, the sucrose content of roots infected with *P. nicotianae* var. *parasitica* was significantly lower than values for control roots and those infected with *P. citrophthora*.

Seedling roots infected with *P. citrophthora* contained significantly less starch than control roots, but the differences were only statistically significant 6 weeks after infestation at 16 and 24°C. At 16°C, infected roots contained an average of 1.73 µg and at 24°C contained 4.21 µg of sucrose per g of root tissue compared to 3.91 µg and 7.52 µg in control roots. After 6 weeks, *P. nicotianae* var. *parasitica* infestation significantly reduced the starch content in infected roots at 24 and 30°C, to 4.30 µg and 1.27 µg per g of root tissue compared to average values of 7.52 µg and 5.42 µg for control roots; however at 24°C, 12 weeks after infestation with this pathogen, starch content was increased to 3.22 µg compared to the average control value of 1.84 µg.

The major difference between carbohydrate utilization in roots infected with *P. nicotianae* var. *parasitica* and *P. citrophthora* was after a 6-week period of time at 16°C when *P. citrophthora*, but not *P. nicotianae* var. *parasitica*, reduced starch content and increased sucrose content of seedling roots compared to the con-

trols. *P. nicotianae* var. *parasitica* but not *P. citrophthora* increased starch levels at 24°C and reduced glucose levels at 30°C.

DISCUSSION

Zoospores of *P. citrophthora* and *P. nicotianae* var. *parasitica* encysted on freshly excised field roots in the laboratory year round but, surprisingly, the greatest number were found on winter roots (November and January). This was especially unexpected for *P. nicotianae* var. *parasitica*, which is not normally active in the field during winter months (13). However, winter roots generally contain the highest reserves of stored starches (15,16,36). Despite the differences in climate among the four locations, there were no significant differences in number of encysted zoospores of either pathogen. This may be explained by the fact that during the 2 years of this experiment, climate, especially the minimum winter temperatures, varied little among the sites except for the Yuma site, which was much warmer.

Excised field roots can also be infected by zoospores year round in the laboratory. This indicates that the absence of infection by *P. citrophthora* in the summer, in regions with a Mediterranean climate, is probably not caused by lack of a suitable carbon source for the fungus in the roots. However, results from this research clearly indicate that *P. nicotianae* var. *parasitica* infection is more abundant in summer roots, whereas *P. citrophthora* infection appears to be more abundant in winter roots even under controlled temperature

conditions in the laboratory. This suggests that the physiology or microbiology associated with citrus roots, at least partially, accounts for the seasonality of infection in the field by these two *Phytophthora* species and not climate, temperature, or other abiotic factors. Citrus trees could secrete a phytoalexin, inhibiting infection by *P. citrophthora* in the summer. Afek and Sztejnberg (2,3) studied the production of the phytoalexin scoparone by the bark of citrus trees and the corresponding resistance to gummosis caused by *P. citrophthora*. Afek and Sztejnberg (1) also found that citrus trees produced the most scoparone at 25°C and produced progressively less as temperatures decreased. They detected no scoparone at 10 or 5°C. To date, no evidence of production of scoparone by citrus feeder roots has been found, but this does not eliminate the possibility that this phytoalexin may be the cause of lower infection by *P. citrophthora* in the summer when it is produced in significant quantities. Winter soil temperatures in California are too low for scoparone production, hence *P. citrophthora* infection would not be inhibited during this time.

There were no consistent differences among the four locations in the amount of *P. nicotianae* var. *parasitica* mycelium that grew in the excised roots; however, the roots from Pauma Valley consistently contained less *P. citrophthora* mycelium than those from the other locations. This is in accordance with the finding that *P. citrophthora* has not been isolated from Pauma Valley (29), and the soil in Pauma

Table 1. Analysis of root health and carbohydrate content of citrus seedlings, grown at different temperatures, 6 and 12 weeks after infestation with *Phytophthora citrophthora* or *P. parasitica*^x

Temp.	Week	Treatment	% Healthy roots	Infection ng mycelium/g root	Root weight (g)	Shoot weight (g)	Glucose µg/g root	Sucrose µg/g root	Starch µg/g root
16°C	6	Control	95.3 a ^y	11 b	1.06 a	1.47 a	4.94 a	15.46 b	3.91 a
		<i>P. citrophthora</i>	15.7 c	89 a	0.66 b	1.23 a	2.72 b	33.41 a	1.73 b
		<i>P. nicotianae</i> var. <i>parasitica</i>	63.5 b	85 a	0.91 a	1.14 a	2.52 b	14.80 b	3.05 a
16°C	12	Control	89.7 a	0.7 c	2.53 a	1.75 a	3.96 a	20.82 b	2.66 a
		<i>P. citrophthora</i> ^z	5.7 c	161 a	0.70 b	1.16 a	0.30 b	34.09 a	2.44 a
		<i>P. nicotianae</i> var. <i>parasitica</i>	64.17 b	97 b	1.49 ab	1.28 a	0.28 b	26.86 ab	2.30 a
24°C	6	Control	99.0 a	8.5 c	1.04 a	1.13 a	7.04 a	25.10 a	7.52 a
		<i>P. citrophthora</i>	43.2 b	148 a	0.36 b	0.49 b	4.69 b	8.15 b	4.21 b
		<i>P. nicotianae</i> var. <i>parasitica</i>	98.2 a	67 b	1.13 a	1.07 a	4.65 b	5.82 b	4.30 b
24°C	12	Control	98.6 a	16 c	4.76 a	7.00 a	2.54 a	25.94 a	1.85 b
		<i>P. citrophthora</i>	67.4 b	161 a	3.07 a	4.63 a	0.32 b	17.95 ab	1.52 b
		<i>P. nicotianae</i> var. <i>parasitica</i>	97.7 a	113 b	3.19 a	3.72 a	0.64 b	8.97 b	3.22 a
30°C	6	Control	89.5 a	1.0 b	1.74 a	2.39 a	5.62 a	7.23 b	5.42 a
		<i>P. citrophthora</i>	66.7 b	104 a	1.57 ab	2.26 a	0.63 b	15.79 a	3.37 ab
		<i>P. nicotianae</i> var. <i>parasitica</i>	84.6 a	123 a	1.30 b	1.95 a	0.37 b	12.63 a	1.27 b
30°C	12	Control	91.0 a	4.1 b	4.03 a	4.96 a	1.04 a	26.10 a	2.60 a
		<i>P. citrophthora</i>	89.9 a	134 a	3.91 a	4.89 a	0.97 a	19.97 a	2.35 a
		<i>P. nicotianae</i> var. <i>parasitica</i>	62.5 b	132 a	3.32 b	4.31 a	0.24 b	29.44 a	2.75 a

^x Each value represents the mean of at least nine replicated values.

^y Treatments followed by the same letter do not differ significantly according to a Waller-Duncan *k*-ratio *t* test at *P* = 0.05 each week and temperature analyzed separately.

^z All seedlings died in one trial, treatment repeated 3 times.

Valley contains microorganisms which inhibit *P. citrophthora* infection (8).

The starch, glucose, and sucrose content of the excised roots could not be correlated in any way with the seasonality of infection by the two *Phytophthora* species. The carbohydrate content determined for the excised citrus roots did not fit the expected pattern of high starch, low sugars in the winter and low starch, high sugars in the summer reported by others (9,10,16). This may be partly attributable to the small differences in climate between locations and to the mild winters observed during the study. Other factors such as nutritional status, irrigation, fruit set, disease, and parasitic nematode levels also affect root carbohydrate content (16). In years of low fruit production, root carbohydrate levels are higher (15,36). Fruit production was not monitored during the 2 years in which the experiment was conducted, but Figure 4 shows that groves with high glucose or starch in 1 year had low values the following year. This finding can be explained by the phenomenon of alternate bearing to which Valencia oranges are subject. Trees have a high yield 1 year and a low yield the next (11,15,36). The yearly fluctuations in carbohydrate content did not show any significant effect on infection by the two *Phytophthora* species.

Parasitic nematode populations on the roots reduce the carbohydrate content (16) and, conversely, roots with high carbohydrate content support larger nematode populations (9). The nematode populations were not monitored, although it was obvious as the experiment progressed that increasing nematode populations was heavily damaging the feeder roots from the Riverside trees. Despite the lack of seasonal variability of carbohydrates, the *Phytophthora* species still showed seasonal patterns which suggests that root carbohydrates are not the main cause of seasonal infection by *P. citrophthora* and *P. nicotianae* var. *parasitica*.

Citrus seedlings grown under conditions of varying heat and light in the greenhouse and in growth chambers tend to display more symptoms of infection by *P. citrophthora* than *P. nicotianae* var. *parasitica*. The roots of seedlings infected with *P. citrophthora* contained more *Phytophthora* mycelium and had more visible signs of root damage than those infected with *P. nicotianae* var. *parasitica*, except when grown at 30°C. However, citrus seedlings in the greenhouse are still growing in a juvenile mode, and they lack the seasonal changes in physiology such as carbon partitioning in the roots that occurs in mature trees. *P. citrophthora* infestation also caused greater differences in carbohydrate levels in seedling roots than *P. nicotianae* var. *parasitica*. *P. nicotianae* var. *parasitica* is generally dormant in southern California as winter soil temperatures drop below the minimum level needed to sup-

port growth. This was supported by the findings that the pathogen did not significantly alter root carbohydrate content at lower temperatures.

When the changes in root carbohydrate content caused by *Phytophthora* infection were examined, it was found that both *P. nicotianae* var. *parasitica* and *P. citrophthora* were able to grow in citrus roots and extract carbohydrates at the temperature range tested (16 to 30°C). However, *P. citrophthora* was able to extract more starch than *P. nicotianae* var. *parasitica* at 16°C, whereas *P. nicotianae* var. *parasitica* was able to extract more glucose than *P. citrophthora* at 30°C. Also, *P. citrophthora* was unable to extract starch from roots at 30°C, but *P. nicotianae* var. *parasitica* was able to extract glucose at 16°C. The sucrose metabolism of citrus seedling roots by the two species of *Phytophthora* was similar to that of glucose. Both sugars are photosynthetic products of the host plants, and the amount present in the roots is dependent on the amount of light available. *Phytophthora nicotianae* var. *parasitica* removed less sucrose than *P. citrophthora* at 16°C and more of the sugar.

These results suggest that although each organism is ecologically suited to its niche, *P. nicotianae* var. *parasitica* is a parasite of summer roots when glucose is plentiful and *P. citrophthora* is a parasite of winter roots when starch is plentiful. However, the carbohydrate content in the roots is not the cause of the seasonality of the fungal infections. This finding is corroborated by the fact that both species of *Phytophthora* grow equally well in amylopectin or glucose throughout most of their temperature range (7).

In conclusion, there are clear seasonal variations in the degree of infection caused by *P. nicotianae* var. *parasitica* and *P. citrophthora* on excised roots despite the fact that roots were under identical temperature conditions in the laboratory. *P. citrophthora* infection was highest on the excised winter roots, whereas *P. nicotianae* var. *parasitica* infection was greatest on summer roots. Temperature therefore, is eliminated as a primary cause of seasonal variation in *Phytophthora* infection. Glucose, sucrose, and starch content of roots were also not correlated with the seasonality of infection. These factors suggest that either some other aspect of root physiology, such as the secretion of a phytoalexin or rhizosphere microorganisms, are affecting the ability of these two pathogens to infect citrus roots. Although *P. citrophthora* uses more starch at low temperatures and *P. nicotianae* var. *parasitica* uses more glucose at higher temperatures, this is thought to be more a consequence of the two organisms using the resources available in their winter and summer niches than a cause for their seasonality.

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