Characterization of *Phytophthora* spp. Causing Outbreaks of Citrus Brown Rot in Florida

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ABSTRACT

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Epidemics of citrus brown rot from 1994 to 1997 in the south-central and east-coast citrus areas of Florida were characterized and the causal *Phytophthora* spp. identified. Two species of *Phytophthora*, *P. palmivora* and *P. nicotianae*, were consistently associated with brown rot. Epidemics caused by *P. palmivora* appeared to be initiated on immature fruit dropped on the orchard floor. The soilborne fungus infected and sporulated on these fruit and was then disseminated to fruit above 1 m in the canopy. In contrast, infection by *P. nicotianae*, the common cause of root rot, was confined to the lowest 1 m of the canopy. Fruit infected by *P. palmivora* produced large amounts of ellipsoidal sporangia available for

splash dispersal, whereas those infected by *P. nicotianae* produced far fewer spherical sporangia. Isolates from brown rot epidemics were compared with *P. nicotianae* from citrus in Florida and Texas, *P. citrophthora* in California, *P. palmivora*, and selected *Phytophthora* spp. from other hosts. Brown rot symptoms produced by the different pathogenic citrus isolates on inoculated fruit were indistinguishable. Morphology, mating behavior, and isozyme patterns of brown rot isolates from 1988 to 1997 matched *P. palmivora* from citrus roots, other host plants, and other locations, but were different from characterized isolates of *P. citrophthora* in California and *P. nicotianae* in Florida and Texas. Cellulose acetate electrophoresis of the isozyme glucose-6-phosphate isomerase rapidly identified the causal citrus pathogen from infected fruit and soil isolation plates. Although *P. palmivora* is an aggressive pathogen of citrus roots, bark, and fruit, populations in orchard soils were low compared with *P. nicotianae*.

Brown rot epidemics of early-season orange varieties have occurred sporadically in the fall in the east-coast and south-central citrus areas of Florida since the early 1950s (9,17). Epidemic seasons are commonly associated with periods of prolonged wetness exceeding 7 days and with temperatures in the diurnal range of 23 to 32°C coinciding with the maturation (i.e., color development) of early- and mid-season oranges and grapefruit (5,6,9). Under these circumstances, fruit infected by *Phytophthora nicotianae* Breda de Haan (synanamorph *P. parasitica* Dastur), the common causal agent of foot rot and root rot in citrus orchards, is restricted to the lower portion of the canopy below 1 m in height. *P. nicotianae* is a minor brown rot pathogen because it produces few sporangia on the fruit surface, and thus, propagules are not available to be splashed to fruit higher in the tree.

Whiteside (18) reported on the factors related to the restricted occurrence of brown rot during September to October 1968 and 1969, caused by a *Phytophthora* sp. that spread high (>1 m) into the tree canopy and rapidly produced abundant sporangia on infected fruit. The causal fungus from several orchards was identified as *P. citrophthora* (R. E. Smith & E. H. Smith) Leonian. The restricted nature of the epidemics was attributed to the sporadic occurrence of prolonged rainfall events in the fall when early- and mid-season varieties are susceptible to infection (18). The limited distribution of *P. citrophthora* also explained why the outbreaks were confined to certain orchard locations, even though susceptible varieties and favorable conditions for disease occurred in other regions of the industry.

In fall 1994 and 1995, outbreaks of a *Phytophthora* sp. resembling *P. palmivora* (Butler) Butler were reported in certain locations throughout the south-central and coastal regions of Florida

(6). In one case, in the aftermath of Hurricane Gordon, several hundred hectares were affected with losses up to 90%, while more typical losses were in the 30% range over areas of 10 to 20 ha. Although *P. palmivora* has been recovered from soil in Florida in two east-coast locations (20), this *Phytophthora* sp. was previously only reported as a widespread citrus pathogen in the warm tropics, but with no supporting data (8). In greenhouse and in vitro studies, Zitko and Timmer (19) demonstrated that *P. palmivora* was a more aggressive and competitive pathogen of citrus roots, stems, and fruit tissues than was *P. nicotianae*.

The purpose of this study was to elucidate the cause of epidemics of brown rot in Florida from 1994 to 1997. The primary objective was to isolate and characterize *Phytophthora* spp. from several brown rot epidemics using morphological methods, pathogenicity and symptoms on citrus fruit, mating behavior, and cellulose acetate electrophoresis (CAE) for isozyme analysis. In addition, the distinguishing features between the epidemiology of *P. palmivora* and *P. nicotianae* in orchards with a history of brown rot epidemics are described together with a diagnostic method to rapidly identify which *Phytophthora* sp. caused the outbreak.

MATERIALS AND METHODS

Isolations from brown rot-affected fruit. From September to December 1994, fruit of Hamlin sweet orange (*Citrus sinensis* (L.) Osbeck) with a light brown, leathery decay was sampled from eight brown rot epidemics in Polk County in the central region of the Florida citrus industry and in Hardee, Desoto, and Hendry counties in the south-central region. In January 1995, white grapefruit (*C. paradisi* Macf.) with postharvest brown rot was obtained from a packinghouse in Indian River County on the east coast. From August to October 1995, an additional 22 orchards of Hamlin orange, one orchard of Temple tangor (*C. reticulata* Blanco hybrid), and blocks of Hamlin, Midsweet, and Pineapple sweet orange in the same location were sampled in Polk, Highlands, and Hardee counties. From June to November 1995, daily rainfall measure-

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ments were recorded in five of the orchards in southern Polk and Highlands counties where the first symptoms of disease were confirmed by fungal isolations from fruit.

Isolations of *Phytophthora* spp. from symptomatic fruit were made by placing pieces of the affected fruit rind without surface-sterilization onto plates of selective PARPH (pimaricin-ampicillin-rifampicin-pentachloronitrobenzene-hymexazol) agar modified as previously described (15). Isolation of *Phytophthora* spp. was also accomplished by placing one to three fruit in a resealable plastic bag with sterile distilled water and shaking the bag to wash the sporangia from the fruit surface. The rinse water was examined under the light microscope for the presence of sporangia, and sporangial characteristics such as the abundance, shape, and length-to-breadth ratio were noted. The rinse water was then spread directly on modified PARPH medium. In general, five isolates of *Phytophthora* spp. were collected from each location.

Isolations from fallen fruit and soil. Isolations of *Phytophthora* spp. from symptomless green fruit (trap fruit) or from soil were made in a heavily diseased orchard in southern Polk County (Bereah) from 1995 to 1997. In September 1995 and August 1997, immature green fruit of Hamlin orange approximately 4 cm in diameter was used to trap *Phytophthora* spp. from the soil in the orchard. Symptomless fruit were placed on the ground under trees with brown rot symptoms on the lower 1 m of the canopy in a 10-ha area of the orchard. After 72 h, 10 fruit each from seven locations in the orchard were combined in a resealable plastic bag and transported immediately to the laboratory. The fruit-washing method described above was used for isolation of *Phytophthora* spp., except that the bags were placed on a rotary shaker at 100 rpm for 1 h prior to plating the rinse water on PARPH.

In July and September 1996 and 1997, before and after the appearance of brown rot in the orchard, 22 100-cm³ soil samples were combined into resealable plastic bags for eight 0.1-ha locations within the same orchard. For isolations, subsamples of soil were spread onto PARPH medium using procedures previously developed for estimating populations of *P. nicotianae* (15). After a 3-day incubation at 27°C, colonies that were smaller and more compact than *P. nicotianae* were counted and isolated for further study.

Mating and pathogenicity tests. Cultures were maintained and fungal structures were produced on clarified V8 juice agar (CV8-A) or broth (CV8-B) as described by Mitchell et al. (11). For sporangium production, 2-day-old cultures in CV8-B were washed with sterile distilled water and incubated for two additional days at room temperature. Cultures were chilled for 15 min at 5°C and then returned to room temperature to induce zoospore production. Chlamydospores were produced by the method of Tsao (16) and separated from mycelium by repeated blending and low-speed centrifugation for observations.

Matings were conducted on CV8-A in the dark at 27°C by pairing isolates from brown rot epidemics with isolates of *P. citrophthora* from citrus, with previously characterized A1 and A2 mating types of *P. palmivora* from citrus and cacao, and with *P. nicotianae* from citrus (Table 1) (20).

For fruit inoculations, mature Valencia sweet orange fruit were collected from the field, rinsed with water, and surface-disinfested by wiping with 70% ethanol. Fruit were placed in closed plastic chambers on wire mesh stands to suspend them above a 2-cm-deep water reservoir. Five 0.05-ml droplets containing 10⁵ zoospores per ml were placed on the surface of each fruit without wounding. Chambers were incubated in the laboratory at 25 to 27°C for 7 days. The number of fruit with brown rot symptoms was counted, and re-isolations for identification of the fungus from infected fruit tissue were performed as described in the previous and following sections.

CAE. A CAE method developed by Goodwin et al. (2) was used for isozyme analysis with modifications. Sterilized cellophane disks were placed on the surface of corn meal agar (CMA) and incubated for 1 day to insure that no bacterial contamination occurred before seeding of the cellophane surface of the agar with *Phytophthora* spp. The CMA cultures were grown at 27°C for 3 to 4 days prior to scraping the mycelium from the surface with a spatula for transfer to a microcentrifuge tube. The mycelium was ground for 30 s with a tissue homogenizer that closely fit the contours of the microcentrifuge tube and then frozen at -5 or -80°C for short- and long-term storage, respectively. Several different enzyme and running buffer combinations were tested with 45 to 85 min run times: glucose-6-phosphate dehydrogenase, glucose-6-phosphate iso-

TABLE 1. *Phytophthora* spp. isolated from citrus in Florida, California, Texas, and from other hosts

Isolate	Species	Origin and isolation date	Host/source	Reference
P29	<i>P. palmivora</i>	Florida 9/94	Citrus fruit	This study
P44b	<i>P. palmivora</i>	Florida 10/94	Citrus fruit	This study
P48	<i>P. palmivora</i>	Florida 1/95	Citrus fruit	This study
P67-1	<i>P. palmivora</i>	Florida 8/95	Citrus fruit	This study
P99-59-1	<i>P. palmivora</i>	Florida 9/95	Citrus fruit	This study
P101-16	<i>P. palmivora</i>	Florida 9/96	Citrus fruit	This study
P107-9	<i>P. palmivora</i>	Florida 2/97	Citrus soil	This study
EB-1	<i>P. palmivora</i>	Florida 9/88	Citrus fruit	Literature citation 18
EB-3	<i>P. palmivora</i>	Florida 9/92	Citrus fruit	G. E. Brown
Plt B	<i>P. palmivora</i>	Florida 10/88	Citrus soil	Literature citation 18
Sh-I	<i>P. palmivora</i>	Florida 12/89	Citrus soil	Literature citation 18
PL-10	<i>P. palmivora</i>	Costa Rica 12/92	Cacao pod	D. J. Mitchell
P66	<i>P. palmivora</i>	Florida 10/92	Milkweed <i>Morrenia odorata</i>	Literature citation 1, ATCC 52158
Dd-1	<i>P. nicotianae</i>	Florida 1/91	Citrus soil	L. W. Timmer
Riv-1	<i>P. nicotianae</i>	Florida 6/88	Citrus soil	L. W. Timmer
P101-2	<i>P. nicotianae</i>	Florida 9/96	Citrus fruit	This study
P95-01	<i>P. nicotianae</i>	Texas 1995	Citrus soil	M. Skaria
P92-19	<i>P. nicotianae</i>	Texas 1992	Citrus soil	M. Skaria
P91-28	<i>P. nicotianae</i>	Texas 1991	Citrus foliage	M. Skaria
M140	<i>P. citrophthora</i>	California 1988	Citrus soil	J. A. Menge
M189	<i>P. citrophthora</i>	California 1990	Citrus roots	J. A. Menge
M259	<i>P. citrophthora</i>	California 1993	Citrus bark	J. A. Menge
M263	<i>P. citrophthora</i>	California 1994	Citrus bark	J. A. Menge
M214	<i>P. citricola</i>	California 1991	Avocado	J. A. Menge
M265	<i>P. citricola</i>	California 1994	Avocado	J. A. Menge
M224	<i>P. citricola</i>	California 1991	Alfalfa	J. A. Menge
M256	<i>P. cactorum</i>	California 1993	Not determined	J. A. Menge
PA3-1	<i>P. arecae</i>	Florida 1986	Bamboo palm <i>Chamaedorea</i> hybrid	Literature citation 14, ATCC 64558
P94-15B	<i>P. capsici</i>	New Mexico 1994	Chili pepper	M. Skaria

merase, isocitrate dehydrogenase, hexokinase, malate dehydrogenase, malate dehydrogenase NADP⁺, mannose-6-phosphate isomerase, peptidase, and phosphoglucosyltransferase. The running buffers and reagents were as recommended by Hebert and Beaton (7). Electrophoretic phenotypes were identified by measuring the distance, in millimeters, of migration from the loading well for a selected band for each enzyme. Setting that distance as 100, other bands were designated by dividing their distance of migration by the selected band and multiplying the ratio by 100.

RESULTS

Description of brown rot epidemics. Outbreaks of brown rot in 1994 and 1995 were most prevalent from August to October in the south-central area of the industry in orchards surrounded by native oak, pine, and palm hammocks that are seasonally flooded. The orchards were usually level and often supported trees more than 5 m in height that formed hedge rows that restricted air movement. In late August and early September 1995, epidemics in five orchards of 20- to 40-year-old Hamlin orange trees occurred after 9 to 12 rain events of at least 2.5 mm in 21 days prior to brown rot symptoms (data not shown). Subsequent epidemics in the same locations in late September and early October 1995 appeared after five to seven rain events over 7- to 10-day periods. Rainfall per event often, but not always, increased prior to the epidemic.

Fruit with brown rot collected more than 1 m high in the tree canopy produced high densities of monopapillate, ellipsoidal sporangia from the surface, whereas fruit from brown rot confined to below 1 m in height in the canopy formed a few, spherical sporangia on the surface. *Phytophthora* spp. isolated from brown rot distributed higher in the tree produced small compact colonies with sporangia present on PARPH medium 3 days after isolation. Colonies from fruit in locations where brown rot was distributed low in the

canopy were fast growing with no sporangia produced on PARPH medium after 3 days.

Isolations from fallen fruit and soil. In September 1995 and August 1997 at Bereah, four of seven and four of six samples of asymptomatic green fruit yielded *Phytophthora* spp. when collected 72 h after they were placed on the orchard floor. *P. palmivora* and *P. nicotianae* were each recovered from two of the four fruit samples positive for *Phytophthora* spp. at both sampling times. In August 1996, before brown rot symptoms were observed in the orchard, *P. palmivora* was not recovered from the soil. However, soil populations of *P. nicotianae* were high (60 propagules per cm³). In October 1996, brown rot was present at low levels and *P. nicotianae* populations were lower (15 propagules per cm³) compared with populations in August. *P. palmivora* was recovered from four of eight soil samples, ranging from 1 to 4 propagules per cm³. In July and August 1997, *P. palmivora* was detected in 3 of 22 soil samples on each occasion, ranging from 2 to 14 propagules per cm³. Soil populations of *P. nicotianae* were significantly (*t* test, *df* = 15, *P* ≤ 0.05) lower in 1997 (11 propagules per cm³) than in 1996.

Morphological and pathological characterization. When cultured in CV8-B, isolates from brown rot collected more than 1 m high in the tree canopy yielded sporangia that were highly caducous and ellipsoidal with a length/breadth (L/B) ratio of 1.6 to 1.8 and readily produced chlamydospores in submerged broth cultures at 18°C. Sporangia from isolates recovered from brown rot distributed near the soil surface were spherical, noncaducous, and had a L/B of 1.2 to 1.3 in CV8-B (P101-2) (Table 2). Isolates with spherical sporangia were indistinguishable morphologically from *P. nicotianae*, the species commonly associated with root rot and foot rot of citrus in Florida (5,6). Isolates with ellipsoidal sporangia from 32 of the 36 brown rot epidemics surveyed were similar to soilborne isolates from orchards on the east coast collected in 1988 and 1989 and identified as *P. palmivora* (Table 2) (20). Florida brown rot

TABLE 2. Morphological characteristics, mating behavior, and isozyme phenotypes for glucose-6-phosphate isomerase (GPI) and malate dehydrogenase (MDH) of *Phytophthora* spp. isolated from citrus in Florida, Texas, California, and from other hosts

Isolate	Species	Sporangial characteristics	Mating type ^a	Isoenzyme phenotypes ^b	
				GPI	MDH
P29	<i>P. palmivora</i>	Ellipsoid, caducous	A1	97/106/112	108/120
P44b	<i>P. palmivora</i>	Ellipsoid, caducous	A1	97/106/112	108/120
P48	<i>P. palmivora</i>	Ellipsoid, caducous	A1	97/106/112	108/120
P67-1	<i>P. palmivora</i>	Ellipsoid, caducous	A1	97/106/112	108/120
P99-59-1	<i>P. palmivora</i>	Ellipsoid, caducous	A1	97/106/112	108/120
P101-16	<i>P. palmivora</i>	Ellipsoid, caducous	A1	97/106/112	108/120
P107-9	<i>P. palmivora</i>	Ellipsoid, caducous	A1	97/106/112	108/120
EB-1	<i>P. palmivora</i>	Ellipsoid, caducous	A1	97/106/112	108/120
EB-3	<i>P. palmivora</i>	Ellipsoid, caducous	A1	97/106/112	108/120
Plt B	<i>P. palmivora</i>	Ellipsoid, caducous	A1	97/106/112	108/120
Sh-1	<i>P. palmivora</i>	Ellipsoid, caducous	A1	97/106/112	108/120
PL-10	<i>P. palmivora</i>	Ellipsoid, caducous	A2	97/106/112	108/120
P66	<i>P. palmivora</i>	Ellipsoid, weakly caducous	A2	97/106/112	108/120
Dd-1	<i>P. nicotianae</i>	Spherical, noncaducous	A1	100	100/112
Riv-1	<i>P. nicotianae</i>	Spherical, noncaducous	A2	100	100/112
P101-2	<i>P. nicotianae</i>	Spherical, noncaducous	A1	100	100/112
P95-01	<i>P. nicotianae</i>	Spherical, noncaducous	A2	100	100/112
92-19	<i>P. nicotianae</i>	Spherical, noncaducous	ND	100	100/112
P91-28	<i>P. nicotianae</i>	Spherical, noncaducous	A1	100	100/112
M140	<i>P. citrophthora</i>	Highly variable, weakly caducous	...	94/107	104/116
M189	<i>P. citrophthora</i>	Highly variable, weakly caducous	...	94/107	104/116
M259	<i>P. citrophthora</i>	Highly variable, weakly caducous	...	94/107	104/116
M263	<i>P. citrophthora</i>	Highly variable, weakly caducous	...	94/107	104/116
M214	<i>P. citricola</i>	Ovoid-obpyriform, noncaducous	H	65	100/112
M265	<i>P. citricola</i>	Ovoid-obpyriform, noncaducous	H	65	100/112
M224	<i>P. citricola</i>	Ovoid-obpyriform, noncaducous	H	94	96/114
M256	<i>P. cactorum</i>	Ovoid, caducous	ND	97	94/111
PA3-1	<i>P. arecae</i>	Ovoid-obpyriform, caducous	A1	108	108/120
P94-15B	<i>P. capsici</i>	Ellipsoid, noncaducous	ND	106	104/114

^a ND = not determined, and H = homothallic.

^b Electrophoretic phenotypes were identified by measuring the distance, in millimeters, of migration from the loading well for a selected band for each enzyme. Setting that distance as 100, other bands were designated by dividing their distance of migration by the selected band and multiplying the ratio by 100.

isolates also closely resembled *P. palmivora* isolates from cacao (*Theobroma cacao*) in Costa Rica (PL10) and milkweed vine (*Morrenia odorata*) in Florida (P66). The latter *P. palmivora* isolate, now marketed as a biological control agent (Devine) for milkweed vine, was originally identified as *P. citrophthora* (1).

Sporangia from brown rot isolates did not resemble *P. citrophthora* isolates from California, which were weakly caducous and highly variable in shape, size, and L/B ratio (1.2 to 1.9) (Table 2). *P. citrophthora* isolates produced bipapillate as well as monopapillate sporangia in CV8-B cultures, but did not form chlamydospores in submerged culture. Although isolates from brown rot epidemics prior to 1994 were morphologically similar to *P. palmivora*, in Florida they were routinely identified as *P. citrophthora* (e.g., EB-1, EB-3) (cf. 18) as reported for epidemics in the 1960s (18).

Pathogenicity of putative *P. palmivora* isolates was confirmed by zoospore inoculation of Valencia orange fruit. Disease symptoms were the same as those produced by isolates of *P. nicotianae* from Florida (Dd-1, Riv-1) and Texas (P91-28), and *P. citrophthora* isolates from California (M259, M189, and M263) (Table 1). *P. palmivora* isolates from cacao and milkweed vine were not pathogenic to fruit after repeated inoculation attempts. All putative *P. palmivora* isolates from Florida brown rot were A1 and mated readily with the A2 isolates from cacao and milkweed vine, as well as with A2 isolates of *P. nicotianae* (Table 2). Isolates of *P. citrophthora* failed to produce oospores in pairings with other species of either mating type or among themselves.

CAE. The CAE method for isozyme analysis identified each morphological species examined as a unique electrophoretic type, with only a few exceptions (Table 2). Several different enzyme and running buffer combinations were tested, with the following producing adequate activity after 45- to 85-min run times: glucose-6-phosphate isomerase (GPI) in Tris glycine buffer and malate dehydrogenase (MDH) in citric acid-4-(3-aminopropyl) morpholine buffer. Other enzyme-buffer combinations failed to give adequate activity or resolution consistently. The three citrus pathogens produced distinctive banding patterns with GPI (Fig. 1). The single band produced by *P. nicotianae* was used as the standard to calculate the pattern phenotypes for species identification purposes. *P. palmivora* produced three bands similar to that reported for *P. infestans* (2): one more intense band between the other two bands. In the case of *P. citrophthora*, the pattern was a primary band and secondary band of lesser intensity.

The same comparisons were made for the isoenzyme patterns produced by MDH (Table 2). The results for the two-enzyme analysis were reinforcing. Each *Phytophthora* sp. produced a unique migration distance or banding pattern for each enzyme, with two exceptions (Table 2, Fig. 2). The MDH phenotype for *P. palmivora* was similar to the two isolates of *P. arecae* from bamboo palm (*Chamaedorea hybrid*), and *P. nicotianae* was similar to the two *P. citricola* isolates from avocado (Tables 1 and 2).

The GPI and MDH patterns for brown rot isolates collected from 1995 to 1997 were indistinguishable from each other and from isolates of *P. palmivora* from citrus soil and milkweed vine

in Florida (Tables 1 and 2) and cacao in Costa Rica (Fig. 1). Furthermore, isolates from 1988 and 1992 epidemics of brown rot in Florida produced the same pattern as isolates from more recent ones (Table 1). The patterns for isolates of *P. nicotianae* from citrus soil and foliar epidemics in Texas were the same as for isolates of *P. nicotianae* from soil and fruit in Florida (Figs. 1 and 2). The patterns for *P. citrophthora* from citrus roots and bark in California were identical and distinct from *P. palmivora* and *P. nicotianae* (Figs. 1 and 2). Other *Phytophthora* spp. that do not attack citrus but are found in the same agricultural areas of California, Florida, and Texas produced phenotypes unique from the citrus pathogens, except as noted above (Fig. 2).

The utility of CAE as a rapid diagnostic tool was demonstrated for isolations from both fruit and soil. Fruit from inoculation tests with mycelial growth on the surface was scraped and the mycelium transferred to a microcentrifuge tube. The samples from *P. nicotianae*-, *P. palmivora*-, and *P. citrophthora*-infected fruit produced the same patterns as the isolates used for inoculations and were easily distinguished from one another (Figs. 1 to 3). Isolates P66 and PL10 that did not produce symptoms on fruit also did not give a detectable GPI pattern from an extract of the rind at the inoculation site. Single colonies on isolation plates were cut out from the agar to evaluate CAE for diagnosis of unknown colonies. Colonies that were compact and dense (*P. palmivora* phenotype) were compared with colonies from the same plates that were diffuse and faster growing (*P. nicotianae* phenotype). The GPI patterns were compared with culture-produced mycelium of each species on the same gels. The patterns were true to type for each *Phytophthora* sp. and readily distinguishable.

DISCUSSION

P. palmivora has been reported as a pathogen of citrus in the warm tropics of the Americas, but is not widely recognized as an important problem (8). Conditions for brown rot epidemics in Florida in 1994, 1995, and 1997 were cloudy, periodically wet

Fig. 2. Cellulose acetate electrophoresis of the isozyme glucose-6-phosphate isomerase for citrus pathogens in Florida, California, and Texas compared with other papillate *Phytophthora* spp. from the same regions. Lane 1 is *P. palmivora* isolate P48; lane 2 is *P. nicotianae* isolate Dd-1; lanes 3 to 5 are isolates *P. citrophthora* M259, M189, and M263; lanes 6 and 7 are *P. nicotianae* isolates P91-28 and P92-19; lane 8 is *P. cactorum*; lanes 9 and 10 are *P. citricola* M214 and M265 from avocado; lane 11 is *P. citricola* M224 from alfalfa; and lane 12 is *P. capsici* P94-15B from chili pepper.

Fig. 1. Cellulose acetate electrophoresis of the isozyme glucose-6-phosphate isomerase for brown rot isolates from Florida compared with isolates of *Phytophthora nicotianae* from Florida and Texas, isolates of *P. citrophthora* from California, and *P. palmivora* from another location and host plant. Lanes 1 to 3 are *P. nicotianae* isolates Dd-1, P101-2, and P95-01; lanes 4 and 5 are *P. citrophthora* isolates M259 and M189; lanes 6 to 11 are *P. palmivora* isolates P44b, P48, P67-1, P99-59-1, P101-16, and P107-9; and lane 12 is *P. palmivora* isolate PL-10 from cacao in Costa Rica.

Fig. 3. Cellulose acetate electrophoresis of the isozyme glucose-6-phosphate isomerase for mycelium scrapped from the surface of brown-rotted fruit of Valencia orange inoculated with citrus pathogens. Lanes 1 and 2 are *Phytophthora nicotianae* isolates Riv-1 and Dd-1; lanes 3 to 5 are *P. citrophthora* isolates M259, M189, and M140; and lanes 6 to 10 are *P. palmivora* isolates P99-59-1, P29, P48, EB-1, and P67-1.

weather with temperatures ranging from 23 to 32°C in late summer to late fall, coincident with the final stages of fruit expansion and development of a colored rind. At an optimum temperature of 27 to 30°C, infection by *P. palmivora* required only 1 to 3 h of moisture on the surface of fruit (L. W. Timmer and S. E. Zitko, unpublished data). Favorable conditions for fruit infection in late August and early September were 9 to 12 rain events for approximately 3 weeks prior to the development of brown rot symptoms in orchards with poor air movement. Epidemics recurred in the same locations in late September and early October after fewer rain events within shorter periods. Rainfall per event often, but not always, increased prior to the epidemic, confirming that duration and periodicity of fruit wetness were important for brown rot development (9). Diseases appeared to spread laterally if infected fruit did not fall to the ground but remained hanging on the tree as mummies late in the fall (J. H. Graham, unpublished data).

Brown rot epidemics have occurred sporadically in localized areas in the south-central and east-coast production areas since the 1950s and 1960s, but not until severe outbreaks from 1994 to 1997 in the same regions was there further investigation of the causal *Phytophthora* spp. Whiteside (18) identified isolates from brown rot high in the tree canopy as *P. citrophthora*, distinct from *P. nicotianae*, the cause of root rot and fruit infection near the ground. In this study, we confirmed that the cause of recent epidemics was not commonly occurring *P. nicotianae*, but *P. palmivora*, a newly reported species causing brown rot in Florida. *P. palmivora* was first detected in Florida from soil samples in two east-coast orchards by Zitko et al. (20). They concluded that the fungus was restricted in occurrence in Florida citrus orchards, but had the potential to become a more serious pathogen of bark and fruit because it produced more severe infection of fibrous roots more rapidly than *P. nicotianae* (19). Similarities of location, severity, and restricted occurrence between the current and earlier outbreaks of brown rot in Florida (9,17) suggest that earlier epidemics may have been caused by *P. palmivora*, rather than *P. citrophthora* as previously reported (18). *P. citrophthora* has yet to be associated with recent epidemics and confirmed in Florida orchard soils.

The morphology, mating behavior, and CAE isozyme analysis of brown rot isolates from 1988 to 1997 matched previously identified isolates of *P. palmivora* from citrus roots, other host plants, and other locations, but differed from *P. citrophthora* in California and *P. nicotianae* in Florida and Texas. The isozyme GPI provided optimum activity and resolution with small quantities of mycelium and gel run times of 45 to 75 min. Since the number of genetic loci that specify the isozymes for each fungal genotype is unknown, the complex banding patterns for some of the *Phytophthora* spp. cannot be strictly interpreted. GPI is a dimeric enzyme in *P. infestans* (2). Homozygous genotypes form a single band (e.g., *P. nicotianae*) (Fig. 1), whereas heterozygous genotypes have three bands: two homodimer bands and one heterodimer band of twice the intensity halfway between the other two bands (e.g., *P. palmivora*) (Fig. 1). The three citrus pathogens produced patterns of one to three bands with GPI, whereas the isolates produced less distinctive patterns of two bands with MDH.

Oudemans and Coffey (12) and Mchau and Coffey (10) analyzed up to 19 different isoenzyme loci, including GPI and MDH, to conclude that *P. palmivora* and *P. nicotianae* had the lowest levels and *P. citrophthora* a much higher level of genetic diversity among 12 papillate species of *Phytophthora* studied. Oudemans and Coffey (12) designated the isolate of *P. palmivora* from milkweed vine included in our study as electrophoretic type (ET) 4. Although *P. palmivora* is polymorphic at the GPI locus, ET4 was the most commonly occurring ET among the 100 isolates analyzed. Thus, citrus isolates of *P. palmivora* are the same ET as the majority of isolates from subtropical and tropical woody plant hosts (12). Citrus isolates of *P. palmivora* are distinguishable by GPI phenotype from isolate PA3-1 of *P. arecae* from bamboo palm in south Florida, as predicted from the earlier isozyme study that included the same

isolate (12). The isolate of *P. arecae* from "citrus soil" evaluated in that study was *P. arecae* isolate PA3-1 (14), isolated from an artificially infested soil from a citrus inoculation trial conducted by Zitko et al. (20), and thus does not represent a citrus isolate of this species. The distinct GPI phenotypes for avocado and alfalfa isolates of *P. citricola* from California indicate that use of one or two isozyme loci for species identification should proceed with caution. Until more representative worldwide populations of *Phytophthora* spp. from citrus are evaluated, isozyme analysis should only be applied for analysis of Florida isolates of citrus pathogens.

Because isolates of *P. palmivora* in Florida were monomorphic at the chosen isozyme locus, CAE with GPI provided a rapid, inexpensive, and unambiguous diagnosis of the causal *Phytophthora* spp. of brown-rotted fruit. Mycelium scraped from the surface of one or two infected fruit held overnight in a resealable plastic bag was sufficient to extract for analysis of GPI activity. The GPI patterns for the three major citrus pathogens were very different and distinct from other papillate *Phytophthora* spp. that might be present on other hosts in citrus-growing regions. This diagnostic capability was applicable when brown rot caused by *P. palmivora* was in the early stages (less than 1 m high in the canopy) and the symptoms were indistinguishable from those caused by *P. nicotianae*. Also, washing the fruit surface to recover high numbers of ellipsoidal sporangia versus low numbers of spherical sporangia was useful for quick diagnosis, although shape of sporangia from fruit was quite variable. Otherwise, isolation of pure cultures from fruit for sporangium production required 2 weeks to make definitive observations of morphology.

Likewise, CAE was applicable for accurate identification of *P. palmivora* colonies on soil isolation plates. Colony morphology of *P. palmivora* was compact compared with spreading, faster-growing colonies of *P. nicotianae* on PARPH selective medium. Ellipsoidal sporangia developed within 3 days with *P. palmivora*, whereas sporangium production by *P. nicotianae* was delayed and sparse. Individual colonies of each species were cut directly from PARPH agar plates and processed for detection of GPI.

P. citrophthora is the well-recognized pathogen of fruit and trunk bark in coastal, Mediterranean regions with wet, foggy winters and temperatures ranging from 18 to 25°C (6). These conditions are quite different from the reported temperature optima for the Florida brown rot and root rot isolates, which range from 23 to 30°C (18,20). At the early stage of conducive conditions in August and September, fruit that fell to the ground for various reasons appeared to be significant for buildup of inoculum prior to the first symptoms of brown rot in the tree. Fallen fruit acted as a trap to move *P. palmivora* to the aerial phase from the soil phase, where it existed at low incidence and populations relative to *P. nicotianae*. *P. palmivora* infected fallen fruit and developed sporangia within 48 to 72 h (L. W. Timmer and S. E. Zitko, unpublished data). When the fruit surface was impacted by rain drops falling from the canopy, sporangia had the potential to disperse. *P. nicotianae* did not disperse in this manner, though it was also isolated from fallen fruit. This species produced few sporangia on the surface of diseased fruit even after infection was well developed.

In Florida, fruit and foliage on the lower skirt of the canopy often hangs near the soil surface, yet fruit and leaf infection by *P. nicotianae* is not common, because surface soils are well drained and water does not puddle for prolonged periods. Most infection of fruit by *P. nicotianae* near the soil surface is due to splash of infested soil directly onto the fruit, which rarely exceeds 1 m in height. Thus, in Florida, the occurrence of brown rot symptoms above 1 m was a good indication that the causal fungus was *P. palmivora*.

In 1997, on the east coast near where *P. palmivora* was first isolated in Florida, *P. palmivora* and *P. nicotianae* were both present at very high populations in soil (>40 propagules per cm³). Both species were isolated from severe root rot on structural roots of citrus initially attacked by *Diaprepes abbreviatus*. Larvae of this root weevil, originally introduced into Florida from Puerto Rico in

the 1950s, deeply etch the root bark, which predisposes the structural roots to infection by *P. palmivora* as well as the more common *P. nicotianae* (4,13). In these situations, *P. palmivora* is a more aggressive and damaging pathogen than *P. nicotianae* alone, even breaking the resistance of the normally resistant rootstock Swingle citrumelo (3,4). These observations appear to support Zitko and Timmer's (19) contention that, even though *P. palmivora* is a more aggressive pathogen, it does not compete in the soil under most circumstances. That is, *P. palmivora* only appeared to compete well when the host roots were predisposed by severe wounding caused by larvae of *D. abbreviatus*. Elsewhere in brown rot-affected orchards, *P. palmivora* existed at low populations on fibrous roots, presumably in competition with *P. nicotianae*, until it was baited out of soil and spread to fruit under conducive conditions for sporulation and dispersal. Another suggestion, by Zitko et al. (20), for the localized occurrence of *P. palmivora* was that the pathogen is introduced into orchards from native vegetation such as hammocks of palms, but has not yet infested nursery sites where much wider distribution in the industry may occur.

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