Comparison of ELISA Techniques and Standard Isolation Methods for *Phytophthora* Detection in Citrus Orchards in Florida and California

L. W. TIMMER, University of Florida, IFAS, CREC, Lake Alfred 33850; J. A. MENGE, University of California, Riverside 92521; S. E. ZITKO, University of Florida, IFAS, CREC, Lake Alfred 33850; E. POND, University of California, Riverside 92521; S. A. MILLER, Agri-Diagnostics Associates, Cinnaminson, NJ 08077; and E. L. V. JOHNSON, University of California, Riverside 92521

ABSTRACT

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

Commercial enzyme-linked immunosorbent assay (ELISA) kits were evaluated for detection of *Phytophthora* spp. in fibrous roots and soil in citrus orchards in Florida and California, and compared to root rot evaluations, percent infection in root pieces, and dilution plating of soil on selective media. In Florida, root ELISA values with kits D and E were correlated with percent root infection and propagule densities of *Phytophthora parasitica* on an orchard basis. In California, root ELISA values with kit D were correlated with propagule densities of *Phytophthora citrophthora* on an orchard basis. Kit D was more effective than kit E for the detection of *Phytophthora* spp. in fibrous roots because of high background values with kit E. Soil ELISA values with kit E were not related to other measurements of *Phytophthora* activity. Variability of root ELISA values and propagule densities in single-core samples was of the same order of magnitude. Kit D was much less reactive to *P. citrophthora* hyphae than to *P. parasitica*. The kit D ELISA values from fibrous roots provided as effective a measure of *Phytophthora* activity as the currently used propagule densities from soil.

Phytophthora parasitica Dastur and *Phytophthora citrophthora* R.E. Sm. & E.H. Sm. are important pathogens of citrus worldwide, causing trunk and crown rots, fibrous root rot, and brown rot of fruit. Crown rot occurs below ground on trees on susceptible rootstocks, and foot rot and gummosis result from infections above the bud union. Fibrous root rot usually does not cause severe tree decline; but it reduces tree vigor, fruit size, and yield, even on many rootstocks resistant to bark infection (7,9,21,29). In Florida, where *P. parasitica* is the main pathogen, fungicide treatment with metalaxyl or fosetyl Al reduced soilborne populations of *Phytophthora* spp. and increased fibrous root density and yields (21,29). Similar results were reported from California, where *P. parasitica* (9,13) or *P. citrophthora* (23) were the main pathogen species. Treatment is costly and economically justified only where *Phytophthora* populations are high, the orchard is planted on a susceptible rootstock, soil conditions are conducive to root rot, and tree condition indicates significant fibrous root damage.

Originally, detection of *Phytophthora* spp. depended on the use of leaf and fruit baits (5,7). These methods could be used quantitatively (30) but were laborious and time-consuming. More recently, selective media and plating procedures have been developed that allow quantitative isolation of *Phytophthora* spp. from citrus soils (4,6,11,13,17,28). Populations can be estimated from a composite sample of 20–40 cores per 4-ha orchard. In California, *Phytophthora* populations are determined per gram of rhizosphere soil, and treatment of orchards is recommended when propagule densities in the rhizosphere exceed 15–20 propagules per gram (9). In Florida, methods differ because of the nature of the soils. No thresholds have been established, but generally 0–5 propagules per cubic centimeter has been considered low, 5–15 moderate, and more than 15 high (26).

Recently, enzyme-linked immunosorbent assay (ELISA) has been used ex-

Present address of S. A. Miller: Ohio State University, OARDC, Wooster 44691.

Florida Agricultural Experiment Station Journal Series R-02720.

Accepted for publication 8 April 1993.

tensively for the detection of soilborne pathogens (15,16). In some cases, the primary goal was to distinguish root rots with similar symptoms which are caused by different pathogens (12,19,20). Rapid detection allows immediate application of the most appropriate fungicide or other control measures. In other cases, the goal was to quantitate low levels of a pathogen or to detect the organism in the early stages to achieve better control (1,2,22). The ELISA systems for *Phytophthora* spp. based on monoclonal antibodies, as developed by Agri-Diagnostics Associates (Cinnaminson, NJ 08077), have proven to be specific and highly sensitive (1,2,12,16,19,20,22). These systems readily detect infections in roots (2,12) and have been used to detect zoospores in irrigation water (12) and low levels of *Phytophthora* in soil from soybean fields prior to planting (22).

In citrus, ELISA has been used successfully to detect *Phytophthora* in roots (24,25), but no attempt was made to quantitate infection. The purposes of the current study were the following: 1) to evaluate the utility of tissue kits D and E from Agri-Diagnostics Associates for detection of *Phytophthora* in roots, and of the soil kit E for detection in soil; 2) to compare the ELISA systems with other measures of *Phytophthora* activity such as root infection, percent healthy roots, and propagule density determinations of field samples on selective media; and 3) to determine the proper tissues and sample numbers to maximize the utility of the system as an aid in management decisions. A preliminary report of portions of this work has been presented (27).

MATERIALS AND METHODS

Comparison of detection techniques for *P. parasitica*. The level of activity of *P. parasitica* was evaluated in 19 citrus orchards in Florida from June to October 1990 by determining the percentage of root rot, the percentage of roots infected, and the propagules per cubic centimeter, and comparing them to various ELISA procedures. Five trees were selected arbitrarily from each orchard, and five soil cores of 930 cm^3 each were collected from each tree. Fibrous roots were screened from the five cores, and composite samples of roots and soil were prepared for each tree. To determine the percentage of root rot, 100 root tips were selected from each tree and rated as rotted or healthy. A total of 100 root pieces from each tree, about 1.0–1.5 cm long, were plated on PARPH medium developed by Kannwischer and Mitchell (6) and Mitchell et al (17) using the ingredient concentrations described by Timmer et al (28). Petri dishes were incubated for 3 days at 27 C, and the percentage of root pieces from which *P. parasitica* was recovered was determined. Propagule densities in the soil were determined by plating soil on the PARPH medium using the procedure described by Timmer et al (28). A single determination was made for each tree using five dishes per sample, and the results were expressed as propagules per cubic centimeter.

In California, samples of *P. citrophthora* were gathered between January and March 1988 from 21 orchards located throughout the citrus-producing regions of the state. Ten trees were selected for sampling in each orchard (4.5–18 ha). Two adjacent trees were sampled from each corner of the orchard and two from the middle. Samples of *P. parasitica* were taken from 217 individual trees from two orchards, the McKellar orchard (navel oranges [*Citrus sinensis* (L.) Osbeck] on rough lemon [*C. jambhiri* Lush.] rootstock), Visalia, California, and the Kiel orchard (navel orange on sweet orange rootstock) from Highland, California, from June to August in 1987 and 1988. Soil samples were collected from the wetted edge of the irrigation zone closest to the north side of individual citrus trees. Samples were obtained to a depth of 30 cm with a 7.5-cm-diameter bucket auger. The top 8 cm of soil was discarded. Each sample consisted of soil that was taken from two holes per tree, and only soil adhering to the fibrous roots (rhizosphere soil) was collected. Rocks and visible roots were excluded from the soil samples. Rhizosphere samples were placed into plastic bags and assayed as quickly as possible. If delays of more than 3 days were necessary, samples were stored at 15 C. A soil dilution assay (10) was performed to quantitate populations of *Phytophthora* by plating rhizosphere soil on PARPH medium (17). Propagule counts were determined from two subsamples of four dishes each, from two combined soil samples from each tree, and expressed as propagules per gram of rhizosphere soil. While soil samples were being assayed, root samples were taken for ELISA and for rhizosphere soil dilution assays. Two soil cores 2.5 cm diameter × 25 cm deep were removed from the same areas described above. Roots were separated from each combined soil sample by screening with a 2-mm screen. Roots were immersed in 1% (w/v) 2,3,5-triphenyl tetrazolium chloride for 24 hr at room temperature (22–25 C) in the dark. Root portions (with the exception of root tips) which stained pink, red, or red-brown were considered living. Yellow-brown roots or root portions were considered dead (8). Total length of roots, length of healthy roots, and percent healthy roots were determined by the line intersect method of Newman (18).

In Florida and California, ELISA procedures were carried out with D and E kits and procedures provided by Agri-Diagnostics Associates (kits are now available from Neogen Corporation, Lansing, MI 48912). Both kits are double antibody ELISAs containing a combination of polyclonal and monoclonal antibodies that are reactive with species in the genus *Phytophthora*. The specificities of these antibodies have been described previously (16). Specialized buffers for extraction of soil samples were provided in the kit.

For tissue ELISA, fibrous roots (those less than 2 mm in diameter) from each sample were cut into 1–2 cm pieces and mixed thoroughly; and 0.5 g, fresh weight, was placed in a resealable bag with 2.0 ml (1:5 dilution) of extraction buffer. In Florida, root pieces were triturated by passing a hand-held ball bearing grinder over the bag until the root cortex was reduced to a pulp. In California, root pieces were triturated to a dry powder with liquid N_2 with a porcelain mortar and pestle. The triturate was placed in a test tube and incubated for 30 min with 2 ml of kit-supplied extract buffer. The triturate was transferred to a microfuge tube and centrifuged at approximately 12,000 rpm for 5–10 min. For each sample, 100 µl of the supernatant was dispensed into a well of a 96-well immunoassay plate.

For soil ELISA, 80 g of soil was added to a 125-ml plastic bottle, which was filled with water and shaken vigorously for 20 sec. Water was then added carefully to form a positive meniscus at the bottle orifice. After 10 min, organic debris floating on the top of the bottle was collected with a fine mesh screen and placed in a plastic capsule with a ball bearing. The sample of organic debris was triturated with a dentist's amalgam shaker. The contents were then placed in a test tube along with the empty capsule and centrifuged for 5 min at 3,000 rpm. A filter was inserted into the tube, and the supernatant was forced up through the filter; 100 µl of the filtrate was dispensed into each well of the immunoassay plates.

The distribution of viable propagules of *P. parasitica* in the fractions of soil prepared for soil ELISA was determined. The entire residue fraction, 10 ml of the water, and 10 ml of the residue-free soil, were added separately to 90 ml of 0.25% water agar and plated on PARPH medium as described previously (28). Ten milliliters of the original soil prior to fractionation was similarly processed for comparative purposes. Five replicate samples of each fraction and the original soil were assayed in each of the three experiments.

All 19 root samples from Florida were assayed with *Phytophthora* tissue kit D, and 14 were also assayed with tissue kit E. All soil assays were conducted with soil kit E. Samples were incubated in the precoated immunoassay plates for 20 min at room temperature while being

shaken on a microplate adapter for a vortex mixer. Plate cells were emptied and washed five times with the kit-supplied wash solution in an automated plate washer. One-hundred microliters of enzyme-antibody conjugate were added to each empty well and incubated and washed as above. One-hundred microliters of substrate were added, and plates were incubated for 10 min followed by the addition of 50 μl of stop solution. Absorbance values were determined at 405 nm with an SLT-Lab Instruments ELISA plate reader or an Emax Maxline Microplate Reader version 2.01.

All samples were tested in duplicate wells. For samples from Florida, the reading of a third nonsensitized well was subtracted from the mean of the duplicate sample wells. The concentration of *Phytophthora* protein in each sample was calculated from a standard curve of absorbance values produced by preparations containing 0, 5, 10, and 20 ng/ml of soluble protein from *P. parasitica* provided by Agri-Diagnostics. In California, 100-μl samples of the substrate were added to each of four wells which were left empty as substrate blanks. The mean of these substrate readings was subtracted from all other readings. The results from California were reported directly as absorbance values at 405 nm. Where absorbance values higher than 3.0 were obtained, the original sample was diluted 1:10 with extraction buffer, and the ELISA procedure was repeated as before.

Regression analyses were used to determine the relationship between the detection techniques.

Sample variability for root ELISA and propagule determinations. An orchard of sweet orange on rough lemon rootstock near Frostproof, Florida, and one of sweet orange on sweet orange rootstock near Lakeland, Florida, were selected to determine core-to-core variability. Twenty-five cores of 930 cm^3 each were collected arbitrarily in each orchard, and the roots and soil from each core were separated. The soil was then assayed on PARPH medium as described above to determine the propagule density for each core. Root samples from each core were assayed by tissue ELISA with the D kit as described above.

Soil and roots from the Frostproof orchard and two orchards of sweet orange on sour orange rootstock near Arcadia, Florida, were used to determine subsample variability. Composite samples of several liters of soil and roots were collected from each orchard. Fibrous roots were separated from larger roots and debris, chopped into about 1-cm lengths, and mixed. The soil was thoroughly mixed by hand. Ten or 11 subsamples of 0.5 g of roots and 10 ml of soil were processed as above for root ELISA and soil propagule determinations.

Standard errors, coefficients of variability, and correlation coefficients, where appropriate, were calculated and used to compare the variability and comparability of the two methods.

Comparative reactivity of hyphae of *Phytophthora* spp. Hyphal mats of *P. parasitica* and *P. citrophthora* were produced in liquid cultures by the method of Menyonga and Tsao (14). Test mats were also grown on corn meal, V8 juice, and potato-dextrose broth (14). After 6 days the mats were removed, rinsed twice in distilled water, blotted dry, and weighed. Four to eight mats were weighed after blotting and then again after drying to determine the moisture content of the hyphae and to enable calculations for converting wet weight to dry weight. The hyphal mats were triturated to a dry powder with liquid N_2 with a porcelain mortar and pestle. Known quantities of *Phytophthora* hyphae, ranging from 0.1 to 0.001 μg, were used as substrate and assayed as described above with kit D.

RESULTS

Comparison of detection techniques. The relationships of the ELISA values for the tissue kits D and E and soil kit E, the percent roots infected, and the percent root tips rotted were compared to the standard soil propagule assay (Fig. 1). The coefficients of determination for all measured parameters for the 19 Florida orchards are presented in Table 1. There was a significant correlation between propagule densities as determined by soil assays and the percent root pieces infected, the percent root tips rotted and tissue kit assay values, but not with the soil kit assays (Fig. 1). Tissue kit E produced very high values (Fig. 1), but background values were unacceptably high. Nevertheless, values were highly related to those obtained with tissue kit D ($R^2 = 0.72$) (Table 1).

When Florida soils were fractionated for the soil ELISA procedure, variable amounts of organic debris were recovered from the different soils. In some

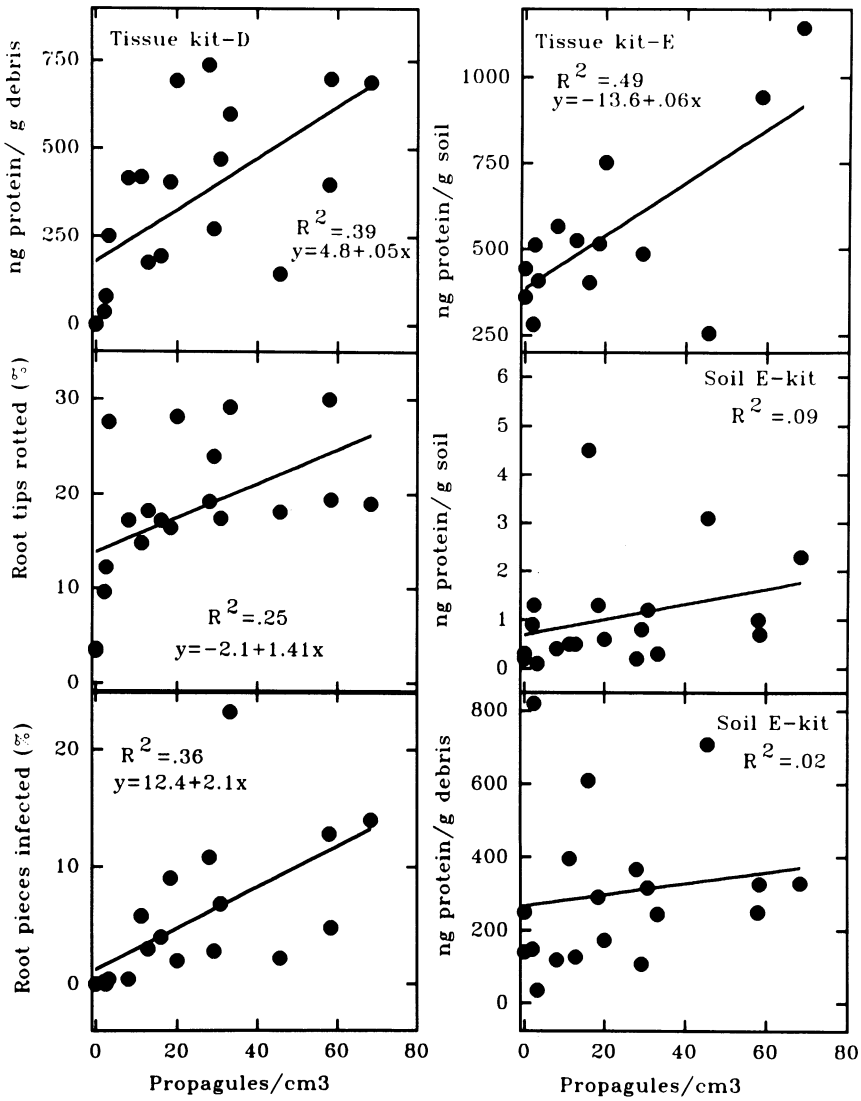

Fig. 1. Relationship of detection methods to the number of propagules of *Phytophthora parasitica* in 19 Florida citrus orchards.

soils, the quantity of residue was so great that it was difficult to collect the total amount; whereas in others, relatively little residue was present. Thus, ELISA results are presented as nanograms of *Phytophthora* protein per gram of organic debris as well as per gram of soil (Fig. 1, Table 1). When the number of viable propagules was determined in the fractions prepared for soil ELISA, 25–60% were found in the organic residue. Most of the remainder were found in the extracted soil, with few or none in the water fraction (Table 2).

With *P. citrophthora* in California, ELISA kit D values were related (R^2 = 0.41) with propagule densities on a per orchard basis (Fig. 2). Tissue kit D values with *P. parasitica* were correlated (R^2 = 0.32) with propagule densities from individual trees but not with total root length, the length of live roots, or the percent live roots (Table 3).

Variability of propagule density and root ELISA values. When core-to-core variability was compared in a Florida orchard with moderate propagule densities, the coefficients of variability were of the same order of magnitude for both methods (Table 4). Where populations were lower, root ELISA values were more variable than were propagule densities. In both orchards, there was a significant correlation between the results of the two procedures.

In tests to determine subsample variability, propagule densities were very low in the Frostproof orchard, and no *Phytophthora* was detected by root ELISA in this experiment. In Arcadia 1, propagule densities and ELISA values were high and did not vary greatly among subsamples (Table 5). Where ELISA values and propagule densities were lower, more variability among subsamples was encountered. The subsample variability was of the same order of magnitude with the two techniques.

Reactivity of mycelia of *Phytophthora* spp. *P. parasitica* mycelia produced on corn meal, V8 juice, or potato-dextrose broth gave nearly identical results with those of kit D per gram of dry weight. The detection limit for kit D approached 1 ng dry weight of *P. parasitica* hyphae. With the D kits, *P. citrophthora* mycelia were less than one-tenth as reactive as *P. parasitica* mycelia (Table 6).

DISCUSSION

When commercial kits D and E were compared for assay of citrus root tissue, kit D usually gave values that were much

Table 1. Coefficients of determination (R^2) for the measured parameters for detection of *Phytophthora* spp. considering the mean for each of 19 orchards

				Roots		Soil	
Measured parameter	Root rot (%)	Roots infected (%)	Propagules /cm³ soil	Kit D ng protein /g root	Kit E ng protein /g root	Kit E ng protein /g debris	Kit E ng protein /g soil
Root rot	...	0.26*[a]	0.25*	0.36**	0.04[ns]	0.02[ns]	0.00[ns]
Roots infected	0.26*	...	0.36**	0.39**	0.44**	0.00[ns]	0.00[ns]
Propagules/cm³	0.25*	0.36**	...	0.39**	0.44**	0.03[ns]	0.09[ns]
Root kit D	0.36**	0.39**	0.39**	...	0.72*	0.01[ns]	0.02[ns]
Root kit E	0.04[ns]	0.44**	0.44**	0.72**	...	0.00[ns]	0.00[ns]
Soil kit E (debris)	0.02[ns]	0.00[ns]	0.03[ns]	0.01[ns]	0.00[ns]	...	0.44**
Soil kit E (bulk)	0.00[ns]	0.00[ns]	0.09[ns]	0.02[ns]	0.00[ns]	0.44**	...

[a]* = Significant at $P \leq 0.05$; ** = significant at $P \leq 0.01$; ns = not significant.

Table 2. Distribution of viable propagules of *Phytophthora parasitica* in soil fractionated for soil enzyme-linked immunosorbent assay (ELISA)

	Exp. 1		Exp. 2		Exp. 3	
Sample	Prop/ cm³	Prop/ 80 g soil	Prop/ cm³	Prop/ 80 g soil	Prop/ cm³	Prop/ 80 g soil
Field soil	12.0 ± 1.4	960	48.8 ± 13.4	3,904	18.0 ± 5.8	1,440
Fraction						
Organic debris	10.0 ± 2.9	1,000	39.2 ± 15.7	3,920	2.4 ± 1.7	2,400
Water	0.0 ± 0.0	0	8.4 ± 6.3	840	0.0 ± 0.0	0
Remaining soil	8.4 ± 1.8	672	66.0 ± 23.3	5,280	10.0 ± 5.8	800
Total		1,672		10,040		3,200
Total as % of field soil		174		257		222
Organic debris (% of total)		60		39		25

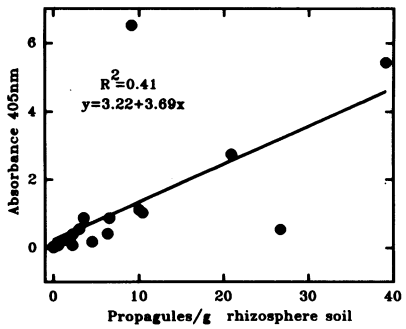

Fig. 2. Relationship of propagule densities of *Phytophthora citrophthora* in rhizosphere soils to the absorbance values using the root enzyme-linked immunosorbent assay (ELISA) in 21 California citrus soils.

Table 3. Coefficients of determination (R^2) for the tissue kit D enzyme-linked immunosorbent assay (ELISA) values for the measured parameters from 217 citrus trees in two orchards in southern California from June to August in 1987 and 1988

Measured parameter	R^2
Living roots (%)	0.01[ns a]
Total root length (cm/cm³ soil)	0.02[ns]
Propagules *Phytophthora parasitica*/g rhizosphere soil	0.32**
Healthy root length (cm/cm³ soil)	0.01[ns]

[a]ns = Not significant; ** = significant at $P \leq 0.01$.

Table 4. Core-to-core variability of propagule determination on a selective medium and *Phytophthora* protein as determined by root enzyme-linked immunosorbent assay (ELISA) kit D in Florida

	Lakeland		Frostproof	
	Propagules /cm³	Protein (ng) /g root	Propagules /cm³	Protein (ng) /g root
Mean	12.3	252	4.1	144.0
Number of cores (*n*)	25	25	25	25
SE	2.9	43	1.4	8.6
CV[a] (%)	118.0	86	166.0	298.0
Correlation coefficient (Propagule vs. ELISA)	$r = 0.57$, $P = 0.003$		$r = 0.86$, $P \leq 0.0001$	

[a]Coefficient of variation.

lower and related more consistently with the other measures of *Phytophthora* activity in citrus soils. Background in the nonsensitized wells was higher with kit E than with kit D. Although kit E has been used successfully to quantitate levels of *Phytophthora* spp. in other systems (1,2), kit D was more suitable for species of *Phytophthora* detected in citrus soils and was used for all subsequent experiments in this study.

Root rot is not generally considered a reliable indicator of *Phytophthora* activity, because it can also be induced by anaerobiosis brought about by flooding, by freeze damage to the canopy, or by other factors. Most of the root rot in the Florida orchards was probably induced by *Phytophthora* infection given the absence of other factors known to induce root rot. The sole exception may be one orchard near Lake Alfred, which was freeze-damaged and had a high percentage of root rot but a low percentage of root infection and low propagule densities. The California tests did not indicate a close relationship between propagule densities and the percent living roots, total root length, or healthy root length. In addition to salinity and anaerobiosis which may cause root rot, two different *Phytophthora* spp. are active at different seasons in California and may be responsible for the lack of correlation in this state. Trees with roots severely rotted by *P. citrophthora* during the winter usually do not show high populations of *P. parasitica* during the summer. Even in carefully controlled greenhouse experiments, the percentage of total root length rotted was only slightly correlated with *P. parasitica* populations; whereas the percentage of root tips rotted was much more strongly correlated with *P. parasitica* populations (3). Although the percentage of roots infected is a reliable indicator of current activity of the pathogen, a high percentage of infection indicates that favorable conditions have existed recently and may not reflect long-term tendencies in the orchard.

Propagule densities are the measure of activity currently used to assist decisions on fungicide treatment programs (13,26). They are apparently related to old root infections and often, but not always, are correlated with recent *Phytophthora* root rot (3). In Florida, the results with ELISA kit D were correlated with propagule densities, as well as with root rot and percent roots infected. Using the current Florida criteria of 0–5 propagules per cubic centimeter as low, 5–15 as moderate, and more than 15 as high, five of the 19 Florida orchards had low populations, three moderate, and 11 high. If we arbitrarily designated less than 150 ng of *Phytophthora* protein low, 150–300 ng moderate, and greater than 300 ng high, then: 1) four of five orchards judged low by propagule counts would also be low by ELISA, the other would be moderate; 2) one of the three orchards judged moderate by propagule counts would be moderate, the others high; and 3) eight of 11 orchards judged high by propagule counts would be high, two moderate, and one low.

Because no precise threshold has been established for either technique (10,13, 21), and given the sample variability with both techniques (28; Table 4), the two methods probably yield equally reliable information. It should be noted that Florida soil samples are taken arbitrarily, and propagule densities are based on total soil volume. Because California samples are taken from the rhizosphere and propagule density is based on dry weight of soil, the propagule numbers in the rhizosphere are approximately 1.5–3.5 higher than those in Florida citrus soil. However, results with *P. parasitica* from California confirm the results from Florida and indicate that with kit D, a Florida moderate threshold value which corresponds to 15–20 propagules of *P. parasitica* per gram of rhizosphere soil in California would fall between 220 and 300 ng of *Phytophthora* protein per gram of root. Because *P. citrophthora* hyphae is 5–10 times less reactive to kit D than is *P. parasitica* and because we do not have good protein standards from the manufacturer of the kits for *P. citrophthora,* it is probably not wise to establish a threshold for it at this time. Nevertheless, in the results from California, the ELISA indicated high populations of *P. citrophthora* from two of the three groves with more than 15 propagules of *P. citrophthora* per gram of soil and from only one of 16 groves with less than 15 propagules per gram.

Results of the soil ELISA were not related to any other measure of *Phytophthora* activity in citrus orchards in Florida (Tables 1 and 4). The soil ELISA may accurately measure the *Phytophthora* protein in the sample extract placed in the well, but other factors may influence its relationship to the other variables measured. First, soil residues may contain living and dead structures of the fungus. Soil ELISA would measure living, dormant, or dead material, whereas the other techniques would measure only germinable propagules. In addition, a variable portion of the propagules remained in the extracted soil (Table 2). Preparation of samples for soil ELISA nearly doubled the total number of propagules detected by assay on selective media compared to direct plating of the soil samples (Table 2). This is probably due to separation of large propagules during the vigorous shaking used in the extraction process. Soil ELISA has been used successfully to detect and quantitate *Phytophthora megasperma* Drechs. in soybean fields (22). However, the vagaries of the system mentioned above make it difficult to use this system for citrus orchards.

The root ELISA system seems applicable to the assessment of *Phytophthora* populations in citrus orchards. The sample and subsample variability of root ELISA and propagule determinations appear to be of the same order of magnitude (Tables 4 and 5). Both systems give highly variable results where populations are low, but there is little need to obtain precise estimates in this situation. Currently, 20–40 cores per 4-ha orchard are recommended for propagule determinations (26,28). Given that sample variabilities of ELISA and of propagule determinations are of the same order of magnitude, a like number of samples should adequately assess *Phytophthora* levels by ELISA.

In other studies, the root ELISA system successfully detected *Phytophthora* in air- and oven-dried roots (Timmer and Zitko, *unpublished*). Air-drying did not

Table 5. Subsample variability of propagule determinations on selective media and *Phytophthora* protein as determined by root enzyme-linked immunosorbent assay (ELISA) kit D in Florida

	Arcadia 1		Arcadia 2	
	Propagules /cm^3	Protein (ng) /g root	Propagules /cm^3	Protein (ng) /g root
Mean	71.5	722	23.0	213
n	11	11	10	10
SE	4.2	160	3.6	152
CV[a] (%)	19.3	22.2	49	71

[a] Coefficient of variation.

Table 6. Comparison of enzyme-linked immunosorbent assay (ELISA) values using the tissue kit D with known amounts of *Phytophthora citrophthora* and *P. parasitica* hyphae

Dry wt *Phytophthora* hyphae (μg)	ELISA—optical density (405 nm)[a]	
	P. parasitica	*P. citrophthora*
0.1	>3.0	0.62 ± 0.08
0.01	1.45 ± 0.28	0.46 ± 0.04
0.001	0.49 ± 0.23	0.34 ± 0.01

[a] Means of two experiments ± standard deviations.

affect ELISA values, whereas oven-drying reduced absorbance of positive samples, but also reduced background readings. Drying has many advantages in sample handling. Roots can be dried and mailed to laboratories for assay, obviating the need to ship bulky soil samples which must be kept moist. Oven-drying of roots allows shipment of samples across national borders where transport of living pathogens is not permitted.

The root ELISA system with kit D accurately determined the amount of *P. parasitica* hyphae produced in vitro (Table 6). Since hyphae produced under several different nutrient regimes gave nearly identical ELISA results, *Phytophthora* hyphae produced in different citrus rootstocks should react similarly to that produced in vitro.

Soil propagule determinations and root ELISA both provide useful information on *Phytophthora* activity in citrus orchards. The choice of method should depend on materials and personnel available, the ease of sample collection and processing, and the cost of the procedures.

ACKNOWLEDGMENTS

We thank Becky L. King for excellent technical assistance. This research was supported in part by the Ciba-Geigy Corporation. Kits and equipment for ELISA were provided by Agri-Diagnostics Associates.

LITERATURE CITED

1. Ali-Shtayeh, M. S., MacDonald, J. D., and Kabashima, J. 1991. A method for using commercial ELISA tests to detect zoospores of *Phytophthora* and *Pythium* species in irrigation water. Plant Dis. 75:305-311.
2. Benson, D. M. 1991. Detection of *Phytophthora cinnamomi* in azalea with commercial serological assay kits. Plant Dis. 75:478-482.
3. Danderand, L.-M. C. 1990. The influence of *Fusarium solani* on citrus root rot caused by *Phytophthora parasitica* or *Phytophthora citrophthora.* Ph.D. diss. University of California, Riverside.
4. Feld, S. J., Menge, J. A., and Stolzy, L. H. 1990. Influence of drip and furrow irrigation on Phytophthora root rot of citrus under field and greenhouse conditions. Plant Dis. 74:21-27.
5. Grimm, G. R., and Alexander, A. F. 1973. Citrus leaf pieces as traps for *Phytophthora parasitica* from soil slurries. Phytopathology 63:540-541.
6. Kannwischer, M. E., and Mitchell, D. J. 1978. The influence of a fungicide on the epidemiology of black shank of tobacco. Phytopathology 68:1760-1765.
7. Klotz, L. J., and DeWolfe, T. A. 1958. Technique for isolating *Phytophthora* spp. which attack citrus. Plant Dis. Rep. 52:675-676.
8. Lutz, A. L. 1987. Propagule survival and population fluctuations of *Phytophthora parasitica* and control of root rot in irrigated citrus groves. Ph.D. diss. University of California, Riverside.
9. Lutz, A. L., and Menge, J. A. 1986. Citrus root health. II. Phytophthora root rot. Citrograph 72:33-39.
10. Lutz, A. L., and Menge, J. A. 1991. Population fluctuations and the numbers and types of propagules of *Phytophthora parasitica* that occur in irrigated citrus groves. Plant Dis. 75:173-179.
11. Lutz, A. L., Menge, J. A., and Ferrin, D. M. 1991. Increased germination of propagules of *Phytophthora parasitica* by heating citrus soils sampled during winter. Phytopathology 81:865-872.
12. MacDonald, J. D., Stites, J., and Kabashima, J. 1990. Comparison of serological and culture plate methods for detecting species of *Phytophthora, Pythium,* and *Rhizoctonia* in ornamental plants. Plant Dis. 74:655-659.
13. Menge, J. A. 1986. Use of systemic fungicides on citrus. Citrograph 71:245-250, 252.
14. Menyonga, J. M., and Tsao, P. H. 1966. Production of zoospore suspensions of *Phytophthora parasitica.* Phytopathology 56:359-360.
15. Miller, S. A., and Martin, R. R. 1988. Molecular diagnosis of plant disease. Annu. Rev. Phytopathol. 26:409-432.
16. Miller, S. A., Rittenburg, J. H., Peterson, F. P., and Grothaus, G. D. 1990. Development of modern diagnostic tests and benefits to the farmer. Pages 15-20 in: Monoclonal Antibodies in Agriculture. A. Schots, ed. Pudoc, Wageningen, Netherlands.
17. Mitchell, D. J., Kannwischer-Mitchell, M. E., and Zentmyer, G. A. Isolating, identifying and producing inoculum of *Phytophthora* spp. Pages 63-66 in: Methods for Evaluating Pesticides for Control of Plant Pathogens. K. D. Hickey, ed. American Phytopathological Society, St. Paul, MN.
18. Newman, E. I. 1966. A method of estimating the total length of root in a sample. J. Appl. Ecol. 3:139-145.
19. Pscheidt, J. W., Burket, J., and Fischer, S. 1990. Use of Phytophthora specific immunoassay kits in a plant disease clinic. (Abstr.) Phytopathology 80:962.
20. Rittenburg, J. H., Petersen, F. P., Grothaus, G. D., and Miller, S. A. 1988. Development of a rapid, field usable immunoassay format, for detection and quantitation of *Pythium, Rhizoctonia,* and *Sclerotinia* spp. in plant tissue. (Abstr.) Phytopathology 78:1516.
21. Sandler, H. A., Timmer, L. W., Graham, J. H., and Zitko, S. E. 1989. Effect of fungicide applications on populations of *Phytophthora parasitica* and on feeder root densities and fruit yields of citrus trees. Plant Dis. 73:902-906.
22. Schmitthenner, A. F. 1990. Predicting yield loss from *Phytophthora megasperma* f. sp. *glycinea* in soybean with a quantitative immunoassay of *Phytophthora* in soil. (Abstr.) Phytopathology 80:962.
23. Sjoerdsma, J. D., Menge, J. A., and Johnson, E. L. V. 1988. Ecology of *Phytophthora citrophthora* root rot of citrus. (Abstr.) Phytopathology 78:1519.
24. Skaria, M., and Gonzalez, G. 1991. ELISA screening of citrus feeder roots for *Phytophthora* root rot. (Abstr.) Phytopathology 81:689.
25. Skaria, M., and Miller, S. A. 1989. A rapid test for detecting *Phytophthora* spp. in citrus. J. Rio Grande Val. Hortic. Soc. 42:63-65.
26. Timmer, L. W., Graham, J. H., Sandler, H. A., and Zitko, S. E. 1988. Populations of *Phytophthora parasitica* in bearing citrus orchards in Florida and response to fungicide applications. Citrus Ind. 69(11):40-44, 54.
27. Timmer, L. W., Menge, J. A., Zitko, S. E., Pond, E., and Miller, S. A. 1991. Comparison of ELISA with other assays for *Phytophthora* spp. in citrus orchards in Florida and California. (Abstr.) Phytopathology 81:1213.
28. Timmer, L. W., Sandler, H. A., Graham, J. H., and Zitko, S. E. 1988. Sampling citrus orchards in Florida to estimate populations of *Phytophthora parasitica.* Phytopathology 78:940-944.
29. Timmer, L. W., Sandler, H. A., Graham, J. H., and Zitko, S. E. 1989. Phytophthora feeder root rot of bearing citrus: fungicide effects on soil populations of *Phytophthora parasitica* and citrus tree productivity. Proc. Fla. State Hortic. Soc. 102:5-9.
30. Tsao, P. H. 1960. A serial dilution end-point method for estimating disease potentials of citrus *Phytophthora* in soil. Phytopathology 50:717-724.