Detection of *Phytophthora cinnamomi* in Azalea with Commercial Serological Assay Kits

D. M. BENSON, Department of Plant Pathology, North Carolina State University, Raleigh 27695-7616

ABSTRACT

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Two commercial serological assay kits were compared to a culture plate method for detection of *Phytophthora cinnamomi* in root samples from inoculated azaleas. Both the multiwell E kit and the rapid assay F kit detected *P. cinnamomi* on azalea roots beginning 1 wk after inoculation. Agreement between immunoassay kits and culture plate results for detection of *P. cinnamomi* was most consistent beginning 3–5 wk after inoculation. Root symptoms, but not foliar symptoms, of Phytophthora root rot were evident during this period. There was a positive correlation between root rot severity in greenhouse trials and root sample absorbance (multiwell) or meter reading (rapid assay) but not between symptom severity and immunoassay results. Although color reactions in the rapid assay detectors became increasingly darker after completion of the test, results after 5 min were as reliable as those after 60 min, since readings for uninoculated controls used to determine test thresholds also increased with time. The multiwell kit detected *P. cinnamomi* in root samples containing as little as 1.0% infected root tissue. In a commercial nursery survey, 5 and 15% of the azalea root samples at two nurseries had positive ELISA values that were unconfirmed by culture plate. The rapid assay kit detected *P. cinnamomi*, was easy to use, and gave results in a short time.

The use of enyzme-linked immunosorbent assay (ELISA) to detect plant pathogens in samples of diseased plant tissue and soil enables rapid and specific disease diagnosis (4–10,12,13). Use of a rapid ELISA such as the immunoassay Phytophthora F kit (Agri-Diagnostics Associates, Cinnaminson, NJ) offers commercial growers and integrated pest management professionals the potential to detect pathogens in crops rapidly without the need for time-consuming culture plate methods. Benefits of rapid detection of pathogens by ELISA as described by MacDonald et al (6) are the timely selection of appropriate fungicides to control the pathogen present in an ornamental crop and the avoidance of applications of fungicides that do not have activity toward that pathogen.

Use of trade names does not imply endorsement by the North Carolina Agricultural Research Service of the products named or criticism of similar ones not mentioned.

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ELISA kits in multiwell format were used successfully to detect *Phytophthora cryptogea* Pethybr. & Lafferty in inoculated sage roots and other *Phytophthora* spp. from several ornamental species growing in commercial nurseries (6). In another study (9), detection of *P. cinnamomi* Rands, *P. citricola* Sawada, and *P. parasitica* Dastur from clinic samples of azaleas (*Rhododendron* L.) was similar for an ELISA in the multiwell format and the time-consuming apple-baiting bioassay.

Research with ELISA for detection of *Phytophthora* spp. in ornamentals has evaluated the Phytophthora D kit (Agri-Diagnostics Associates), which does not contain antibodies that react with antigen from *P. cinnamomi* (6), and an ELISA kit in the multiwell format that will not be offered to commercial growers (6,9). The purpose of this study was to: 1) compare two ELISA kit formats—the multiwell Phytophthora E kit and the rapid assay Phytophthora F kit—with a culture plate method for detection of *P. cinnamomi* in azalea under greenhouse conditions, 2) evaluate the multiwell kit for azaleas inoculated and grown under nursery conditions, 3) evaluate the multiwell kit by testing azalea root samples from commercial growers, and 4) estimate the sensitivity of the kits. A brief report has been presented (1).

MATERIALS AND METHODS

Nursery experiment. Ten-month-old azalea cultivar Hinodegiri plants were potted in 2.6-L containers with a pine bark:sand medium (3:1, v/v) with micronutrient (C-trel, Coors Farm Supply, Smithfield, NC) at 0.9 kg/m^3, lime at 3.3 kg/m^3, and triple superphosphate at 1.2 kg/m^3. Plants were inoculated with isolates of *P. cinnamomi* pathogenic to azalea, including 100 from H. A. J. Hoitink (Ohio State University) and 101 (ATCC 46292), 116, and 128 from rhododendron and 150 from azalea in North Carolina. Isolates were grown separately in oat grain culture for 4 wk at 25 C, then mixed as inoculum. Plants received 0, 1, 3, 9, 30, or 90 oat grains per pot. Colonized oat grains were introduced at three locations around the root ball of the transplants except at the lowest two rates. A plastic pot label marked each location, so these areas could be avoided in subsequent root sampling.

Experimental design and sampling. Four plants per inoculum rate were used for each replication. Enough replications were set up for sampling over a 14-wk period, starting 1 wk after inoculation and continuing at 3, 5, 7, 9, 11, 13, and 14 wk. At each sampling date a replication was chosen for assay at random. Three samples per pot were collected with a 1.7-cm-diameter sampling tube to a depth of about 15 cm from areas of the pot away from initial inoculum introduction. Samples containing roots and media were bulked for each plant.

Sample preparation and culture plate method. Bulked samples from each plant were washed under running water to remove media particles. Each root sample then was divided into two clumps of about 0.2 g each. One clump was further divided into five subclumps for culturing on PPP medium (2). The other half of the root sample was used for immunoassay as described below.

ELISA protocol. An ELISA kit in multiwell format (Phytophthora E kit) was used to assay samples for *P. cinnamomi*. Root samples ground on abrasive pads supplied with the kit were suspended in an extraction solution. Extract solution (0.1 ml) was pipetted to each of three microwells per sample and incubated for 10 min with gentle agitation on an orbital shaker. A standard protocol supplied by Agri-Diagnostics for use with the multiwell immunoassay kits was followed. The positive-negative threshold for declaring absorbance readings positive for *P. cinnamomi* was the mean plus three times the standard deviation of the absorbance of the uninoculated controls (14).

Immunoassay sensitivity. Sensitivity was tested by regression analysis of absorbance reading vs. inoculum rate, with values for uninoculated controls excluded, and by adding known amounts of infected root pieces to root samples from healthy plants. A line-intercept method was used to count root pieces in samples of a known weight collected from healthy plants (11). The routine root sample weight used for ELISA of 0.2 g had a mean root length of 1,232 cm. Percentages of infected roots added to healthy root samples were 0, 0.1, 0.5, 1.0, and 5.0 by length.

Greenhouse experiments. The nursery experiment was repeated in the greenhouse during the fall of 1989. The experimental design was similar except that the Hinodegiri azaleas were 5 mo old when transplanted from 6.5-cm-diameter pots into 10-cm-diameter pots and only inoculum rates of zero, one, three, and nine colonized oat grains per pot were used. At sampling, root and foliar symptoms of Phytophthora root rot were rated, fresh top weight was measured, and the entire root system was collected for assay. Root rot severity was assessed as 1 = healthy roots, 2 = fine roots necrotic, 3 = coarse roots necrotic, 4 = crown rot, and 5 = dead plant. Foliar symptoms were assessed as 1 = healthy foliage, 2 = chlorosis, 3 = severe chlorosis, and 4 = necrosis and wilting. Samples were collected at 1, 3, 4, 5, and 8 wk. Plants were maintained by drip irrigation that cycled 15 min, two times per day.

A second set of greenhouse experiments was conducted during the spring of 1990 to compare results of the multiwell (Phytophthora E kit) format and culture plate method with a commercial, on-site rapid assay kit (flow-through ELISA, Phytophthora F kit). The experimental procedure was the same as that described above for the greenhouse. The entire root system was divided for culture and for ELISA. The half for ELISA was ground on the abrasive pad as before, then placed in an extraction bottle with the filter tip and extraction solution provided with the rapid assay kit. Extract solution was then used for both multiwell and rapid assay tests.

The rapid assay kit utilizes a flow-through device with three closely spaced test spots. One is a positive control, one is a negative control, and one is the sample detector. Root extract and kit reagents were applied to the flow-through device according to instructions supplied with the kit. Color intensity in the sample detector was quantified with a light meter (Agrimeter II, Agri-Diagnostics Associates) after 5–15 min, then at various intervals up to 60 min. The positive-negative threshold for declaring meter readings positive for *P. cinnamomi* was the sample mean plus two times the standard deviation of the uninoculated control (14). The more liberal test threshold for the rapid assay kit was chosen because standard deviations for uninoculated control samples were an order of magnitude greater than those for samples with the multiwell format.

Commercial nursery survey. A block of approximately 400 Hinodegiri azalea plants was selected at random in two commercial nurseries in October 1989. From each block, 20 randomly selected plants in 2.6-L containers were sampled as described above. The combined sample cores from each sampled plant were split for culture and multiwell ELISA assay.

RESULTS

Comparison of the multiwell Phytophthora E kit and culture method in the nursery. One week after inoculation, two plants were positive for *P. cinnamomi* by culture assay and two different plants were positive by ELISA in the multiwell format (Fig. 1A). At 3 wk, all plants sampled at 90 oat grains per plant were positive by culture and two of four by ELISA (Fig. 1B). As inoculum rate decreased, number of positives by culture or ELISA also decreased. By week 5, some plants at all inoculum concentrations were positive by culture (nine plants) or by ELISA (17 plants). The highest number of unconfirmed ELISA positives was observed at week 5. Unconfirmed ELISA positives ranged from zero to two (0–10%) at the other sampling dates (Table 1). After week 5, there was very close agreement between positives identified by culture and by ELISA (Fig. 1C–E). Between week 3 and week 11, the number of cultures positive for *P. cinnamomi* increased from nine to 12, whereas ELISA positives increased from six to 13 plants of 24 sampled (Table 1). By week 13, positives increased to 16 for cultures and 18 for ELISA. The regression equation for the number of positives by culture vs. the number by ELISA over the experiment was $y = -0.112 + 1.12x$ ($P = 0.01$).

The positive negative threshold values for declaring an ELISA positive were 0.473, 0.253, 0.120, 0.199, 0.647, 0.084, 0.278, and 0.059 for weeks 1, 3, 5, 7, 9, 11, 13, and 14, respectively (Fig. 1). The test threshold value of 0.647 for week 9 was high because three of four uninoculated control plants had absorbance values of 0.343–0.491. Because *P. cinnamomi* was not isolated from these control plants, the seemingly high values were included in calculation of the test threshold value. In general, positive controls included in each set of ELISA tests were always near 1.0 and negative controls ranged from 0.033 to 0.169 with the exception of week 1, in which 20-min incubation times resulted in about double the values for the test threshold and positive and negative controls.

The number of plants showing foliar symptoms of Phytophthora root rot was similar to the number of positives determined by either assay method at weeks 7, 9, and 11 (Table 1). At weeks 13 and 14, the number of plants showing symptoms was fewer than the number in which *P. cinnamomi* was detected.

Results were similar when this experiment was repeated in the greenhouse in the fall of 1989 (*data not shown*). At week 1, three of 12 inoculated plants were positive by culture and none by ELISA. At week 3, however, eight and seven of 12 plants were positive by culture and

Table 1. Detection of *Phytophthora cinnamoni* on azalea cultivar Hinodegiri inoculated with colonized oat grains and grown in pine bark:sand medium (3:1) based on symptomatology, culture plate method, and ELISA absorbance value

Week	Number positive for *P. cinnamoni*[a]			Matches[b]		
	Symptoms	Culture	ELISA	Symptoms/ culture	Culture/ ELISA	Symptoms/ ELISA
1	...	2	2	...	0	...
3	...	9	6	...	6	...
5	...	9	17	...	9	...
7	12	10	10	7	9	7
9	10	10	9	10	9	10
11	12	12	13	9	11	9
13	13	16	18	13	16	13
14	14	16	18	14	16	14

[a] Foliar symptoms of Phytophthora root rot, isolation of *P. cinnamoni* by culture plate on PPP medium (2), and detection of *P. cinnamoni* with a multiwell immunoassay kit (Phytophthora E kit) from an assay of 20 inoculated plants at each sampling date.
[b] Number of times one detection procedure agreed with another.

ELISA, respectively. Symptoms on roots were most severe at the highest inoculum rate (nine oat grains per plant) beginning at week 4. Foliar symptoms were not evident until week 8 regardless of inoculum rate. At week 4, 11 and eight plants were positive for *P. cinnamomi* by culture and ELISA, respectively. By week 8, all inoculated plants were judged positive by both methods.

Comparison of rapid assay F kit, multiwell E kit, and culture method in the greenhouse. Development of Phytophthora root rot on azaleas was assessed on the basis of fresh top weight, foliar symptoms, and root symptoms. Azalea top weight did not begin to increase until week 7 across all inoculum rates. By week 9, however, there was a difference ($P = 0.05$) in top weight between the uninoculated control and plants inoculated at nine oat grains per plant (Fig. 2A). Foliar symptoms, as in a previous greenhouse experiment, did not develop until week 7 (Fig. 2B), but root symptoms began to develop by week 3 and increased steadily in severity over time and inoculum rate (Fig. 2C).

Five of 12 inoculated plants were judged positive in week 1 with the rapid assay kit, and *P. cinnamomi* was recovered from seven inoculated plants (Fig. 3A). In contrast, no plants were judged positive with the multiwell kit at week 1 (Fig. 4A). At week 3, the pathogen was recovered in culture from all inoculated plants at the high inoculum rate and both ELISA kits detected *P. cinnamomi* from each plant at the high inoculum rate (Figs. 3B and 4B). By week 5, and thereafter, *P. cinnamomi* was cultured from all inoculated plants at all inoculum rates and both ELISA kits also detected *P. cinnamomi* from all inoculated plants with the exception of the rapid assay kit at week 7 (Figs. 3C–E and 4C–E). The regression of meter readings for the rapid assay kit with absorbance readings for the multiwell kit was $y = 9.86 + 10.26x$ ($P = 0.0001$) when data from the uninoculated control were excluded. Results in a second experiment were similar.

The intensity of the purple color on the test spot of the rapid assay detector increased with time after completion of the test. Indeed, a positive slope was found for regression of meter reading and time after the test, where $y = 10.57 + 0.226x$ ($P = 0.0001$). As the equation indicates, meter readings were higher as times increased from 5 to 60 min. However, the relatively higher readings for the uninoculated controls at corresponding times resulted in higher test threshold values for judging readings positive.

Immunoassay sensitivity. Sensitivity of the assay kits was tested by regression analysis for meter reading or absorbance reading for plants vs. inoculum rate. Data for week 5 at 5-min readings were

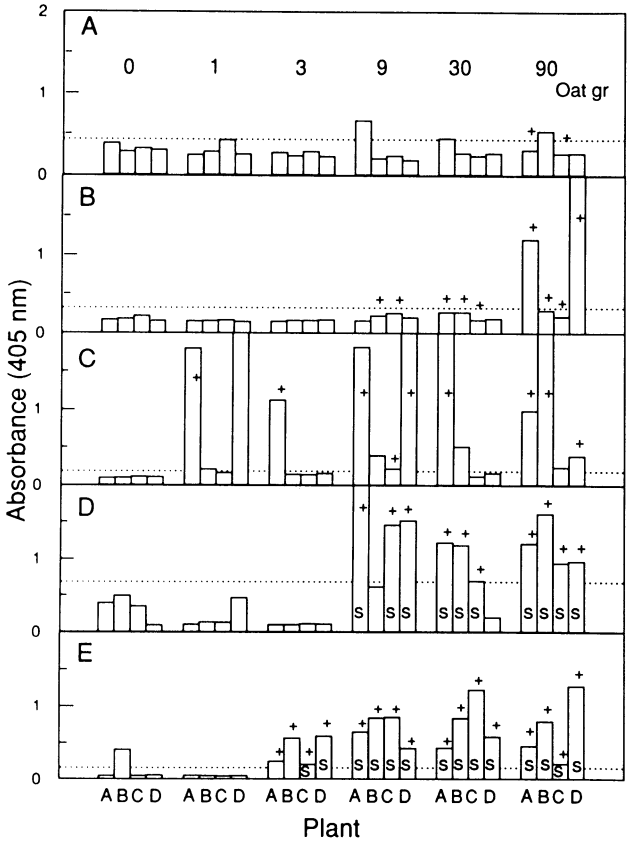

Fig. 1. Absorbance values for multiwell ELISA detection of *Phytophthora cinnamomi* in azalea cultivar Hinodegiri inoculated with 0, 1, 3, 9, 30, or 90 colonized oat grains per plant in a research nursery: **(A)** week 1, **(B)** week 3, **(C)** week 5, **(D)** week 9, and **(E)** week 14. Each plant assayed is represented. Dashed line is positive-negative threshold for judging a result positive based on the mean of the uninoculated control plus three times the standard deviation. Plus signs indicate root samples from which *P. cinnamomi* was cultured. The letter S indicates that a particular plant expressed foliar symptoms of Phytophthora root rot.

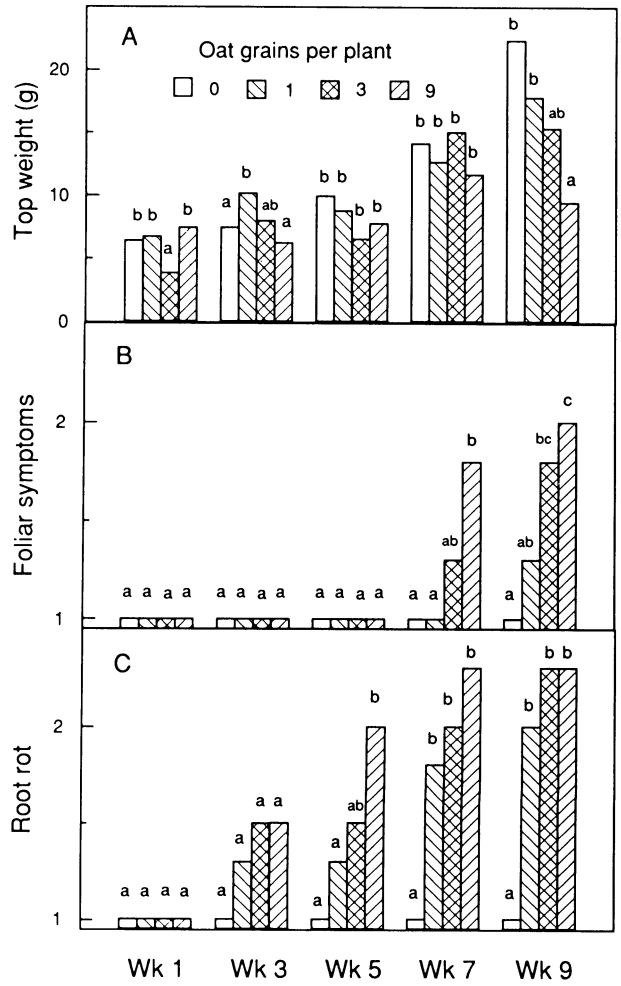

Fig. 2. Comparison at weeks 1, 3, 5, 7, and 9 of **(A)** fresh top weight, **(B)** foliar symptoms of Phytophthora root rot, and **(C)** root rot symptoms for azalea cultivar Hinodegiri plants inoculated with zero, one, three, or nine oat grains per plant colonized by *Phytophthora cinnamomi*. Plants were grown in a greenhouse with drip irrigation. Foliar symptom rating: 1 = healthy foliage, 2 = chlorosis, 3 = severe chlorosis, and 4 = necrosis and wilting. Root rot rating: 1 = healthy roots, 2 = fine roots necrotic, 3 = coarse roots necrotic, 4 = crown rot, and 5 = dead plant. Bars capped with the same letter within a specific week after inoculation are not significantly different according to the Waller-Duncan k-ratio t test ($k = 100$, $P = 0.05$).

used because the difference in root rot severity among inoculum rates was greatest at this time; the uninoculated control was excluded. Neither the equation for the rapid assay kit ($y = 16.44 - 0.159x$) nor that for the multiwell kit ($y = 1.25 - 0.019x$) was significant.

In a second approach, symptom and root rot severities for all plants in the experiment, regardless of inoculum rate or time after inoculation, were regressed with corresponding meter or absorbance reading. The regressions for symptom severity with ELISA values were not significant for either assay format. However, the regressions of root rot severity with ELISA value for both ELISA formats were significant. The equations were $y = 0.46 + 5.45x$ ($P = 0.0003$) for the rapid assay kit and $y = -0.246 + 0.518x$ ($P = 0.0001$) for the multiwell kit.

Sensitivity of the multiwell E kit was also tested by adding infected root pieces to root samples from healthy (uninoculated) plants to create different proportions of infected root tissue per sample. In culture, *P. cinnamomi* was recovered from 46% of the 1-cm-long infected root pieces used to make up the portion added to the healthy root sample. Absorbance values with standard deviations were 0.034 ± 0.004, 0.106 ± 0.077, 0.129 ± 0.068, and 0.630 ± 0.276, respectively, for 0, 0.1, 0.5, and 1.0% infected roots per total root sample. For the 1.0% infected root sample, an average of 11.6 cm of infected roots was added to 1,162 cm of healthy roots. With the average test threshold value of 0.264 from the uninoculated, control plants at the eight sampling dates in the nursery experiment (Fig. 1) used as a conservative test threshold, a sample containing 1% infected root tissue was the limit of detection of *P. cinnamomi* with the multiwell kit.

Commercial nursery survey. None of the 20 azaleas in each block of plants sampled at two commercial nurseries had symptoms of Phytophthora root rot, and *P. cinnamomi* was not recovered in culture. At nursery A, one plant had an absorbance reading of 0.911; the rest were less than 0.120. As a control, two uninoculated plants and two at 90 oat grains per plant from the previously described nursery test were included at the time of assay (October 1989). The uninoculated plants had absorbance readings less than 0.130, whereas the inoculated plants had readings near 0.900. At nursery B, three sampled plants had absorbance readings of 0.443, 0.320, and 2.000; the rest were less than 0.190. A set of uninoculated and inoculated control plants included from the nursery test had absorbance readings of about 0.080 and 1.600, respectively.

DISCUSSION

Differences in detection of *P. cinnamomi* within 1 wk of inoculation of azaleas were found among the ELISA

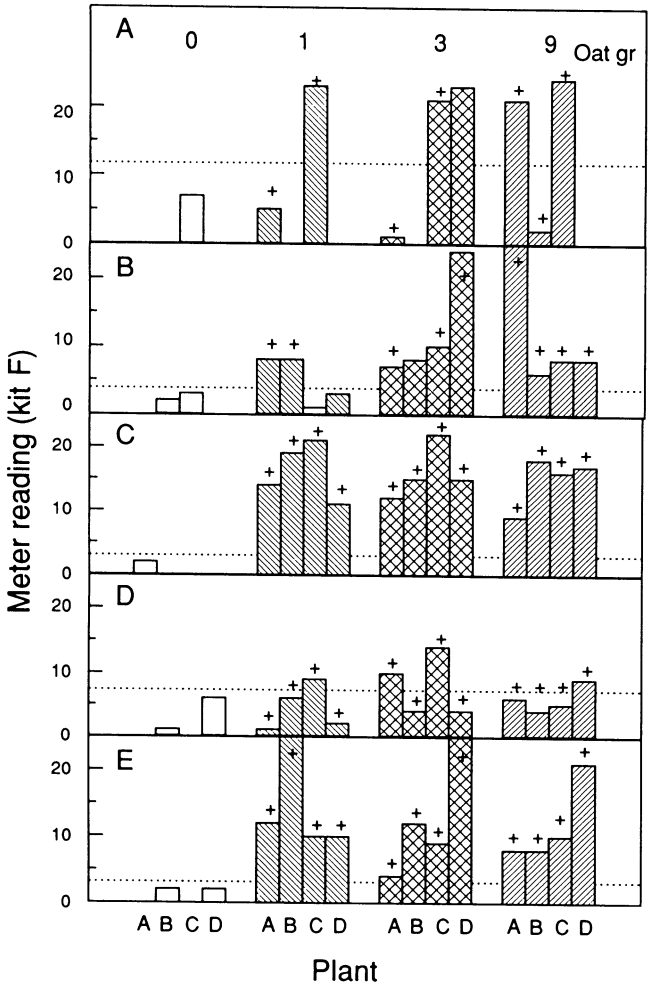

Fig. 3. Meter readings for a rapid assay (Phytophthora F kit) to detect *Phytophthora cinnamomi* in root samples of azalea cultivar Hinodegiri plants inoculated with zero, one, three, or nine colonized oat grains per plant in a greenhouse. Readings were made **(A)** 10 min after completion of the assay for week 1, **(B)** 15 min after for week 3, **(C)** 5 min after for week 5, **(D)** 5 min after for week 7, and **(E)** 5 min after for week 9. Each plant assayed is represented. Dashed line is positive-negative threshold for judging a result positive based on the mean of the uninoculated control plus two times the standard deviation. Plus signs indicate root samples from which *P. cinnamomi* was cultured.

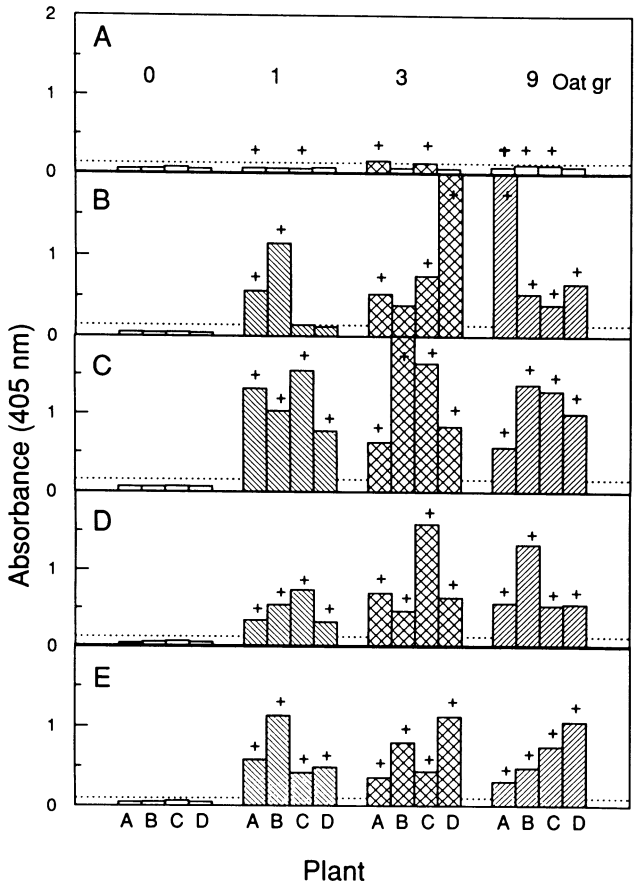

Fig. 4. Absorbance values for a multiwell ELISA kit (Phytophthora E kit) to detect *Phytophthora cinnamomi* in azalea cultivar Hinodegiri plants inoculated with zero, one, three, or nine colonized oat grains per plant in a greenhouse: **(A)** week 1, **(B)** week 3, **(C)** week 5, **(D)** week 7, and **(E)** week 9. Each plant assayed is represented. Dashed line is positive-negative threshold for judging a result positive based on the mean of the uninoculated control plus three times the standard deviation. Plus signs indicate root samples from which *P. cinnamomi* was cultured.

kits and the culture plate method. For the nursery test (Fig. 1A), the multiwell kit and the culture method each detected two infected plants at week 1, although not the same plants. In the greenhouse test (Fig. 4A), the multiwell kit did not detect any infected plants but the culture method detected seven. In contrast, the rapid assay kit detected five positive root samples in week 1 (Fig. 3A) from the same root extract solutions as used in the multiwell screen, although one of these was not detected by the culture plate method. Differences in detection of *P. cinnamomi* between ELISA and the culture plate method within 1 wk of inoculation may be caused by sampling error when root colonization by *P. cinnamomi* is low (6).

Detection of *P. cinnamomi* beginning 3–5 wk after inoculation and before plants showed foliar symptoms of Phytophthora root rot was similar for both ELISA kits and the culture plate method. Early detection of pathogens, so that appropriate management strategies can be initiated, is one prerequisite for commercial adoption of any immunoassay detection kit. *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *tritici* J. Walker was detected in root samples of 6-day-old wheat seedlings with ELISA (3). MacDonald and Stites (5) found that ELISA readings were positive for a *Phytophthora* sp. only if roots of container-grown plants had lesions. In the present study, root rot symptoms were present beginning 3–4 wk after inoculation, at which time roots tested positive with the ELISA kits.

El-Nashaar et al (3) found a positive correlation between log value of take-all inoculum concentration and absorbance reading of infected wheat roots. In the present study, a quantitative relation between inoculum rate and either meter reading (rapid assay kit) or absorbance (multiwell kit) was not found. However, a quantitative relation was found when actual root rot severities but not symptom severities were regressed with readings from either test format. Apparently, inoculum was not equally effective at a given rate. This may have been due to the mixture of isolates of *P. cinnamomi* used for inoculum. Although all isolates used cause root rot, the severity of root rot caused by a given isolate and the ability of the ELISA kits to detect antigen in a given isolate may have varied.

The sensitivity of the multiwell kit for detection of *P. cinnamomi* was as low as 1% infected roots per azalea root sample, based on a conservative test threshold. A detection limit of 1% infected tissue per sample also was reported for *P. cryptogea* on chrysanthemun roots with the multiwell Phytophthora D kit (6). In the present study, the level of sensitivity may be even lower, as *P. cinnamomi* was cultured from only about one-half of the infected root pieces used to make up the 1.0% infected root samples.

With the rapid assay kit, meter readings at 5–15 min were suitable for judging positive ELISA reactions based on a positive negative threshold of the uninoculated control mean plus two times the standard deviation. Over four of the five weekly assays of uninoculated control plants in the greenhouse (Fig. 3), meter readings made within 15 min of the test averaged 1.12 with a standard deviation of 1.8 when the meter was calibrated with the 1989 calibrator. A newer calibrator released in 1990 and designed to give higher absolute meter readings was used with week 9 samples. Mean reading for uninoculated control samples with the 1990 calibrator at 5 min was 5.8 with a standard deviation of 1.7, compared with a mean of 1.0 and a standard deviation of 1.2 for the 1989 calibrator. Even though meter readings were higher with the 1990 calibrator, a corresponding higher positive negative threshold value did not result in more ELISA positive reactions (*unpublished*). Until a larger number of ornamental crops and *Phytophthora* spp. have been evaluated with the rapid assay kit, it may be difficult to suggest a standardized test threshold value for judging ELISA reactions positive with the rapid assay F kit.

P. cinnamomi could not be detected by culture plate from root samples of Hinodegiri azalea at the two nurseries surveyed, although ELISA values were high for one sample at nursery A and three samples at nursery B. The report by MacDonald et al (6) on the comparison of serological and culture plate methods for detection of *Phytophthora, Pythium*, and *Rhizoctonia* in root samples from commercial nurseries illustrates the problems associated with interpretation of immunoassay results. The unconfirmed positives at the two nurseries could be due to detection of nonviable pathogen antigen or to interference of fungicides applied before culture plate assay (6). Positive ELISA results from the nursery samples were not expected, however, since root rot severity was correlated with ELISA results in the greenhouse and no root rot symptoms were evident in these samples.

No evidence of cross-reaction of the ELISA kits was found in the experiments conducted at the nursery or in the greenhouse. No color reactions in microwells or rapid assay detectors for uninoculated control samples were observed. Likewise, values for meter or absorbance readings for these samples were low. In the commercial nursery survey, one sample at nursery A and three samples at nursery B had high absorbance readings. Failure to isolate *P. cinnamomi* in culture from the other half of these root samples should not be construed as a cross-reaction, however, because of the possibility of nonviable pathogen antigen or fungicide interference, as mentioned above.

With its ease of use, ability to detect *P. cinnamomi* before foliar symptom development, and short time needed for completion, the rapid assay offers several advantages over the culture plate method for pathogen detection. Commercial acceptance of rapid assay kits will depend not only on these features but also on the economics of assay cost.

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