Detecting Multiple Species of *Phytophthora* in Container Mixes from Ornamental Crop Nurseries

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ABSTRACT

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A baiting bioassay was developed to detect species of *Phytophthora*, i.e., those typically associated with ornamental crops, in container mixes that are used routinely in producing container-grown landscape plants. Both fresh and air-dried subsamples of container mixes were baited to improve detection of species that persist as dormant propagules. Leaf disks of *Camellia japonica* detected *Phytophthora* spp. most frequently and consistently, but intact needles of shore juniper also were effective baits and less likely to become contaminated. *Phytophthora* spp. were detected at baiting durations of 24, 48, and 72 h; both detection and contamination were greatest at 72 h. To minimize problems from contamination and maximize detection, camellia leaf disks and shore juniper needles were used simultaneously; half of the baits were removed at 24 h and the other half were removed at 72 h. Baiting at temperatures of 15, 20, and 25°C did not have a dramatic effect on detection; however, *Phytophthora* spp. occasionally were detected more frequently at 20 and 25°C than at 15°C. Both camellia leaf disks and shore juniper needles were colonized readily by zoospores of *P. cinnamomi*, *P. nicotianae* (= *P. parasitica*), *P. cryptogea*, and *P. citricola* but were not colonized as readily by zoospores of *P. cactorum*. Disks from leaves of *C. sasanqua* and six cultivars of *C. japonica* were effective as baits; however, some differences among camellia types occurred. *P. cinnamomi*, *P. nicotianae*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, and *P. cactorum* have been detected in naturally infested container mixes using this baiting bioassay.

Phytophthora spp. cause root rot of many woody ornamental crops—such as Japanese andromeda, azalea, boxwood, camellia, juniper, pine, and rhododendron—but they also may cause stem dieback and foliar blight depending on the species of host and pathogen involved (3,7,11,18,27). These are some of the most frequently occurring and economically important diseases of ornamental crops grown in nurseries in South Carolina (1) and elsewhere (3,13,18,19). Species of *Phytophthora* associated with woody ornamental crops in nurseries include *P. cactorum*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. gonapodyoides*, *P. heveae*, *P. lateralis*, *P. megasperma*, *P. nicotianae (*synonym = *P. parasitica)*, and *P. syringae* (3,11,13,18). In South Carolina, *P. cinnamomi* and *P. nicotianae* are the species most commonly recovered from diseased ornamental plants (6). Detection of *Phytophthora* spp. in nursery container mixes can be important in diagnosing disease problems occurring at commercial nurseries. A procedure for detecting *Phytophthora* spp. consistently and effectively in container mixes before symptoms develop could be a key factor in development and implementation of an integrated management strategy for Phytophthora diseases and in the prevention of epidemics.

Detection of *Phytophthora* spp. in soil typically has relied on direct isolation, i.e., by plating soil on selective media, or on indirect isolation, i.e., by baiting soil with susceptible plants or plant parts. Immunological and nucleic acid techniques also have been developed (2,10). Tsao (26) and, more recently, Erwin and Ribeiro (7) have published excellent reviews on this topic. Although there has been some success in detecting *Phytophthora* spp. in soil using newer technologies, such as enzyme-linked immunosorbent assay (ELISA; 2) and DNA probes (10), these approaches tend to be specific for individual species and have not had broad application. Therefore, these methods would not be useful for routinely testing nursery container mixes for a variety of *Phytophthora* spp. Both baiting bioassays and soil plating probably are capable of detecting several species of *Phytophthora* simultaneously in a given sample. However, baiting bioassays have certain advantages over soil plating. Greater quantities of soil can be assayed using baiting methods, thereby increasing the likelihood of detecting populations present at low densities (4,16). They also are more successful than soil plating methods for detecting homothallic species of *Phytophthora*, which often survive as dormant oospores (16). Because various homothallic species can attack ornamental crops (e.g., *P. cactorum*, *P. citricola*, *P. heveae*, *P. megasperma*, and *P. syringae)* and, therefore, may be present in nursery container mixes, a baiting bioassay should be more useful than a direct soil plating method for routinely detecting *Phytophthora* spp. in container mixes.

Numerous baiting systems have been developed to detect both heterothallic and homothallic species of *Phytophthora* in soil (7,26). Most baiting systems have been designed to detect usually one, but occasionally several, species from soil around a specific host plant, and pieces of that host plant—especially leaf pieces and intact fruits—are the most commonly used bait (7). However, availability of leaves and fruits can be limited due to their seasonal nature. Leaf pieces have been successful baits for various species of *Phytophthora* on a number of crops; however, leaf-piece baits, with a perimeter of wounded cells, are more likely to be colonized by contaminating microorganisms (e.g., *Pythium* spp.) than baits having an intact perimeter (16).

Previously, a variety of bait types have been used for detecting *Phytophthora* spp. (primarily *P. cinnamomi*) from soil around woody ornamental crops and trees, including lupine radicles and seedlings; needles of cedar and pine; leaf disks of rhododendron, azalea, and eucalyptus; cotyledons of eucalyptus; and apple and pear fruits (7,26). Only one baiting bioassay has been developed specifically for use on nursery-grown ornamental plants (19). Few bioassays have been developed specifically to detect a variety of species of *Phytophthora* from different hosts. Dance et al. (4) developed a procedure using pine needle segments or lupine radicles that detected 14 species, including many of those commonly attacking woody ornamentals. To date, no baiting bioassay has been developed specifically to detect the variety of species of *Phytophthora* known

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to attack woody ornamental plants in soilless container mixes that routinely are used in nurseries.

Pretreating soil subsamples before baiting has improved detection of *Phytophthora* spp. in several bioassays. Air drying and then remoistening soil subsamples prior to baiting enhanced detection of homothallic species (14,16,25), and detection of *P. nicotianae*, a heterothallic species, was enhanced by uniformly moistening soil subsamples before baiting (15). Temperature and baiting duration also may affect detection of *Phytophthora* spp. in baiting bioassays (7).

Our objective in this study was to develop a bioassay using inexpensive, readily available baits to routinely detect the variety of species of *Phytophthora* that typically occur around ornamental plants growing in nursery container mixes. This bioassay may be useful as a scouting tool in developing an integrated pest management program for commercial nurseries. Preliminary results have been reported (8,9).

MATERIALS AND METHODS

Standard procedure. All container mixes used in these experiments were naturally infested with *Phytophthora* spp. They were obtained from the Clemson University Plant Problem Clinic and were stored in polyethylene bags at room temperature (22 to 25°C) until used, which usually was within 1 to 4 weeks. Container mixes had been submitted to the clinic along with plant samples for diagnosis of plant disease problems and, therefore, usually contained roots or pieces of roots from the plants in question. Mixes were typical of those used at commercial nurseries in the southeastern region of the United States; they contained primarily pine bark (75 to 90%) and sand (25 to 10%) and were free of field soil. In all experiments, bait materials were surface disinfested with 10% bleach solution (0.5% sodium hypochlorite), rinsed in distilled water, and blotted dry prior to use. When leaf disks were used, whole leaves were surface disinfested before the disks (7 mm in diameter) were removed with a standard hole punch.

Unless stated otherwise, container mixes were baited as follows: 30 cm^3 of each mix was added to a deep glass petri plate (90 by 20 mm) and flooded with 50 ml of distilled water. Three replicate plates were used for each treatment, and five baits were floated in each plate. Baited mixes were held at a constant temperature in an incubator for 24 to 72 h in the dark. Baits then were removed, blotted on paper towels, and placed on modified PARPH, a medium selective for *Phytophthora* spp. that contains the antimicrobial amendments pimaricin, ampicillin, rifampicin, pentachloronitrobenzene (PCNB), and hymexazol (17). The modified medium was prepared by adding 50 ml of clarified V8 juice and 15 g of Difco Bacto Agar, instead of cornmeal agar as the basal medium, to 950 ml of distilled water. Clarified V8 juice was prepared by mixing 1 g of $CaCO_3$ with each 100 ml of V8 juice, centrifuging the mixture at 7,000 rpm for 10 min, and decanting and saving the supernatant in 50-ml aliquots, which were frozen at –20°C until used. Each liter of basal medium was amended, after autoclaving and cooling to 50 to 55°C in a water bath, with 5 mg of pimaricin (also known as natamycin) as Delvocid Instant (Gist-Brocades Food Ingredients Inc., Menomonee Falls, WI), 250 mg of ampicillin sodium salt (Fisher Scientific, Fairlawn, NJ), 10 mg of rifamycin SV sodium salt (Sigma-Aldrich, St. Louis), 50 mg of PCNB as Terraclor 75WP (Uniroyal Chemical Company, Inc., Middlebury, CT), and 50 mg of hymexazol (99.5%; Sankyo Co. Ltd., Tokyo). Each amendment was dissolved or suspended in 5 ml of sterile, distilled water in a culture tube. The medium, designated PARPH-V8, was dispensed into standard disposable plastic petri plates (90 by 15 mm), and plates were stored in a refrigerator (at 4°C in darkness) until used.

Isolation plates containing baits were held at 20°C in the dark, and the baits were examined for *Phytophthora* spp. and contaminating microorganisms (usually *Pythium* spp. or bacteria) over a 7-day period. The number of baits from which *Phytophthora* spp. or contaminants grew were counted, and percentages for detection and contamination were calculated. Representative isolates of *Phytophthora* spp. were transferred to fresh PARPH-V8 medium to obtain pure cultures. Isolates eventually were transferred to cornmeal agar slants and stored at 15°C for later identification. All data were analyzed with SAS statistical software for Windows (release 6.11, Statistical Analysis Systems, Inc., Cary, NC).

Fresh versus air-dried container mix. Experiments to investigate various baiting parameters used both fresh container mixes (i.e., as collected and received at the clinic) and subsamples of these mixes that first were air dried and then remoistened before flooding and adding baits. To air dry, 30 cm^3 of each mix were placed in a deep glass petri plate and left uncovered at room temperature for 3 days. Then, each container mix subsample was stirred with a spatula while adding just enough distilled water to moisten the mix without leaving standing water. Plates were covered and held for 3 days at the temperature to be used for baiting.

Baiting parameters. To determine the most effective bait for detecting *Phytophthora* spp. consistently and without interference from contaminants, pine needle segments (*Pinus* sp.); leaf disks of silver-dollar or spiral eucalyptus (*Eucalyptus cinerea*), camellia (*Camellia japonica* cv. Governor Mouton), and azalea (*Rhododendron* sp.); whole needles of shore juniper (*Juniperus conferta* cv. Blue Pacific); and whole leaves of gumpo azalea (*Rhododendron eriocarpum* cv. Gumpo White) and Japanese holly (*Ilex crenata* cv. Helleri) were tested. A total of 8 to 10 container mixes were used to evaluate potential baits, including both fresh and air-dried subsamples of each mix. Baiting was conducted at 22°C for 24 h, and the experiment was conducted twice. In one trial, Japanese holly leaves were not used.

The effect of temperature during the bioassay procedure on detection of *Phytophthora* spp. was investigated. Temperatures of 15, 20, and 25°C were compared using five container mixes. Five camellia leaf disks and five shore juniper needles were floated in each replicate plate (i.e., 10 baits total per plate). Subsamples of each mix were baited simultaneously at all three temperatures for 48 h. The experiment was conducted three times, and, in one trial, both fresh and air-dried mixes were used.

The effect of the duration for which baits were floated and exposed for colonization on detection of *Phytophthora* spp. also was examined. Baiting durations of 24, 48, and 72 h were compared using five container mixes. A total of 15 camellia leaf disks and 15 shore juniper needles were floated in each replicate plate at 22°C. Five camellia leaf disks and five shore juniper needles were removed and plated on PARPH-V8 medium at 24, 48, and 72 h. The experiment was conducted twice, and fresh and air-dried container mixes were used in both trials.

For baiting parameter experiments, percentage data were transformed to arcsine-square root values prior to analyses to stabilize variances (24). The transformed data were analyzed by two-way analyses of variance (ANOVA), and treatment means were separated by Fisher's protected least significant difference (LSD) with $P \leq 0.05$. However, data are reported as untransformed percentages in tables and figures.

Sensitivity of baits to *Phytophthora* spp. The susceptibility and relative sensitivity of camellia leaf disks and shore juniper needles to zoospores of different species of *Phytophthora* likely to be encountered in nurseries were evaluated. In all, 24 isolates were used: 5 isolates each of *P. cinnamomi* (from two nursery container mixes, field soil, and ginkgo from South Carolina and cranberry from Oregon); *P. nicotianae* (from five container mixes from South Carolina nurseries); *P. citricola* (from black walnut, sugar maple, and rhododendron from Wisconsin and Mugo pine and field soil from New York); *P. cactorum* (from forsythia, Fraser fir, and lilac from Wisconsin and rhododendron and field soil from New York); and 4 isolates of *P. cryptogea* (from two nursery container mixes in South Carolina, an apple tree from New York, and a cherry tree from Wisconsin). These isolates are part of

a permanent collection maintained by S. N. Jeffers at Clemson University. All isolates were grown for 72 h at 25°C on V8 juice agar (V8A; 200 ml of V8 juice, 2 g of $CaCO_3$, and 15 g of Difco Bacto Agar in 800 ml of distilled water). A total of 10 plugs (5 mm) were taken from the edge of each actively growing colony and transferred to a deep glass petri plate. Next, 20 ml of a 1.5% nonsterile soil extract solution (16) was added to each plate, and the plates were placed at room temperature (22 to 24°C) under continuous fluorescent light for 24 h. Isolates were examined at 30 to 50× with a dissecting microscope for the presence of sporangia on V8A plugs and motile zoospores in the solution. The number of sporangia per plug for each isolate was estimated and recorded. Isolates that had not released sufficient quantities of zoospores were chilled for 1 to 2 h at 2 to 4°C and then placed at room temperature for at least 60 min to stimulate zoospore formation and release.

After all isolates had released zoospores, 30 ml of distilled water was added to each plate, bringing the total volume to 50 ml. A total of 10 camellia leaf disks and 10 shore juniper needles were floated in each plate, and plates were held at 25°C in the dark for 72 h. Five camellia leaf disks and five shore juniper needles were removed and placed on PAR-V8 selective medium (i.e., PARPH-V8 without PCNB and hymexazol; 17) at 24 and 72 h. Isolation plates were held at 20°C in the dark and were examined over a 7-day period for *Phytophthora* spp. growing from baits. The number of baits from which *Phytophthora* spp. grew was recorded for each isolate.

This experiment was repeated twice at reduced inoculum concentrations, once with three and then again with only one 5-mm agar plug. In these trials, five camellia leaf disks and five shore juniper needles were used in each plate, and all 10 baits were removed to PAR-V8 medium at 72 h. These experiments with three plugs and one plug each were conducted twice. Data for the four or five isolates of each species were combined for all trials and are summarized as the percentages of camellia leaf disks and shore juniper needles detecting each species of *Phytophthora*.

Different types of camellia as baits. An experiment was conducted to determine if leaves of different camellia species and cultivars differ in effectiveness as baits for *Phytophthora* spp. *Camellia* spp. are evergreen; therefore, only leaves from the previous season were used to avoid using tender, succulent leaves formed during the current season. Leaves were collected from six cultivars of *C. japonica*, a species very susceptible to Phytophthora root rot (cvs. Governor Mouton, Pink Perfection, Marjorie Magnificent, Debutante, Kumagai, and Lady Clair) and from *C. sasanqua*, a species considered resistant to Phytophthora root rot (18,20). For each container mix, 30 cm^3 of mix was added to each of seven deep glass petri plates (one plate for each camellia species or cultivar) and 50 ml of distilled water was added to each plate to thoroughly flood mix subsamples. A total of 20 leaf disks (7 mm) from one camellia species or cultivar were floated in a plate. Plates were placed at 25°C for 72 h, and then baits were removed and placed on plates of PARPH-V8 medium, which were held at 20°C in the dark for up to 7 days. The number of baits from which *Phytophthora* spp. grew was recorded for each camellia type in each container mix. The experiment initially was conducted (and repeated) with three container mixes in summer, when shoots on camellia trees were actively elongating. The experiment was conducted (and repeated) again with six different container mixes in the fall when shoots no longer were actively elongating. Efficacies of the different camellia types were compared in 2-by-7 contingency tables for each container mix independently. Fisher's Exact Two-Tail test ($P \leq 0.05$) for binomial data was used to determine if there was a difference among camellia types in numbers of baits detecting *Phytophthora* spp. for each container mix.

RESULTS

The baiting bioassay detected *P. cinnamomi*, *P. nicotianae*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, and *P. cactorum* in naturally infested container mixes from South Carolina nurseries. The modified selective medium, PARPH-V8, was as effective as the standard PARPH medium (with cornmeal as the basal medium) for isolating and recovering *Phytophthora* spp. from baits. However, the colony morphology of several species differed somewhat on the new medium. The main advantage of the modified medium was that *Phytophthora* spp. tended to sporulate more readily on the V8 juice-based medium than on the cornmeal-based medium, which aided in distinguishing certain species directly on isolation plates.

Fresh versus air-dried container mix. The condition of the container mix at the time of baiting (i.e., whether fresh or air-dried and remoistened) occasionally affected the results obtained in the bioassay. Different species of *Phytophthora* were detected in fresh and air-dried subsamples of three naturally infested container mixes. In one mix, *P. cinnamomi* was detected in the fresh subsamples and *P. citricola* was detected in the air-dried and remoistened subsamples. In a second mix, *P. cinnamomi* was detected in the fresh subsamples, and *P. nicotianae* was detected in the air-dried subsamples. In a third mix, an unidentified isolate of *Phytophthora* was detected in the air-dried subsamples but not in the fresh subsamples. The isolate was identified tentatively as *P. citricola*, but we were unable to confirm its identity.

Baiting parameters. Each of the parameters examined (i.e., bait type, baiting duration, and temperature) significantly affected the results of the bioassay. In two-way ANOVAs with a baiting parameter and container mix as factors (Tables 1 to 3), interactions often were significant ($P \leq 0.05$), indicating that the effect of a given parameter was not consistent for all container mixes. However, because our objective was to identify a standard baiting procedure that could be used for all container mixes, main effects of each parameter were evaluated. In all three experiments on baiting parameters (Tables 1 to 3), the main effect of container mix always was significant ($P \leq 0.001$). This was expected, because mixes came from different crops at various nurseries and, therefore, had different microflora. Differences among mixes will not be discussed further.

In preliminary experiments to compare various types of baits, pine needle segments, azalea leaf disks, and gumpo azalea leaves did not detect *Phytophthora* spp. as consistently or effectively as other potential baits and frequently were contaminated with interfering microorganisms. In addition, pine needle segments frequently sank, and gumpo azalea leaves did not maintain adequate surface contact with the agar when placed on selective medium. For these reasons, pine needle segments, azalea leaf disks, and gumpo azalea leaves were eliminated as candidate baits.

Leaf disks of silver-dollar eucalyptus and camellia, whole needles of shore juniper, and whole leaves of Japanese holly were effective in early experiments and, therefore, were compared further. Holly leaves were not included in one trial. All baits were relatively easy to handle and manipulate; however, eucalyptus and camellia leaf disks occasionally sank during the baiting process, and Japanese holly leaves tended to curl and did not always maintain adequate contact with the selective medium. Shore juniper needles were the easiest to handle and manipulate.

These baits differed significantly in detection of *Phytophthora* spp. and in contamination by other microorganisms (Table 1, Fig. 1). The percentage of camellia leaf disks detecting *Phytophthora* spp. was consistently the highest and usually was significantly greater than those for the other baits tested (Table 1). In all trials combined (Fig. 1), camellia leaf disks were the most effective bait; 49% of camellia leaf disks used in all trials detected *Phytophthora* spp. Overall, the percentages of shore juniper needles, eucalyptus leaf disks, and Japanese holly leaves detecting *Phytophthora* spp. were 37, 30, and 29%, respectively (Fig. 1). Camellia leaf disks also detected *Phytophthora* spp. most consistently; in only one container mix was a species of *Phytophthora* detected by one of the other baits (juniper needles) and not by camellia leaf disks. However, regardless of

the species of plant from which baits came, leaf disks usually were contaminated more frequently than whole needles or leaves (Table 1, Fig. 1). In all trials combined, 35 and 40% of camellia and eucalyptus leaf disks, respectively, were contaminated, whereas only 20% of shore juniper needles and 25% of Japanese holly leaves were contaminated (Fig. 1). Although camellia leaf disks often were more contaminated than other baits, the level of contamination typically was not severe enough to interfere

Table 1. Incidence of *Phytophthora* spp. and contaminating microorganisms on leaf disks of camellia and silver-dollar eucalyptus and whole leaves of shore juniper and Japanese holly used as baits in a bioassay of both fresh and air-dried container mixes[w]

	Incidence of *Phytophthora* spp. (%)[x]				Incidence of contamination (%)[x]			
	Fresh mix		Air-dried mix		Fresh mix		Air-dried mix	
Bait type	**Initial**	**Repeat**	**Initial**	**Repeat**	**Initial**	**Repeat**	**Initial**	**Repeat**
Camellia leaf disks	58 a	86 a	29 a	22 a	19 b	28 b	40 a	49 a
Eucalyptus leaf disks	59 a	33 c	15 c	13 b	32 a	43 a	39 a	46 a
Juniper needles	47 b	56 b	22 b	23 a	23 b	21 bc	19 b	18 c
Holly leaves	…[y]	44 bc	21 b	22 a	…[y]	16 c	26 b	32 b
ANOVA: *P* values[z]								
Bait × mix interaction	0.005	<0.001	<0.001	0.065	<0.001	0.003	0.112	<0.001
Bait main effect	0.012	<0.001	<0.001	0.032	0.027	<0.001	<0.001	<0.001
Mix main effect	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[w] Samples from 8 to 10 container mixes naturally infested with *Phytophthora* spp. were used; each mix was assayed fresh (as collected) and after air-drying and then remoistening. Mixes were assayed twice—once in an initial trial and then again in a repeat of that trial.

[x] Percentages of baits from which either *Phytophthora* spp. or contaminating microorganisms grew on PARPH-V8 selective medium. Percentages are calculated from the number of baits positive out of five in each replicate and are the overall means of all the replicates used for all of the container mixes. Within each column, means with different letters are significantly different based on Fisher's protected least significant difference ($P \leq 0.05$).

[y] Japanese holly leaves were not tested in this trial.

[z] ANOVA = analysis of variance. *P* values are for *F* statistics of interactions and main effects from two-way analyses of variance. Proportions of baits were transformed to arcsine-square root values before analysis.

Table 2. Incidence of *Phytophthora* spp. and contaminating microorganisms on five camellia leaf disk and five shore juniper needle baits that were floated over flooded container mixes for 24, 48, or 72 h at 22°C in a baiting bioassay[x]

	Incidence of *Phytophthora* spp. (%)[y]				Incidence of contamination (%)[y]			
	Fresh mix		Air-dried mix		Fresh mix		Air-dried mix	
Duration (h)	**Initial**	**Repeat**	**Initial**	**Repeat**	**Initial**	**Repeat**	**Initial**	**Repeat**
24	40 b	42 b	17 b	25 b	37 b	15 c	25	31
48	45 b	47 b	17 b	38 a	33 b	25 b	28	32
72	61 a	59 a	30 a	31 b	58 a	45 a	27	36
ANOVA: *P* values[z]								
Duration × mix interaction	0.066	0.004	0.011	0.329	0.424	0.003	0.954	0.559
Duration main effect	<0.001	<0.001	0.021	0.003	<0.001	<0.001	0.598	0.566
Mix main effect	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[x] Samples from five container mixes naturally infested with *Phytophthora* spp. were used; each mix was assayed fresh (as collected) and after air-drying and then remoistening. Mixes were assayed twice—once in an initial trial and then again in a repeat of that trial.

[y] Percentages of baits from which either *Phytophthora* spp. or contaminating microorganisms grew on PARPH-V8 selective medium. Percentages are calculated from the number of baits positive out of 10 in each replicate and are the overall means of all of the replicates used for all of the container mixes. Within each column, means with different letters are significantly different based on Fisher's protected least significant difference ($P \leq 0.05$).

[z] ANOVA = analysis of variance. *P* values are for *F* statistics of interactions and main effects from two-way analyses of variance. Proportions of baits were transformed to arcsine-square root values before analysis.

Table 3. Incidence of *Phytophthora* spp. and contaminating microorganisms on five camellia leaf disk and five shore juniper needle baits that were floated over flooded container mixes for 48 h at 15, 20, and 25°C in a baiting bioassay[x]

	Incidence of *Phytophthora* spp. (%)[y]				Incidence of contamination (%)[y]			
	Fresh mix			Air-dried mix	Fresh mix			Air-dried mix
Temperature (°C)	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 1**	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 1**
15	85	27 b	39	19 b	22 b	14	27	45
20	79	45 a	42	39 a	44 a	11	21	46
25	83	46 a	41	49 a	34 a	19	21	47
ANOVA: *P* values[z]								
Temperature × mix interaction	<0.001	<0.001	0.038	0.054	<0.001	0.664	0.288	<0.001
Temperature main effect	0.485	<0.001	0.877	<0.001	0.001	0.406	0.499	0.933
Mix main effect	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[x] Samples from five container mixes naturally infested with *Phytophthora* spp. were used in each trial; mixes in Trial 1 were assayed both fresh (as collected) and after air-drying and then remoistening.

[y] Percentages of baits from which either *Phytophthora* spp. or contaminating microorganisms grew on PARPH-V8 selective medium. Percentages are calculated from the number of baits positive out of 10 in each replicate and are the overall means of all of the replicates used for all of the container mixes. Within each column, means with different letters are significantly different based on Fisher's protected least significant difference ($P \leq 0.05$).

[z] ANOVA = analysis of variance. *P* values are for *F* statistics of interactions and main effects from two-way analyses of variance. Proportions of baits were transformed to arcsine-square root values before analysis.

with detection, which was not true for eucalyptus leaf disks. Shore juniper needles had the least amount of contamination in all trials.

Duration of baiting (i.e., the length of time baits were floated over flooded container mixes) affected both detection of *Phytophthora* spp. and incidence of contaminants (Table 2, Fig. 2). Significantly more baits detected *Phytophthora* spp. at 72 h than at 24 or 48 h in three of four trials; however, in one trial, detection was better at 48 h than at 24 or 72 h. Baits left for 72 h also were significantly more contaminated ($P \leq 0.001$) in two trials. A comparison of baiting durations from all trials combined corroborates these results (Fig. 2). Based on a total of 600 bait pieces (i.e., 300 camellia leaf disks and 300 shore juniper needles) used to bait five mixes in four trials, the percentage of baits detecting *Phytophthora* spp. was higher when baits were floated over flooded container mixes for 72 h (45%) than for either 48 (37%) or 24 h (31%). However, 72-h baits also had more contamination (42%) than 48-h (30%) or 24-h baits (27%).

Temperature during baiting did not have a dramatic effect on detection of *Phytophthora* spp. or incidence of contamination; it significantly affected detection in two trials and contamination in only one trial (Table 3). When detection was affected, more baits detected *Phytophthora* spp. at 20 or 25°C than at 15°C ($P \leq 0.001$). Results were similar when the effect of temperature was compared for all trials, which included a total of 600 baits used to assay five container mixes in four trials (Fig. 3). The percentages of baits detecting *Phytophthora* spp. were 55, 51, and 43% at 25, 20, and 15°C, respectively. The percentages of baits contaminated (30, 31, and 27%, respectively) were similar for all temperatures.

Sensitivity of baits to *Phytophthora* spp. All isolates used, except for *P. cinnamomi*, readily formed sporangia on V8A plugs and released zoospores in nonsterile soil extract solution after 24 h under fluorescent lights. A few isolates of *P. cinnamomi* did not form sporangia until 3 to 6 h after soil extract solution had been removed and replaced with distilled water. Isolates of *P. cinnamomi* were refrigerated at 2 to 4°C for 60 min after sporangia formed and then moved to room temperature for at least 60 min, which induced zoospore formation and release. The number of sporangia formed varied with the species (Table 4). Isolates of *P. cinnamomi* formed less than 50 sporangia per plug; isolates of *P. nicotianae*, *P. citricola*, and *P. cryptogea* formed between 51 and 500 sporangia per plug; and isolates of *P. cactorum* were very prolific and formed over 500 sporangia per plug. Sporangia of all isolates released ample zoospores based on microscopic examination.

In experiments using 1, 3, and 10 mycelium plugs (Table 4), all camellia and shore juniper baits were colonized by every isolate of *P. nicotianae*, *P. citricola*, and *P. cryptogea* regardless of the concentration of zoospores present. Shore juniper needles were somewhat less sensitive to isolates of *P. cinnamomi*. In the one-plug experiment, all camellia leaf disks were colonized, but only 74% of shore juniper needles were

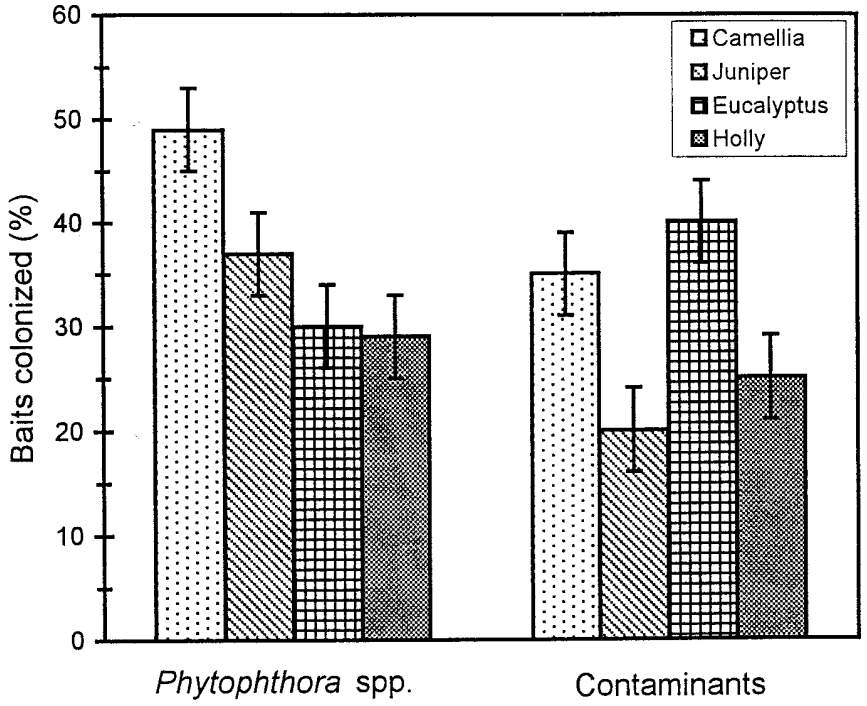

Fig. 1. Overall comparison of the effectiveness of leaf disks of camellia and silver dollar eucalyptus, whole needles of shore juniper, and whole leaves of Japanese holly for detecting *Phytophthora* spp. and contaminating microorganisms in naturally infested container mixes with a baiting bioassay. Data are based on a total of 555 baits (for camellia, eucalyptus, and juniper) or 420 baits (for holly) used to assay 8 to 10 container mixes four times. Bars are 95% confidence intervals, which ranged from ±3.3 to ±4.3%.

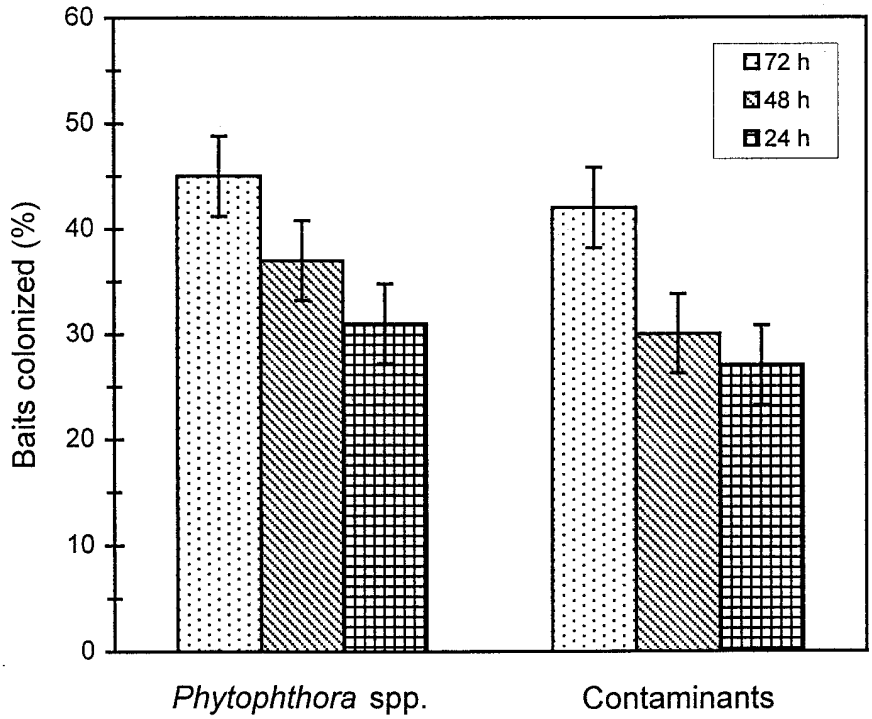

Fig. 2. Overall comparison of the effect of baiting durations of 24, 48, and 72 h on colonization of camellia leaf disks and shore juniper needles by *Phytophthora* spp. and contaminating microorganisms in a baiting bioassay. Data are based on a total of 600 baits (300 camellia and 300 juniper) for each duration that were used in assaying five naturally infested container mixes four times. Bars are 95% confidence intervals, which ranged from ±3.6 to ±4.0%.

colonized; the percentage of juniper needles colonized was approximately the same for each of the five isolates of *P. cinnamomi* used. Camellia and juniper baits were not as sensitive to isolates of *P. cactorum* as to those of other species; only 88 to 64% of the camellia and juniper baits were colonized by *P. cactorum* in the 10-, 3-, and 1-plug experiments. These lower percentages of baits colonized were due to two isolates of *P. cactorum*. One isolate colonized juniper baits more frequently than camellia baits (90 compared to 20%, respectively) in the 1-plug experiment. The other isolate did not colonize any of the baits in the 1-plug experiment despite the presence of abundant zoospores, and colonized only 10% of the baits in the 3-plug experiment and 50% of the baits in the 10-plug experiment. The other three isolates of *P. cactorum* colonized all of the baits in the 10-, 3-, and 1-plug experiments.

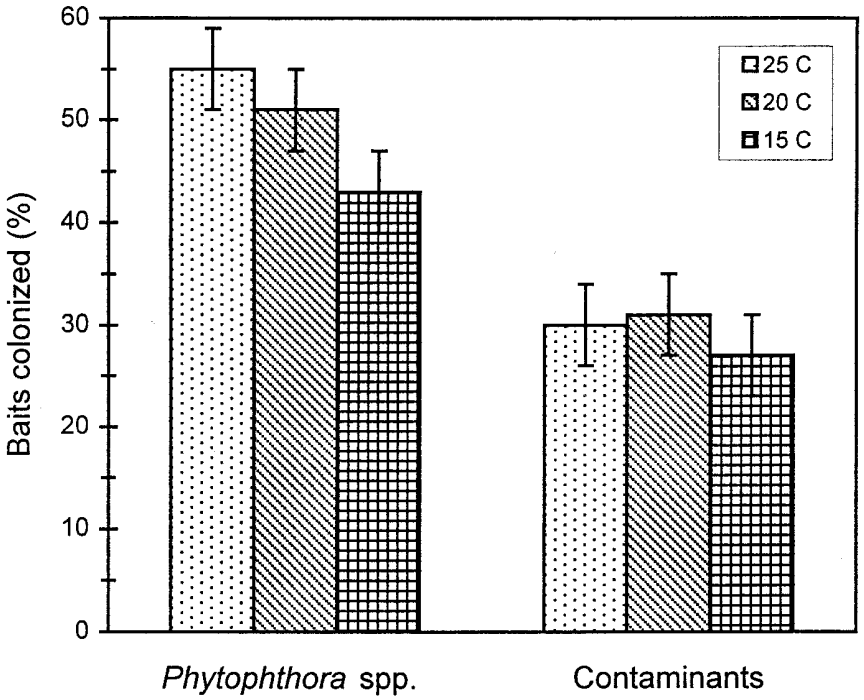

Fig. 3. Overall comparison of the effect of temperatures of 15, 20, and 25°C on colonization of camellia leaf disks and shore juniper needles by *Phytophthora* spp. and contaminating microorganisms in a baiting bioassay. Data are based on a total of 600 baits (300 camellia and 300 juniper) for each temperature that were used in assaying five naturally infested container mixes four times. Bars are 95% confidence intervals, which ranged from ±3.6 to ±4.0%.

Table 4. Sensitivity of camellia leaf disk and shore juniper needle baits to zoospores of different species of *Phytophthora*[v]

Species[x]	No. of plugs[y]	Sporangia[z]	Baits infected (%)[w]	
			Camellia leaf disks	Juniper needles
P. cinnamomi	10	++	100	100
	3	++	100	100
	1	+	100	74
P. nicotianae	10	+++	100	100
	3	+++	100	100
	1	+++	100	100
P. citricola	10	+++	100	100
	3	+++	100	100
	1	+++	100	100
P. cryptogea	10	+++	100	100
	3	+++	100	100
	1	+++	100	100
P. cactorum	10	++++	88	88
	3	++++	82	82
	1	++++	64	78

[v] Baits were floated over flooded V8A plugs of four or five isolates of each species of *Phytophthora* for 3 days at 25°C and then were placed on PARPH-V8 selective medium at 20°C for up to 7 days.
[w] Percentage of baits from which *Phytophthora* spp. grew on PARPH-V8. Five baits of each type were used for each isolate, and the experiments with three plugs and one plug were repeated. Results are based on total number of baits used for all isolates per species in all experiments.
[x] Five isolates each of *P. cinnamomi, P. nicotianae, P. citricola,* and *P. cactorum* and four isolates of *P. cryptogea* were used. Each isolate was tested in a separate petri plate.
[y] Number of 5-mm V8A plugs flooded with 1.5% nonsterile soil extract solution in a deep glass petri plate for each isolate used per species.
[z] Relative abundance of sporangia formed on individual V8A plugs: + = <10, ++ = 10–50, +++ = 51–500, ++++ = >500.

Different types of camellia as baits. Leaf disks from *C. sasanqua* and six cultivars of *C. japonica* were compared as baits using three naturally infested container mixes in the summer, when plants from which leaves were picked were physiologically active (i.e., shoots were elongating). However, the number of baits detecting *Phytophthora* spp. varied significantly among camellia types, and the results from the initial and repeat trials were inconsistent (*data not presented*). Sometimes, a cultivar or species that detected *Phytophthora* spp. consistently in one trial failed to do so partially or entirely in the repeat trial.

Therefore, the experiment was conducted again in the fall when plants were less active physiologically (i.e., shoots had set terminal buds), using six different naturally infested container mixes (Table 5). Two of the mixes (Table 5, mixes B and F) apparently had very low populations of *Phytophthora* spp. because only a small number of baits—regardless of camellia type—detected *Phytophthora* spp. in these two container mixes. Results from two independent trials were consistent and statistically similar (Fisher's Exact Two-Tail test), so data were combined for comparisons. Overall, the types of camellia used did not greatly affect detection of *Phytophthora* spp. However, significant differences were detected in three of the six container mixes. In one of the mixes where a low population level existed (Table 5, mix B), only a few of all of the baits from three cultivars detected *Phytophthora* spp. In the other two mixes (Table 5, mixes C and E), *C. sasanqua* baits detected *Phytophthora* spp. less frequently than cultivars of *C. japonica.* Cv. Marjorie Magnificent detected *Phytophthora* spp. less frequently than the other cultivars of *C. japonica* in one mix (Table 5, mix E). *C. japonica* cv. Governor Mouton and *C. sasanqua* were the only two types to detect *Phytophthora* spp. in all six container mixes. The other cultivars detected *Phytophthora* spp. in four or five of the mixes.

DISCUSSION

Throughout this study and routinely in our laboratory, we successfully have used a modified version of the PARPH selective medium that has become popular among researchers who work with *Phytophthora* spp. (7,17). This modified version contains dilute (50 ml/liter) clarified and buffered V8 juice instead of the standard cornmeal as the basal medium. V8 juice media are among the most popular and frequently

used worldwide for growing and culturing *Phytophthora* spp. (7). This modification of the medium proved to be very useful for routine isolations; although colony morphologies of individual species were somewhat different on PARPH-V8 than on standard PARPH. Homothallic species (e.g., *P. citricola, P. megasperma*) readily produced oospores directly on isolation plates of PARPH-V8, and mycelium plugs taken from PARPH-V8 plates that were flooded with nonsterile soil extract solution and placed under lights produced sporangia in 24 to 48 h. In addition, sporangia seemed to form on this agar medium more frequently than on standard PARPH. Consequently, tentative identification of isolates could be made directly on isolation plates, which usually was not possible with standard PARPH because most species produce only mycelium on this medium. Previously, others have recognized the advantages of V8 juice as a nutrient source in media selective for *Phytophthora* spp. Shew (22) substituted dilute V8A for cornmeal agar as the basal medium in PARPH, and Schmitthenner (21) used dilute V8 juice in the selective medium he developed and used routinely.

Use of both fresh and air-dried subsamples of nursery container mixes increased the overall diversity of *Phytophthora* spp. detected. Air drying container mix subsamples before baiting almost always eliminated detection of *P. cinnamomi*, a heterothallic species. *P. nicotianae*, another heterothallic species, frequently was detected only in the fresh samples but occasionally was detected in subsamples that were air dried and remoistened; in one mix, *P. nicotianae* was detected only in the air-dried and remoistened sample. Therefore, propagules of *P. nicotianae* appeared to withstand air drying better than those of *P. cinnamomi*. Enhanced detection of *P. nicotianae* when soil samples are premoistened for a period before baiting has been reported previously (15). In two container mixes, *P. citricola* was detected only in mix subsamples that were air dried and remoistened prior to baiting—probably because this species is homothallic and readily produces oospores. Others (14,16,25) have clearly shown that air drying and then remoistening soil subsamples enhances detection of homothallic species—presumably by stimulating germination of dormant oospores.

Although pine needles (4) and azalea leaf disks (23) have been used successfully to bait soils for *Phytophthora* spp., they were not as effective as other baits in our preliminary experiments and, therefore, were eliminated as candidate baits. Eucalyptus leaf disks, first used by Linderman and Zeitoun (19), are the baits used by the Clemson University Plant Problem Clinic and some other diagnostic clinics to detect *Phytophthora* spp. in soil samples (S. N. Jeffers, *personal observation*). This bait type was effective much of the time but was not as effective or as consistent as camellia leaf disks for detection of *Phytophthora* spp. All types of leaf disks were prone to contamination, particularly by *Pythium* spp., which could interfere with detection of *Phytophthora* spp. Contamination by *Pythium* spp. previously has been reported to be a problem with eucalyptus leaf-disk (19) and needle baits (4).

Whole-leaf or needle baits were less frequently contaminated than leaf-disk baits, but they also detected *Phytophthora* spp. less often. Intact surfaces of whole-leaf or needle baits may be less prone to infection by zoospores than the wounded perimeter of leaf-disk baits. In fact, the small wounded areas at the base of whole leaves or needles where they had been removed from stems were the sites most frequently infected by *Phytophthora* spp. as well as by contaminating microorganisms. Similar findings were reported for cedar and pine-needle baits (4). Previously, Jeffers and Aldwinckle (16) reported that using baits with an intact surface reduced the amount of contamination on baits; they found that the intact surfaces of apple cotyledon baits usually were not colonized by contaminating *Pythium* spp. but readily were infected by zoospores of *P. cactorum*. Of the whole-leaf baits tested in our study, intact needles of shore juniper (*Juniperus conferta* cv. Blue Pacific) were most successful overall.

Camellia leaf disks detected *Phytophthora* spp. most frequently and consistently of all the baits evaluated. These baits frequently were contaminated, but the contamination usually was limited and did not interfere with detection of *Phytophthora* spp. However, camellia leaf disks failed to detect *Phytophthora* spp. in one container mix when shore juniper needles successfully detected the pathogen. Based on this fact and the realization that one type of bait probably will not detect a variety of species equally well, we recommend baiting with both camellia leaf disks and shore juniper needles together to enhance detection of the range of species of *Phytophthora* that might be present in nursery container mixes. The importance of using a combination of baits was confirmed in the bait sensitivity experiments. Camellia leaf-disk baits were more sensitive than shore juniper needles to isolates of *P. cinnamomi*, and one isolate of *P. cactorum* colonized shore juniper needles more frequently than camellia leaf disks. The advantage of using more than one bait to assay a given soil sample when multiple species of *Phytophthora* are present has been noted previously (4,12). However, the importance of using different bait types simultaneously to detect a variety of *Phytophthora* spp. has not been emphasized.

The conditions used during the baiting bioassay significantly affected detection of *Phytophthora* spp. Holding baits for 72 h over flooded container mixes improved detection of *Phytophthora* spp. but also increased the incidence of contamination compared to 24 or 48 h. In contrast, others have found baits floated for 24 h detected *P. cinnamomi* as well as or better than those floated for longer durations (19,23). Our results indicate that *Phytophthora* spp. were detected successfully at 24, 48, or 72 h, but the 72-h duration should be used to maximize detection when baiting nursery container mixes for a variety of *Phytophthora* spp. However, to avoid unforeseen interference from contaminating microorganisms and still take advantage of the longer baiting duration, half of the baits can be removed at 24 h and the other half removed at 72 h.

Temperatures of 15, 20, or 25°C during baiting did not have a major effect on detection. *Phytophthora* spp. occasionally were detected more frequently at 20 and 25°C than at 15°C. This range includes normal room temperature, so baiting could be done on a laboratory bench if incubators are not available. Temperature also has affected other baiting bioassays (4,7,16); in one study, detection of *P. cinnamomi* was better at 20 than at 25°C (19).

Table 5. Comparison of leaf disks from *Camellia sasanqua* and six cultivars of *C. japonica* as baits in a bioassay for detecting *Phytophthora* spp. in six naturally infested container mixes (designated A through F)[x]

	Frequency of detection[y]					
Camellia type	**A**	**B**	**C**	**D**	**E**	**F**
C. sasanqua	39	2	30	40	34	1
C. japonica cultivars						
Governor Mouton	40	1	40	40	39	2
Pink Perfection	40	0	40	40	39	0
Kumagai	38	0	39	38	39	2
Lady Clair	40	6	40	40	40	0
Debutante	39	0	40	40	40	1
Marjorie Magnificent	38	0	39	40	30	0
P value[z]	0.624	0.001	0.001	0.140	0.001	0.624

[x] Leaf disks were floated over flooded container mixes for 3 days at 25°C and then were placed on PARPH-V8 selective medium at 20°C for up to 7 days.

[y] Number of baits out of 40 detecting *Phytophthora* spp. Each mix was baited with 20 leaf disks of each species or cultivar in two independent trials; results were similar so data were combined.

[z] *P* values are from Fisher's Exact Test (2-tailed) for binomial data.

Camellia leaf disks and shore juniper needles both were attacked and colonized very readily by zoospores of *P. cinnamomi*, *P. nicotianae*, *P. cryptogea*, and *P. citricola* but were not colonized as readily by zoospores of *P. cactorum*—particularly when zoospores were present at lower concentrations. Although all camellia leaf disks and juniper needles were colonized by three isolates of *P. cactorum*, one isolate colonized none and another isolate colonized only some of the baits. Therefore, a bioassay using camellia leaf disks and shore juniper needles may not always detect the isolates of *P. cactorum* present in nursery container mixes. If *P. cactorum* is suspected in a given container mix sample, it would be advisable to use a bait that is more susceptible to *P. cactorum* along with camellia leaf disks and shore juniper needles.

The species or cultivar of camellia did not appear to greatly affect detection of *Phytophthora* spp. by leaf-disk baits. *C. japonica* cv. Marjorie Magnificent and *C. sasanqua* were the only types that detected *Phytophthora* spp. less frequently than the others even though sufficient inoculum was present—excluding the two container mixes with very low levels of *Phytophthora* spp. Because *C. sasanqua* is resistant to *P. cinnamomi* (18,20), it is not surprising that leaf disks from this species were colonized by *Phytophthora* spp. less often than leaf disks of *C. japonica*, which is susceptible. However, *C. sasanqua* was one of the only two camellia types that detected *Phytophthora* spp. in all of the container mixes assayed. So, this species may be less sensitive overall but appears to be more consistent as a bait than some of the cultivars of *C. japonica*. Although *C. sasanqua* and *C. japonica* cv. Marjorie Magnificent detected *Phytophthora* spp. less frequently than the other types in two of the container mixes, a majority of the leaf disks from both of these types still were colonized by *Phytophthora* spp. Consequently, leaves from any type of camellia probably can be used successfully for baiting nursery container mixes for *Phytophthora* spp. The variability in the initial trial using different types of camellia, which was conducted in early summer, may have been related to the physiological activity of the plants at that time of year. When the experiment was repeated in the fall, there was much less variability among camellia types and between trials; results in the initial and repeat trials were consistent. Consequently, camellia leaf-disk baits should be used cautiously during early summer months, when shoots on plants are actively elongating, and it is advisable also to include another bait type at this time of year.

In summary, the following bioassay procedure can be used routinely to detect *Phytophthora* spp. in naturally infested nursery container mixes. Independently bait three to five replicate subsamples of both fresh and air-dried-then-remoistened container mixes using six camellia (*C. japonica*) leaf disks and six shore juniper (*J. conferta* cv. Blue Pacific) needles per replicate at 20 to 25°C. Remove half of the baits (i.e., three leaf disks and three needles) at 24 h and put these on PARPH-V8 selective medium. Remove and isolate from the other half of the baits at 72 h. Incubate isolation plates at 20°C in the dark, and examine plates periodically over 7 days. This bioassay successfully has detected *P. cinnamomi*, *P. nicotianae*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, and, to a lesser extent, *P. cactorum*.

Our baiting bioassay is the first to be developed specifically for detecting multiple species of *Phytophthora* in naturally infested mixes that are used to produce container-grown ornamental nursery crops. Only one other bioassay (19) has targeted container-grown nursery crops. The bioassay described here could be a valuable tool for research, routine diagnosis, and as a component of an integrated management strategy for Phytophthora diseases on ornamental crops. It can detect *Phytophthora* spp. in container mixes before aboveground symptoms on plants have developed (6); thus, there is the potential to identify problems and initiate disease management actions before losses are incurred. The bioassay has been shown to be effective under field use. It was used to detect *Phytophthora* spp. at ornamental crop nurseries throughout South Carolina (6). This baiting bioassay also may have applications beyond those in ornamental crop nurseries. Recently, camellia leaf disks and a modification of the procedure described here were used to detect *P. erythroseptica* in potato field soils in South Australia (5).

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