A Dipstick Immunoassay for the Specific Detection of *Phytophthora cinnamomi* in Soils

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We thank E. O'Gara, P. Pentland, and B. Philip for assistance during this work and those who provided soil samples.
Accepted for publication 9 August 1994.

ABSTRACT

Cahill, D. M., and Hardham, A. R. 1994. A dipstick immunoassay for the specific detection of *Phytophthora cinnamomi* in soils. Phytopathology 84:1284-1292.

A dipstick immunoassay that is specific for *Phytophthora cinnamomi* was developed for use in soils. Azo dye detection of monoclonal antibody-labeled cysts attached to a nylon membrane provided a rapid, sensitive assay suitable for field use. There was no cross-reaction with other *Phytophthora* and *Pythium* species in controlled environment assays or with soil or other organic matter that adhered to the membrane. The assay was as sensitive as a *Eucalyptus sieberi* baiting assay and, when run together with the baiting assay, was quantitative for an infested soil-water suspension from 2.5×10^2 to 5×10^3 zoospores per milliliter. The assay was used successfully to detect *P. cinnamomi* in a wide range of soil samples collected from beneath a diverse range of host species. There are several advantages to using the dipstick assay compared with traditional procedures: familiarity with *Phytophthora* taxonomy is not required; the assay can be performed by unskilled personnel; and soil rather than infected plant tissues can be assayed. Field testing of the assay showed that in kit form, it could be used as a reliable diagnostic tool to replace or augment current isolation and detection methods. The dipstick assay should find broad use for the detection of *P. cinnamomi* in soil from forests and plant communities and in the horticultural and ornamental crops affected by this pathogen.

Additional keywords: disease diagnosis, Oomycetes, soilborne pathogens.

Phytophthora cinnamomi Rands is a plant pathogen of worldwide distribution (30) and causes disease in a large and diverse range of plant species (2,28–31). This soilborne pathogen seriously affects many horticultural, ornamental, and forestry crops. It is widespread in natural ecosystems, especially those in the southwest and southeast regions of Australia, and has a devastating impact not only on the flora but also the fauna of those regions (20,27). Current control strategies require precise knowledge of the distribution of the pathogen. This has been achieved largely by the testing of soil samples with one or more isolation and identification techniques. While it is possible to isolate and identify *P. cinnamomi* directly from soil and plant tissue within 2–3 days when a high population level is present (13,23), the use of selective media and a sound knowledge of *Phytophthora* taxonomy is required. More often, isolation from soil is by use of a bait plant, such as cotyledons of *Eucalyptus sieberi* L. A. S. Johnson or whole fruit of pear or avocado. Subsequent plating, after several days, onto one or more selective media can be followed 2–3 days later by identification with morphological criteria (9,26,29).

Immunological assays that have been developed for detection of *Phytophthora* spp. in plant tissues have the potential to reduce identification time to hours. These assays have, however, suffered from cross-reactivity with related genera, have reacted poorly and inconsistently with *P. cinnamomi,* and require careful sampling of infected host tissues (1,22). Similar assays developed for use in soils also have been hindered by cross-reactivity, both to soil particles and other *Phytophthora* species (16,17). Molecular probes that are specific for *Phytophthora* spp. (8,21), and *P. cinnamomi* in particular (6,15), recently have been developed. Although they are highly sensitive and specific, their practical use requires expensive, sophisticated equipment and trained laboratory personnel.

We previously described the development of a rapid, sensitive immunoassay that was specific for *P. cinnamomi* (4). The assay is based on the phenomenon of chemotaxis and electrotaxis to attract zoospores in solution to a membrane attached to a plastic dipstick and is designed for field use. This paper describes the practical use of the dipstick immunoassay under a variety of conditions and with soil collected in association with a wide range of plant hosts.

MATERIALS AND METHODS

Fungal isolates and zoospore production. Isolates of all fungi used in the experiments were from an extensive collection housed at the Research School of Biological Sciences, Australian National University. Source and collection details can be found in Gabor et al (7). Isolates used for inoculation were *P. cinnamomi* A2 mating type (H1000), *P. cinnamomi* A1 mating type (H1065), *P. citricola* Sawada (H1017), *P. cryptogea* Pethybr. and Lafferty (H1125), *P. nicotianae* Breda de Haan var. *nicotianae* (H1109), *Pythium aphanidermatum* (Edson) Fitzp. (H200 or H201), *Pythium butleri* L. Subramanian (H202), and *Pythium irregulare* Buisman (H204). Isolates were maintained on V8 juice agar and subcultured regularly.

Zoospores were produced from isolates of *Phytophthora* and *Pythium* with either a shake-culture method (3) (*P. cinnamomi* and *P. n. nicotianae*) or with a soil extract method (12) (*P. cryptogea, Pythium aphanidermatum, Pythium irregulare,* and *Pythium butleri*). For *P. citricola,* zoospores were produced by a combination of these methods. Approximately 1×10^5 zoospores per milliliter were consistently produced.

Plants. Eight species of plants from a range of families were used in inoculation experiments. Seeds of *E. marginata* Donn ex Sm. and *E. calophylla* R. Br. were germinated in moistened vermiculite. Young eucalypt seedlings and seedlings of *Lycopersicon esculentum* Mill. were transferred to a sterile peat-sand-acidic top soil mix in 150-mm standard plastic pots and grown in a Conviron constant environment chamber (Controlled Environments, Winnipeg, Manitoba, Canada) at 14 h of daylight and 10 h of night; 70–80 $\mu E/m^2 \cdot s$; relative humidity, 50%; and temperatures of 24 C during the day and 18 C at night. Plants of *Correa*

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alba Andrews, *Banksia serrata* L., *E. sieberi,* and *Kunzea ambigua* (Sm.) Druce were transferred to pots of sterile soil mix and kept in a glasshouse. They were then transferred to the environmental chamber 2–4 wk prior to inoculation. Plants of *Pinus radiata* D. Don. were collected from the field and transferred to sterile potting mix before they were placed in the glasshouse. Plants were from 3 mo (*L. esculentum*) to 18 mo (*P. radiata*) old when used in experiments.

Infestation of soil with *Phytophthora* and *Pythium* species. Plants of *L. esculentum, B. serrata,* and *P. radiata* were used in initial experiments to determine the soil infestation levels at which the dipstick assay could be used to detect the presence of *P. cinnamomi.* The soil in the pots was infested with a single dilution from a series of 10-fold dilutions (3×10^0 to 3×10^5 per pot) of a *P. cinnamomi* zoospore suspension. Treatments were duplicated, and the experiment was performed at least twice for each species. Three weeks after inoculation, a sample of soil was collected from each pot and tested for the presence of *P. cinnamomi* by using cotyledon baiting in conjunction with the dipstick assay.

Soil in pots containing *E. sieberi, C. alba,* or *K. ambigua* was infested with *Phytophthora* or *Pythium* species alone or with a combination of other *Phytophthora* and *Pythium* species with or without *P. cinnamomi.* In experiments with *E. sieberi,* 18 plants were used; 32 plants of each other species were used. Treatments were duplicated, and plants were arranged randomly in the controlled environmental cabinet. Immediately prior to inoculation, the soil within pots was watered to saturation. The soil was then infested by placing three 1-ml aliquots of separate zoospore suspensions (10^4/ml) of the *Phytophthora* or *Pythium* species, singly or in combination, equally spaced around the pot on the soil surface. Soil in control pots was treated with distilled water. Pots were allowed to drain freely, and the soil was then watered again to saturation. Plants were watered daily throughout the experiments.

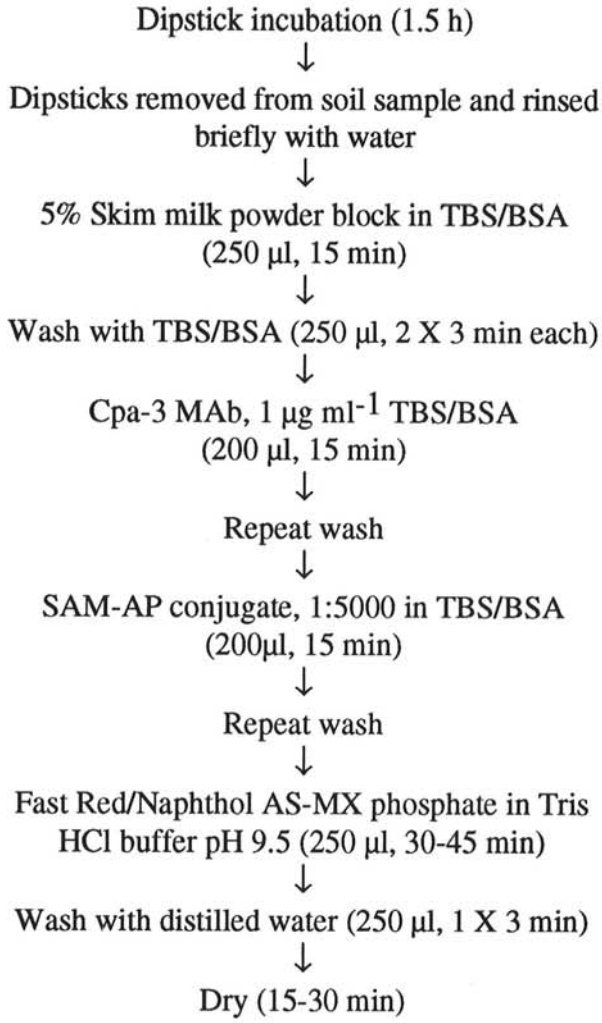

Fig. 1. Protocol for the detection of *Phytophthora cinnamomi* with the dipstick immunoassay. TBS/BSA = Tris-buffered saline, pH 7.4, with 1% (w/v) bovine serum albumin; MAb = monoclonal antibody; and SAM-AP conjugate = sheep-anti-mouse immunoglobulin G alkaline phosphatase conjugate.

Soil sampling and baiting. At appropriate times after inoculation, soil samples (approximately 60 g) were collected from each pot at a depth of 5–10 cm. Individual samples were then divided into three approximately 20-g subsamples and then placed in plastic cups (70 mm in diameter and 45 mm high). To each subsample, 120 ml of single glass-distilled water was added. The soil and water were then mixed for 1 min with a Handy swab applicator stick (BDF Australia Ltd., New South Wales).

Soil samples were baited with cotyledons excised from 3-wk-old seedlings of *E. sieberi* grown in vermiculite in flats (18) or, in some experiments, with needles of young *P. radiata* seedlings (10). Three pairs of cotyledons, adaxial side down, or five 1.5-cm segments of pine needles were placed on the surface of each replicate soil sample slurry. Soil samples were then incubated on the laboratory bench for 60–72 h.

After incubation, soil samples were cold-shocked to induce zoospore release from sporangia (3) by placing them in a cold room at 4 C for 20–30 min.

Dipstick immunoassay for *P. cinnamomi.* Soil samples that had been subjected to cold shock were returned to the laboratory bench, and three dipsticks (4) were floated, membrane side down, on the liquid surface. Dipsticks remained in place for 1.5 h and were then removed and gently washed with distilled water from a wash bottle. The dipstick immunoassay was conducted essentially as previously described (4) (Fig. 1). The assay utilized a monoclonal antibody, Cpa-3, specific for *P. cinnamomi* that recognizes an antigen located on the cyst periphery (11). The assay was modified for use with soils by using as the secondary antibody sheep-anti-mouse Ig (immunoglobulin) G conjugated to alkaline phosphatase (SAM-AP conjugate; Silenus Laboratories, Hawthorn, Australia). Detection of bound secondary antibody was with a mixture of 1% (w/v) 4-chloro-2-methylbenzenediazonium salt (Fast Red TR; Sigma-Aldrich, Castle Hill, New South Wales) in 70% dimethylformamide in water and 1% (w/v) naphthol AS-MX phosphate (Sigma-Aldrich) in 100% dimethylformamide. These solutions were added to 0.1 M Tris-HCl substrate buffer (0.1 M NaCl and 5 mM $MgCl_2 \cdot 6H_2O$, pH 9.5). In the presence of the diazonium salt, an essentially insoluble azo dye (red in color) was formed after removal of the phosphoryl group from naphthol AS phosphate by the alkaline phosphatase (14). All incubations were performed in 96-well, flat-bottomed enzyme-linked immunosorbent assay (ELISA) trays (Disposable Products, Technology Park, South Australia). Dipsticks were

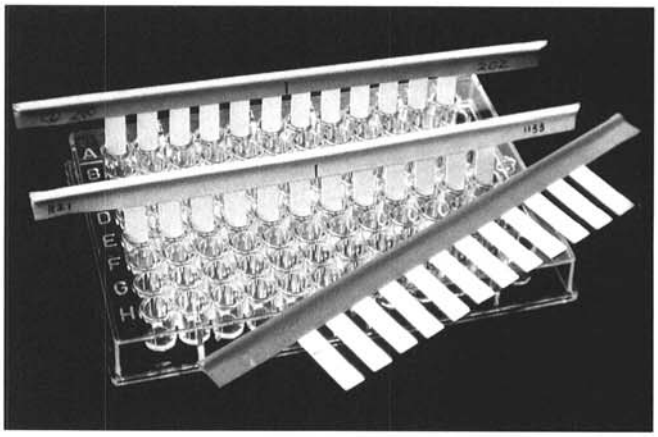

Fig. 2. Dipsticks aligned for use with a 96-well enzyme-linked immunosorbent assay tray for the incubation and washing steps.

aligned on adhesive tape in strips of 12 with a 96-well ELISA tray as a template (Fig. 2) and then subjected to immunoassay. After the final wash in distilled water, the dipsticks were dried at room temperature on the laboratory bench or in a fume hood (15 min). Dipsticks were then observed with a stereo dissecting microscope or a hand lens (20×) for the presence of red pink cysts.

To determine the level of nonspecific binding, a negative control in which the Cpa antibody was replaced by a nonimmune mouse IgG monoclonal antibody (Sigma-Aldrich) at 10 μg/ml was routinely included in the assay.

Plating of bait material and dipsticks onto selective media. Cotyledon baits and a single dipstick per soil sample cup were plated onto the *Phytophthora*-selective medium, PARPH (pimaricin, ampicillin, rifampicin, pentachloronitrobenzene, hymexazol, and cornmeal agar), as modified by Kellam and Coffey (12), or for the isolation of *Pythium* and *Phytophthora* species, onto the same medium without hymexazol (PARP). Identities of the *Phytophthora* and *Pythium* species that were reisolated were determined by morphological characteristics (24). The presence of *P. cinnamomi* was further confirmed by reisolating putative isolates onto V8 juice agar, inducing production and release of zoospores (3), and then retesting the pure zoospore suspension with the dipstick immunoassay.

Effect of baiting with *E. sieberi* cotyledons. The effect of the addition of a bait to the soil slurry on detection of *P. cinnamomi* with the dipstick assay was tested. The soil used was from a bulk soil sample that was collected from pots infested previously with *P. cinnamomi* in the constant environment cabinet. Thirteen to 15 replicate soil test cups were prepared with and without cotyledons of *E. sieberi* and tested by the dipstick assay after 0, 16, 24, 48, 72, and 96 h. Three cotyledons were placed in each replicate bait treatment cup at the start of the experiment. At each time point, four dipsticks were placed on the slurry surface of each baited and unbaited sample. After the cold-shock period, three dipsticks from each cup were processed through the immunoassay ($n = 39$–45 at each time point), and the remaining dipstick ($n = 13$–15) was placed onto selective medium. Also, at each time point, the cotyledons from bait treatments ($n = 39$–45) were plated onto selective medium.

Positively labeled cysts and any background spots were counted on each dipstick at each time point for each soil slurry treatment, and means and ranges were determined. Petri plates with selective medium containing cotyledons and dipsticks were observed for 2–3 days for the presence of *P. cinnamomi* hyphae. The experiment was repeated.

Sensitivity of the dipstick immunoassay. Sensitivity of the routine assay was determined in vitro with a zoospore suspension dilution series (1×10^0 to 1×10^5 zoospores per milliliter) prepared in the plastic test cups. One hundred and twenty milliliters of zoospore suspension was prepared in triplicate for each dilution. Three cotyledons of *E. sieberi* were then placed on the surface of the suspension in each test cup. After 72 h of incubation in the light at 20 C, the test cups were cold-shocked, and three dipsticks were floated on the surface of the suspension. Dipsticks remained in place for 1.5 h, and then from each test cup, two dipsticks were taken through the immunoassay and the third was plated onto PARPH medium. For those dipsticks taken through the immunoassay, cysts on each were counted and means and standard errors for each treatment calculated. All cotyledons from each cup were plated onto selective medium, and plates with cotyledons and dipsticks were observed for up to 4–5 days for the presence of *P. cinnamomi* hyphae. The experiment was repeated twice.

Comparison of the dipstick immunoassay with ELISA for detection of *P. cinnamomi*. An ELISA kit for detection of *Phytophthora* spp. in plant tissues was purchased from Neogen Corporation (Lansing, MI). These kits were formerly manufactured and sold by Agri-Diagnostics Associates (Cinnaminson, NJ). The kit purchased was the "*Phytophthora*" kit, which is equivalent to the former "*Phytophthora* E" kit of Agri-Diagnostics. ELISA was performed according to the manufacturer's instructions, and results were compared with those obtained by the dipstick immunoassay. For ELISA, duplicate root samples (approximately 0.2 g, fresh weight) were collected from 6-mo-old plants of *C. alba* and *K. ambigua* that were grown and inoculated with *Phytophthora* and *Pythium* as previously described. The root samples tested were those that were obviously discolored and/or showed the presence of lesions. Each root sample was tested by ELISA in triplicate, and the mean optical density at 405 nm was calculated.

Testing the dipstick assay with field soils. Preliminary testing of the assay with field soils used samples that were collected from two local sites with a history of infestation by *P. cinnamomi*. The first site was located in the Currowan State Forest, New South Wales, in an area where dead and dying *Macrozamia communis* L. A. S. Johnson were observed in the understory of a forest dominated by *E. maculata* Hook. Twenty-four soil samples (approximately 1 kg each) were collected from beneath the crown of chlorotic *M. communis*. Samples were returned to the laboratory; each soil sample was mixed by hand; and then three 20-g subsamples were tested by cotyledon baiting and the dipstick immunoassay for the presence of *P. cinnamomi* as previously described. Twelve sites within the Australian National Botanic Garden, Canberra, Australian Capital Territory, were sampled similarly, and the soil was tested. Other soils were received from Western Australia (two collections each from gardens, national parks, and a state forest) and from New South Wales (one collection from a commercial protea grower) (Table 1).

As part of a program to test the performance of the dipstick immunoassay, prototype kits were prepared and sent to personnel around Australia. As a result of this testing, 142 soil samples were returned for our independent analysis. Samples were received from 32 different areas throughout Australia (Table 1) where previous isolations or the presence of disease symptoms suggested that *P. cinnamomi* was present. Soils that were tested for the presence of *P. cinnamomi* ranged from sands, loams and sandy loams, krasnozem, and clays of varying color. Potting mixes and garden soils of variable constituents, but usually of high organic content, were also tested. Samples were collected from beneath native vegetation, from soil in which horticultural, ornamental, and forest species were growing, and from potted plants in nurseries.

RESULTS

Characteristics of the cotyledon and pine needle baiting assays and the dipstick immunoassay. Soil samples collected from plants grown under constant environmental conditions and from the field contained root and organic material that acted as reservoirs of inoculum. Observations of roots in the samples, the cotyledon baits, and soil within the soil-slurry cups showed that sporangia of *P. cinnamomi* and the other *Phytophthora* and *Pythium* species formed on roots within 24–48 h. Although zoospores were few in number, germinating cysts were seen at the edges of cotyledon baits. By 48 h, sporangia in a range of developmental stages were present on the edges of the cotyledon baits, and zoospores were present in greater abundance. After 72 h of incubation, there were greater numbers of sporangia around the bait margins, and zoospore release from most sporangia had occurred. Zoospores continued to be released into suspension over the following 24 h.

Each *Phytophthora* and *Pythium* species released zoospores into the soil suspension that were attracted to, and encysted upon, the dipstick membrane floating at the liquid surface. On completion of the immunoassay, cysts of *P. cinnamomi* attached to the dipstick membrane were readily visualized with either a hand-held lens (20×) or a dissecting microscope by the specific, localized production of the insoluble Fast Red precipitate. Cysts of the other species were not labeled (4), but their presence was confirmed by plating dipsticks onto PARPH and PARP media. Most adherent soil particles and organic matter had been washed off during the immunoassay procedure, but the few particles that remained on the dipstick membrane were easily distinguished from *P. cinnamomi* cysts (Fig. 3).

TABLE 1. Testing the dipstick assay with soil samples from a range of host associations, soil types, and geographical locations from within Australia

Collection site[a]	Associated plant species	Soil type	Source	Samples tested	Cotyledon bait[b]	Dipstick plating[b]	Dipstick immunoassay[b]
Currowan State Forest, NSW	*Macrozamia communis*	Sandy loam	Field collection	24	4/24	3/24	3/24
National Botanic Gardens, ACT	*Telopea* sp., *Grevillea* sp., *Banksia* spp.	Variable, mulch high organic	Field collection	12	4/12	4/12	4/12
Robertson, NSW	Protea cultivars	Red volcanic	R. Harris	3	3/3	3/3	3/3
Jarrah forest, WA	Jarrah forest species	Lateric sands	G. Hardy	6	5/6	5/6	5/6
Southwest National Parks, WA	Jarrah forest, heathland	Sandy loams	M. Stukely	14	11/14	11/14	11/14
Jarrah forest, bauxite mines, WA	Jarrah forest, heathland	Laterites	G. Hardy	12	7/12	7/12	7/12
Orange, NSW	*Eucalyptus* sp., *Banksia* spp.	Loams and clays	G. Gurr	4	0/4	1/4	1/4
Robertson, NSW	Protea cultivars	Red volcanic	R. Harris	4	1/4	1/4	1/4
Anglesea, VIC	*Xanthorrhoea* sp., heathland	Sandy clays	S. Laidlaw	6	4/6	4/6	4/6
Various, NSW	*Castanea* sp., *Banksia* sp., *Pinus* spp.	Sandy loams, brown and black clays	M. Dudzinski	6	6/6	6/6	6/6
Walnut orchard, SA	*Juglans regia* L.	Sandy loam, sandy clay loam	G. Walker	4	0/4	0/4	0/4
Various sites, NSW	*Rhododendron* sp., *Grevillea* sp., Waratah, nursery plants	Potting mixes, garden soils	G. Stovold	6	0/6	1/6	1/6
Kinglake NP, VIC	*Xanthorrhoea australis* R. Br.	Krasnozem	M. Duncan	6	5/6	5/6	5/6
Lenswood Horticultural Centre, SA	*J. regia*, *Castanea* sp.	Sandy loams	B. Hall	8	1/8	1/8	3/8
Various sites, NSW	Eucalypt forest	Sandy loams and clays	B. Wild	6	0/6	0/6	0/6
Alstonville, NSW	Avocado, *Leucospermum* sp.	Horticultural soils	S. Darnell	6	3/6	2/6	5/6
Currumbin, Mount Tamborine, QLD	Avocado	Clay loams, krasnozem	H. Ogle	4	4/4	4/4	4/4
Jarrah forest, Alcoa minesite, WA	*Banksia grandis* Willd., *Xanthorrhoea preissi* Endl. in Lehm.	Gravel/loam, clay loams	G. Woodman	4	1/4	1/4	1/4
Jarrah forest, WA	*B. grandis*, *X. preissi*, *Macrozamia* sp.	Lateritic gravels	B. Smith	4	2/4	2/4	2/4
Jarrahdale, Jandakot, WA	*Dryandra sessilis* (Knight) Domin.	Gravel/loamy sands	F. Tay	4	4/4	4/4	4/4
	B. grandis, *B. attenuata* R. Br.	Sand					
Southern TAS	*Leucodendron* sp., protea cultivars, native species	Clay loams, peat, sand	G. Johnstone	5	2/5	2/5	3/5
Mount Tamborine, Currumbin, QLD	Avocado	Krasnozem, clay loam	K. Pegg	4	4/4	3/4	3/4
Mornington Peninsula VIC	*X. australis*	Sandy podzol	I. Smith	4	3/4	3/4	3/4
National Botanic Gardens, ACT	Native species	Various mulched soils	S. Donaldson	6	3/6	3/6	3/6
Kinglake, VIC	Native species	Loams/sandy loams	T. Price	4	3/4	2/4	2/4
Northern NSW	Chickpea, pea, lucerne	Red and black clays	R. Beardsell	4	0/4	0/4	0/4
Grampians National Park, VIC	*Eucalyptus baxteri*, *Grevillea* spp., *Xanthorrhoea* sp., *Hakea* sp.	Sandy loams	D. Guest	3	2/3	2/3	2/3
Near Brisbane, QLD	Protea cultivars, *Leucospermum* sp.	Sandy loam, krasnozem, red earths	L. Turnbull	5	0/5	0/5	0/5
Dwellingup, WA	*Eucalyptus marginata*	Lateric	I. Bennett	4	0/4	0/4	0/4
Busselton/Capel, WA	*E. marginata*, *B. grandis*	Sand, laterite	N. Malacjzuk	5	0/5	0/5	0/5
Near Sydney, NSW	Native species	Sandy loams, garden soil	D. Backhouse	4	1/4	1/4	1/4
Wilsons Promontory, Grampians, VIC	Native species	Sand, sandy loams	G. Weste	10	7/10	7/10	7/10

[a]ACT = Australian Capital Territory; NSW = New South Wales; QLD = Queensland; SA = South Australia; TAS = Tasmania, VIC = Victoria; and WA = Western Australia.
[b]Data represent the number of samples that were positive for *Phytophthora cinnamomi* compared with the total number of samples tested.

Dipsticks that were used as negative controls had low levels of nonspecific binding, and only occasionally were one or two positively labeled spots (possibly caused by precipitation of the Fast Red dye) observed on the dipstick membrane. In subsequent experiments, dipsticks that had four or fewer spots or cysts were classed as negative for *P. cinnamomi,* and those with greater than or equal to five spots or cysts were classed as positive for *P. cinnamomi.*

Infestation of soil in pots with a known density of zoospores in suspension followed by testing the soil for the presence of *P. cinnamomi* by the dipstick immunoassay showed that an initial inoculum of as few as 3×10^2 zoospores per pot were required for positive detection. Baiting of soil with cotyledons also enabled the detection of *P. cinnamomi* at that level of infestation. There were differences in the level at which a positive detection was made that depended on the plant species growing in the soil. Thus, for *B. serrata,* 3×10^2 zoospores per pot were required before positive detection with either the dipstick assay or cotyledon baiting, whereas in pots containing *P. radiata* and *L. esculentum*, between 3×10^2 and 3×10^3 zoospores per pot were required.

Sensitivity of the dipstick immunoassay. The in vitro tests designed to determine the limit of detection of the dipstick immunoassay when used in the routine soil cup assay with added bait showed the lower limit of detection was 2.5×10^2 zoospores per milliliter (Table 2). The number of cysts per dipstick increased with increasing initial density of zoospores in suspension from 1×10^2 to 5×10^4 zoospores per milliliter (Fig. 4). Hyphal growth from dipsticks (Fig. 5) and cotyledons that were plated onto the selective medium also showed a lower limit of detection of 2.5×10^2 zoospores per milliliter.

Baited vs. unbaited assays. Few cysts of *P. cinnamomi* were detected on dipsticks 16 h after soil samples were prepared with or without cotyledon baits (Table 3). At this time, one sample was shown to be positive after dipsticks were plated, and *P. cinnamomi* was detected in three samples by cotyledon baiting. At 24 h, there were several dipsticks that had five or more labeled spots per membrane; however, the mean number of cysts per dipstick was below that which could be regarded as a positive detection (Table 3). *P. cinnamomi* was recovered from dipsticks plated onto PARPH from both baited and unbaited samples, and nine samples were recorded as positive from cotyledon plating. By 48 h, 12 of 13 samples were shown to contain *P. cinnamomi* by the dipstick assay in the presence of baits. Eight of 13 samples without baits were shown by the dipstick assay to be positive. Twelve of 13 samples were shown to be positive by cotyledon baiting. At 48 h, the mean number of positively labeled cysts on dipsticks from baited samples was 14.3, and the number of cysts on individual dipsticks ranged from zero to 88. In contrast, dipsticks from samples run without baits generally had cyst numbers that were lower than the threshold value. By 72 h, all samples were shown to be positive for *P. cinnamomi* with cotyledon baiting, the dipstick assay of baited soils, and plating of dipsticks from baited soils. At this time, the maximum number of cysts for all time points tested was observed on dipsticks run

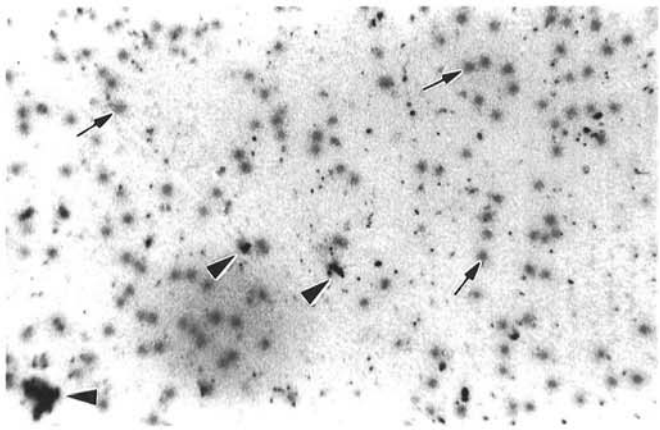

Fig. 3. The dipstick membrane with numerous adherent *Phytophthora cinnamomi* cysts (arrows) and some soil particles (arrowheads) (100×).

TABLE 2. Sensitivity of the dipstick immunoassay when used with cotyledon baiting compared with plating dipsticks and cotyledons onto selective medium

Zoospores per milliliter[a]	Dipstick immunoassay[b,c]	Plating onto selective medium[c,d]	
		Dipsticks	Cotyledons
1×10^5	6/6	3/3	9/9
1×10^4	6/6	3/3	9/9
5×10^3	6/6	3/3	9/9
1×10^3	6/6	3/3	9/9
5×10^2	6/6	3/3	9/9
2.5×10^2	6/6	3/3	9/9
1×10^2	0/6	0/3	0/9
5×10^1	0/6	0/3	0/9
1×10^1	0/6	0/3	0/9
1×10^0	0/6	0/3	0/9
0	0/6	0/3	0/9

[a] Zoospores were diluted to concentration in 120 ml of distilled water in the test cups.
[b] Two dipsticks per test cup were processed through the immunoassay.
[c] Data represent the number of positive assays or isolations compared with the total number of tests from a single experiment.
[d] One dipstick and three cotyledons per test cup were plated onto PARPH medium.

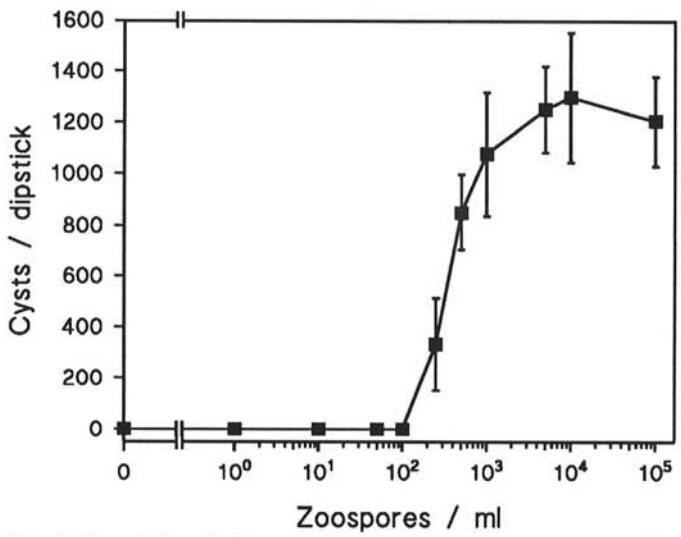

Fig. 4. The relationship between the number of zoospores per milliliter of solution baited with cotyledons and the number of cysts found per dipstick. Each data point is the mean ± standard error ($n = 6$).

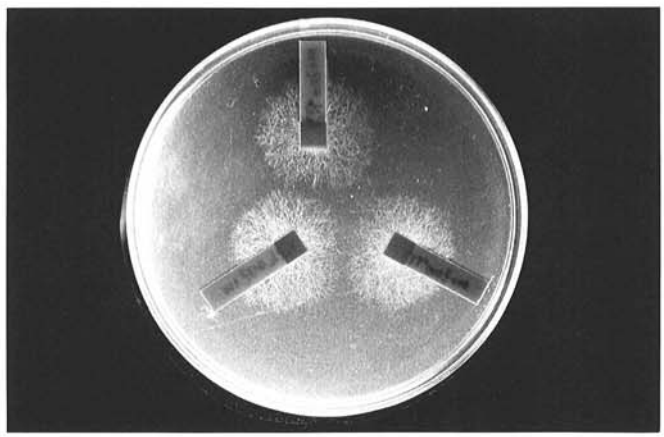

Fig. 5. Growth of hyphae of *Phytophthora cinnamomi* from dipsticks plated onto PARPH selective medium.

with baits, although there was some variation in numbers between dipsticks. In samples without baits, the mean number of cysts was well above the threshold value, but numbers on individual dipsticks varied from one to 31. After 96 h of incubation of soil slurries, all dipsticks were positive for *P. cinnamomi,* whether or not baits were present. Plating of dipsticks and cotyledons taken from soil samples incubated with baits showed all samples to be infested at this time. Plating of dipsticks from unbaited soils showed 11 samples to be positive.

Effectiveness of the dipstick immunoassay for detection of *P. cinnamomi* in artificially inoculated soils. Seedlings of *E. calophylla* (resistant) and *E. marginata* (susceptible) were used in preliminary pot experiments to determine whether *P. cinnamomi* alone in soil could be detected by the dipstick immunoassay. Three weeks after soil infestation, *E. marginata* showed symptoms typical of infection by *P. cinnamomi*—root lesions were well developed; shoots were wilted; leaves were chlorotic; and deaths had occurred. *E. calophylla* developed only restricted root lesions and no other symptoms. Uninoculated control plants remained healthy. When the dipstick assay was used with soil samples taken from pots containing plants of *E. marginata* and *E. calophylla* that had been infested with *P. cinnamomi,* numerous labeled cysts were present on each dipstick (data not shown). Cotyledons and pine needles that were plated onto the selective medium confirmed the presence of *P. cinnamomi.* Soil taken from control pots was negative in each assay.

Further testing of the specificity of the dipstick immunoassay used soil samples taken from pots containing *E. sieberi* (susceptible) with and without *P. cinnamomi* and containing 3×10^4 zoospores per pot (initial density) of other *Phytophthora* or *Pythium* species (Table 4). Three weeks after soil infestation with *P. cinnamomi,* all plants (except one that died some weeks later) showed symptoms of disease. Plants in soil that was infested with *P. n. nicotianae* alone or in combination with the other *Phytophthora* species also showed disease symptoms. In each soil sample tested with the dipstick assay that did not contain *P. cinnamomi,* the assay was negative. Species of *Phytophthora* or *Pythium* that had been used singly or with *P. cinnamomi* were readily reisolated from the cotyledon baits 2–4 days after plating onto selective media. The pine needle baits gave inconsistent isolation results with species other than *P. cinnamomi* and were therefore not used in further experiments.

A third series of experiments with *C. alba* and *K. ambigua* as the plant hosts included detailed reisolation procedures and

TABLE 3. Effect of baiting soil slurries with *Eucalyptus sieberi* cotyledons on the detection of *Phytophthora cinnamomi* with the dipstick immunoassay and a comparison with plating of dipsticks and cotyledons onto a selective medium[a]

Soil incubation time[b] (h)	Dipstick immunoassay[c]		Number of cysts per dipstick[d,e]		Dipsticks plated[c]		Cotyledons plated[c]
	Baited	Not baited	Baited	Not baited	Baited	Not baited	
1	3/15[f]	4/15[f]	0.3 ± 0.6 (0–2)	0.3 ± 0.5 (0–1)	0/15	0/15	0/15
16	1/14[f]	4/14[f]	0.1 ± 0.5 (0–1)	0.5 ± 0.9 (0–2)	1/14	0/14	3/14
24	5/14[f]	9/14[f]	0.7 ± 1.4 (0–5)	1.4 ± 1.9 (0–7)	3/14	2/14	9/14
48	12/13[f]	8/13[f]	14.3 ± 25.5 (0–88)	1.8 ± 2.1 (0–5)	10/13	2/13	12/13
72	13/13	13/13[f]	111.6 ± 136.4 (14–516)	11.4 ± 9.5 (1–31)	13/13	10/13	13/13
96	13/13	13/13	67.9 ± 60.4 (10–224)	96.6 ± 60.2 (25–208)	13/13	11/13	13/13

[a] Results of two experiments were similar and the data for one experiment is shown.
[b] The soil was infested with 3×10^4 zoospores of *P. cinnamomi* (A1 and A2 mating types), *P. citricola*, and *Pythium aphanidermatum.*
[c] Data represent the number of soil samples recorded as positive for *P. cinnamomi* of the total number of samples tested.
[d] Mean number with standard error ($n = 39$–45).
[e] Number in parentheses is the range of values.
[f] Data contain some samples where numbers of cysts on the dipstick membrane were below that required for positive detection.

TABLE 4. Comparison of the dipstick immunoassay with cotyledon and pine needle baiting of soils infested[a] with *Phytophthora* and *Pythium* species and containing *Eucalyptus sieberi* seedlings

Phytophthora or *Pythium* species	Absence or presence of *P. cinnamomi* A2	Detection of *P. cinnamomi*[b]			Plant health[e]
		Dipstick assay[c]	Baiting assays[d]		
			Cotyledon	Pine needle	
P. cinnamomi A1	Absent	+	+	+	Dead
	Present	+	+	+	Healthy[f]
P. nicotianae var. *nicotianae*	Absent	−	−	−	Dead
	Present	+	+	+	Dying
P. citricola	Absent	−	−	−	Healthy
	Present	+	+	+	Chlorotic
P. cryptogea	Absent	−	−	−	Healthy
	Present	+	+	+	Dead
All *Phytophthora* spp.[g]	Absent	−	−	−	Dead
	Present	+	+	+	Dead
Pythium aphanidermatum	Absent	−	−	−	Healthy
	Present	+	+	+	Dead
Pythium irregulare	Absent	−	−	−	Healthy
	Present	+	+	+	Dying
Both *Pythium* species	Absent	−	−	−	Healthy
	Present	+	+	+	Dead
Controls	Absent	−	−	−	Healthy
	Absent	−	−	−	Healthy

[a] Soil was tested 3 wk after infestation.
[b] + = *P. cinnamomi* detected, and − = *P. cinnamomi* not detected.
[c] The assay was performed in the presence of baits.
[d] Baits were plated onto selective media.
[e] Assessed at time of sampling.
[f] Plant died several months after completion of the experiment.
[g] Excluding *P. cinnamomi* A1.

a comparison with a commercial ELISA test. Soils were infested with different *Phytophthora* and *Pythium* species either singly or in combination. Because these plant species behaved similarly to inoculation and the results of the dipstick immunoassay and dipstick and cotyledon plating were similar, only results for *C. alba* are shown. After 3 wk, plants showed varying symptoms (Table 5). Symptoms were evident as chlorosis and plant death, notably in those plants that had been exposed to *P. cinnamomi* but also in those exposed to *P. n. nicotianae*. In pots that contained *P. cinnamomi* (A1 or A2 mating type), *P. cinnamomi* was detected with the dipstick immunoassay. Plating of cotyledon baits and dipsticks onto selective medium also showed the presence of *P. cinnamomi* in each case. Where *P. cinnamomi* was not present in the soil, the dipstick assay was negative, and plating of cotyledon baits and dipsticks confirmed the absence of *P. cinnamomi*. The dipstick immunoassay was able to readily distinguish *P. cinnamomi* when the five other species of *Phytophthora* and *Pythium* were present in the soil and sporulating. The presence on the dipstick membrane of cysts of the other species was shown by plating dipsticks onto the selective media and identifying the hyphae that grew from them.

The commercial *Phytophthora* ELISA gave inconsistent results (Table 5). Absorbance values recorded for individual root samples ranged from approximately 0.1 to as high as 2.7 absorbance units after the 10-min color-development period. Only five root samples from 18 *Phytophthora*-infested soils were positive for *Phytophthora* spp. when tested with the ELISA, even though the assay is reported to detect all the *Phytophthora* species present. There was considerable variation in ELISA readings between duplicate plants; and in several treatments, one plant was shown to be positive in the ELISA and the other negative. The ELISA also detected *Pythium butleri* in roots when it was the sole inoculum. Inoculation with all species of *Phytophthora* and *Pythium* gave rise to high ELISA absorbance values, and all root samples tested were positive.

Performance of the dipstick immunoassay in infested field soils. Limited sampling of local soils and several samples received for preliminary testing of the dipstick immunoassay showed that by using the assay we were able to detect *P. cinnamomi* in a range of soil types (Table 1). The testing program was then widened to encompass samples from throughout the range of *P. cinnamomi* within Australia and thus covered a wide variety of geographical locations, soil types, and associated host species. In total (including those soils assayed in the preliminary tests), 201 soil samples were tested. Ninety-four samples were determined to be positive by the dipstick immunoassay, 90 by cotyledon baiting and plating, and 88 by plating dipsticks onto selective medium.

Agreement between isolation with the dipstick immunoassay and cotyledon baiting was extremely good, and there were few instances where the dipstick immunoassay failed to detect *P.*

TABLE 5. Effectiveness of the dipstick immunoassay for detection of *Phytophthora cinnamomi* in soil from pots containing *Correa alba* in the presence of other *Phytophthora* and *Pythium* species[a]

Species	Absence or presence of *P. cinnamomi* A2[a]	*P. cinnamomi* isolation[b]			Neogen ELISA[d]	Species reisolated from cotyledons and dipsticks	Plant health
		Dipstick immunoassay	Cotyledon plating[c]	Dipstick plating[c]			
P. cinnamomi A2	Present	+	+	+	0.497	*P. cinnamomi*	Chlorotic
	Present	+	+	+	0.191	*P. cinnamomi*	Chlorotic
P. cinnamomi A1	Present	+	+	+	0.402	*P. cinnamomi*	Dead
	Present	+	+	+	0.866*	*P. cinnamomi*	Dead
	Absent	+	+	+	0.117	*P. cinnamomi*	Dead
	Absent	+	+	+	0.567*	*P. cinnamomi*	Chlorotic
P. n. nicotianae[e]	Present	+	+	+	1.215*	*P. cinnamomi*, *P. n. nicotianae*	Chlorotic
	Present	+	+	+	0.272	*P. cinnamomi*, *P. n. nicotianae*	Dead
	Absent	−	−	−	2.315*	*P. nicotianae*	Chlorotic
	Absent	−	−	−	0.107	*P. nicotianae*	Chlorotic
P. citricola	Present	+	+	+	0.149	*P. cinnamomi*, *P. citricola*	Chlorotic
	Present	+	+	+	0.731*	*P. cinnamomi*, *P. citricola*	Chlorotic
	Absent	−	−	−	0.301	*P. citricola*	Chlorotic
	Absent	−	−	−	0.108	*P. citricola*	Chlorotic
P. cryptogea	Present	+	+	+	0.223	*P. cinnamomi*, *P. cryptogea*	Dead
	Present	+	+	+	0.383	*P. cinnamomi*, *P. cryptogea*	Chlorotic
	Absent	−	−	−	0.144	*P. cryptogea*	Chlorotic
	Absent	−	−	−	0.118	*P. cryptogea*	Chlorotic
Pythium aphanidermatum	Present	+	+	+	0.509*	*P. cinnamomi*, *Pythium aphanidermatum*	Chlorotic
	Present	+	+	+	0.168	*P. cinnamomi*, *Pythium aphanidermatum*	Chlorotic
	Absent	−	−	−	0.243	*Pythium aphanidermatum*	Healthy
	Absent	−	−	−	0.118	*Pythium aphanidermatum*	Healthy
Pythium butleri	Present	+	+	+	0.111	*P. cinnanomi*, *Pythium butleri*	Chlorotic
	Present	+	+	+	0.231	*P. cinnamomi*, *Pythium butleri*	Chlorotic
	Absent	−	−	−	0.294	*Pythium butleri*	Chlorotic
	Absent	−	−	−	0.742*	*Pythium butleri*	Healthy
All species	Present	+	+	+	1.559*	All species reisolated	Dead
	Present	+	+	+	0.552*	All species reisolated	Chlorotic
	Absent	−	−	−	2.704*	All species except *P. cinnamomi*	Chlorotic
	Absent	−	−	−	2.471*	All species except *P. cinnamomi*	Dead
Controls	Absent	−	−	−	0.138	Nothing isolated	Healthy
	Absent	−	−	−	0.387	Nothing isolated	Healthy

[a] Data are for duplicate plants in each treatment.
[b] + = *P. cinnamomi* was detected or isolated, and − = *P. cinnamomi* was not detected or isolated.
[c] Cotyledons and dipsticks were plated onto *Phytophthora* (PARPH)- or *Pythium* (PARP)-selective media and observed 2–3 days later for the presence of hyphae.
[d] Enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions. Absorbance at 405 nm. An asterisk indicates a positive detection in ELISA at 3 × SD of control values (1).
[e] *P. nicotianae* var. *nicotianae*.

cinnamomi when it was shown by cotyledon baiting to be present. The reverse, i.e., the dipstick immunoassay recording more positives than cotyledon baiting, also occurred. Whether or not *P. cinnamomi* was isolated, other *Phytophthora* (e.g., *P. megasperma, P. nicotianae, P. cryptogea* [syn. *P. drechsleri*]) and *Pythium* species were often isolated from both dipstick and cotyledon plating (data not shown). Nonspecific background labeling (assessed with a negative control and/or soil known not to contain *P. cinnamomi*) on the dipstick membrane was extremely low throughout the testing.

DISCUSSION

We have shown that the monoclonal antibody-based dipstick immunoassay is a sensitive, specific assay for the detection of *P. cinnamomi* in soils. The assay was shown previously to be specific for *P. cinnamomi* by extensive laboratory screening of isolates of *P. cinnamomi* (A1 and A2 mating types) and isolates of other *Phytophthora, Pythium,* and *Saprolegnia* species (4). The most successful dipstick format identified in the previous study, a plastic dipstick (25 × 5 mm) with a 5-mm square of nylon or nitrocellulose membrane glued to one end, has now been shown to be highly successful for use with soils. The nitrocellulose membrane may be coated with a chemoattractant (for example, aspartic acid) to attract zoospores from suspension, or a positively charged nylon membrane may be used without application of a chemoattractant (4). Use of the latter membrane has several advantages over the use of membranes coated with chemoattractant. For example, preparation of the dipsticks was simplified; possible degradation of the attractant was avoided; and establishment of the relatively short-term attractant gradient within solution was not required. In addition, differences between compounds in their attractiveness to zoospores of different species within the Oomycetes have been shown (5,9). Zoospores from the species we have used in both previous screening (4) and in the pot experiments described here were all found to be attracted to the positively charged nylon membrane.

The dipstick immunoassay has a number of clear advantages over conventional detection procedures. With appropriate modification, the assay can be used as a field test and would not require agar media or a microscope. Knowledge of oomycete taxonomy is not required, because the presence or absence of the end product, red pink spots (*P. cinnnamomi* cysts) on a pale background, gives a yes or no answer immediately. Results can be obtained more quickly than with conventional isolation and detection methods, i.e., within 60–72 h when medium to high inoculum levels are involved, with or without bait plants. The actual immunoassay time can be as short as 1.5 h with the present protocol after dipsticks have been incubated with soil samples. If the assay were to be made direct by using an enzyme-labeled primary antibody, then time may be further reduced compared with an indirect assay. Success of the assay requires that living inoculum be present in the soil being tested, thus reducing the possibility of false positives. A further advantage is that the presence of *P. cinnamomi* can be determined directly from soil. In many instances, it is easier and less time consuming to collect and sample soil rather than to select specific, infected plant tissues. Use of the dipstick assay to detect *P. cinnamomi* need not wait until visible symptoms appear on host plants; we have shown that even though host plants may be healthy in appearance, *P. cinnamomi* may be present in the soil (or in host roots).

There are distinct advantages to using the dipstick immunoassay, and optimum sensitivity and consistency would be afforded if it were used in combination with one of the more common baiting assays (29), especially when the level of inoculum in the soil is low. The dipstick assay was quantitative when used with cotyledons of *E. sieberi* over the range of 2.5×10^2 to 5×10^3 zoospores per milliliter. However, because sporulation (and hence zoospore release) occurred on root pieces and other organic material present in unbaited soils, the assay may be used successfully without baiting. The presence of bait material served two functions in the development of the assay: 1) to increase inoculum (zoospore) levels in suspension and 2) to confirm the presence of *P. cinnamomi* in the samples by plating the bait onto a selective medium.

If a baiting assay were employed in addition to the dipstick immunoassay, then familiarization with the baiting assay to the degree we have demonstrated for *E. sieberi* cotyledon baiting may be necessary. For example, knowing the timing of sporangium production to maximize release of zoospores was critical to the success of the dipstick assay when used with the bait material. Reducing the temperature of the solution in which sporangia of *P. cinnamomi* were produced induced the near-synchronous release of zoospores. The time at which the dipsticks were placed on the soil slurry to optimize zoospore capture could therefore be judged with considerable accuracy. The minimum number of zoospores required to infect bait material successfully may also vary. We found in our test-cup system that 2.5×10^2 zoospores per milliliter or greater were required for infection of *E. sieberi* cotyledons. In a similar baiting assay, Halsall (10) found 50% of *E. sieberi* cotyledons were infected by *P. drechsleri* at a density of 3×10^2 zoospores per milliliter (approximately 10^4 zoospores per milliliter were required for 100% infection of baits). In assays carried out with a range of container types, we have shown that, depending on the size and shape of the container, as few as 40 zoospores per milliliter are sufficient for detection of *P. cinnamomi* by the dipstick immunoassay (4).

The dipstick assay now has been shown to work well with a diverse range of soil types collected from an equally diverse range of host plant associations. Soils included rich, red brown krasnozems, red orange laterites, brown and black clays, loams, sandy loams, sands, and a variety of garden soils. Adherence of soil particles and other soil matter to the dipstick membrane was minimal, and when the membrane was thoroughly air dried, there was little or no background color. Host plants have included those of native forests (e.g., *Eucalyptus, Banksia,* and *Xanthorrhoae* species), horticultural species (e.g., *Persea* spp. [avocado] and *Castanea* sp. [chestnut]), and ornamental species (e.g., protea cultivars and *Leucospermum* spp.).

Monoclonal antibody-based immunoassays, including dipstick-type assays, have been developed successfully for only a limited number of soilborne plant pathogens. For example, Thornton et al (25), using lyophilized mycelial fragments as the immunogen, developed a number of different immunoassays that were specific for *Rhizoctonia solani* in soil. Several of the monoclonal antibody-based assay kits originally developed by Agri-Diagnostics Associates are reasonably specific for the target fungi and include kits directed to *Phytophthora, Pythium, Sclerotinia,* and *Rhizoctonia* species. However, cross-reactivity with related species and other genera is a problem, especially with the *Phytophthora* kit (22).

In our hands, the dipstick assay was considerably more reliable than a commercial ELISA to detect *P. cinnamomi.* In all instances where *P. cinnamomi* was present in the soil, the dipstick assay was found to be positive. Conversely, where *P. cinnamomi* was absent, the dipstick assay was negative. The ELISA showed considerable variation between replicate plants and often failed to detect *P. cinnamomi* (and/or the other *Phytophthora* species) when their presence was shown by the dipstick immunoassay, cotyledon plating, and declining health of the plants. The ELISA was positive in several instances where *Pythium* was the only inoculum; hence, as has been found by others (1,16), this assay cross-reacts with *Pythium* species. The ELISA was strongly positive for samples of soils that had been infested with several *Phytophthora* and *Pythium* species. The ELISA did have the advantage of a short assay time from collection of the sample to reading the results, but this advantage was outweighed by sample-to-sample variation and the need for careful (and hence time-consuming) selection of sample material. Field use of the ELISA would also be limited in forests where selection of diseased plant material (especially from large, woody species) is difficult.

The dipstick assay described here for the isolation and identification of *P. cinnamomi* in soils could almost certainly be adapted for use with other species of *Phytophthora* or *Pythium,* provided that specific monoclonal antibodies to the target organism could

be generated. With little modification, the assay could also be used to detect other microorganisms, such as some bacteria, which are motile or have a motile phase. Identifying the presence of *Phytophthora* to the genus level may often be sufficient because control methods for many *Phytophthora* species are similar. In some areas (for example, the native forests and heathlands of southwestern Australia), plants are susceptible to and are killed by a number of *Phytophthora* species. In such situations, a genus-specific test would be valuable. We envisage that a *Phytophthora* diagnostic kit that had the dipstick assay as its basis would contain a number of species-specific tests as well as a genus-specific test.

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