Techniques

Exploitation of Zoospore Taxis in the Development of a Novel Dipstick Immunoassay for the Specific Detection of *Phytophthora cinnamomi*

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ABSTRACT

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Species-specific monoclonal antibodies (MAbs) were used to develop a dipstick immunoassay for the detection of *Phytophthora cinnamomi*. The assay is based on the phenomena of chemotaxis and electrotaxis to attract zoospores in suspension to a membrane where they encyst and are detected by immunoassay. A chemotaxis assay was used to identify compounds that could be incorporated into the dipstick. The most attractive compounds included several amino acids, alcohols, phenolics, and isovaleraldehyde. Some compounds, such as pectin, aspartic acid, and the phytohormone abscisic acid, caused encystment in addition to attraction. Zoospores also exhibited strong electrotaxis to a positively charged nylon membrane. As few as 40 zoospores per milliliter were detected with the dipstick assay in an assay time of less than 45 min. Immunolabeled cysts attached to the membrane were observed with the naked eye or with low-power magnification after silver enhancement of a gold-labeled secondary probe or after an enzyme color reaction. Screening of 24 MAbs raised to aldehyde-fixed zoospores demonstrated that there were nine MAbs that reacted strongly with *P. cinnamomi* cysts. Two MAbs were revealed to be species specific from an extensive screening of isolates of *P. cinnamomi* (15 isolates of mating type A1 and 29 of mating type A2), of other *Phytophthora* species (21 species or varieties of *Phytophthora* comprising 73 isolates), and of other oomycetous genera (11 species of *Pythium* encompassing 14 isolates and one isolate each of three species of *Saprolegnia*).

Additional keywords: diagnosis, disease detection, zoosporic fungi.

Phytophthora cinnamomi Rands is one of the most important plant pathogens found throughout the tropical and temperate zones (35,37). It affects an increasingly diverse range of species from a wide variety of plant families (36). Hosts include economically important horticultural crops such as avocado, pineapple, and macadamia, ornamental species, and several valuable timber species. Effects of this fungus on the endemic flora of southeastern Australia and the southwestern part of Western Australia, where some plant species are on the brink of extinction, are devastating examples of the impact of an introduced pathogen on a flora composed of many susceptible species (32,35,36). Control of this pathogen and improved understanding of its biology must be based in part on information on the location and density of inoculum in soil. To date, and depending on the method used, relatively difficult and time-consuming procedures were required to first isolate and then identify *P. cinnamomi* from soil. It is possible to isolate and identify *P. cinnamomi* within 2–3 days (20), but most procedures have involved baiting of soil samples for up to 7–10 days followed by plating of the infected bait onto one or more selective media. After growth on the selective medium for several days, identification must be confirmed by someone familiar with *Phytophthora* taxonomy (14,34).

Currently, the most versatile and useful diagnostic assays are those based on antibodies that specifically recognize the target organism. These assays have been used with great success with plant-pathogenic viruses and bacteria (16) and a range of fungi (9). Polyclonal antibodies have been used for the detection of *P. cinnamomi*. Antibodies were produced that permitted detection of chlamydospores of *P. cinnamomi* in soil, but the assay suffered from high background binding of the antibodies to soil particles and lacked species specificity (26). Similar procedures were used to produce antibodies that labeled *Phytophthora* zoospore cysts and germ tubes, but again these were not species specific (23). Several immunoassays were developed for a number of important plant pathogens including *Phytophthora* (28). For example, monoclonal antibodies (MAbs) were used to detect cysts of *Phytophthora* and *Pythium* collected from irrigation water on filter pads (1). These assays all have been based on the use of fungal mycelium or mycelial fractions as the immunogen but have also shown considerable lack of specificity. A collection of MAbs raised to aldehyde-fixed zoospores of *P. cinnamomi*, which showed genus, species, and isolate specificities in preliminary studies (18), has great potential for the development of a species-specific immunodiagnostic test for *P. cinnamomi*.

Perhaps the simplest form of diagnostic immunoassay is based on an antigen absorbed by, or a capture antibody adsorbed to, a dipstick. These assays involve movement of the dipstick from the test solution through solutions containing a labeled antibody and then into a final solution that allows visualization of the bound antibody. This form of immunoassay has been used successfully to detect several plant pathogens (5,10,11,27) and forms the basis of many medical diagnostic assays (22,33). The great advantages of the dipstick-type assays over other antibody-based assays are that they can be carried out quickly, cheaply, and without specialized instrumentation, and a reliable diagnosis can be performed by unskilled workers.

In this paper, we describe the development and use of a dipstick immunoassay that is specific for *P. cinnamomi*. The assay incorporates a novel use of zoosporic chemotaxis and/or electrotaxis followed by detection by species- or genus-specific MAbs.

MATERIALS AND METHODS

Fungal isolates. The *P. cinnamomi* isolate (A2 mating type, 6BR, H1000) used throughout this work was originally isolated

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from the Brisbane Ranges, Victoria, Australia. Sources and culture conditions for this and all other isolates of *Phytophthora, Pythium,* and *Saprolegnia* have been detailed by Gabor et al (13), and each isolate has been assigned a culture collection identification number (13). Four other isolates tested in the present study but not recorded by Gabor et al are listed in Table 1.

Zoospore production. Zoospores of *P. cinnamomi* (H1000) were produced axenically (6), and zoospores of all other fungi were produced with the method of Dolan and Coffey (12). Numbers of zoospores in suspensions ranged from 1×10^3 to 5×10^5/ml.

MAbs. MAbs from a previously described collection (17,18) were used. The MAbs were grouped according to labeling patterns on zoospores and cysts: Zt binds to the surface of the anterior, tinsel flagellum; Zg binds to a restricted area in the ventral groove and to mastigonemes on the anterior flagellum; Cpa binds to the cyst coat material; Lpv binds to the contents of large peripheral vesicles; Cpw binds to the cyst wall; ZCp binds to the surface of both zoospores and cysts; and Vsv and Gvv bind to the contents of ventral surface vesicles located around the groove region.

Chemotaxis assay. A wide range of analytical-grade chemical compounds, including sugars, amino acids, phenolic compounds, alcohols, organic acids, and plant hormones, was tested. The assay for determining the chemoattractiveness of compounds for zoospores of *P. cinnamomi* was modified from an agar-filled tube technique (21). A chemotaxis chamber was formed by attaching two 5-μl microcapillary tubes longitudinally, 13 mm apart, in the center of a glass microscope slide (25.4 × 72.6 mm) and then placing a glass coverslip (22 × 22 mm) over the capillary tubes to form an open-ended chamber (240 μl in volume). Zoospore suspension was carefully introduced into the chamber from a glass pipette. The test substance, dissolved in distilled water, was drawn into a 5-μl capillary tube and inserted 7 mm into one end of the chamber. A tube that contained distilled water alone was inserted into the opposite end of the chamber and served as the control in each test. Four slides were prepared for each test substance. Slides were immediately transferred to a humidity chamber that was then kept in the light at room temperature (approximately 25 C). After incubation for 20 min, slides were removed from the chamber and heated for 5 s on a hot plate to immobilize the zoospores. Counts of zoospores within the capillary tubes were made from images produced from a videographic printer (Sony (Australia), North Ryde, New South Wales) coupled to a monitor and video camera mounted on a Zeiss Photomicroscope III (Carl Zeiss, Camperdown, New South Wales). Zoospores were counted individually by an electronic colony counter (Manostat, New York, NY). The mean numbers of zoospores in the test and control capillaries were calculated, and depending on the ratio of the number of zoospores in the capillary containing the attractant to the number of zoospores in the capillary without attractant, the test compounds were rated as not attractive (ratio of 1:1), weakly attractive (1.1–1.5:1), moderately attractive (1.6–2.5:1), or strongly attractive (>2.5:1). Tests were performed at least twice on each test substance.

Dipstick design. In preliminary experiments, we used a range of solid supports on which zoospores were able to encyst and adhere. Supports included plastic, glass, filter paper, an Immunostick (Nunc, Denmark), nitrocellulose membranes (plain nitrocellulose and supported nitrocellulose [Bio-Rad, North Ryde, New South Wales], Hybond-C and Hybond Super-C [Amersham Australia, North Ryde, New South Wales], zeta-probe and zeta-probe GT nylon membranes, both positively charged [Bio-Rad], and Hybond-N and Hybond-N+, the latter positively charged [Amersham]), and a polyvinylidene difluoride membrane (Bio-Rad). These supports were variously treated for use in several assay types. In some assays, an attractant was added to the membrane square, which in several tests was attached to one end of a plastic support by clear nail varnish. In other assays, the attractant was added to filter paper strips or mixed with gelatin, poly-L-lysine, or glucose and applied to the solid support. The effectiveness of the dipstick format in attracting and capturing the zoospores was assessed by counting the number of cysts that had adhered to the dipsticks after a 30-min incubation in a zoospore suspension (10^4/ml). Dipsticks that did not contain attractant served as controls.

Dipstick immunoassay. Several direct and indirect immunoassays were assessed in preliminary experiments. In initial tests, the presence of cysts on the dipstick was tested with an alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibody enzyme reaction to produce a colored solution, but these assays were successful only when high numbers of cysts (500–1,000) were attached to the membrane (data not shown). Methods based on the formation of an insoluble, colored precipitate on and around the cysts were far superior. An indirect immunoassay with a colloidal gold (10 nm)-labeled secondary antibody (AuroProbe BL GAM immunoglobulin [Ig] G plus IgM [H + L], Amersham) and subsequent silver enhancement (IntenSE BL silver enhancement kit, Amersham) was used routinely in all subsequent experiments. During development of the method, assays were run in 96-well enzyme-linked immunosorbent assay (ELISA) trays, and the total assay time was 3.5–4 h. The time taken for the assay was reduced to less than 45 min without loss of sensitivity by reducing incubation times and by increasing the concentrations of the primary and secondary antibodies.

For the routine assay, a dipstick was placed in 200 μl of zoospore suspension for 30 min and transferred to 250 μl of 5% skim milk (Carnation, Sydney, New South Wales) in Tris-buffered saline (TBS), pH 7.4, with 0.1% gelatin for 15 min. The dipstick was washed twice for 3 min in 250 μl of TBS containing 0.8% bovine serum albumin (TBS-BSA) (Sigma Chemical Co., Castle Hill, New South Wales) and placed into 200 μl of genus- or species-specific MAb appropriately diluted in TBS-BSA. After incubation for 45 min, the dipstick was washed twice for 3 min in 250 μl of TBS-BSA and placed into 200 μl of gold-labeled goat anti-mouse IgG plus IgM (H + L) for 45 min. The dipstick was then washed once in 250 μl of TBS-BSA for 3 min, once in 250 μl of TBS for 3 min, and in 250 μl of distilled water for 1 min. The dipstick then was transferred to 250 μl of silver enhancement reagent. Within 60–70 min, an intense black precipitate formed on and around cysts, which had bound the primary antibody. The reaction was stopped by immersing the dipstick in distilled water for 1–2 min. The dipstick was then air-dried, and cysts were observed unmagnified or with a 10× hand lens. Dipsticks were critically examined with a stereo dissecting microscope with zoom optics (maximum magnification 67.5×) and incident illumination. All steps in the protocol were conducted in a laboratory at room temperature under constant lighting conditions. In assays for which counts of the total number of cysts on each dipstick were required, the procedure used for counting cysts in the chemo-

TABLE 1. Isolates of *Phytophthora cinnamomi* screened in the present study in addition to those described by Gabor et al (13)

Isolate	Alternate code	Mating type	Host	Location	Source[a]
H1075	A2190	A2	*Pinus pseudostrobus*	Nompia, Papua New Guinea	M. Dudzinski, CSIRO Forestry, Australian Capital Territory
H1076	A2189	A2	*Persea americana*	Mt. Hagen, Papua New Guinea	M. Dudzinski
H1083	A2182	A2	*Araucaria cunninghamii*	Wau, Papua New Guinea	M. Dudzinski
H1090	A119	A1	*Castanopsis* sp.	Mauki, Bulolo, Papua New Guinea	F. Arentz, ANUTECH Pty. Ltd., Canberra, Australian Capital Territory

[a] CSIRO = Commonwealth Scientific and Industrial Research Organisation.

taxis chambers was followed. Dipsticks were attached to microscope slides with adhesive tape and illuminated from above with a dissecting microscope light source.

Application of the chemoattractant. Aspartic acid or glutamic acid was dissolved in distilled water at 95 C. A 1-μl aliquot of a suitably diluted solution of the amino acid was placed in the center of the membrane square. The dipsticks were then used immediately or stored desiccated. To test the effectiveness of these two compounds after application to the dipstick membrane, dipsticks were incubated in a zoospore suspension for 30 min, and cysts were counted as described above.

MAb screening. Twenty-four MAbs, selected from the immunofluorescence assay as potentially diagnostic from an original collection of 35 MAbs (13), were screened for the ability to bind to cysts in the dipstick assay. Affinity purified antibodies (1 or 10 μg/ml) or hybridoma supernatants (neat or 50% dilution in TBS-BSA) were used in the indirect immunoassay, and each was scored after silver enhancement of the secondary probe relative to a nonimmune mouse IgG negative control (10 μg/ml) for its ability to bind to cysts that were attached to the dipstick membrane and to produce a visible precipitate. MAbs were tested on duplicate dipsticks with *P. cinnamomi* (6BR, H1000) cysts.

Cross-reactivity of *Phytophthora* and related genera. Cross-reactivity of the MAbs with cysts of isolates of *Phytophthora, Pythium,* or *Saprolegnia* was tested with the dipstick immunoassay. The Cpa (Cpa-2, 1 μg of the purified MAb per milliliter; Cpa-3, 25% dilution of hybridoma supernatant; and Cpa-7, 50% dilution of hybridoma supernatant), ZCp-2 (50% dilution of hybridoma supernatant), and Cpw-4 (10 μg of purified MAb per milliliter) MAbs were tested. Nonimmune mouse IgG (10 μg/ml) was used as the negative control for each isolate. All MAbs were diluted in TBS-BSA. MAb Vsv-1, which recognizes an antigen within the ventral surface vesicles of zoospores in each of the genera, was used as a check for the presence of cysts of *Pythium* and *Saprolegnia* on the dipstick membrane, since cysts of these genera were not labeled by the MAbs tested. The dipsticks were joined in strips of 12 so that they could be used in a 96-well ELISA plate assay.

Sensitivity of the dipstick assay. Three procedures were compared and used to determine the dipstick assay sensitivity. A dilution series of zoospores from an initial concentration of 10^4 zoospores per milliliter was used for each. In the first procedure, the zoospore suspension was diluted with distilled water to a range of concentrations in a total volume of 12 ml and was added to a 10-ml Erlenmeyer flask so that the surface of the zoospore suspension was approximately halfway up the neck (12 mm internal diameter) of the flask. In the second procedure, 10 ml of zoospore suspension was dispensed into small glass petri dishes (40 mm diameter). A single dipstick was placed, membrane side down, on the surface of the zoospore suspension in each flask or petri dish. In a third procedure, dipsticks were used in an ELISA tray assay in which zoospores were diluted with distilled water to form a dilution series. Dipsticks were placed vertically into 200 μl of zoospore suspension within the well of the tray. After incubation for 30 min in the zoospore suspensions, dipsticks were removed and subjected to immunoassay. Cysts on the dipstick membrane were counted as described above.

TABLE 2. Relative attractiveness of compounds for zoospores of *Phytophthora cinnamomi* determined by the "swim-in" chemotaxis assay

Compound	Attractiveness[a]	Compound	Attractiveness
Sugars (10 mM)		Amino acids (1 mM)	
D-Mannose	−	L-Aspartic acid	+++[b]
D-Xylose	−	D-Aspartic acid	+++
D-Ribose	−	L-Methionine	++
D-Fructose	−	D-Methionine	−
D-Lactose	−	L-Glutamic acid	+++[b]
D-Glucose	−	L-Arginine	++
Sucrose	−	Guanidine	+
D-Galactose	−	Glycine	−
L-Fucose	−	L-Cysteine	+
D-Cellobiose	−	L-Lysine	−
L-Rhamnose	−	L-Leucine	−
L-Arabinose	−	L-Phenylalanine	−
Phenolic compounds (10 mM)		L-Tyrosine	−
		Phytohormones (10 mM)	
Caffeic acid	+[b]	Kinetin	−
Hydroxybenzoic acid	−	Benzylaminopurine	−
Ferulic acid	++	Isopentenyladenine	−
Syringic acid	+[b]	± Abscisic acid	+[b]
Gallic acid	−[b]	Gibberellic acid (GA3)	−
Gentisic acid	+[b]	Indolbutyric acid	+[b]
p-Coumaric acid	−	Napthalene acetic acid	+[b]
Vanillin	+	Alcohols (25 mM)	
Coumarin	−	Ethanol	+++
Phloroglucinol	−	Methanol	++
Rutin	−	Isopropanol	+++
Myrcetin	−	Pectin and derivatives	
Kaempferol	−	D-Galacturonic acid (100 μg/ml)	−
Organic acids (10 mM)		Polygalacturonic acid (100 μg/ml)	+
Maleic	+		
L-Malic	+	Pectin (100 μg/ml)	++[b]
Citric	+[b]	Aldehyde (1 mM)	
Succinic	−	Isovaleraldehyde	+++
Fumaric	+	Miscellaneous	
L-Ascorbic	++	Casein (100 μg/ml)	−[b]
Valeric	+	Casein hydrolysate (100 μg/ml)	
Isovaleric	+		+
Folic	−	V8 juice (20%)	+
Acetic	−	V8 broth (5%)	+
		Gelatin (10%)	++

[a] Attractiveness was determined as the ratio of the number of *P. cinnamomi* zoospores and cysts in capillary tubes containing the compound compared with the number in capillaries without the compound: − = ratio of 1:1; + = ratio of 1.1–1.5:1; ++ = 1.6–2.5:1; +++ = >2.5:1.

[b] Also caused some encystment of zoospores.

RESULTS

Selection of chemoattractant(s). Compounds that were chemotactically attractive to zoospores of *P. cinnamomi* were identified in the "swim-in" assay (Table 2). Many of the compounds tested caused rapid accumulation of zoospores within the capillary tubes. The compounds included those known to occur in the rhizosphere, root exudates, and roots of many plant species (8,25). Of the compounds tested, several amino acids, alcohols, phenolic compounds, and isovaleraldehyde were highly attractive. The amino acids aspartic acid and glutamic acid were especially attractive (Table 3). Some compounds, including pectin, syringic acid, abscisic acid, aspartic acid, and glutamic acid, also caused encystment. Sugars were generally not attractive except at relatively high concentrations (>100 mM) (data not shown). Preliminary experiments with several of the compounds run in dilution series demonstrated that there was a concentration for each compound (usually >100 mM) above which zoospores were repelled or en-

TABLE 3. Comparison of amino acids (1 mM) in the chemotaxis assay for attractiveness to *Phytophthora cinnamomi* zoospores

Amino acid	Number of zoospores in capillaries[a]		Ratio[b]
	Control	With amino acid	
L-Aspartic	24.6 ± 11.2	128.2 ± 32.0	5.2:1***
D-Aspartic	30.2 ± 7.7	116.8 ± 18.3	3.9:1***
L-Methionine	26.6 ± 11.8	41.4 ± 15.0	1.6:1
D-Methionine	102.4 ± 10.2[c]	99.4 ± 21.3[c]	1.0:1
L-Glutamic	20.0 ± 7.3	78.2 ± 18.8	3.9:1**
L-Arginine	32.8 ± 6.4	147.4 ± 37.1	4.5:1***

[a] Mean and standard errors of counts of zoospores in one field of view from four replicates from two separate experiments.

[b] Ratio calculated as number of zoospores in the capillaries containing the amino acid divided by the number in capillaries without amino acid. Means were compared by analysis of variance. ** = Significantly different from controls, $P < 0.05$; *** = significantly different from controls, $P < 0.01$.

[c] Mean and standard errors of counts of zoospores from two fields of view.

cysted rapidly and a concentration below which zoospores were not attracted. For example, concentrations of aspartic and glutamic acids that caused the greatest accumulation of zoospores within the capillary tubes differed; the concentration was 0.1 mM for aspartic acid (L or D configuration) and 1 mM for glutamic acid (Fig. 1). On the basis of their attractiveness and encystment-inducing properties, aspartic and glutamic acids, arginine, pectin, and ethanol were chosen for use in development of the dipstick assay.

Dipstick format. Zoospores were attracted to and encysted upon all the solid supports used (Table 4). The presence of an attractant absorbed into a membrane, mixed with the coating substance, or dried to the support surface increased the number of cysts bound to the support compared with controls that did not contain attractant. Cyst numbers were higher on the nitrocellulose and nylon membranes treated with attractant than on other support types and coatings. Coated supports, such as the Immunostick, glass slides, and the plastic dipstick, attracted zoospores, but the coating material usually did not bind well and was lost after several washes. This made the coatings inappropriate for use in an immunoassay. In contrast, cysts adhered well to the membrane surfaces and were not removed by washing.

The most successful and useful format was the plastic dipstick (5 × 20 mm) with a square (5 × 5 mm) of nitrocellulose or nylon membrane glued to one end. A variety of membrane types was tried, but the nylon membranes, which are more hydrophilic than is nitrocellulose, enabled more even dispersal and rapid absorption of the water drop containing the dissolved attractant. The polyvinylidene difluoride membrane, although used successfully by others (9), was unsuitable because it must be wetted with methanol before use. Positively charged nylon membranes attracted zoospores in greater numbers with or without added chemoattractant than did neutral membrane. The zeta-probe nylon membrane, which carries a high-density quaternary amine charge, was especially attractive.

These plastic dipsticks with attached membranes had several advantages over the other supports and coatings. They could be used vertically in or horizontally on a zoospore suspension and could be used in 96-well ELISA trays either as single sticks or, when correctly aligned on a length of adhesive tape, in strips of up to 12. The use of multiple dipstick strips enabled the processing of many samples simultaneously and considerably eased the logistics of the immunoassay.

MAb screening. There was no labeling by MAbs of the Zt group of *P. cinnamomi* (6BR, H1000) cysts that had adhered to the dipstick membrane. There were very small localized areas of labeling with each of the Zg MAbs (possible labeling of the water expulsion vacuole) (*unpublished*), but these could be seen only under high-power magnification (>100×). All the Lpv MAbs gave weak to very weak, diffuse labeling patterns. In contrast, seven of the Cpa MAbs reacted moderately or strongly with material coating the cyst surface. Three of the Cpa MAbs (Cpa-5, Cpa-8, and Cpa-12) showed no labeling or only weak labeling. The Cpw-4 and ZCp-2 MAbs reacted moderately and the Gvv MAb only weakly in the assay.

Five MAbs were selected on the basis of the intensity of labeling of cysts in the dipstick assay and on supplementary information from immunofluorescence and ELISA studies (13): MAbs Cpa-2, Cpa-3, and Cpa-7 (putative species specific), and Cpw-4 and ZCp-2 (putative genus specific). These MAbs were used in further studies to test for specificity in the dipstick assay.

Screening *Phytophthora* and related genera. Forty-four isolates of *P. cinnamomi*, including 15 A1 mating type and 29 A2 mating type obtained from throughout Australia and including isolates from Papua New Guinea and Japan, 21 species or varieties of *Phytophthora* encompassing 75 isolates, 11 species of *Pythium* encompassing 13 isolates, and three species of *Saprolegnia* (one isolate each), were tested against the five selected MAbs with the dipstick assay (Tables 5 and 6). All three Cpa MAbs labeled cysts of the *P. cinnamomi* isolates but did not label any cysts from isolates of the other *Phytophthora* species. In contrast, MAbs Cpw-4 and ZCp-2 labeled all *Phytophthora* isolates. Slight variation occurred among the *P. cinnamomi* isolates in labeling intensity of the cysts, but no difference occurred between A1 and A2 mating types. The putative species-specific MAbs labeled cysts more strongly than did the putative genus-specific MAbs. MAbs Cpa-3 and Cpa-7 did not label any cysts from the isolates of *Pythium* and *Saprolegnia* tested (Table 6). MAb Cpa-2 did, however, cross-react weakly with *P. aphanidermatum, P. butleri, P. debaryanum, P. irregulare,* and *S. declina.* MAb ZCp-2 reacted weakly with *P. debaryanum* and *P. irregulare.* MAb Cpw-4 reacted weakly with *P. middletonii.* MAb Vsv-1 reacted with all isolates of *Pythium* and *Saprolegnia.*

Dipstick assay sensitivity. The shape of the container holding the zoospore solution influenced the number of cysts found on the dipstick membrane. Over the range of dilutions of the zoospore suspension, more cysts were found on dipsticks used in the conical flask assay than in the petri dish or ELISA plate assays. The sensitivity of the dipstick assay was determined from a dilution series as the highest dilution of zoospores that could be detected. For the conical flask assay, 40 zoospores per milliliter was the lowest concentration at which one or more cysts were found on the dipstick membrane after the 30-min incubation period (Fig. 2). Minimum detection limits were 156 and 312 zoospores per milliliter for the petri dish and ELISA plate assays, respectively. Results from the ELISA plate assay are, however, not directly comparable to those of the other two assays, because the dipsticks were placed vertically in the zoospore solution. A single cyst attached to the dipstick membrane was the minimum required for a positive identification, although in practice probably no fewer than 5–10 cysts per membrane would be the minimum necessary to be confident of an identification.

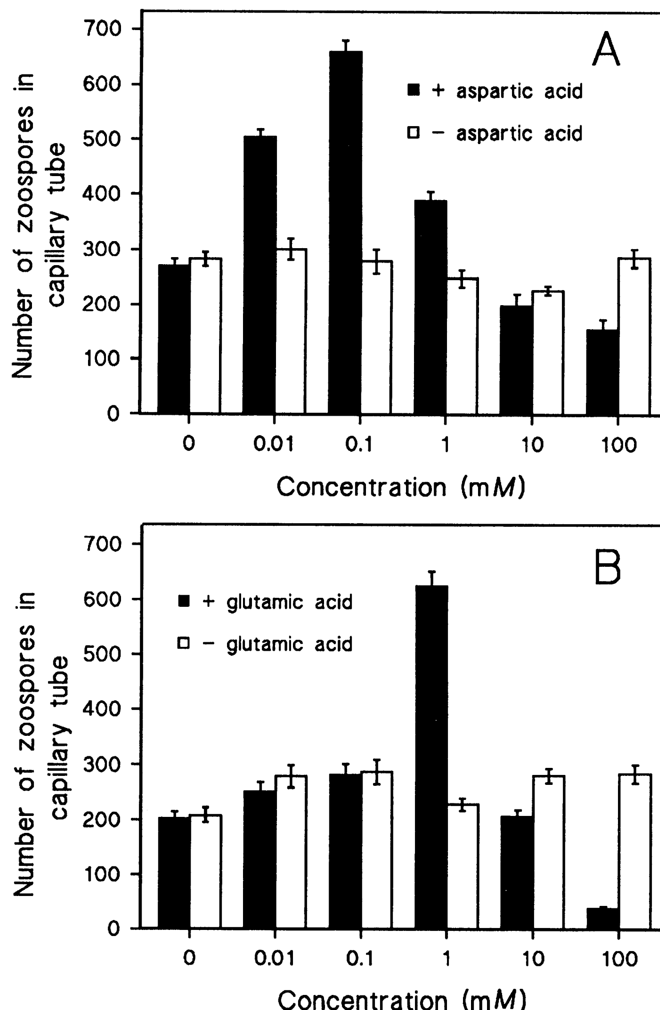

Fig. 1. Concentration-dependent effect of **A,** aspartic acid and **B,** glutamic acid in the "swim-in" chemotaxis assay. Standard errors of the mean of four replicates in two separate experiments are indicated.

DISCUSSION

We have used MAbs that recognize antigens on the cyst surface to develop a rapid dipstick immunoassay that is specific for *P. cinnamomi.* The basis for this assay is a novel application of the phenomenon of chemotaxis, and with the selection of appropriate capture membranes, this can be augmented by electrotaxis to a positive charge. That both of these taxes can be used has implications for the success of the assay. Diffusion of a chemoattractant enables a "long-distance" (several millimeters), short-term (minutes), chemical gradient to be established, whereas the presence of a positive charge in and around the immediate vicinity of the membrane serves as a "close-range," longer-term (hours) attractant. The assay has shown no cross-reactivity with many isolates of other *Phytophthora, Pythium,* and *Saprolegnia* species. At present, we have two MAbs from the Cpa group that are species specific and could be used in a diagnostic kit.

Zoospores of oomycetous fungi are attracted by a variety of chemical compounds that includes amino acids, sugars, alcohols, aldehydes, and phenolic compounds (2,7,19,21,29). Attraction of *P. cinnamomi* zoospores to several phenolic acids and phytohormones has not been previously reported. In a similar assay, other phenolic compounds, especially the isoflavones diadzein and genistein, were both potent and specific attractants of zoospores of *P. sojae* (29). Of the 65 compounds and mixtures tested in the present study, 32 attracted zoospores of *P. cinnamomi* to some degree at a concentration of 25 mM or less. The alcohols, amino acids, and isovaleraldehyde were the most attractive substances. We have also demonstrated that aspartic acid and glutamic acid can induce both attraction and encystment of zoospores of *P. cinnamomi* in a manner similar to that found for zoospores of *P. aphanidermatum* (19). Aspartic acid was used as the attractant in our assays because it was a potent attractor and induced encystment.

The sensitivity of the dipstick assay was determined to be as few as 40 zoospores per milliliter in a conical flask. This level of sensitivity is similar to that of an immunoassay for zoospores in irrigation water (1) and detection of ascospores of *Venturia inaequalis* (4) and greater than that of a chemoluminescence-based assay for bacteria in soil (31). The real sensitivity of the dipstick assay probably is fewer than 40 zoospores per milliliter, because zoospores in an unstirred solution are not randomly distributed. Over time, they tend to aggregate at the air-water interface, possibly because of negative geotaxis and/or positive aerotaxis. Movement of zoospores to the surface of a liquid can occur quickly (within minutes) in still solutions. The concentration of zoospores near the interface will thus depend on the initial concentration of zoospores in suspension, the volume of suspension, and the surface area of the interface. Zoospore concentration at the surface will be in inverse proportion to the surface area. The concentration of zoospores at the interface also increases with time. Theoretically, to maximize the number of zoospores at the interface in a given volume of suspension, the container should be a conical shape (ideally a frustum of a cone). The sloping internal walls of a conical container could direct upwardly moving zoospores to the small surface area of the opening and hence greatly increase the concentration of zoospores around the air-water interface. Furthermore, chances that a zoospore will encounter the chemical

TABLE 4. Effectiveness of various solid supports, assays, coatings, and chemoattractants for the capture and assay of zoospores of *Phytophthora cinnamomi*

Solid support	Assay format	Coating	Test compound[a]	Ratio[b]
Nitrocellulose membrane	Square (5 × 5 mm) floated on zoospore suspension	Nc[c]	Aspartic acid	++
		Nc	Arginine	+
		Nc	Glutamic acid	+++
		Nc	V8 broth	+
		Nc	Pectin	++
	Strips (2 × 10 mm long) in chemotaxis chamber	Nc	Aspartic acid	++
		Nc	Glutamic acid	+++
		Nc	Ethanol	+++
	Strips (5 × 10 mm long) in ELISA[d] tray	Nc	Aspartic acid	+
		Nc	Glutamic acid	++
		Nc	Pectin	+++
		Nc	Ethanol	++
Immunostick	Immersed in zoospore suspension	Nc	Aspartic acid	+
		Nc	Glutamic acid	+
		Nc	Pectin	+
		5% Glucose	Aspartic acid	+
		5% Glucose	Glutamic acid	++
		5% Glucose	Pectin	+
		10% Gelatin	Aspartic acid	+
		10% Gelatin	Glutamic acid	++
		10% Gelatin	Pectin	++
		Poly-L-lysine	Aspartic acid	+
		Poly-L-lysine	Glutamic acid	+++
		Poly-L-lysine	Pectin	++
Plastic dipstick (5 × 20 mm long)	ELISA tray	10% Gelatin	Glutamic acid	+
		Poly-L-lysine	Glutamic acid	+
Plastic dipstick (5 × 20 mm long)	Nitrocellulose membrane (5 × 5 mm)	Nc	Aspartic acid	+++
		Nc	Glutamic acid	+++
	Nylon membrane (5 × 5 mm)	Nc	Aspartic acid	+++
		Nc	Glutamic acid	+++
	Nylon membrane (5 × 5 mm)	Nc	No attractant	+++
Filter paper	Strips (2 × 10 mm long) in chemotaxis chamber	Nc	Aspartic acid	++
		Nc	Glutamic acid	++
		Nc	Ethanol	++
Glass microscope slide	Immersed in zoospore suspension	10% Gelatin	Aspartic acid	++

[a] Test compounds were assayed at 0.1 mM aspartic acid, 0.1 mM arginine, 1 mM glutamic acid, 25 mM ethanol, 5% V8 broth, and 100 μg of pectin per milliliter.
[b] Number of cysts on the solid support with the attractant divided by the number of cysts on the solid support without attractant. + = Ratio of 1.1–1.5:1; ++ = 1.6–2.5:1; +++ = > 2.5:1.
[c] Not coated.
[d] Enzyme-linked immunosorbent assay.

and electrical gradients associated with the dipstick will be enhanced by a small surface area. In determining the sensitivity of the dipstick assay, the change in concentration of zoospores in suspension over time must be considered. In our dilution series experiments, the concentration of zoospores stated is that of the initial, stirred solution.

After the 30-min incubation period, during which zoospores were exposed to the dipstick, it is not likely that every cyst stuck to the dipstick membrane possessed a completely formed cell wall, even though this process takes only a few minutes; thus, a range of cyst developmental states probably was present. Use of the MAbs must take this into account, because the permeability of

TABLE 5. Reaction of *Phytophthora cinnamomi* mating types and other *Phytophthora* species to species- and genus-specific monoclonal antibodies (MAbs) in the dipstick assay

Species		Species-specific MAbs[a]			Genus-specific MAbs	
Mating type	Isolate	Cpa-2	Cpa-3	Cpa-7	ZCp-2	Cpw-4
P. batemanensis	H1015	−	−	−	+	+
P. boehmeriae	H1026	−	−	−	+	+
P. cactorum	H1016	−	−	−	+	+
	H1039[b]	−	−	−	+	+
P. cambivora	H1045	−	−	−	+	+
	H1153	−	−	−	+	+
P. cinnamomi						
A2	H1000	+	+	+	+	+
A2	H1001	+	+	+	+	+
A1	H1003	+	+	+	+	+
A2	H1004	+	+	+	+	+
A2	H1005	+	+	+	+	+
A2	H1006	+	+	+	+	+
A2	H1007	+	+	+	+	+
A2	H1008	+	+	+	+	−
A2	H1009	+	+	+	+	+
A2	H1010	+	+	+	+	+
A2	H1011	+	+	+	+	+
A1	H1012	+	+	+	+	+
A2	H1060	+	+	+	+	+
A2	H1064	+	+	+	+	+
A1	H1065	+	+	+	+	+
A1	H1066	+	+	+	+	−
A1	H1067	+	+	+	+	+
A2	H1068	+	+	+	+	+
A1	H1069[b]	+	+	+	+	+
A2	H1070	+	+	+	+	+
A1	H1072[b]	+	+	+	+	−
A2	H1073	+	+	+	+	+
A2	H1074	+	+	+	+	+
A2	H1075	+	+	+	+	+
A2	H1076	+	+	+	+	+
A2	H1077	+	+	+	+	+
A2	H1079	+	+	+	+	+
A2	H1080	+	+	+	−	+
A2	H1081	+	+	+	+	+
A2	H1082	+	+	+	+	+
A2	H1083	+	+	+	+	+
A2	H1084[b]	+	+	+	+	+
A1	H1085	+	+	+	+	+
A1	H1087	+	+	+	+	+
A1	H1090	+	+	+	+	+
A1	H1092	+	+	+	+	−
A1	H1094	+	+	+	+	+
A1	H1096	+	+	+	+	+
A1	H1113	+	+	+	+	+
A2	H1115	+	+	+	+	+
A2	H1116	+	+	+	+	+
A1	H1117	+	+	+	+	+
A2	H1118[b]	+	+	+	+	+
A2	H1119	+	+	+	+	+
P. citricola	H1017	−	−	−	+	+
	H1046	−	−	−	+	+
	H1049	−	−	−	+	+
	H1056	−	−	−	+	+
	H1132	−	−	−	+	+
	H1134	−	−	−	+	+
	H1135	−	−	−	+	−
	H1136	−	−	−	+	+
	H1137	−	−	−	+	+
	H1157	−	−	−	+	+
P. citrophthora	H1018	−	−	−	+	+
P. cryptogea	H1050	−	−	−	+	+
	H1051	−	−	−	+	+
	H1120	−	−	−	+	+
	H1122	−	−	−	+	+
	H1123	−	−	−	+	+
	H1124	−	−	−	+	+
	H1125	−	−	−	+	+
	H1126	−	−	−	+	+
P. drechsleri	H1025	−	−	−	+	+
	H1127	−	−	−	+	+
	H1128	−	−	−	+	+
	H1129	−	−	−	+	+
	H1130	−	−	−	+	+
P. erythroseptica	H1019	−	−	−	+	−
	H1020	−	−	−	+	−
	H1162	−	−	−	+	+
P. heveae	H1030[b]	−	−	−	+	+
P. meadii	H1031	−	−	−	+	+
P. megasperma	H1035	−	−	−	+	+
	H1040	−	−	−	+	NT
	H1043	−	−	−	+	+
	H1163	−	−	−	+	+
	H1164	−	−	−	+	+
P. megasperma var. *megasperma*	H1139	−	−	−	+	NT
	H1140	−	−	−	+	+
P. megasperma var. *sojae*	H1052	−	−	−	+	+
	H1141	−	−	−	+	NT
	H1142	−	−	−	+	+
	H1143	−	−	−	+	+
	H1144	−	−	−	+	+
P. nicotianae	H1042	−	−	−	+	+
	H1105	−	−	−	+	+
	H1106	−	−	−	+	NT
	H1107	−	−	−	+	+
	H1108	−	−	−	+	+
P. nicotianae var. *nicotianae*	H1032	−	−	−	+	+
	H1041[b]	−	−	−	+	+
	H1097	−	−	−	+	+
	H1098[b]	−	−	−	+	+
	H1100	−	−	−	+	+
	H1102	−	−	−	+	+
	H1103	−	−	−	+	+
	H1109	−	−	−	+	+
	H1111	−	−	−	+	+
	H1112[b]	−	−	−	+	+
	H1145	−	−	−	+	+
	H1146	−	−	−	+	+
	H1147	−	−	−	+	+
	H1148	−	−	−	+	+
	H1149	−	−	−	+	+
P. nicotianae var. *parasitica*	H1033[b]	−	−	−	+	+
	H1099[b]	−	−	−	+	+
P. palmivora	H1022	−	−	−	+	+
	H1104	−	−	−	+	+
P. sojae	H1169[b]	−	−	−	+	+
	H1170[b]	−	−	−	+	+
P. syringae	H1055	−	−	−	+	+
P. vignae	H1034	−	−	−	+	+

[a] Labeling was assessed by comparison with controls that used a nonimmune mouse MAb. + = Cysts labeled by the MAb; − = cysts not labeled by the MAb; NT = not tested.

[b] Isolate was run in duplicate. All other isolates were run singly.

TABLE 6. Reaction of *Saprolegnia* and *Pythium* species to species- and genus-specific monoclonal antibodies (MAbs) in the dipstick assay

		MAbs[a]					
		Species specific			Genus specific		Marker
Species	Isolate	Cpa-2	Cpa-3	Cpa-7	ZCp-2	Cpw-4	Vsv-1
Saprolegnia diclina	H301[b]	+	–	–	–	–	+
S. ferax	H302	–	–	–	–	NT	+
S. parasitica	H303	–	–	–	–	–	+
Pythium acanthicum	H212	+	–	–	–	NT	+
P. aphanidermatum	H201[b]	+	–	–	–	–	+
	H200	+	–	–	–	–	+
P. butleri	H202	+	–	–	–	–	+
P. coloratum	H218	–	–	–	–	–	+
P. debaryanum	H215	+	–	–	+	NT	+
P. irregulare	H203[b]	+	–	–	+	NT	+
	H204	+	–	–	+	NT	+
P. middletonii	H213	–	–	–	–	+	+
P. periplocum	H206	–	–	–	–	–	+
P. rostratum	H211	–	–	–	–	NT	+
P. ultimum var. *sporangiiferum*	H216	–	–	–	–	–	+
P. ultimum var. *ultimum*	H217	–	–	–	–	NT	+

[a] Labeling was assessed by comparison with controls that used a nonimmune mouse MAb. + = Cysts labeled by the MAb; – = cysts not labeled by the MAb; NT = not tested.
[b] Isolates were tested twice. All other isolates were tested once.

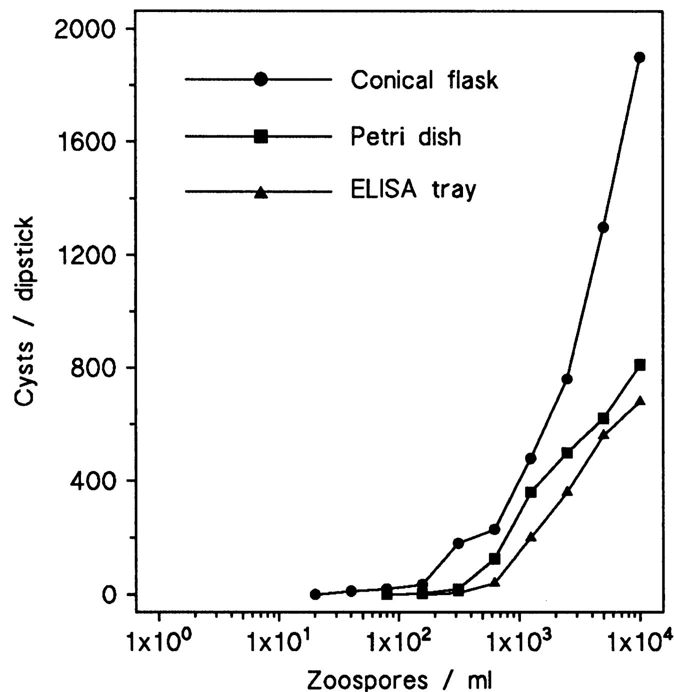

Fig. 2. Effect of container type on the number of cysts attached to the dipstick membrane after a 30-min incubation period.

the newly formed cyst wall will determine access to certain antigenic sites. Weak labeling by the Zt, Zg, and Lpv MAbs in the MAb screening assay was consistent with either a loss of the relevant target antigens or to inaccessibility of the cyst interior. That there was any labeling at all by the Zg and Lpv MAbs probably indicates the population of cysts that did not have completely formed cell walls. Thus, the Zt, Zg, and Lpv MAbs offered little potential for development of the dipstick diagnostic assay. In immunofluorescence assays of zoospores and cysts of *P. cinnamomi* and ELISA assays that used mycelium, Cpa and Lpv MAbs were species specific and useful for diagnosis (13).

The immunoassay we used routinely involved silver enhancement of a colloidal gold-labeled secondary antibody. This detection system produced an intense signal of high contrast that was easily distinguished by eye, hand-held lens, or dissecting microscope. The cyst coat material, which is immunologically distinct from material that aids in adhesion of cysts (15), spread out around the perimeter of the cyst during assay incubation and hence increased the amount and area of labeling and increased the signal.

In preliminary experiments (data not shown) in which soil was infested with *P. cinnamomi,* the colloidal gold-silver enhancement technique allowed us to readily distinguish cysts from adhering soil and organic particles. The possibility of confusion with other particles adhering to the dipstick membrane, however, was completely eliminated with an alkaline phosphatase-labeled secondary antibody with napthol phosphate-fast red as the substrate and indicator dye. The pink-red cysts were easily distinguished from all other adherent matter. We have also used fast blue, which gives an insoluble light blue product, and bromochloroindolyl phosphate-nitro blue tetrazolium, which gives an insoluble purple reaction product, to differentiate cysts of *P. cinnamomi* on dipsticks that have been used in soil samples.

The length of time we used to run the routine assay was 3.5–4 h to maximize labeling and detection in the screening assays. For development into a diagnostic kit, assay time could be reduced considerably. In particular, use of alkaline phosphatase-conjugated secondary antibody and fast dyes decreased the signal development time to only a few minutes. A biotin-streptavidin-based immunoassay also has the potential to both increase the signal and reduce assay time. With a biotin-labeled secondary antibody and streptavidin labeled with colloidal gold (15 nm), the silver enhancement time was reduced to one-third that of when a gold-labeled secondary antibody was used, although the background signal was increased (data not shown). Process time can also be greatly reduced by making the assay direct rather than indirect by using, for example, a colloidal gold-labeled primary antibody that can then be enhanced. Duration of the procedure was reduced with no loss in sensitivity when this technique was employed with a Cpa primary antibody conjugated to 10-nm gold (gift of J. Dearnaley, Australian National University).

Several immunologically based assays for the detection of plant pathogens are currently available in kit form, and several assays that involve use of monoclonal antibodies as the detection system have been reported (9,27). Cross-reactivity, however, has limited the use of many of these assays, which have immunogens based on whole cells or cell walls and soluble surface antigens. Similarly, the usefulness of commercial diagnostic tests for the detection of *Phytophthora*, and of *P. cinnamomi* in particular, has been severely hampered by cross-reactivity both within the genus and to other closely related genera such as *Pythium* and *Peronospora* (1,3,24,30). The power of the dipstick immunoassay described in this paper lies in the specificity of the MAbs, originally raised to aldehyde-fixed zoospores and cysts, which apparently contain unique antigenic components and show far greater specificity than

either the polyclonal or monoclonal antibodies previously reported.

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