A Method for Using Commercial ELISA Tests to Detect Zoospores of *Phytophthora* and *Pythium* Species in Irrigation Water

M. S. ALI-SHTAYEH, Associate Professor, Department of Biological Sciences, An-Najah National University, Nablus, West Bank, via Israel; J. D. MacDONALD, Associate Professor, Department of Plant Pathology, University of California, Davis 95616; and J. KABASHIMA, Farm Advisor, University of California Cooperative Extension, 1000 S. Harbor Blvd., Anaheim 92805

ABSTRACT

Ali-Shtayeh, M. S., MacDonald, J. D., and Kabashima, J. 1991. A method for using commercial ELISA tests to detect zoospores of *Phytophthora* and *Pythium* species in irrigation water. Plant Dis. 75:305-311.

Commercially available ELISA tests for detecting *Phytophthora* and *Pythium* species were used to detect propagules of these genera in water samples. Samples were filtered through 0.45-μm filters to concentrate propagules before extraction. Heating filter residues to 100 C for 5 min yielded extracts that were equal or superior in reactivity to those obtained by liquid nitrogen disruption. As few as 30–40 zoospore cysts were detected in filter pad extracts. Detection sensitivity (propagules per liter) depends on the volume of water that can be passed through the filters. A water sample collected in late winter from a recycling pond in a northern California nursery did not contain any viable propagules of pythiaceous fungi or detectable antigen. A similar water sample collected in early spring from a southern California nursery contained 442 viable propagules per liter of water. The species recovered on agar media were *Phytophthora parasitica, P. citrophthora, P. cryptogea*, and an unidentified *Phytophthora* species, in addition to *Pythium coloratum, P. rostratum, P. middletonii, P. ultimum* var. *sporangiferum*, and Pythium 'L' group. Reactivity of filter extracts in Phytophthora ELISA tests was proportional to the quantity of filter residue extracted. All of the *Phytophthora* species recovered reacted positively with Phytophthora kits and negatively with Pythium kits. All of the *Pythium* species recovered also reacted positively with Phytophthora and negatively with Pythium kits. With a relatively simple filter-extraction procedure, the ELISA kits effectively detected *Phytophthora* and *Pythium* in water samples. Although the tests are fallible at the genus level, they still could be used in commercial nurseries, where there is no known acceptable tolerance for these genera in irrigation water.

Additional keywords: *Chrysanthemum* ×*morifolium, Gardenia jasminoides, Hibiscus rosa-sinensis, Juniperus sabina*, monoclonal antibody, root disease

For several years, many nurseries in California have been required to substantially limit irrigation runoff. Regulations controlling runoff have been enacted by local governments and water districts in an effort to force greater water use efficiency or in response to public concerns that the drain water may be contaminated with nutrient or pesticide leachates. Because the regulations pertaining to runoff are enacted and enforced locally, the existence and stringency of regulations vary greatly across the state.

Where irrigation runoff is strictly limited, nurseries typically trap runoff water in large ponds on the nursery grounds. As the ponds fill to capacity, water is pumped out and reused for irrigation to prevent overflow. Recycled water is usually blended with fresh water to dilute accumulated salts, but the inputs of fresh water must be managed carefully to assure they do not exceed the storage capacity of the holding pond. Recycled water is used primarily on established plants growing outdoors and is excluded from use in some parts of the nursery (e.g., for propagation).

As nurseries are being required to limit runoff, many are installing recycling systems. However, there are many reports attributing the introduction or spread of *Phytophthora* and *Pythium* species in field and orchard crops to the use of contaminated or recycled irrigation water (4,7,9,16,18,19,23,24,27). Root diseases caused by species of *Phytophthora* and *Pythium* are persistent and sometimes serious problems throughout the nursery industry. Nursery operators are aware of the disease risks associated with recycled water (3), and many have responded by increasing the prophylactic use of fungicides or by installing elaborate water treatment facilities that may include filters, clarifiers, and chlorine injectors.

All changes in irrigation management, fungicide use, and water treatment have been devised and implemented without knowledge of specific pathogens, pathogen populations, seasonal fluctuations, or treatment efficacy within the nursery environment. Given the diversity of nursery size, geographic location, crops under cultivation, water quality, irrigation practices, and many other factors, universal guidelines seem unlikely. Risks, and the effectiveness of management practices, need to be assessed on a nursery-by-nursery basis. Thus, there is need for an effective, simple pathogen detection method that can be used by nursery operators or their consultants.

The most commonly employed methods for detecting pythiaceous fungi involve baiting, culture plating, or a combination of these (20). Although existing methods can be quite effective, they also are labor-intensive and cumbersome and require knowledge of fungal taxonomy (20). Detection methods utilizing monoclonal antibodies can be much easier to use, and antibodies developed by Agri-Diagnostics Associates (Cinnaminson, NJ) have been reported to be sensitive, quantitative detectors of *Phytophthora megasperma* f. sp. *glycinea* and *P. parasitica* Dastur var. *nicotianae* in soil assays (6,12,14,17). We have worked with prototype kits produced by Agri-Diagnostics Associates for diagnosis of *Phytophthora, Pythium*, and *Rhizoctonia* root rots in nursery crops (8) and felt they may have value in irrigation water assays.

The objectives of this research were to develop methods for utilizing ELISA tests to detect pythiaceous fungi in irrigation waters and to test their sensitivity and specificity. ELISA tests were compared with culture-plating in assays of runoff water collected from two commercial nurseries.

MATERIALS AND METHODS

ELISA tests and procedures. Antibodies were obtained from Agri-Diagnostics Associates in standard ELISA multiwell format. To detect *Phytophthora* species, we used Phytophthora E kits. These kits contain a mixture of two monoclonal lines, one that reacts strongly with many common species of *Phytophthora* (but not with *P. cinnamomi* Rands) and one that reacts strongly with *P. cinnamomi* as well as other species of *Phytophthora*. To detect *Pythium* species, we used Pythium C kits. These contain a monoclonal antibody that gives broad genus-level detection and that also reacts with some *Phytophthora* species.

Accepted for publication 19 September 1990 (submitted for electronic processing).

Filter pads containing fungal propagules were prepared for ELISA analysis by one of four methods. In initial experiments, extracts were frozen by chilling a mortar and pestle with liquid nitrogen, placing a filter pad containing zoospores in the chilled mortar, and adding a small volume of liquid nitrogen. The frozen filter pad was ground into a fine powder, which was placed into a chilled glass tissue grinder. A known volume of kit-supplied extraction buffer was added, and the powder was homogenized. The homogenate was centrifuged to 1,800 *g* for 30 min at 4 C. The supernatant was transferred to a chilled glass tube and frozen into pellets by adding drops of the extract to liquid nitrogen. The frozen pellets were stored in microcentrifuge tubes at −15 C.

Two other extraction methods that could be more easily used by nursery operators were also tested. One involved freezing the filter pads containing fungal propagules in a standard −15 C freezer for 60 min. After freezing, the pads were immersed in 2 ml of room-temperature extraction buffer and vortexed for 60 sec. The filter pad then was removed and the extraction solution adjusted to a final volume of 2 ml with extraction buffer.

Because the antigens detected by these kits are heat-stable, and because kit instructions suggest that the reactivity of some mycelial extracts can be enhanced by brief heating, the other method involved heat extraction. Filter pads containing propagules were cut into small pieces and placed in 2 ml of distilled water in a test tube and heated to 100 C for approximately 5 min in a boiling water bath. After the liquid cooled to room temperature, 0.5 ml of extraction buffer concentrate was added to each tube, and the tubes were shaken on a vortex mixer for 10–15 sec. The pieces of filter pad then were lifted out of the solution, pressed against the side of the tube to drain retained liquid, and removed.

As a comparison to the above methods, ELISA tests also were run on untreated zoospores by immersing filter pads directly in 2 ml of extraction buffer, vortexing for 60 sec, and removing the filter.

After extracts were prepared, 100 µl of each sample was dispensed into the wells of a sensitized microtiter plate. Plates were incubated for 10 min at room temperature with continuous shaking using a microplate adapter (Scientific Industries, Inc., Bohemia, NY) on a vortex mixer, then washed five times with kit-supplied wash solution. Enzyme-conjugated antibody (100 µl) was added to each well, and the incubation and washing process was repeated. This was followed by addition of 100 µl of substrate to each well and, after 10 min of incubation for color development, 50 µl of stop solution. Absorbance values were determined at 405 nm using a Titertek Multiskan 3100 ELISA plate reader (Flow Laboratories, McLean, VA). The kit-supplied positive and negative controls were included in each multiwell plate, along with extracts from blank filter controls. Nonsensitized wells were always included to detect possible interference. Test thresholds (the absorbance values above which reactions were considered positive) were the mean absorbance plus three standard deviations of the blank filter controls (8).

Determination of ELISA test sensitivity. To determine how many zoospores had to be present on filter pads to ensure detection, a series of experiments were done in which known numbers of zoospores were added to filters before ELISA processing. The isolates used in these experiments included two isolates of *Phytophthora citrophthora* (R.E. Smith & E.H. Smith) Leonian (isolate 34-3-3, recovered from walnut and obtained from S. M. Mircetich, USDA-ARS, University of California, Davis, and isolate Ph603, recovered from irrigation water near Nablus, West Bank), an isolate of *P. parasitica* (isolate Ph605, recovered from irrigation water near Nablus, West Bank), and an isolate of *Pythium coloratum* Vaartaja (isolate 6-H-12, recovered from *Hibiscus rosa-sinensis* L. 'Whitewings' from southern California).

Cultures were grown for 3 days at 25 C under continuous light in plastic petri dishes containing V-8 juice agar. Then, a 1–1.5 cm wide ring was cut from the outermost edge of the agar culture and the plate was flooded with sterile 2.5% soil extract. Flooded cultures were held under continuous light at room temperature for 3–5 days. Cultures then were

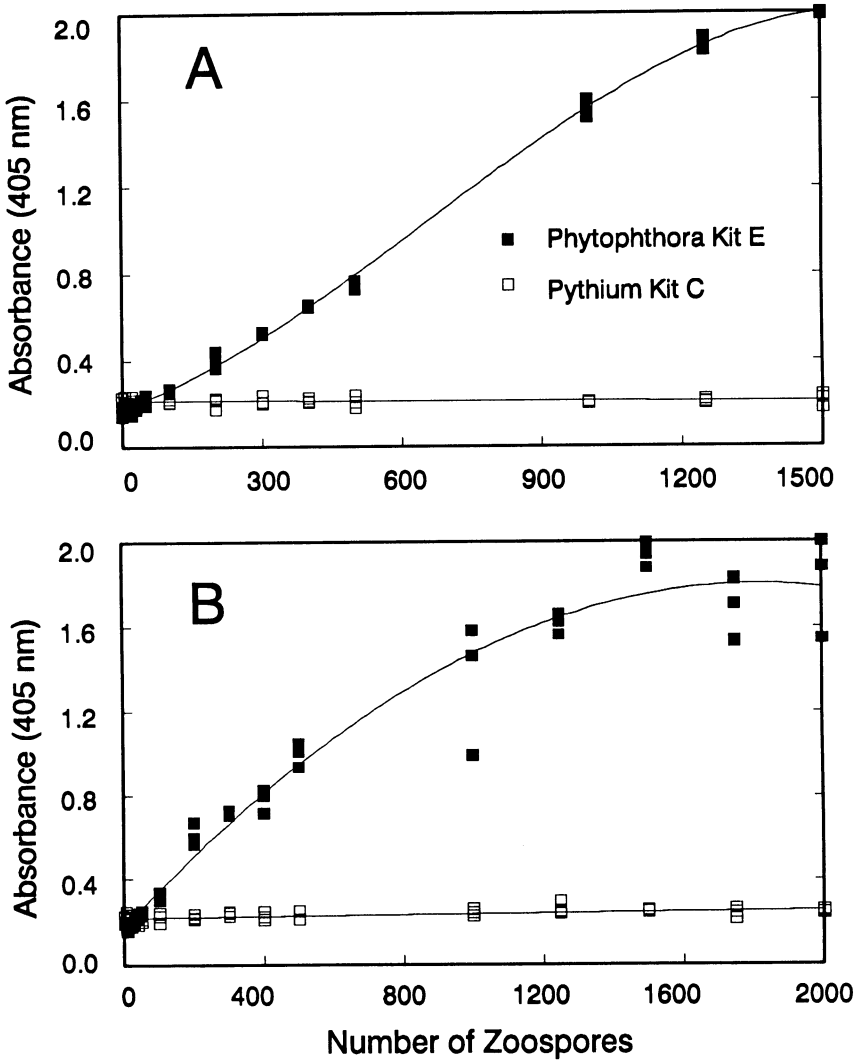

Fig. 1. Relationship between the numbers of zoospore cysts on filter pads and the absorbance of extracts obtained after disruption in liquid nitrogen. **(A)** Reaction of *Phytophthora citrophthora* isolate Ph603 in the Phytophthora E kit ($Y = 0.175 + 0.8 \times 10^{-3}X + 1.25 \times 10^{-6}X^2 - 6.30 \times 10^{-10}$; $r^2 = 0.998$) and Pythium C kit (slope not significantly different from zero). Threshold absorbance for a positive detection was 0.196 for kit E and 0.249 for kit C. **(B)** Reaction of *Pythium coloratum* isolate 6-H-12 in the Phytophthora E kit ($Y = 0.172 + 0.002X - 4.93 \times 10^{-7}X^2$; $r^2 = 0.97$) and Pythium C kit (slope not significantly different from zero). Threshold absorbance for a positive detection was 0.208 for kit E and 0.206 for kit C. Equations and coefficients of determination (r^2) were based on values from three replicate samples, shown as points on the regression lines.

rinsed two or three times with sterile distilled water (SDW), after which 10 ml of SDW was added to each plate. The plates were incubated under continuous light for an additional 2–3 days, after which abundant sporangia were present. To induce zoospore release, cultures were rinsed two or three times with SDW, and 10 ml of fresh SDW was added to each plate. Plates then were chilled to 6 C for 60 min and rewarmed to room temperature. Zoospore release typically occurred within 20–30 min of rewarming. The concentration of zoospores in the suspensions was determined with a hemacytometer.

The relationship between zoospore concentration and ELISA reaction intensity was determined by collecting known numbers of *P. citrophthora* 603 and *Pythium coloratum* zoospores on separate 47-mm-diameter, 0.45-μm membrane filters (Millipore Corp., Bedford, MA) and extracting antigen from the filter pads using the liquid nitrogen method described above. Frozen pellets of each species were thawed as needed and volumetrically diluted to obtain a range of extracts representing different zoospore concentrations (0–2,000). Extract dilutions from both species were pipetted into the *Phytophthora* and *Pythium* microtiter plates to compare their reactivity.

Experiments also were done to determine whether the antigenic activity of the extracts was significantly influenced by extraction method. Filter pads containing zoospores of *P. citrophthora* 603 were extracted by both the liquid nitrogen and the heating method, whereas pads containing zoospores of *P. citrophthora* 34-3-3 and *P. parasitica* 605 were extracted using each of the four methods. Volumetric dilutions were prepared from each sample and pipetted into both *Phytophthora* and *Pythium* microtiter plates (isolate 603) or just into *Phytophthora* plates (isolates 34-3-3 and 605).

In all these experiments, three replicate dilutions were prepared for each species vs. extraction method vs. ELISA test combination, and each experiment was repeated in its entirety two or three times.

Quantification of pythiaceous fungi in nursery water samples. Samples of irrigation runoff water were collected from the recycling pond of a northern California nursery in early March and from a southern California nursery in late April. Water samples (12–16 L) were collected by holding opened 4-L plastic bottles 2–3 cm below the water surface and allowing water to flow in. When filled, the bottles were sealed with screw-top caps and returned to the laboratory. The samples appeared colored but relatively free of sediments. Evaluation of the first sample was completed before the second sample was collected.

The contents of the collection bottles from each nursery were recombined in

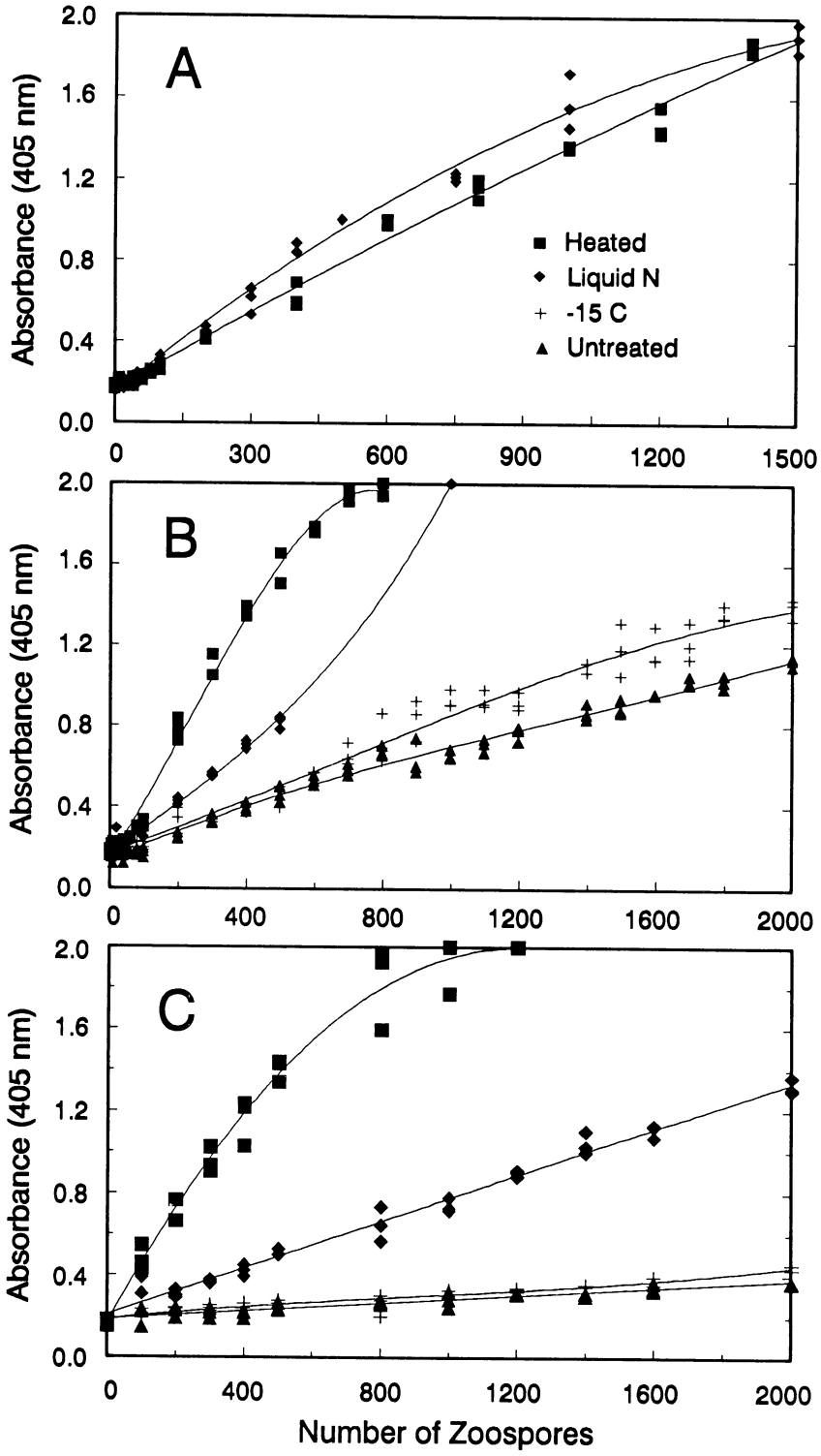

Fig. 2. Influence of extraction method on the absorbance of zoospore cyst extracts in Phytophthora E kits. **(A)** *Phytophthora citrophthora* isolate Ph603 extracted by heating ($Y = 0.161 + 1.3 \times 10^{-3}X - 1.07 \times 10^{-7}X^2$; $r^2 = 0.994$) and liquid nitrogen ($Y = 0.145 + 0.002X - 4.45 \times 10^{-7}X^2$; $r^2 = 0.991$). Threshold absorbance for a positive detection was 0.217 for heated and 0.197 for liquid nitrogen extracts. **(B)** *P. citrophthora* isolate 34-3-3 extracted by heating ($Y = 0.145 + 0.002X + 3.26 \times 10^{-6}X^2 - 4.29 \times 10^{-9}X^3$; $r^2 = 0.993$), liquid nitrogen ($Y = 0.175 + 1.2 \times 10^{-3}X - 6.48 \times 10^{-9}X^2 + 6.58 \times 10^{-10}$; $r^2 = 0.997$), freezing to −15 C ($Y = 0.171 + 6.0 \times 10^{-4}X + 1.33 \times 10^{-7}X^2 - 6.88 \times 10^{-11}$; $r^2 = 0.98$), or without treatment ($Y = 0.138 + 8.0 \times 10^{-4}X - 2.58 \times 10^{-7}X^2 + 6.54 \times 10^{-11}$; $r^2 = 0.988$). Threshold absorbance for a positive detection was 0.197 for liquid nitrogen and 0.189 for all other extracts. **(C)** *Phytophthora parasitica* isolate 605 extracted by heating ($Y = 0.201 + 0.003X - 1.20 \times 10^{-6}X^2$; $r^2 = 0.98$), liquid nitrogen ($Y = 0.261 + 3.4 \times 10^{-4}X + 2.37 \times 10^{-7}X^2 + 5.07 \times 10^{-11}$; $r^2 = 0.98$), freezing ($Y = 0.177 + 2.4 \times 10^{-4}X - 1.55 \times 10^{-7}X^2 + 5.07 \times 10^{-11}$; $r^2 = 0.96$), or without treatment ($Y = 0.189 + 1.0 \times 10^{-4}X$; $r^2 = 0.86$). Threshold absorbance for a positive detection was 0.211 for liquid nitrogen and heated extracts and 0.259 for all other extracts. Equations and coefficients of determination (r^2) were based on values from three replicate samples, shown as points on the regression lines.

a large plastic tub to form a single water sample that was thoroughly mixed before filtration through 0.45-μm filters. Depending on the objectives of individual experiments, different volumes (up to 1.75 L) of irrigation water were filtered through each pad. Filter pads were either used fresh or frozen in a −15 C freezer until needed.

To determine the number of viable propagules present in the samples, 200 ml was filtered through each of five filters. The filters then were placed in separate test tubes, each containing 6 ml of sterile 0.09% water agar, and the material on the filters was suspended by vortex mixing. Aliquots (1 ml) of the suspension in each test tube were pipetted into each of six petri dishes containing VP3 selective medium (2). The suspension was spread evenly over the agar surface, and plates were incubated in the dark for approximately 30 hr at 25 C to allow colony development. Young colonies then were counted and a random sample (totaling approximately 30% of all recovered) was transferred to pure cultures for taxonomic identification. Identification of *Phytophthora* species was based on the keys of Tucker (21), Waterhouse (25), Newhook et al (15), and Ho (5). Identification of *Pythium* species was based on the keys of Middleton (10), Waterhouse (26), van der Plaats-Niterink (22), and Ali-Shtayeh (1).

The amount of antigen present in the water samples was quantified by ELISA tests. Propagules were recovered by filtering 10 L of water through six to eight filters (approximately 1.25–1.75 L per filter pad). The filters containing propagules were cut into small pieces and bulked into one sample for heat extraction. Thus, the 2 ml of extract solution remaining after filter pad removal contained the total antigen recovered from 10 L of irrigation water. Aliquots of this solution (0–100 μl) were added to reciprocal volumes of extract buffer (100–0 μl) to yield 100-μl volumes of extract solution containing the amount of antigen recoverable from different volumes of irrigation water (0–500 ml). These solutions were added to the wells of *Phytophthora* and *Pythium* microtiter plates, with three replicate dilutions made for each water volume equivalent.

Because the water samples were colored, indicating the presence of dissolved materials, two experiments were done to determine if materials in the irrigation water interfered with the ELISA reactions. In one series of experiments, 200 ml of irrigation water (from the northern California nursery) was drawn through a filter pad immediately before a small volume of SDW containing a known number of *P. citrophthora* 603 zoospores. An identical volume of zoospore suspension was filtered through a second filter pad, through which 200 ml of SDW had earlier been drawn. After heat extraction of antigens, volumetric dilutions were made from each extract solution to yield extracts representing 0–700 zoospores. Three replicate wells were used for each zoospore concentration, and the entire experiment was repeated using *P. parasitica* 605.

In another experiment, 500 zoospores of *P. citrophthora* 603 were filtered onto each of a series of filters through which different volumes (0–200 ml) of irrigation water had already been filtered. Heated extracts from these filters, and from filters through which irrigation water had been drawn but no zoospores added, were prepared and added to microtiter plates. Three wells were filled with each extract solution.

Data analysis. Data were analyzed using PC SAS version 6.03 (SAS Institute, Inc., Cary, NC). Data were tested for normality using the SAS UNIVARIATE procedure, and main effects were analyzed using the General Linear Models procedure. Treatment means and confidence intervals were modeled using SAS PROC REG procedures.

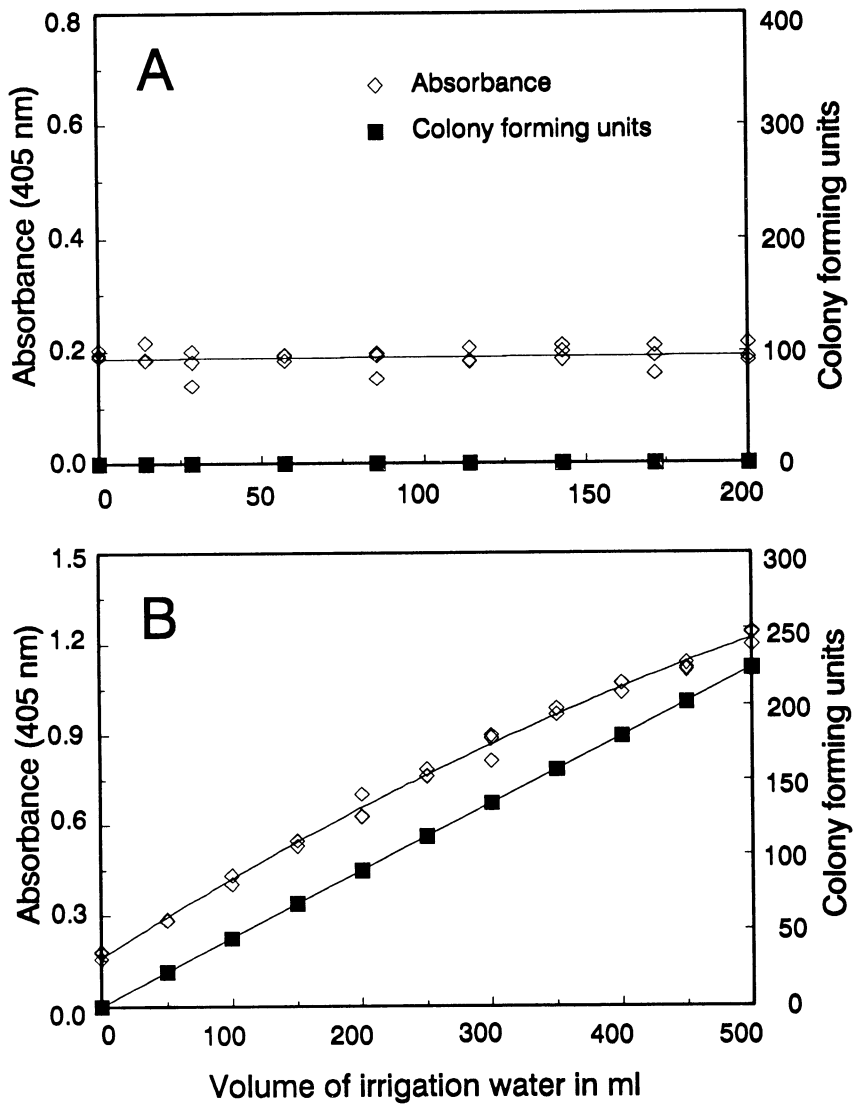

Fig. 3. Detection of propagules of pythiaceous fungi in water samples collected from two commercial nurseries. Right axis shows the numbers of viable *Phytophthora* plus *Pythium* propagules predicted in different volumes; numbers are based on propagules recovered from cultured water samples. Left axis shows the absorbance values of filter pad extracts in Phytophthora E kits that represent the amount of material recovered from different volumes of water. **(A)** Northern California water sample showing the absence of viable propagules or detectable antigen. Threshold absorbance for a positive detection was 0.224. **(B)** Southern California water sample showing the relationship between absorbance ($Y = 0.1597 + 6.2 \times 10^{-3}X - 6.34 \times 10^{-6}X^2$; $r^2 = 0.996$) and numbers of viable propagules in the water samples. Threshold absorbance for a positive detection was 0.183.

RESULTS

Determination of ELISA test sensitivity. When filter pads containing *P. citrophthora* 603 zoospores were disrupted by freezing in liquid nitrogen, the Phytophthora E kit gave reactions significantly ($P < 0.05$) above background when extracts contained the equivalent of 30 zoospores (Fig. 1A). Absorbance values increased nearly linearly as zoospore numbers increased from approximately 150 to 1,200 and began to level

off at zoospore numbers >1,200. These same extracts gave no detectable reaction in the Pythium C kits, even when large numbers of zoospores were represented in the extracts (Fig. 1A). Extracts from *Pythium coloratum* zoospores reacted much like the *P. citrophthora* extracts. Absorbance values in the Phytophthora kits were significantly ($P < 0.05$) above background when there were approximately 40 zoospores represented in the extracts, the slope of the line relating absorbance to zoospore number began to level off at zoospore numbers >1,000, and there was no significant reaction with the Pythium C kit (Fig. 1B).

The antigenicity of extracts was significantly influenced by extraction method. When filter pads containing zoospores of *P. citrophthora* 603 were extracted by liquid nitrogen or heating treatments, there was no significant difference in their reaction intensities at any zoospore concentration (Fig. 2A). However, when all four extraction methods were compared (using *P. citrophthora* 34-3-3 and *P. parasitica* 605), substantial differences were observed (Fig. 2B and C). Relative to extracts made from untreated filter pads, freezing the pads to −15 C increased the antigenicity of extracts either slightly (Fig 2B) or not at all (Fig. 2C). Relative to extracts obtained by liquid nitrogen disruption, those obtained from untreated or −15 C frozen zoospores were significantly less reactive and those obtained by heating were significantly more reactive (Fig. 2B and C). Comparison of the absorbance values of zoospore extracts obtained by heating or liquid nitrogen treatment showed that isolate 34-3-3 of *P. citrophthora* reacted much more strongly with the antibodies in the Phytophthora E kit than did isolate 603 (Fig. 2A and B). Because the heat treatment of filters was relatively simple and yielded highly reactive extracts, this method of extraction was used in all subsequent experiments, even those using filters that had been stored frozen at −15 C.

Quantification of pythiaceous fungi in nursery water samples. When the residue retained on filter pads was resuspended and spread onto VP3 medium, no colonies developed on any plate seeded with material from the northern California water sample. This contrasts with the 88.4 ± 4.4 propagules recovered from 200-ml aliquots of the southern California water sample. This is equivalent to 442 ± 22 propagules per liter, of which 16% were *Phytophthora* (including *P. parasitica, P. citrophthora, P. cryptogea* Pethybr. & Lafferty, and an unidentified species) and 84% were *Pythium* (64% were *P. coloratum*, 17% were *P. rostratum* E.J. Butler, and the balance were *P. middletonii* Sparrow, *P. ultimum* Trow var. *sporangiferum*, and *Pythium* 'L' group). Microscopic examination of young, developing colonies often revealed their zoospore origins.

The ELISA test results partially confirmed the culture plate findings. Aliquots from the northern water sample yielded no detectable reactions and aliquots from the southern sample yielded reactions with absorbance values that were positively related to the volume assayed (Fig. 3A and B). However, positive reactions were obtained only in the Phytophthora kits; Pythium kits gave negative results for both water samples.

When zoospores of *P. citrophthora* 603 and *P. parasitica* 605 were collected on clean filters or on filters through which 200 ml of irrigation water from the northern California nursery had previously been filtered, there was no detectable difference in the ELISA reaction intensity at equivalent zoospore dilutions (Fig. 4A and B). When extracts were prepared from inoculated or uninoculated filter pads through which different volumes of irrigation water had been passed, there was no detectable effect of irrigation water on the reactivity of the extracts (Fig. 5).

DISCUSSION

The commercially available ELISA tests used in these experiments were able to detect relatively small numbers of *Phytophthora* and *Pythium* zoospores in water samples. For example, when zoospores were concentrated onto filter pads and antigens were extracted by brief heating, the Phytophthora E kits gave significant ($P < 0.05$) positive reactions with as few as 30–40 zoospores on a filter pad. Because the water samples we collected from commercial nurseries did not contain visible amounts of suspended materials, we were able to filter up to

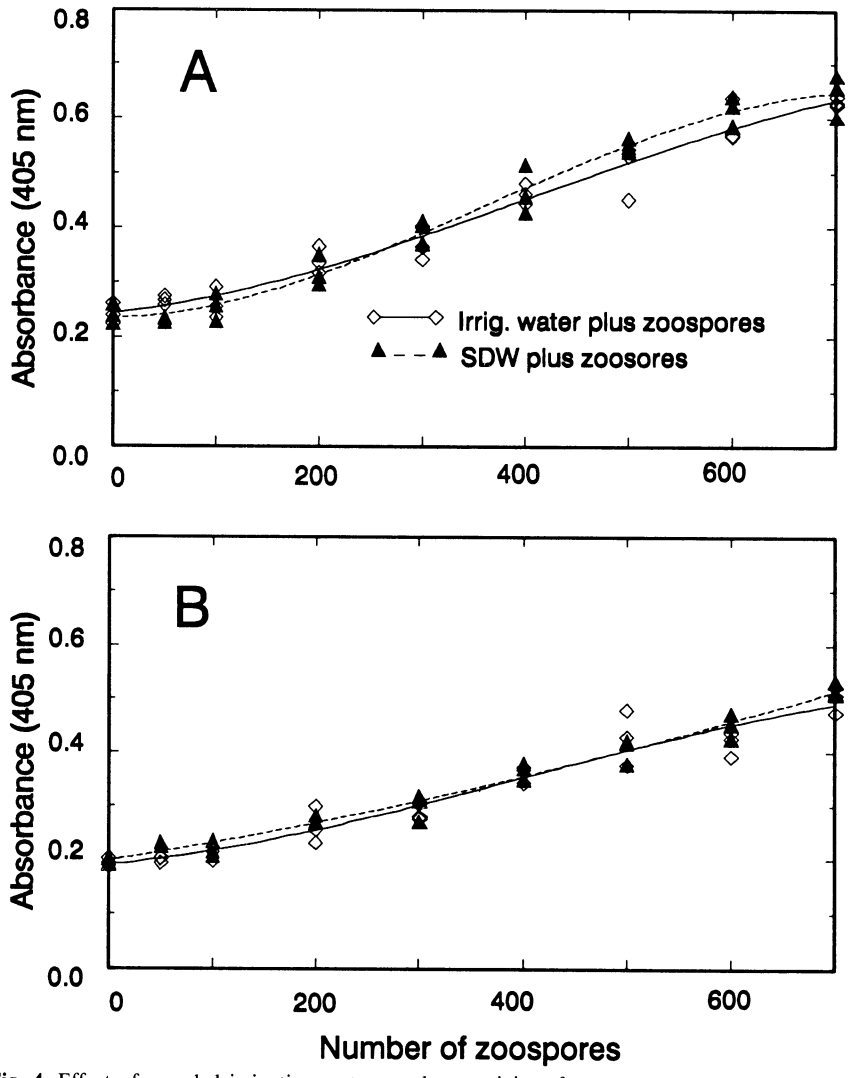

Fig. 4. Effect of recycled irrigation water on the reactivity of zoospore cyst extracts. Known numbers of zoospore cysts were collected on filters through which 200 ml of sterile distilled water (solid symbols) or untreated irrigation water (northern California sample, open symbols) had previously been passed. **(A)** Reaction of *Phytophthora citrophthora* (isolate Ph603) in extracts from SDW ($Y = 0.237 - 3 \times 10^{-6}X + 2.57 \times 10^{-6}X^2 - 2.41 \times 10^{-9}$; $r^2 = 0.978$) and recycled irrigation water ($Y = 0.245 + 2 \times 10^{-4}X + 1.371X^2 - 1.16 \times 10^{-9}$; $r^2 = 0.966$). Threshold absorbance for a positive detection was 0.253. **(B)** Reaction of *Phytophthora parasitica* (isolate Ph605) in extracts from SDW ($Y = 0.198 + 3 \times 10^{-4}X + 2.13 \times 10^{-7}X^2$; $r^2 = 0.978$) and recycled irrigation water ($Y = 0.193 + 1 \times 10^{-4}X + 1.13 \times 10^{-6}X^2 - 9.82 \times 10^{-10}$; $r^2 = 0.943$). Threshold absorbance for a positive detection was 0.224.

1.75 L through a single filter pad. This gave an effective detection capability of 17–23 zoospores per liter.

In soil assays using ELISA tests, Jones and Shew (6) detected < 2.5 propagules of *P. p. nicotianae* per gram of soil. The much lower detection threshold we found ($1.7–2.3 \times 10^{-2}$ zoospores per milliliter) can be attributed to the filtration process, which served to concentrate zoospores onto the filters. The detection limits reported here, however, may not apply to all water samples, since the volume of water that can be passed through a single filter depends on the amount of suspended materials it carries. We have encountered samples where it is difficult to pass more than 200 ml through a filter before it becomes plugged. Although it is possible to combine several such filters into a single sample for extraction, there are limits as to how many can be combined in the 2-ml extraction volume. We have found one or two filters to be optimum, with more than five difficult to manage. Detection also could vary with propagule type. All our experiments and water assays (to the extent we could determine) involved zoospore cysts. Although the tests can detect other propagule forms, i.e., oospores (12,13), different propagule forms could possess different amounts of the antigens detected by these tests and thereby influence sensitivity.

We also found that different species, and even different isolates of the same species, can differ in their reaction intensity in the Phytophthora kits, suggesting that detection limits may vary according to the mixture of species present in water samples. Indeed, the number of propagules and the mixture of species in irrigation water can vary greatly with time of year (19), and this may partly explain the differences we observed between the northern and southern water samples. The southern sample was collected later in the year and from a warmer climate than the northern sample. A sample from the same northern California nursery collected in August 1990 contained 1,415 ± 105 propagules per liter (*unpublished*). Seasonal fluctuations in pathogen populations could be an important consideration in management decisions and are a subject of ongoing research.

Heating the filter residues to 100 C gave as good an antigen extraction as freezing and grinding in liquid nitrogen. Since heat extraction could be more generally used in assays of water samples, it was the principal method used in these experiments. The heat stability of the *Phytophthora* and *Pythium* antigens detected by these kits is noted in their accompanying literature. While simplifying extraction, the stability of the antigens suggests that the kits could react with antigen extracted from nonviable propagules. However, data relating the antigenicity of filter extracts and number of viable propagules recovered from irrigation water suggest that if dead propagules were present, they were not sufficiently numerous to affect the ELISA results. Also, experiments with known numbers of viable zoospore cysts and known volumes of irrigation water showed no detectable background in the northern California water sample.

While it was possible to detect relatively low numbers of propagules in water samples, the antibodies in the Phytophthora E and Pythium C kits have characteristics that must be considered in interpreting test results. Chief among these is the breadth of reactivity of the antibodies. As genus-level tests, they cannot be used to detect particular species within either genus. However, it may be possible to enhance species specificity by combining the ELISA tests with a baiting procedure, as has been done recently in soil assays for *Phytophthora* (17). A variety of plants or plant parts has been used to trap particular pathogens from soil or water samples (4,7,9,16,18–20,23), and baiting can have the added benefit of increasing detectable antigen through pathogen colonization of the bait. Although baiting methods can be inconvenient, use of ELISA to analyze the baits can allow the usual time-consuming culturing and identification steps to be bypassed.

Interpretation of test results also must take into account cross-reactions that can occur between antibodies in the Pythium C and Phytophthora E kits and certain species of Phytophthora and Pythium, respectively (13). We did not observe cross-reactions with any of the *Phytophthora* species used in our experiments or recovered from nursery water samples; all reacted positively in the Phytophthora E kits and negatively in the Pythium C kits. In contrast, all five *Pythium* species we recovered from the southern California water sample cross-reacted with the *Phytophthora* antibodies and failed to react with the *Pythium* antibody. This was observed in the initial water assays, where positive reactions developed only in the Phytophthora kits and in subsequent assays of zoospores harvested from individual, representative isolates (*data not shown*). Although the Pythium C kits did not react with the *Pythium* propagules present in the southern water sample, we regard this as an artifact of the particular species present at the time of sampling. The kits are known to react strongly with several other species of *Pythium* (11).

The inability of the Pythium C kit antibodies to react with *Pythium coloratum* and *P. rostratum* is known and was reported in the kit instructions. But this apparently is the first report that these antibodies also do not react with some isolates of *Pythium middletonii, P. u. sporangiferum*, and *Pythium* 'L' group. It was interesting to note that while all of these *Pythium* species lacked detectable amounts of the antigen recognized by the Pythium C kit antibody, they all shared the antigen(s) detected by the Phytophthora E kit antibodies. In fact, we found that zoospore cysts from our isolate of *Pythium coloratum* gave reactions very similar to those of *P. citrophthora*.

Pythium coloratum and *P. rostratum* were the most commonly encountered species in the southern California water sample, followed by the *Phytophthora* species and relatively small numbers of other *Pythium* species. In greenhouse inoculations, we have found isolates of *P. coloratum* and isolates of all the *Phytophthora* species (*P. cryptogea, P. parasitica*, and *P. citrophthora*) to be

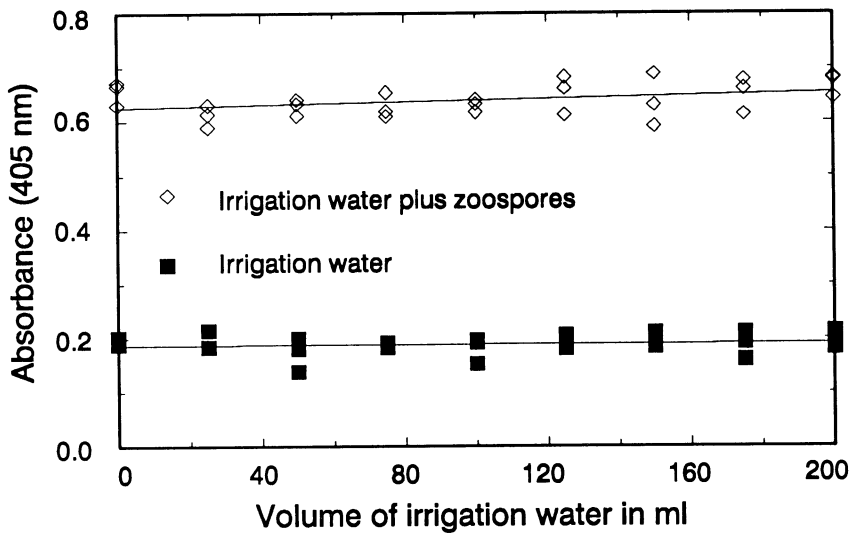

Fig. 5. Effect of recycled irrigation water on the reactivity of zoospore cyst extracts. Extracts were made from filter pads through which differing volumes of untreated irrigation water (northern California sample) had been passed before the addition of known numbers of *Phytophthora citrophthora* (isolate 603) zoospore cysts ($Y = 0.626 + 1 \times 10^{-4}X$; $r^2 = 0.096$). Threshold absorbance for a positive detection was 0.296.

pathogenic to one or more of the following plants: *Chrysanthemum* ×*morifolium* Ramat. 'Paragon', *Juniperus sabina* L. 'tamariscifolia', *H. rosa-sinensis* 'Whitewings', and *Gardenia jasminoides* Ellis 'Mystery' (*unpublished*). We have not yet determined whether the other *Pythium* species are pathogenic to common nursery crops. It seems logical to assume, however, that in a closed recycling system, the relative numbers of propagules present will be related to the pathogenic activity of the organisms. Propagules of saprophytic or weakly pathogenic species would be less numerous than those of aggressive pathogens. This may not be the case in more "open" water supplies (i.e., rivers, streams, surface canals), where saprophytic and aquatic species may be abundant. The antibodies used in the Phytophthora and Pythium kits have not been tested for their reactivity to such diverse species.

In earlier experiments, we found the Pythium and Phytophthora kits to be sensitive, effective diagnostic aids (8). Their use in assays of irrigation water, where many pythiaceous species may be encountered simultaneously, can make test interpretation more ambiguous than the narrower application of disease diagnosis. But, although the Pythium C and Phytophthora E kits cannot distinguish individual species and are affected by some cross-reactivity, they still can provide nursery operators with useful information. Used together, these kits can detect many common pathogenic *Phytophthora* and *Pythium* species. The significance of this capability lies in the fact that nursery recycling systems are essentially "closed loops," and there are no data describing "acceptable" levels of either genus in nursery crops. The confined nature of the containerized root system, the potential aggressiveness of these fungi, and the threat of introducing them into new areas with nursery crops indicate a unique situation where threshold is zero. Any detection is regarded as cause for corrective action, and early, accurate detection is required for control measures to have maximum beneficial effect. With a relatively simple method for detecting pathogens, nursery operators may be able to time or localize treatment procedures to greatly reduce pathogen dispersal and improve nursery sanitation.

ACKNOWLEDGMENTS

This research was supported in part by the California Association of Nurserymen. We wish to thank the Fullbright Program Council for International Exchange of Scholars for supporting the first author while this work was done. We also wish to thank Agri-Diagnostics Associates, Cinnaminson, New Jersey, for donation of all serological materials used in these experiments, and Janet Stites and Lisa Ferguson, for assistance with parts of the research.

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