Occurrence of *Phytophthora* Species in Recirculated Nursery Irrigation Effluents

J. D. MacDONALD, Professor, Department of Plant Pathology, University of California, Davis 95616; M. S. ALI-SHTAYEH, Associate Professor, Department of Biological Sciences, An-Najah National University, Nablus, West Bank, via Israel; J. KABASHIMA, Farm Advisor, University of California Cooperative Extension, South Coast Field Station, 7601 Irvine Blvd., Irvine 92718; and J. STITES, Staff Research Associate, Department of Plant Pathology, University of California, Davis 95616

ABSTRACT

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Water samples were collected from effluent holding ponds at one northern and two southern California nurseries that practice the capture and recirculation of irrigation runoff water. Nursery effluent samples were collected approximately monthly over a 12-mo period and aliquots filtered through 0.45-μm Millipore filters. Filter residues were resuspended and dispersed onto selective agar media in petri dishes to estimate the numbers of viable propagules of *Phytophthora* spp. or total pythiacious fungi. Propagule numbers varied greatly from month to month at each nursery location. *Pythium* propagules were consistently the most numerous, ranging from 500 to 1,500 per liter, whereas the number of *Phytophthora* spp. propagules ranged from 0 to 400 per liter. At the northern California nursery, propagule numbers were lowest during winter months and highest during warm seasons. Seasonal fluctuations in inoculum load were not apparent in the southern California nurseries. *P. citrophthora* was the most commonly detected *Phytophthora* sp. Other species frequently recovered included *P. citricola, P. cinnamomi*, and *P. cryptogea*. Isolates of *P. parasitica, P. megasperma*, and *P. syringae* were recovered less frequently. Water samples also were tested for *Phytophthora* spp. using commercially available ELISA tests. The ELISA reaction intensity of filter pad extracts was correlated with the numbers of propagules estimated to be on the filters, but the correlation was stronger at some times than at others. This is believed to reflect temporal differences in water sample quality or species mixtures.

Over the past decade, many federal, state, and local regulations have been adopted to control the disposition of irrigation wastewater. Such regulations serve to promote water conservation and reduce the amounts of nitrate and pesticide leachates released into the environment where they can contaminate surface or groundwater resources. Although agriculture is classified generally as a "nonpoint source" of nitrogen pollution, many nursery and glasshouse operations have been classified as "point sources" of pollution. The runoff water from some nurseries is routinely monitored, and growers have been fined for releasing water that exceeds specified nitrogen limits.

While the need to conserve limited water resources and prevent the pollution of drinking water supplies or aquatic ecosystems is self-evident, the procedures for managing irrigation effluents are not. Many nurseries facing tailwater discharge restrictions have installed reservoirs to collect and hold effluents. However, as effluent holding ponds fill to capacity, water must be pumped out to prevent overflow and illegal runoff. The water removed from the ponds is pumped back into the nursery irrigation system and reused. Recirculating this wastewater does provide some economic benefit to growers in the form of reduced water and nitrogen purchases, but it also introduces a potential risk of spreading plant pathogens.

The spread of plant pathogens via contaminated irrigation water is a well-documented problem. For example, Shokes and McCarter (14) found that species of *Phytophthora, Pythium, Rhizoctonia*, and *Fusarium* were common in Georgia irrigation ponds. They suggested that apparent failures of field fumigations were actually the result of pathogen reintroduction via contaminated irrigation water. Contaminated surface irrigation waters (i.e., canals or ditches) also have been reported as sources of *Phytophthora* inoculum in citrus orchards in the United States (18,25) and apple orchards in Canada (10). Even river waters in California (11), Australia (23), the West Bank of Jordan (2), and South Africa (6,22) have been shown to be infested with propagules of *Phytophthora* and *Pythium* species, which then may be introduced to orchard or nursery crops.

Although pathogen contamination of rivers and canals is acknowledged as a widespread and significant problem that has been linked to disease establishment in many crops, it probably represents an overall lesser risk of disease than the recirculation of crop effluents. This is because a small number of infected plants in a nursery crop could release large numbers of propagules into irrigation leachates, which then would be recirculated back to susceptible crops. Clearly, the reuse of untreated crop effluents poses disease risks.

In a previous publication (3), we described a method for using ELISA test kits to detect *Phytophthora* spp. in water samples. Some of the experiments described in that publication utilized nursery effluent waters and revealed that *Phytophthora* propagules were indeed present in effluent ponds. Because the earlier study involved only a few isolated water samples, this study was undertaken to gain a broader perspective of the potential problem. Our objectives were to quantify the abundance of pythiacious fungi in recirculation systems, to determine whether there were significant seasonal fluctuations in populations, and to identify the *Phytophthora* species most commonly found in recirculation systems.

MATERIALS AND METHODS

Water samples were collected from effluent holding ponds at three commercial nurseries (one in northern and two in southern California) over a 12-mo period starting in July 1990. The nurseries were selected as being typical of large (>80 ha) container operations that have installed recycling systems. Effluent samples were collected from each nursery at approximately 1-mo intervals by filling four 3.8-L plastic jugs with effluent water at the point where crop effluents flowed into the holding pond. After transport to the laboratory, the four jugs were emptied into large plastic tubs so that there was a single 15.2-L water sample from each nursery.

The bulked water samples were stirred vigorously and eight 100-ml aliquots were removed from each and filtered through separate 0.45-μm Millipore filters. The used filter pads were placed individually into test tubes containing 6 ml of 0.09% sterile water agar, and each test tube was agitated on a vortex mixer to resuspend the filter residue. Then, 1 ml of the resulting suspension was pipetted into each of six 9-cm-diameter petri dishes containing selective agar medium, and the suspensions were spread uniformly over the surface of the medium with a glass rod. Four of the filter suspensions were spread onto VP3 medium (1) to estimate the total numbers of pythiacious fungi, and four were spread onto VP3 medium amended with 50 mg/

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L of hymexazol (VP3H) to estimate numbers of *Phytophthora* propagules. Plates were incubated for 48 hr in darkness, after which they were washed under running tap water to remove solid residues and the numbers of developing colonies were counted.

After being counted, 15–20% of the colonies growing on VP3H medium were picked off at random and transferred first to PARP medium (8) and then to Difco cornmeal agar (CMA) and potato-dextrose agar (PDA) for subsequent identification. The proportion of each *Phytophthora* species in the isolate subsampling was used to estimate the numbers of propagules of the various species in the original water samples.

Isolates were identified on the basis of colony morphology, growth-temperature relationships, and morphology and dimensions of sporangia, oogonia, and antheridia. To determine colony morphology, a 5-mm-diameter mycelial plug of each isolate was transferred to CMA and PDA. Three replicate plates of each medium were used for each isolate and the plates were incubated for 48–96 hr at 25 C, after which they were examined for growth characters. CMA cultures also were incubated at 4, 25, and 35 C for 72 hr, then measured to determine linear growth rates.

Sporangial formation was induced by growing colonies for 3 days on V8 juice agar (12) under fluorescent light at room temperature, then cutting agar blocks from the advancing edge of the colonies. Agar blocks were transferred to petri dishes containing either sterile distilled water, a sterilized mixture of creek water and distilled water (1:2), or a sterile 2.5% soil extract. Two or three sterile hemp seeds were placed on the blocks incubated in distilled water, and one or two autoclaved grass leaves (2–3 cm long) were placed on the blocks incubated in the creek water/distilled water mixture. Cultures were usually incubated for 3–7 days and examined periodically for sporangial production and zoospore release. To induce zoospore release, cultures were rinsed two or three times with sterile distilled water, chilled at 6 C for 30–60 min, and then kept at room temperature for 15–30 min.

To examine sexual reproductive structures, isolates were grown on cleared V8 agar medium (CV8A) supplemented with β-sitosterol (30 mg/L) and thiamin-HCl (1 mg/L) (12), then incubated in the dark for 2–3 wk at 25 C. Agar cultures that had been incubated in darkness were examined for the presence of oospores. Isolates that failed to produce sex organs in single or paired cultures were subsequently grown on plates of the same media with known A1 and A2 isolates of *Phytophthora cinnamomi* Rands, *P. drechsleri* Tucker, and *P. cryptogea* Pethybr. & Lafferty. Cultures were incubated in the dark for 2–3 wk and examined microscopically. Species identification was based on the keys of Tucker (21), Waterhouse (24), Newhook et al (13), and Ho (7).

In addition to being cultured on selective agar media, each water sample was tested for *Phytophthora* spp. by ELISA (Phytophthora Kit E in multiwell format, Agri-Diagnostics Associates, Cinnaminson, NJ; currently available from Neogen Corp., Lansing, MI). Four effluent aliquots each of 25, 50, 100, 200, and 400-ml volumes were filtered through 0.45-μm Millipore filters. Each filter pad (four at each volume) was individually tested for the presence of *Phytophthora* using ELISA procedures described previously (3). Separate filter pads, through which distilled water was passed, served as controls for establishing the ELISA positive/negative test threshold. ELISA results were compared to culture plate results to determine their

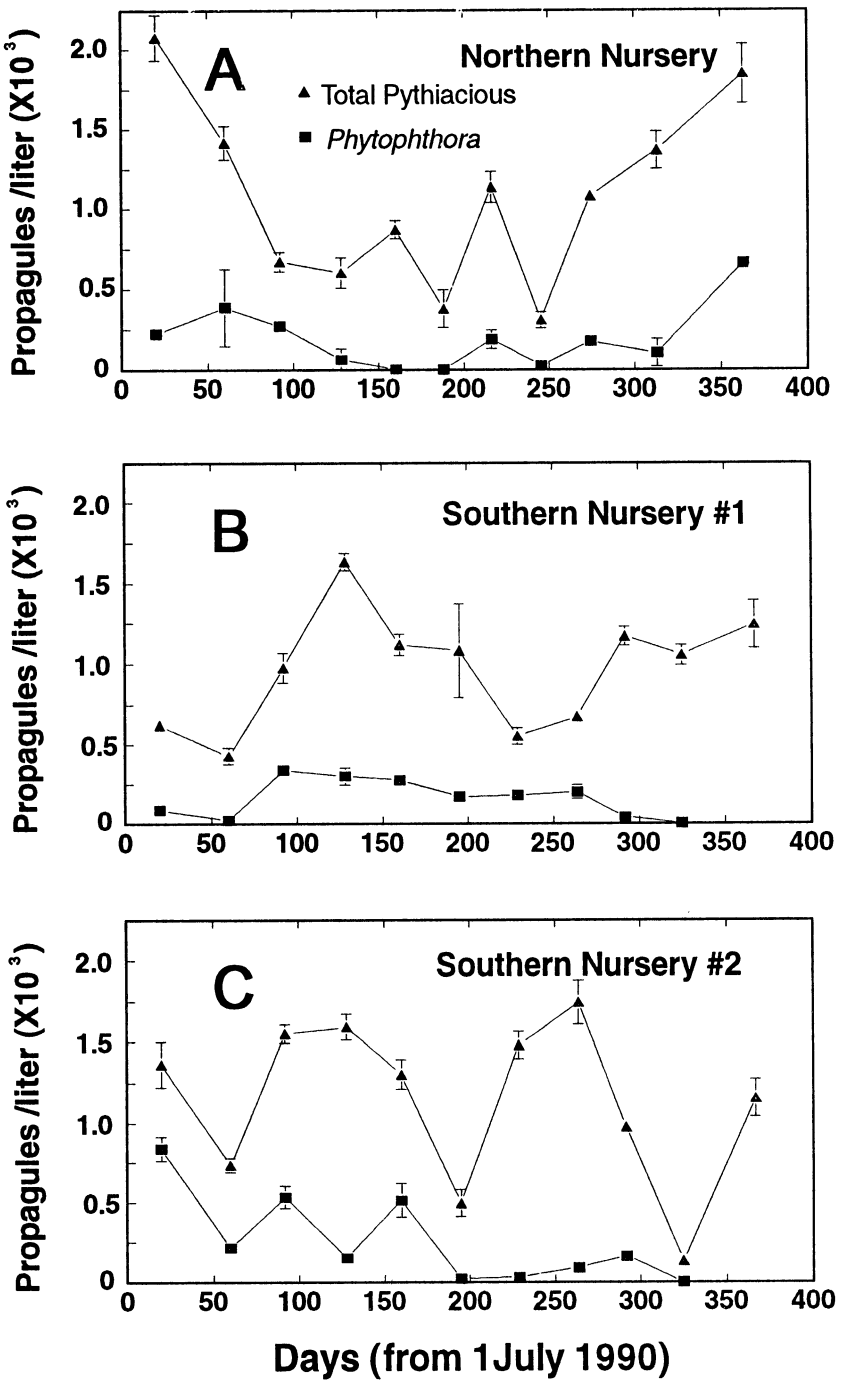

Fig. 1. Numbers of propagules of pythiacious fungi in effluent samples from **(A)** one northern and **(B and C)** two southern California nurseries practicing recirculation. Effluent samples were collected at intervals spanning 12 mo and filtered through 0.45-μm Millipore filters. Trapped residues were resuspended and spread onto two selective agar media, one for pythiacious fungi and one for *Phytophthora* spp. Propagule numbers were obtained from the number of colonies that developed on the media. Each point is the mean of four replicate assays. Vertical lines indicate ± 1 SD. Where no vertical bars are visible, the SD was smaller than the symbol.

degree of correlation over time.

Data were analyzed using PC SAS 6.03 (SAS Institute, Cary, NC). Data were tested for normality by the UNIVARIATE procedure, and main effects were analyzed by the General Linear Models procedure. ELISA treatment means were modeled by the PROC REG procedure, with propagule numbers as the independent variable and absorbance (405 nm) as the dependent variable.

RESULTS

Wide monthly variations were observed in the numbers of propagules in the recycling ponds (Fig. 1). *Pythium* propagules were consistently the most numerous, generally ranging from 500 to 1,500 propagules per liter. *Phytophthora* spp. generally ranged from 0 to 400 propagules per liter. Although there were large month-to-month differences in the numbers of propagules detected, there did appear to be some broad patterns. For example, the numbers of propagules detected in the northern California pond were lowest from late fall through the winter and highest during warmer times of the year (Fig. 1A). In fact, during the winter months, there were very few viable *Phytophthora* propagules detected (Fig. 1A), a result that agreed with previous findings (3).

In the two southern California nurseries, changes in propagule numbers did not appear to be seasonally related (Figs. 1B and C). For example, in southern nursery No. 1, *Phytophthora* populations appeared to be highest during the fall, winter, and spring and lowest during the warm summer months (Fig. 1B). However, southern nursery No. 2, approximately 5 km away, did not show a similar pattern (Fig. 1C), suggesting that propagule fluctuations were not related to ambient nursery or water temperatures.

The identification of *Phytophthora* spp. recovered on VP3H medium revealed that propagules of *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian were the most numerous at all nurseries, at almost all times of the year (Fig. 2). At the northern California nursery, propagules of this species were most abundant in the summer and spring and least abundant during the winter (Fig. 2A). Although less numerous, propagules of *P. citricola* Sawada exhibited a similar pattern and large numbers of *P. cinnamomi* Rands propagules were detected only once, in the winter (Fig. 2A).

Whereas propagules of *P. citrophthora* dominated those recovered from southern nursery No. 1 (Fig. 2B), large numbers of *P. citricola* and *P. cinnamomi* propagules also were recovered, as well as smaller numbers of *P. cryptogea, P. megasperma* Drechs., and *P. parasitica* Dastur (Fig. 2B). In contrast to the northern nursery and southern nursery No. 1, *P. citrophthora* was clearly the dominant species in southern nursery No. 2 (Fig. 2C). Propagules of this species were, on several occasions, two to three times more numerous than at the other two nurseries, and while propagules of other *Phytophthora* species were detected in the effluent pond, their numbers were insignificant relative to those of *P. citrophthora* (Fig. 2C).

When the results of the ELISA procedures were compared with those of the culture plating, we found a positive but variable relationship between ELISA reaction intensity and the numbers of propagules estimated to be on the filters (as determined from corresponding culture plate results). For example, at southern nursery No. 1, there was a strong positive relationship between ELISA reaction intensity and propagule numbers in December (Fig. 3). In February, however, the slope of the regression line was significantly lower (Fig. 3). As a result of such month-to-month variation, the yearlong results from southern nursery No. 1 showed a positive relationship between ELISA reactions and propagule numbers, but the coefficient of correlation was relatively low (Fig. 4). ELISA results from the other nurseries were similarly variable (*data not shown*).

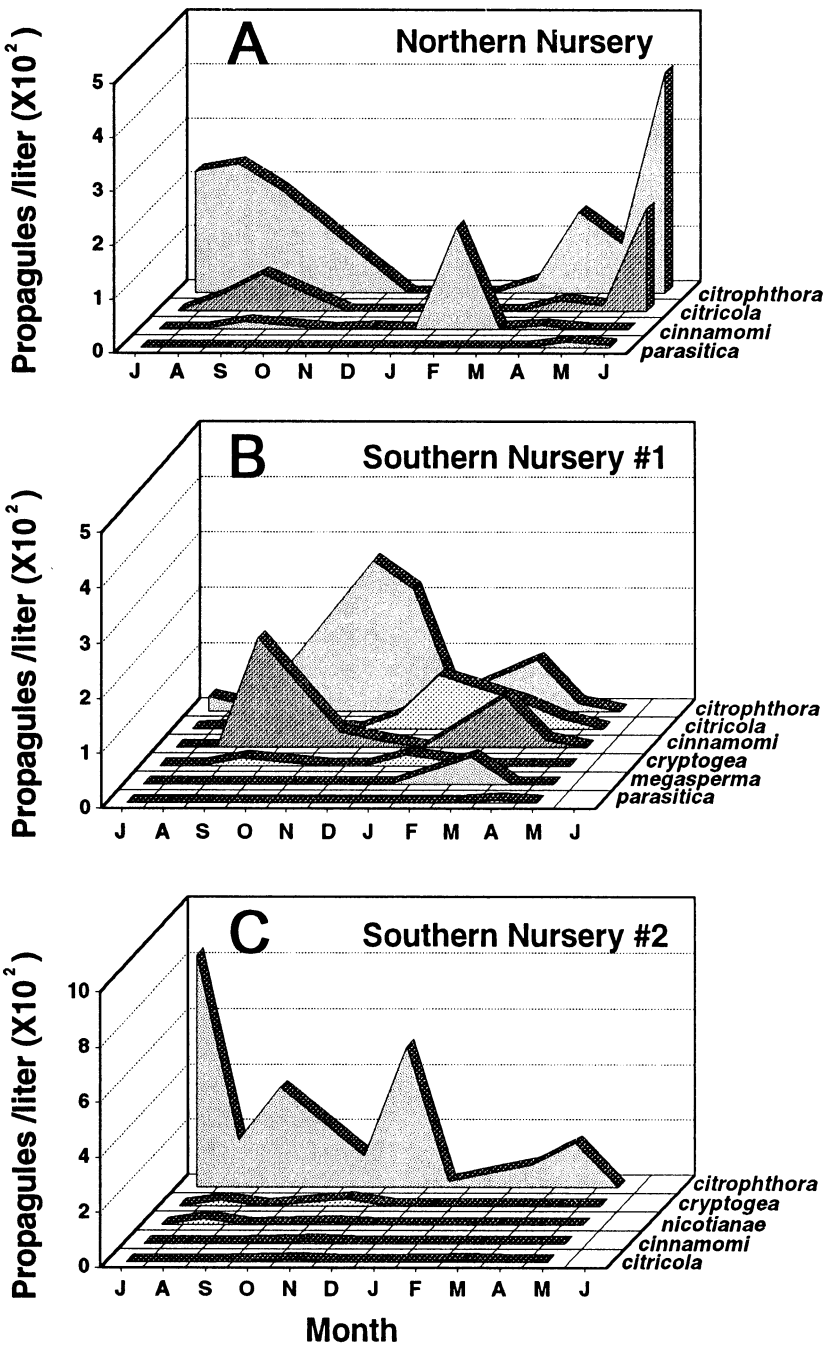

Fig. 2. Numbers of propagules of various *Phytophthora spp.* in effluent samples from **(A)** one northern and **(B and C)** two southern California nurseries practicing recirculation. Propagule numbers were estimated by subculturing randomly selected colonies that developed on a selective agar medium and identifying the fungi to species. The proportion of each species in the subculture collection was used to estimate its relative number in the original population.

DISCUSSION

Our assay results showed that nursery effluents contained numerous propagules of pythiacious fungi, including several *Phytophthora* spp. that are known to be pathogenic to nursery crops. While we did not attempt to identify the many *Pythium* isolates recovered from effluents (Fig. 1), we previously reported the presence of *Pythium coloratum* Vaartaja, *P. rostratum* E.J. Butler, *P. middletonii* Sparrow, and *P. ultimum* Trow var. *sporangiferum* Drechs. in effluents collected from one of the nurseries included in this study (3). There is some evidence that one or more of these *Pythium* species can be pathogenic to nursery plants (3).

The numbers of propagules detected in effluent waters fluctuated widely over the sampling period (Fig. 1). Many factors may have contributed to this variation. For example, the specific sections of the nurseries being irrigated differed from sample time to sample time. Because some plant species grown in nurseries are more susceptible to Phytophthora root rots than others, individual blocks of plants can differ in their inherent potential for root disease. Since the probability of detecting *Phytophthora* propagules in an effluent sample is dependent upon the number of diseased plants within the irrigated area, much of the variation we observed (Fig. 1) may have been an artifact of irrigation patterns or disease control efforts that affected pathogen-loading of effluents. Additionally, plants in some sections of the nurseries were packed "can-tight," while those in other sections were widely spaced to encourage spreading growth of tops. Where plants are widely spaced, a significant amount of sprinkler-applied irrigation water lands on the ground between plants and runoff water contains a smaller fraction of pot effluent. Finally, irrigation volumes pumped in nurseries differ on a daily, weekly, and/or monthly basis, depending upon climatic conditions. High flow rates (during warm weather when plants are actively transpiring) yield large volumes of runoff, but the runoff often contains large amounts of erosional sediments as well as a greater portion of fresh water added to offset evapotranspirational losses. Low flow rates (during cool or cloudy weather) produce smaller volumes of runoff that tend to contain less sediments. All of these factors could have influenced either the numbers of propagules in the effluent samples or our ability to successfully recover them. It was not possible to account for or control such factors or to accurately determine their effects on propagule counts.

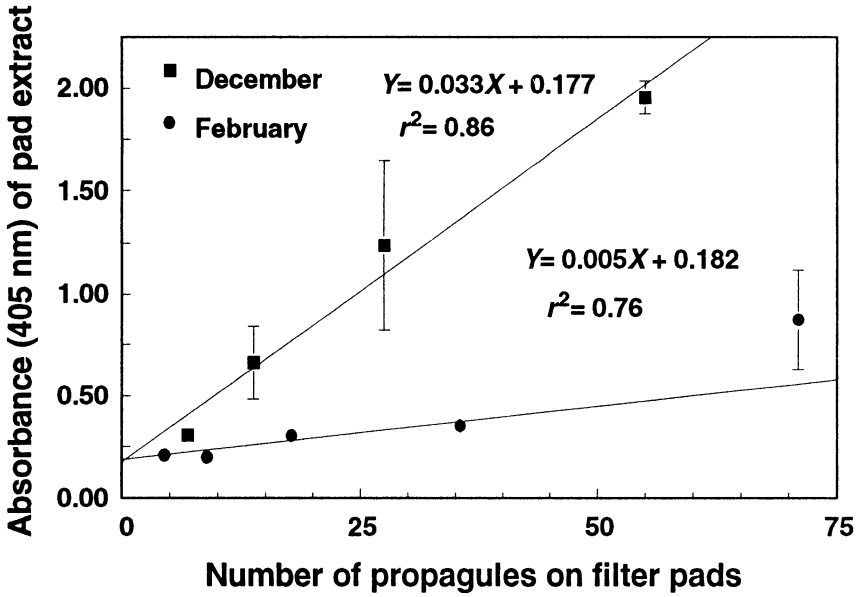

Fig. 3. Comparison of the ELISA reaction intensity of effluent samples collected at two different times from one nursery location. Different volumes of effluent were filtered through parallel sets of 0.45-μm Millipore filters. Residue extracts were prepared from one set of filters, and the concentration of *Phytophthora* antigen was determined using commercial ELISA test kits. Residues from the remaining filters were cultured onto selective agar media to estimate the corresponding numbers of propagules. Each point is the mean of four replicate assays. Vertical lines indicate ± 1 SD. Where no vertical bars are visible, the SD was smaller than the symbol.

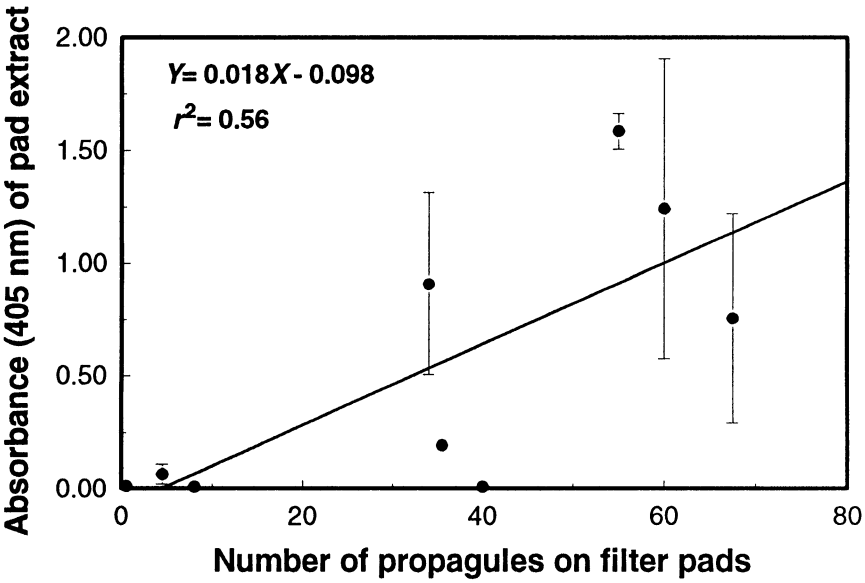

Fig. 4. Comparison of the ELISA reaction intensity of effluent samples collected at various times throughout the year from a nursery practicing recirculation. Effluent samples were filtered through parallel sets of 0.45-μm Millipore filters. Residue extracts were prepared from one set of filters, and the concentration of *Phytophthora* antigen was determined using commercial ELISA test kits. Residues from the other filters were cultured onto selective agar media to estimate the corresponding numbers of propagules. Each point is the mean of four replicate assays. Vertical lines indicate ± 1 SD. Where no vertical bars are visible, the SD was smaller than the symbol.

We also believe there may have been a persistent error in our estimates of propagule numbers caused by the effluent filtration and plating procedure that we used. Some *Phytophthora* spp. and propagule forms are inhibited more than others by the antibiotics used in selective media (19,20), so that we may have detected fewer viable propagules than were actually present. We tested this by producing and adding known numbers of *P. cryptogea* zoospore cysts to soil extract solutions and then attempting to recover the propagules using the filtration/plating method. We found that our assay detected only one-fifth to one-tenth of the expected numbers of propagules (*unpublished*), suggesting that cysts either were "injured" by the filtration process, were inefficiently recovered from filter pads, or failed to germinate efficiently on the selective media.

While this indicates a potential error in our assay method, the relatively small standard deviations throughout the sampling period (Fig. 1) suggest that errors were fairly consistent. Also, the

laboratory-produced zoospore cysts used in our recovery tests may not truly reflect the behavior of propagules produced in nature. It does seem likely, however, that the numbers of propagules reported here (Fig. 1) underrepresent the actual numbers in effluent waters.

We previously reported that commercially available ELISA tests could detect as few as 30–40 zoospore cysts of *P. citrophthora* and *P. parasitica* trapped on Millipore filters (3). When we compared the intensity of ELISA reactions with the numbers of *Phytophthora* propagules estimated to be on the filter pads, we found that the detection threshold varied from sample to sample. In some samples, the intensity of the ELISA reaction was significantly above background when there were as few as 15–20 propagules predicted to be on the filter pads (Fig. 3, December data). At other times, 75–100 propagules were required to yield reactions that were significantly above background (Fig. 3, February data).

Several factors may have contributed to the variable relationship between propagule numbers and ELISA reaction intensity. First, as noted above, the filtration/plating assays may have incorrectly predicted the numbers of propagules on the pads (although we believe that assay errors were consistent across time). Second, the mixture of species differed over time (Fig. 2). Such differences in species mixtures could alter the relationship between propagule numbers and reaction intensity, since species differ in their reactivity with the ELISA antibodies (3,9). Third, water samples contained more sediments at some times than at others. These sediments were trapped on the filter pads and can (particularly organic particulates) interfere with the ELISA reactions.

It was clear from the culture plating and ELISA results (Figs. 1, 3, and 4) that, for many reasons, assays of nursery effluents are inherently "noisy." In the case of ELISA procedures, it may be better to regard the tests as qualitative indicators rather than as quantitative measures. However, while the exact numbers of propagules in effluents may be uncertain, these results clearly demonstrate that there are numerous viable propagules in runoff water.

While the recirculation of *Phytophthora*- and *Pythium*-contaminated water is assumed to pose a disease risk, the degree of risk associated with the inoculum levels detected in this study is presently unknown. The water pumped from recycling ponds is typically filtered through sand-filled canisters to remove large particles that could foul irrigation distribution systems. In limited sampling, we have found that such filtering reduces the numbers of viable propagules returned to crops by a factor of 2–10 (*unpublished*). Unfortunately, there are virtually no data allowing prediction of the disease threat posed by continually exposing plants to such low levels of inoculum.

Treating crop effluents to eliminate pathogens is technically possible, but most of the research on this subject has involved hydroponic-type cropping systems (e.g., 4,5,17,26) or postharvest processing (e.g., 15,16). However, treatments that may effectively disinfest water used in hydroponic growing systems or packinghouse dip tanks cannot be applied directly to the problem of disinfesting nursery or greenhouse effluents. The volumes of water used and the irrigation pumping rates in large nurseries ($190–750 \times 10^3$ L/hr at peak rates) are much greater than in hydroponic systems. And while hydroponic systems may recirculate (and treat) water several times a day, the tailwater "cycle-back" times in nurseries are comparatively slow. Finally, nursery tailwater can be very turbid, containing large amounts of organic and mineral solids. All these factors combine to reduce the efficacy of water treatments such as chlorination, ozonation, UV irradiation, and microfiltration. Furthermore, it is unlikely (within the range of economic feasibility) that any disinfestation method could eliminate all viable propagules from recycled water. In the absence of reliable disinfestation processes, growers generally have relied on prophylactic fungicide applications to minimize the potential for disease establishment in susceptible crops. They also restrict the use of recirculated water to well-established hardy plants and exclude it from areas used for propagation.

The requirements that force nurseries to capture and recirculate crop effluents clearly introduce the possibility of spreading pathogens within crops. There is a real need to develop information leading to the establishment of rational, economical water quality standards (i.e., acceptable propagule residuals), effective disinfestation methods, and reliable monitoring procedures.

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