MANAGING THE PLANT PATHOGEN, *PHYTOPHTHORA*, TO IMPROVE ACCEPTANCE OF RECYCLING TECHNOLOGY IN ORNAMENTAL NURSERIES

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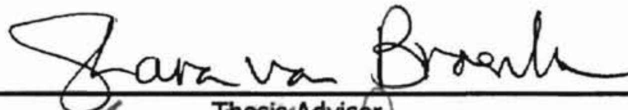

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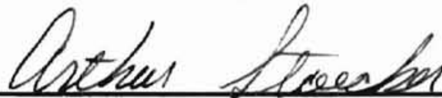

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CHAPTER I

LITERATURE REVIEW

Restrictions on water supplies (Higginbotham and Block, 1991), increased regulation (Koval and Moen, 1991), and public concern about the environment (Kuack, 1990) have compelled growers and nurserymen to search for alternative water management practices. Contamination of water supplies by runoff carrying fertilizers and pesticides from nurseries and greenhouses has become a state and national concern (Brubaker, 1991; USEPA, 1992). The need for increased control over water availability and water quality while meeting environmental objectives has led many nurseries and greenhouses to examine the potential of recycling irrigation water as a solution.

Greenleaf Nursery Company, located in eastern Oklahoma, is a nationally competitive wholesale nursery, serving customers across the United States, and in parts of Canada and Mexico. Additional production sites in Texas and North Carolina rank Greenleaf as one of the largest nurseries in the United States. Lake Tenkiller operated by the Army Corps of Engineers, Tulsa District, Tulsa, Oklahoma (Jobe, 1996), is adjacent to Greenleaf Nursery, and is its primary source of water. It is also part of the Illinois River Basin Scenic River System.

In 1989, the Illinois River Irrigation Tailwater Project was implemented. The project was designed to limit the release of nutrients and pesticides from the commercial nurseries in the basin (ODA, 1994). Maintaining water quality is important for preserving the scenic river, drinking water sources, and the recreational value of the river and lake. Greenleaf was one of three nurseries that instated this voluntary, proactive project.

Topographic features play an instrumental role in the design and layout of a recycling system. With a capture and recycle system, retention basins are designed to retain runoff from

the nursery and to hold storm water. Although no retention limits have been set for Oklahoma, other states require that ½ to 1 inch of storm water be retained for discharging (von Broembsen, 1998). Currently, statewide standards do not exist for nursery effluents, but data from the Illinois River Project could be used as a guideline to set realistic standards for Oklahoma (von Broembsen, 1998).

Nursery owners are acknowledging water quality issues by considering systems which capture and recycle irrigation water onsite and which offer a number of important benefits. Recycling can conserve 35-50% (Clover, 1995; Skimina, 1987) of irrigation water in comparison to a 70-80% loss before installation of a recycle\reuse system (USEPA, 1992). However, problems with recycling do exist. Plant pathogens are also recycled with the irrigation water, resulting in increased levels of both pathogens and disease (Clover, 1995; Daughtry, 1989; Thomson and Allen, 1974; Whitcomb, 1990).

Disease caused by these waterborne pathogens is a limiting factor for growers. As a nursery begins to recirculate their water, increased loss to disease may occur (Clover, 1995; Steadman *et al*, 1975). Growers need information on the effects of disease increase and its significance, better methods of disease management, and new water handling techniques and how to implement them. An important prerequisite to managing plant pathogens in irrigation water is to be able to detect and measure them in water.

Detection and Quantification of *Phytophthora* spp. in Irrigation Water. Water sampling and leaf baits are common techniques to detect the presence of *Phytophthora* in nursery irrigation water. MacDonald *et al* (1994a) collected nursery effluent from three commercial nurseries in California. The samples were collected at one-month intervals in 3.8-L plastic jugs. After transporting the samples back to the laboratory, they were filtered through 0.45-μm Millipore filters. A suspension was made by placing the filter into a test tube containing 6 ml of 0.09% sterile water agar (MacDonald *et al*, 1994a), then the filter residue was resuspended via a vortex mixer. Filter suspensions were spread to a selective agar medium, VP3, and VP3 medium amended with 50 mg/L of hymexazol to estimate total pythiacious fungi and

total *Phytophthora* colonies respectively. The fungi were then identified by colony morphology, growth-temperature relationships, and morphology (MacDonald *et al*, 1994a).

Leaf baiting, particularly with lemon leaf baits, has been used to detect presence of *Phytophthora* zoospores in soil and water. Although the baits detect Phytophthora, they do not give an indication to the degree of infestation (Tsao, 1960). Zoospores infest baits by accumulating along the cut edges of floating leaf pieces, they then germinate forming mycelium and sporangia (Grimm and Alexander, 1973). The leaf pieces are then plated to a selective media for identification of the disease plant pathogen.

In 1959, Klotz *et al.* determined lemon baits were the most satisfactory trapping agents for use in their system research. Thomson and Allen (1974) also used sieves and citrus leaf baits in Arizona to isolate *Phytophthora* in irrigation water. Then in 1976, continuing research by Thomson and Allen found 35% and 95% of leaf baits in contact with infested water for 10 minutes and 1 hour respectively, were infected with *Phytophthora*.

ELISA (enzyme-linked immunosorbant assay) test kits for detecting plant pathogens are commercially available for use by growers. The serological kits allow rapid detection versus the days or weeks required for a diagnosis when using a standard culture plating method. In 1990, MacDonald, Stites, and Kabashima compared an ELISA kit (Agri-Diagnostics Associates of Cinnaminson, NJ) to standard culture plating for their ability to detect species of *Phytophthora* and *Pythium*.

The research indicated the ELISA kits had excellent potential to be used as a diagnostic tool. Phytophthora D kits and Pythium C kits (Agri-Diagnostics) were used for the study. MacDonald, Stites, and Kabashima (1990) discovered that the Phytophthora D kits reacted weakly with *Phytophthora cinnamomi* and with some *Pythium* spp, but the kits strongly detected most *Phytophthora* spp. The Pythium C kits indicated broad genus detection including a weak reaction with some *Phytophthora* spp.

In 1991, Benson compared the multiwell Phytophthora E kit, and the rapid assay Phytophthora F kit (Agri-Diagnostics Associates, Cinnaminson, NJ) to a culture plate method.

The Phytophthora E kit was developed to detect *P. cinnamomi*. No cross-reactivity of the ELISA kits was found in nursery or greenhouse experiments. Benson (1991) concluded that commercial acceptance depends not only on ease of use, ability to detect, and a short time frame, but also on assay cost.

Also in 1991, Ali-Shtayeh, MacDonald, and Kabashima used the Phytophthora E and the Pythium C kits (Agri-Diagnostics) for their experiments. Cross-reactions were not observed with any of the *Phytophthora* spp. The samples reacted positively with the Phytophthora E kits and negatively with the Pythium C kits. However, the *Pythium* spp. failed to react with the Pythium C kits, and reacted positively with the Phytophthora kit. The research also indicated different species and even different isolates of the same species differed in their reaction intensity in the Phytophthora kits. This suggested that the kits ability to detect may vary with the amount of species present in water samples. Even though the kits are unreliable at the genus level, they still can be used for rapid detection in commercial nurseries where these genera are not acceptable in irrigation runoff (Ali-Shtayeh et al, 1991).

Pscheidt *et al.* (1992) evaluated 17 species of *Phytophthora* and their reactivity to Phytophthora kit E (Agri-Diagnostics). *Phytophthora* reacted with the kits, but differences of sensitivity between and within species were noted. *Pythium* cross-reactions with the kits make accurate diagnosis between genera difficult. However, Pscheidt *et al.* (1992) supported the kits as a useful diagnostic tool.

Factors Affecting the Occurrence of *Phytophthora* spp. in Irrigation Water. The genus *Phytophthora*, a plant pathogen often found in irrigation water, contains many fungal plant pathogens known to kill or damage a wide variety of economically important plants, including food crops and ornamental plants (Carlile and Watkinson, 1994; Erwin and Ribeiro, 1996; Zentmyer, 1983). *Phytophthora* spp. produce motile biflagellate zoospores and most species can also produce oospores or chlamydospores for survival in extreme conditions (Carlile 1983; Erwin and Ribeiro, 1996). These pathogens can invade through natural openings, or by penetration of

living tissue such as roots. Some species are host specific while others have a broad host range (Erwin and Ribeiro, 1996).

Research has also indicated electrotaxis (Carlile, 1983; Carlile and Watkinson, 1994; Troutman and Wills, 1964) and chemotaxis (Deacon and Donaldson, 1993; Zentmyer, 1961) attract the zoospores to roots. The motility of the zoospores allow these fungi to locate infection sites on roots, but passive movement by water can carry them longer distances and is a very important means of spread. Therefore, to be able to control *Phytophthora* populations, release of propagules into runoff and their survival in irrigation water as well as the effects of the environment on these processes must be understood.

Water temperature was the primary focus of studies conducted during the 1950's through the 1980's on fluctuating *Phytophthora* populations in irrigation water. Thomson and Allen (1974) stated the development of *Phytophthora* spp. in irrigation water was seasonal and dependent on water temperature. Their study used "feathered" Rough Lemon (*Citrus jambhiri*) as leaf bait to detect *Phytophthora* populations in irrigation water in citrus groves in Arizona over a one-year period. *P. parasitica* and *P. citrophthora* were recovered from wastewater and recycled water. *P. parasitica* was isolated when water temperatures ranged from 20 to 30°C while *P. citrophthora* was only recovered at temperatures below 23°C.

Another study conducted by Klotz, Wong and Dewolfe (1959) isolated *P. citrophthora, P. parasitica,* and *P. syringae* from irrigation water. Their study involved 12 canals, and three reservoirs in southern California, which supplied irrigation water to citrus groves. Klotz, Wong and Dewolfe also found the *Phytophthora* spp. recovered varied with seasonal changes in the temperature of the water. *P. syringae* was prevalent when the water temperatures ranged from 6 to 17°C, and *P. parasitica* was isolated during the summer and early fall (17 to 31°C). The key difference between this study and Thomson and Allen's study is *P. citrophthora* was recovered throughout the year in the Florida study.

Recent research has explored other factors besides water temperature for *Phytophthora* fluctuations. Ristaino (1991) stated in a comparison of two fields with inoculated plots disease

reduced yield by 43% in plots frequently irrigated versus 37% in plots irrigated less frequently. Her conclusion from this study was that final disease incidence and the rate of disease increase were significantly greater in those infested plots irrigated more frequently.

The most recent findings suggest fluctuations in disease populations may not be related to ambient nursery or water temperatures. A study conducted by MacDonald, Ali-Shtayeh, Kabashima, and Stites (1994a) entailed holding ponds at three commercial nurseries in California. The ponds were monitored for *Phytophthora* propagules. Two of the nurseries were 5 km from each other in southern California while the third nursery was in northern California where some broad patterns emerged. The number of propagules detected was lowest from late fall through winter and highest during the warmer months. In contrast at one of the southern nurseries *Phytophthora* populations were lowest during the summer months, and highest during the fall, winter, and spring. The second southern nursery did not show a similar pattern. A similarity all three nurseries shared was *P. citrophthora* was the dominant species.

Managing Disease in Recycling Systems. The most important ways that disease can be managed in recycling systems are cultivation of resistant cultivars, appropriate ways of using recycled water, and disinfection of recycled water before re-use. Resistant cultivars are selected and then used to replace disease susceptible cultivars in production as a method for disease management in the nursery. Currently, very little information exists on the effectiveness of methods to use recycled water. Some nurseries feel that dilution with clean water will reduce *Phytophthora* to levels that will not have significant adverse effects. An additional technique is to use recycled water only on established plants and exclude its use from disease sensitive areas such as propagation (Ali-Shtayeh *et al*, 1991). Disinfection of recycled water may be necessary. Effective methods of disinfection are available, but are costly and require intense management.

Common disinfection methods include chlorine, ozone, and ultra-violet (UV) radiation. Chlorine injection is the least expensive way to control organisms (Daughtry, 1989), although precautions must be used when handling this potentially dangerous chemical. Chlorine and ozone are oxidizing agents which degrade the cell walls and membranes of microbes, while UV

radiation damages essential nucleic acids (MacDonald *et al*, 1994a). Ozone and UV radiation eliminate pathogenic organisms from the water, but do not remove vital nutrients (Kuack, 1990). Nursery tailwater can be quite turbid, containing organic and mineral solids, reducing the effectiveness of water treatments such as chlorination, ozone and UV (MacDonald *et al*, 1994a). Flow rate and contact time are crucial elements in the success of these disinfection methods.

Given the diversity of nursery size, geographic location, water quality, irrigation demands, financial resources, and crops under cultivation, a single method of controlling pathogens in recycled irrigation water is not going to be applicable to every nursery (Ali-Shtayeh *et al*, 1991; MacDonald *et al*, 1994b). In addition, there is more to be learned about the effects of irrigation systems on disease population levels, quality comparison of captured runoff water to water that is stored and diluted, threshold levels of plant pathogens, and easy yet reliable means of disease detection.

CHAPTER II

DETECTION AND QUANTIFICATION OF *PHYTOPHTHORA* SPP. IN IRRIGATION WATER

INTRODUCTION

When capturing and reusing irrigation water plant pathogens are also recycled, resulting in increased levels of pathogens and disease (Daughtry, 1989; Thomson and Allen, 1974; Whitcomb, 1990). Methods for detection and quantification of plant pathogens in recycled irrigation water are necessary to manage pathogen populations. A number of methods for detecting and quantifying *Phytophthora* spp. in irrigation water have been shown effective.

Citrus leaves have been previously used to recover *Phytophthora* spp. from irrigation water (Grimm and Alexander, 1973; Thomson and Allen, 1976). In this method leaf pieces are plated to a *Phytophthora* selective medium. However, since northern nurseries may not include citrus varieties in their production, it would be useful if other types of leaves, readily available to northern nursery growers could be used. In this manner, nurseries could use leaf baiting for detection of *Phytophthora* by taking leaves from plants in production at their nursery or greenhouse. Although the baits detect *Phytophthora*, they do not give an indication to the degree of infestation (Tsao, 1960).

Another technique for nurserymen to detect *Phytophthora* levels is by using ELISA (enzyme linked immuno sorbent assay) kits. The kits could be used for rapid detection and determination of the presence of a specific genus in irrigation water or in leaf baits. Although the kits may be unreliable at distinguishing between *Pythium* and *Phytophthora*, they could be used where either genera is not acceptable in runoff (Ali-Shtayeh *et al*, 1991). For the kits to be useful to growers their use must be accurate and cost-effective. Commercial acceptance

depends not only on ease of use, ability to detect, and a short time frame, but also on assay cost (Benson, 1991).

Qualitative recovery of *Phytophthora* propagules from irrigation water by filtering propagules from known volumes of water, plating filters to *Phytophthora* selective media, and then counting colonies has been shown (von Broembsen, 1984). In these studies, several methods of detecting and quantifying *Phytophthora* spp. in irrigation water were compared and evaluated. These methods included the 'use' of leaf baits in collaboration with either plating to selective media or with ELISA kits, and the plating of propagules to selective media either by plating inverted filters through which irrigation water had been passed or direct plating.

MATERIALS AND METHODS

Maintenance of experimental cultures. Cultures were maintained in a culture collection on malt extract agar (15 g Difco Bacto-Agar and 10 g Difco Malt Extract per liter of distilled water). Isolates used for experimental purposes were P022, *Phytophthora cinnamomi* from *Leucadendron comosum*, P065, *P. citricola* from hibiscus; P068, *P. parasitica* from *Catharanthus roseus*; P083, *P. cactorum* from strawberry (obtained from Mike Benson, North Carolina State University); 5A-2NG, *P. citrophthora* from rhododendron; and G981-13, *Pythium sp.* from nursery irrigation runoff.

Production of zoospores. Cultures were grown on clarified V8 juice agar (V8A; Ribeiro, 1978) for 3-5 days. Ten 2-mm square blocks were transferred to 100 x 15 mm petri plates containing 10 ml clarified V8 juice broth (V8 broth; Ribeiro, 1978) and incubated at 24°C for 24 h. The V8 broth was then removed and the mycelial mats were rinsed twice with 10 ml of a mineral salt solution (MSS; 3.08 g of $Ca(NO_3)_2 * 4 H_2O$; 1.49 g of $MgSO_4 * 7 H_2O$; 0.51 g KNO_3), and then incubated in 10 ml of MSS per plate at 20-21°C under fluorescent lights. After 24 h, the MSS was removed and replaced with 10 ml fresh MSS. Four days later, the MSS was removed and the mats were washed twice with 10 ml sterile distilled water (SDW). The mats were chilled in 11 ml SDW per plate for 45 min at 5°C, then warmed to room temperature;

zoospores were released in 1–3 h. An aliquot of zoospore suspension was encysted by vortexing for 90 s and counted using a hemacytometer to estimate the concentration of zoospores.

Isolation of pythiaceous fungi from water by filtration. A series of volumes of water (effectively a dilution series without any addition of water) were filtered through 47-mm Nuclepore® polycarbonate membrane filters held in a Nuclepore® filter funnel assembly (Costar Scientific Corporation, Cambridge, MA), with three replicate filtrations for each volume (modified after von Broembsen, 1984). Filters were then inverted and placed in 60-mm petri plates containing a selective media, either P_{10}VP (Tsao and Ocana, 1969) for total pythiaceous fungi or P_{10}VPH (Masago *et al.*, 1977) for total *Phytophthora* spp. The plates were incubated at 24°C for 24 h, the filters removed, and the plates returned to the incubator for 24 h. Then the number of colonies developing on each plate were recorded for each volume and the number of colony forming units/L (cfu/L) were calculated.

Comparison of recovery of *Phytophthora* zoospores by filtration and by direct plating. Isolates of four *Phytophthora* spp., P022, P065, P068 and P083, were used to produce suspensions of motile zoospores as previously described. Half of each preparation was encysted by vortexing and concentrations were determined as previously described. Suspensions of motile and encysted zoospores of each species were diluted with SDW to a concentration of 10 or 100 zoospores per ml. For filtration recovery, 10 ml containing an estimated 100 zoospores was passed though each filter following the specified procedure. For direct plating, 1 ml containing an estimated 100 zoospores was pipetted to 60- mm plates of P_{10}VP, swirled to distribute the suspension, and then incubated at 24°C. After 24 h, the number of colonies per plate was recorded. All treatments were replicated three times.

Screening of potentially suitable leaf baits for recovery of four *Phytophthora* spp. Leaves of trifoliate orange, hibiscus, euonymus, and rhododendron were chosen for preliminary studies. Three forms of leaf baits, whole leaves, leaf pieces and cut leaves, were evaluated for recovery efficiency. Zoospore suspensions of four isolates of *Phytophthora* (P022, P065, P068 and P083) were produced and concentrations were determined as described above.

Water with known concentrations of zoospores was prepared by dilution of zoospore suspensions of either motile or encysted zoospores with SDW in 1-L beakers to give a final volume of 1 L. Whole leaves (one leaf/ beaker) and leave pieces (ten 1-cm pieces/beaker) were floated in infested water or in SDW in beakers for 48 h at room temperature (20-21°C) on a laboratory bench, then removed and plated to $P_{10}VP$. Plates were examined for growth of fungal colonies after 48 h. Treatments were replicated twice and the experiment was repeated, but cut leaves were also included in the second experiment. Cut leaves of each plant type were prepared by trimming away leaf margins, then cutting into leaves so as to produce tabs with three cut surfaces but attached to the main body of the leaf by the fourth side. Only the tabs of these cut leaves were plated onto selective media to determine recovery of *Phytophthora* spp.

Recovery by rhododendron and lemon leaf baits of zoospores of four *Phytophthora* spp. Two cut leaves each of rhododendron, 'Nova Zembla' and lemon 'Meyers' with 10 tabs per leaf were used to bait motile or encysted zoospores from 1 L of water infested with 1 zoospore/ml for P022, P065 and P068, but 100 zoospores/ml for P083. Tabbed leaves were also used to bait SDW to serve as controls. After 48 h the leaves were removed, and leaf tabs were plated to $P_{10}VP$. The plates were incubated at 24°C for 48 h, and results recorded as the number of infected leaf pieces out of 20.

Comparison of recovery by direct plating, filtration, and leaf baiting of three concentrations of zoospores. *Phytophthora parasitica,* P068, was used to produce zoospore suspensions and concentrations determined as previously described. Half of the suspension was vortexed to encyst the zoospores. Both motile and encysted zoospores were diluted with SDW to achieve concentrations 1, 10 or 100 cells/ml.

For recovery by direct plating, 10 ml of suspension was pipetted to three 60-mm plates of $P_{10}VPH$ for 1 and 10 cells/ml, but only 1 ml for 100 cells/ml for both motile and encysted zoospores. Therefore, 100 cells were plated from the 10 cells/ml and 100 cells/ml zoospore suspension, but only 10 cells could be plated to the 1 cell/ml assay due to the holding capacity of the 60-mm petri plate. The suspension was swirled to distribute cells on the selective media, and

incubated at 24°C. After 24 h excess water was drained from plates to which 10 ml suspensions had been added and the number of colonies on all plates was recorded. Each of the six treatments was replicated three times.

For recovery by filtration, 100, 10 and 1 ml aliquots of zoospore suspension containing 1, 10 or 100 cells/ml respectively were filtered and filters were plated to P_{10}VPH media as previously described. This assured that regardless of the concentration, 100 cells were assayed for each of three replicates per dilution treatment.

For recovery by leaf baiting, two leaves each of rhododendron and lemon were cut to have 10 tabs each. The tabbed leaves were used to bait three replicate 1 L water samples containing 1, 10 or 100 cells/ml for 48 h at 20-21°C for both motile and encysted zoospore treatments. Tabbed leaves were also placed in similar volumes of SDW to serve as controls. Once the incubation was complete, the leaf baits were removed from the beakers, and the leaf tabs from these were plated to P_{10}VPH media and incubated at 24°C. Results were recorded after 48 h as the number of infested leaf pieces per leaf.

Alert® Phytophthora detection kits (Neogen Lansing, MI) were also tested using a now outdated "cup" version. Two tabs from each of two leaves for each of the three replicates were used to bait the 100 cells/ml samples for motile and encysted zoospores as in the above experiment. The leaves were removed and ground using the Extrak® Pads included in the Alert detection kit. The kit instructions were followed to complete the sampling procedure and results were recorded as either negative or positive.

Experiment I. Irrigation water samples were taken at six different sites at Greenleaf Nursery Company (GLN) in 3.78 L plastic bottles and transported back to the laboratory in an ice chest. For each of the six sites, two 1-L sub-samples were baited with one cut leaf each of lemon and rhododendron. After 24 h, a second set of leaf baits were marked to distinguish them from the first set and floated in each beaker. After an additional 24 h, all leaf tabs were plated to P_{10}VPH and incubated at 24°C for 24 h. Final results were recorded as number of infected leaf

pieces out of 10. The concentration of *Phytophthora* spp. in these samples was also determined using the standard filtration assay described previously.

Experiment II. Experiment I was repeated at a later date with water samples from the same sites at Greenleaf Nursery Company. This time, however, two sets of tabbed leaves of each plant variety were added to water samples at the same time. After 24 h, one leaf of each plant variety was removed for plating of the tabs and after 24 h further incubation, the remaining set of leaves was removed for plating. Final recovery was determined after 48 h incubation from time of plating and results were recorded as the number of infected leaf pieces per leaf.

Evaluation of Agden® water filtration kit and Alert® well kits for detection of *Phytophthora* spp. in irrigation water. A commercially available complete water filtration kit (Product code WK1000, Adgen Diagnostics, Scotland) for use in conjunction with Alert Phytophthora or Pythium detection kits to assay irrigation water was supplied by Neogen Corporation, (Lansing, MI). The water filtration kit consisted of a filter assembly unit with a 500 ml reservoir and a 1000 ml receiving vessel, 50 cellulose-nitrate Millipore filter membranes (0.45 µm pore size), a hand-operated vacuum pump, and all the other equipment and supplies needed to recover fungal propagules on filters and to prepare the filters for assay using the Alert kits.

Irrigation water samples were collected from Greenleaf Nursery at three different sites designated RH, D1A and D5E and transported back to the laboratory for processing. The concentrations of *Phytophthora* spp. in these samples were determined by Nuclepore filtration and plating of filters to selective medium as previously described. Measured volumes from each sample were also passed through the Agden® filter assembly according to the kit instructions. Only 200 ml of water would pass through for two of the samples (RH and D5), but the recommended 1 L volume was filtered for the third sample (D1A). A 30-ml volume of sample RH was also filtered using the Nuclepore® filtration assembly with a polycarbonate filter to determine if polycarbonate filters would be suitable for use with the Alert® kits. Filters, through which samples had been passed, were prepared for assaying with the Alert kits according to the Agden® kit instructions. Each filter was cut into ten strips of similar width and the strips were

heated for 5 min in 2 ml water in a glass tube in a beaker of boiling water. The tube was removed and allowed to cool for several minutes. Then filter pieces were transferred to an extraction bottle of an Alert kit.

Evaluation of Alert® kits for detection of *Phytophthora* spp. in leaf pieces. *Phytophthora citrophthora* (isolate 5A-2NG) and a *Pythium* sp. (isolate G981-13) were grown out on corn meal agar (17 g Difco corn meal agar in 1000 ml SDW). Squares of rhododendron leaves (ca 1 cm^2) were then placed on the plates and incubated for 48 h to allow infestation of the leaf pieces. Alert® Phytophthora detection kits were used to assay the leaf pieces according to the kit instructions, except that one set each of the leaf pieces were treated before grinding and extracting by boiling in SDW as described above or microwaving for 60 s at high power in 2 ml SDW. Sets of four unheated leaf pieces infested with each of the test isolates were used as controls.

Data handling and analysis. Data from all experiments was entered into data sets in Excel (Microsoft, Redmond, WA) and analyzed using the Microsoft Excel statistical analysis package (developed by GreyMatter International, Inc. Cambridge, MA).

RESULTS

Recovery of zoospores of four *Phytophthora* spp. by filtration. The filtration assay method showed no significant difference between recovery of motile and encysted zoospores for the four *Phytophthora* spp. tested. There were slight but significant differences in recovery amongst the *Phytophthora* spp. (Figure 2.1). The overall mean recovery was 23.7 % of expected recovery based on hemacytometer counts.

Screening of potentially suitable leaf baits. The first experiment showed that all four leaf varieties were able to detect at least two *Phytophthora* spp. (Table 2.1). However, hibiscus and rhododendron detected best, recovering 4 and 3 *Phytophthora* spp., respectively. Baiting with cut leaf pieces resulted in greater colonization than did baiting with whole leaves. The second experiment, which included tabbed leaves, showed that hibiscus and rhododendron again had the greatest recovery efficiency. Whole leaves were unable to recover *Phytophthora*

spp. in this experiment while tabbed leaves showed a significantly better recovery than did cut pieces. In both experiments, it was observed that leaves of euonymus and trifoliate orange were often pre-colonized with other fungi, making then less suitable for baiting. While hibiscus recovery was actually best, whole leaves or even large pieces would not lie flat on the isolation medium, and were less suitable for use as tabbed, cut leaves. Using tabbed rhododendron leaves was the best baiting method overall of those tested.

Comparison of baiting with rhododendron and lemon leaves. Tabbed leaves of both rhododendron and lemon were able to detect *Phytophthora* spp. at low (1 zoospore/ml) concentrations (Table 2.2). However, rhododendron leaves detected all three *Phytophthora* spp. (*P. cinnamomi, P. citricola,* and *P. parasitica*) tested at this level, while lemon did not detect *P. cinnamomi.* Both rhododendron and lemon leaves recovered *P. cactorum* at a high (100 zoospores/ml) concentration very well, but recovery by rhododendron leaves was significantly better (Figure 2.2). Recovery of *P. cactorum* from water infested with encysted zoospores was significantly less than from water infested with motile zoospores. No Phytophthora was recovered from uninfested water controls by rhododendron or lemon leaves.

Comparison of recovery by direct plating, filtration, and leaf baiting of water with three concentrations of zoospores. Recovery of encysted *P. parasitica* zoospores by direct plating was 27.0% of that estimated to be present based on hemacytometer counts in this experiment (Fig. 2.3) There was no significant difference between recovery by filtration assay compared with direct plating or between recovery of motile zoospores compared with encysted zoospores. Since 100 zoospores were assayed for each concentration x volume treatment, the recovery data for both motile and encysted zoospores by direct plating and filtration was analyzed by considering all three concentration x volume treatments as one treatment with nine replications. Using a two-factor ANOVA with replication, there was no significant difference between these treatments; that is, the volume containing the 100 zoospores did not affect quantitative recovery by either of these methods.

There was no significant difference in the recovery of *Phytophthora* zoospores by rhododendron and lemon leaf baits for either motile or encysted zoospores; therefore, we compared recovery of motile zoospores and encysted zoospores combining lemon and rhododendron data. Recovery of motile zoospores was significantly higher ($P < 0.001$) than recovery of encysted zoospores (Fig. 2.4).

Exposure time of leaf baits in irrigation water. Leaf baits placed in irrigation water samples 24 h after a similar set had been placed in these same samples but removed at the same time did not become infested to the same level (data not shown). It was not clear whether this was due to the shorter exposure time, or to the first set of baits having already baited most of the *Phytophthora* in the water samples. A second experiment eliminated the latter possibility by starting both exposure periods at the same time. In this experiment there was no significant difference between a 24 h and a 48 h exposure period in the recovery of *Phytophthora* spp. by either lemon or rhododendron leaves (Table 2.3). There was a slightly better recovery by lemon than rhododendron at 24 h. For this experiment the actual concentrations of *Phytophthora* spp. as determined by the filtration assay were 600, 0, 330, 3, 470 and 1000 cfu's/L for irrigation water samples 1-6 respectively.

Evaluation of Alert® kits for detection of *Phytophthora* spp. in irrigation water and in leaf baits. The concentrations of *Phytophthora* spp. in the three irrigation water samples, RH, D1A and D5E, from GLN were 600, 0 and 880 cfu/L, respectively. Assays using the Agden® filter assembly and cellulose-nitrate filters, gave positive reactions for the D1A and D5E samples. The RH sample gave an inconclusive result. The fourth test using the Nuclepore filtration assembly and polycarbonate filter for a smaller (30 ml) aliquot of sample RH tested positive.

Boiling and microwaving leaf pieces infested with *Phytophthora citrophthora* isolate 5A-2NG gave positive results while unheated pieces gave inconclusive results. Only one of the duplicate wells for each test gave a faintly positive reaction while the other well for each of these techniques was negative. For the leaf pieces infested with *Pythium* (isolate G981-13), boiled

pieces gave a distinctly positive reaction while the microwaved and untreated pieces gave either negative or inconclusive results. Table 2.4 summarizes these results.

DISCUSSION

The overall recovery efficiency of laboratory produced zoospores using the filtration method was 23.7% compared to direct plating. This is slightly better than the 10-20% recovery efficiency reprinted by MacDonald *et al* (1994) for a different filtration method. The method has proven to be a reliable method of estimating the number of propagules in irrigation water. Direct plating of actual irrigation water for quantifying *Phytophthora* propagules in irrigation water was often unsuccessful due to suspended sediment, algae, and bacteria present in the runoff irrigation water.

Hibiscus and rhododendron demonstrated similar recovery efficiency; however, rhododendron was selected over hibiscus. Hibiscus leaves curled and did not make good contact with the selective media. Rhododendron proved to be a hardier leaf and the material can be found in production at most northern nurseries. The leaves laid flat when plated to media, and survived the high water temperatures in irrigation water for 48 h. For 1997, both lemon and rhododendron were floated at Greenleaf Nursery to compare the leaves in field sampling (see Chapter 3). Lemon leaf baits recovered *Phytophthora* efficiently, but high water temperatures made the leaves mushy and difficult to plate in mid-summer.

The water sampling kit was easy to use and would be convenient for nurseries or greenhouses to use on-site. The kit instructions suggested filtering 1-L water samples, but this volume was not feasible for turbid runoff from a nursery setting. The Alert® kits showed cross-reactivity with *Phytophthora* and *Pythium*, and would not be useful in differentiating between the genera. Recommendation of the kit would solely be for a nursery or greenhouse wanting to detect presence of both *Phytophthora* and *Pythium*.

Table 2.1. Comparison of four potential leaf baits for recovery[a] of four Phytophthora spp.: P022, P065, P068 and P083.

Experiment I.

Isolate	Trifoliate Orange		Hibiscus		Euonymus		Rhododendron	
	whole	cut	whole	cut	whole	cut	whole	cut
P022	0	0	0	(+)	0	0	(+)	(++)
P065	0	0	0	(+)	0	0	0	0
P068	(+)	(++)	(+)	(+)	0	(+)	(+)	(++)
P083	0	(+)	0	(+)	0	(+)	0	(++)

Experiment II.

Isolate	Trifoliate Orange			Hibiscus			Euonymus			Rhododendron		
	whole	cut	tabbed	whole	cut	tabbed	whole	cut	tabbed	whole	cut	tabbed
P022	0	0	0	0	0	(+)	0	0	0	0	0	(+)
P065	0	(+)	0	0	0	0	0	0	0	0	0	(+)
P068	0	0	(+)	0	(+)	(+)	0	0	0	0	(+)	(+)
P083	0	(++)	(++)	0	(++)	(+)	0	(+)	(++)	0	(++)	(++)

[a] (+) = 1-5 infested leaf pieces out of 10 or 1-10 infested leaf pieces out of 20 for Trifoliate Orange and Euonymus feathered leaves on $P_{10}VP$.
(++) = 6-10 infested leaf pieces out of 10 or 11-20 infested leaf pieces out of 20 for Trifoliate Orange and Euonymus feathered leaves on $P_{10}VP$.

Table 2.2. Comparison of baiting recovery[a] with Rhododendron and Lemon Leaves using both motile and encysted Phytophthora zoospores.

	P022	P065	P068
Lemon—Motile	0	(+)	(+)
	0	0	(+)
	0	0	(+)
Lemon—Encysted	0	0	0
	0	0	0
	0	0	(+)
Rhododendron—Motile	0	0	(++)
	(+)	0	(+)
	0	0	0
Rhododendron—Encysted	(+)	(+)	0
	0	0	0
	0	0	0

[a] (+) = 1-5 infested leaf pieces out of 10 on $P_{10}VP$.
(++) = 6-10 infested leaf pieces out of 10 on $P_{10}VP$.

Table 2.3. Mean Recovery of *Phytophthora* spp. with 24 h versus 48 h Exposure Time in Irrigation Water with Rhododendron and Lemon Leaves[a]

	24 h		48 h	
Sample Number Rhododendron	Lemon	Rhododendron	Lemon	
1	8.0	5.0	7.0	5.5
2	0.5	0.0	0.0	0.0
3	6.0	4.0	5.0	2.0
4	0.5	0.0	0.0	0.0
5	8.0	6.0	4.5	3.5
6	8.0	3.0	6.5	4.0

[a] = number of infested leaf pieces out of 10; mean of two replicates

Table 2.4. Results Summary of Phytophthora Alert Kits from Field and Laboratory Experiments

	Untreated	Boiled	
		Phytophthora	Pythium
Irrigation Water Filter Strips[a]	Negative or Inconclusive	Positive	Positive
Leaf Bait Pieces[b]	Negative	Positive	Positive

[a] = either cellulose-nitrate Millipore filter membrane or polycarbonate filter
[b] = Rhododendron leaf pieces

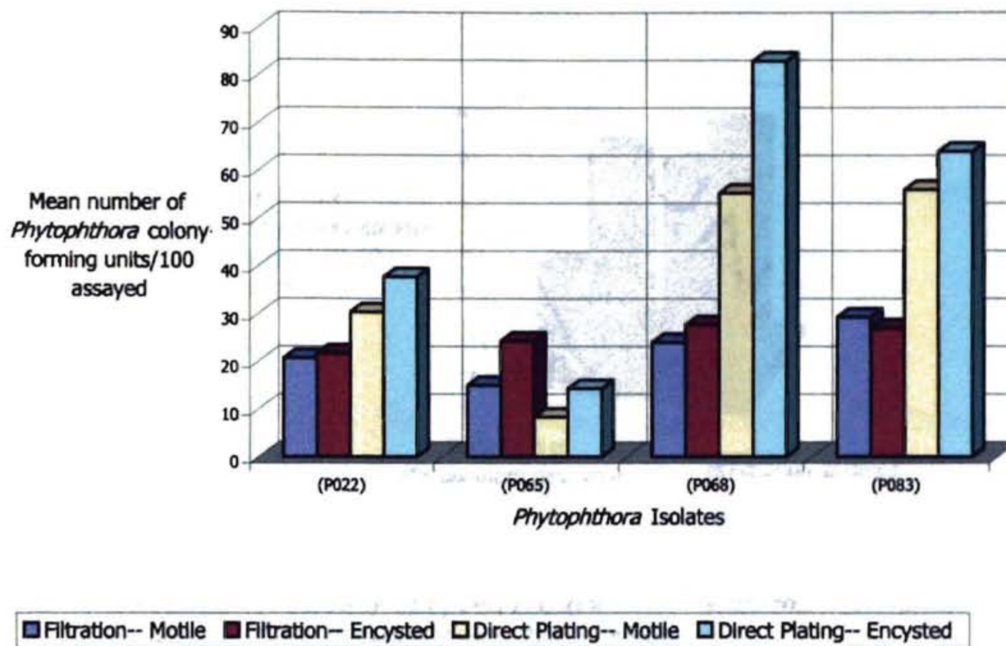

Fig. 2.1 Comparison of the Efficiency of Recovery by Filtration versus Direct Plating for four *Phytophthora* spp.

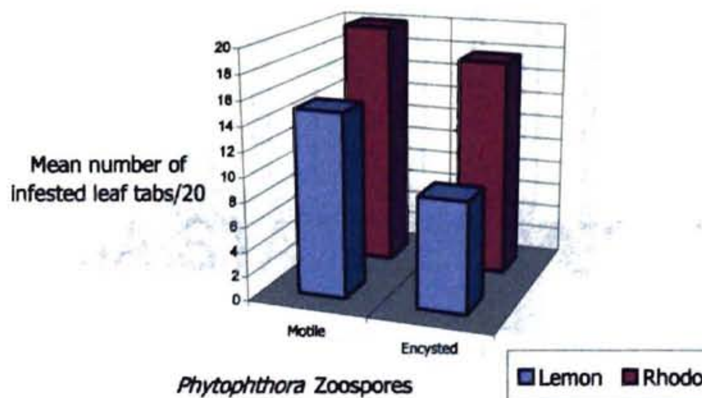

Fig. 2.2 Baiting Efficiency of Rhododendron and Lemon Leaves Using Motile and Encysted *Phytophthora cactorum* (P083) Zoospores.

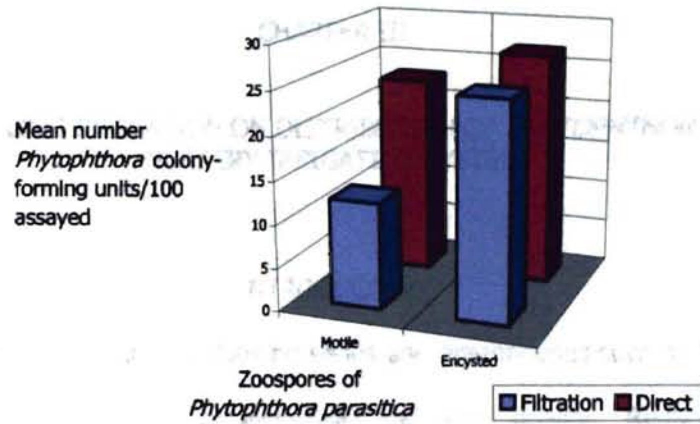

Fig 2.3 Comparison of Motile or Encysted Zoospores of *Phytophthora parasitica* from Water Using Filtration Versus Direct Plating

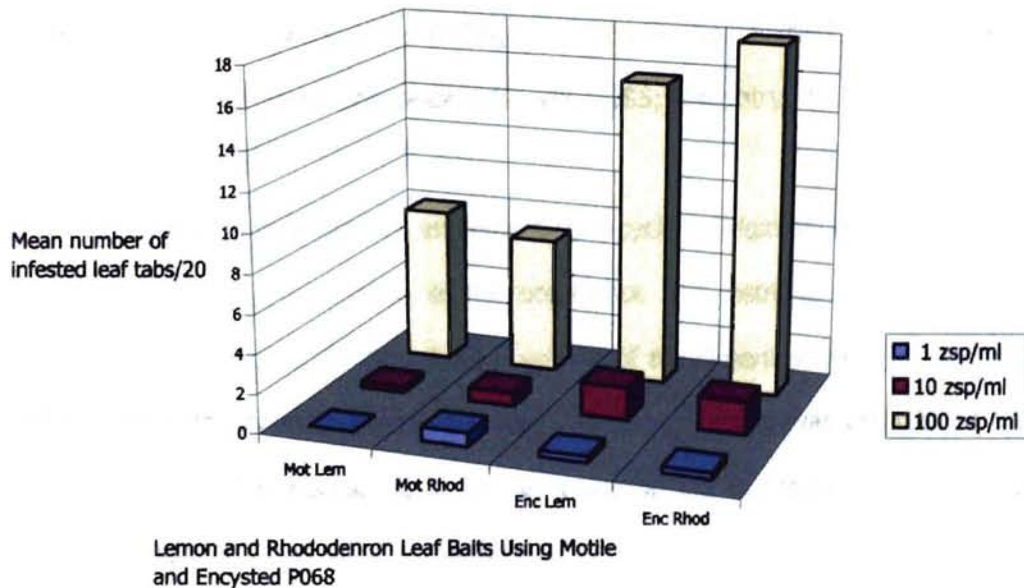

Fig. 2.4 Comparison of the Recovery Efficiency of Motile and Encysted *Phytophthora parasitica* (P068) With 1, 10, and 100 zoospores/ml by Using Lemon and Rhododendron.

CHAPTER III

EFFECTS OF RECYCLING IRRIGATION ON DISTRIBUTION OF *PHYTOPHTHORA* SPP. WITHIN A NURSERY IRRIGATION SYSTEM

INTRODUCTION

Recycling irrigation water provides nurseries and greenhouses such as Greenleaf Nursery Company increased control over water availability and water quality. Reducing or eliminating runoff from nurseries and greenhouses also preserves the quality of water resources. Additional benefits of recycling irrigation water include reduced water costs and a decline in nitrogen costs (MacDonald *et al*, 1994a; MacDonald *et al*, 1994b; Skiminia, 1987). However, along with the irrigation water and nutrients, plant pathogens may also be recycled and this can result in increased levels of pathogens and disease (Clover, 1995; Daughtry, 1989; Thomson and Allen, 1974; Whitcomb, 1990).

To examine the effects of implementing recycling irrigation on populations of plant pathogens, a study was undertaken of the occurrence and distribution of an indicator plant pathogen, *Phytophthora*, within the irrigation system of the Greenleaf Nursery Company before and after implementation of recycling irrigation. A baseline study was established to measure the levels of *Phytophthora* occurring at various points in the irrigation system throughout the 1997 growing season. Two different methods of sampling for *Phytophthora* in irrigation water were also evaluated. The effects of implementation of recycling, which took place in late 1997, on the levels of *Phytophthora* within the system were then monitored during the 1998 season. This chapter reports on these 1997 and 1998 field studies, the effectiveness of the different sampling methods and discusses the implications of the findings for disease management.

MATERIALS AND METHODS

Field Sampling Design. Greenleaf Nursery Company has a diverse topography with numerous retention basins (Fig. 3.1). To establish an effective method for sampling this system, evaluation of the basins and how they are interconnected is necessary. Many of the basins can be pumped to larger basins or diluted with water from the lake. During the 1997 season, the sampling design placed emphasis on pathogen levels in runoff compared with source water. However, during the 1998 season, the emphasis was switched to sampling for pathogen levels in irrigation water as delivered to plants in production.

Pre-recycling (1997). The objectives for 1997 were to evaluate methods for detecting and monitoring *Phytophthora* in recirculating irrigation systems, and to establish baselines for population levels in different components of the irrigation system. Six sites were selected for water collection and leaf bait sampling. Water from Site 2, Lake Tenkiller (source of nursery's irrigation water), and from Site 4, the main storage dam from water pumped from Lake Tenkiller, was sampled at the point of use on the production blocks (Fig. 3.1). Site 4 also received some off-site runoff containing low levels of *Phytophthora* spp. The remaining four sites were collected from runoff channels from nursery production areas. Site 1 was runoff from the propagation area and Phytophthora-sensitive plants while sites 3, 5, and 6 were runoff sites from the nursery beds.

Recycling (1998). Sampling sites were modified to place more emphasis on sampling water at the point of use and less on sampling runoff ditches. This shift from sampling runoff sites to sampling at sites of delivery was made to determine how disease levels were being affected by storage before reuse. In 1998, the entire nursery with the exception of the propagation/sensitive area used recycled water. Water was sampled at two runoff sites, RH and RSP, which flow into Basins 3 and 5 respectively (Fig. 3.2). D1A, D22G, D5E and D34D are sampling sites for water delivered from Lake Tenkiller, Basin 3, Basin 5 and the upper storage basin respectively. The upper storage basin received some recycled water from Basin 3 and some off-site runoff, but most of its water was pumped from Lake Tenkiller.

Recovery by filtration. Water samples were collected in a 3.78 L container and were taken intermittently over a 20 minute time period from flowing water from either runoff channels or from irrigation risers. Temperature, pH, and electrical conductivity (EC) were recorded in the field at each site. The samples were then transported back to the laboratory in ice chests. The samples were individually assayed using the filtration method described in Chapter 2 with P_{10}VPH as the selective medium. The number of colony-forming units per plate was recorded and up to five representative isolates were selected from each site for sporulation. The isolates were first transferred to a fresh P_{10}VPH plate, incubated at 24 °C, then CMA and finally grown on V8A. Small squares (5 mm^2) were then cut from the leading edge of the actively growing culture on V8A and placed into 60 x 15 mm Petri dishes filled with either 5 ml of MSS or 5 ml non-sterile soil extract solution (15 g sandy loam soil in 1 L of distilled water, agitated with magnetic stirrer for 24 h, and filtered through Whatman No. 1 filter paper). These plates were incubated under fluorescent lights at 20-21°C for 2-4 days. Once sporulated, the isolates were submitted to Sharon L. von Broembsen for identification to the species level.

Leaf Baiting of Irrigation Water. Bags for deploying leaf baits were made with a nylon mesh material cut 20 x 40 cm, which was folded and fastened with staples. The leaves were then tabbed as previously described and inserted into the bags along with a stone for weight. The top of the bags were closed with fishing line, and fastened to plastic milk cartons which served as floats. Samples were floated in runoff ditches and basins secured with ropes. After an exposure period the leaf baits were collected and placed in Ziplock® bags and transported back to the laboratory in ice chests. Leaf tabs were removed, and plated to P_{10}VPH media, and incubated for 24 h at 24°C. Each bag was recorded separately with results reported as the number of infested leaf tabs out of 20.

For the pre-recycling monitoring (1997), leaf baits were floated at Greenleaf Nursery for 48 h of continual re-exposure. Leaf baits were floated at all sampling sites (Fig. 3.1). Two bait bags containing two tabbed leaves of rhododendron were deployed at each site containing two tabbed leaves of lemon and two tabbed leaves of rhododendron. For 1998 (recycling) two bait

bags were deployed at four sites: Lake Tenkiller pump station (BL), Basin 3 (BH), Basin 5 (BSP), and the upper-storage basin (BUS) (Fig. 3.2). Bait bags were submerged for 24 h of continual re-exposure.

Water Quality Assessment. Temperature, pH and electrical conductivity (EC) were recorded in the field at the water sampling site and the leaf bait site at the time of sampling using the Cole-Parmer® 19815-00, Basic Conductivity Meter and Basic Conductivity/TDS Meter (Vernon Hills, IL) and the Cole-Parmer® 59002-00 pH/mV/Temperature Meter (Vernon Hills, IL). The probes for these hand-held meters were directly inserted into the retention basins or flowing water.

RESULTS

Recovery by filtration.

Pre-recycling (1997). Phytophthora levels recorded during the 1997 season are given in Fig. 3.3. Very high sediment levels in runoff samples from Site 6 occasionally interfered with the assay method. However, samples from the other 3 runoff sites, 1, 3, and 5, gave consistent results. The runoff samples had extremely high *Phytophthora* levels averaging about 450 cfu/L over the season with some samples exceeding 1000 cfu/L. In contrast, water pumped onto blocks from Site 2, the lake source, or Site 4, the upper storage dam was very clean. *Phytophthora* spp. were recovered from Site 2 on two sampling dates and from Site 4 three times early in the season. Concentrations were 5-7 cfu/L in these instances. The species recovered at Greenleaf Nursery over the 1997 season were *P. citrophthora, P. cryptogea, P. citricola, P. parasitica,* and *P. cinnamomi* (Table 3.1).

Recycling (1998). Phytophthora levels recorded during the 1998 season are given in Fig. 3.4. The runoff sites, RH and RSP, had exceptionally high *Phytophthora* levels averaging about 600 cfu/L over the season with some samples from both sites reaching 1000 cfu/L. Two of the sites delivering recycled water, D5E and D34D, did not exceed 190 cfu/L at any sampling, but the third site D22G, reached 270 and 400 cfu/L for the April and July sampling respectively. No Phytophthora was recovered from water delivered from the lake source at site D1A. Water

delivered from the storage dam, at site D34D, had extremely low concentrations of *Phytophthora* (20 cfu/L in the instances where *Phytophthora* was recovered. The species recovered at Greenleaf Nursery over the 1998 season *P. citrophthora, P. cryptogea, P. citricola,* and *P. parasitica* (Table 3.1).

Leaf Baiting.

Pre recycling (1997). Leaf baits were an extremely sensitive method of detecting *Phytophthora* spp. For 1997 baits exposed at runoff sites in moving water consistently showed almost total saturation at each sampling (Fig. 3.5). These results reflect both the extremely high levels recorded at these sites through filtration recovery from a 3.8 L sample, and the effect of continual exposure of the baits to large volumes of infested water over a 48 h period. The leaf baits were very useful as a qualitative, screening technique to detect the presence of *Phytophthora* spp. at low levels in water samples. Leaf baits often detected *Phytophthora* spp. at the surface of the two "clean" water sites (Site 2 and Site 4) even when no *Phytophthora* was recovered from a corresponding 3.78 L water sample taken at the beginning of the 48 h exposure period. Rhododendron and lemon leaves were equally effective in recovering *Phytophthora* spp. However, towards the end of the summer when water temperatures were exceptionally high, the integrity of lemon leaves deteriorated during the 48 h exposure.

Recycling (1998). As noted in Chapter 2, laboratory experiments showed no significant difference between a 24 h and a 48 h exposure period for leaf baits. Also, rhododendron and lemon leaves proved to be equally effective in recovering *Phytophthora* spp. Therefore, only rhododendron leaves, which were readily available at Greenleaf Nursery, were floated in the leaf bait bags at sites BL, BH, BSP and BUS (Fig. 3.2). The leaf baits demonstrated equal recovering efficiency at lower *Phytophthora* levels as in 1997. The two main retention basin sites (BH and BSP) had considerably higher levels of *Phytophthora* than either the upper storage basin (BUS) or the Lake Tenkiller source site (BL). These results correspond to the number of *Phytophthora* spp. recovered by the filtration method from a 3.78 L water sample taken at the end of each 24 h

exposure period. Throughout the season the rhododendron leaves did not show signs of deterioration during the 24 h exposure.

Water Quality Assessment. Data for water temperature, pH, and EC are given in Tables 3.2, 3.3, and 3.4 respectively. For the 1997 season the temperature ranged from 13° C to 33.6° C and the pH varied from 7.14 to 9.24. The EC was typically 200-500 μS/cm. In 1998 the temperature ranged from 4.8 to 33.3° C, the pH from 6.73 to 9.54, and the EC averaged about 300 μS/cm. There was no apparent relationship between water quality factors and *Phytophthora* levels.

DISCUSSION

One key to finding a suitable management program for *Phytophthora spp.* in a recycling irrigation system is to be able to monitor *Phytophthora* spp. within the system. Two methods for detecting and monitoring this plant pathogen, filtration and leaf baiting, have been demonstrated to be appropriate for this purpose. Using the filtration method, runoff sites were shown to have extremely high levels of *Phytophthora*. During 1997 levels of *Phytophthora spp.* averaged about 450 cfu/L, and in 1998 the amount of *Phytophthora* was about 270 cfu/L. These numbers were comparable to the 0-400 *Phytophthora* propagules/L recovered by MacDonald *et al* (1994) in California. The upper storage basin did not exceed 110 cfu/L before or after recycling was implemented, and few *Phytophthora* isolates were recovered from the lake source during 1997 only. This is valuable information since the lake is the main source for irrigation water for the nursery, and the upper storage basin, which hold 35 million gallons, is the main storage basin for the nursery.

Leaf baits were also evaluated to provide a qualitative estimate of the level of *Phytophthora* spp. present in a recycling irrigation system. Lemon leaves have been used as leaf baits for *Phytophthora* spp. (Klotz *et al.*, 1959), but Greenleaf Nursery Company in Oklahoma does not produce lemon trees, as is typical of other northern nurseries. Laboratory studies (Chapter 2) showed that rhododendron was equally effective as a leaf bait when compared with lemon leaf baits. The two types of leaf baits were equally effective in the field in 1997, so only

rhododendron leaf baits were used for the 1998 sampling. Overall, the leaf baits proved to be a useful qualitative tool for detecting the presence of *Phytophthora* spp. in recycling irrigation water. The leaf baits were capable of detecting the presence of the plant disease pathogen even at very low levels of *Phytophthora*.

In 1974, Thomson and Allen stated the development of *Phytophthora* spp. in irrigation water used for citrus groves was seasonal and dependent on water temperature. This research indicated there is no statistical relationship between the pH, EC or temperature and population levels of *Phytophthora* spp.

Table 3.1 Percent of sampling dates select species of *Phytophthora* were recovered from Greenleaf Nursery Company from 12 sampling dates in 1997 and 5 sampling dates in 1998.

	1997 (%)	1998 (%)
Phytophthora cinnamomi	8.3	0
Phytophthora citricola	41.7	20
Phytophthora citrophthora	100	80
Phytophthora cryptogea	83.3	100
Phytophthora parasitica	66.7	40

Table 3.2 Seasonal Temperature (Celsius) Variation at Greenleaf Nursery Company for 1997 and 1998.

1997 Data												
	April-1	April-15	April-29	May-13	May-27	June-10	June-24	July-8	July-22	August-5	Sept-4	Oct-9
Site 1	18.4	14.8	15.4	21.5	20.7	22.4	30.0	26.6	30.3	26.0	25.6	19.7
Site 2	16.5	13.0	15.2	19.3	19.4	24.3	28.3	26.0	29.7	26.5	26.7	22.4
Site 3	16.9	18.0	21.8	23.6	20.3	27.0	31.2	25.7	33.6	29.5	26.7	22.5
Site 4	16.7	15.0	17.3	21.2	19.2	24.2	27.5	27.3	31.8	30.1	30.6	21.8
Site 5	15.6	15.8	19.5	20.6	19.4	26.3	29.3	25.9	31.3	27.9	28.0	20.5
Site 6	19.8	14.5	17.3	23.1	19.0	30.5	27.5	27.8	29.2	28.6	30.2	21.5
Site X	16.4	16.5	18.5	20.7	20.9	25.7	28.9	26.7	34.8	29.6		

1998 Data					
	March-1	April-7	July-7	Sept-9	Oct-6
D1A		15.5	31.3	29.1	21.7
RH	7.0	23.1	33.3	23.5	21.1
D22G		17.8	31.9	27.4	19.9
RSP	4.8	17.9	30.6	26.0	20.0
D5E		17.5	30.7	26.7	19.9
D34D		17.3	31.1	28.4	21.3

Table 3.3 Seasonal pH Variation by Date and Site at Greenleaf Nursery Company for 1997 and 1998.

1997 Data												
	April-1	April-15	April-29	May-13	May-27	June-10	June-24	July-8	July-22	August-5	Sept-4	Oct-9
Site 1	8.4	7.9	7.26	8.72	7.14	7.18	8.41	8.25	7.92	8.47	8.82	6.82
Site 2	8.59	7.83	7.93	8.93	8.1	8.41	9.05	8.69	8.46	7.52	8.51	7.4
Site 3	8.75	8.76	8.49	8.79	7.94	8.72	9.09	7.92	9.24	8.7	8.43	7.96
Site 4	8.49	9.12	9.17	8.12	7.81	7.57	7.58	8.04	8.53	7.32	9.0	7.49
Site 5	8.14	7.99	8.2	8.02	7.22	7.61	8.12	8.68	9.13	8.89	9.2	7.23
Site 6	8.03	7.2	7.31	8.55	7.38	8.3	7.31	7.72	6.91	8.22	9.21	6.24
Site X	7.9	8.27	9.17	8.34	7.07	7.67	7.27	9.08	9.79	8.47		

1998 Data					
	March-1	April-7	July-7	Sept-9	Oct-6
D1A		7.24	9.19	8.8	7.42
RH	7.74	9.03	9.13	9.42	8.13
D22G		7.91	8.14	7.57	6.71
RSP	7.55	8.04	9.04	9.07	7.98
D5E		7.56	8.49	8.16	7.06
D34D		8.01	8.43	7.79	6.85

Table 3.4 Seasonal EC (uS/cm) Variation at Greenleaf Nursery Company for 1997 and 1998.

1997 Data	April-29	May-13	May-27	June-10	June-24	July-8	July-22	August-5	Sept-4	Oct-9
Site 1	245	256	398	468	327	252	791	277	258	271
Site 2	197	225	226	208	96.1	177.3	158.6	349	153	191.8
Site 3	268	297	247	238	197	254	303	286	446	207
Site 4	208	229	253	235	224	282	265	248	220	226
Site 5	227	272	233	252	250	237	281	213	170	210
Site 6	411	277	336	218	281	358	390	373	285	421
Site X	247	309	222	271	323	305	237	274		

1998 Data	March-1	April-7	July-7	Sept-9	Oct-6
D1A		195.7	135	231	199.9
RH	294	134.6	378	461	181.5
D22G		262	329	385	190.5
RSP	165.1	410	337	332	197
D5E		322	322	409	187
D34D		257	265	390	228

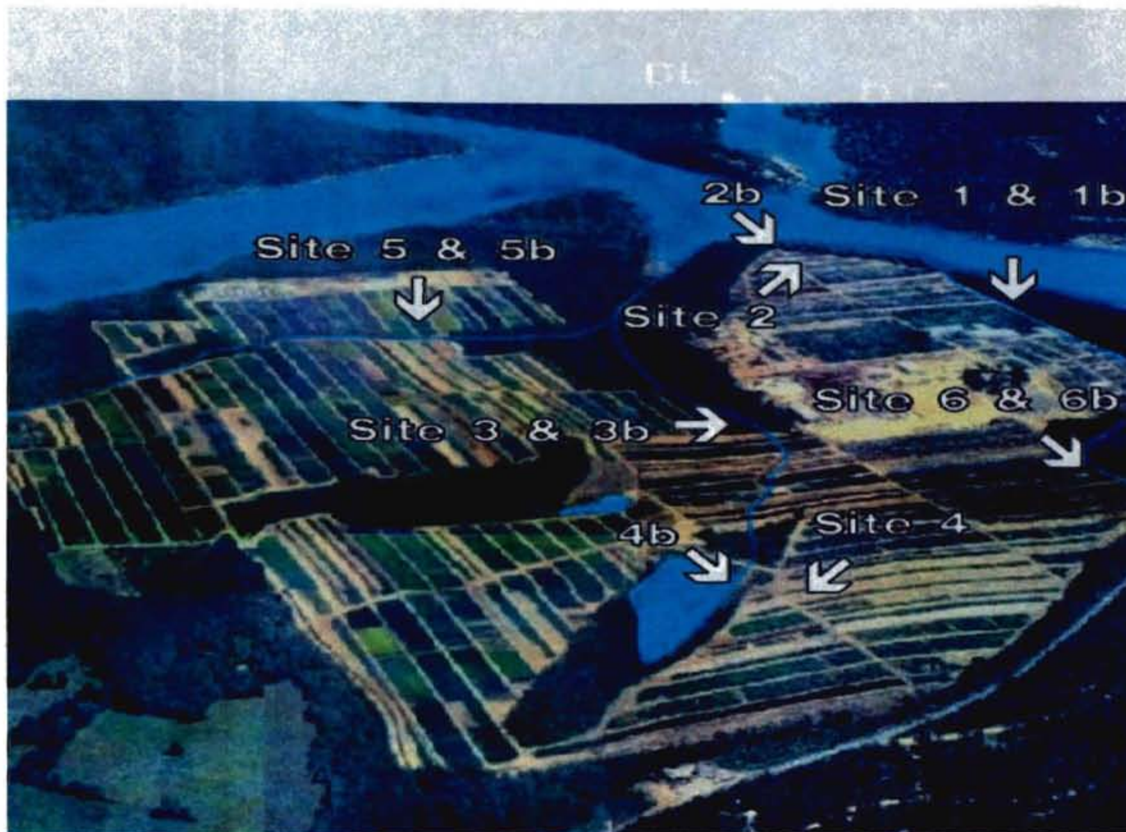

Fig. 3.1 Map of Greenleaf Nursery Company Showing Six Water Sampling Sites for 1997.

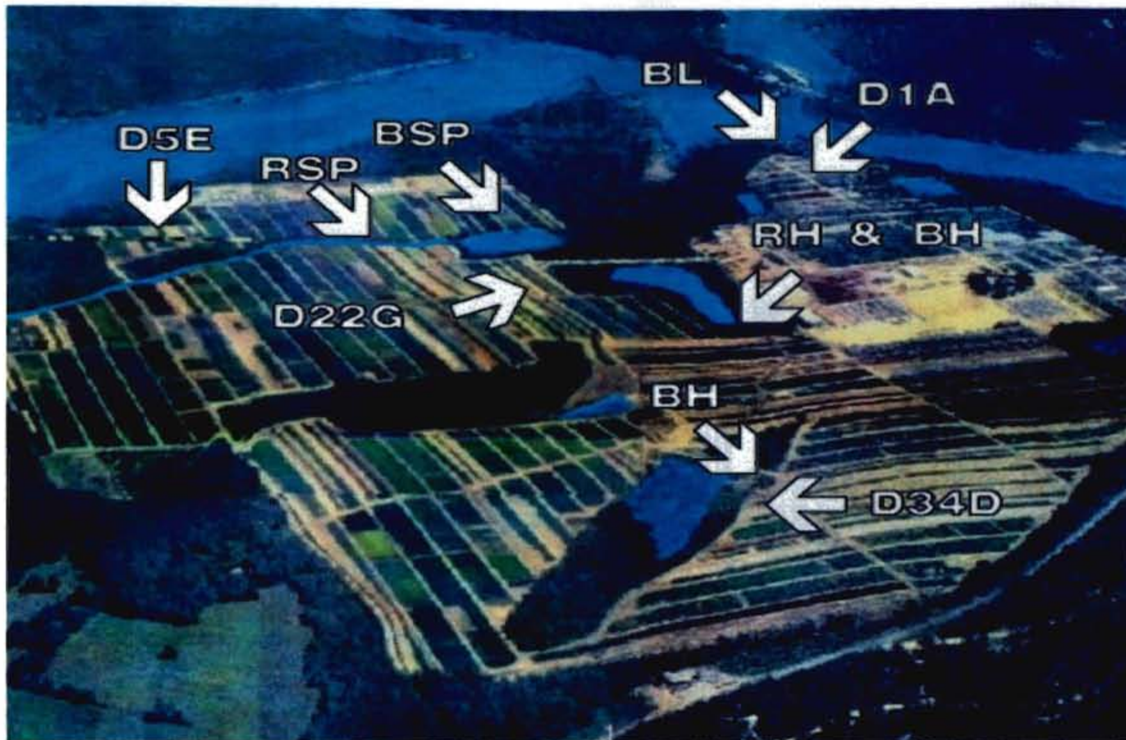

Fig. 3.2 Map of Greenleaf Nursery Company Showing Six Water Sampling Sites for 1998.

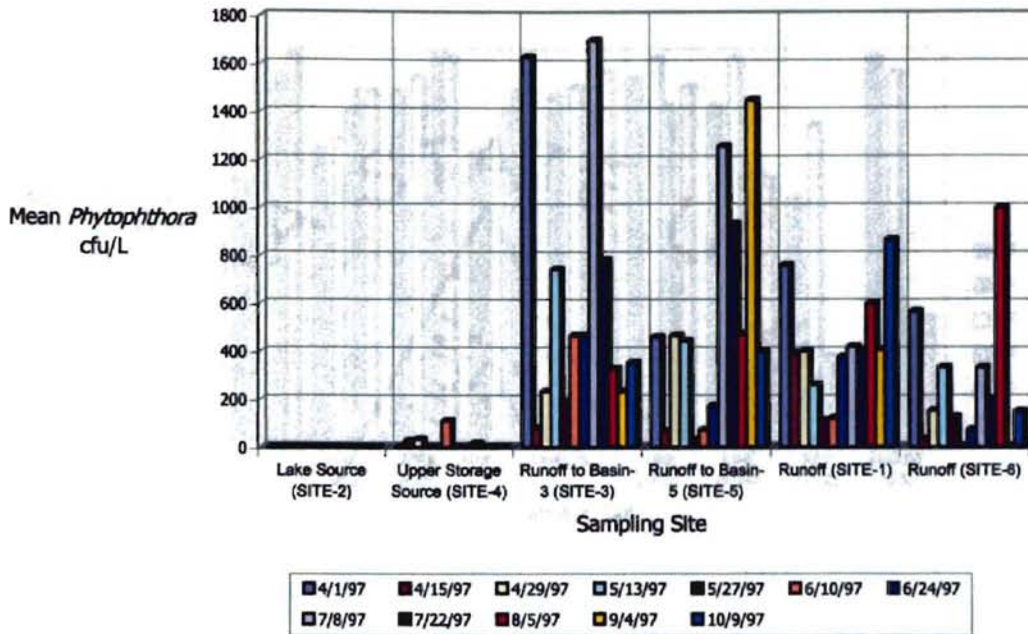

Fig. 3.3 1997 Mean Total *Phytophthora* per Liter for 12 Sampling Dates at Six GLN Sites. Two of the sites are source water and the remaining sites are runoff samples.

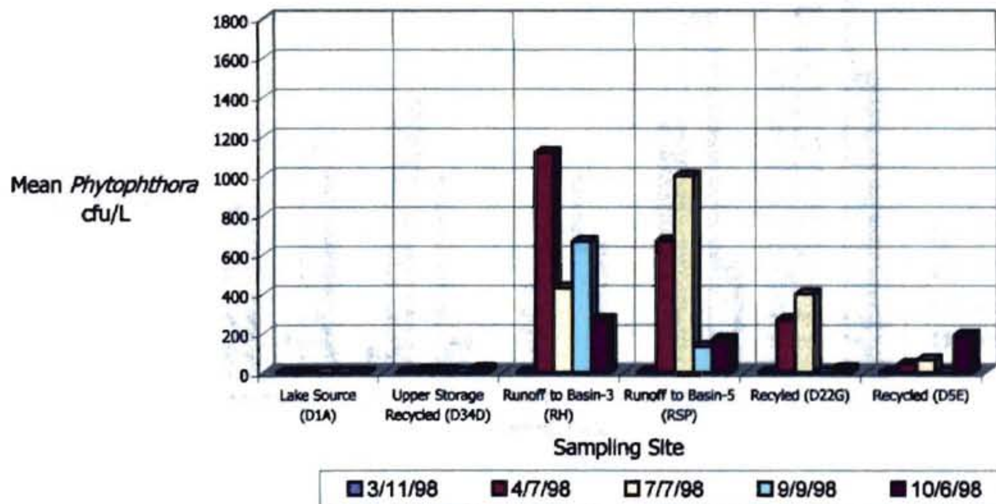

Fig. 3.4 1998 Mean Total *Phytophthora* per Liter for 5 Sampling Dates at Six GLN Sites. The lake was the only source water site while D22G, D5E and D34D are recycled water as applied to nursery beds. The runoff to basin 3 and 5 was also sampled.

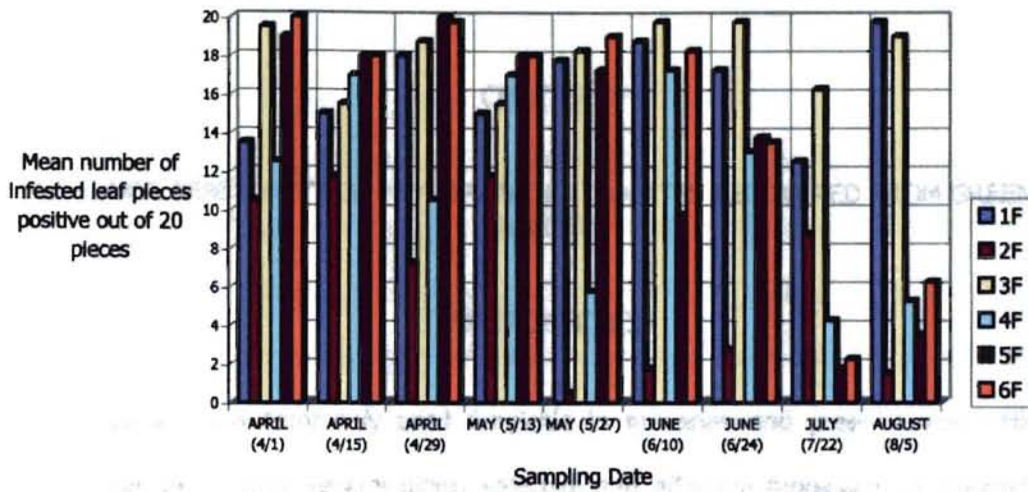

Fig. 3.5 Mean Positive Number of infested leaf pieces/20 for 1997.

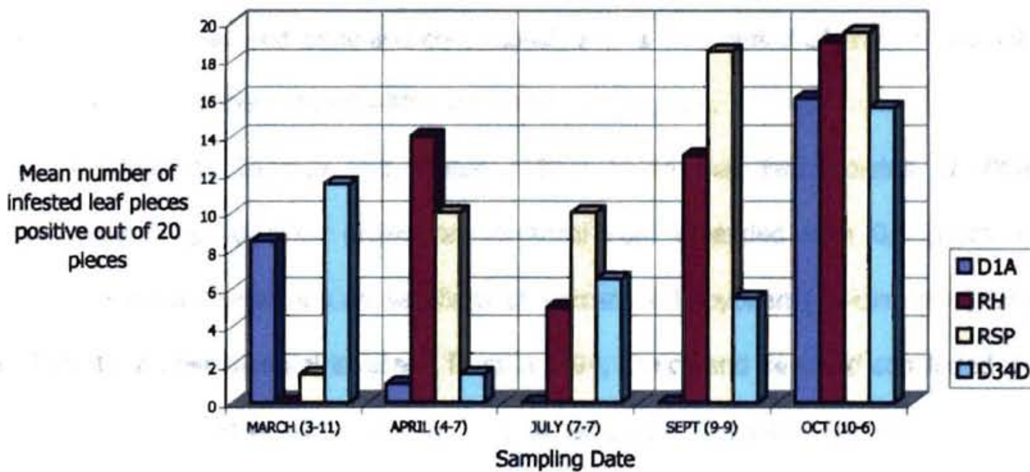

Fig. 3.6 Mean Positive Number of Infested Leaf Pieces/20 for 1998.

CHAPTER IV

METALAXYL SENSITIVITY OF PHYTOPHTHORA ISOLATES RECOVERED FROM GREENLEAF NURSERY

INTRODUCTION

Metalaxyl is a commonly used fungicide in nurseries and greenhouses. Historically, metalaxyl was the first of several highly selective and effective fungicides (acylalanine family) toxic to *Phytophthora* spp. to be developed (Erwin, 1983). However, towards the end of 1979, the first instances of metalaxyl failure under field conditions occurred. Schwinn (1983) published some traits that cases of metalaxyl resistance had in common. First, all had occurred under high disease pressure and on highly susceptible cultivars. Second, resistance had developed only where metalaxyl was used alone and continuously over a long period of time; it had not occurred when metalaxyl had been mixed with a conventional fungicide.

A study by Lamboy and Paxton (1992) found that two isolates of *Phytophthora megasperma* f. sp. *glycinea* grown on cornmeal agar amended with 0.1 μg/ml metalaxyl, displayed a greater virulence on seedlings of Harosoy 63 soybean (*Glycine max*) treated with metalaxyl than their parental isolates. Then in 1994, Csinos and Bertrand concluded that typical field application rates of metalaxyl may not be adequate to controls isolates of *Phytophthora parasitica* Dastur var. *nicotianae* on tobacco (*Nicotiana tabacum* L.) that have a low level of sensitivity. However, this study did not conclude whether this variation was natural or due to decreased sensitivity by regular use of metalaxyl.

In 1996, Goodwin, Sujkowski and Fry amended rye agar with 5 and 10 μg of metalaxyl per ml to determine sensitivity. The criterion for insensitivity established for this study was the ability of any isolate to grow more than 40% of the control on 5 μg/ml metalaxyl medium; all

other isolates were recorded as sensitive. Then in 1997, Fabritius, Shattock and Judelson used medium amended with 0, 0.5, and 5.0 μg/ml of metalaxyl. The relative insensitivity of each strain was determined by calculating the ratio of mean radial growth on the media amended with metalaxyl to that on non-amended media.

It was important to determine if Greenleaf Nursery Company had resistant *Phytophthora* isolates of metalaxyl for several reasons. If resistant isolates were present a change in fungicidal use would prove to be more effective in controlling fungal plant pathogens such as *Phytophthora* should increased disease occur following implementation of recycling. Resistant isolates might also be spread by recycled irrigation water to other crops and other areas in the nursery where metalaxyl would then be ineffective

MATERIALS AND METHODS

Isolates were selected from plant, root and soil samples collected in July 1997, April 1998 and July 1998 from plant areas with a history of disease losses caused by *Phytophthora* spp. Thirty-five *Phytophthora* isolates consisting of five *Phytophthora* spp. (Table 4.1) were tested for metalaxyl sensitivity. The isolates were grown out in pure culture on CMA plates. Then 6 mm diameter agar plugs containing mycelial growth of each isolate was transferred to 3 replicate plates of CMA amended with 0, 0.5, and 5 μg/ml active ingredient metalaxyl. The plates were incubated in the dark at 24°C and radial growth was recorded at 24 h, 3 days, and 6 days later. The relative insensitivity of each isolate was determined by calculating the ratio of mean radial growth on the CMA amended with metalaxyl to growth on non-amended CMA.

RESULTS

Phytophthora citrophthora isolates averaged 83.8% radial growth on CMA amended with 0.5 μg/ml active ingredient metalaxyl compared to non-amended CMA (Fig. 4.1), and were generally sensitive at that concentration. However, moderate levels of insensitivity was demonstrated on the 5.0 μg/ml metalaxyl plates for some isolates. Twenty-two *P. citrophthora* isolates were reduced on average by 49.4%. Overall, the growth of twelve isolates grew more

than 50% and were considered insensitive. Four other *Phytophthora* spp. were also tested for metalaxyl sensitivity (Table 4.2). All isolates were sensitive to metalaxyl.

DISCUSSION

At Greenleaf Nursery Company some *Phytophthora* isolates were found that were insensitive to relatively low levels of metalaxyl. Spread of these insensitive isolates to other parts of the nursery where crops could develop a higher risk to these insensitive pathogens is a concern. To decrease the possibility of further metalaxyl insensitivity in the nursery, alternative fungicides should be considered. Mefenoxam has been commercially marketed to replace metalaxyl. The mode of action of mefenoxam is similar to that of metalaxyl. Continuing studies on the effect of mefenoxam on *Phytophthora* isolates will determine any cross reactivity between metalaxyl and mefenoxam.

Table 4.1 Isolates collected from Greenleaf Nursery Company on July 1997, April 1998, and July 1998

Species	Isolate	Host
P. citrophthora	1G-R-1	Rhododendron
	3D-A	Rhododendron
	2D-A	Rhododendron
	3D-L	Rhododendron
	1G-L-1	Rhododendron
	2D-L	Rhododendron
	5A-2G	Rhododendron
	5A-1(R)1	Rhododendron
	5A-3S	Rhododendron
	GLN R-8	Rhododendron
	5A-3R	Rhododendron
	5A-2R	Rhododendron
	5A-4(R)1	Rhododendron
	5A-2NG	Rhododendron
	G98G-9(1)	Rhododendron
	G98 S-9	Rhododendron
	3N-Astem	Rhododendron
	G98G(10)	Rhododendron
	G98G-4(1)	Rhododendron
	G98 S-7	Rhododendron
	GLN R-9	Rhododendron
	GLN R-7	Rhododendron
P. citricola	4G-L-1	Dogwood
	4G-R-1	Dogwood
	5G-R-1	Dogwood
	10A-1G	Dogwood
	10A-3G	Dogwood
	5A-4S	Rhododendron
P. cinnamomi	1D-A	Rhododendron
	5N-L	Dogwood
P. dreschsleri	G98 S-14	Dogwood
	G98 S-13	Dogwood
	G98 S-11A	Dogwood
	GLN R-14	Dogwood
P. cryptogea	2G-1R	Willow Oak

Table 4.2 Percentage of Greenleaf Nursery Company isolates of the mean radial growth on CMA amended with metalaxyl (0.5 ug/ml and 5.0 ug/ml) versus the non-amended CMA.

Species	Isolate	0.5 ug/ml metalaxyl (%)	5.0 ug/ml metalaxyl (%)
citricola	4G-L-1	26.1	4.5
	4G-R-1	48.6	9.6
	5G-R-1	33.5	6.2
	10A-1G	16.3	7.6
	10A-3G	17.9	6.5
	5A-4S	0	0
cinnamomi	1D-A	11.9	0
	5N-L	0	0
dreschsleri	G98 S-14	0	0
	G98 S-13	0	0
	G98 S-11A	0	0
	GLN R-14	4.1	0
cryptogea	2G-1R	26.6	16.5

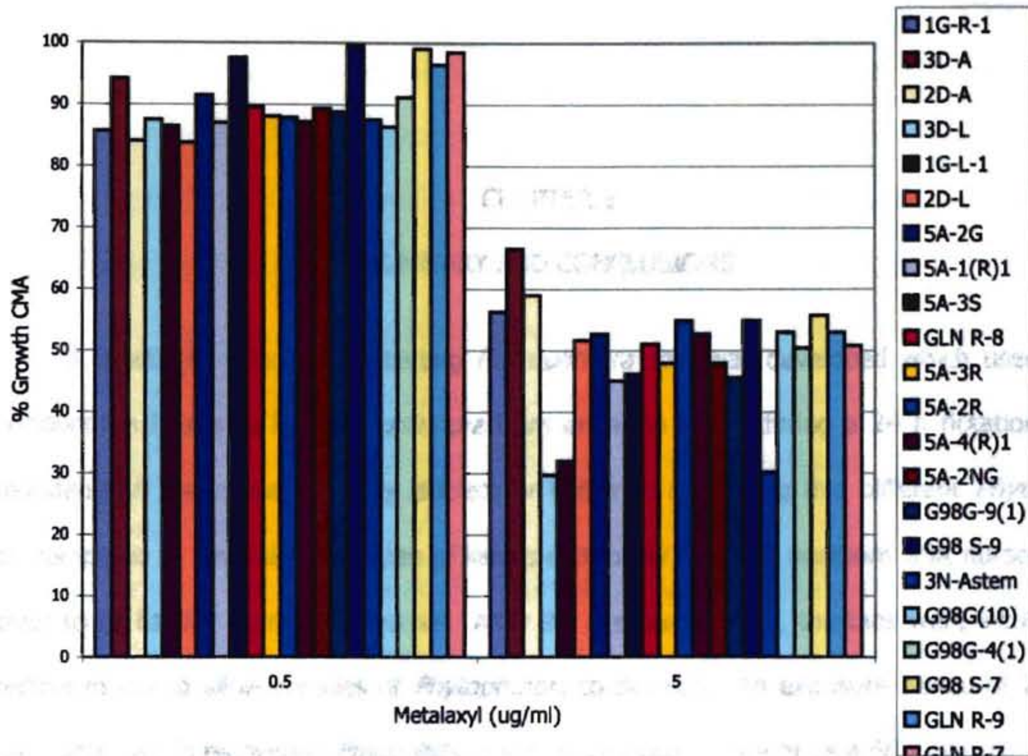

Fig. 4.1 Radial growth of *Phytophthora citrophthora* on 0.5 ug/ml and 5.0 ug/ml active ingredient metalaxyl.

CHAPTER V

SUMMARY AND CONCLUSIONS

A practical method for detecting *Phytophthora* spp. was developed which used tabbed rhododendron leaves to bait *Phytophthora* from irrigation water during a 24 h flotation period. Rhododendron leaves were equally efficient or better at recovering five different *Phytophthora* spp. compared to several other types of leaves commonly found in northern USA nurseries, and proved to be hardier than lemon leaves. After the exposure period, the tabs were incubated on selective media to allow colonies of *Phytophthora* to develop. An exposure period of 24 h was equally effective in recovering *Phytophthora* spp. from irrigation water as a 48 h period.

The efficiency of leaf baiting to recover known concentrations of *Phytophthora* zoospores in water was compared to the recovery efficiency with an established filtration technique. Direct plating of water containing these same concentrations of zoospores served as a control treatment. For the filtration method, inverted filters were also incubated on selective medium for isolation of colonies. Although leaf baiting was not as rigorously quantitative as the filtration method, certain advantages of this method such as readily available baiting material, exposure of leaf baits to larger volumes of water, and ease of either submitting leaves to a diagnostic laboratory or using ELISA kits for on-site detection of *Phytophthora* make it useful for nurseries.

Phytophthora Alert® kits were tested for ability to detect *Phytophthora* in irrigation water and leaf baits. Cross-reactivity with *Pythium* was observed with the *Phytophthora* kits, and these kits would not be useful in differentiating between the genera. The kits could be used by a nursery or greenhouse wanting to detect presence of both Phytophthora and Pythium. Potentially, kits will be developed in the future that allow detection of only *Phytophthora*.

The indicator plant pathogen, *Phytophthora*, was monitored before and after recycling was implemented at Greenleaf Nursery Company. From April to October 1997, twelve samples were collected twice a month from four irrigation runoff sites and two source water sites. From irrigation runoff, high levels of *Phytophthora* averaging 450 colony forming units (cfu)/L were recorded over the season, but the source water samples averaged 5-7 cfu/L. Then in 1998, sampling sites were modified to place more emphasis on stored water. Five samplings from March to October sampled two irrigation runoff sites, one source water site, and three recycled water sites. Colony-forming units/L were 450, 0 and 68 for the irrigation runoff, source water, and recycled sites, respectively.

For both seasons, leaf baits were added to the study to include a user-friendly method of monitoring *Phytophthora* spp. present in the recycling irrigation system. In 1997, rhododendron and lemon leaves were floated in retention basins for 48 h. Towards the end of the summer, the integrity of the lemons leaves deteriorated during the 48 h exposure. Lemon and rhododendron leaves were equally effective in recovering *Phytophthora* spp. Laboratory experiments showed no significant difference between a 24 h and 48 h exposure; therefore, only rhododendron leaves, which were readily available at Greenleaf Nursery, were floated for 24 h in retention basins during 1998. The leaf baits were capable of detecting the presence of the plant disease pathogen even at very low levels of *Phytophthora*.

Isolates from Greenleaf Nursery Company demonstrated moderate levels of insensitivity to metalaxyl; 34% of the isolates grew greater than 50% of growth rates of the non-amended CMA on 5.0 μg/ml active ingredient metalaxyl. *Phytophthora* isolates insensitive to metalaxyl can be recycled in irrigation water and redistributed to other crops. Spread of these insensitive isolates to other parts of the nursery is a concern. To decrease the possibility of further metalaxyl insensitivity in the nursery, alternative fungicides should be considered.

The methods for detection and monitoring of *Phytophthora* spp. have been demonstrated to provide much needed management information for growers and nurserymen. High levels of *Phytophthora* could warrant disinfection or non-use of recycled water from fast turnover

retention basins. For instance, in 1998 recycled water was sampled at irrigation risers delivering water pumped from three retention basins that differed notably in capacity and turnover. Samples of water delivered from the largest basin, a 35,000 million gallon storage basin (D34D), yielded a mean of only 8 colony forming units (cfu)/L. Samples of water delivered from two much smaller retention basins, D5E and D22G, were 60 and 138 cfu/L respectively. D5E water is pumped from the larger of the two basins, which has a slower turnover rate. D22G water is pumped from a shallow basin with a fast turnover rate. Larger retention basins with slower turnover rates should allow for settling, physical and biological degradation, and dilution of *Phytophthora* in recycled irrigation water. The information presented regarding the concentrations of *Phytophthora* spp. in water delivered from retention basins is a significant result. It is a first big step in helping nurseries make informed choices about how captured water can be handled to decrease disease risk.

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