Control of Pythium blight on turfgrass: Isolate characterization and strategies to delay fungicide resistance in Pythium aphanidermatum

Guangbin Peng
Iowa State University

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Control of Pythium blight on turfgrass:
Isolate characterization and strategies to delay fungicide resistance in

*Pythium aphanidermatum*

by

Guangbin Peng

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Major Professors: Mark L. Gleason and Forrest W. Nutter, Jr.

Iowa State University

Ames, Iowa

2000
Graduate College
Iowa State University

This is to certify that the Master’s thesis of
Guangbin Peng

has met the thesis requirements of Iowa State University

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For the Graduate College
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Pythium blight, a serious disease of turfgrass, is caused by *Pythium* spp., including *P. aphanidermatum*, an oomycete fungus. Tank-mixing strategies, which deploy a mixture of two or more fungicides with different modes of action, have been used to suppress problems of fungicide resistance. However, few studies have examined how interactions within *P. aphanidermatum* populations may affect the proliferation of resistance. The isolates present in populations of *P. aphanidermatum* may vary in their fitness characteristics, and those characteristics may play an important role in competition among isolates. Different fungicide treatments impose characteristic selection pressures on *Pythium* populations, which may differentially affect the survival of resistant and sensitive isolates. In order to improve effectiveness of current strategies, the influence of interaction of fitness characteristics and fungicide treatments on proliferation of fungicide-resistant isolates needs to be elucidated.

A collection of 44 field isolates of *P. aphanidermatum* was used to investigate variation in fitness characteristics. Growth rate of mycelia, sporulation capacity, and aggressiveness on perennial ryegrass, along with sensitivity to mefenoxam, were quantified. Sixteen isolates showed complete resistance to mefenoxam. In 28 sensitive isolates, mefenoxam sensitivity was not correlated with the other three fitness characteristics. A wide variation in fitness characteristics was detected among the isolates.

In the second phase of the research, two test populations were constructed by mixing two mefenoxam-sensitive isolates possessing different fitness characteristics with a mefenoxam-resistant isolate in appropriate ratios. Changes in the proportion of resistant isolates within each population under six fungicide treatments were monitored during five cycles of selection on perennial ryegrass under controlled conditions. The rate of proliferation of
mefenoxam-resistant isolates was lower under application of a half-rate fungicide mixture of mefenoxam with propamocarb than under application of mefenoxam fungicide alone. No significant difference in the change of proportions of resistant isolates was detected between a test population containing a relatively fast-growing mefenoxam-sensitive isolate and a test population containing a relatively slow-growing isolate in any fungicide treatment.

The results from this study indicated that half-rate tank mixing could delay the proliferation of mefenoxam-resistant isolates in a *P. aphanideramtum* population. An effect of fitness characteristics on rate of proliferation of mefenoxam resistance was not demonstrated.
INTRODUCTION

**Fungicide resistance.** Fungicide application is a powerful tool for disease control in agroecosystems. In recent decades, under pressure from environmental concerns, pesticide manufactures have developed fungicides with increasingly specific modes of action. However, resistance to these fungicides by target fungi has appeared. The first few cases of fungicide resistance were reported in the late 1960s, such as resistance of *Venturia inaequalis*, the apple scab pathogen, to dodine, and resistance of *Erysiphe cichoracearum*, a powdery mildew pathogen, to dimethirimol (4,42). Since then, resistance problems have developed in four major fungicide classes: benzimidazoles, dicarboximides, phenylamides and demethylation inhibitors (32).

Although the biological mechanisms of resistance vary from case to case (15), one of the most important mechanisms is to modify the target sites (usually biosynthesis or metabolic pathways) attacked by fungicides in the pathogen populations. In many cases, the target site is a step in a biochemical pathway, often controlled by several genes or even a single gene. For example, the benzimidazole fungicides act by inhibiting tubulin biosynthesis, a process under single-gene control that is prone to frequent mutation leading to resistance (13,44). Dicarboximide fungicides act by affecting one enzyme of DNA synthesis in fungi (29). Metalaxyl, the first phenylamide fungicide, targets ribosomal RNA synthesis in fungi in the order Peronosporales by inhibiting the activity of endogenous RNA polymerase, which is controlled by a single gene (14). Demethylation inhibitor (DMI) fungicides were thought to be at low risk for resistance development, but evidence showed that DMI resistance is inherited as a single gene (6). The single-site fungicides are highly effective at low rates but also possess a high risk of resistance development. In contrast, fungicides with
multi-site activity have relatively low risk of inducing resistance, but often require much
higher use rates and may have greater toxicity to non-target organisms than single-site
fungicides.

Generally, proliferation of fungicide resistance at a given location is a consequence of
evolutionary change in a pathogen population under the selection pressure of fungicides. In
the absence of fungicides, a pathogen population may experience selection for fitness traits
such as rapid mycelial growth, high sporulation capacity, and long-term viability of survival
structures. However, if the population is genetically heterogeneous, isolates with a wide
range of different fitness characteristics may coexist. Some isolates in the population may
resist a particular fungicide by modifying the target site of the fungicide. The proportion of
resistant isolates will increase in the population under continuous selection pressure imposed
by presence of this fungicide. Control failure becomes evident when the resistant isolates
dominate the population (10).

Management strategies developed to deal with fungicide resistance problems include:
risk assessment during development of fungicides; using an integrated disease management
program to reduce disease pressure, and thereby the need for fungicide applications; using at-
risk, single-site fungicides only when absolutely necessary, preferably early in an epidemic,
thereby minimizing pathogen exposure; alternating or tank-mixing at-risk fungicides with
fungicides having different modes of action; and avoiding repeated or curative use of at-risk
fungicides (23). Although these precautions have been partially successful, they have many
limitations. The risk of resistance development is difficult to predict due to complex
interactions among microorganisms and fungicides. Furthermore, managing resistance with
full-rate mixtures is at odds with the public desire to reduce pesticide use. The value of some
strategies to delay resistance, such as extending the interval between sprays and alternation vs. mixing of fungicides with different modes of action, is still debated among plant pathologists (23). More research is needed to help solve resistance development problems. In a review article in 1995, Russell pointed out that the dynamics of resistance development are still poorly understood and that a multitude of interacting factors could influence the development and the survival of resistant isolates (32). Only by obtaining detailed information about each influencing factor will it be possible to produce more rational, integrated disease control strategies while minimizing the threat of fungicide resistance.

**Pythium blight and *Pythium aphanidermatum***. Pythium blight is one of the most devastating diseases on golf courses in the United States. It can kill susceptible turfgrass under conditions of high relative humidity (>90%) and day-time temperatures ≥ 30°C followed by night temperatures > 20°C (24). It can strike turfgrass in the southern U.S. year-round, but causes serious problems in the northern states only during midsummer. The most common warm-weather Pythium blight pathogen is *Pythium aphanidermatum* (10). Other pathogenic species include *P. ultimum*, *P. graminicola*, and *P. arrhenomanes*. Although some warm-season grasses such as bermudagrass (*Cynodon* spp.) and bentgrass (*Agrostis* spp.) are susceptible to these pathogens, the major hosts are the cool-season grasses, such as perennial ryegrass (*Lolium perenne*), tall fescue (*Festuca arundinacea*), Kentucky bluegrass (*Poa pratensis*), and annual bluegrass (*Poa annua*).

Pythium blight typically appears as white, fuzzy mycelium growing on the blades of infected turfgrass in the early morning. When the spots dry, the grass blades collapse and turn a light brown or reddish brown. Under disease-favorable weather, the initial spots can grow rapidly and devastate large areas of susceptible greens or fairways within 24 hours (10).
Mowing practices and irrigation can also influence the pattern of disease on golf courses. Infected plant tissue, zoospores and other propagules can be spread on mowers and in irrigation water, resulting in characteristic spatial patterns of symptom development. Heavy nitrogen fertilizer application and low mowing height before or during disease-favorable weather also increase host susceptibility (10).

Although little is known about the genetics of *Pythium* spp., they are considered homothallic species (43). Morphological characteristics of this species include inflated filamentous sporangia, intercalary antheridia, and aplerotic oospores (43). Since the 1960s, most ecological research on *P. aphanidermatum* has focused on survival of oospores in soil, germination of oospores, chemotaxis of zoospores, and methods to quantify propagule density in soil (2, 33, 34, 39, 40, 41, 46). These studies provided basic tools for further investigations at the population level.

The disease cycle of Pythium blight is complex. The mycelium gives rise to sporangia, which are asexual reproduction structures that either germinate directly by producing one to several germ tubes or produce zoospore-containing vesicles. The zoospores, when released from vesicles, swarm for several minutes and then form cysts, which germinate by producing germ tubes. The germ tubes can penetrate host tissues and start new infections. Under stressful environmental conditions, the mycelium can give rise to spherical oogonia and club-shaped antheridia. After fertilization, oogonia produce thick-walled oospores, which are resistant to adverse temperature and moisture conditions and serve as the survival and resting stage of the pathogen (18). Oospores germinate in a manner similar to that of sporangia (3).

**Management practices.** Currently, control of Pythium blight is based on timing of fungicide application to precede disease-favorable weather. A disease forecasting system for
Pythium blight developed in Pennsylvania indicated that the two most important weather variables were 1) a maximum daily temperature higher than 30°C and 2) at least 14 hours of RH>90% with a minimum temperature higher than 20°C (28). However, fungicides are sometimes applied as curative treatments after an epidemic has begun. Other cultural management practices include increasing the mowing height, increasing air movement by removal of surrounding vegetation and the use of electric fans, and reducing nitrogen fertilization rates. Using disease-resistant grass species may be another way to control the disease. However, grass species selection depends on regional adaptability of grass, its suitability for specific uses and its availability.

Metalaxyl is among the most effective fungicides against Pythium blight. Metalaxyl displays both curative and protectant activity against several fungi in the Peronosporales. Metalaxyl interferes with functioning of the polymerase-template complex by inhibiting endogenous nuclear RNA polymerase activity (14). This molecular-level disruption leads to inhibition of mycelial growth and suppression of sporangial and oospore germination. It has been found that rRNA synthesis remains unaffected in metalaxyl-resistant isolates, and the inability of metalaxyl to inhibit endogenous RNA polymerase activity of nuclei isolated from these isolates suggests that a target site change is responsible for resistance (12).

Appearance of metalaxyl resistance has typically followed several years of intensive metalaxyl application. The first case of resistance under field conditions was reported in Israel with Pseudoperonospora cubensis on cucumber in 1980, two years after metalaxyl entered the market. Several years later, other cases of resistance occurred in vegetable pathosystems, such as Phytophthora infestans on potatoes. Common features of these
outbreaks were that the product was used by itself, disease pressure was high, and the product was used curatively (25).

The intensive use of metalaxyl on golf courses has also led to resistance problems. The first case of metalaxyl resistance of _Pythium_ spp. in the United States was reported on a golf course in Pennsylvania in 1983 (34). In 1986 and 1987, several cases of failure of metalaxyl in _Pythium_ blight control were documented in perennial ryegrass on golf course fairways (25). However, occurrence of metalaxyl resistance in _Pythium_ blight have been relatively rare on golf courses. It has been documented on fewer than 50 golf courses despite widespread use of metalaxyl (M.Agnew, Novartis Crop Protection, personal communication). Other cases of oomycete resistance in field crops have been reported in several states (25).

Cross-resistance occurs among all metalaxyl-related products on the market now, including mefenoxam (Subdue Maxx®), a stereoisomer of metalaxyl which has the same mode of action as metalaxyl. Currently, mefenoxam is registered for use on turfgrass in the U.S., whereas the metalaxyl registration has been discontinued.

**Strategies to delay resistance to metalaxyl.** Reduction of selection pressure on pathogen populations is an important principle in combating fungicide resistance. This principle is applied to delay resistance of systemic fungicides, including metalaxyl. One way to reduce selection pressure is to decrease fungicide applications, for example by increasing the interval between spray applications. However, it can be difficult to prevent or delay the development of resistance while achieving effective disease control.

Using additional effective fungicides with different modes of action can relieve the selective pressure of metalaxyl and related compounds on pathogen populations. Alternation
and tank-mixing are two strategies based on this idea. Few studies have indicated, however, that alternation of metalaxyl with other fungicides could be an effective strategy for suppressing resistance (35). The possibility of survival of resistant isolates after application of an unrelated fungicide under field conditions cannot be overlooked. Thick-walled oospores have shown excellent survival ability. As a result, the survival of metalaxyl-resistant isolates after application of unrelated fungicides could eventually lead to a high level of resistance when metalaxyl use is resumed.

Multiple-site fungicides have very low risk of resistance (32). Tank mixtures of narrow-mode-of-action fungicides having different modes of action may behave like multiple-site fungicides. Field and greenhouse experiments by Sanders and coworkers suggested that half-rate mixtures of metalaxyl with propamocarb, mancozeb, or fosetyl Al provided excellent control of Pythium blight on perennial ryegrass and delayed the increase of resistance in *Pythium aphanidermtum* populations (36). However, no further evaluations of this strategy have been published.

In order to improve the effectiveness of strategies to delay the development of fungicide resistance, interaction of fungicides in mixtures and the population dynamics of pathogens need to be investigated further. First, the interaction of fungicides in mixtures can be complex. Both synergistic and antagonistic interactions of fungicides against Pythium blight have been found (11). Therefore, the control effect of a mixture of two fungicides may not depend solely on properties of each fungicide, but also on their interactions. Even if two fungicides in the mixture are selected carefully to obtain excellent disease control, the effectiveness of the mixture in delaying resistance is still in question.
Second, fungal population dynamics are influenced by many factors. The composition of the target population may affect the proliferation of resistance. Within a population, isolates may differ in fitness characteristics (e.g., growth rate of mycelia, sporulation capability) and in responses to fungicides. Under natural or fungicide-driven selection pressure, changes in composition of the population may be influenced not only by resistance, but also by other fitness traits. It would be valuable to know the diversity of fitness traits in target populations of *P. aphanidermatum* in order to reduce the risk of resistance to certain fungicides.

The purposes of the present study were to 1) characterize the radial growth, sporulation, aggressiveness on host and mefenoxam sensitivity of a range of *P. aphanidermatum* isolates and 2) test the hypotheses that the rate of proliferation of mefenoxam resistance is affected by a) the fitness characteristics of the isolates present and b) the pattern of fungicide use.

**Organization of thesis.** Two parts are included in the main body of this thesis. Each part has its own Introduction, Materials and Methods, Results and Discussion. An abstract and a general introduction precede the main body, and a general conclusion and a reference list follow the main body.
PART I: CHARACTERIZATION OF *Pythium aphanidermatum* ISOLATES SENSITIVE OR RESISTANT TO MEFENOXAM
INTRODUCTION

*Pythium aphanidermatum* is one of the major pathogens causing Pythium blight on turfgrass. Control of Pythium blight has been achieved by application of fungicides along with appropriate cultural practices (3, 10,18, 26,38). Metalaxyl is among the most widely used fungicides against Pythium blight. However, metalaxyl resistance has appeared in *P. aphanidermatum* populations on many golf courses since the first control failure was reported in Pennsylvania in 1983 (34). A closely related fungicide, mefenoxam, recently replaced metalaxyl, but metalaxyl-insensitive isolates of *P. aphanideramtm* are equally insensitive to mefenoxam (30).

Management strategies deployed to deal with fungicide resistance are based on the idea that a balance between resistant and sensitive isolates in the pathogen population can be maintained under certain fungicide application regimes (23, 32, 36). If such a balance exists, resistant isolates will not become dominant and effective disease control can still be achieved by applying fungicides. The success of these strategies depends on the outcome of competition among resistant and sensitive isolates when the fungicide in question is present or absent. The outcome of the competition is determined by the relative fitness of individual isolates in the population. Consequently, quantifying and comparing fitness characteristics of *Pythium aphanidermatum* is an important first step toward understanding the proliferation and persistence of mefenoxam resistance and improving the effectiveness and durability of Pythium blight management strategies (19,20).

Fitness characteristics can include any factor that contributes to survival and reproduction. The choice of fitness characteristics, however, is based on available knowledge about the organism under study. Fitness characteristics of many pathogen populations have
been detailed in the past 10 years. For example, studies of *Phytophthora infestans* on
different hosts (20, 21, 22) found different sporulation capacities and levels of aggressiveness
among metalaxyl-sensitive and metalaxyl-resistant isolates. Few such studies have been done
on *Pythium* spp., partly because the complex biology of this pathogen group makes them
relatively difficult to study (43). Although some progress has been made in characterizing
diversity within *Pythium* populations, most studies have focused on variability in
pathogenicity and fungicide sensitivity (1,5,9,45). No thorough profile of isolate fitness
characteristics has been made for any species in this genus. In a population of *Pythium*
aphanidermatum, the growth rate of mycelia, sporulation capacity of colonies,
aggressiveness of isolates on host plants, and their sensitivity to mefenoxam may influence
the population dynamics under the selective pressure of fungicides. Quantification of these
factors is a first step toward development of more effective methods to monitor and predict
the development of resistance. This report quantifies these four characteristics for a
collection of 44 *Pythium aphanidermatum* isolates, analyzes the relationships among these
factors, and discusses implications of the results for disease management.
MATERIALS AND METHODS

Isolates of Pythium aphanidermatum. Of a total of 44 field isolates (Table 1), 14 were isolated from turfgrass samples sent to our laboratory by cooperators during the past 3 years. *P. aphanidermatum* was isolated from symptomatic grass blades by surface sterilizing with 0.5% NaOCl for 30 seconds followed by a sterile distilled water rinse, then plating tissue segments on a selective medium (7). The plates were incubated under 14 hr light at 30° C. After incubation for 2 days, colonies formed on the media, and hyphal tips of the colonies were transferred to 2% water agar. Preliminary species identification was accomplished by observing the sexual structures under light microscopy at 400X magnification (43). Thirty isolates were provided by colleagues at Novartis Crop Protection, Zeneca Crop Protection and various universities. All isolates were confirmed as *P. aphanidermatum* by consultation with Dr. H.D. Shew, Department of Plant Pathology, North Carolina State University. Perennial ryegrass, tall fescue, and bentgrass on golf courses and lawns in 14 states were the main sources of isolates. Several additional isolates came from sugar beets. Pure cultures were maintained on corn meal agar (CMA) for up to 3 months at room temperature. To avoid contamination, all isolates on CMA were re-transferred onto fresh media every three months.

Growth rate of mycelia. A 5-mm-diameter agar disk from the edge of a 3-day-old culture was placed in the center of a 15-cm-diameter CMA plate and incubated at 22° C under 14 hr light and 10 hr darkness per day. At 12, 18, 24, 36, and 48 hr, colony radii were measured from the center of the disk to the margin of the colony in four different directions each 90° apart. Colony radius was represented as the mean of the four measurements. Growth rates were calculated by regression analysis of radius vs. time. Three replicate plates were measured for each isolate, and the experiment was repeated twice.
**TABLE 1. Characteristics of isolates of *Pythium aphanidermatum***

<table>
<thead>
<tr>
<th>No.</th>
<th>Origin of isolates</th>
<th>Original hosts*</th>
<th>Growth rate (cm/day)</th>
<th>Sporulation (oospores/mm²)</th>
<th>Disease severity (%)</th>
<th>Sensitivity to mefenoxam (EC₅₀ mg/L)</th>
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<td>49 b-i</td>
<td>47 c-g</td>
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</tr>
<tr>
<td>24</td>
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<td>50 c-e</td>
<td>0.41 bc</td>
</tr>
<tr>
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<td>60 bc</td>
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<tr>
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</tr>
<tr>
<td>28</td>
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<td>1.89 qr</td>
<td>57 bc</td>
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</tr>
<tr>
<td>29</td>
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<td>2.25 f-l</td>
<td>40 c-j</td>
<td>45 c-g</td>
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<tr>
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<td>NA</td>
</tr>
<tr>
<td>40</td>
<td>CA RG</td>
<td></td>
<td>2.25 f-l</td>
<td>46 b-j</td>
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<td></td>
<td>1.91 q-r</td>
<td>29 g-j</td>
<td>30 e-j</td>
<td>1.13 a-c</td>
</tr>
<tr>
<td>42</td>
<td>CA PR</td>
<td></td>
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<td>42 d-h</td>
<td>0.78 a-c</td>
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</tr>
<tr>
<td>44</td>
<td>IN BG</td>
<td></td>
<td>2.33 b-j</td>
<td>55 b-e</td>
<td>57 b-d</td>
<td>NA</td>
</tr>
</tbody>
</table>

Means with the same letters are not significantly different (Waller-Duncan K ratio test K=100)

N=9 for growth rate, N=4 for sporulation, N=6 for disease severity, N=12 for EC₅₀

* BG-Kentucky bluegrass, COB-colonial bentgrass, CRB-creeping bentgrass, PG-Perennial ryegrass, RG-ryegrass, SB-sugar beet, TF-tall fescue, TG-turfgrass (species unknown)

** NA- No EC₅₀ value, since those isolates are completely resistant to mefenoxam
**Sporulation capacity.** Reproductive ability among isolates was estimated as the number of oospores per unit area. Double-layer agar plates were made to quantify this characteristic. First, 1-ml of molten water agar (WA) was pipetted into a 3-cm-diameter plate to form a thin, uniform layer on the bottom of the plate. One ml of molten CMA was then pipetted onto the surface of the cooled WA layer and three sterilized, 2-mm-diameter wooden dowels were placed 120° apart in the cooling CMA equidistant (1 cm) from the center of the plate. After the CMA solidified, the dowels were withdrawn, creating three wells in the top layer of the agar. Four days after a 2-mm-diameter agar disk containing an isolate of *P. aphanidermatum* was placed in the center of the plate and incubated at 22° C in darkness, oogonia formed in the water agar layer. Usually, an oogonium produced an oospore after being fertilized by an antheridium. Therefore, the number of oospores per unit area was estimated by counting the number of oogonia in each well on the agar plate at 100X magnification after 7 days of incubation. Two replicated plates were counted per isolate. The experiment was conducted twice.

**Disease severity (aggressiveness) on perennial ryegrass.** Perennial ryegrass (cv. SR4100) was grown from seed in 6-inch plastic pots (0.5 g seeds/pot). The grass was clipped and fertilized by a nitrogen fertilizer (6-0-0) weekly. After 28 days, a 1-cm-diameter CMA agar disk from the edge of a 3-day-old culture of *P. aphanidermatum* was transferred to the base of the ryegrass at the center of each of three pots per isolate. Each pot was then sealed in a transparent plastic bag and transferred to a growth chamber at 70 to 90% relative humidity with 14 hr of light at 32° C and 10 hr of darkness at 22° C. Experimental design was a randomized complete block design with three replications. After a 4-day incubation period,
the magnitude of the blighted area was estimated visually as a percentage of the seeded area of each pot. The experiment was repeated three times.

**Sensitivity to mefenoxam in vitro.** Approximately 20 ml CMA amended with 0, 0.005, 0.05, 0.5, or 50 mg a.i. mefenoxam /L at 40\(^\circ\)C was dispensed into 10-cm-diameter plates. A 5-mm-diameter CMA agar disk was transferred from the edge of a 3-day-old culture of an isolate onto the center of replicated agar plates at each mefenoxam concentration. The plates were then incubated in a growth chamber at 22\(^\circ\)C in darkness. After 24 hr, colony diameter (cm) was measured as described previously. \(EC_{50}\), the concentration of mefenoxam inhibiting mycelial growth by 50% compared to growth on unamended CMA, was estimated by fitting a regression line of percent inhibition plotted against the log-transformed fungicide concentration. The experiment was repeated five times.

**Single-spore cultures and tests.** Genetic diversity within populations of *Pythium* spp. has been documented previously (16). In the present study, possible phenotypic variation within a field isolate was considered. In order to detect variation, two sets of single-spore cultures were made for isolate #18 (mefenoxam-sensitive) and isolate #11 (mefenoxam-resistant). First, a 7-day-old culture of each isolate on WA was liquefied by blending with sterilized distilled water in a Waring blender. A ten-fold serial dilution was then made from the liquid, and 1 ml at 1:1000 dilution was spread on selective agar (7). Individual oospores could be observed under a compound microscope at 100X magnification. Colonies formed from oospores were then transferred to CMA for characterization. Six single-spore cultures were made for isolate #18 and seven cultures for isolate #11. Characterization was done for all 13 single-spore cultures by using the same methods as described for field isolate characterization.
RESULTS

Fitness characteristics. Growth rate among isolates ranged from 1.71 to 2.50 cm/day (Table 1). Most isolates (36 of 44) had growth rates $\geq 2.02$ cm/day (Figure 1A). The number of oospores/mm$^2$ for all isolates ranged from 22 to 87 (Table 1). Most isolates (42 of 44) had fewer than 60 oospores /mm$^2$ (Figure 1B). Disease severity on perennial ryegrass ranged among isolates from 5% to 80% (Table 1). Most isolates had disease severity between 33% and 63%, whereas four isolates had severity $>63\%$ and four had severity $<33\%$ (Figure 1C).

Sensitivity to mefenoxam. Two categories of growth rate response to mefenoxam concentration were found. Fifteen isolates showed response curves similar to that of isolate #11 (Figure 2), indicating that growth rates of these 16 isolates were not reduced under any mefenoxam concentration. They were considered to be mefenoxam-resistant isolates. Twenty-seven isolates showed dose-response curves similar to that of isolate #18 (Figure 2), and were considered to be mefenoxam-sensitive isolates. $EC_{50}$ values of those sensitive isolates ranged from 0.18 mg/L to 1.64 mg/L (Table 1), and 23 of 28 had $EC_{50} < 1.06$ mg/L (Figure 1D).

Characterization of single-spore culture. Fitness characteristics were quantified for single-spore cultures of two isolates. Statistical tests showed at least one difference for each characteristic among seven single-spore cultures of isolate #11 (Table 2), but all were uniformly resistant to mefenoxam. The growth rates of six single-spore cultures of isolate #18 were not significantly different, but values of sporulation capacity, aggressiveness on perennial ryegrass, and $EC_{50}$ were significantly different (Table 3).
Figure 1. Variation of fitness characteristics among 44 isolates of *Pythium aphanidermatum*. A. growth rate, B. sporulation capacity, C. disease severity, and D. EC$_{50}$ values for sensitive isolates.
Figure 2. The response curves of isolates #11 (a mefenoxam-resistant isolate) and isolate #18 (a mefenoxam-sensitive isolate) after log-transformation. Each data point was the mean of six replications.
TABLE 2. Fitness characteristics of single-spore cultures of isolate #11.

<table>
<thead>
<tr>
<th>No. of Culture</th>
<th>Growth rate (cm/hr.)</th>
<th>Sporulation capacity (number of oospores/mm²)</th>
<th>Severity (%)</th>
<th>Sensitivity to mefenoxam</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.10</td>
<td>14</td>
<td>55</td>
<td>R</td>
</tr>
<tr>
<td>2</td>
<td>0.10</td>
<td>15</td>
<td>50</td>
<td>R</td>
</tr>
<tr>
<td>3</td>
<td>0.085</td>
<td>11</td>
<td>45</td>
<td>R</td>
</tr>
<tr>
<td>4</td>
<td>0.084</td>
<td>8</td>
<td>70</td>
<td>R</td>
</tr>
<tr>
<td>5</td>
<td>0.09</td>
<td>12</td>
<td>25</td>
<td>R</td>
</tr>
<tr>
<td>6</td>
<td>0.10</td>
<td>21</td>
<td>50</td>
<td>R</td>
</tr>
<tr>
<td>7</td>
<td>0.09</td>
<td>10</td>
<td>75</td>
<td>R</td>
</tr>
</tbody>
</table>

\[ F = 4.41 \ (P = 0.005) \quad F = 1.83 \ (P = 0.13) \quad F = 8.65 \ (P < 0.001) \]

TABLE 3. Fitness characteristics of single-spore cultures of isolate #18.

<table>
<thead>
<tr>
<th>No. of Culture</th>
<th>Growth rate (cm/hr.)</th>
<th>Sporulation capacity (number of oospores/mm²)</th>
<th>Severity (%)</th>
<th>EC₅₀ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.08</td>
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<td>0.46</td>
</tr>
<tr>
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<td>0.32</td>
</tr>
<tr>
<td>3</td>
<td>0.08</td>
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<td>25</td>
<td>0.44</td>
</tr>
<tr>
<td>4</td>
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<td>4</td>
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</tr>
<tr>
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<td>0.09</td>
<td>9</td>
<td>12</td>
<td>0.38</td>
</tr>
<tr>
<td>6</td>
<td>0.09</td>
<td>18</td>
<td>33</td>
<td>0.45</td>
</tr>
</tbody>
</table>

\[ F = 1.25 \ (P = 0.35) \quad F = 4.04 \ (P = 0.02) \quad F = 19.21 \ (P < 0.001) \quad F = 1.82 \ (P = 0.18) \]
DISCUSSION

Correlation coefficients of EC$_{50}$ of sensitive isolates with the other three fitness characteristics (growth rate, sporulation capacity, and aggressiveness) were not significant (P=0.05) among the isolates (data not shown). The results agreed with those of Brantner and Windels on variability in sensitivity of $P$. aphanidermatum to metalaxyl (5). However, a study by Kadish et al showed superior fitness characteristics of metalaxyl-resistant isolates of $Phytophthora$ infestans (19). Thus, sensitivity to phenylamide fungicides may not necessarily be related to other fitness characteristics, and this kind of relationship may vary among fungal species. Absence of a significant correlation between growth rate and sporulation capacity indicated that faster-growing isolates may not produce more spores than slower-growing isolates. A significant (P=0.01) correlation between growth rate in vitro and aggressiveness on perennial ryegrass was detected (r=0.62), but the correlation between sporulation capacity and aggressiveness was not significant (P=0.05). Since the aggressiveness test was performed by inoculation with an agar plug, the size of diseased area in the pots was determined mainly by the growth rate of mycelia from the agar plug rather than sporulation capacity. Furthermore, production of oospores is a response to unfavorable conditions, whereas conditions in the growth chamber were optimal for mycelial growth. Therefore, isolate fitness was tested under only one scenario for each characteristic, whereas the survival and proliferation of isolates in a natural population, under varying environmental conditions, are probably influenced by many fitness characteristics.

Characterization of single-spore cultures showed that considerable variability exists within field isolates. Similar heterogeneity has been reported in other $Pythium$ spp. (16). However, sensitivity to mefenoxam among single-spore cultures of the resistant isolate did
not vary. On the other hand, the variability of EC$_{50}$ values of single-spore cultures of sensitive isolates showed a much smaller range of sensitivity than the range among field isolates. This could be explained by the mechanism of resistance (14,32). Isolates of *Pythium aphanidermatum* achieve complete resistance to mefenoxam by possessing a single resistance gene. The resistance gene may be present in all oospores produced by the mycelia of resistant isolates. In sensitive isolates, where the resistance gene is absent, sensitivity to mefenoxam displayed a range of variation similar to those for other fitness characteristics. At the population level, resistance to mefenoxam varies due to variation in the proportions of resistant isolates in the population. Although field isolates were used for characterization of fungal population, a field isolate can be considered to be a population of single-spore cultures. The field isolates showed particular fitness characteristics that differed from its component single-spore genotypes. However, characteristics of the field isolates were consistent from replication to replication, indicating that field isolates behaved in a replicable manner despite their heterogeneity. Instead of single-spore cultures, field isolates were used to in our trials in order to represent population-level behavior of the pathogen, but results from the single-spore assessments made it clear that testing field isolates alone does not comprehend the full range of genetic variability in a population.
PART II: INFLUENCE OF FUNGICIDE DEPLOYMENT STRATEGIES ON
PROLIFERATION OF RESISTANCE OF *Pythium aphanidermatum*
TO MEFENOXAM
INTRODUCTION

Research on microbial competition is sparse, because population dynamics of microorganisms are not easily quantified (17,27). However, *Pythium* spp. have suitable characteristics for studying effects of fitness on competition since they have quantifiable components of fitness, such as mycelial growth and rate of development of reproductive structures (31).

Studies of the development of resistance in *Pythium aphanidermatum* populations by Sanders et al (35,36) did not consider the possible influence of differential isolate fitness on competition among isolates. Proliferation of resistance is a possible outcome of competition among resistant and sensitive isolates in a population. The fitness characteristics of isolates could influence the outcome of competition. For example, it is possible that differences in mycelial growth rate among fungicide-sensitive isolates could affect the rate at which resistant isolates become dominant, or could even prevent such dominance. Alternatively, greater spore production by resistant than sensitive isolates could accelerate proliferation of resistance. Cohen and Caffey (8) and Kadish and Cohen (20) quantified fitness components of metalaxyl-sensitive and metalaxyl-resistant populations of *Phytophthora infestans* such as infection frequency, size of lesion areas, and sporulation capacity. They found that metalaxyl-resistant isolates were more competitive than metalaxyl-sensitive isolates. Their results indicated that resistance developed rapidly after intensive application of metalaxyl fungicides on pathogen populations containing resistant isolates of superior fitness. No evidence has been presented indicating whether mefenoxam-resistant isolates are more or less competitive than mefenoxam-sensitive isolates of *P. aphanidermatum*, but evaluation of
competitive fitness may yield insights that improve the effectiveness of resistance management.

Fungicide deployment strategy is another factor influencing competition in fungal populations. It has been suggested that alternating or mixing fungicides with different modes of action may delay the development of resistance (32,35). Sanders et al. (1985) showed that reduced-rate fungicide mixtures could delay resistance to metalaxyl in populations of *P. aphanidermatum* on turfgrass (36). No additional studies of these fungicide strategies have been published, but a better understanding of interactions between fungal isolates and fungicides is a key to developing more effective strategies to delay the proliferation of resistance.

We constructed two populations of *P. aphanidermatum* having predetermined proportions of isolates sensitive and resistant to mefenoxam. Changes in the proportion of resistant isolates were quantified to assess the rate of proliferation of resistance under various fungicide deployment strategies. The results from the two mixed (resistant and sensitive) populations were analyzed to compare the effectiveness of the fungicide strategies and influence of isolate fitness on delaying the proliferation of resistance to mefenoxam.
MATERIALS AND METHODS

Host plants. Perennial ryegrass seeds (cv. SR4100, Seed Research of Oregon, Corvallis, Oregon) were planted in 6-inch pots at a rate of 0.6 gram/pot. The seeds were distributed evenly on the surface of pasteurized media (1:2:1 peat: perlite: soil), and a thin layer of the media was added to cover the seeds. The grass was grown in a greenhouse under 14 hr light and 10 hr darkness per day for 4 wk and was clipped and fertilized once a week with a nitrogen (6-0-0) fertilizer. The grass was clipped by hand at a height of 3 cm. The height of grass when used for experiments was 4 to 5 cm.

Constructing populations. One mefenoxam-resistant isolate (R) and two mefenoxam-sensitive isolates (S1, S2) of *P. aphanidermatum* were chosen for the test populations (Table 4). Two sensitive isolates were selected because their mycelial growth rates were significantly different (P=0.05). The value of growth rate of the resistant isolate was intermediate between the two sensitive isolates. Mefenoxam sensitivity was not significantly different between S1 and S2. In addition to three single-isolate populations of

<table>
<thead>
<tr>
<th>ID.</th>
<th>Isolate no.</th>
<th>Growth rate (cm/24hrs)</th>
<th>Sporulation capacity (oospores/mm²)</th>
<th>Disease Severity (%)</th>
<th>EC₅₀ mefenoxam (mg/L)</th>
<th>EC₅₀ propamocarb (mg/L)</th>
</tr>
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<tr>
<td>S1</td>
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<td>63</td>
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<tr>
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<td>#18</td>
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<td>24</td>
<td>33</td>
<td>0.44</td>
<td>26.5</td>
</tr>
<tr>
<td>R</td>
<td>#11</td>
<td>2.30</td>
<td>42</td>
<td>45</td>
<td>-</td>
<td>25.2</td>
</tr>
<tr>
<td>LSD (P=0.05)</td>
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<td>16</td>
<td>11</td>
<td>0.66</td>
<td>7.35</td>
<td></td>
</tr>
</tbody>
</table>

Note: EC₅₀ to propamocarb were quantified by using the same method as EC₅₀ to mefenoxam.
S1, S2 and R, two mixed populations, S1R and S2R, were constructed by mixing each with isolate R in specified proportions.

Obtaining Inocula. Each of the three isolates was used to inoculate twenty 6-inch pots of 4-wk-old perennial ryegrass by transferring a 1-cm-diameter CMA agar disk from the edge of a 3-day-old culture to the soil surface at the center of a pot. The pots were then covered with transparent plastic bags and incubated in a growth chamber under 14 hr light at 32°C and 10 hr darkness at 24°C. After 7 days, the bags were removed and the blighted grass was allowed to air dry. Dried foliage from 20 pots of each isolate was harvested separately and ground for 3 min in a Waring blender.

Bioassay of populations. Infested foliage was homogenized in a Waring blender for 3 min with sterile distilled water (0.5 g dry wt / 100 ml), and six 1-ml aliquots of each homogenate were spread onto two types of selective agars. An additional 1-ml aliquot was used to make a 1:10 dilution, and six 1-ml of aliquots were plated on these selective agars. One selective agar was that of Burr and Stanghellini (7) and the other was made by amending this agar with 50 mg/L mefenoxam. Resistant and sensitive isolates were distinguished by counting the number of colonies on the two types of selective agar after 48 hr incubation at 38°C in darkness.

After the number of propagule per gram dry weight of grass blades was determined by assaying populations of the three separate isolates, test populations with 0.1% incidence of the resistant isolate (1R:999S1 or 1R:999S2) were obtained by mixing appropriate weights of dried grass containing R with that containing S1 or S2, respectively. The proportion of the resistant isolate in populations (PR) was verified by assaying the mixed populations before they were applied to inoculate pots of grass at the beginning of the cycling experiments. Two
mixed populations (S1R and S2R) and three single-isolate populations (S1, S2 and R) were used as inoculum sources in the following experiments.

**Population cycling experiments.** The five test populations constructed above were used to inoculate perennial ryegrass in pots. Each population was treated with five different fungicide applications, including full rate mefenoxam, half rate mefenoxam, full rate propamocarb, half rate propamocarb, a half-rate mixture of both fungicides, and tap water. The application rates were calculated based on the label rate of Sudue Maxx ® (1.0 fl oz /1000ft²) and Banol 6E ® (2.0 fl oz/1000ft²), and expressed as the amount of fungicides sprayed on 15 pots that were placed into a 3-ft² rectangle. The full rates used were 0.06 ml / 3 ft² for mefenoxam and 0.12 ml / 3 ft² for propamocarb. In each cycle, there were a total of 30 treatment combinations and 3 replications. Ninety pots were arranged in a growth chamber at 70 to 90% relatively humidity with 14 hr light at 32° C and 10 hr darkness at 22° C daily in a randomized complete block design. The populations were cycled through inoculation, fungicide regimes, incubation, and harvest/assay for five sequential 15-day cycles in the growth chamber. The cycling experiment was repeated once.

**Procedure of cycling experiment.** Procedures for this experiment was similar to those of Sanders et al. (1985). Fifty ml of homogenate of one test population containing oospores, zoospores, segment of mycelia and grass debris was poured onto a 2.5-cm-diameter area in the center of a 15-cm pot of perennial ryegrass. Eighteen pots were used for each population. After inoculation, all pots were covered with transparent plastic bags and transferred to the growth chamber. After 4 days, the plastic covers were removed and fungicide treatments were applied. Seven days after fungicide applications, the pots were re-covered with plastic bags and incubated for 4 days in the same chamber. At the end of incubation, the plastic
covers were removed and grass blades were harvested at the base and homogenized in 100 ml sterile distilled water per 0.5 g fresh wt of grass. A portion of the homogenate was assayed as described above, and another portion of the homogenate was used to inoculate grass in a new set of pots for the next cycle. The five populations were cycled under six fungicide regimes through five sequential 15-day cycles in the growth chamber (Figure 3).

**Figure 3.** Procedure for cycling experiment and bioassay of populations.
RESULTS

Single-isolate populations. No change in the proportion of resistance ($P_R$) was detected in the three single-isolate populations under any of the six treatments (data not shown). $P_R$ in the R population was equal to one at the beginning of the cycling and fluctuated around one during five cycles, whereas $P_R$ in the S1 and S2 populations remained at zero during all five cycles. Disease severity data showed differences under different fungicide treatments. Disease severity caused by any of these populations remained higher than 80% during the five cycles under application of propamocarb at either full or half rate. Disease caused by population S2 was reduced to < 20% after three cycles under application of half-rate mefenoxam and was totally eliminated after three cycles under application of full-rate mefenoxam or a half-rate mixture of mefenoxam and propamocarb. Population S1 caused disease severity similar to that of population R. Neither was suppressed by any fungicide treatment.

Mixed populations of isolates. In mixed populations, $P_R$ was affected strongly by fungicide treatments. $P_R$ in population S1R increased dramatically under application of half-rate and full-rate mefenoxam (Figure 4A). By the end of the fourth cycle, the value of $P_R$ was approached 1, and indicating complete dominance of resistance in the population. Under application of the half-rate mixture of mefenoxam and propamocarb, the increase of $P_R$ was slowed, and $P_R$ reached 0.3 at the end of the fifth cycle. There was little change in $P_R$ under application of propamocarb or tap water (Figure 4A). Disease severity caused by population S1R increased during each cycle. The least disease occurred under application of the half-rate mixture (Figure 4B). In population S2R, a similar change in $P_R$ was detected. The fastest increases were found under application of mefenoxam (Figure 5A). Under application of full-
rate mefenoxam, $P_R$ reached one by three cycles. At the end of fifth cycle, the value of $P_R$ was 0.35 under application of half-rate mixture and 0.14 under application of tap water. No change was detected in the populations under application of propamocarb. Disease severity caused by population S2R increased continuously (Figure 5B). A slower increase in disease severity was found in the population under application of full-rate mefenoxam.
Figure 4. Change of PR and severity in population S1R under fungicide treatments
A. PR in population S1R   SEM=0.07 n=90
B. Severity caused by population S1R   SEM=0.03 n=90
Figure 5. Change of $P_R$ and severity in population S2R under fungicide treatments
A. $P_R$ of population S2R  $SEM=0.07$  $n=90$
B. Severity caused by population S2R  $SEM=0.10$  $n=90$
DISCUSSION

The goal of the cycling experiment was to investigate the influence of competition among isolates with different fitness characteristics in populations and fungicide treatments on the change of PR in two mixed populations. In order to detect the difference among each treatment combination, the following non-linear equation was used to fit the curves:

\[ Y = \frac{1}{1 + \exp[-(a + b \cdot X) \}} \]  

(1)

In equation (1), variable \( Y \) is the value of PR and \( X \) is the number of the cycle. Parameter “a” controls the location of the curve, and “b” is the slope of the curve when \( Y=0.5 \), which reflects the rate of change of PR.

After fitting equation (1) to all curves of populations S1R and S2R (Figure 4A, 5A), the values of \( a \) and \( b \) were compared among six fungicide treatments and two populations. All the values of parameter “a” were less than zero (Table 5), indicating that PR was small at the beginning of cycles (since \( y=0.001 \), “a” must be less than zero). No significant differences were detected among most treatments and between the two mixed populations.

TABLE 5. Comparison of interceptions of curves (parameter “a”) among treatments and populations

<table>
<thead>
<tr>
<th>Populations</th>
<th>fp</th>
<th>fm</th>
<th>hp</th>
<th>hm</th>
<th>hh</th>
<th>LSD(P=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1R</td>
<td>-3.68</td>
<td>-1.23</td>
<td>-5.31</td>
<td>-9.42</td>
<td>-5.23</td>
<td>-4.43</td>
</tr>
<tr>
<td>S2R</td>
<td>-14.66</td>
<td>-14.43</td>
<td>-6.22</td>
<td>-3.58</td>
<td>-6.64</td>
<td>-5.69</td>
</tr>
<tr>
<td>LSD(P=0.05)</td>
<td>27.25</td>
<td>29.76</td>
<td>2.00</td>
<td>8.35</td>
<td>13.27</td>
<td>8.74</td>
</tr>
</tbody>
</table>

Note: fp- full-rate propamocarb, fm- full-rate mefenoxam, hp- half-rate propamocarb, hm- half-rate mefenoxam, hh- half-rate mixture of mefenoxam and propamocarb, tw-tap water. N=3 for each cell mean.
Differences among values of parameter “b” indicated differential responses of populations under the various fungicide regimes. There were no significant differences in values of “b” between applications of propamocarb and tap water, indicating that $P_R$ was not affected by propamocarb (Table 6). Under application of mefenoxam, the value of “b” was higher than for other fungicide treatments in both populations. The values of “b” in population S1R was significantly lower under application of the half-rate mixture of mefenoxam and propamocarb than under application of half-rate mefenoxam. In population S2R, the value of “b” under application of the half-rate mixture was significantly lower than for full-rate mefenoxam. These results quantitatively confirmed the finding by Sanders et al in 1985 that the half-rate mixtures of metalaxyl with propamocarb could delay the proliferation of a resistant isolate (36). The value of this delay in proliferation of mefenoxam resistance may be questionable. However, since $P_R$ increased steadily and was >0.4 after five cycles of selection, the half-rate mixtures strategy may merely delay, not prevent, proliferation of resistant isolates.

We were unable to detect an impact of isolate fitness on proliferation of resistance since there was no significant difference between the two mixed populations, S1R and S2R, in any fungicide treatment. This result may be explained in three possible ways. First, the isolate fitness characteristics not related to fungicide resistance may have had only minor impact on competition in populations compared to the impact of fungicides. Second, variability in isolate sensitivity to propamocarb (Table 4) may complicate the outcome of competition among isolates. We should perhaps select the isolates with the similar sensitivity to propamocarb in order to simply the experimental system. Third, grass homogenates sampled to quantify the value of $P_R$ contained oospores, zoospores, hyphal segments, and grass debris.
TABLE 6. Comparison of slopes of curves (parameter “b”) among treatments and populations

<table>
<thead>
<tr>
<th>Fungicide treatment</th>
<th>Populations</th>
<th>fp</th>
<th>fm</th>
<th>hp</th>
<th>hm</th>
<th>hh</th>
<th>tw</th>
<th>LSD(P=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1R</td>
<td>0.17</td>
<td>2.78</td>
<td>-0.07</td>
<td>5.13</td>
<td>1.01</td>
<td>0.16</td>
<td>3.06</td>
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<tr>
<td></td>
<td>S2R</td>
<td>0.12</td>
<td>9.75</td>
<td>-0.37</td>
<td>4.54</td>
<td>1.97</td>
<td>0.65</td>
<td>7.11</td>
</tr>
<tr>
<td></td>
<td>LSD(P=0.05)</td>
<td>0.37</td>
<td>13.99</td>
<td>0.33</td>
<td>8.18</td>
<td>5.08</td>
<td>1.69</td>
<td></td>
</tr>
</tbody>
</table>

Note: fp- full-rate propamocarb, fm- full-rate mefenoxam, hp- half-rate propamocarb, hm- half-rate mefenoxam, hh- half-rate mixture, tw-tap water. N=3 for each cell mean.

Sampling from these homogenate caused the large experimental error that could obscure the true difference between two populations.

Disease severity data did not correlated closely with changes in PR, except for the strong impact of mefenoxam on population S2. Effective control on Pythium blight with a half-rate mixture of metalaxyl and propamocarb was demonstrated in field trials by Sanders et al (36) but not under the controlled conditions of our experiment. This difference may result from the fact that environmental conditions in our experiment were uniformly disease-favorable, whereas field conditions are likely to be more variable. In our trial, P. aphanidermatum grew so rapidly that differences in aggressiveness among treatments might have been obscured by rating disease development at a single time interval. The effectiveness of half-rate mixture on Pythium blight needs to be investigated further by measuring disease severity more frequently under different fungicide regimes.

Further studies are needed to investigate the interactions among fungicides and survival of Pythium aphanidermatum in order to improve resistance management strategies. More powerful quantitative tools, such as molecular markers, and further understanding of
the basic biology of the pathogen may also help to gain further insight into population-level competition.
CONCLUSIONS

Resistance to mefenoxam in populations of *Pythium aphanidermatum* led to ineffective control of Pythium blight following application of mefenoxam fungicide. Competition between resistant and sensitive isolates under different fungicide regimes plays an important role in development of resistance.

In this work, variation of fitness characteristics among isolates of *P. aphanidermatum* was quantified. Growth rate of mycelia, sporulation capacity, and aggressiveness on perennial ryegrass showed wide variation among isolates. Based on sensitivity to mefenoxam, two categories, 16 isolates completely resistant to mefenoxam and 28 sensitive isolates were characterized. Significant variation in the values of EC$_{50}$ was detected among sensitive isolates. No correlation was found between mefenoxam sensitivity and any of three fitness characteristics. This finding was consistent with results of Brantner and Windel (5).

In the population cycling experiment, the two test populations constructed by mixing a sensitive isolate and a resistant isolate showed similar population dynamics under all fungicide treatments. The rate of change in proliferation of resistant isolates was significantly slower under application of a half-rate mixture of mefenoxam and propamocarb than under application of mefenoxam alone. This finding supports those of Sanders et al. (36) and confirms that reduced-rate tank mixing can delay fungicide resistance, but the durability of this strategy is questionable. The effect of fitness characteristics on development of resistance remains unclear. A large number of isolates need to be characterized in order to select appropriate isolates to construct test populations. More effective techniques such as molecular markers could help to quantify population components during cycling experiments and elucidate population dynamics more accurately and precisely.
REFERENCES CITED


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