Fitness Attributes of *Pythium aphanidermatum* with Dual Resistance to Mefenoxam and Fenamidone

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Abstract

Pythium aphanidermatum is the predominant species causing Pythium root rot on commercially grown poinsettias in North Carolina. Resistance to mefenoxam is common in populations of *P. aphanidermatum* but resistance to fenamidone and other quinone outside inhibitor fungicides has only just been reported in greenhouse floriculture crops. The in vitro sensitivity to the label rate of mefenoxam (17.6 µl active ingredient [a.i.]/ml) and fenamidone (488 µl a.i./ml) was determined for 96 isolates of *P. aphanidermatum*. Isolates were assigned to four fungicide phenotypes: mefenoxam-sensitive/fenamidone-sensitive (MefS, FenS), mefenoxam-sensitive/fenamidone-insensitive (MefS, FenR), mefenoxam-insensitive/fenamidone-sensitive (MefR, FenS), and mefenoxam-insensitive/fenamidone-insensitive (MefR, FenR). In all, 58% of isolates were insensitive to one (MefR, FenS = 36% and MefS, FenR = 16%) or both fungicides (MefR, FenR = 6%). A single point mutation in the cytochrome *b* gene (G143A) was

identified in fenamidone-insensitive isolates. Mycelial growth rate at three temperatures (20, 25, and 30°C), in vitro oospore production, and aggressiveness on poinsettia were evaluated to assess relative fitness of sensitive and insensitive isolates. Isolates with dual insensitivity to mefenoxam and fenamidone had reduced radial hyphal growth at 30°C and produced fewer oospores than isolates sensitive to one or both fungicides. Isolates sensitive to both fungicides produced greater numbers of oospores. Aggressiveness on poinsettia varied by isolate but fungicide phenotype was not a good predictor of aggressiveness. These results suggest that populations of *P. aphanidermatum* with dual resistance to mefenoxam and fenamidone may be less fit than sensitive populations under our imposed experimental conditions but populations of *P. aphanidermatum* should continue to be monitored in poinsettia production systems for mefenoxam and fenamidone insensitivity.

Pythium root rot, caused by *Pythium aphanidermatum*, is an ongoing challenge for commercial poinsettia (*Euphorbia pulcherrima* Willd. ex Kotzch) producers in North Carolina. Long production seasons and common irrigation practices, including recirculating and flood systems, make management of this disease challenging. Most commercially available poinsettia cultivars are susceptible or only partially resistant to Pythium root rot (Lookabaugh et al. 2017). Therefore, disease management is achieved through a combination of sanitation practices and preventative fungicide applications. Historically, preventative applications of mefenoxam have been the most effective means of controlling Pythium root rot on poinsettia. Mefenoxam is an enantiomer of phenylamide metalaxyl that is transported systemically in plants and inhibits synthesis of ribosomal RNA in oomycetes. The site-specific mode of action of mefenoxam increases the risk of resistance development in target populations of oomycetes (Brent and Holloman 2007).

Resistance to metalaxyl was first reported in isolates of *Pythium* sampled from turfgrass in 1984 (Sanders 1984). Mefenoxam-insensitive species of *Pythium* have been sampled from many cultivated plants throughout the world, including floriculture crops (Aegerter et al. 2002; Del Castillo Múnera and Hausbeck 2016; Lookabaugh et al. 2015; Moorman and Kim 2004; Moorman et al. 2002). In spite of these findings, mefenoxam is still widely used throughout the floriculture industry due to the limited number of fungicides that provide protection against Pythium root rot and other oomycete diseases. Recent research has identified alternative fungicides for

control of Pythium root rot caused by *P. aphanidermatum* on poinsettia (Lookabaugh 2017). Fenamidone provided suppression of Pythium root rot disease that was comparable with mefenoxam. The quinone outside inhibitor (QoI) class of fungicides targets the cytochrome *b* gene (*cytb*) located within the mitochondrial bc1 ubiquinol oxidase complex III and inhibits electron transfer required for cellular respiration and production of ATP (Anke et al. 1977; Bartlett et al. 2002; Gisi et al. 2002). Fenamidone and other strobilurins (Fungicide Resistance Action Committee group 11), including azoxystrobin and pyraclostrobin, are not commonly used for control of Pythium root rot but are applied to manage foliar diseases such as Botrytis blight and powdery mildew. Previously, in vitro insensitivity to fenamidone, azoxystrobin, and pyraclostrobin was identified in isolates of *P. aphanidermatum* (Lookabaugh 2017). Additionally, 1 of 25 isolates screened in this study was insensitive to both mefenoxam and fenamidone. The occurrence of QoI-insensitive and dual-insensitive isolates in populations of *P. aphanidermatum* is not currently known.

Implementation of antiresistance strategies for mefenoxam has been challenging due to the general lack of inexpensive alternatives. If QoI are to be used for Pythium root rot control and resistance management, it is important to assess the frequency of insensitive isolates in populations of *P. aphanidermatum* to mitigate potential risks for resistance development before these fungicides are incorporated into control programs.

Mutations associated with fungicide resistance may have adverse pleiotropic effects, or fitness costs, that may become apparent in the absence of selection pressure. Fitness is defined as an organism's ability to contribute to the gene pool in subsequent generations, which is measured as a function of its ability to grow, reproduce, and survive through repeated life cycles (Nelson 1979; Pringle and Taylor 2002; Vanderplank 1982). The establishment and evolution of fungicide resistance in plant pathogen populations is largely dependent on pathogen fitness and has important implications for disease management (Pamell et al. 2005; Peever and Milgroom 1994). Assessing fitness of fungicide-insensitive isolates relative to sensitive isolates may help to better predict whether insensitive isolates will persist in the absence of selection pressure (Chapara et al. 2011; Crute and Harrison 1988).

In general, limited information is available on potential fitness costs associated with fungicide insensitivity in oomycetes. Most studies have focused on phenylamide resistance in species of *Phytophthora*,

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including *Phytophthora infestans*, *P. capsici*, *P. nicotianae*, and *P. erythroseptica*, and downy mildew pathogens *Bremia lactucae* and *Pseudoperonospora cubensis* (Café-Filho and Ristaino 2008; Chapara et al. 2011; Cohen et al. 1983; Day and Shattock 1997; Hu et al. 2008; Timmer et al. 1998; Ziogas et al. 2006). Sensitive isolates of *Pythium aphanidermatum* are continually recovered in greenhouse production facilities that have long histories of mefenoxam use and often cooccur with insensitive isolates (Lookabaugh et al. 2015). A continuous influx of sensitive isolates from propagative material via gene flow or, alternatively, competition of insensitive isolates with sensitive isolates could account for this observation (Dowley et al. 1995; Williams and Gisi 1992). The relative fitness of isolates of *P. aphanidermatum* insensitive to mefenoxam and fenamidone has not been previously examined and is unknown.

The objectives of this study were to (i) assess in vitro sensitivity to mefenoxam and fenamidone in isolates of *P. aphanidermatum* obtained from infected hosts, (ii) identify the molecular basis for QoI insensitivity in isolates of *P. aphanidermatum*, and (iii) assess selected fitness attributes in fungicide-sensitive and -insensitive isolates of *P. aphanidermatum*, including their aggressiveness on poinsettia.

Materials and Methods

Sensitivity of *P. aphanidermatum* to mefenoxam and fenamidone. The 96 isolates of *P. aphanidermatum* used in this research were collected during a survey of North Carolina floriculture greenhouses or from samples submitted to the Plant Disease and Insect Clinic at North Carolina State University (Lookabaugh et al. 2015). Cultures had been placed into 1.5-ml cryovials containing sterile deionized water or sterile water with hemp seed for storage in the dark at 25°C. Isolates were recovered from storage by dispensing tube contents onto clarified V8 juice agar (CV8A; 50 ml of clarified V8 juice and 15 g of Bacto agar; Becton, Dickinson, and Co.). Isolates were maintained on 2% water agar for the duration of the experiments. For sensitivity assessments, CV8A was amended with label rates of commercial formulations of fenamidone (Fenstop, 488 µl active ingredient [a.i.]/ml) or mefenoxam (Subdue MAXX, 17.6 µl a.i./ml) and dispensed in Petri plates (100 by 15 mm in diameter). CV8A not amended with fungicides served as a control. Salicylhydroxamic acid (50 mg/ml) was added to CV8A containing fenamidone. For each isolate, a 5-mm-diameter plug excised from the leading edge of an actively growing colony was placed mycelium-side down in the center of a fungicide-amended or -nonamended Petri plate containing CV8A. There were three replicate plates for each fungicide-isolate combination. Plates were arranged in a completely randomized design and incubated in the dark at 25°C for 27 h. Percent mycelial inhibition was calculated by dividing mean radial mycelial growth on fungicide-amended medium by mean radial mycelial growth on nonamended medium, subtracting from 1, and multiplying by 100. The experiment was conducted twice.

Data from both experiments were combined and descriptive statistics were calculated using PROC MEANS procedures (SAS 9.4; SAS Institute). Isolates were assigned to four fungicide phenotypes: mefenoxam-sensitive/fenamidone-sensitive (MefS, FenS), mefenoxam-insensitive/fenamidone-sensitive (MefR, FenS), mefenoxam-sensitive/fenamidone-insensitive (MefS, FenR), and mefenoxam-insensitive/fenamidone-insensitive (MefR, FenR).

Molecular characterization of G143A mutation in *P. aphanidermatum*. A set of *cytb* gene-specific primers was developed using a publicly available sequence (accession number AX577562.1) in GenBank (National Center for Biotechnology Information). Primers PAcyt-F (5'-AATGATGGCAACAGCTTTCA-3') and PAcyt-R (5'-TGGTGTTTTCATAGGATTTGCTT-3') were designed to amplify a 387-bp fragment of the *cytb* gene region using Primer 3 software.

Polymerase chain reaction (PCR) was performed directly from mycelium using Phire Plant Direct PCR Master Mix (Thermo Scientific). The 96 isolates were transferred onto 9-cm-diameter Petri plates containing potato dextrose agar (Becton, Dickinson, and Co.) and incubated at 25°C for 5 days. Approximately 0.1 g of mycelium was harvested from each agar plate with a sterile pipette tip and transferred to tubes containing 20 µl of sterile distilled water.

A pipette tip was used to gently grind mycelium and samples were incubated at room temperature for 20 min. The supernatant (mycelial suspension) was collected for amplification with direct PCR, as described below.

PCR was carried out in a MyCycler Thermal Cycler (Bio-Rad Laboratories Inc.) with 2× Phire Plant Direct PCR Master Mix (25 µl), 0.5 µM forward and reverse primer (1 µl each), 1.25 µl of mycelial suspension, and water to obtain a final reaction volume of 50 µl. The cycling conditions were an initial denaturation period at 98°C for 5 min; 35 cycles at 98°C for 5 s, 53°C for 5 s, and 72°C for 20 s; with a final extension at 72°C for 1 min. PCR products were purified with ExoSAP-IT (USB Corporation). Amplicons were submitted to ETON Bioscience (Research Triangle Park) for Sanger sequencing. Nucleotide sequences were trimmed and contigs were assembled, aligned, and analyzed using CLC Workbench (Qiagen Bioinformatics).

Fitness of *P. aphanidermatum* with varying fungicide phenotypes.

A subset of 20 isolates of *P. aphanidermatum* sampled from poinsettia, garden mum (*Chrysanthemum* sp.), or tomato (*Solanum lycopersicum*) (Table 1) representing a range of fungicide phenotypes (MefS, FenS; MefR, FenS; MefS, FenR; and MefR, FenR) was arbitrarily selected and used to evaluate mycelial growth rate at three temperatures, oospore production on nutrient medium, and aggressiveness on poinsettia.

Influence of temperature on in vitro mycelial growth of *P. aphanidermatum*.

A 5-mm-diameter plug of each isolate was placed mycelium-side down in the center of a Petri plate containing nonamended V8 juice agar. Cultures were grown in the dark at 20, 25, or 30°C and colony diameter was measured after 24 h. Three replicate plates were used for each temperature (20, 25, and 30°C)–isolate (PA1 to PA20) combination. The experiment was conducted twice.

Runs were not significantly different; therefore, data from both runs of the experiment were combined and analysis of variance was performed using PROC GLIMMIX procedures (SAS 9.4; SAS Institute). Fungicide phenotype, temperature, and their interaction were treated as fixed effects while replicates and runs were treated as random effects. The fungicide phenotype–temperature interaction was significant; therefore, least squares means were calculated for the interactions and simple effects of phenotype groups were analyzed within treatments to determine significant differences. Least square

Table 1. Isolates of *Pythium aphanidermatum* used in fitness experiments and their in vitro sensitivity to mefenoxam and fenamidone

Isolate	Host	Fungicide phenotype ^z
PA1	Poinsettia	MefS, FenS
PA2	Poinsettia	MefR, FenS
PA3	Poinsettia	MefS, FenR
PA4	Poinsettia	MefR, FenR
PA5	Poinsettia	MefR, FenR
PA6	Poinsettia	MefR, FenR
PA7	Poinsettia	MefR, FenR
PA8	Poinsettia	MefS, FenS
PA9	Poinsettia	MefR, FenS
PA10	Poinsettia	MefR, FenS
PA11	Mum	MefS, FenR
PA12	Mum	MefS, FenR
PA13	Mum	MefS, FenR
PA14	Mum	MefS, FenR
PA15	Mum	MefS, FenS
PA16	Mum	MefS, FenS
PA17	Tomato	MefS, FenS
PA18	Tomato	MefS, FenS
PA19	Tomato	MefS, FenS
PA20	Tomato	MefS, FenS

^z Isolates were designated as sensitive or insensitive to label rates of mefenoxam (MefS and MefR, respectively; 17.6 µl active ingredient [a.i.]/ml) and fenamidone (FenS and FenR, respectively; 488 µl a.i./ml) and assigned to one of four fungicide phenotypes.

means were separated using Fisher's protected least significant difference (LSD; $\alpha = 0.05$) where appropriate.

Oospore production by 20 isolates of *P. aphanidermatum*. Agar plugs measuring 5-mm in diameter were excised with a cork borer from the leading edge of an actively growing colony of each *P. aphanidermatum* isolate. Plugs were placed mycelium-side down in the center of each 9-cm-diameter Petri plate containing V8 juice medium, with three replicate plates for each isolate. After plates were incubated at 25°C for 96 h, three agar plugs (3 mm in diameter) were removed, placed individually onto glass slides, and flattened with a cover slip. A light microscope was used to view the plugs at $\times 200$ magnification and oospores were counted. Oospore counts from each of the three agar plugs were averaged for each replicate plate. The experiment was conducted twice.

Runs were not significantly different; therefore, data from both runs of the experiment were combined and analysis of variance was performed using PROC GLIMMIX procedures (SAS 9.4; SAS Institute). Fungicide phenotype was treated as a fixed effect while run and replicate were treated as random effects. Least-squares means were calculated for each fungicide phenotype to determine significant differences among phenotypes ($P < 0.05$) and separated using Fisher's protected LSD ($\alpha = 0.05$) where appropriate.

Aggressiveness of four isolates of *P. aphanidermatum* on poinsettia in vegetative state. Unrooted cuttings of the susceptible cultivar Prestige Red were placed in Oasis Wedge growing media (Smithers-Oasis North America) and maintained under mist irrigation until roots emerged from the bottom of the foam wedge. Rooted plugs of poinsettia were transferred to new 15-cm plastic pots containing Fafard 4P (Fafard) peat-based potting media. Once transplanted, plants were fertilized with 5 g of Multicote 4 (14-14-16; Haifa Chemicals Ltd.) sprinkled evenly on the pot surface and watered in.

Four isolates of *P. aphanidermatum*, representing each fungicide phenotype—PA1 (MefS, FenS), PA2 (MefR, FenS), PA3 (MefS, FenR), and PA4 (MefR, FenR)—were used in this experiment. Inoculum was prepared by transferring four 4-mm-diameter agar disks from an actively growing culture of *P. aphanidermatum* into 125-ml flasks of twice-autoclaved long-grain rice (25 g of rice and 18 ml of deionized water), and cultures were incubated for 5 days at 25°C. Flasks were shaken twice daily to promote uniform colonization of rice grains and prevent clumping. Plants were inoculated with one of the four isolates 6 days after transplanting; noninoculated plants were included as controls. Three replicate plants per inoculation treatment were arranged in a randomized complete block design and the experiment was conducted twice. Plants were transplanted 21 March 2016, inoculated 27 March 2016, and harvested 6 May 2016 in experiment (run) 1 and transplanted 14 June 2016, inoculated 20 June 2016, and harvested 27 July 2016 in experiment (run) 2.

Fig. 1. In vitro sensitivity of 96 isolates of *Pythium aphanidermatum* to mefenoxam (17.6 μ l active ingredient [a.i.]/ml) and fenamidone (488 μ l a.i./ml). Isolates having percent mycelial inhibitions less than 50% were considered insensitive. Isolates were assigned to a fungicide phenotype based on sensitivity (S) or insensitivity (R) to mefenoxam (Mef) or fenamidone (Fen).

Plants were maintained under natural light cycles so they remained in a vegetative state for the duration of the experiment.

At the end of each experiment, shoot symptoms were evaluated on a disease severity scale of 1 = healthy plant, 2 = slightly stunted, 3 = chlorosis, moderate stunting or defoliation, 4 = wilting or severe stunting, and 5 = dead (Lookabaugh et al. 2015). Plants were carefully inverted, the pot was removed, and roots were observed with the growth media intact. Root rot was visually assessed based on the size and integrity of the root ball and root color. The rating scale was 1 = healthy white roots with root ball completely intact; 2 = 25% root rot, some root discoloration present; 3 = 50% root rot, brown discoloration evident throughout root system, root ball integrity fairly weak, root cortex sloughed off easily; 4 = 75% root rot, brown dead roots evident throughout root system, root ball integrity severely compromised, very few white roots, and 5 = 100% brown dead roots, root ball has lost all integrity.

Aggressiveness of 20 isolates of *P. aphanidermatum* on poinsettia in reproductive state. Unrooted cuttings of Prestige Red were rooted and transplanted as described above. Each of the 20 isolates previously characterized for sensitivity to mefenoxam and fenamidone (Table 1) was used to inoculate plants approximately 3 weeks after transplanting. Noninoculated plants were included as controls. Inoculum production and inoculations were carried out as previously described. The treatments were arranged in randomized complete blocks with three replicate plants per treatment. Root rot and shoot symptoms were rated as described above. Two runs of the experiment were conducted simultaneously at two locations approximately 1.5 km apart. Plants were transplanted 30 August 2016, inoculated 15 September 2016, and harvested 14 December 2016 at both locations. Plants were maintained under natural light cycles for the duration of this experiment. Short day lengths triggered bract color change and plants entered reproductive states.

Statistical analysis. Each of the two inoculation experiments had two runs. Runs were not significantly different; therefore, data from both runs were combined for each experiment. Analysis of variance and correlation analysis were performed using PROC GLIMMIX and PROC CORR procedures (SAS 9.4; SAS Institute). Isolate was treated as a fixed effect while run and block were treated as random effects. Root rot ratings and disease severity ratings were highly correlated (Spearman's $R > 0.90067$, $P < 0.0001$) and averaged to obtain a disease severity score for each isolate. Least-square means were calculated for each isolate to determine significant differences among isolates ($P < 0.05$) and separated using Fisher's protected LSD ($\alpha = 0.05$) where appropriate.

Results

Sensitivity of *P. aphanidermatum* to mefenoxam and fenamidone. The 96 isolates evaluated for sensitivity to mefenoxam and fenamidone varied in response. Isolates with a mean percent inhibition less than 50% were considered insensitive. In total, 58% of isolates were insensitive to one (MefR, FenS = 36% and MefS, FenR = 16%) or both fungicides (MefR, FenR = 6%) (Fig. 1). Mefenoxam and fenamidone completely inhibited mycelial growth of sensitive isolates (mean inhibition = 100%). Fifteen isolates were identified as insensitive to fenamidone and six isolates were insensitive to both mefenoxam and fenamidone.

Molecular characterization of G143A mutation in *P. aphanidermatum*. A single nucleotide mutation (G→C) in codon 143 of the *cytB* region of the genome (GGT→GCT) was present in all 21 FenR isolates. This mutation was not present in 75 FenS isolates. Partial nucleotide sequences for the *cytB* gene from one FenS isolate and one FenR isolate used in this study were deposited in GenBank.

Influence of temperature on growth of *P. aphanidermatum*. Variation in colony diameter was significant for fungicide group ($P = 0.0028$) and temperature ($P < 0.0001$). Colony diameter was greatest at 30°C. Averaged across three temperatures, MefR, FenS isolates had higher radial mycelial growth than isolates belonging to the other fungicide phenotypes, whereas isolates insensitive to both fungicides (MefR, FenR) exhibited the least radial mycelial growth across the three temperatures tested (Table 2). A significant

phenotype–temperature interaction ($P = 0.0455$) was observed. At 30°C, isolates insensitive to both mefenoxam and fenamidone (MefR, FenR) had the least mycelial radial growth compared with the other isolates ($P = 0.0025$, Table 2), whereas no differences among fungicide phenotypes were observed at 20 or 25°C.

Oospore production by *P. aphanidermatum*. Oospore production varied among fungicide phenotypes ($P < 0.0001$). Isolates sensitive to both fungicides (MefS, FenS) produced significantly more oospores than isolates insensitive to one (MefR, FenS or MefS, FenR) or both (MefR, FenR) fungicides (Table 3). Isolates insensitive to both fungicides produced the fewest oospores, whereas no differences were observed between the isolates insensitive to only one fungicide.

Aggressiveness of four isolates of *P. aphanidermatum* on poinsettia in vegetative state. Aggressiveness varied by isolate ($P = 0.0005$). Plants inoculated with MefS isolates (MefS, FenS and MefS, FenR) had higher disease severity scores than plants inoculated with the dually insensitive isolates (MefR, FenR) (Fig. 2). Fungicide sensitivity had no impact on aggressiveness among the plants inoculated with FenR isolates (MefS, FenR and MefR, FenR).

Aggressiveness of 20 isolates of *P. aphanidermatum* on poinsettia in reproductive state. Isolates varied in aggressiveness on poinsettia ($P < 0.0001$) but the mean aggressiveness of fungicide phenotypes did not differ ($P = 0.4715$; Table 4). Four MefS isolates were the most aggressive: PA14 (chrysanthemum; MefS, FenR), PA17 (tomato; MefS, FenS), PA19 (tomato; MefS, FenS), and PA3 (poinsettia; MefS, FenR). Plants inoculated with isolates PA6 (poinsettia; MefR, FenR) or PA15 (chrysanthemum; MefS, FenS) exhibited minor stunting symptoms in some replicates and no symptoms were observed in plants inoculated with either PA16 (chrysanthemum; MefS, FenS) or PA12 (chrysanthemum; MefS, FenR).

Single degree-of-freedom linear contrasts indicated that tomato isolates produced higher disease severity scores than isolates from poinsettia or garden mum (mean difference of 1.02 ± 0.3 , $P = 0.0021$). Isolates PA17 and PA19 were originally collected from tomato and among the most aggressive isolates. The lowest disease severity scores were observed on plants inoculated with isolates from garden chrysanthemum (mean difference of 1.14 ± 0.4 , $P = 0.0027$). Among isolates originally collected from mum, PA12 and PA16 (LSmean = 1.0) caused no symptoms in poinsettia whereas PA14 (LSmean = 4.8) was the most aggressive isolate and caused plant mortality in most replicates.

Discussion

The frequency of insensitivity to the QoI fenamidone was high in the population of *P. aphanidermatum* sampled from plants grown in commercial greenhouses in North Carolina. Among the isolates screened in this study, 22% were insensitive to label rates of fenamidone and possessed the G143A mutation. The presence of the G143A mutation in plant pathogens has been associated repeatedly with high levels of resistance and loss of efficacy in the field (Fraaije et al. 2006; Kildea et al. 2010; Lesniak et al. 2011). This is the first report of QoI resistance conferred by the G143A mutation in *P. aphanidermatum* isolates collected from greenhouse crops.

In all, 17 of 21 QoI-resistant isolates (71%) were collected from a single facility in North Carolina and originally isolated from

poinsettia and garden chrysanthemum. Intensive use of QoI fungicides for managing Botrytis blight and powdery mildew on poinsettia and continued use of these products year-round on other hosts probably led to selection and establishment of QoI-resistant populations in that facility. These isolates were collected over a period of 5 years, with sensitive isolates collected concurrently during this period. The long-term stability of QoI resistance in greenhouse populations of *P. aphanidermatum* in the absence of selection pressure is unknown. Regardless, to prevent selection of insensitive strains, fenamidone or other QoI should be used only in programs where they are mixed or rotated with a different mode-of-action fungicide (Brent and Holloman 2007). Three other QoI-resistant isolates were collected from garden chrysanthemum at two additional facilities. This indicates that QoI resistance is not limited to a single location; however, the prevalence of insensitive strains in those facilities and in the industry generally remains unknown due to lack of intensive spatial and temporal sampling.

Rapid growth of MefR isolates compared with MefS isolates has been reported in *Phytophthora infestans*, *P. nicotianae*, and *P. erythroseptica* (Hu et al. 2008; Porter et al. 2007; Timmer et al. 1998). However, other studies reported no differences between sensitive and insensitive isolates of *P. capsici*. Reduced fitness of MefR isolates was observed in isolates of *P. infestans* sampled from potato from Europe (Café-Filho and Ristaino 2008; Davidse et al. 1989; Day and Shattock 1997; Dowley and O'Sullivan 1985). In the current studies, MefR, FenS isolates had the highest mean radial mycelial growth in the temperature studies, while isolates insensitive to fenamidone (MefS, FenR) or both fungicides (MefR, FenR) had a reduced mean mycelial radial growth. Mefenoxam insensitivity did not correspond with increased aggressiveness on poinsettia but the ability of MefR isolates to grow as vegetative mycelium at high temperatures may provide a selective advantage by promoting substrate colonization independent of a susceptible host. Growth studies to evaluate substrate colonization by isolates of *Pythium aphanidermatum* with varying fungicide sensitivity phenotypes are warranted.

Dual insensitivity to mefenoxam and QoI fungicides has not been reported previously in populations of *P. aphanidermatum* sampled from plants in commercial floriculture greenhouses. In this study, 6% of the isolates tested belonged to this fungicide resistance group. Isolates with dual insensitivity had smaller mean radial mycelial

Table 3. Differences in in vitro oospore production by isolates of *Pythium aphanidermatum* belonging to four fungicide resistance groups

Fungicide phenotype ^y	n	Oospore counts $\pm$ standard error ^z
MefS, FenS	8	37.1 $\pm$ 2.9 a
MefR, FenS	3	27.3 $\pm$ 4.5 b
MefS, FenR	5	28.5 $\pm$ 2.6 b
MefR, FenR	4	15.2 $\pm$ 2.1 c
$P > F$	...	$P < 0.0001$

^y Twenty isolates were assigned to fungicide phenotype based on sensitivity (S) or insensitivity (R) to mefenoxam (Mef) or fenamidone (Fen).

^z Values represent data from two runs averaged across three replicates per run. Least square means followed by the same letter are not significantly different (Fisher's protected least significant difference; $\alpha = 0.05$).

Table 2. Colony diameter of 20 isolates of *Pythium aphanidermatum* grown on nonamended V8 juice medium at 20, 25, or 30°C for 24 h^y

Fungicide phenotype ^z	n	Temperature			Group LSmean
		20°C	25°C	30°C	
MefS, FenS	8	24.5 $\pm$ 1.17 a	48.9 $\pm$ 2.13 a	65.6 $\pm$ 2.11 a	46.3 $\pm$ 1.07 B
MefR, FenS	3	26.5 $\pm$ 1.91 a	55.6 $\pm$ 3.47 a	69.4 $\pm$ 3.45 a	50.5 $\pm$ 1.75 A
MefS, FenR	5	24.6 $\pm$ 1.47 a	46.9 $\pm$ 2.68 a	63.4 $\pm$ 2.68 a	44.9 $\pm$ 1.36 BC
MefR, FenR	4	26.6 $\pm$ 1.65 a	45.8 $\pm$ 3.00 a	53.5 $\pm$ 2.99 b	41.9 $\pm$ 1.52 C
$P > F$	...	0.6347	0.1547	0.0025	...
Temperature LSmean	...	25.5 $\pm$ 1.25 A	49.3 $\pm$ 1.25 B	62.9 $\pm$ 1.25 C	...

^y Values represent data from two runs averaged across three replicates per run $\pm$ standard error. Least square means followed by the same letter are not significantly different (Fisher's protected least significant difference; $\alpha = 0.05$).

^z Isolates were assigned to fungicide phenotype based on sensitivity (S) or insensitivity (R) to mefenoxam (Mef) or fenamidone (Fen).

growth than other isolates only at 30°C. Similarly, MefR isolates of *Plasmopara viticola* exhibited reduced fitness on grape leaves only at high temperatures of 30°C (Corio-Costet 2012). These results suggest that isolates of *Pythium aphanidermatum* insensitive to both mefenoxam and QoI may have reduced fitness at higher temperatures. Furthermore, differences in radial growth of MefR, FenS isolates, MefS, FenS isolates, and MefS, FenR isolates were not observed at 30°C, which is near the optimal temperature for mycelial growth of *P. aphanidermatum* (Middleton 1943). Poinsettia is most vulnerable to infection early in the production cycle, soon after transplant in August and September, when greenhouses are hot. Reduced fitness of dual-insensitive isolates in hot conditions may result in

Fig. 2. Differences in aggressiveness of four isolates of *Pythium aphanidermatum* when artificially inoculated on poinsettia in vegetative state. Isolates were assigned to a fungicide phenotype based on sensitivity (S) or insensitivity (R) to mefenoxam (Mef) or fenamidone (Fen). Scores were averaged across two runs and three replicates per run. Least square means with the same letter are not significantly different (Fisher's least significant difference; $\alpha = 0.05$).

these isolates being less problematic and easier to control early in the production cycle. Preventative fungicide drenches should begin at transplant to ensure plant protection.

Isolates of *P. aphanidermatum* with dual fungicide insensitivity produced fewer oospores than the other isolate groups, indicating loss of sexual reproductive fitness in dually insensitive isolates. This may explain why sensitive isolates persist in facilities that regularly use mefenoxam or fenamidone because oospores are the primary survival structure produced by *P. aphanidermatum*. Similarly, a previous study observing sexual fitness of MefR and MefS isolates of *Phytophthora infestans* showed that oospore production was depressed when two MefR isolates were crossed, whereas oospore production was highest in crosses of two MefS isolates (Hanson and Shattock 1998). However, in another study, MefR isolates of *P. erythroseptica* produced 6 to 20 times more oospores than MefS isolates but sensitive isolates produced more zoospores (Porter et al. 2007). Additional studies are needed to determine whether insensitivity to one or more fungicides reduces asexual reproduction in *Pythium aphanidermatum* and to evaluate the influence of oospore morphology and production in plant tissue and potting medium on epidemiology.

Aggressiveness has been used as an indicator of noncompetitive fitness of fungicide-insensitive isolates (Café-Filho and Ristaino 2008). When a limited number of isolates was evaluated in this study ($n = 4$) for their aggressiveness on poinsettia, plants inoculated with MefS isolates had higher disease severity scores than plants inoculated with MefR isolates but no differences were observed between FenS and FenR isolates. However, when a larger sample of 20 isolates was evaluated, fungicide sensitivity was not a clear predictor of pathogen aggressiveness. The different outcomes of these studies could be attributed to the genetic background of individual isolates rather than the fungicide phenotype. The mixed reproductive system of *P. aphanidermatum* coupled with a nearly continuous supply of susceptible host material allows this pathogen to maintain large population sizes year round. Additionally, movement of pathogen strains

Table 4. Differences in aggressiveness of 20 isolates of *Pythium aphanidermatum* when artificially inoculated on poinsettia in reproductive state

Isolate	Host	Fungicide phenotype ^y	Disease severity score $\pm$ standard error ^z
PA1	Poinsettia	MefS, FenS	3.8 $\pm$ 0.6 abc
PA8	Poinsettia	MefS, FenS	3.8 $\pm$ 0.6 abc
PA15	Mum	MefS, FenS	1.2 $\pm$ 0.6 ef
PA16	Mum	MefS, FenS	1.0 $\pm$ 0.6 f
PA17	Tomato	MefS, FenS	4.5 $\pm$ 0.6 ab
PA18	Tomato	MefS, FenS	3.8 $\pm$ 0.6 abc
PA19	Tomato	MefS, FenS	4.5 $\pm$ 0.6 ab
PA20	Tomato	MefS, FenS	3.6 $\pm$ 0.6 abc
Mean	...	...	3.3 $\pm$ 0.3
PA2	Poinsettia	MefR, FenS	3.8 $\pm$ 0.6 abc
PA9	Poinsettia	MefR, FenS	2.7 $\pm$ 0.6 cde
PA10	Poinsettia	MefR, FenS	2.8 $\pm$ 0.6 cd
Mean	...	...	3.1 $\pm$ 0.4
PA3	Poinsettia	MefS, FenR	4.5 $\pm$ 0.6 abc
PA11	Mum	MefS, FenR	3.9 $\pm$ 0.6 abc
PA12	Mum	MefS, FenR	1.0 $\pm$ 0.6 f
PA13	Mum	MefS, FenR	4.2 $\pm$ 0.6 abc
PA14	Mum	MefS, FenR	4.8 $\pm$ 0.6 a
Mean	...	...	3.7 $\pm$ 0.3
PA4	Poinsettia	MefR, FenR	3.2 $\pm$ 0.6 bcd
PA5	Poinsettia	MefR, FenR	2.8 $\pm$ 0.6 cd
PA6	Poinsettia	MefR, FenR	1.8 $\pm$ 0.6 def
PA7	Poinsettia	MefR, FenR	4.1 $\pm$ 0.6 abc
Mean	...	...	3.0 $\pm$ 0.4
$P > F$			
For isolates	...	...	<0.0001
For phenotype	...	...	=0.4715

^y Isolates were assigned to fungicide phenotype based on sensitivity (S) or insensitivity (R) to mefenoxam (Mef) or fenamidone (Fen).

^z Values represent combined data from two runs and were averaged across three replicates per run. Isolate least square means followed by the same letter are not significantly different (Fisher's protected least significant difference; $\alpha = 0.05$).

on diseased propagative material increases the chance for genotype flow between facilities, thereby increasing the overall evolutionary potential of *P. aphanidermatum* (McDonald and Linde 2002). Other unknown evolutionary processes may counteract selection pressures imposed by fungicide use that influence isolate aggressiveness.

Fitness components may vary greatly depending on the nature of disease and life history of plant-pathogenic organisms. Isolates of *P. aphanidermatum* insensitive to only one fungicide do not consistently demonstrate a loss of fitness in the parameters investigated in this study. In the absence of selection pressure, populations of *P. aphanidermatum* sensitive to fungicides may become established and persist in production systems. Alternatively, isolates that were insensitive to both fungicides demonstrated reduced mycelial growth and oospore production under our imposed environmental conditions. Dual-insensitive populations may be rare throughout the industry, suggesting that most populations of *P. aphanidermatum* can be well managed with sound fungicide programs. However, these findings may not be characteristic of other species of *Pythium* that coexist with *P. aphanidermatum* in greenhouse production systems. Widespread incidence of mefenoxam insensitivity combined with the recent observation of QoI insensitivity reinforces the need for integrated control strategies and fungicide rotations to minimize losses due to fungicide insensitivity.

Literature Cited

- Aegerter, B. J., Greathead, A. S., Pierce, L. E., and Davis, R. M. 2002. Mefenoxam-resistant isolates of *Pythium irregulare* in an ornamental greenhouse in California. *Plant Dis.* 86:692.
- Anke, T., Oberwinkler, F., Steglich, W., and Schramm, G. 1977. The strobilurins—New antifungal antibiotics from the basidiomycete *Strobilurites tenacellus*. *J. Antibiot.* 30:806-810.
- Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. 2002. The strobilurin fungicides. *Pest Manage. Sci.* 58:649-662.
- Brent, K. J., and Holloman, D. W. 2007. Fungicide Resistance, The Assessment of Risk. FRAC Monogr. No. 2 (second, revised edition). CropLife International, Brussels.
- Café-Filho, A. C., and Ristaino, J. B. 2008. Fitness of isolates of *Phytophthora capsici* resistant to mefenoxam from squash and pepper fields in North Carolina. *Plant Dis.* 92:1439-1443.
- Chapara, V., Taylor, R. J., Pasche, J. S., and Gudmestad, N. C. 2011. Competitive parasitic fitness of mefenoxam-sensitive and -resistant isolates of *Phytophthora erythroseptica* under fungicide selection pressure. *Plant Dis.* 95:691-696.
- Cohen, Y., Reveuni, M., and Samoucha, Y. 1983. Competition between metalaxyl-resistant and sensitive strains of *Pseudoperonospora cubensis* on cucumber plants. *Phytopathology* 73:1516-1520.
- Corio-Costet, M.-F. 2012. Fungicide resistance in crop protection: Risk and management. Pages 159-160 in: *Fungicide Resistance in Plasmopara viticola* in France and Anti-Resistance Measures. T. S. Thind, eds. CABI, Wallingford, UK.
- Crute, I. R., and Harrison, J. M. 1988. Studies on the inheritance of resistance to metalaxyl in *Bremia lactucae* and on the stability and fitness of field isolates. *Plant Pathol.* 37:231-250.
- Davidse, L. C., Henken, J., Van Dalen, A., Jaspers, A. B. K., and Mantel, B. C. 1989. Nine years of practical experience with phenylamide resistance in *Phytophthora infestans* in The Netherlands. *Neth. J. Plant Pathol.* 95:197-213.
- Day, J. P., and Shattock, R. C. 1997. Aggressiveness and other factors relating to displacement of populations of *Phytophthora infestans* in England and Wales. *Eur. J. Plant Pathol.* 103:379-391.
- Del Castillo Múnera, J., and Hausbeck, M. K. 2016. Characterization of *Pythium* species associated with greenhouse floriculture crops in Michigan. *Plant Dis.* 100:569-576.
- Dowley, L. J., Cook, L. R., and O'Sullivan, E. 1995. Development and monitoring of an anti-resistance strategy for phenylamide use against *Phytophthora infestans*. Pages 130-136 in: *Phytophthora infestans* 150. L. J. Dowley, E. Bannon, L. R. Cooke, T. Keane, and E. O'Sullivan, eds. Boole Press Ltd., Dublin, Ireland.
- Dowley, L. J., and O'Sullivan, E. 1985. Monitoring metalaxyl resistance in populations of *Phytophthora infestans*. *Potato Res.* 28:531-533.
- Fraaije, B. A., Burnett, F. J., Clark, W. S., and Luca, J. A. 2006. Development and field testing of fungicide anti-resistance strategies, with particular reference to strobilurins QoI group of fungicides. HGCA Proj. Rep. 392. Home-Grown Cereal Authority, London.
- Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Manage. Sci.* 58:859-867.
- Hanson, K., and Shattock, R. C. 1998. Effect of metalaxyl on formation and germination of oospores of *Phytophthora infestans*. *Plant Pathol.* 47:116-122.
- Hu, J. H., Hong, C. X., Stromberg, E. L., and Moorman, G. W. 2008. Mefenoxam sensitivity and fitness analysis of *Phytophthora nicotianae* isolates from nurseries in Virginia, USA. *Plant Pathol.* 33:347-354.
- Kildea, S., Dunne, B., Mullins, E., Cooke, L. R., Mercer, P. C., and O'Sullivan, E. 2010. Pyraclostrobin reduces germ tube growth of QoI-resistant *Mycosphaerella graminicola* pycnidiospores and the severity of Septoria leaf blotch on winter wheat. *Plant Pathol.* 59:1091-1098.
- Lesniak, K. E., Proffier, T. J., Beckerman, J. L., and Sundin, G. W. 2011. Occurrence of QoI resistance and detection of the G143A mutation in Michigan populations of *Venturia inaequalis*. *Plant Dis.* 95:927-934.
- Lookabaugh, E. C. 2017. Integrated strategies for managing *Pythium* root rot and fungicide-insensitive strains of *Pythium aphanidermatum* in poinsettia. Doctoral dissertation, North Carolina State University.
- Lookabaugh, E. C., Ivors, K. M., and Shew, B. B. 2015. Mefenoxam sensitivity, aggressiveness, and identification of *Pythium* species causing root rot on floriculture crops in North Carolina. *Plant Dis.* 99:1550-1558.
- Lookabaugh, E. C., Whipker, B., and Shew, B. B. 2017. Evaluation of poinsettia cultivars for resistance to *Pythium* root rot caused by *Pythium aphanidermatum*. *HortTechnology*. 27:805-812.
- McDonald, B. A., and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annu. Rev. Phytopathol.* 40:349-379.
- Middleton, J. T. 1943. The taxonomy, host range and geographic distribution of the genus *Pythium*. *Mem. Torrey Bot. Club* 20:1-171.
- Moorman, G. W., Kang, S., Geiser, D. M., and Kim, S. H. 2002. Identification and characterization of *Pythium* species associated with greenhouse floral crops in Pennsylvania. *Plant Dis.* 86:1227-1231.
- Moorman, G. W., and Kim, S. H. 2004. Species of *Pythium* from greenhouses in Pennsylvania exhibit resistance to propamocarb and mefenoxam. *Plant Dis.* 88:630-632.
- Nelson, R. R. 1979. The evolution of parasitic fitness. Pages 23-46 in: *Plant Disease—An Academic Treatise*, Vol. IV. J. G. Horsfall and E. B. Cowling, eds. Academic Press, New York.
- Parnell, S., Gilligan, C. A., and Van den Bosch, F. 2005. Small-scale fungicide spray heterogeneity and the coexistence of resistant and sensitive pathogen strains. *Phytopathology* 95:632-639.
- Peever, T. L., and Milgroom, M. G. 1994. Lack of correlation between fitness and resistance to sterol biosynthesis-inhibiting fungicides in *Pyrenophora teres*. *Phytopathology* 84:515-519.
- Porter, L. D., Miller, J. S., Nolte, P., and Price, W. J. 2007. *In vitro* somatic growth and reproduction of phenylamide-resistant and -sensitive isolates of *Phytophthora erythroseptica* from infected potato tubers in Idaho. *Plant Pathol.* 56:492-499.
- Pringle, A., and Taylor, J. W. 2002. The fitness of filamentous fungi. *Trends Microbiol.* 10:474-481.
- Sanders, P. L. 1984. Failure of metalaxyl to control *Pythium* blight on turfgrass in Pennsylvania. *Plant Dis.* 68:776-777.
- Timmer, L. W., Graham, J. H., and Zitko, S. E. 1998. Metalaxyl-resistant isolates of *Phytophthora nicotianae*: Occurrence, sensitivity, and competitive parasitic ability on citrus. *Plant Dis.* 82:254-261.
- Vanderplank, J. E. 1982. *Host-Pathogen Interactions in Plant Disease*. Academic Press, New York.
- Williams, R. J., and Gisi, U. 1992. Monitoring pathogen sensitivity to phenylamide fungicides: Principles and interpretation. *Bull. OEPP/EPPO Bull.* 22:299-306.
- Ziogas, B. N., Anastasios, N. M., Dimitrios, I., Theodosios, A., and Stavroula, B. 2006. A high multi-drug resistance to chemically unrelated oomycete fungicides in *Phytophthora infestans*. *Eur. J. Plant Pathol.* 115:283-292.