Fungicide Resistance in *Pythium* and *Phytophthora* from Ornamentals in Georgia©

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INTRODUCTION

The majority of root and crown diseases on ornamental crops are caused by oomycete pathogens, including species of *Pythium* and *Phytophthora*. Both Pythium and *Phytophthora* cause root, crown, stem, and foliage blights. Symptoms often include root softening, sloughing, darkening of roots, crowns and stems, wilting, foliage chlorosis, leaf drop, stem dieback, and leaf and petiole blighting.

Oomycete pathogens or "water molds" as they are commonly called, which also includes downy mildew causing pathogens, are unique and are not true fungi. They are more closely related to brown algae than fungi. One of the major differences between oomycetes and true fungi is in their cell wall components. Oomycete cell walls are composed of β-1,3 and β-1,6 glucans, whereas true fungi cell walls are composed of chitin. This is an important distinction because the mode of action of many fungicides is to act on and inhibit chitin cell wall biosynthesis. Since Oomycete cell walls do not contain chitin, these products have no activity on these pathogens. This has resulted in a limited number of commercially available fungicides with activity against *Pythium*, *Phytophthora*, and downy mildew diseases.

The predominant fungicide used against *Pythium* and *Phytophthora* diseases in ornamentals has been the phenylamide systemic fungicide, metalaxyl, which was replaced by mefenoxam (the R-enantiomer of metalaxyl), and marketed under the trade names of Subdue 2E and Subdue Maxx (Syngenta Crop Protection, Inc., Greensboro, North Carolina), respectively. Metalaxyl was registered for use in the United States of America in 1980 and within 4 years fungicide resistance in *Pythium* causing turf blight was identified (Sanders, 1984).

Mefenoxam fungicide resistance, or rather insensitivity, has been noted in several states in *Pythium* and *Phytophthora* species causing root and crown rots of ornamental plants. In Pennsylvania, 32.5% of the 120 *Pythium* isolates recovered from infected plants were insensitive to mefenoxam (Moorman et al., 2002). Eleven species of *Pythium* were identified from the 120 isolates. The most common species were *P. irregulare* and *P. aphanidermatum* of which 36.8 and 37.5% of these species, respectively, were insensitive to mefenoxam. In North Carolina, three species of *Phytophthora* (*P. nicotianae*, *P. cryptogea*, and *P. palmivora*) were recovered as the predominant species infecting floriculture crops (Hwang and Benson, 2005). Although, all isolates of *P. palmivora* were still sensitive to mefenoxam, 100% of the *P. cryptogea* and 21% of *P. nicotianae* isolates were insensitive. In a more recent North Carolina study, *P. nicotianae*, *P. drechsleri*, *P. cryptogea*, and *P. tropicalis* were the most commonly recovered *Phytophthora* species from floriculture corps, of which 66% of these *Phytophthora* isolates were insensitive or intermediate in resistance to mefenoxam (Olson and Benson, 2011).

These studies would suggest that mefenoxam insensitivity is widespread within floriculture production. However, another study involving multiple states in the southeastern USA concluded that across six states and 488 isolates that only 6% of the *Phytophthora* isolates were insensitive to mefenoxam (Olson et al., 2013). The viability of mefenoxam as a valuable tool in managing *Pythium* and *Phytophthora* root diseases is of great concern. The objectives of this study was (1) to identify species of *Pythium* and *Phytophthora* from symptomatic plants within both floriculture and woody ornamental crops in Georgia, and (2) to evaluate the recovered isolates for mefenoxam sensitivity.

MATERIALS AND METHODS

Isolate Collection

From 2010-2011, plant samples exhibiting symptoms of root or crown rot were collected from 17 wholesale ornamental production facilities (nine specializing in container-grown woody shrubs and eight specializing in floriculture or herbaceous crops). Discolored root and/or crown tissue were washed with tap water to remove rooting substrate, blotted dry, and placed on filter paper for selection and direct isolation. Symptomatic tissue sections were plated onto V8-PARP medium [15 g Bacto agar (Becton, Dickerson and Co., Sparks, Maryland); 50 ml clarified V-8 juice (Campbells, Camden, New Jersey); 67 mg 75% PCNB (Terraclor; Chemtura, Middlebury, Connecticut); 400 µl pimaracin (Sigma-Aldrich, St. Louis, Missouri); 250 mg ampicillin (Sigma-Aldrich, St. Louis, Missouri); 10 mg rifampicin (Sigma-Aldrich, St. Louis, Missouri) in 950 ml deionized water] (Jeffers and Martin, 1986). Plates were incubated in the dark at 22°C for up to 10 days. Putative *Pythium* and/or *Phytophthora* colonies were transferred onto new V8-PARP plates. After 24 to 72 h, actively growing colonies were then transferred by hyphal tip to new V8-PARP or non-amended V8-agar (15 g of Bacto agar; 100 ml of clarified V8 juice; 900 ml of deionized water) plates to obtain an axenic culture.

Morphological Identification

Isolates were grown on V8-agar for 72 h. Two agar plugs from the leading edge of the suspected *Pythium* and *Phytophthora* colony were transferred to a 35-mm plastic petri dish and flooded with non-sterile soil extract solution (NSES). Non-sterile soil extract solution was prepared by stirring 15 g of loamy field soil in 1 L of distilled water using a magnetic stirrer for at least 4 h and allowing the solution to settle overnight. The supernatant was decanted and centrifuged for 10 min at 8,000 rpm. If needed, the solution was vacuumed filtered to remove any debris left in the solution. Plates were then grown at 22-24°C for 24-72 h and examined for sporangia and morphological characteristics. Isolates resembling either *Pythium* or *Phytophthora* were then prepared for internal transcribed spacer (ITS) sequencing.

Identification with Internal Transcribed Spacer Sequencing

The ITS region (ITS1, 5.8S, and ITS2) of the rDNA of each isolate was sequenced for DNA-based identification. Suspected *Pythium* and *Phytophthora* isolates were grown on V8-agar at 22-24°C for 72 h. Hyphae was scraped and/or lightly touched with a 200-µl pipette tip. The tip was then placed into a 0.5-ml PCR tube containing a PuReTaq Ready-To-Go™ PCR Bead (GE Healthcare), 1 µl of 10 µM ITS-1 primer (5'-TCCGTAGGTG AACCTGCGG-3'), 1 µl of 10 µM ITS-2 primer (5'-GCTGCGTTCTTCATCGATGC-3'), and 23 µl of sterile nuclease-free water and mixed by gently pipetting up and down several times. Total PCR reaction volume was 25 µl. Thermal cycling conditions consisted of an initial denaturation at 94°C for 5 min; followed by 34 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min; and a final extension step of 72°C for 5 min, followed by a 4°C hold (Moorman et al., 2002). To improve sequencing results and for confirmatory testing, ITS-2 primer was replaced with ITS-4 (5'-TCCTCCGCTTA TTGATATGC-3') in the PCR reaction. Amplification products were confirmed with gel electrophoresis (1% molecular grade agarose; 100V for 55-60 min). PCR products were purified using QIAquick Purification Kit (Qiagen, Inc., Valencia, California) and submitted to the Georgia Genomics Facility (Athens, Georgia). Isolate DNA was stored and maintained at -20°C. DNA sequences were aligned and manually edited using Geneious software (Biomatters Ltd., Auckland, New Zealand). Internal transcribed spacer sequences were BLAST analyzed in GenBank (National Center for Biotechnology Information, Bethesda, Maryland) and the *Phytophthora* Database (http://www.phytophthoradb.org/).

Mefenoxam Sensitivity Assays

All isolates were screened for sensitivity to mefenoxam in vitro by amending V8-agar (50 ml clarified V8 juice; 15 g Bacto agar; 950 ml deionized water) with 100 μg a.i./ml of mefenoxam by suspending Subdue Maxx (Syngenta Crop Protection, Greensboro, North Carolina) in water and distributing it in molten agar prior to pouring into 35-mm plastic petri plates and evaluating mycelia growth compared to the growth of the same isolate on non-amended medium. Agar plugs (7 mm in diameter) were cut from the leading edge of a 3-4 day old isolate culture and inverted onto the center of mefenoxam-amended and non-amended plates. Two non-amended and two mefenoxam-amended plates for each isolate was incubated at 22°C in the dark for 24-48 h depending upon isolate growth rate. Plates were evaluated macroscopically. Mycelial growth was measured from the inoculated plug edge to the edge of the colony along two radii per plate. Isolates that grew ≥50% of the non-fungicide amended control plates (EC_{50} > 100 ug a.i./ml) were considered insensitive. Isolates that grew <50% as compared to the control were considered to be sensitive.

Isolates were further tested depending on their results from the initial screening. If the isolate was determined to be sensitive (growth <50%), it was further evaluated at tested at a concentration of 10 μg a.i./ml. If isolates were considered to be insensitive (growth ≥50%), then they were further evaluated at 500 and 1000 μg a.i./ml.

RESULTS AND DISCUSSION

Out of the 152 symptomatic samples collected, oomycete root pathogens were recovered from 80% of them. Either no pathogen or a non-oomycete pathogen was recovered from the remaining samples. Of the 121 oomycete isolates recovered, 39 were identified as *Phytophthora* spp., 77 as *Pythium* spp., and five as *Phytopythium* spp. The *Phytophthora* species identified included *P. nicotianae*, *P. pini*, *P. undulata*, *P. cinnamomi*, *P. citrophthora*, *P. palmivora*, *P. dreschsleri*, and *P. cryptogea*, with *P. nicotianae* being the most prevalent (30% of the *Phytophthora* isolates). Approximately, 21% of the *Phytophthora* isolates could not be identified to species based upon morphology or DNA sequencing. This is not uncommon and has been seen in previous studies (Hwang and Benson, 2005; Olson and Benson, 2011; Olsen et al., 2013).

Pythium species recovered included *Pythium irregulare*, *P. myriotylum*, *P. aphanidermatum*, *P. monospermum*, *P. chamaehyphon*, *P. vexans*, *P. diclinum*, *P. cucurbitacearum*, *P. zingiberis*, and *P. acanthophoron*. *Pythium irregulare* was the most prevalent and accounted for 12.5% of the identifiable species. The majority of the *Pythium* isolates recovered (approximately 50%) could not be identified to the species level.

Of note in this study is the identification of five *Phytopythium* isolates recovered from diverse symptomatic plants including *Coreopsis lanceolata*, *Hydrangea arborescens*, *Rosmarinus officinalis*, *Tagetes patula*, and *Thymus praecox* from three production facilities. *Phytopythium* is a relatively new taxonomic genus whose members were classified as clade K species of *Pythium*, and have more characteristics similar to *Phytophthora* than other *Pythium* species. *Pythium litorale* and *Pythium heliocoides* are now classified as *Phytopythium* species (Robideau et al., 2011) and both were recovered in this study. In recent studies, *Phytopythium heliocoides* was found to be pathogenic to begonia in Virgina (Yang, et al., 2013) and *P. litorale* was pathogenic to squash in Georgia (Parkunan and Ji, 2013).

Across all oomycete isolates in this study, 45.5% were mefenoxam insensitive. Insensitivity was identified in 27.2% of the *Phytophthora* isolates. All isolates *of P. undulata* and *P. palmivora* recovered from three production facilities and 57% of the unidentifiable *Phytophthora* spp. were insensitive to mefenoxam. However, all *P. nicotianae* and *P. pini* isolates, which accounted for over 50% of the total number of *Phytophthora* isolates recovered, were sensitive to mefenoxam.

Mefenoxam insensitivity was found in 58.4% of the *Pythium* isolates recovered. Approximately 28% of all *Pythium* isolates identified were *P. irregulare*, *P. myriotylum*,

and *P. aphanidermatum*. Of these species, only one of the 21 isolates (<5%) was insensitive to mefenoxam. Most of the insensitive isolates were uncommon or unidentifiable *Pythium* species. In addition, all of the *Pythopythium* isolates recovered were mefenoxam insensitive.

The seemingly high occurrence (45.5%) of mefenoxam-insensitive *Phytophthora*, *Pythium*, and *Phytopythium* isolates recovered in this study would suggest that the usefulness of mefenoxam to manage oomycete root diseases is questionable. However, many *Pythium* species are known to be saprobic. It is plausible that many of the unidentifiable *Pythium* isolates recovered, of which the majority were mefenoxam insensitive, are saprobic and not plant pathogenic. Until pathogenicity is proven, the high occurrence of mefenoxam insensitivity, particularly within *Pythium* and *Phytopythium* isolates, may be misleading.

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