Characterization of *Phytophthora* spp. on Floriculture Crops in North Carolina

H. A. Olson and D. M. Benson, Department of Plant Pathology, North Carolina State University, Raleigh 27695

Abstract

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Isolates of *Phytophthora* in floriculture crops were collected from North Carolina commercial greenhouse facilities in 2007 and 2008, identified, and characterized for mating type and mefenoxam sensitivity. In all, 163 isolates from 13 host species at 11 locations were identified primarily as *Phytophthora nicotianae* (59%), *P. drechsleri* (23%), *P. cryptogea* (9%), and *P. tropicalis* (4%). Multiple *Phytophthora* spp. were found at five locations. DNA sequencing was more reliable for differentiating *P. cryptogea* and *P. drechsleri*. Only the A1 mating type of *P. drechsleri* was collected; however, both mating types of *P. nicotianae*, *P. cryptogea*, and *P. tropicalis* were found. Overall, 66% of

Phytophthora isolates were resistant or intermediate in resistance to mefenoxam at 1 µg a.i./ml. Three groups of *P. drechsleri* isolates had effective concentration of mefenoxam providing 50% growth inhibition (EC₅₀) estimates over 700 µg a.i./ml. EC₅₀ estimates for *P. nicotianae* ranged from 246 to 435 µg a.i./ml. Isolates of *P. nicotianae* exhibited phenotypic heterogeneity at several locations, suggesting multiple separate introductions to the facilities. In contrast, *P. nicotianae* and *P. drechsleri* isolates from two locations were phenotypically identical to a previous survey, suggesting that the facilities have *Phytophthora* spp. established onsite.

The wholesale value of the United States floriculture crop was estimated to be \$4.22 billion in 2008 (52), with North Carolina being the fifth-largest producer of bedding, garden, and potted flowering plants. In the United States, over 38,000,000 m² of greenhouse space are utilized in the production of floriculture crops. Many diseases are problematic in greenhouse systems, including root and crown rot and foliar blight caused by multiple species of *Phytophthora* (12). Diseases caused by *Phytophthora* spp. are an ongoing problem in greenhouse production because of the difficulty in managing them (10). Movement of plant material between production facilities allows for introduction of the pathogen (32). The greenhouse environment provides conditions favorable for growth and sporulation of *Phytophthora* spp. (10). In addition, facilities utilizing recycled irrigation water provide ample opportunities to disperse motile zoospores (23,46,47). Finally, year-round production is common in greenhouse facilities; thereby, providing a continual supply of new hosts for infection (10).

Recently, research on the genus *Phytophthora* has received renewed interest within landscape and ornamental plant pathology, particularly with the broad range of host plants affected, the discovery of new species, and the movement of *P. ramorum* in planting material (10). Because of this increased interest, surveys of nursery and greenhouse facilities have been carried out in several states (11,24,25,27,32,43,53,55). During 2001 and 2002, a survey of greenhouse production facilities in North Carolina was conducted (27). Isolation from 41 symptomatic plant species at 29 production locations resulted in 483 isolates of *Phytophthora* from annual vinca (*Catharanthus roseus*), African violet (*Saintpaulia ionantha*), dusty miller (*Senecio cineraria*), English ivy (*Hedera helix*), gerbera daisy (*Gerbera jamesonii*), lavender (*Lavandula angustifolia*), pansy (*Viola × wittrockiana*), and petunia (*Petunia × hybrida*) at seven locations. The isolates were identified as *Phytophthora nicotianae* (57%), *P. cryptogea* (38%), and *P. palmivora* (5%) and, subsequently, were characterized for mating type and

resistance to mefenoxam, the active ingredient of a fungicide frequently used in the management of *Phytophthora* spp. Only the A1 mating type of *P. cryptogea* and *P. palmivora* were recovered, whereas both mating types of *P. nicotianae* were collected from North Carolina greenhouses. Only 56 of 273 isolates (21%) of *P. nicotianae* exhibited mefenoxam resistance at 1 µg a.i./ml. In contrast, all isolates of *P. cryptogea* were resistant or intermediate in resistance. Although surveys such as this provided much needed information on *Phytophthora* spp. within the ornamental industry, the surveys only provided data on *Phytophthora* spp. for one point in time. Detailed monitoring of pathogen populations over time can provide information valuable to disease management. Fungicide resistance can have a tremendous impact for growers faced with this problem in their greenhouse facilities. Detecting changes in the distribution of fungicide-resistant isolates can be integral to making recommendations for fungicide rotation programs. The potential for sexual reproduction and development of new pathogen genotypes can be predicted by examining changes in the distribution of mating types in organisms that outcross. Little is known about the dynamics of *Phytophthora* spp. through time in greenhouse floriculture production.

Since the initial work with *P. cryptogea* by Tompkins et al. (49,50), this species has been reported on over 40 ornamental species (13). Similarly, *P. drechsleri* has been reported on over 20 floriculture hosts, including multiple hosts which overlap with *P. cryptogea* (14). Differentiating these two species can be quite difficult because of highly similar morphological characteristics (12). For the previous survey of North Carolina greenhouses (27), a restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) was used to identify 184 isolates as *P. cryptogea*. The analysis was developed originally to separate *P. cryptogea* from *P. citrophthora* with the *Hae*III restriction enzyme (40). The authors noted that the restriction digest could not separate *P. cryptogea* from the closely related *P. erythrosetica* (40). *P. drechsleri*, also a species closely related to *P. cryptogea* (4,9), was not included in the development of the restriction analysis. In addition, no reference isolates of *P. drechsleri* were included as comparisons in the RFLP analysis conducted to identify isolates from the 2001 to 2002 North Carolina survey (27). Considering this, the validity of the RFLP for differentiating *P. cryptogea* and *P. drechsleri* is unknown.

The objectives of this study were to survey North Carolina greenhouse facilities for *Phytophthora* spp., identify the species present, characterize the isolates for mating type and mefenoxam

Corresponding author: H. A. Olson, E-mail: heather.a.olson@gmail.com

Current address of H. A. Olson: Virginia Tech, Hampton Roads AREC, Virginia Beach 23455.

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resistance, and compare the results with the 2001 to 2002 survey to identify changes over time. In addition, the previously developed RFLP analysis and DNA sequencing were compared to confirm the reliability of the RFLP method for distinguishing *P. cryptogea* and *P. drechsleri*.

Materials and Methods

Isolate collection. During 2007 and 2008, plants exhibiting symptoms of root rot, crown rot, or foliar blight were sampled at 25 wholesale greenhouse facilities in 17 North Carolina counties. Initially, greenhouse facilities were selected from the 2001 to 2002 survey; additional greenhouses were selected arbitrarily. Locations A, B, C, F, and K in the present survey correspond to locations A, B, D, C, and E, respectively, from the 2001 to 2002 survey (27). The sampling methods from the 2001 to 2002 survey were followed, except for the number of plants collected from an epidemic. During the 2001 to 2002 survey, up to 120 plants were collected from an epidemic (27), whereas only up to 12 plants were collected during the 2007 and 2008 survey. An epidemic was defined as symptomatic plants of the same species and age occurring within a growing block at a location. Symptomatic plants were placed in labeled plastic bags, which were placed in coolers for transportation and storage until samples were processed in the laboratory. Root, stem, or leaf tissues were selected for direct isolation. Root samples were washed under running water to remove potting mix. Symptomatic tissues were plated onto cornmeal agar (CMA; BD Diagnostics, Sparks, MD) amended with pimarin, ampicillin, rifamycin, and pentachloronitrobenzene (PARP) with and without hymexazol (H) (30). Plates were incubated at 20 to 25°C in the dark for up to 10 days. Putative *Phytophthora* colonies were transferred to new CMA-PARP(H). After 48 to 72 h, an actively growing hyphal tip was transferred to CMA to establish an axenic culture. For long-term storage, four 7-mm agar disks from an actively growing culture were placed into a 2-ml screw-cap microcentrifuge tube containing 1 ml of sterile deionized water and two twice-autoclaved hemp seeds. Cultures were incubated for 5 to 7 days at 20 to 25°C and then stored at 15°C (32). An isolate was defined as a culture of *Phytophthora* spp. isolated from an individual plant within an epidemic, and a group was defined as all of the isolates of *Phytophthora* recovered from an epidemic.

Morphological identification of *Phytophthora* spp. Colony and sporangia morphology were observed on CMA after incubating for 5 to 7 days at 20 to 25°C under continuous fluorescent light. If sporangia had not formed by 7 days, cultures were flooded with 5% clarified V8 broth (50 ml of clarified V8 juice and 950 ml of sterile deionized water) (15) and incubated under continuous fluorescent lighting for an additional 2 to 7 days. Clarified V8 juice was prepared by centrifuging 100 ml of V8 juice containing 1 g of CaCO₃ at 7,000 rpm for 10 min and collecting the supernatant (15).

Identification of *Phytophthora* spp. with ITS sequencing. The ITS region (ITS1, 5.8S, and ITS2) of the rDNA of each isolate of *Phytophthora* was sequenced for DNA-based identification. Each isolate was grown in pea broth for 5 to 7 days at 20 to 25°C. Three 7-mm agar disks of an isolate were transferred to 25 ml of pea broth in a 250-ml flask. Pea broth was prepared by autoclaving 120 g of frozen green peas in 500 ml of deionized water for 5 min. After cooling, the solution was filtered through several layers of cheesecloth to remove the peas. Deionized water was added to bring the total volume to 1 liter. Aliquots (25 ml) of pea broth were placed into 250-ml flasks, which were subsequently autoclaved for 30 min. Mycelia mats were harvested by vacuum filtration, placed in microcentrifuge tubes, and stored at -20°C. DNA was extracted from mycelia with the PUREGENE DNA Isolation Kit (Qiagen, Valencia, CA) for 10 to 20 mg of fungal tissue with a protocol optimized for *Phytophthora* spp. (K. Ivors, *personal communication*). Extracted DNA was stored at -20°C.

Polymerase chain reaction (PCR) amplification of the ITS region was performed with the ITS4 (5' TCCTCCGCTTATTGATATGC) and ITS6 (5' GAAGGTGAAGTCGTAACAAGG) primers (9). Reactions were performed in 25- μ l volumes and were com-

posed of 5 μ l of 5 $\times$ GoTaq Green Flexi buffer (Promega Corporation, Madison, WI), 1.5 μ l of 25 mM MgCl₂, 0.5 μ l of 40 mM dNTP mix (10 mM each dNTP; Promega Corporation), 0.25 μ l of each 50 μ M primer, 0.25 μ l of GoTaq DNA polymerase (5 U/ μ l; Promega Corporation), 16.25 μ l of nuclease-free water, and 1 μ l of extracted DNA. Thermal cycling conditions consisted of an initial denaturation of 94°C for 3 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension step of 72°C for 10 min. Amplification products were confirmed and concentrations estimated visually with gel electrophoresis (1% agarose; 95V for 75 min). PCR products were purified with the Wizard SV Gel and PCR Clean-up System (Promega Corporation), then submitted to the Yale University DNA Analysis Facility (New Haven, CT) for sequencing. DNA sequences were aligned and manually edited with Sequencher 4.9 (GeneCodes Corporation, Ann Arbor, MI). The ITS sequence of each isolate was subjected to BLAST analysis in the Phytophthora Database and GenBank (National Center for Biotechnology Information, Bethesda, MD).

Determination of mating type of heterothallic *Phytophthora* spp. Each collected isolate was paired with a known A1 and A2 mating type of either *P. capsici* (isolates OP97 and SP98 obtained from K. Lamour, University of Tennessee) or *P. nicotianae* (isolates ROC-BAK19 and SUR-SND7 obtained from D. Shew, North Carolina State University). A 7-mm agar plug of the unknown isolate and the known isolate were placed 2 cm apart on either V8 agar (100 ml of clarified V8 juice, 900 ml of sterile deionized water, and 15 g of agar) amended with 30 mg of β -sitosterol or rapeseed-extract malt agar (500 ml of rapeseed extract, 500 ml of sterile deionized water, 1 g of malt extract, and 17 g of Bacto agar; BD Diagnostics; 51). Rapeseed extract was prepared by boiling 100 g of rapeseed in 1 liter of deionized water for 30 min. The suspension was cooled to room temperature and the rapeseed was removed by filtration. Plates were incubated at 20 to 25°C in the dark and observed weekly for 5 weeks for the formation of sexual structures.

Mefenoxam sensitivity of *Phytophthora* isolates. Each isolate of *Phytophthora* was grown on CMA plates containing mefenoxam at 0, 1, and 100 μ g a.i./ml (Subdue Maxx; Syngenta, Greensboro, NC; 27). A 7-mm agar plug from an actively growing *Phytophthora* culture was placed in the center of each of three replicate plates of each mefenoxam concentration. The plates were incubated in the dark at 20 to 25°C. The diameter of hyphal growth was measured perpendicularly when growth on nonamended plates had reached the edge of the plate or when 10 days had passed for slow-growing isolates. Mefenoxam sensitivity was determined as the percentage of growth at a given mefenoxam concentration compared with growth on the nonamended plate (growth on mefenoxam/growth on nonamended $\times$ 100). At a given concentration, isolates were defined as (i) sensitive if hyphal growth was little or none, (ii) intermediate if hyphal growth was less than 50% compared with the growth on the nonamended plate, or (iii) resistant if hyphal growth was equal to or greater than 50% of growth on the nonamended plate. Each isolate was tested twice using a completely randomized design. Data were pooled based on the homogeneity of variances, and the proportion of isolates of each species that were sensitive, intermediate in resistance, or resistant was calculated.

Growth inhibition. The effective concentration of mefenoxam providing 50% growth inhibition (EC₅₀) was determined for isolates of *Phytophthora* that initially grew with mefenoxam at 1 μ g a.i./ml (16,27). Isolates within species were divided into groups based on location, host of origin, and initial mefenoxam response. Up to three representative isolates per group were selected arbitrarily and grown on three replicate CMA plates containing a range of mefenoxam concentrations of 0.1, 0.2, 0.5, and 1 μ g a.i./ml; 100, 200, and 500 μ g a.i./ml; or 200, 500, and 1,000 μ g a.i./ml. Hyphal growth was measured perpendicularly when growth on the nonamended control plate reached the plate edge. The percent growth inhibition was determined and converted to a probability scale (probit). The mean across isolates within a group was determined.

The experiment was conducted twice and the data pooled based on homogeneity of variances. A log-probit regression analysis was conducted with fungicide concentration expressed on the logarithmic scale (SAS 9.2; SAS Institute, Cary, NC). The resulting regression equations were used to interpolate the EC₅₀ for each group of isolates.

Comparison of molecular methods. The RFLP analysis used to identify isolates collected in the 2001 to 2002 North Carolina survey as *P. cryptogea* (27) was compared with sequencing of the ITS region to confirm reliability of the RFLP method. Four isolates from the 2001 to 2002 survey and four isolates from the present survey were selected arbitrarily for the RFLP. In addition, the reference isolates of *P. cryptogea* (American Type Culture Collection [ATCC]15402 and ATCC15403) from the 2001 to 2002 North Carolina survey (27) and a well-characterized isolate of *P. cryptogea* (p1088/ATCC46721) (50) and *P. drechsleri* (p1087/ATCC46724) (48), obtained from the World Oomycete Genetic Resource Collection, were included in the RFLP. DNA extraction, and PCR amplification of the ITS region were conducted as described above. For the RFLP analysis, the PCR product was digested with the *Hae*III restriction enzyme following published methods (27,40). Each restriction digest was performed twice and visualized by gel electrophoresis (1.5% agarose; 55 mA for 2 h).

Results

Collection and species identification of *Phytophthora* isolates. In total, 1,228 symptomatic tissue samples from 39 plant species were collected from 25 wholesale greenhouse production facilities

in North Carolina (Table 1). *Phytophthora* spp. were isolated from 13 host plant species, including annual vinca, blue daze (*Evolvulus glomeratus*), bugleweed (*Ajuga reptans*), calibrachoa (*Calibrachoa × hybrida*), cushion spurge (*Euphorbia polychroma*), dusty miller, fuchsia (*Fuchsia × hybrida*), gardenia (*Gardenia jasminoides*), gerbera daisy, gloxinia (*Sinningia speciosa*), petunia, pothos (*Epipremnum aureum*), and verbena (*Verbena × hybrida*) at 11 locations. In total, 163 isolates of *Phytophthora* were collected (Table 1). Based on morphological characteristics and sequencing of the ITS region, the isolates were identified as *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. nicotianae*, *P. tropicalis*, and an undescribed species (Table 2). A comparison of these results with the results of the 2001 to 2002 North Carolina survey is illustrated in Table 3.

In total, 97 isolates of *P. nicotianae* (59% of total isolates collected) were collected from annual vinca, calibrachoa, cushion spurge, dusty miller, fuchsia, gardenia, gerbera daisy, petunia, and verbena at six locations. *P. drechsleri* comprised 23% of isolates and was collected from fuchsia and gerbera daisy at two locations. In all, 16 isolates of *P. cryptogea* (9%) were obtained from blue daze, dusty miller, gerbera daisy, and verbena plants retrieved from 5 of 11 locations. *P. tropicalis* was collected from gloxinia, pothos, and verbena at three locations, resulting in seven isolates (4%). Six isolates of an undescribed species were collected from bugleweed at a single location, and a single isolate of *P. citrophthora* was collected from petunia. Two or more species of *Phytophthora* were isolated at 5 of 11 greenhouse facilities. At three of these locations (B, F, and G), two species (*P. cryptogea* and *P. drechsleri*, *P. nico-*

Table 1. Plant species, number of plants and locations sampled, and number of *Phytophthora* isolates collected during a survey of floriculture crops in North Carolina greenhouse facilities in 2007 and 2008

Plants	Number of		
	Samples	Locations	Isolates
African violet, <i>Saintpaulia ionantha</i>	11	1	0
Annual vinca, <i>Catharanthus roseus</i>	75	5	1
Begonia, <i>Begonia</i> spp.	58	6	0
Blue anise sage, <i>Salvia guaranitica</i>	12	1	0
Blue daze, <i>Evolvulus glomeratus</i>	9	1	1
Bugleweed, <i>Ajuga reptans</i>	11	1	6
Calibrachoa, <i>Calibrachoa × hybrida</i>	106	7	18
Celosia, <i>Celosia crista</i>	36	4	0
Cushion spurge, <i>Euphorbia polychroma</i>	24	2	10
Dahlia, <i>Dahlia × hybrida</i>	23	2	0
Duranta, <i>Duranta erecta</i>	10	1	0
Dusty miller, <i>Senecio cineraria</i>	69	4	36
Dwarf papyrus, <i>Cyperus</i> spp.	12	1	0
English ivy, <i>Hedera helix</i>	17	2	0
Fuchsia, <i>Fuchsia × hybrida</i>	10	1	7
Gardenia, <i>Gardenia jasminoides</i>	10	1	7
Geranium, <i>Pelargonium × hortorum</i>	1	1	0
Gerbera daisy, <i>Gerbera jamesonii</i>	297	15	40
Gloxinia, <i>Sinningia speciosa</i>	5	2	2
Gomphrena, <i>Gomphrena globosa</i>	10	1	0
Japanese pachysandra, <i>Pachysandra terminalis</i>	11	1	0
Lantana, <i>Lantana camara</i>	26	3	0
Lavender, <i>Lavandula angustifolia</i>	12	1	0
Lisianthus, <i>Eustoma grandiflorum</i>	12	1	0
Melampodium, <i>Melampodium paludosum</i>	7	1	0
Mexican heather, <i>Cuphea hyssopifolia</i>	10	1	0
New Guinea impatiens, <i>Impatiens</i> spp.	20	2	0
Pothos, <i>Epipremnum aureum</i>	5	1	2
Periwinkle, <i>Vinca minor</i>	10	1	0
Petunia, <i>Petunia × hybrida</i>	154	9	19
Primrose, <i>Primula × polyantha</i>	5	1	0
Shamrock, <i>Oxalis triangularis</i>	11	1	0
Shrimp plant, <i>Justicia brandegeana</i>	10	1	0
Snapdragon, <i>Antirrhinum majus</i>	39	3	0
Summer snapdragon, <i>Angelonia angustifolia</i>	10	1	0
Sweet potato vine, <i>Ipomoea batatas</i>	10	1	0
Tickseed, <i>Coreopsis</i> spp.	8	1	0
Verbena, <i>Verbena × hybrida</i>	51	5	14
Wishbone flower, <i>Torenia fournieri</i>	11	1	0

tianae and *P. citrophthora*, and *P. nicotianae* and *P. drechsleri*, respectively) coexisted within the same epidemic although, in each instance, the individual species were isolated from separate plants (Table 2).

Comparison of RFLP and DNA sequencing. All of the known and suspect isolates of *P. cryptogea* and *P. drechsleri* tested yielded the same RFLP pattern following digestion with *Hae*III. Sequencing of the ITS region and subsequent BLAST analysis identified isolates p1087, ATCC15403, NCB88, NCB103, CRGB8, FWGB5, FDM11, and FWDM19 as *P. drechsleri* (Table 4). Only isolates p1088, ATCC15402, NCB108, and NCB110 were identified as *P. cryptogea* by sequencing (Table 4). A subsequent virtual RFLP of the DNA sequences of the known isolates using Sequencher 4.9 showed that *Hae*III produced almost identical banding patterns in *P. cryptogea* (fragment sizes 696, 73, 49, 6, and 4 bp) and *P. drechsleri* (fragment sizes 695, 73, 49, 6, and 4 bp). After obtaining these results, the ITS region of all isolates from the 2001 to 2002 survey originally identified as *P. cryptogea* were sequenced and subjected to BLAST analysis. All isolates were identified as *P. drechsleri* by DNA sequencing and will be referred to as such during the remaining discussion.

Mating type of *Phytophthora* isolates. Only the A1 mating type of *P. drechsleri* was recovered from North Carolina greenhouse facilities (Table 2). In contrast, both the A1 and A2 mating types of *P. cryptogea*, *P. nicotianae*, and *P. tropicalis* were recovered. Of the 97 isolates of *P. nicotianae*, 78 isolates (80%) were the A1 mating type and 19 isolates (20%) were A2 (Fig. 1). The A1 mating type of *P. nicotianae* was collected from calibrachoa, cushion spurge, dusty miller, gerbera daisy, and petunia at five locations, and the A2 mating type was isolated from annual vinca, gardenia, gerbera daisy, and verbena at four locations (Table 2). At two locations, both mating types of *P. nicotianae* were collected; however, isolates of each mating type were from separate epidemics. At location G, the A1 mating type was isolated from fuchsia and the A2 mating type from annual vinca. Similarly, at location H, the A1 mating type of *P. nicotianae* was isolated from cushion spurge and the A2 mating type from verbena (Table 2). Isolates of the A1 and A2 mating types from each facility were paired in culture and found to produce abundant oospores. Of the 16 isolates of *P. cryptogea*, 13 isolates (80%) were the A2 mating type, which were collected from blue daze, gerbera daisy, and dusty miller at three locations, and 3 isolates were the A1 mating type and were col-

Table 2. Location, host of origin, and phenotypic characteristics of isolates of *Phytophthora* collected during 2007 and 2008 from floriculture crops in North Carolina

Group ^b	Location	Host	<i>Phytophthora</i> spp.	Isolates ^c	MT ^d	Mefenoxam sensitivity ^a		EC ₅₀ ^e
						1 µg a.i./ml	100 µg a.i./ml	
1	A	Dusty miller	<i>P. nicotianae</i>	26	A1	Resistant	Resistant	415
2	A	Gerbera daisy	<i>P. nicotianae</i>	2	A1	Sensitive	Sensitive	NA
3	F	Petunia	<i>P. nicotianae</i>	9	A1	Resistant	Resistant	353
4	F	Petunia	<i>P. nicotianae</i>	9	A1	Resistant	Resistant	429
5	F	Calibrachoa	<i>P. nicotianae</i>	8	A1	Resistant	Resistant	427
6	F	Gardenia	<i>P. nicotianae</i>	7	A2	Sensitive	Sensitive	NA
7	G	Fuchsia	<i>P. nicotianae</i>	4	A1	Resistant	Resistant	363
8	G	Annual vinca	<i>P. nicotianae</i>	1	A2	Sensitive	Sensitive	NA
9	H	Cushion spurge	<i>P. nicotianae</i>	10	A1	Sensitive	Sensitive	NA
10	H	Verbena	<i>P. nicotianae</i>	9	A2	Sensitive	Sensitive	NA
11	I	Gerbera daisy	<i>P. nicotianae</i>	1	A2	Sensitive	Sensitive	NA
12	I	Verbena	<i>P. nicotianae</i>	1	A2	Sensitive	Sensitive	NA
13	K	Calibrachoa	<i>P. nicotianae</i>	10	A1	Resistant	Intermediate	247
14	B	Gerbera daisy	<i>P. drechsleri</i>	29	A1	Resistant	Resistant	341
15	G	Gerbera daisy	<i>P. drechsleri</i>	3	A1	Resistant	Resistant	727
16	G	Fuchsia	<i>P. drechsleri</i>	3	A1	Resistant	Resistant	910
17	G	Gerbera daisy	<i>P. drechsleri</i>	1	A1	Resistant	Resistant	755
18	B	Gerbera daisy	<i>P. cryptogea</i>	2	A1	Sensitive	Sensitive	NA
19	C	Blue daze	<i>P. cryptogea</i>	1	A2	Sensitive	Sensitive	NA
20	E	Gerbera daisy	<i>P. cryptogea</i>	2	A2	Sensitive	Sensitive	NA
21	I	Verbena	<i>P. cryptogea</i>	1	A1	Sensitive	Sensitive	NA
22	J	Dusty miller	<i>P. cryptogea</i>	10	A2	Sensitive	Sensitive	NA
23	B	Gloxinia	<i>P. tropicalis</i>	2	A2	Sensitive	Sensitive	NA
24	F	Verbena	<i>P. tropicalis</i>	3	A1	Intermediate	Sensitive	0.49
25	K	Pothos	<i>P. tropicalis</i>	2	A2	Intermediate	Sensitive	0.31
26	F	Petunia	<i>P. citrophthora</i>	1	A0	Sensitive	Sensitive	NA
27	D	Bugleweed	<i>Phytophthora</i> sp.	6	A2	Sensitive	Sensitive	NA

^a Isolates were (i) sensitive if hyphal growth was little to none, (ii) intermediate in resistance if hyphal growth was less than 50% of growth of control cultures, or (iii) resistant if hyphal growth was greater than 50% of growth of control cultures.

^b A group is defined as isolates of the same species of *Phytophthora* collected from a single plant species at the same location on a survey date.

^c Number of isolates.

^d MT = mating type.

^e Concentration (µg a.i./ml) of mefenoxam providing 50% growth inhibition (EC₅₀) for isolates that grew with mefenoxam at 1 µg a.i./ml. NA = not assessed.

Table 3. Comparison of survey results between the 2007 to 2008 present North Carolina survey and a 2001 to 2002 North Carolina survey

Survey year	Number of locations		Number of plant species		Total number of	
	Sampled	Isolated ^a	Sampled	Isolated ^a	<i>Phytophthora</i> spp.	Isolates collected ^b
2007–2008	25	11	39	13	6	163
2001–2002 ^c	29	7	41	8	3	483

^a Locations or plant species where *Phytophthora* spp. were isolated.

^b Differences in isolate numbers reflect differences in sampling methods between the two surveys. During the 2007 to 2008 survey, up to 12 plants per epidemic were collected, whereas up to 120 plants were collected from an epidemic during the 2001 to 2002 survey (27). An epidemic was defined as symptomatic plants of the same species and age occurring within a growing block at a location.

^c Previous survey by Hwang and Benson (27).

lected from gerbera daisy and verbena at two locations (Table 2; Fig. 1). For *P. tropicalis*, three of the seven isolates were the A1 mating type and were recovered from verbena at a single location. The remaining four isolates from gloxinia and pothos were the A2 mating type (Table 2; Fig. 1).

Mefenoxam sensitivity responses. For all *Phytophthora* spp., isolates collected from the same epidemic were homogeneous in their reaction (sensitive, intermediate, or resistant) to mefenoxam. Overall, 107 of 163 isolates of *Phytophthora* (66%) were resistant or intermediate in resistance to mefenoxam at 1 µg a.i./ml, and 102 of the 107 isolates also were resistant or intermediate in resistance at 100 µg a.i./ml. For *P. nicotianae*, 68% of isolates were resistant to mefenoxam at 1 µg a.i./ml (Fig. 2). For mefenoxam at 100 µg a.i./ml, 58% of isolates were resistant and 10% were intermediate in resistance. Resistant isolates were found at four of the six facilities from which *P. nicotianae* was collected (Table 2). At three of the facilities, both resistant and sensitive isolates were recovered; however, the resistance response depended on the host of origin within the facility. At location A, resistant isolates were collected from dusty miller and sensitive isolates from gerbera daisy and, at location F, resistant isolates were recovered from petunia and calibrachoa and sensitive isolates from gardenia (Table 2). Likewise, resistant isolates were collected from fuchsia and sensitive isolates from annual vinca at location G. The EC₅₀ estimates for isolates of *P. nicotianae* that were resistant to mefenoxam at 1 µg a.i./ml were similar and ranged from 247 to 429 µg a.i./ml (Fig. 3; Table 2).

Regardless of location or host, all isolates of *P. cryptogea* collected from North Carolina greenhouses were sensitive to mefenoxam at 1 µg a.i./ml. In contrast, all isolates of *P. drechsleri* were resistant to mefenoxam at 1 and 100 µg a.i./ml (Fig. 2). Three groups of *P. drechsleri* isolates from the same location (groups 15, 16, and 17) had EC₅₀ estimates over 700 µg a.i./ml (Fig. 3; Table 2). Group 14 had an EC₅₀ estimate of 341 µg a.i./ml. For *P. tropicalis*, five of seven isolates were intermediate in resistance to mefenoxam at 1 µg a.i./ml, and the remaining two isolates were sensitive. For mefenoxam at 100 µg a.i./ml, all *P. tropicalis* isolates were sensitive (Fig. 2; Table 2). For *P. tropicalis*, the EC₅₀ estimates were 0.49 and 0.31 µg a.i./ml (groups 24 and 25, respectively; Fig. 3; Table 2).

Discussion

Five described species (*P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. nicotianae*, and *P. tropicalis*) and one undescribed species of *Phytophthora* were collected and identified during the 2007 to 2008 survey of North Carolina greenhouse facilities. In contrast, only *P. drechsleri*, *P. nicotianae*, and *P. palmivora*, were isolated during the 2001 to 2002 North Carolina survey (27). Our results indicate that digestion with the *Hae*III restriction enzyme does not have the resolution needed to distinguish *P. cryptogea* from *P. drechsleri*. Based on this and the DNA sequencing results,

it appears that isolates identified as *P. cryptogea* during the 2001 to 2002 North Carolina survey are actually *P. drechsleri*. *Phytophthora* spp. were isolated from more greenhouse facilities in the present survey compared with the previous survey, suggesting that the incidence of *Phytophthora* spp. has increased in the North Carolina greenhouse industry. Of the 17 facilities that were visited in both surveys, only five greenhouses had *Phytophthora* spp. present during both surveys. At two of the five facilities, the species of *Phytophthora* and the mating type and mefenoxam sensitivity were the same over the two surveys. This suggests that these two facilities could have *Phytophthora* spp. established onsite and should focus their management emphasis on eradication. In contrast, at facilities where multiple species of *Phytophthora* were recovered or where isolates exhibiting different phenotypes were recovered, introduction of *Phytophthora* spp. on new planting material may be more important.

P. nicotianae remained the most prevalent species of *Phytophthora* in North Carolina floriculture crops, comprising 59% of isolates in the present survey and 57% of isolates in the previous survey (27). *P. nicotianae* is a well-documented pathogen in floriculture crops grown in greenhouse production. In the present survey, *P. nicotianae* was isolated from nine hosts, all of which have been reported previously (1,5,12,20,39,54). Petunia and annual vinca were the only two hosts common to both North Carolina

Fig. 1. Proportion of A1 and A2 mating types of *Phytophthora nicotianae*, *P. drechsleri*, *P. cryptogea*, and *P. tropicalis* as determined by three replicate pairings with tester isolates of known mating type in culture. Numbers in parentheses on the x-axis indicate the number of isolates of a species tested.

Table 4. Comparison of identifications of *Phytophthora cryptogea* and *P. drechsleri* isolates obtained by restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA using the *Hae*III restriction enzyme and by sequencing of the ITS region

Isolate	Isolate information	Identification method	
		RFLP	Sequencing
p1088/ATCC46721 ^a	Well-characterized isolate of <i>P. cryptogea</i>	<i>P. cryptogea</i>	<i>P. cryptogea</i>
p1087/ATCC46724 ^a	Well-characterized isolate of <i>P. drechsleri</i>	<i>P. cryptogea</i>	<i>P. drechsleri</i>
ATCC15402 ^b	2001–2002 <i>P. cryptogea</i> control	<i>P. cryptogea</i>	<i>P. cryptogea</i>
ATCC15403 ^b	2001–2002 <i>P. cryptogea</i> control	<i>P. cryptogea</i>	<i>P. drechsleri</i>
NCB108	2007–2008 North Carolina from gerbera daisy	<i>P. cryptogea</i>	<i>P. cryptogea</i>
NCB110	2007–2008 North Carolina from gerbera daisy	<i>P. cryptogea</i>	<i>P. cryptogea</i>
NCB88	2007–2008 North Carolina from gerbera daisy	<i>P. cryptogea</i>	<i>P. drechsleri</i>
NCB103	2007–2008 North Carolina from gerbera daisy	<i>P. cryptogea</i>	<i>P. drechsleri</i>
CRGB8 ^b	2001–2002 North Carolina from gerbera daisy	<i>P. cryptogea</i>	<i>P. drechsleri</i>
FWGB5 ^b	2001–2002 North Carolina from gerbera daisy	<i>P. cryptogea</i>	<i>P. drechsleri</i>
FDM11 ^b	2001–2002 North Carolina from dusty miller	<i>P. cryptogea</i>	<i>P. drechsleri</i>
FWDM19 ^b	2001–2002 North Carolina from dusty miller	<i>P. cryptogea</i>	<i>P. drechsleri</i>

^a Isolate from World Oomycete Genetic Resource Collection (48,50).

^b Isolate from 2001 to 2002 survey of North Carolina greenhouses (27).

surveys. During the previous North Carolina survey, 38% of isolates were *P. drechsleri* (27), while only 23% of isolates were *P. drechsleri* in the present survey. *P. drechsleri* was isolated from fuchsia and gerbera daisy. Although fuchsia is a common host for *P. nicotianae* in greenhouse production (21,39), it appears that fuchsia has not been reported as a host of *P. drechsleri*. Gerbera daisy was a host of *P. drechsleri* during the 2001 to 2002 North Carolina survey (27) and has been reported as a host in other areas of the United States, Mexico, and Australia (4,18,41,42). *P. cryptogea* was recovered from more facilities than *P. drechsleri* but only represented 9% of *Phytophthora* isolates. At the four locations where only one to two isolates of *P. cryptogea* were recovered from an epidemic, growers reported treating with fungicides prior to our sampling, which may have impacted isolations because all the collected isolates of *P. cryptogea* were sensitive to mefenoxam. *P. cryptogea* was isolated from gerbera daisy, dusty miller, blue daze, and verbena. Gerbera daisy and dusty miller are commonly reported ornamental hosts for *P. cryptogea* throughout the world (18,28,29,31,36,44,49,56); however, no reports of blue daze or verbena as hosts have been found. Recently described in 2001, *P. tropicalis* was found on gloxinia, pothos, and verbena in this survey (2). This species has been reported with increasing frequency on ornamental hosts (11,19,22,24,35,37). In particular, *P. tropicalis* has caused significant problems in the production of pothos (33,37). The isolates of *P. tropicalis* collected during this survey grow quickly, produce abundant sporangia within 72 h, and release

zoospores readily during minor temperature fluctuations, all characteristics providing advantages in the greenhouse system. Based on this, *P. tropicalis* should be considered a species with emerging importance in greenhouse production. A single isolate of *P. citrophthora* was collected from petunia during this survey. Although this species is common on many woody ornamental hosts in nursery production (11,43,55), *P. citrophthora* is not a significant species in greenhouse production. In the present study, two species of *Phytophthora* coexisted within the same epidemic at three locations. In the previous survey of North Carolina greenhouses, there were no instances where two species of *Phytophthora* were isolated from the same epidemic (27).

Of the 163 isolates of *Phytophthora* collected during this survey, 66% were resistant or intermediate in resistance to mefenoxam at 1 μg a.i./ml. In contrast, 48% of isolates were resistant or intermediate in resistance in the previous North Carolina survey (27). This suggests that the prevalence of mefenoxam resistance in *Phytophthora* spp. is increasing in the North Carolina greenhouse industry. The most significant increase in mefenoxam resistance occurred in *P. nicotianae*. In the previous North Carolina survey, only 21% of isolates of *P. nicotianae* were resistant to mefenoxam

Fig. 2. Proportion of *Phytophthora nicotianae*, *P. drechsleri*, *P. cryptogea*, and *P. tropicalis* isolates exhibiting sensitivity (S), intermediate resistance (Int), and resistance (R) to mefenoxam at A, 1 μg a.i./ml and B, 100 μg a.i./ml based on percent growth inhibition on mefenoxam-amended medium. Numbers in parentheses on the x-axis indicate the number of isolates of a species tested.

Fig. 3. Concentration-response curves for hyphal growth inhibition of *Phytophthora* isolates collected from North Carolina floriculture crops by mefenoxam. Percent inhibition of growth by mefenoxam was transformed to a probability, and the concentration was transformed to the logarithmic scale. A, Isolates of *Phytophthora tropicalis* collected from verbena (group 24) and pothos (group 25). B, Isolates of *P. drechsleri* recovered from gerbera daisy (groups 14, 15, and 17) and fuchsia (group 16). C, Isolates of *P. nicotianae* collected from dusty miller (group 1), petunia (groups 3 and 4), calibrachoa (groups 5 and 13), and fuchsia (group 7).

at 1 and 100 µg a.i./ml (27), whereas 68% of isolates were resistant or intermediate in resistance to mefenoxam at 1 and 100 µg a.i./ml in the present study. A similar increase was observed in Virginia (25), suggesting that mefenoxam resistance in *P. nicotianae* found in ornamentals may be of an increasing concern in other states, as well. All isolates of *P. drechsleri* from this survey were resistant to mefenoxam at 1 and 100 µg a.i./ml. Similar levels of mefenoxam resistance were reported in the previous survey, where all isolates were resistant or intermediate in resistance to 1 µg a.i./ml and only 5 of the 184 isolates of *P. drechsleri* were sensitive to mefenoxam at 100 µg a.i./ml (27). At one location where *P. drechsleri* was recovered during both North Carolina surveys, the level of mefenoxam resistance was sustained over the 6-year period between surveys. This suggests that mefenoxam-resistant isolates are not being replaced by mefenoxam-sensitive isolates in the greenhouse system. Hu et al. (25) found that mefenoxam-resistant isolates of *P. nicotianae* outcompeted mefenoxam-sensitive isolates in the absence of mefenoxam. In addition, the mefenoxam-resistant isolates were significantly more fit in all aspects except mycelial growth (25).

Interestingly, *P. nicotianae* group 7 and *P. drechsleri* group 16 were collected from the same epidemic (i.e., growing block) of Phytophthora root rot on fuchsia at location G but have significantly different EC₅₀ estimates (mefenoxam at 363 and 910 µg a.i./ml, respectively). This occurrence suggests that these two species may have differing predispositions to developing resistance to mefenoxam. Variations in sensitivity have been observed within and between other *Phytophthora* spp. with isolates collected before the commercial deployment of metalaxyl. Hunger and Hansen (26) found that 12 of 35 isolates of *P. megasperma* were highly tolerant to metalaxyl at 1 and 10 µg a.i./ml. Similarly, Coffey and Bower (7) reported significant variation in responses to metalaxyl among 15 isolates of *P. capsici* from pepper, which ranged from 39.1 to 85.6 µg a.i./ml for metalaxyl. In addition, most isolates of *P. capsici* were more tolerant to metalaxyl at 0.1 and 1 µg a.i./ml than isolates of *P. parasitica* (i.e., *P. nicotianae*). In a similar study, Coffey et al. (8) found that most isolates of *P. citricola* were more tolerant of metalaxyl than isolates of *P. cinnamomi*.

Though in vitro assays are utilized frequently to examine fungicide resistance, there is some question as to whether in vitro fungicide resistance translates to fungicide resistance during commercial use. Screening for mefenoxam resistance using mefenoxam-amended media appears to be a good indicator of mefenoxam resistance in ornamentals produced in greenhouses. Ferrin and Rohde (17) observed that metalaxyl failed to control Phytophthora root rot in annual vinca inoculated with an isolate of *P. nicotianae* having an EC₅₀ of metalaxyl of 717.4 µg a.i./ml. More recently, Hu et al. (25) found that mefenoxam provided complete control of mefenoxam-sensitive isolates of *P. nicotianae* in geranium; however, a complete loss of control by mefenoxam was observed in geranium plants inoculated with mefenoxam-resistant isolates. Similarly, two mefenoxam-resistant isolates of *P. drechsleri* collected during the present survey caused 100% mortality in gerbera daisy treated with mefenoxam (Subdue Maxx at 0.08 ml/liter) (3). All of the facilities where mefenoxam-resistant isolates were collected during our survey reported regular use of mefenoxam and had significant epidemics of Phytophthora root rot present. The overall high percentage of mefenoxam resistance exhibited in the present study suggests that North Carolina commercial floriculture growers have a high likelihood of experiencing a loss of control when using mefenoxam and that other chemistries should be used in the management of *Phytophthora* spp.

All of the species of *Phytophthora* recovered during this survey were heterothallic. Like the previous North Carolina survey (27), all isolates of *P. drechsleri* were the A1 mating type. Similarly, Lamour et al. (32) reported only the A1 mating type in their survey of floriculture crops in select U.S. greenhouse facilities. Though both mating types of *P. cryptogea* and *P. tropicalis* were recovered from floriculture crops, both mating types were not present within the same facility, which greatly reduces the opportunity for sexual reproduction to occur. In contrast, both mating types of *P. nicotianae*

were collected within the same production facility at two locations. When crossed, the isolates within a facility were highly compatible and produced abundant oospores, suggesting that there is a chance of sexual reproduction should they come in contact. During the previous survey, all isolates of *P. nicotianae* within a facility were the same mating type (27). Asexual reproduction appears to be the primary means of reproduction in greenhouse systems. Oospores can be important survival structures, particularly in species that do not produce chlamydospores (12); however, because production is year-round in greenhouse facilities, new plant material is constantly introduced, providing new host material, which allows the asexual life cycle to perpetuate. This likely reduces the importance of oospores in the life cycle because long-term survival structures are less likely to be needed to allow the persistence of *Phytophthora* spp. in the greenhouse.

The diversity of *Phytophthora* spp. prevalent in floriculture crops in North Carolina commercial greenhouse facilities has increased in the past 5 years, particularly with the occurrence of *P. tropicalis* on gloxinia, verbena, and pothos. In addition, *Phytophthora* spp. were recovered from a wider range of hosts compared with the previous North Carolina survey (27). The overall diversity of species of *Phytophthora* on floriculture crops is limited compared with woody ornamentals. For example, over 15 species of *Phytophthora* have been reported from hybrid rhododendron (*Rhododendron* spp.) alone (6,11,34,38,43,45,53,55). Even though the diversity of *Phytophthora* spp. is limited in floriculture crops, most species, with the exception of *P. cryptogea*, have developed mefenoxam resistance, presumably through frequent fungicide applications on highly susceptible hosts. Most notably, *P. nicotianae*, the most prevalent species recovered, had over 50% of the isolates resistant to mefenoxam at 100 µg a.i./ml. Growers will continue to face challenges for fungicide rotation schemes as the occurrence of mefenoxam-resistant isolates increases and as new fungicides with a high risk of developing resistance are introduced.

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