ABSTRACT

LOOKABAUGH, EMMA CHRISTINE. Integrated strategies for managing Pythium root rot and fungicide-insensitive strains of *Pythium aphanidermatum* in poinsettia. (Under the direction of Drs. Barbara Shew and Marc Cubeta).

Pythium aphanidermatum is the predominant species causing Pythium root rot of commercially grown poinsettia in North Carolina. Pythium root rot is managed primarily through a combination of sanitation practices and preventative fungicide applications, but growers need additional tools, such as host resistance and rotation programs, to implement a more integrated approach to disease management. Commercially available poinsettia cultivars were inoculated with *P. aphanidermatum* three weeks after transplant and evaluated for resistance to Pythium root rot. Most cultivars were susceptible to Pythium root rot and none were completely resistant. However, several cultivars demonstrated partial resistance to Pythium root rot. Interspecific hybrid cultivars, including 'Luv U Pink', had higher levels of partial resistance when compared to conventional cultivars. These results suggest that partial resistance in poinsettia could be used in combination with fungicide applications to limit disease caused by *P. aphanidermatum*. Growers prefer to use mefenoxam for Pythium root rot control because of its high efficacy and low cost, but resistance to mefenoxam is widespread in populations of *Pythium*. Other products that could be used instead of or in combination with mefenoxam are not commonly used to control Pythium root rot on poinsettia. To identify candidate fungicides for Pythium root rot control, the efficacy of 10 fungicides was assessed on seven poinsettia cultivars inoculated with *P. aphanidermatum.* One experiment examined control with a single application of each fungicide made at transplant and another experiment examined repeat applications of the fungicides made

throughout the experiment. Treatments containing etridiazole, mefenoxam, fenamidone, and cyazofamid provided excellent control of Pythium root rot across all cultivars in both experiments. Mefenoxam and fenamidone, a quinone-outisde inhibitor (QoI), were the most efficacious among the fungicides tested against Pythium root rot on poinsettia. Four isolates of *P. aphanidermatum* previously collected from plants grown in commercial greenhouses also were evaluated for *in vitro* sensitivity to 10 fungicides. Etridiazole, fosetyl-al, and potassium phosphite completely inhibited mycelial growth of all isolates, whereas sensitivity varied in response to mefenoxam, cyazofamid, propamocarb, fenamidone, azoxystrobin, and pyraclostrobin. Twenty-one additional isolates were evaluated for *in vitro* sensitivity to three QoI fungicides. Seven isolates were insensitive to all three QoI fungicides and one isolate was insensitive to all QoIs and mefenoxam. It is not known whether insensitivity to QoI fungicides is widespread in populations of *Pythium*. *In-vitro* sensitivity or insensitivity to the label rate of mefenoxam (17.6 µl a.i/ml) and fenamidone (488 µl a.i./ml) was tested on 96 isolates of *P. aphanidermatum* and isolates were assigned to four fungicide resistance groups. Fifty-eight percent of isolates were insensitive to one (MefR, FenS=36%; MefS, FenR=16%) or both fungicides (MefR, FenR = 6%). A single point mutation in the cytochrome-b gene (G143A) was identified in all fenamidone-insensitive isolates. Mycelial growth at three temperatures, oospore production *in vitro*, and aggressiveness on poinsettia were evaluated to assess relative fitness of mefenoxam and fenamidone sensitive and insensitive isolates. Isolates insensitive to both mefenoxam and fenamidone had less radial mycelial growth at 30°C and produced fewer oospores *in vitro* than isolates sensitive to one or both fungicides, whereas isolates sensitive to both fungicides produced the most oospores *in vitro*.

Aggressiveness on poinsettia varied by isolate but fungicide resistance profiles were not always predictive of *in vivo* aggressiveness. These results suggest that populations of *P. aphanidermatum* with dual resistance to mefenoxam and fenamidone may be a threat in poinsettia production but may also be less fit than sensitive populations at higher temperatures. Additionally, this is the first report of insensitivity to QoI fungicides and dual insensitivity to QoIs and mefenoxam in isolates of *P. aphanidermatum* sampled from greenhouse floriculture crops. Since mefenoxam and fenamidone have high risk for resistance development, treatment programs that incorporate products with two or more modes of action were evaluated on cultivars with varying levels of partial resistance. Treatment programs using single applications (tank mixes) at transplant were compared with rotations of two or three fungicide applications. All six tank mix and rotation programs prevented Pythium root rot on all cultivars inoculated with insensitive isolates, even when mefenoxam and fenamidone were included in the rotation. These results provide growers with a choice of fungicide programs for use in their facilities.

Integrated strategies for managing Pythium root rot and fungicide-insensitive strains of *Pythium aphanidermatum* in poinsettia

by
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DEDICATION

I dedicate this work to my parents. I finally finished, WOOHOO!

BIOGRAPHY

Emma Lookabaugh was born in Sumter, SC on January 14, 1988. As the daughter of an Air Force pilot, she had the opportunity to live all over the US before landing in North Carolina. Emma attended North Carolina State University, where she double-majored in biological sciences and plant biology and had a minor in genetics. She graduated Valedictorian, Summa Cum Laude in May, 2010 and is a member of Phi Beta Kappa and Phi Kappa Phi national honor societies

Emma became interested in plant pathology during her sophomore year when she took a summer job Plant Disease and Insect Clinic (PDIC). Unbeknownst to Shawn Butler at the time, this would be the job that changed her career path and led her to pursue Master's and doctoral degrees in Plant Pathology, under the direction of Dr. Barbara Shew. For her Master's research, Emma characterized *Pythium* species collected from floriculture greenhouse operations in North Carolina. For her doctoral work, she explored integrated management strategies to control Pythium root rot on poinsettia caused by *Pythium aphanidermatum*. In addition to her thesis research, Emma continued working in the PDIC, where she was responsible for tomato disease diagnosis under the direction of Dr. Frank Louws.

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I am incredibly grateful for the constant support and friendship offered by my graduate student peers. I made it through a little blood, a lot of sweat, and a few tears with amazing friends by my side.

I offer my deepest appreciation to the folks at the Plant Disease and Insect Clinic for putting up with me for the past nine years. I especially want to thank Shawn Butler. You changed my path and I wouldn't be where I am today if you hadn't given me that job all those years ago. You taught me most of what I know about disease diagnosis and I will be

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CHAPTER 1

Evaluation of poinsettia cultivars for resistance to Pythium root rot

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Abstract

Pythium root rot is one of the most serious diseases of commercially grown poinsettia (*Euphorbia pulcherrima* Willd. ex Kotzch) and *Pythium aphanidermatum* (Edson) Fitzp. is the predominant species causing Pythium root rot in North Carolina. Under favorable environmental conditions, *P. aphanidermatum* causes stunting, root rot, wilting, defoliation, and in severe cases, plant death. Traditionally, Pythium root rot has been managed primarily through a combination of strict sanitation practices and preventative fungicide applications, but host resistance has not been considered a useful method for managing root rot in poinsettia. To determine if host resistance could play a role in the integrated management of Pythium root rot, information on the susceptibility of commercial poinsettia cultivars is needed. Commercially available poinsettia cultivars were inoculated with *P. aphanidermatum* three weeks after transplant and evaluated for resistance to Pythium root rot two months later. Thirty-four cultivars were evaluated for resistance in 2014 and 58 cultivars were evaluated in 2015, for a total of 62 cultivars evaluated. Twenty-nine cultivars were evaluated in both years. The majority of cultivars were susceptible to Pythium root rot and none were completely resistant. However, several cultivars demonstrated partial resistance to Pythium root rot. Interspecific hybrid cultivars, including 'Luv U Pink', had a higher level of

partial resistance when compared to conventional cultivars. Partial resistance varied across bract color, response time, and plant vigor groupings. Overall, six of 13 partially resistant cultivars identified in 2015 had red bracts. These results indicate that growers should be able to choose among several red bract cultivars that have higher level partial resistance to Pythium root rot than others.

Pythium root rot is one of the most common diseases of commercially grown poinsettia (*Euphorbia pulcherrima*) and *Pythium aphanidermatum* is the predominant species causing Pythium root rot in North Carolina (Lookabaugh et al., 2015). Under favorable environmental conditions, *P. aphanidermatum* causes stunting, root rot, wilting, defoliation, and in severe cases, plant death. Traditionally, Pythium root rot has been managed primarily through a combination of strict sanitation practices and preventative fungicide applications, but long production seasons and common irrigation practices make this disease difficult to control. Unfortunately, only a few fungicides are efficacious against *P. aphanidermatum* and resistance to mefenoxam, the primary active ingredient used to control Pythium root rot, is common in *Pythium* populations (Lookabaugh et al., 2015; Moorman et al., 2002; Moorman and Kim, 2004). To meet these challenges, growers need additional tools to implement a more integrated approach to disease management.

Host resistance is among the most important tools for integrated disease management, but disease resistance has not been widely used in floricultural crops, including poinsettia (Daughtrey and Benson, 2005). Commercial poinsettia cultivars have not been systematically evaluated for their response to Pythium root rot, nor has the genetic basis of

resistance been explored in university research (Trejo et al, 2012). Complete resistance to Pythium root rot is unknown in cultivated poinsettia and generally is lacking in other ornamental species (Munéra and Hausbeck 2015). Resistance to Pythium root rots is partial, i.e., quantitatively expressed, in geranium (Chagnon and Belanger, 1991), common bean (Lucas and Griffiths, 2004), soybean (Bates et al., 2008), and wheat (Higginbothom et al., 2004). Caladium (*Caladium* x *hortulanum* Birdsey) is one of the few floriculture species evaluated for resistance to Pythium root rot*;* seven of 42 cultivars expressed partial resistance when inoculated with *Pythium myriotylum* (Deng et al., 2005 a and b).

Complete resistance or immunity often is found in wild relatives of cultivated plants, which are used to develop resistant cultivars through hybridization and selection. Among many examples, closely related wild species are sources of resistance to Fusarium wilt in tomato (Gonzalez-Cendales, 2016), rusts in small grains (Miedaner and Korzun, 2012), and late blight resistance in potato (Fry, 2008). It is not known if wild and cultivated relatives of poinsettia are sources of resistance to Pythium root rot, or if interspecific hybridization can offer increased resistance to Pythium root rot. However, poinsettia breeding programs recently have incorporated interspecific hybrids of *Euphorbia pulcherrima* and the uncultivated *E. cornastra* or *E. fulgens* into commercial production lines (Ecke et al., 2004). These cultivars have been introduced primarily for their novel growth habits, which feature numerous, small, flat bracts. As with other poinsettia cultivars, information about their resistance or susceptibility to Pythium root rot is lacking.

Growers and breeders could benefit from information about the response of poinsettia cultivars to Pythium root rot to avoid cultivars that are highly susceptible and to integrate

partially resistant cultivars into their disease management programs. The objective of this research was to evaluate commercially available poinsettia cultivars and hybrids for resistance to Pythium root rot caused by *P. aphanidermatum.*

Materials and Methods

Isolate selection and inoculum preparation

The isolate of *Pythium aphanidermatum* used in this study (PA1, mefenoxam-sensitive) was isolated from rotted poinsettia roots sampled from a plant growing in a commercial greenhouse in North Carolina. Previous research demonstrated that this isolate was highly aggressive on poinsettia (Lookabaugh et al., 2015). Inoculum was prepared by transferring four 4-mm-diameter agar disks from an actively growing culture into 125-ml flasks containing twice-autoclaved long-grain rice (25 g rice and 18 ml deionized water). Cultures were incubated for 5 days at 25°C (Holmes and Benson, 1994). Flasks were shaken twice daily to promote uniform colonization of rice grains and prevent clumping.

Experimental design, inoculation, and resistance evaluation

Experiments were conducted over two years to evaluate poinsettia cultivars for resistance to Pythium root rot. Thirty-four cultivars were evaluated in 2014 and 58 cultivars were evaluated in 2015. In 2014, all cultivars tested came from Dümmen Orange (including Red Fox and Ecke Ranch; n = 34), whereas three sources were represented in 2015 (Dümmen Orange, n = 33; Syngenta, n = 23; Beekenkamp, n = 2). A few hybrid and numbered cultivars also were included. Cultivars represented an assortment of horticultural traits, including response time (early, middle, and late), vigor (compact, medium, and tall), and bract color (red, white, pink, and novelty) as determined by information provided by the propagator

(Tables 1.1 and 1.2). Rooted cuttings were donated from commercial propagators and transplanted to 15-cm pots containing Fafard 4P potting medium (Fafard, Agawam, MA). Plants were inoculated by placing six *P. aphanidermatum* colonized rice grains evenly in the potting media around the plant base (1-cm deep and 2.5-cm from the stem) ca. 3 weeks after transplanting. Four replicate plants were inoculated for each cultivar and non-inoculated plants were included as controls. A randomized complete block design with four blocks was used in each trial. Plants were fertilized with 5 g of Multicote 4 (14-14-16) (Haifa Chemicals ltd. Haifa, Israel), maintained under drip irrigation, and exposed to natural light cycles. To control whiteflies, plants were treated with dinotefuran (Safari, 1.25 ml per liter; Valent USA Corporation, Walnut Creek, CA) prior to inoculation. Experiment one was transplanted 15 Aug. 2014 and evaluated 23 Nov. 2014. Experiment two was transplanted 13 Aug. 2015 and evaluated 16 Nov. 2015. Plants were soft-pinched approximately 3 weeks after inoculation.

At the end of the experiments, disease severity was evaluated on a visual scale of 1 = healthy plant, 2 = slightly stunted, 3 = chlorosis, moderate stunting and/or defoliation, 4 = wilting and/or severe stunting, and 5 = dead (Lookabaugh, et al., 2015). Plants were inverted, the pot was removed, and roots were observed with the substrate staying intact. Root rot severity was visually assessed based on the size and integrity of the root ball and root color. The rating scale used 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 lost all

integrity (Parker and Benson, 2013). Root rot ratings and above-ground disease severity ratings were averaged to obtain a combined disease severity score for each cultivar.

Analysis of variance and correlation were performed using PROC GLIMMIX and PROC CORR procedures (SAS 9.4, SAS Institute, Cary, NC). Cultivar was treated as a fixed effect and block was treated as a random effect. Least-squares means were calculated for each cultivar to determine significant differences among cultivars ($P < 0.05$) and were separated using paired t-tests where appropriate. Horticultural traits, including response time, vigor, and bract color, were used to group cultivars and means were separated using paired t-tests where appropriate. Contrasts also were performed to compare mean disease severity scores of hybrid cultivars with traditional cultivars. Data from each year were analyzed separately.

Twenty-nine cultivars were represented in both 2014 and 2015. A separate analysis of variance was performed on cultivar data combined over years. Cultivar was treated as a fixed effect while block and trial were considered random effects. Least-squares means were calculated for each cultivar to determine significant differences and were separated with paired t-tests. Population quartiles and percentiles were computed using PROC UNIVARIATE.

Results and Discussion

All cultivars developed root rot, but root rot and above-ground symptoms varied across cultivars. Spearman's rank correlation analysis was performed to determine relationships between root rot ratings and disease severity ratings for each experiment. Ranks of cultivars by root rot ratings and disease severity ratings were correlated in both

experiments (2014, $r = 0.43$, $P < 0.001$; 2015, $r = 0.86$, $P < 0.001$), so root rot ratings and above-ground disease severity ratings were averaged to obtain a combined disease severity score for each observation. Disease severity scores (DSS) were used to separate cultivar means and to categorize partial resistance in each experiment (Tables 1.1 and 1.2).

Experiment one: disease resistance, 2014. All cultivars exhibited symptoms but disease severity varied by cultivar. The majority of cultivars exhibited severe root rot and above-ground symptomology, including severe stunting, foliar chlorosis, wilting, and defoliation (Table 1.1). The symptoms were so severe that these plants would not be suitable for sale. 'Sparkling Punch' was the least resistant (lsmean DSS = 4.8) with plant mortality in two replicate plants 8 days after inoculation (Figure 1.1). Hybrid cultivars 'Princettia Max White' and 'Luv U Pink' had the lowest disease severity scores (lsmean DSS = 2.1). These cultivars exhibited slight stunting and minor root damage. Some plants of 'Princettia Max White' and 'Luv U Pink' were asymptomatic, but *P. aphanidermatum* was readily isolated from roots. Asymptomatic plants or plants with minor above-ground symptoms probably could be sold, but if overlooked in production systems, they could harbor pathogen propagules and serve as a source of inoculum for more susceptible plants or subsequent crops.

Relationships among horticultural traits and resistance, 2014. Cultivars were grouped by response time, color group, and vigor to analyze the association of partial resistance with these horticultural traits. Hybrid cultivars were not included in the analysis. No differences in disease severity scores were observed across response time ($P = 0.0736$), bract color ($P = 0.3347$), or vigor groupings ($P = 0.8646$) (data not shown).

Experiment two: disease resistance, 2015. Fifty-eight cultivars were evaluated in 2015, representing lineages from three breeding companies. Seven cultivars ('Advent Red', 'Jubilee White', 'Jubilee Pink', 'Winter Rose Dark Red', 'Sparkling Punch', 'Ice Punch', and 'Red Glitter') had mean severity scores of 4.9 with complete loss of root systems occurring in all plants (Table 1.2). Three hybrid cultivars, 'Luv U Pink', 'EK HC 70B', and 'EK HC 68B' were among the twelve cultivars with disease severity scores not different from 'Carousel Red', which had the lowest disease severity score (lsmean DSS = 1.4).

Relationships among horticultural traits and resistance, 2015. Excluding hybrids, single-degree-of-freedom linear contrasts indicated that cultivars from Dümmen Orange (including Red Fox and Ecke Ranch; n = 29) had more disease than cultivars from Syngenta (n = 23; mean difference of 1.65 units ± 0.08, $P < 0.001$). Due to observed differences in disease resistance, cultivars from each source were analyzed separately. Among cultivars from Dümmen Orange, significant differences were observed among cultivars belonging to different response time ($P = 0.0041$) and vigor groups ($P = 0.0101$) (Table 1.3). Cultivars belonging to the early-season response groups (n = 5, lsmean = 3.3) had the lowest disease severity scores. Medium vigor cultivars had higher disease severity scores (n = 19, lsmean = 4.0) than compact (n = 8, lsmean = 3.3) cultivars. No differences in disease severity scores were observed among bract color groups ($P = 0.6292$).

Among cultivars from Syngenta, significant differences were observed across bract color groups ($P = 0.0152$), but not among response or vigor groups. White bract (n = 4) and novelty bract (n = 5) cultivars had the highest disease severity scores (lsmean = 2.9) whereas red bract cultivars had the lowest disease severity scores (lsmean = 2.4) (Table 3). Novelty

and white cultivars often are the result of color sports or spontaneous mutations. Color sports often are genetically similar to their parental lines and can even revert back to their parental color, so it is unclear whether mutations associated with color affect mechanisms of disease susceptibility. Only two cultivars were represented from Beekenkamp and these were excluded from the trait analyses.

Disease resistance in hybrid cultivars. *Euphorbia* hybrids including 'Luv U Pink', 'Princettia Max White' and two numbered cultivars had the lowest disease severity scores compared to conventional cultivars (Tables 1.1 and 1.2). In 2014, linear contrasts indicated that mean difference between hybrid and conventional cultivars was 0.76 units ± 0.11 ($P < 0.001$); in 2015 the mean difference was 1.37 units ± 0.15 ($P < 0.0001$). Typical symptoms observed on hybrids were slight stunting and minimal root damage. These results suggest that hybrid cultivars may have higher levels of partial resistance to Pythium root rot when compared to conventional cultivars. However, 'Princettia Dark Pink' had higher disease severity scores in 2014 (Table 1.1) and 2015 (Table 1.2) than other hybrid cultivars. This cultivar exhibits a compact, mounding growth habit as opposed to the tall, vigorous growth habit associated with 'Princettia Max White', 'Luv U Pink', and the two numbered cultivars 'EK HC 68B' and 'EK HC 70B'. The parental lines of 'Princettia Dark Pink' and the 'Luv U' series may differ in levels of partial resistance.

Disease resistance in cultivars tested in 2014 and 2015. Twenty-nine cultivars were represented in both 2014 and 2015. Disease severity scores for the two experiments were moderately correlated with relatively higher severity ratings overall in 2015 compared with 2014 ($r = 0.4448$, $P = 0.0156$; Figure 1.2). Two cultivars ('Infinity Red' and 'Jubilee Pink')

had disease severity scores that were notably different in 2015 compared to 2014. In 2014, 'Infinity Red' (DSS lsmean = 2.4) and 'Jubilee Pink' (DSS lsmean = 2.3) were not different from the best performing cultivars 'Luv U Pink' and 'Princettia Max White' (DSS lsmean = 2.1). However, in 2015, 'Infinity Red' (DSS lsmean = 4.2) and 'Jubilee Pink' (DSS lsmean = 4.9) were among cultivars with the highest disease severity scores. The reasons for changes in response are not known, but these results illustrate the importance of repeating resistance evaluation experiments before making recommendations to breeders or growers.

The combined ranking data were used to group cultivars by their performance in both years (Table 1.4). Based on least-square means separation, six cultivars ('Sparkling Punch', 'Red Glitter', 'Advent Red', 'Winter Rose Dark Red', 'Jubilee Red', and 'Jubilee White') were among the least resistant to Pythium root rot and belonged to the 75th percentile for disease severity (Q3 = DSS 3.88 to 4.88). Planting these cultivars should be discouraged in facilities with recurring Pythium root rot problems. Five cultivars ('Luv U Pink', 'Autumn Leaves', 'Princettia Dark Pink', 'Premium Ice Crystal', and 'Visions of Grandeur') exhibited partial resistance to Pythium root rot and were in the 25th percentile of disease severity (Q1 = DSS 2.0 to 3.06). Pythium root rot on these cultivars may be easier to control with routine fungicide applications than cultivars with little to no partial resistance. These cultivars also could serve as resistant checks in future screening trials.

These results indicate that poinsettia cultivars differ in their resistance to Pythium root rot caused by *P. aphanidermatum* and that some cultivars, breeding lines, and lineages may have more resistance than others. The modern era of poinsettia production began with the seedling cultivar 'Oak Leaf' in 1923. From the 1920's to 1960's, all commercial cultivars

originated primarily as sports (somatic mutations) of 'Oak Leaf' (Ecke et al., 2004). In the 1960's intensive breeding programs began and new cultivars were introduced. Poinsettia is intolerant of inbreeding and most cultivars are genetically heterogeneous due to outcrossing. To insure commercial uniformity, poinsettia is vegetatively propagated. These breeding procedures and tendency of poinsettia to undergo somatic mutations have resulted in the commercial introduction of many cultivars of uncertain genetic background (Parks and Moyer, 2004). Additionally, information on the genetic background of many cultivars is largely proprietary, which makes identifying lineages with increased disease resistance particularly challenging.

Horticultural trait associations with Pythium root rot resistance responses were not consistent across breeding companies. For Dümmen Orange, early-season cultivars with compact habits were associated with lower levels of disease, but bract color was not associated with disease severity. In contrast, color was more predictive of partial resistance among cultivars from Syngenta; cultivars with red bracts had the lowest disease severity scores among the company's cultivars. Overall, six of 13 partially resistant cultivars identified in 2015 had red bracts. Cultivars with red bracts consistently ranked among the top selling varieties and red bracts were not associated with high disease severity scores in either 2014 or 2015. These results indicate that growers should be able to choose among several red bract cultivars that have higher level partial resistance to Pythium root rot than others.

It is unknown if there are any genetic linkages among response time, color, or vigor and Pythium root rot resistance. In a previous study, poinsettia cultivars with red bracts were more susceptible to powdery mildew (*Oidium* spp. Link) than those with pink, variegated,

and white bracts (Celio and Hausbeck, 1997). Further genetic analyses are needed to identify parental lines associated with the phenotypic expression of Pythium root rot resistance observed in partially resistant cultivars.

While most cultivars were susceptible to Pythium root rot caused by *P. aphanidermatum*, we identified several cultivars with partial resistance to disease, including 'Luv U Pink', 'Autumn Leaves', 'Carousel Red', and 'Mira Red'. The finding that hybrid cultivars have partial resistance to Pythium root rot is encouraging for breeders that are interested in incorporating interspecific hybrids into their programs. These cultivars could serve as parental lines for developing conventional cultivars with improved partial resistance to Pythium root rot. The probability of finding complete resistance to Pythium root rot is low among commercial poinsettia lines, but screening wild *Euphorbia* species against *Pythium* could potentially identity additional novel sources of resistance. Although *P. aphanidermatum* is the predominant cause of Pythium root rot in North Carolina, other species may predominate in other production regions (Múnera and Hausbeck, 2016). Therefore, breeders should assess putative sources of resistance against other species of *Pythium* known to cause root rot in poinsettia.

Growers can use the results of this study to guide them when making cultivar selections. Selecting cultivars with partial resistance could be particularly useful in facilities with recurring Pythium problems, or in facilities that may be at risk for pathogen introduction and spread, such as those with recirculating irrigation systems. Since none of the cultivars screened were completely resistant to Pythium root rot, these results emphasize the need for

integrated pest management strategies that combine sanitation, host resistance, and fungicides to maximize disease control.

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Table 1.1. Differences in poinsettia cultivar responses to Pythium root rot caused by *Pythium aphanidermatum* in a trial conducted in 2014.

Cultivar	Traits[a]	Root rot rating ± S.E.[b]	Disease severity rating ± S.E.	Disease severity score ± S.E.[c]
Sparkling Punch	MNM	4.8 ± 0.2	4.8 ± 0.2	4.8 ± 0.2 a
Red Glitter	MNM	4.1 ± 0.2	4.0 ± 0.2	4.0 ± 0.2 b
Enduring Red	MRC	4.1 ± 0.2	4.0 ± 0.2	4.0 ± 0.2 b
Jubilee Red	MRM	4.1 ± 0.2	4.0 ± 0.2	4.0 ± 0.2 b
Advent Red	ERT	4.1 ± 0.2	3.8 ± 0.2	3.9 ± 0.2 bc
Winter Rose Dark Red	LRC	4.1 ± 0.2	3.5 ± 0.2	3.8 ± 0.2 bcd
Infinity Polar	MWM	3.8 ± 0.2	3.5 ± 0.2	3.6 ± 0.2 bcde
Premier White	EWC	4.1 ± 0.2	3.0 ± 0.2	3.5 ± 0.2 bcdef
Prestige Early Red	MRM	4.1 ± 0.2	3.0 ± 0.2	3.5 ± 0.2 bcdef
Viking Red	MRM	4.1 ± 0.2	3.0 ± 0.2	3.5 ± 0.2 bcdef
Enduring White	MWC	3.8 ± 0.2	3.0 ± 0.2	3.4 ± 0.2 cdefg
Jester Red	ERC	3.6 ± 0.2	3.0 ± 0.2	3.3 ± 0.2 defgh
Prestige Red	MRM	4.1 ± 0.2	2.5 ± 0.2	3.3 ± 0.2 defgh
Jubilee White	MWM	3.8 ± 0.2	2.8 ± 0.2	3.3 ± 0.2 defgh
Polly's Pink	MPM	3.6 ± 0.2	3.0 ± 0.2	3.3 ± 0.2 defgh
Classic White	MWT	3.6 ± 0.2	2.8 ± 0.2	3.1 ± 0.2 efghi
Majestic Red	MRM	3.3 ± 0.2	3.0 ± 0.2	3.1 ± 0.2 efghi
Jester White	EWC	3.8 ± 0.2	2.3 ± 0.2	3.0 ± 0.2 fghi
Princettia Dark Pink	EPC	4.1 ± 0.2	2.0 ± 0.2	3.0 ± 0.2 fghi
Monet Early	MNC	3.8 ± 0.2	2.3 ± 0.2	3.0 ± 0.2 fghi
Autumn Leaves	ENC	3.8 ± 0.2	2.3 ± 0.2	3.0 ± 0.2 fghi
Orange Spice	LNC	3.6 ± 0.2	2.3 ± 0.2	2.9 ± 0.2 ghij
Brilliant Red	MRM	3.1 ± 0.2	2.8 ± 0.2	2.9 ± 0.2 ghij
Visions of Grandeur	MNT	3.3 ± 0.2	2.3 ± 0.2	2.8 ± 0.2 hijk
Ice Punch	MNM	3.1 ± 0.2	2.5 ± 0.2	2.8 ± 0.2 hijk
Red Soul	MRM	3.6 ± 0.2	2.0 ± 0.2	2.8 ± 0.2 hijk
Polar Bear	MWM	3.6 ± 0.2	2.0 ± 0.2	2.8 ± 0.2 hijk
Premium Ice Crystal	ENC	3.3 ± 0.2	2.0 ± 0.2	2.6 ± 0.2 ijkl
Infinity Red	MRM	3.1 ± 0.2	1.8 ± 0.2	2.4 ± 0.2 jkl
Jubilee Pink	MPM	3.3 ± 0.2	1.3 ± 0.2	2.3 ± 0.2 kl
Premier Red	ERC	3.3 ± 0.2	1.0 ± 0.2	2.2 ± 0.2 l
Premium Red	ERM	2.6 ± 0.2	1.8 ± 0.2	2.2 ± 0.2 l
Princettia Max White	EWT	2.3 ± 0.2	2.0 ± 0.2	2.1 ± 0.2 l
Luv U Pink	LPT	2.3 ± 0.2	2.0 ± 0.21	2.1 ± 0.2 l
	P > F	*< 0.0001*	*< 0.0001*	*< 0.0001*

[a] Horticultural traits as determined by the propagator included response time (early=E, middle=M, late=L), bract color (red=R, white=W, pink=P, and novelty=N), and vigor (compact=C, medium=M, and tall=T).
[b] Least square means of four replicate root rot ratings and disease severity ratings (1=healthy to 5=dead)
[c] Disease severity scores were obtained by averaging root rot ratings and disease severity ratings for each observation. Least square means with same letter are not different based on paired t-tests ($\alpha = 0.05$).

Table 1.2. Differences in poinsettia cultivar responses to Pythium root rot caused by *Pythium aphanidermatum* in a trial conducted in 2015.

Cultivar	Traits[a]	Root rot[b] rating ± S.E.	Disease severity rating ± S.E.	Disease severity score ± S.E.[c]
Advent Red	ERT	5.0 ± 0.3	4.9 ± 0.3	4.9 ± 0.3 a
Jubilee White	MWM	5.0 ± 0.3	4.9 ± 0.3	4.9 ± 0.3 a
Jubilee Pink	MPM	5.0 ± 0.3	4.9 ± 0.3	4.9 ± 0.3 a
Winter Rose Dark Red	LRC	5.0 ± 0.3	4.9 ± 0.3	4.9 ± 0.3 a
Sparkling Punch	MNM	5.0 ± 0.3	4.9 ± 0.3	4.9 ± 0.3 a
Ice Punch	MNM	5.0 ± 0.3	4.9 ± 0.3	4.9 ± 0.3 a
Red Glitter	MNM	5.0 ± 0.3	4.9 ± 0.3	4.9 ± 0.3 a
Jubilee Red	MRM	4.7 ± 0.3	4.4 ± 0.3	4.6 ± 0.3 ab
Ruby Frost	MNM	4.7 ± 0.3	4.4 ± 0.3	4.6 ± 0.3 ab
Infinity Polar	MWM	4.5 ± 0.3	4.4 ± 0.3	4.4 ± 0.3 ab
Majestic Red	MRM	4.5 ± 0.3	4.2 ± 0.3	4.3 ± 0.3 abc
Classic White	MWT	4.5 ± 0.3	3.9 ± 0.3	4.2 ± 0.3 abcd
Infinity Red	MRM	4.2 ± 0.3	4.2 ± 0.3	4.2 ± 0.3 abcd
Prestige Red	MRM	4.0 ± 0.3	3.7 ± 0.3	3.8 ± 0.3 bcde
Viking Red	MRM	4.2 ± 0.3	3.4 ± 0.3	3.8 ± 0.3 bcde
Brilliant Red	MRM	3.5 ± 0.3	3.7 ± 0.3	3.6 ± 0.3 cdef
Enduring White	MWC	3.7 ± 0.3	3.4 ± 0.3	3.6 ± 0.3 cdef
Monet Early	MNC	3.7 ± 0.3	3.4 ± 0.3	3.6 ± 0.3 cdef
Prestige Early Red	MRM	3.7 ± 0.3	3.4 ± 0.3	3.6 ± 0.3 cdef
Jester Red	ERC	3.7 ± 0.3	3.2 ± 0.3	3.4 ± 0.3 defg
Majestic Pink	MPM	3.5 ± 0.3	3.4 ± 0.3	3.4 ± 0.3 defg
Premium Red	ERM	3.5 ± 0.3	3.4 ± 0.3	3.4 ± 0.3 defg
Mira White	EWM	4.0 ± 0.3	2.9 ± 0.3	3.4 ± 0.3 defg
Titan White	EWM	4.0 ± 0.3	2.9 ± 0.3	3.4 ± 0.3 defg
Red Soul	MRM	4.0 ± 0.3	2.7 ± 0.3	3.3 ± 0.3 efgh
Enduring Red	MRC	4.0 ± 0.3	2.4 ± 0.3	3.2 ± 0.3 efghi
Polar Bear	MWM	3.5 ± 0.3	2.9 ± 0.3	3.2 ± 0.3 efghi
Mars Red	MRM	3.2 ± 0.3	3.2 ± 0.3	3.2 ± 0.3 efghi
Mars Marble	MNM	3.2 ± 0.3	3.2 ± 0.3	3.2 ± 0.3 efghi
Solar Red	MRM	3.5 ± 0.3	2.7 ± 0.3	3.1 ± 0.3 efghij
Sigma	ERC	3.7 ± 0.3	2.4 ± 0.3	3.1 ± 0.3 efghij
Polly's Pink	MPM	3.0 ± 0.3	2.9 ± 0.3	2.9 ± 0.3 fghijk
Advantage Red	MRM	3.0 ± 0.3	2.9 ± 0.3	2.9 ± 0.3 fghijk
Visions of Grandeur	MNT	3.0 ± 0.3	2.4 ± 0.3	2.7 ± 0.3 ghijkl
Premium Ice Crystal	ENC	3.5 ± 0.3	1.9 ± 0.3	2.7 ± 0.3 ghijkl

Table 1.2 continued.

Venus Hot Pink	EPM	2.7 ± 0.3	2.7 ± 0.3	2.7 ± 0.3 ghijkl
Titan Red	ERM	3.2 ± 0.3	2.2 ± 0.3	2.7 ± 0.3 ghijkl
Mars White	MWM	3.0 ± 0.3	2.2 ± 0.3	2.6 ± 0.3 hijklm
Mars Pink	MPM	3.0 ± 0.3	2.2 ± 0.3	2.6 ± 0.3 hijklm
Cortez Early Red	LRM	3.0 ± 0.3	1.9 ± 0.3	2.4 ± 0.3 hijklm
Cinnamon Star	ENM	3.0 ± 0.3	1.9 ± 0.3	2.4 ± 0.3 hijklm
Early Mars Red	ERM	3.0 ± 0.3	1.9 ± 0.3	2.4 ± 0.3 hijklm
Princettia Dark Pink	EPC	2.7 ± 0.3	1.9 ± 0.3	2.3 ± 0.3 jklmno
Sonora White Glitter	LNC	3.0 ± 0.3	1.7 ± 0.3	2.3 ± 0.3 jklmno
Red Elf	ERC	3.2 ± 0.3	1.4 ± 0.3	2.3 ± 0.3 jklmno
Early Orion Red	ERM	2.5 ± 0.3	1.9 ± 0.3	2.2 ± 0.3 klmnop
Titan Pink	EPM	3.0 ± 0.3	1.4 ± 0.3	2.2 ± 0.3 klmnop
White Star	EWM	3.0 ± 0.3	1.2 ± 0.3	2.1 ± 0.3 lmnop
Orion Red	ERM	2.5 ± 0.3	1.7 ± 0.3	2.1 ± 0.3 lmnop
Autumn Leaves	ENC	2.2 ± 0.3	1.7 ± 0.3	1.9 ± 0.3 lmnop
Saturnus Red	MRM	3.0 ± 0.3	0.9 ± 0.3	1.9 ± 0.3 lmnop
Neva	MRM	1.7 ± 0.3	2.2 ± 0.3	1.9 ± 0.3 lmnop
Luv U Pink	LPT	1.7 ± 0.3	1.9 ± 0.3	1.8 ± 0.3 mnop
EK HC 70B	LPT	1.7 ± 0.3	1.9 ± 0.3	1.8 ± 0.3 mnop
Cortez Burgundy	LNM	1.7 ± 0.3	1.9 ± 0.3	1.8 ± 0.3 mnop
Mira Red	ERM	1.7 ± 0.3	1.7 ± 0.3	1.7 ± 0.3 nop
EK HC 68B	LPT	1.7 ± 0.3	1.4 ± 0.3	1.6 ± 0.3 op
Carousel Red	MRC	2.0 ± 0.3	0.9 ± 0.3	1.4 ± 0.3 p
	P > F	*< 0.0001*	*< 0.0001*	*< 0.0001*

[a] Horticultural traits as determined by the propagator included response time (early=E, middle=M, late=L), bract color (red=R, white=W, pink=P, and novelty=N), and vigor (compact=C, medium=M, and tall=T).

[b] Least square means of four replicate root rot ratings and disease severity ratings (1=healthy to 5=dead)

[c] Disease severity scores were obtained by averaging root rot ratings and disease severity ratings for each observation. Least square means with same letter are not different based on paired t-tests ($\alpha = 0.05$).

Table 1.3. Relationships between horticultural traits of poinsettia and resistance to Pythium root rot in 2015.

	Mean Disease Severity Scores[a]	
Trait	**2015 Dümmen Orange**	**2015 Syngenta**
Response		
Early	3.3 ± 0.25 c	2.6 ± 0.11
Middle	3.9 ± 0.15 b	2.8 ± 0.15
Late	4.9 ± 0.50 a	2.3 ± 0.22
P > F	*P = 0.0041*	*P = 0.0789*
Color		
Red	3.8 ± 0.17	2.4 ± 0.11 b
White	4.1 ± 0.25	2.9 ± 0.19 a
Pink	3.8 ± 0.31	2.5 ± 0.22 ab
Novelty	3.7 ± 0.22	2.9 ± 0.17 a
P > F	*P = 0.6292*	*P = 0.0152*
Vigor		
Compact	3.3 ± 0.22 b	2.3 ± 0.19
Medium	4.0 ± 0.16 a	2.7 ± 0.09
Tall	3.9 ± 0.30 ab	…
P > F	*P = 0.0101*	*P = 0.1285*

[a] Disease severity scores were obtained by averaging four root rot ratings and disease severity ratings within a trait. Least square means with same letter are not different based on paired t-tests (α = 0.05).

Table 1.4. Differences in poinsettia cultivar responses to Pythium root rot caused by *Pythium aphanidermatum* using combined data from 2014 and 2015.

Cultivar	**Disease Severity Score ± S.E.**[a]	**Level (Quantile)**[b]
Sparkling Punch	4.88 ± 0.27 a	
Red Glitter	4.50 ± 0.27 ab	95th (4.5)
Advent Red	4.44 ± 0.27 abc	
Winter Rose Dark Red	4.38 ± 0.27 abcd	
Jubilee Red	4.31 ± 0.27 abcde	
Jubilee White	4.13 ± 0.27 abcdef	
Infinity Polar	4.06 ± 0.27 bcdefg	
Ice Punch	3.88 ± 0.27 bcdefgh	75th (Q3, 3.88)
Majestic Red	3.75 ± 0.27 bcdefghi	
Classic White	3.69 ± 0.27 cdefghi	
Viking Red	3.69 ± 0.27 cdefghi	
Enduring Red	3.63 ± 0.27 defghi	
Jubilee Pink	3.63 ± 0.27 defghi	
Prestige Early Red	3.56 ± 0.27 efghij	50th (3.56)
Prestige Red	3.56 ± 0.27 efghij	
Enduring White	3.50 ± 0.27 fghijk	
Jester Red	3.38 ± 0.27 fghijkl	
Infinity Red	3.31 ± 0.27 ghijkl	
Monet Early	3.31 ± 0.27 ghijkl	
Brilliant Red	3.25 ± 0.27 hijklm	
Polly's Pink	3.13 ± 0.27 hijklm	
Red Soul	3.06 ± 0.27 ijklm	25th (Q1, 3.06)
Polar Bear	3.00 ± 0.27 ijklm	
Premium Red	2.81 ± 0.27 jklm	
Visions of Grandeur	2.75 ± 0.27 lmn	
Premium Ice Crystal	2.69 ± 0.27 lmn	
Princettia Dark Pink	2.69 ± 0.27 lmn	
Autumn Leaves	2.50 ± 0.27 mn	5th (2.5)
Luv U Pink	2.00 ± 0.27 n	

[a] Disease severity scores were obtained by averaging root rot ratings and disease severity ratings for each observation. Least square means represent data from two years with four replicates per year. Means with same letter are not different based on paired t-tests ($\alpha = 0.05$).

[b] Estimated quantiles and percentiles for the population

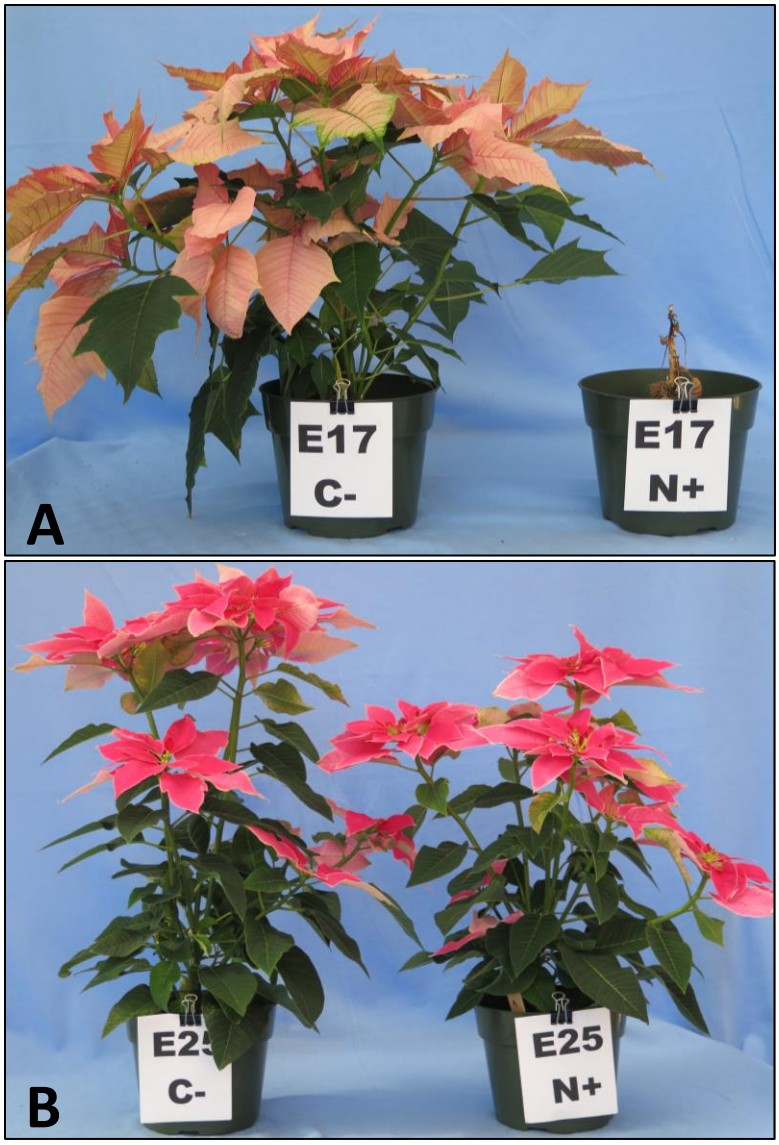

Figure 1.1. Differences in Pythium root rot disease severity on two cultivars with varying levels of partial resistance. **A)** Sparkling Punch, susceptible cultivar and **B)** Luv U Pink, partially resistant hybrid cultivar.

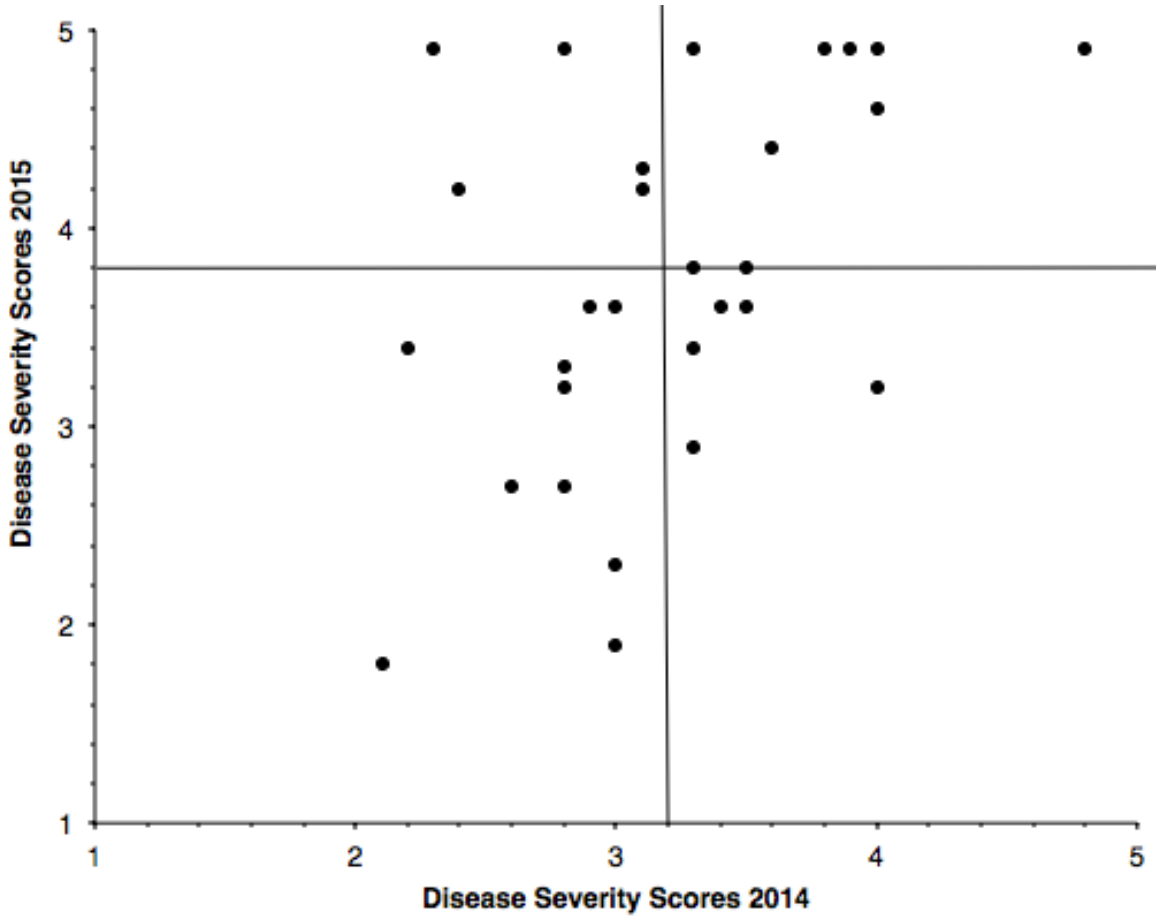

Figure 1.2. Disease severity scores of 29 cultivars evaluated for resistance to Pythium root rot caused by *P. aphanidermatum* in both 2014 and 2015. Disease severity scores were obtained by averaging root rot ratings and disease severity ratings, both visually rated on a 1 to 5 scale. Disease severity scores were moderately correlated ($r = 0.44$, $P = 0.0156$). Means for each year are indicated by solid black lines.

CHAPTER 2

Developing integrated management strategies to control Pythium root rot on poinsettia

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Abstract

Pythium aphanidermatum is the predominant species causing Pythium root rot of commercially grown poinsettia in North Carolina. Pythium root rot is managed primarily through a combination of sanitation practices and preventative fungicide applications. Growers prefer to use mefenoxam for root rot control because of its high efficacy and low cost, but resistance is common in greenhouse populations of *Pythium*. Alternative fungicides such as etridiazole are expensive, particularly if repeated applications are needed. Other products with activity against *P. aphanidermatum* are not commonly used on poinsettia. Because of the limitations associated with chemical control, this research was conducted to determine whether Pythium root rot could be better managed with an approach that integrates applications of diverse fungicide chemistries with host resistance. Twenty-five isolates of *P. aphanidermatum* cultured from plants growing in commercial greenhouses were evaluated for *in vitro* sensitivity to 10 fungicides. Etridiazole, fosetyl-al, and potassium phosphite completely inhibited mycelial growth, whereas isolates varied in response to mefenoxam, cyazofamid, propamocarb, fenamidone, azoxystrobin, and pyraclostrobin *in vitro*. Seven isolates were insensitive to all three quinone outside inhibitors (QoIs) and one isolate was insensitive to QoIs and mefenoxam. Next, greenhouse studies were conducted to assess efficacy of fungicide treatments in seven poinsettia cultivars inoculated with *P. aphanidermatum.* One study examined control with a single fungicide application made at transplant and another study examined repeat applications made throughout the experiment. Treatments containing etridiazole, mefenoxam, fenamidone, and cyazofamid provided control of Pythium root rot across all cultivars in both experiments. Azoxystrobin,

pyraclostrobin, and propamocarb did not control disease on susceptible cultivars, but suppressed disease on partially resistant cultivars. These results suggest that partial resistance in poinsettia could be used in combination with fungicides to limit disease caused by *P. aphanidermatum*. Additionally, this is the first report of insensitivity to QoIs and dual insensitivity to QoIs and mefenoxam in *P. aphanidermatum* isolates collected from greenhouse floriculture crops.

Poinsettia ranks second in the United States for potted flowering plant sales, with an estimated wholesale value of $140 million, and is the most popular holiday flower worldwide. North Carolina is the second largest producer of poinsettia with over 4.3 million pots produced in 2015 (USDA, 2016). Limiting losses from disease is critical, as growers must meet market demands for quality while minimizing costs of production.

In a survey to determine the prevalence of *Pythium* spp. in commercially grown floriculture crops in North Carolina, *Pythium aphanidermatum* was the predominant species causing root rot in poinsettia (Lookabaugh et al. 2015). Long production seasons and common irrigation practices make Pythium root rot particularly challenging to control in greenhouse systems. Pythium root rot is primarily managed through a combination of strict sanitation practices and preventative fungicide applications. Mefenoxam is widely used throughout the industry despite the presence of mefenoxam-resistant strains or species of *Pythium* in many locations and floriculture crops (Aegerter et al. 2002; Lookabaugh et al. 2015; Moorman et al. 2002; Moorman and Kim 2004). Mefenoxam is less costly than

etridiazole, another widely used chemical, which could account for the industry's continued reliance on mefenoxam.

Alternatives to mefenoxam and etridiazole would provide more options for chemical control of Pythium root rot and could help to counter the predominance of mefenoxam-insensitive isolates in populations of *P. aphanidermatum*. However, few chemicals are registered for use against Pythium root rot in floriculture crops (Garzón et al. 2011). It is not clear whether they are efficacious against *P. aphanidermatum* because *Pythium* species vary in their sensitivity to fungicides and specific management recommendations cannot be generalized across *Pythium* species or host plants (Moorman and Kim 2004; Weiland et al. 2014; Múnera and Hausbeck 2015; 2016). Efficacy also can vary significantly depending on application method, timing, rate, and reapplication intervals. It is not known if single fungicide applications at transplant can protect poinsettia throughout the entire production season or if repeat applications are necessary to achieve adequate control. Additionally, the active ingredients in some of these products are highly site-specific, which raises the possibility that growers could inadvertently select for insensitive populations of *P. aphanidermatum* through repeated use on poinsettia or rotational crops.

Given the limitations associated with chemical control, Pythium root rot could be better managed with an approach that integrates chemical control with other methods of disease management, including host resistance. The use of disease resistant varieties is an economical and readily implemented integrated management strategy to limit disease on agronomic crops and can reduce or replace the need for repeat fungicide applications (Bates et al. 2008; Everts 2002; Higginbothom et al. 2004; Kirk et al. 2005; Lucas and Griffiths

2004; Li et al. 2016). Unfortunately, disease resistance has played a limited role in the management of Pythium root rot because resistant cultivars have not been identified or developed in many crops, including poinsettia (Chagnon and Belanger 1991; Deng et al. 2005 a & b). In a recent study, however, 58 commercial poinsettia cultivars were evaluated for resistance to Pythium root rot caused by *P. aphanidermatum* (Lookabaugh 2017). While no cultivars were completely resistant to root rot, several were identified as partially resistant. Interspecific hybrid cultivars, including 'Luv U Pink' and 'Princettia Max White', had higher levels of partial resistance when compared to conventional cultivars, including 'Prestige Red', a popular red-bract cultivar. 'Sparkling Punch', a novelty cultivar, was the least resistant to Pythium root rot among the cultivars tested. These results suggest that partial resistance in poinsettia could be used in combination with fungicide applications to limit disease caused by *P. aphanidermatum*, reduce the number of drenches required to achieve adequate control, and mitigate loss of control due to fungicide insensitivity. The objectives of this study were 1) characterize in-vitro sensitivity of *P. aphanidermatum* to various fungicides, and 2) to explore the combined utility of fungicides and cultivar selections to manage Pythium root rot caused by *P. aphanidermatum*.

Materials and Methods

Experiment 1: In-vitro evaluation of fungicide efficacy on mefenoxam sensitive and insensitive isolates. Nine fungicides were tested at four concentrations (Table 2.1). The fungicide concentrations tested (designated 1x) were selected based on label recommendations and previous efficacy experiments. Additional concentrations (0.5x, 2x, and 5x) were tested to confirm that the 1x rates were appropriate for further testing. SHAM

(50 mg/ml) was added to media amended with the quinone outside inhibitors (QoIs) azoxystrobin, pyraclostrobin, and fenamidone. Four isolates of *P. aphanidermatum* previously collected from diseased poinsettia were tested for sensitivity to the fungicides. Two isolates (PA1 and PA2) were known to be sensitive to mefenoxam at 100 µl a.i./ ml and two isolates (PA3 and PA4) were insensitive (Lookabaugh et al. 2015). Agar plugs from actively growing cultures were transferred to fungicide-amended clarified V8 juice agar (CV8A; 50 ml clarified V8 juice, 15 g Bacto agar [Becton, Dickinson, and Co.]) dispensed in 100 x 15 mm Petri plates, with 3 replicate plates for each fungicide x concentration x isolate combination. Plates were incubated for 27 h and percent mycelial inhibition was calculated by dividing the average mycelial radial growth on fungicide-amended media by the average mycelial radial growth on non-amended media, subtracting from 1, and then 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). The opaque quality of media amended with the 5x rate of cyazofamid reduced scorability and this treatment was not used in the analysis.

Experiment 2: In-vitro fungicide sensitivity. Twenty-one additional isolates of *P. aphanidermatum* were screened for *in-vitro* sensitivity to commercial formulations of six of the nine fungicides (azoxystrobin, pyraclostrobin, fenamidone, mefenoxam, cyazofamid, and propamocarb). Isolates 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). Nine isolates were identified previously

as mefenoxam resistant (Lookabaugh et al. 2015). Fungicides and concentrations were selected based on results of the previous experiment: azoxystrobin (Heritage, 70 µg a.i./ml), pyraclostrobin (Empress Intrinsic, 117 µl a.i./ml), fenamidone (Fenstop, 976 µl a.i./ml), mefenoxam (Subdue MAXX, 35.2 µl a.i./ml), cyazofamid (Segway, 158 µl a.i./ml), and propamocarb (Banol, 2,074 µl a.i./ml). Agar plugs from actively growing cultures were transferred to fungicide-amended clarified V8 juice agar as described above. SHAM (50 mg/ml) was added to QoI-amended media. Plates were incubated for 27 h and percent inhibition of mycelial radial growth was calculated. The entire experiment was conducted twice.

Data from both experiments were combined and analysis of variance was performed using PROC GLIMMIX procedures (SAS 9.4, SAS Institute). Isolates, treatments, and their interaction were treated as fixed effects while replicates and trials were treated as random effects. The isolate x treatment interaction was significant; therefore least squares means (LSmeans) were calculated for the interactions and the simple effects of isolates were analyzed within treatments to determine significant differences. Least square means were separated using paired t-tests where appropriate.

Experiments 3 and 4: Integrated control with fungicides and host resistance. To test the combined utility of cultivar selection and fungicides for disease control, two experiments were conducted. Each experiment was conducted at two greenhouses located approximately 2.5 km apart, for a total of four experiments. Experiment 3 evaluated the efficacy of a single application of ten fungicides applied at the label rate at transplant and was conducted concurrently at both locations (Table 2.2). All planting materials were obtained from

commercial sources (Dümmen Orange). Plants at each location were transplanted and treated 11 Apr 2016, inoculated 12 Apr 2016, and harvested 6 Jun 2016. Experiment 4 evaluated the efficacy of repeated fungicide applications for Pythium root rot control (Table 2.2). The experiment was conducted concurrently at the two greenhouse locations. Plants were transplanted and treated for the first time on 16 Jun 2016, inoculated 17 Jun 2016, and harvested 15 Aug 2016 at both locations.

Experiment 3: Integrated control with host resistance and a single fungicide application. Unrooted cuttings from six cultivars, representing a range of partial resistance ('Princettia Max White', 'Sparkling Punch', 'Freedom Early Red', 'Prestige Red', 'Infinity Red', and 'Infinity Polar') 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 peat-based potting media. The test products were applied by drench (118 ml per pot) at transplant. Once transplanted, the plants were fertilized with 5 g of Multicote 4 (14-14-16) sprinkled evenly on the pot surface and watered in.

Inoculum was prepared by transferring four 4-mm-diameter agar disks from an actively growing culture of *P. aphanidermatum* (isolate PA1) into 125-ml flasks of twice-autoclaved long-grain rice (25 g rice and 18 ml deionized water). This isolate is sensitive to mefenoxam and causes typical symptoms of Pythium root rot in poinsettia including stunting, defoliation, root rot, wilting, and plant mortality (Lookabaugh et al. 2015, Lookabaugh and Shew, 2017). It also was sensitive to all fungicides tested the *in vitro* experiments described above. Cultures were incubated for 5 days at 25°C (Holmes and Benson, 1994) and flasks

were shaken twice daily to promote uniform colonization of rice grains and prevent clumping during incubation. Plants were inoculated 24 h after transplant and treatment with the test materials (Table 2.2). Each of the two experiments had 10 treatments and included non-inoculated, non-treated plants and inoculated, non-treated plants as controls, for a total of 12 treatments. Treatments were arranged in a randomized complete block design with three replications.

Shoot symptoms were evaluated on a disease severity scale of 1 = healthy plant, 2 = slightly stunted, 3 = chlorosis, moderate stunting and/or defoliation, 4 = wilting and/or severe stunting, and 5 = dead (Lookabaugh et al., 2015). Plants were carefully inverted, the pot was removed, and the 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 integrity (Parker and Benson, 2013). Data from both runs of the experiment were combined for analysis.

Experiment 4: Integrated control with host resistance and repeat fungicide applications. Unrooted cuttings from nine cultivars, representing a range of partial resistance (‘Advent Red’, ‘Luv U Pink’, ‘Prestige Red’, ‘Sparkling Punch’, ‘Princettia Max White’, ‘Premium Red’, ‘Infinity Red’, ‘Infinity Polar’, ‘Viking Red’) were placed in Oasis Wedge growing media (Smithers-Oasis North America) and maintained under mist irrigation until

roots emerged from bottom of the foam wedge. Rooted plugs of poinsettia were transferred to new 15-cm plastic pots containing Fafard 4P peat-based potting media. Once transplanted, the plants were fertilized with 5 g of Multicote 4 (14-14-16) sprinkled evenly on the pot surface and watered in.

Plants were inoculated and treatments were applied according to rates and application intervals specified on the product label (Table 2.2). Each of the experiments had 14 treatments: 10 fungicides applied at transplant and reapplied at label intervals; two rotation treatments; an inoculated, non-treated control; and a non-inoculated, non-treated control. The two rotation treatments consisted of mefenoxam at transplant followed by etridiazole at week 6, or mefenoxam at transplant followed by cyazofamid at week 6, followed by mefenoxam at week 8. Treatments were arranged in a randomized complete block design with three replicates. Plants were maintained under drip irrigation and treated with Safari (Dinotefuran; 1.25 ml per liter) three weeks after transplanting to control whiteflies. At the end of the trials, disease severity was evaluated as described previously. Data from both runs of the experiment were combined for analysis.

Statistical analysis, Experiments 3 and 4. Analysis of variance and correlation analysis were performed using PROC GLIMMIX and PROC CORR procedures (SAS 9.4, SAS Institute). In both experiments, root rot ratings and above-ground disease severity ratings were highly correlated (Spearman's $r \geq 0.8985$, $P < 0.0001$). These dependent variables were averaged for each observation to create disease severity scores (DSS) used in subsequent analyses. Cultivar, treatment, and their interactions were treated as fixed effects while trial and block were treated as random effects. If the cultivar x treatment interaction

was significant, the least squares means were calculated for the interactions. Simple effects of fungicide treatments were analyzed within cultivar to determine significant differences. Least square means were separated using paired t-tests where appropriate.

Results

Experiment 1: In-vitro fungicide efficacy on mefenoxam sensitive and insensitive isolates. All rates of fosetyl-al, potassium phosphite, and etridiazole inhibited mycelial growth on all four isolates tested. Mycelial growth of the isolates varied in response to mefenoxam, cyazofamid, propamocarb, and the QoIs (azoxystrobin, pyraclostrobin, and fenamidone) (Table 2.3). QoIs inhibited mycelial growth of isolates PA1, PA3, and PA4, but did not inhibit mycelial growth of isolate PA2 at 0.5 to 5x label concentration. Insensitivity was observed when isolate PA2 was tested on all three QoIs. Mean percent inhibition at the 1x concentration was 46.0 ± 15.02 for azoxystrobin, 20.5 ± 5.98 for pyraclostrobin, and 19.7 ± 7.22 for fenamidone (Table 2.3). Dose-response relationships were not apparent at the concentrations tested. As expected, mefenoxam inhibited mycelial growth of isolates PA1 (mean inhibition at 1x rate = 100%) and PA2 (mean inhibition = 100%) but not isolates PA3 (mean inhibition = 9.42%) and PA4 (mean inhibition = 3.12%). These isolates had been identified previously as mefenoxam insensitive (Lookabaugh et al., 2015). Inhibition varied across isolates on media amended with up to 2x the label rate of cyazofamid (mean inhibition 66.6% ± 16.7, min = 32.9%, max = 100%) and propamocarb (mean inhibition 77.8% ± 8.82, min = 51.5%, max = 100%).

Experiment 2: In-vitro evaluation of isolates for fungicide sensitivity. Percent inhibition of mycelial radial growth varied across isolate and fungicide treatment ($P <$

0.0001). Six isolates in addition to PA2 (PA5, PA13, PA14, PA17, PA19, and PA25) were insensitive *in-vitro* to label rates of all three QoI fungicides tested (Table 2.4). Among QoI-insensitive isolates, inhibition was lowest with fenamidone (18.8 to 28.6% inhibition), followed by pyraclostrobin (33.0 to 43.3%) and azoxystrobin (41.3 to 60.7%). Complete inhibition (100%) was observed in the remaining isolates on all three QoI-amended media. Insensitivity to mefenoxam was confirmed in the eight isolates (in addition to isolates PA3 and PA4) previously identified as insensitive (2.9 to 11.9% inhibition). Inhibition of mycelial growth on media amended with cyazofamid and propamocarb varied across isolates, ranging from 57.6 to 100% for cyazofamid and 51.6 to 75.4% for propamocarb.

Experiment 3: Integrated control with host resistance and a single fungicide application at transplanting. Disease severity and root rot scores were moderate in these experiments. Non-inoculated, non-treated plants did not exhibit Pythium root rot symptoms. Disease severity scores varied among fungicides (main effect $P < 0.0001$) and cultivars ($P < 0.0001$), but fungicide x cultivar effects were not significant (interaction $P = 0.5637$), indicating that effects of fungicide and cultivar were additive. In absence of a fungicide treatment, 'Sparkling Punch' and 'Infinity Polar' had the highest disease severity scores (DSS) (inoculated, non-treated DSS = 4.2) while 'Princettia Max White' had the lowest disease severity score (inoculated, non-treated DSS = 2.2, Figure 2.1). Across all six cultivars tested, mefenoxam, fenamidone, cyazofamid, and etridiazole consistently reduced Pythium root rot. Potassium phosphite and the biological treatment (*Trichoderma* spp.) did not control root rot, with mean disease severity scores not different from those in the inoculated, non-treated plants (Figure 2.2).

Experiment 4: Integrated control with host resistance and repeat fungicide applications. Disease severity and root rot scores were high for both runs of this experiment. Non-inoculated, non-treated plants did not exhibit Pythium root rot symptoms. Variation in disease severity scores were significant for fungicide and cultivar ($P < 0.0001$), but fungicide efficacy differed across cultivars (treatment x cultivar $P < 0.0001$; Table 2.5). Averaged across fungicide treatments, 'Sparkling Punch' had the highest disease severity scores and 'Luv U Pink' had the lowest disease severity scores. Two applications of mefenoxam, fenamidone, and etridiazole, or three applications of cyazofamid reduced Pythium root rot across all cultivars, whereas *Trichoderma* spp., potassium phosphite, and fosetyl-al did not suppress disease on any cultivar. Three applications of propamocarb suppressed disease relative to the untreated controls in all cultivars, but did not provide disease suppression equal to the most effective fungicides (mefenoxam, fenamidone, etridiazole, and cyazofamid). Performance of azoxystrobin and pyraclostrobin varied with cultivar susceptibility. Two applications of pyraclostrobin did not reduce disease in the susceptible cultivars 'Sparkling Punch', 'Advent Red', 'Infinity Polar', and 'Infinity Red', with mean disease severity scores on treated plants not different from the inoculated, non-treated controls. However, treatment with pyraclostrobin reduced Pythium root rot relative to the non-treated controls on the partially resistant cultivars 'Viking Red' and 'Luv U Pink'. Slight disease suppression was observed on 'Prestige Red' and 'Premium Red' treated twice with pyraclostrobin, but not on other cultivars. Compared to non-treated controls, two applications of azoxystrobin reduced disease on the partially resistant cultivar, 'Luv U Pink', and provided some suppression on 'Premium Red', 'Princettia Max White', 'Prestige Red' and

‘Viking Red’. However, azoxystrobin did not reduce disease in the highly susceptible cultivars ‘Sparkling Punch’, ‘Advent Red’, ‘Infinity Polar’, and ‘Infinity Red’, with mean disease severity scores in treated plants not different from those in the inoculated, non-treated plants.

Fungicide rotation treatments controlled disease across all cultivars, with diseases severity scores not different from the healthy control with either rotation. The rotation with mefenoxam at transplant followed by cyazofamid at week 6, followed by mefenoxam at week 8 provided better disease control than three applications of cyazofamid alone on ‘Advent Red’. These treatments were equivalent on other cultivars. Likewise, the rotation of mefenoxam at transplant followed by etridiazole at week 6 provided control equal to two applications of either mefenoxam or etridiazole across all cultivars.

Discussion

This study evaluated the potential for integrated management practices to minimize losses caused by Pythium root rot in poinsettia production systems. Previously reported differences in cultivar resistance to root rot caused by *P. aphanidermatum* were confirmed (Lookabaugh 2017). ‘Sparkling Punch’ was highly susceptible to Pythium root rot, while ‘Luv U Pink’ and ‘Princettia Max White’ consistently demonstrated moderate partial resistance to Pythium root rot in untreated, inoculated controls. Other cultivars were intermediate in their response to Pythium root rot. Surprisingly, ‘Viking Red’, which was intermediate among cultivars in the previous study, expressed partial resistance to Pythium root rot in both runs of Experiment 3 in this study.

Most fungicide labels recommend preventative applications to poinsettia at transplant, followed by one or two additional applications at specified intervals. Previous studies on poinsettia reported that plants inoculated early in the production cycle demonstrated more severe symptoms of Pythium root rot than plants inoculated when more mature (Lookabaugh 2017). In Experiment 3 of this study, transplant drenches of mefenoxam, etridiazole, fenamidone, and cyazofamid provided excellent control of Pythium root rot caused by an isolate of *P. aphanidermatum* (PA1) that was sensitive to these products. These products were effective when used on all cultivars as single applications, but single applications may not suppress Pythium root rot in more challenging conditions. Disease severity scores of non-treated, non-inoculated plants were higher overall in Experiment 4 (mean disease severity score of 4.3) than in Experiment 3 (mean disease severity score of 3.5). Under the higher levels of disease severity in Experiment 4, repeated applications of mefenoxam, fenamidone, etridiazole and cyazofamid resulted in lower disease severity scores than were observed with a single application under less severe conditions in Experiment 3. These results suggest repeated applications may be necessary to achieve maximum protection when disease is severe.

In the current study, rotating cyazofamid with mefenoxam offered additional control of Pythium root rot on the susceptible cultivar 'Advent Red' when compared to three applications of cyazofamid alone. Other studies showed that cyazofamid controlled Pythium blight on perennial ryegrass (*Lolium perenne*) inoculated with *P. aphanidermatum*, but did not suppress Pythium root rot on geranium (*Pelargonium x hortorum*) or snapdragon

(*Antirrhinum majus*) (Koch and Kerns 2012, 2013; Aynardi and Uddin 2015, 2016; Múnera and Hausbeck 2015).

While cyazofamid is not commonly used in rotation with mefenoxam, many growers follow mefenoxam transplant drenches with late-season drenches of etridiazole. In the current study, the rotation with mefenoxam at transplant followed by etridiazole 6 weeks later suppressed disease equal to that of treatments consisting of two applications of either fungicide alone. These results provide support for rotation practices currently being implemented in poinsettia production systems.

In several previous studies, fenamidone consistently provided good to excellent control of Pythium root rot caused by *P. aphanidermatum,* but very few growers report using fenamidone on poinsettia (Parker and Benson 2012, 2013; Múnera and Hausbeck 2015). Fenstop (fenamidone, Bayer CropScience) is expensive relative to other products, and high application rates further discourage most growers from incorporating it into their fungicide programs. Azoxystrobin and pyraclostrobin are affordable but have not performed consistently in efficacy trials, including those reported herein (Parker and Benson 2012; Múnera and Hausbeck 2015). It is possible that the inconsistent performance of these fungicides in earlier trials could be attributed to previously undetected QoI insensitivity in some isolates of *P. aphanidermatum.* However, the isolate used in Experiments 3 and 4 was sensitive to these fungicides when tested *in vitro*. Additional research is needed to address control of Pythium root rot caused by fungicide-insensitive isolates.

Among the isolates of *P. aphanidermatum* screened in this study, 28% were insensitive to QoIs *in vitro*. Insensitivity to three fungicides belonging to the QoI class

(Fungicide Resistance Action Committee [FRAC] Code 11) was observed in seven isolates of *P. aphanidermatum*. The QoIs completely inhibited mycelial growth of the sensitive isolates. One isolate, PA19 exhibited *in vitro* insensitivity to both mefenoxam (phenylamide, FRAC Code 4) and insensitivity to three QoIs tested. To our knowledge, this is the first report of insensitivity to QoIs and dual insensitivity to QoIs and mefenoxam in *P. aphanidermatum* isolates collected from greenhouse floriculture crops. The finding of QoI-insensitive isolates was unexpected and indicates that the options growers have for chemical control of *Pythium* spp. may be fewer than previously assumed. This also reinforces the need for routine screening for fungicide sensitivity in effort to monitor resistance development in pathogen populations over time.

It is not known whether QoI insensitivity is widespread in *Pythium* populations found in poinsettia production systems. QoIs are not commonly used to control Pythium root rot on poinsettia in North Carolina, but products containing azoxystrobin and trifloxystrobin are used to prevent fungal leaf spot diseases, including powdery mildew and botrytis blight (Leonberger and Beckerman 2010; Hausbeck and Werner 2002). All seven insensitive isolates identified in this study were collected from one large greenhouse operation in North Carolina over a period of five years. This grower reported making frequent foliar applications of azoxystrobin to manage powdery mildew on poinsettia while simultaneously applying drenches of mefenoxam and etridiazole for Pythium root rot control. It is possible that these practices inadvertently selected for QoI insensitivity in *Pythium* populations over time. The possibility of off-target effects associated with intensive fungicide programs further

reinforces the need for resistant cultivars and for fungicide programs that incorporate two or more modes of action.

Resistance to propamocarb, the active ingredient in Banol, has been observed in some isolates of *P. aphanidermatum*, *P. irregulare*, and *P. ultimum* (Moorman and Kim 2004). Propamocarb is applied to turfgrass to manage *Pythium* diseases, but usually is ineffective. It is not commonly used to control Pythium root rot in poinsettia production systems. In several previous studies, the EC_{50} of propamocarb *in vitro* ranged from 0.5 to 10 µg/ml for *P. aphanidermatum*, *P. splendens*, *P. irregulare*, and *P. ultimum*, but EC_{50} values close to 1,000 µg/ml or more were found in others (Papavizas et al. 1978; Moorman and Kim 2004). In the current study, propamocarb (Banol) at the label-recommended rate of 1,037 µg/ml did not completely inhibit mycelial growth on amended media. Mycelial growth inhibition in some isolates was as low as 51% on media amended with propamocarb (concentrations ranging from 519 – 5,185 µl a.i./ ml). Banol was not effective in controlling Pythium root rot on several cultivars inoculated with *P. aphanidermatum*. It is not clear if these observations indicate that isolate PA1 used in the disease management experiments was insensitive to propamocarb, or if *P. aphanidermatum* populations in general are not sensitive to propamocarb at the rates used in these trials.

The biological control product Rootshield Plus (*Trichoderma* spp.) did not suppress Pythium root rot in any of the cultivars tested. To achieve consistency with the fungicide treatments, Rootshield Plus was applied as a drench at transplant 24 h prior to inoculation. Other studies have applied biological control agents several days to weeks before inoculation to provide ample time for the biological control agent to colonize the rhizosphere. It is

possible that *Trichoderma* may have been more effective in this trial if allowed to colonize potting media well before inoculation (Garcia-Garza et al., 2003; Harman, 2000). Other studies have reported that *Trichoderma* spp. protected snapdragons against *P. irregulare* but not *P. aphanidermatum* or *P. dissotocum* (Múnera and Hausbeck 2015).

Two phosphonates, fosetyl-al and potassium phosphite, completely inhibited *in vitro* mycelial growth of four isolates of *P. aphanidermatum* at concentrations ranging from 384 to 3,480 µg a.i./ml (fosetyl-al) and 341 to 3,405 µl a.i./ml (potassium phosphite). A similar range of sensitivity to fosetyl-al (1,256 to 1,508 µg/ml) was found in isolates of *P. irregulare, P. sylvaticum,* and *P. ultimum* collected from forest nurseries and a soil drench with fosetyl-al (576 µg a.i./ml) was one of the most effective treatments to control damping-off of Douglas Fir (Weiland et al. 2014). Other studies have found isolates of *P. aphanidermatum* with much lower (35.6 to >171.8 µg/ml) sensitivity ranges (Fenn and Coffey, 1984; Cook et al., 2009). In this study, poinsettia was inoculated with an isolate of *P. aphanidermatum* that was sensitive to fosetyl-al and potassium phosphite *in vitro,* but neither phosphonate controlled disease at label rates (768 µg a.i./ml for fosetyl-al; 681 µg a.i./ml for potassium phosphite). This is in agreement with other studies in which transplant drenches with either fosetyl-al or potassium phosphite failed to suppress Pythium root rot on poinsettia (Hausbeck and Harlan 2012; Parker and Benson 2012). These results demonstrate that *in vitro* sensitivity and EC_{50} values may not accurately reflect or predict disease control with phosphonates.

The efficacy data presented herein can provide growers with new alternatives for the control of *P. aphanidermatum* in poinsettia. Etridiazole (FRAC Code 14) consistently gave

excellent protection against Pythium root rot and the risk of resistance development is low among members of this fungicide class. Cyazofamid and fenamidone are not commonly used in poinsettia production systems, but both controlled disease across all cultivars tested. In the absence of resistant isolates, mefenoxam is still one of the most efficacious products against *Pythium* species. These four products can provide good control of Pythium root rot in poinsettia production systems, and rotating them within or across cropping cycles could reduce the risk of selecting fungicide-resistant populations of *P. aphanidermatum*. In this study, treating partially resistant cultivars with azoxystrobin, pyraclostrobin, and propamocarb provided control superior to that provided by either strategy alone, demonstrating the combined utility of using host resistance and fungicides for integrated management of Pythium root rot in poinsettia. The results of this study can be used to guide growers toward management programs that maximize disease control across both susceptible and partially resistant cultivars.

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Table 2.1. Fungicide products and concentrations evaluated for *in vitro* efficacy against four isolates of *Pythium aphanidermatum*

		Concentrations µg a.i./ml[b]			
Active ingredient[a]	**Trade name**	**0.5x rate**	**1x rate**	**2x rate**	**5x rate**
Fosetyl-Al	Aliette	384	768	1,536	3,840
Propamocarb	Banol	519	1,037	2,074	5,185
Pyraclostrobin	Empress Intrinsic	59	117	234	585
Azoxystrobin	Heritage	18	35	70	175
Fenamidone	Fenstop	244	488	976	2,400
Potassium phosphite	ProPhyt	341	681	1,362	3,405
Cyazofamid	Segway	40	79	158	•••
Mefenoxam	Subdue Maxx	9	18	36	88
Etridiazole	Terrazole WP	91	182	364	910

[a] SHAM (50 mg/ml) was added to QoI fungicides (azoxystrobin, pyraclostrobin, and fenamidone)

[b] Clarified V8 agar was amended with indicated concentrations of each fungicide

Table 2.2. Fungicide treatments used to evaluate the combined utility of fungicides and cultivar selections to manage Pythium root rot on poinsettia in two experiments

			Application timing	
Active ingredient	**Trade name**	**Rate/ L[a]**	**Exp 3**	**Exp 4**
Experiments 3 and 4				
Mefenoxam	Subdue Maxx	0.08 ml	T	T, 6
Etridiazole	Terrazole 35 WP	0.52 g	T	T, 4
Azoxystrobin	Heritage	0.07 g	T	T, 4
Pyraclostrobin	Empress Intrinsic	0.50 ml	T	T, 4
Fenamidone	Fenstop	1.10 ml	T	T, 4
Cyazofamid	Segway	0.23 ml	T	T, 2, 4
Potassium phosphite	Prophyt	1.25 ml	T	T, 2, 4
Fosetyl-Al	Aliette	0.96 g	T	T, 2, 4
Propamocarb	Banol	1.56 ml	T	T, 2, 4
Trichoderma spp.	Rootshield Plus	0.45 g	T	T
Experiment 4 only[c]				
Mefenoxam + etridiazole	Subdue/Terrazole	0.08 ml / 0.52 g		T, 6
Mefenoxam + cyazofamid	Subdue/Segway/Subdue	0.08 ml/ 0.23 ml / 0.08 ml		T, 6, 8

[a] The formulated rate was applied as a soil drench (118 ml per 15-cm pot) at transplant (T) in all experiments. Application was made 24 h prior to inoculation with *Pythium aphanidermatum*. The Root Shield (*Trichoderma* spp.) treatment was applied as a soil drench (236 ml per 15-cm pot).
[b] Timing (in weeks) of applications made in Experiment 4 following the initial application at transplant.
[c] Applied as a rotation in Experiment 4.

Table 2.3. *In-vitro* sensitivity of four isolates of *Pythium aphanidermatum* to nine fungicides

	Percent inhibition of mycelial growth ± S.E.[a]			
Treatment (µg a.i./ml)[b]	**PA1**	**PA2**	**PA3**	**PA4**
Etridiazole				
0.5 x (91)	100	100	100	100
1 x (182)	100	100	100	100
2 x (364)	100	100	100	100
5 x (910)	100	100	100	100
Fosetyl-al				
0.5 x (384)	100	100	100	100
1 x (768)	100	100	100	100
2 x (1,536)	100	100	100	100
5 x (3,840)	100	100	100	100
Potassium phosphite				
0.5 x (340.5)	100	88.5 ± 9.06	100	100
1 x (681)	100	100	100	100
2 x (1,362)	100	100	100	100
5 x (3,405)	100	100	100	100
Pyraclostrobin				
0.5 x (59)	100	19.8 ± 8.44	100	100
1 x (117)	100	20.5 ± 7.07	100	100
2 x (234)	100	25.0 ± 8.51	100	100
5 x (585)	100	27.5 ± 15.02	100	100
Azoxystrobin				
0.5 x (17.5)	100	25.3 ± 8.51	100	100
1 x (35)	100	46.0 ± 15.02	100	100
2 x (70)	100	60.4 ± 18.42	100	100
5 x (175)	100	56.0 ± 12.57	100	100
Fenamidone				
0.5 x (244)	100	25.3 ± 7.18	100	100
1 x (488)	100	19.7 ± 7.22	100	100
2 x (976)	100	21.1 ± 5.79	100	100
5 x (2,400)	100	27.2 ± 6.33	100	100
Mefenoxam				
0.5 x (8.8)	100	100	12.0 ± 6.85	10.1 ± 13.2
1 x (17.6)	100	100	9.4 ± 7.07	3.1 ± 5.81
2 x (35.5)	100	98.2 ± 4.45	11.9 ± 12.97	1.2 ± 6.95
5 x (88)	100	100	19.6 ± 20.89	7.5 ± 11.79

Table 2.3. continued

Propamocarb				
0.5 x (519)	82.4 ± 9.81	75.5 ± 6.68	80.5 ± 3.01	82.2 ± 4.07
1 x (1,037)	84 ± 12.72	74.9 ± 4.79	82.5 ± 1.56	80.9 ± 3.11
2 x (2,074)	79.1 ± 11.62	69.3 ± 9.25	81.5 ± 1.78	80.4 ± 1.38
5 x (5,185)	66.3 ± 9.40	65.5 ± 8.38	78.6 ± 2.01	79.9 ± 10.01
Cyazofamid				
0.5 x (39.5)	74.0 ± 6.04	55.8 ± 10.04	56.2 ± 7.87	80.2 ± 22.3
1 x (79)	70.8 ± 4.11	57.4 ± 18.95	57.7 ± 7.51	79.5 ± 22.9
2 x (158)	64.6 ± 11.47	61.7 ± 7.22	55.9 ± 15.64	88.4 ± 12.8

[a] Percent mycelial growth inhibition ± standard error. Values represent data from two runs and three replicates per run. Isolates PA1 and PA2 are sensitive to mefenoxam; PA3 and PA4 are insensitive to mefenoxam.

[b] Clarified V8 agar was amended with 0.5, 1, 2, and 5x label concentrations of each fungicide. The opaque quality of media amended with the 5x rate of cyazofamid reduced scorability so this treatment was removed.

Table 2.4. *In-vitro* sensitivity of 25 isolates of *Pythium aphanidermatum* to mefenoxam, azoxystrobin, pyraclostrobin, fenamidone, cyazofamid, and propamocarb

	Percent inhibition of mycelial growth ± S.E.[a]					
Isolate	**Mefenoxam** 35.2 µg a.i./ml	**Azoxystrobin** 70 µg a.i./ml	**Pyraclostrobin** 117 µg a.i./ml	**Fenamidone** 976 µg a.i./ml	**Cyazofamid** 158 µg a.i./ml	**Propamocarb** 2,074 µg a.i./ml
PA1	93.4 ± 1.8 b	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	81.8 ± 9.6 ab	61.3 ± 3.0 ef
PA2	92.1 ± 1.8 b	44.0 ± 2.3 d	33.3 ± 2.0 d	19.9 ± 3.5 bc	68.2 ± 9.6 b	65.9 ± 3.0 bcde
PA3	5.4 ± 1.8 de	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	73.3 ± 9.6 ab	63.2 ± 3.0 de
PA4	2.9 ± 1.8 e	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	71.8 ± 9.6 b	73.2 ± 3.0 ab
PA5	100 ± 1.8 a	60.7 ± 2.3 b	34.4 ± 2.0 cd	18.8 ± 3.5 c	72.9 ± 9.6 b	68.1 ± 3.0 abcde
PA6	3.4 ± 1.8 e	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	81.9 ± 9.6 ab	74.2 ± 3.0 a
PA7	3.6 ± 1.8 e	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	78.1 ± 9.6 ab	72.5 ± 3.0 ab
PA8	100 ± 1.8 a	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	75.7 ± 9.6 ab	72.3 ± 3.0 ab
PA9	100 ± 1.8 a	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	74.1 ± 9.6 ab	70.9 ± 3.0 abcd
PA10	100 ± 1.8 a	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	76.9 ± 9.6 ab	71.6 ± 3.0 abc
PA11	100 ± 1.8 a	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	100 ± 9.6 a	74.9 ± 3.0 a
PA12	10.2 ± 1.8 cd	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	76.9 ± 9.6 ab	67.5 ± 3.0 abcde
PA13	100 ± 1.8 a	44.7 ± 2.3 d	34.0 ± 2.0 cd	20.2 ± 3.5 bc	100 ± 9.6 a	51.6 ± 3.0 g
PA14	100 ± 1.8 a	54.1 ± 2.3 c	43.3 ± 2.0 b	28.6 ± 3.5 b	79.1 ± 9.6 ab	54.9 ± 3.0 fg
PA15	100 ± 1.8 a	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	100 ± 9.6 a	63.9 ± 3.0 cde
PA16	100 ± 1.8 a	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	78.2 ± 9.6 ab	70.9 ± 3.0 abcd
PA17	100 ± 1.8 a	41.3 ± 2.3 d	32.9 ± 2.0 d	22.4 ± 3.5 bc	66.9 ± 9.6 b	69.9 ± 3.0 abcd
PA18	100 ± 1.8 a	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	81.7 ± 9.6 ab	71.5 ± 3.0 abc
PA19	5.2 ± 1.8 de	59.7 ± 2.3 bc	38.8 ± 2.0 bc	24.8 ± 3.5 bc	74.1 ± 9.6 ab	63.2 ± 3.0 de
PA20	11.6 ± 1.8 c	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	77.2 ± 9.6 ab	67.8 ± 3.0 abcde
PA21	100 ± 1.8 a	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	81.7 ± 9.6 ab	72.1 ± 3.0 abc
PA22	100 ± 1.8 a	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	72.8 ± 9.6 b	73.4 ± 3.0 ab
PA23	10.1 ± 1.8 cd	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	79.7 ± 9.6 ab	75.4 ± 3.0 a
PA24	11.9 ± 1.8 c	100 ± 2.3 a	100 ± 2.0 a	100 ± 3.5 a	57.6 ± 9.6 b	71.9 ± 3.0 abc
PA25	100 ± 1.8 a	46.7 ± 2.3 d	32.9 ± 2.0 d	22.6 ± 3.5 bc	72.2 ± 9.6 b	69.8 ± 3.0 abcd

[a] Percent mycelial growth inhibition ± standard error of 25 *Pythium aphanidermatum* isolates. Values represent data from two runs and three replicates per run. Isolate least square means followed by the same letter are not significantly according to paired t-tests (α=0.05).

Table 2.5. Differences in severity of Pythium root rot in poinsettia cultivars with varying levels of partial resistance and treated with two or three applications of plant protection products in Experiment 4

	Cultivars[b]									
Treatment[a]	'Sparkling Punch'	'Advent Red'	'Infinity Polar'	'Infinity Red'	'Premium Red'	'Prin. Max White'	'Prestige Red'	'Viking Red'	'Luv U Pink'	**Treatment LSM**
Inoc. control	4.9 a	4.5 a	4.7 a	4.3 a	4.8 a	4.6 ab	4.2 a	3.1 b	3.9 a	**4.3 ± 0.1 A**
Trichoderma spp.	4.9 a	4.8 a	4.8 a	4.3 a	4.8 a	4.8 a	4.0 ab	3.2 b	3.3 ab	**4.3 ± 0.1 A**
Potassium phosphite	4.8 a	4.9 a	4.2 a	4.1 a	4.5 ab	3.8 bc	4.3 a	4.3 a	3.0 bc	**4.2 ± 0.1 A**
Fosetyl-al	4.8 a	4.7 a	4.1 a	3.7 ab	3.5 c	3.5 dc	3.3 bcd	4.3 a	3.4 ab	**3.9 ± 0.1 B**
Pyraclostrobin	4.8 a	4.8 a	4.1 a	4.3 a	3.3 c	4.7 a	3.3 bc	2.1 cd	2.4 cd	**3.8 ± 0.1 B**
Azoxystrobin	4.3 a	4.7 a	4.0 a	4.0 a	3.9 bc	3.0 dc	2.5 d	2.5 bc	1.5 ef	**3.4 ± 0.1 C**
Propamocarb	2.8 b	2.0 b	2.4 b	3.1 b	1.9 d	2.7 d	3.1 cd	2.9 bc	2.1 de	**2.5 ± 0.1 D**
Cyazofamid	1.6 c	2.1 b	1.7 bc	1.1 c	1.4 de	1.3 e	1.4 e	1.0 e	1.2 f	**1.4 ± 0.1 E**
Mef/ etridriazole[c]	1.5 c	1.0 c	1.0 c	1.5 c	1.8 de	1.1 e	1.1 e	1.3 ed	1.0 f	**1.2 ± 0.1 EF**
Mef/ cyazo/ mef[d]	1.3 c	1.0 c	1.1 c	1.1 c	1.1 de	1.2 e	1.3 e	1.6 ed	1.0 f	**1.2 ± 0.1 EF**
Etridiazole	1.6 c	1.2 c	1.3 c	1.0 c	1.2 de	1.1 e	1.0 e	1.0 e	1.0 f	**1.1 ± 0.1 F**
Fenamidone	1.3 c	1.0 c	1.3 c	1.2 c	1.2 de	1.1 e	1.0 e	1.0 e	1.1 f	**1.1 ± 0.1 F**
Mefenoxam	1.1 c	1.3 c	1.4 c	1.0 c	1.0 e	1.0 e	1.2 e	1.1 e	1.0 f	**1.1 ± 0.1 F**
Healthy control	1.4 c	1.0 c	1.0 c	1.0 c	1.0 e	1.0 e	1.0 e	1.0 e	1.1 f	**1.0 ± 0.1 F**
± *S.E.*	*± 0.2*	*± 0.3*	*± 0.3*	*± 0.3*	*± 0.3*	*± 0.3*	*± 0.3*	*± 0.3*	*± 0.3*	
P > F	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*	
Cultivar LSM	**2.9 ± 0.1 A**	**2.8 ± 0.1 AB**	**2.6 ± 0.1 BC**	**2.5 ± 0.1 C**	**2.5 ± 0.1 CD**	**2.5 ± 0.1 CD**	**2.3 ± 0.1 DE**	**2.2 ± 0.1 E**	**1.9 ± 0.1 F**	

[a] The first treatment was applied as a soil drench (118 ml per 15-cm pot) 24 h prior to inoculation with *P. aphanidermatum*. Subsequent applications were made 2, 4, or 6 weeks after transplanting based on label recommendations for each product.
Trichoderma spp. treatment was applied as a soil drench (236 ml per 15-cm pot). [b] Values represent data from two runs and three replicates per run. Least square means followed by the same letter are not different according to paired t-tests (α=0.05). Use letters within a column to compare fungicides within cultivars. Use upper-case bold letters to compare main effect least square means of fungicides or cultivars.
[c] Rotation treatment consisting of mefenoxam at transplant followed by etridiazole at week 6.
[d] Rotation treatment consisting of mefenoxam at transplant followed by cyazofamid at week 6, followed by mefenoxam at week 8.

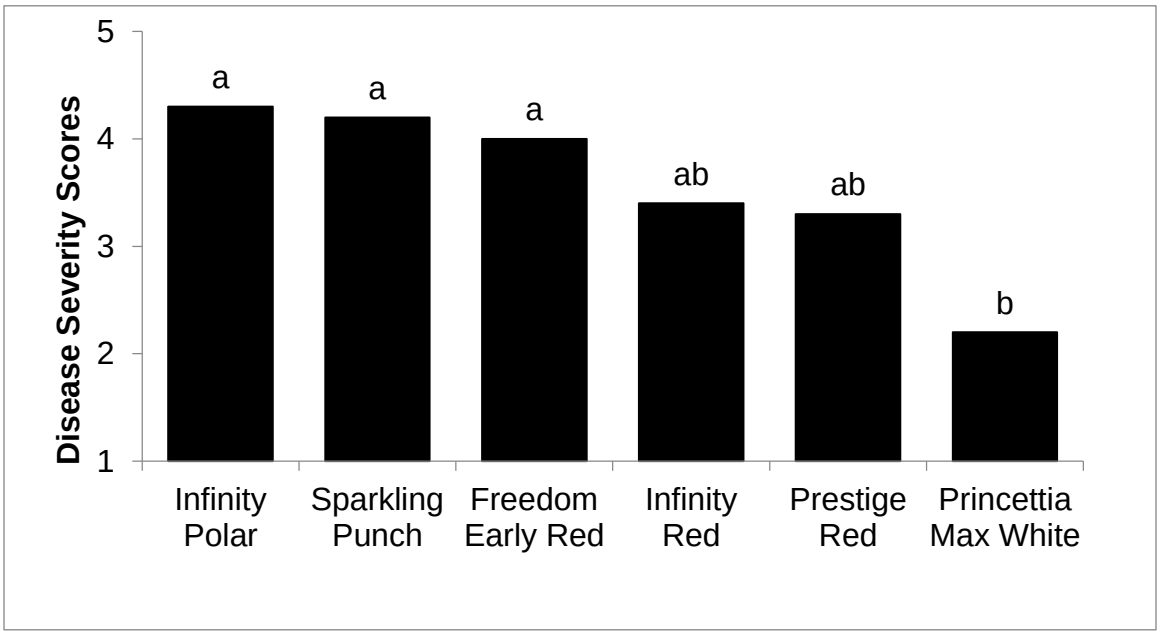

Figure 2.1. Pythium root rot disease severity scores for untreated, non-inoculated poinsettia cultivars, averaged across 3 replicates and runs of Experiment 3. LSmeans followed by the same letter are not significantly different according to paired t-tests (α=0.05).

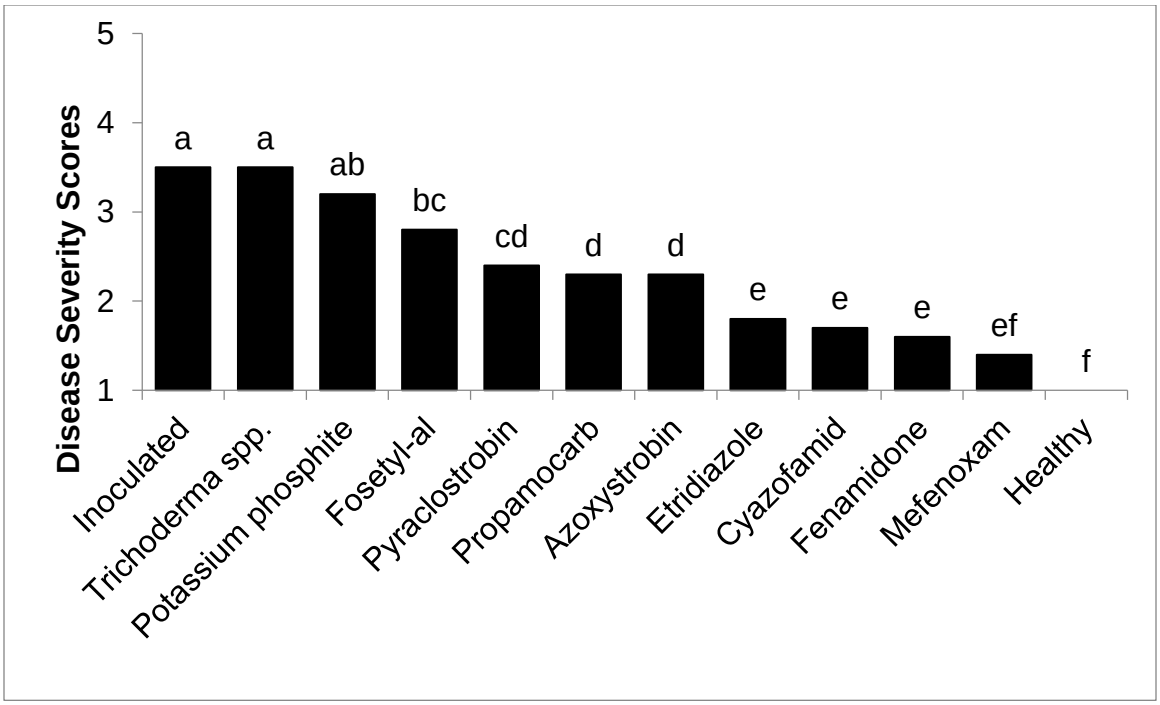

Figure 2.2. Pythium root rot disease severity scores of fungicide treatments averaged across six cultivars of poinsettia, 3 replicates, and runs of Experiment 3. A single fungicide application was made at transplanting. LSmeans followed by the same letter are not significantly different according to paired t-tests (α=0.05).

CHAPTER 3

Integrated management of Pythium root rot of poinsettia caused by fungicide-insensitive strains of *Pythium aphanidermatum*

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Abstract

Pythium aphanidermatum is the predominant species causing Pythium root rot of commercially grown poinsettia in North Carolina. Control strategies for *P. aphanidermatum* rely heavily on fungicides. *In vitro* insensitivity to mefenoxam and fenamidone, a quinone-outisde inhibitor (QoI), has been observed in isolates of *P. aphanidermatum* collected from greenhouses in North Carolina. Seven poinsettia cultivars were treated with label rates of mefenoxam (17.6 µl a.i/ml) or fenamidone (488 µl a.i./ml) and inoculated with isolates of *P. aphanidermatum*. Mefenoxam and fenamidone did not control Pythium root rot in plants inoculated with isolates of *P. aphanidermatum* insensitive to mefenoxam and fenamidone *in vitro*, thereby demonstrating practical resistance to both fungicides. Partially resistant cultivars, 'Luv U Pink' and 'Visions of Grandeur', exhibited less severe symptoms than highly susceptible cultivars when they were inoculated with fungicide-insensitive isolates and treated with mefenoxam or fenamidone. This suggests that partially resistant cultivars may be used to reduce selection pressure on fungicide-sensitive isolates and reduce losses due to fungicide-resistant isolates. Mefenoxam and fenamidone are both considered at high risk for resistance development. Treatment programs that incorporate products with two or more modes of action were evaluated on three cultivars with varying levels of partial resistance. Treatment programs using single applications at transplant were compared with rotations of two or three fungicide applications. All single application and rotation programs prevented Pythium root rot on all cultivars inoculated with insensitive isolates, even when mefenoxam and fenamidone were included in the rotation. These results provide growers with a choice of fungicide programs for use in their facilities.

Pythium aphanidermatum is the predominant species causing Pythium root rot of commercially grown poinsettia in North Carolina. Control strategies for *P. aphanidermatum* rely heavily on fungicides and growers prefer to use mefenoxam for root rot control because of its relatively low cost and high efficacy. Unfortunately, mefenoxam resistance is widespread in greenhouse populations of *Pythium* and both sensitive and resistant isolates often are found concurrently in production systems (Lookabaugh et al. 2015; Moorman et al. 2002; Múnera and Hausbeck 2016). Additionally, *in vitro* insensitivity to three quinone-outside-inhibitors (QoIs) has been found in isolates of *P. aphanidermatum* sampled from poinsettia growing in commercial greenhouses in North Carolina (Lookabaugh and Shew, 2017). Although several QoIs are labeled for Pythium root rot control in poinsettia, they are not widely used for this purpose. However, products containing trifloxystrobin and azoxystrobin often are applied to poinsettia to control botrytis blight and powdery mildew (Leonberger and Beckerman, 2010; Hausbeck and Werner, 2002). It is possible that intensive use of QoIs on poinsettia and alternative rotational crops has inadvertently resulted in selection of QoI-insensitive isolates of *Pythium*.

Practical resistance or field resistance are terms used to describe the observable losses of chemical control due to the presence of fungicide-insensitive or resistant isolates of a plant pathogen (Köller 1991). Mefenoxam-insensitive isolates have been recovered from diseased poinsettia previously treated with mefenoxam, but studies that conclusively demonstrate practical resistance to mefenoxam have not been completed on poinsettia. In the absence of such studies, other explanations for control failures, such as fungicide misapplication,

fungicide degradation over time, or infection prior to fungicide application cannot be eliminated.

Poinsettia is vegetatively propagated through cuttings, which are provided by a limited number of commercial sources. Due to the interconnected nature of the floriculture industry, diseased or infected propagative material can easily move between facilities, providing a pathway for new or repeated pathogen introduction and movement of fungicide-resistant strains. In North Carolina, however, several growers produce cuttings from their own stock plants, which are maintained in vegetative (non-reproductive) growth for this purpose. This practice extends the length of time poinsettias are present in production systems by several months, increases the number of fungicide applications needed for disease control, and increases potential contacts with other infected hosts. The possibility that off-target effects are associated with repeated fungicide applications further reinforces the need for integrated control strategies and for fungicide programs that incorporate products with different modes of action.

The use of disease resistant varieties is an economical and readily implemented integrated management strategy to limit disease on agronomic crops (Lucas and Griffiths, 2004; Li et al., 2016; Bates et al., 2008; Higginbothom et al., 2004). Complete resistance to *Pythium* species is uncommon in most cultivated species of plant and floriculture crops are not regularly screened for partial resistance (Chagnon and Belanger, 1991; Deng et al., 2005 a & b). However, several commercially available poinsettia cultivars appear to possess partial resistance to Pythium root rot (Lookabaugh and Shew, 2017). These results suggest that partial resistance in poinsettia could be used in combination with fungicide applications to

limit disease caused by *P. aphanidermatum* and mitigate loss of control due to fungicide insensitivity.

In recent efficacy trials, etridiazole (FRAC Code 14) consistently gave excellent protection against Pythium root rot (Lookabaugh and Shew 2017). The risk of resistance development is low among members of its class, making it an attractive candidate for use in resistance management programs. Cyazofamid (FRAC Code 21) and fenamidone (QoI, FRAC Code 11) are not commonly used in poinsettia production systems, but both controlled disease when tested on plants inoculated with a fungicide-sensitive isolate of *P. aphanidermatum*. In the absence of resistant isolates, mefenoxam is highly effective against *Pythium* species (Lookabaugh and Shew 2017; Múnera and Hausbeck 2015; Parker and Benson 2013, 2012). These four products can provide good control of Pythium root rot in poinsettia production systems, and rotating or mixing them within or across cropping cycles could reduce the risk of selecting fungicide-resistant populations of *P. aphanidermatum* and corresponding control failures. Coupling fungicide resistance management programs with partially resistant cultivars could further reduce these risks (Wolf, 1981; Chin 1987; Taylor et al. 2008). The objectives of this study were 1) to determine whether inoculation of poinsettia with isolates of *P. aphanidermatum* that exhibit *in vitro* insensitivity to mefenoxam and fenamidone results in control failures under production conditions, 2) to explore the combined utility of fungicides and cultivars with varying levels of partial resistance to manage Pythium root rot caused by fungicide-insensitive isolates of *P. aphanidermatum*, and 3) to develop fungicide programs that suppress Pythium root rot caused by fungicide-

sensitive and resistant isolates of *P. aphanidermatum* on poinsettia cultivars with varying levels of partial resistance.

Materials and Methods

Inoculum production. The four isolates (PA1, PA2, PA3, PA4) used in this study were collected from diseased plants during a survey of North Carolina floriculture greenhouses (Lookabaugh et al. 2015). Isolates were previously tested for mefenoxam (M) and fenamidone (F) sensitivity (S) and insensitivity (R) and included PA1, mefenoxam sensitive, fenamidone sensitive, (MefS, FenS); PA2, mefenoxam insensitive, fenamidone sensitive (MefR, FenS); PA3, mefenoxam sensitive, fenamidone insensitive (MefS, FenR), and PA4, mefenoxam insensitive, fenamidone insensitive (MefR, FenR). 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 rice and 18 ml deionized water). Cultures were incubated for 5 days at 25°C and flasks were shaken twice daily to promote uniform colonization of rice grains and prevent clumping (Holmes and Benson, 1994).

Plant culture. All planting materials were obtained from commercial sources (Dümmen Orange) and cultivars representing a range in partial resistance were selected based on results from previous studies (Lookabaugh and Shew, 2017). Unrooted cuttings of test cultivars 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 peat-based potting media. Once transplanted, plants were fertilized with 5 g of Multicote 4

(14-14-16) sprinkled evenly on the pot surface and watered in. Plants were maintained under drip irrigation and treated with Safari insecticide (Dinotefuran; 1.25 ml per liter) three weeks after transplanting to control whiteflies.

Assessing above-ground disease severity and root rot. Shoot symptoms were evaluated on an above-ground disease severity scale of 1 = healthy plant, 2 = slightly stunted, 3 = chlorosis, moderate stunting and/or defoliation, 4 = wilting and/or severe stunting, and 5 = dead (Lookabaugh et al., 2015). Plants were carefully inverted, the pot was removed and roots were observed with the substrate staying 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 sloughs 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 (Parker and Benson, 2013). In all trials, analysis with PROC CORR procedures (SAS 9.4, SAS Institute) indicated that root rot ratings and above-ground disease severity ratings were highly correlated (Spearman's $r \geq 0.8692$, $P < 0.0001$). These variables then were averaged for each observation to create disease severity scores (DSS) used in all subsequent analyses.

Experiment 1: Practical implications of mefenoxam insensitivity for disease control. This experiment was conducted in the 2015 and 2016 growing seasons. In 2015, plants were transplanted 16 Sept, treated for the first time and inoculated 17 Sept and

harvested 19 Nov. In 2016, plants were transplanted 21 Sept, treated for the first time and inoculated 22 Sept, and harvested 12 Dec.

The experiment included seven poinsettia cultivars in 2015: 'Red Glitter', ''Polly's Pink', 'Polar Bear', 'Visions of Grandeur', Prestige Early Red', 'Jubilee Red', and 'Enduring White'. Seven cultivars were used in 2016, including 'Red Glitter', Polly's Pink', 'Polar Bear', 'Visions of Grandeur', 'Prestige Red', 'Sparkling Punch', and 'Luv U Pink'. Some of cultivars differed between 2015 and 2016 due to availability.

Plants of each cultivar were inoculated with *P. aphanidermatum* isolate PA1, mefenoxam-sensitive (MefS) or PA2, mefenoxam-insensitive (MefR). Inoculated plants received treatments with mefenoxam or were not treated for a total of four treatment combinations per cultivar. Replicated non-inoculated, non-treated plants also were planted as controls but remained disease-free and were not included in the data analysis. Mefenoxam-treated plants were drenched (118 ml per pot) at transplant with Subdue Maxx (0.08 ml/L) and inoculated 24 h later. A reapplication was made 6 weeks later, for a total of two applications. At the end of the trials, disease severity was evaluated as described previously. Treatments were arranged in a randomized complete block design with three blocks.

Statistical analysis, Experiment 1: Data from both trials of the experiment were analyzed separately and analysis of variance and correlations were performed using PROC GLIMMIX and PROC CORR procedures (SAS 9.4, SAS Institute). Cultivar, fungicide, isolate, and their interactions were treated as fixed effects while block was treated as a random effect. The cultivar x treatment and cultivar x isolate interactions were significant and least squares means were calculated for the interactions. Simple effects of treatments

were analyzed within cultivar. Least square means were separated using paired t-tests where appropriate. Single-degree-of-freedom linear contrasts were performed to compare disease severity on plants treated with mefenoxam and not treated with mefenoxam. Population quartiles and percentiles were computed using PROC UNIVARIATE.

Experiments 2 and 3: Practical implications of fenamidone insensitivity or dual fungicide insensitivity for disease control. Two experiments, each with two trials, were conducted at two greenhouses located approximately 2.5 km apart, for a total of four trials. Experiment 2 evaluated practical implications of fenamidone resistance on vegetatively maintained plants and was conducted concurrently at both locations. Plants at each location were transplanted and treated 5 Jul 2016, inoculated 6 Jul 2016, and harvested 25 Aug 2016. Experiment 3 evaluated practical implications of fenamidone resistance on reproductive plants and was conducted concurrently at both locations. Plants were transplanted and treated for the first time on 22 Sept 2016, inoculated 23 Sept 2016, and harvested 12 Dec 2016 at both locations. For experiment 2, six cultivars representing a range of partial resistance were included: 'Red Glitter', 'Freedom Early Red', 'Polly's Pink', 'Polar Bear', 'Enduring Red', and 'Visions of Grandeur'. Due to cultivar availability, 'Freedom Early Red' was replaced with 'Infinity Red' in experiment 3.

Each experiment had three fungicide and four isolate treatment combinations, for a total of 12 treatments. Fungicide treatments included mefenoxam (Subdue Maxx, 0.08 ml/L) or fenamidone (Fenstop, 1.1 ml/L) applied by drench (118 ml per pot) at transplant and an untreated control. The isolates varied in their response to mefenoxam and fenamidone: PA1 was sensitive to both fungicides (MefS, FenS), PA2 was insensitive to mefenoxam but

sensitive to fenamidone (MefR, FenS), PA3 was sensitive to mefenoxam but not fenamidone (MefS, FenR), and PA4 was insensitive to both fungicides (MefR, FenR). Inoculum was produced as described and plants were inoculated 24 h after transplanting. Treatments were arranged in a randomized complete block design with three replicates. Non-inoculated, non-treated plants were maintained as controls but not included in the analysis. At the end of the trials, disease severity was evaluated as described previously.

Statistical analysis, Experiments 2 and 3. Data from trials within experiments were combined before analysis. Analysis of variance and correlation analysis were performed with PROC GLIMMIX and PROC CORR procedures (SAS 9.4, SAS Institute). Cultivar, treatment, isolate, and their interactions were treated as fixed effects while trial and block were treated as random effects. If interactions were significant, least squares means were calculated for the interactions and the simple effects were analyzed to determine significant differences. Least square means were separated using paired t-tests where appropriate.

Experiment 4: Efficacy of fungicide programs for control of Pythium root rot caused by insensitive isolates. The experiment was conducted simultaneously at two greenhouse locations, for a total of two trials. Initial fungicide treatments were applied by drench (118 ml per pot) at transplant. Plants at each location were transplanted and treated 6 Sept 2016, inoculated 7 Sept 2016, and harvested 12 Dec 2016.

Poinsettia cultivars 'Luv U Pink', 'Prestige Red', and 'Red Glitter' were treated with combinations of eight fungicide programs and four isolates, for a total of 32 treatments per cultivar. Fungicide rotation programs were designed to determine whether starting programs with etridiazole or cyazofamid at transplant could minimize damage caused by fungicide-

insensitive isolates. The eight fungicide programs consisted of an inoculated, non-treated control, a non-inoculated, non-treated control, and six fungicide programs (Table 3.1) as follows: 1) Etridiazole at transplant followed by cyazofamid (week 4) and etridiazole (week 8), for a total of three applications; 2) Cyazofamid at transplant followed by mefenoxam (week 4) and cyazofamid (week 10), for a total of three applications; 3) Etridiazole at transplant followed by mefenoxam (week 4), for a total of 2 applications; 4) Tank mix of etridiazole and mefenoxam at transplant followed by cyazofamid (week 6), for a total of two applications; 5) Tank mix of etridiazole and a low label rate of fenamidone at transplant followed by mefenoxam (week 4), for a total of two applications; and 6) a single application of a tank mix of a high label rate of etridiazole and mefenoxam at transplant. The intervals between and total number of fungicide applications, and thus the total number of applications, were based on the product labels.

Inoculum of four isolates of *P. aphanidermatum* either sensitive or resistant to mefenoxam and/or fenamidone (PA1 MefS, FenS; PA2 MefR, FenS; PA3 MefS, FenR; PA4 MefR, FenR) was produced as described above and plants were inoculated 24 h after transplanting. The 96 treatments were arranged in a randomized complete block design with three replicates. At the end of the trials, disease symptoms were evaluated as described previously.

Statistical analysis, Experiment 4. Data from the two trials were combined for analysis. Analysis of variance and correlation analysis were performed using PROC GLIMMIX and PROC CORR procedures (SAS 9.4, SAS Institute). Cultivar, rotation treatment, isolate, and their interactions were treated as fixed effects while trial and block

were treated as random effects. If interactions were significant, least squares means were calculated for the interactions and simple effects were analyzed to determine significant differences. Least square means were separated using paired t-tests where appropriate.

Results

Experiment 1: Practical implications of mefenoxam insensitivity for disease control. Disease severity was high in both years of the experiment (Tables 3.2 and 3.3). Non-inoculated, non-treated plants did not exhibit disease symptoms and were not included in the data analysis. In both experiments, mefenoxam treatment reduced disease severity overall (main effect fungicide $P < 0.0001$). Variations in disease severity were significant for cultivar (main effect $P < 0.0001$) and isolate (main effect $P < 0.0001$, 2015; $P = 0.0003$, 2016), but disease severity differed across cultivars in response to fungicide and isolate combinations. The interaction between fungicide and isolate was also significant ($P < 0.0001$), but the three-way interaction for cultivar x fungicide x isolate was not significant ($P = 0.3554$, 2015; $P = 0.4651$, 2016).

2015 results. Averaged across all cultivars, the sensitive (PA1) and insensitive isolate (PA2) caused similar amounts of disease (DSS 4.0 vs 3.6) in plants that were not treated with mefenoxam, but disease severity differed in plants treated with mefenoxam, which suppressed disease in plants inoculated with PA1 (DSS 1.5) but not PA2 (DSS 3.0; Table 3.2). Differences in disease severity caused by PA1 and PA2 varied by cultivar as indicated by a significant cultivar x isolate ($P = <0.0001$) interaction. Averaged across fungicide treatments, PA2 caused more disease than PA1 on ‘Prestige Early Red’, ‘Polar Bear’,

‘Jubilee Red’, ‘Enduring White’, and ‘Visions of Grandeur’. No differences in disease severity were observed in ‘Red Glitter’ and ‘Polly’s Pink’ inoculated with either isolate.

2016 results. Averaged across cultivars, disease severity scores did not differ on non-treated plants inoculated with either isolate (Table 3.2). Fungicide treatment efficacy varied by cultivar as indicated by a significant cultivar x fungicide treatment ($P = 0.0039$, 2016) interaction. Mefenoxam reduced disease on ‘Red Glitter’ and ‘Polly’s Pink’ but did not suppress disease symptoms on the most susceptible cultivar, ‘Sparkling Punch’, when disease severity scores were averaged across both isolates (Table 3.4). Disease severity scores were generally low on ‘Luv U Pink’, ‘Visions of Grandeur’, ‘Prestige Red’, and ‘Polar Bear’ plants inoculated with either isolate and no differences were observed between treated and non-treated plants (Table 3.4).

Experiments 2 and 3: Practical implications of fenamidone or dual fungicide insensitivity for disease control. Disease severity and root rot scores were high in experiments 2 and 3. Non-inoculated, non-treated plants did not exhibit disease symptoms in either experiment. Overall, results were consistent across both experiments. Disease severity scores varied among cultivars (main effect $P < 0.0001$), fungicide treatments (main effect $P < 0.0001$), and isolates (main effect $P < 0.0001$). Cultivars differed in response to fungicide and isolate treatment combinations as indicated by the interactions of cultivar x fungicide treatment ($P = 0.0014$, vegetative; $P < 0.0001$, reproductive) and cultivar x isolate ($P < 0.0256$, vegetative; $P = 0.0077$, reproductive). The interaction between fungicide and isolate was also significant ($P < 0.0001$). The three-way interaction for cultivar x fungicide x isolate

was not significant in the vegetative experiment but was significant in the reproductive experiment (P = 0.2073, vegetative; P = 0.0041, reproductive).

Averaged across all cultivars and in both experiments, disease severity scores were higher on non-treated plants inoculated with isolate PA1 (MefS, FenS; sensitive to both fungicides) than on plants inoculated with PA4 (MefR, FenR; insensitive to both fungicides, Table 3.5). Mefenoxam suppressed disease on plants inoculated with MefS isolates (PA1 and P3), but not on plants inoculated with MefR isolates (PA2 and PA4) Likewise, fenamidone suppressed disease on plants inoculated with FenS isolates (PA1 and PA2), but not in plants inoculated with FenR isolates (PA3 and PA4). Compared to plants inoculated with the dual-insensitive isolate PA4 (MefR, FenR) and treated with either fungicide, disease severity scores were higher in plants inoculated with isolate PA2 (MefR, FenS) and treated with mefenoxam, or in PA3 (MefS, FenR) and treated with fenamidone (Table 3.5).

Averaged across isolates, treatment efficacy varied by cultivar. In the vegetative plants in experiment 2, mefenoxam suppressed disease in five of the cultivars but did not control disease on highly susceptible 'Red Glitter'. Fenamidone suppressed disease in all six cultivars (Figure 3.1A). In the reproductive stage plants in experiment 3, mefenoxam suppressed disease across in all cultivars, but fenamidone did not control disease in 'Infinity Red.' It also did not affect disease severity in 'Visions of Grandeur', but this was attributed to low overall disease severity in this cultivar (Figure 3.1B).

Experiment 4: Efficacy of fungicide programs for control of Pythium root rot caused by fungicide-insensitive isolates. Disease severity and root rot scores were high in non-treated plants, whereas non-inoculated, non-treated plants did not exhibit disease

symptoms. Disease severity scores varied among cultivars (main effect $P < 0.0001$), fungicide rotation programs (main effect $P < 0.0001$), and isolates (main effect $P < 0.0001$). Cultivars differed in response to fungicide programs (cultivar x program $P < 0.0001$). The fungicide program x isolate interactions ($P = 0.0018$) and the three-way ($P = 0.0157$) interactions also were significant. The interactions can be explained by isolate and cultivar differences within the inoculated, non-treated plants.

Within non-treated plants, significant differences were observed among cultivars ($P < 0.0001$) and isolates ($P = 0.0088$). The cultivar 'Luv U Pink' had lower disease severity scores than 'Red Glitter' or 'Prestige Red' (Figure 3.2). Plants inoculated with the dual-insensitive isolate PA4 (MefR, FenR) had the lowest disease severity scores (2.8), while no differences in disease severity score were observed among cultivars inoculated with isolates PA1 (MefS, FenS), PA2 (MefR, FenS), or PA3 (MefS, FenR; Figure 3.3).

In contrast to the results on non-treated plants, all fungicide programs suppressed disease in all three cultivars tested (Table 3.6). Isolate effects were not significant among the treated plants, regardless of their sensitivity/insensitivity profiles (Table 3.6). A single application at transplant of mefenoxam and etridiazole suppressed disease on all cultivars throughout the remainder of the production season with control equal to treatments featuring repeated fungicide application treatments (Table 3.6).

Discussion

Widespread resistance to mefenoxam in *Pythium* species has been documented in greenhouse floriculture production systems, but resistance to QoIs has only recently been observed in isolates of *P. aphanidermatum* collected from poinsettia (Lookabaugh and Shew,

2017). Most studies have characterized resistant isolates using direct methods, including *in vitro* sensitivity screens and molecular characterization of resistant genotypes, but it is not known if *in vitro* insensitivity corresponds with control failures when fungicides are applied at label rates. Some insensitive isolates used in this study were collected from facilities that had reported control failures after mefenoxam applications. Associations between fungicide failures in the greenhouse and loss of *in vitro* sensitivity have been suspected previously, but have not been verified until now.

Practical resistance or field resistance are terms sometimes used to describe the observable loss of chemical control due to the presence of fungicide-resistant isolates of a pathogen. In greenhouse production systems, practical resistance may be difficult to observe because fungicide-resistant and sensitive isolates often are found concurrently in diseased samples (Lookabaugh et al. 2015). Growers may use several different fungicide products during a production cycle to target more than one disease; for example, QoIs are commonly used to control powdery mildew and Botrytis blight on poinsettia, while mefenoxam is applied to control Pythium root rot. While not commonly used to control Pythium root rot, QoIs have good activity against oomycetes and several are labeled for use on ornamentals, including poinsettia (Parker and Benson 2012; Lookabaugh and Shew 2017). Resistance to mefenoxam could at times be masked by non-target control provided by QoIs, while conversely, use of QoIs may have inadvertently selected for QoI-insensitive strains of *P. aphanidermatum*. In the current study, applying mefenoxam or fenamidone to plants inoculated with isolates insensitive to the respective fungicide failed to suppress Pythium root rot, thereby demonstrating practical resistance to both mefenoxam and fenamidone in *P.*

aphanidermatum. These results reinforce the need for routine fungicide resistance monitoring in greenhouse production systems so that shifts in fungicide resistance can be identified before loss of efficacy occurs.

Among non-treated plants, poinsettia cultivars inoculated with isolates that were sensitive to mefenoxam and fenamidone exhibited more severe disease symptoms than plants inoculated with the isolate resistant to both chemicals. These results suggest that in the absence of fungicides, some isolates sensitive to one or both fungicides may be more aggressive than isolates that are insensitive to both. However, other studies have shown that fungicide insensitivity was not correlated with aggressiveness, (Lookabaugh and Shew 2017).

The use of disease resistant cultivars is a common tool incorporated into integrated management strategies, but complete resistance to *P. aphanidermatum* is lacking in commercial poinsettia cultivars (Lookabaugh and Shew 2017; Trejo et al. 2012). Previous studies have identified cultivars that exhibited partial resistance to Pythium root rot, including the hybrid cultivar 'Luv U Pink'. In the current study, 'Luv U Pink' and the moderately resistant cultivar 'Visions of Grandeur' exhibited fewer disease symptoms than the highly susceptible cultivars when inoculated with fungicide-insensitive isolates and treated with mefenoxam or fenamidone. These results suggest that partially resistant cultivars may be used to reduce selection pressure on fungicide-sensitive isolates and to offset losses due to infection by fungicide-resistant isolates.

Relatively few chemicals have efficacy against *P. aphanidermatum* and several, including mefenoxam, fenamidone, and cyazofamid, are at moderate to high risk for

fungicide resistance development (Kuck et al. 2000). Typically, most growers apply mefenoxam at transplant followed by a second application of mefenoxam or etridiazole 6 weeks later. In more challenging situations, a third application may be made late in the season to ensure post-harvest performance for the consumer. In the current study, mefenoxam and fenamidone provided excellent control of disease caused by fungicide sensitive isolates across all cultivars tested, stressing the importance of resistance management strategies that safeguard the continued use of these chemicals.

Fungicide rotation programs started with etridiazole or cyazofamid at transplant to maximize suppression of mefenoxam or fenamidone insensitive isolates. Etridiazole has demonstrated good to excellent control of Pythium root rot in poinsettia and other floriculture crops and is low-risk for fungicide resistance development. Our results support the use of etridiazole or cyazofamid at transplant to minimize or counteract resistance development in *P. aphanidermatum*.

To our knowledge, this is the first study that evaluated fungicide efficacy against both fungicide-sensitive and insensitive isolates of *P. aphanidermatum*. Treatment programs using single applications (tank mixes) at transplant were compared with rotations of two or three fungicide applications. No significant differences were observed across any of the fungicide rotation treatments, with all programs offering excellent control of Pythium root rot in both partially resistant (‘Luv U Pink’) and susceptible (‘Red Glitter’ and ‘Prestige Red’) cultivars. A single transplant application of mefenoxam plus etridiazole suppressed disease throughout the production season and provided control equal to the programs with repeated fungicide applications. Tank mixes are an alternative to fungicide rotation treatments and can also be

used minimize the risk of fungicide resistance development (Wolf 1981; Urech and Staub 1985; Matheron and Porchas 2013). In poinsettia production systems, tank mixes to control Pythium root rot are not commonly used due to the cost of using two products for two or more applications. However, if tanks mixes allow growers to make fewer total fungicide applications to achieve adequate control, they may prove more economical than repeated applications, which increase the cost of labor. The levels of disease in this trial were considered challenging, with high levels of mortality in non-treated plants of susceptible cultivars. Further studies are needed to determine if single fungicide applications would provide a similar level of control under a range of typical production conditions. Single applications could be inadequate in systems where the pathogen could be spread throughout the production cycle by use of flood irrigation or recirculating water.

Single and repeated applications of mefenoxam or fenamidone alone failed to control Pythium root rot caused by insensitive isolates. However, the fungicide rotation programs provided excellent control of Pythium root rot in plants inoculated with either sensitive or insensitive isolates. These results suggest that the use of mefenoxam or fenamidone alone should be discontinued in facilities where insensitive isolates are found. Tank mixes or fungicide rotations create a diverse environment for pathogens and reduce the likelihood of that fungicide-insensitive isolates will be selected. Mixtures may also delay or prevent the development of insensitive populations in facilities with low sensitive-insensitive ratios (Urech and Staub 1985).

All six rotation programs prevented Pythium root rot on poinsettias inoculated with insensitive isolates, even when mefenoxam and fenamidone were included in the rotation.

These results provide growers with a choice of fungicide programs for use in their facilities. In facilities with high insensitive/sensitive ratios, etridiazole at transplant followed by cyazofamid (week 4) and etridiazole (week 8), does not include any treatments of mefenoxam or fenamidone so the risk of fungicide failures due to insensitive isolates is low.

Similar levels of Pythium root rot control were observed in experiments conducted during vegetative growth and reproductive growth. Control strategies discussed in this paper should offer consistent protection for poinsettia during both stock plant production (vegetative) and seasonal production (reproduction).

P. aphanidermatum has a wide host range and is commonly isolated from garden mum and tomato crops grown in rotation with poinsettia crops in greenhouse production systems, resulting in a near-constant source of host material. Emphasis on training growers in resistance management is especially important in greenhouse production systems because high-risk fungicides may be used to control one or more pathogens on several crops. Since resistance to mefenoxam is known throughout the industry, care must be taken to minimize the spread of resistant isolates between facilities on infected plants or propagation material.

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Table 3.1. Fungicides and application schedules for control of Pythium root rot on three cultivars of poinsettia inoculated with isolates of *P. aphanidermatum* insensitive to mefenoxam, fenamidone, or both fungicides

	Active Ingredients (Rate / Liter)[a]				
Program	**Transplant[b]**	**Week 4**	**Week 6**	**Week 8**	**Week 10**
E / C / E	Etridiazole (0.52 g)	Cyazofamid (0.23 ml)	N/A	Etridiazole (0.52 g)	
C / M / C	Cyazofamid (0.23 ml)	Mefenoxam (0.08 ml)	N/A	N/A	Cyazofamid (0.23 ml)
E / F	Etridiazole (0.52 g)	Fenamidone (1.1 ml)	N/A	N/A	
E + M / C	Etridiazole (0.52 g) + mefenoxam (0.08 ml)	N/A	Cyazofamid (0.23 ml)	N/A	N/A
E + F / M	Etridiazole (0.52 g)+ fenamidone (0.55 ml)	Mefenoxam (0.08 ml)	N/A	N/A	N/A
E + M	Etridiazole (0.74 g) + mefenoxam (0.08 ml)	N/A	N/A	N/A	N/A

[a] Fungicide treatments applied as a soil drench (118 ml per 15 cm pot) at transplant. Application was made 24 h before inoculation. E = etridiazole, C = cyazofamid, M = mefenoxam, F = fenamidone. Dashes (/) denote rotations while plus signs (+) denote tank mixes

[b] Initial treatments were applied at transplant and subsequent applications were made at 4, 6, 8 or 10 weeks after the initial application.

Table 3.2. Differences in Pythium root rot disease severity caused by two isolates of *Pythium aphanidermatum* that are sensitive or insensitive to mefenoxam in 2015 and 2016

	Treatments[a]					
	2015			**2016**		
Isolate[b]	No fungicide	Mefenoxam	**Isolate[c]**	No fungicide	Mefenoxam	**Isolate[c]**
PA1 MefS	4.0 ± 0.2 a	1.5 ± 0.1 b	**2.7 ± 0.1 B**	3.5 ± 0.2 a	1.4 ± 0.1 b	**2.5 ± 0.1 B**
PA2 MefR	3.6 ± 0.2 a	3.0 ± 0.1 a	**3.3 ± 0.1 A**	3.0 ± 0.2 a	3.2 ± 0.1 a	**3.1 ± 0.1 A**
P > F for isolate	*P = 0.1170*	*P < 0.0001*		*P = 0.0808*	*P < 0.0001*	
Treatment	**3.8 ± 0.1 A**	**2.2 ± 0.1 B**		**3.3 ± 0.1 A**	**2.3 ± 0.1 A**	

[a] Mefenoxam (Subdue Maxx, 0.08 ml/L) was applied as a soil drench (118 ml per 15 cm pot) at transplant. Application was made 24 h before inoculation.

[b] MefS = sensitive isolate (PA1), MefR = resistant isolate (PA2)

[c] Above-ground symptoms and root rot were rated individually on a 1 to 5 scale and ratings were averaged to create a disease severity score. Values represent data from three replicates averaged across seven cultivars. Isolate least square means followed by the same letter are not different according to paired t-tests (α=0.05).

Table 3.3. Differences in Pythium root rot disease severity in seven cultivars of poinsettia inoculated with two isolates of *Pythium aphanidermatum* that are sensitive or insensitive to mefenoxam in 2015

	Cultivars[a]							
Isolate[b]	Red Glitter	Prestige Early Red	Polly's Pink	Polar Bear	Jubilee Red	Enduring White	Visions of Grandeur	**Isolate[c]**
PA1 MefS	2.7 ± 0.3 a	3.1 ± 0.3 b	2.6 ± 0.2 a	2.0 ± 0.2 b	3.3 ± 0.3 b	2.7 ± 0.2 b	2.7 ± 0.4 a	**2.7 ± 0.1 B**
PA2 MefR	3.5 ± 0.3 a	4.4 ± 0.3 a	2.4 ± 0.2 a	2.9 ± 0.2 a	4.4 ± 0.3 a	4.0 ± 0.2 a	1.3 ± 0.4 b	**3.3 ± 0.1 A**
P > F for isolate	*P = 0.0755*	*P = 0.0138*	*P = 0.5237*	*P = 0.0131*	*P = 0.0361*	*P = 0.0033*	*P = 0.0370*	
Cultivar	**3.1 ± 0.2 B**	**3.8 ± 0.2 A**	**2.5 ± 0.2 C**	**2.5 ± 0.2 C**	**3.8 ± 0.2 A**	**3.3 ± 0.2 AB**	**2.0 ± 0.2 C**	

[a] Plants received one of two treatments: no fungicide or mefenoxam (Subdue Maxx, 0.08 ml/L) applied as a soil drench (118 ml per 15 cm pot) at transplant. Application was made 24 h before inoculation.

[b] MefS = sensitive isolate (PA1), MefR = resistant isolate (PA2)

[c] Above-ground symptoms and root rot were rated individually on a 1 to 5 scale and ratings were averaged to create a disease severity score. Values represent data from three replicates averaged across two treatments. Isolate least square means followed by the same letter are not different according to paired t-tests (α=0.05).

Table 3.4. Effect of mefenoxam treatment on seven cultivars of poinsettia inoculated with two isolates of *Pythium aphanidermatum* that are sensitive or insensitive to mefenoxam in 2016

	Cultivars[b]							
Treatment[a]	Red Glitter	Prestige Red	Polly's Pink	Polar Bear	Sparkling Punch	Luv U Pink	Visions of Grandeur	**Treatment**
No fungicide	4.8 ± 0.3 a	2.6 ± 0.3 a	3.5 ± 0.2 a	3.4 ± 0.3 a	4.3 ± 0.4 a	2.3 ± 0.3 a	2.0 ± 0.3 a	**3.1 ± 0.1 A**
Mefenoxam	2.5 ± 0.3 b	2.0 ± 0.3 a	1.8 ± 0.2 b	2.6 ± 0.3 a	4.4 ± 0.4 a	1.8 ± 0.3 a	2.0 ± 0.3 b	**2.3 ± 0.1 B**
P > F for treatment	*P = 0.0003*	*P = 0.1280*	*P = 0.0004*	*P = 0.0607*	*P = 0.2388*	*P = 0.2645*	*P = 1.0000*	
Cultivar	**3.7 ± 0.2 A**	**2.3 ± 0.2 CD**	**2.7 ± 0.2 BC**	**3.0 ± 0.2 B**	**3.9 ± 0.2 A**	**2.1 ± 0.2 CD**	**2.0 ± 0.2 D**	

[a] Plants received one of two treatments: no fungicide or mefenoxam (Subdue Maxx, 0.08 ml/L) applied as a soil drench (118 ml per 15 cm pot) at transplant. Application was made 24 h before inoculation.

[b] Above-ground symptoms and root rot were rated individually on a 1 to 5 scale and ratings were averaged to create a disease severity score. Values represent data from three replicates averaged across two isolates. Treatment least square means followed by the same letter are not different according to paired t-tests (α=0.05).

Table 3.5. Differences in Pythium root rot disease severity caused by four isolates of *Pythium aphanidermatum* that are sensitive or insensitive to mefenoxam and/ or fenamidone

	Treatments[a]							
	Experiment 1 (vegetative)				**Experiment 2 (reproductive)**			
Isolate[b]	No fungicide	Mefenoxam	Fenamidone	**Isolate[c]**	No fungicide	Mefenoxam	Fenamidone	**Isolate[c]**
PA1 MefS, FenS	4.6 ± 0.2 a	2.1 ± 0.2 c	1.6 ± 0.2 c	**2.8 ± 0.1 C**	3.8 ± 0.1 a	1.2 ± 0.1 c	1.3 ± 0.1 c	**2.1 ± 0.1 C**
PA2 MefR, FenS	3.8 ± 0.2 b	3.9 ± 0.2 a	1.3 ± 0.2 c	**3.0 ± 0.1 BC**	3.4 ± 0.1 b	3.3 ± 0.1 a	1.4 ± 0.1 c	**2.7 ± 0.1 B**
PA3 MefS, FenR	4.6 ± 0.2 a	1.6 ± 0.2 d	4.6 ± 0.2 a	**3.6 ± 0.1 A**	3.5 ± 0.1 ab	1.2 ± 0.1 c	4.4 ± 0.1 a	**3.0 ± 0.1 A**
PA4 MefR, FenR	3.1 ± 0.2 c	2.7 ± 0.2 b	3.5 ± 0.2 b	**3.1 ± 0.1 B**	2.2 ± 0.1 c	2.8 ± 0.1 b	3.7 ± 0.1 b	**2.9 ± 0.1 A**
P > F for isolate	*P < 0.0001*	*P < 0.0001*	*P < 0.0001*		*P < 0.0001*	*P < 0.0001*	*P < 0.0001*	
Treatment	**4.0 ± 0.08 A**	**2.6 ± 0.08 B**	**2.7 ± 0.08 B**		3.3 **± 0.06 A**	2.1 **± 0.06 C**	2.7 **± 0.06 B**	

[a] Mefenoxam (Subdue Maxx, 0.08 ml/L) and fenamidone (Fenstop, 1.1 ml/L) was applied as a soil drench (118 ml per 15 cm pot) at transplant. Application was made 24 h before inoculation.

[b] MefS, FenS = mefenoxam sensitive, fenamidone sensitive isolate (PA1), MefR, FenS = mefenoxam insensitive, fenamidone sensitive isolate (PA2), MefS, FenR = mefenoxam sensitive, fenamidone insensitive (PA3), MefR, FenR = mefenoxam insensitive, fenamidone insensitive (PA4)

[c] Above-ground symptoms and root rot were rated individually on a 1 to 5 scale and ratings were averaged to create a disease severity score. Values represent data from two runs and three replicates per run averaged across six cultivars. Treatment least square means followed by the same letter are not different according to paired t-tests (α=0.05).

Table 3.6. Differences in Pythium root rot disease severity caused by four isolates of *Pythium aphanidermatum* that are sensitive or insensitive to mefenoxam and/ or fenamidone that received one of six fungicide programs

	Treatments[a, b]						
Isolates[c]	E / C / E	C / M / C	E / F	E + M / C	E + F / M	E + M	Non-treated, non-inoculated
PA1 MefS, FenS	1.3 ± 0.2 a	1.3 ± 0.1 a	1.0 ± 0.1 a	1.2 ± 0.1 a	1.0 ± 0.1 a	1.0 ± 0.1 a	4.2 ± 0.3 a
PA2 MefR,FenS	1.2 ± 0.2 a	1.1 ± 0.1 a	1.2 ± 0.1 a	1.0 ± 0.1 a	1.0 ± 0.1 a	1.3 ± 0.1 a	3.9 ± 0.3 a
PA3 MefS, FenR	1.5 ± 0.2 a	1.0 ± 0.1 a	1.4 ± 0.1 a	1.0 ± 0.1 a	1.3 ± 0.1 a	1.0 ± 0.1 a	3.9 ± 0.3 a
PA 4MefR, FenR	1.1 ± 0.2 a	1.1 ± 0.1 a	1.1 ± 0.1 a	1.0 ± 0.1 a	1.0 ± 0.1 a	1.1 ± 0.1 a	2.8 ± 0.3 b
P > F	***P = 0.7205***	***P = 0.5395***	***P = 0.2745***	***P = 0.3992***	***P = 0.3010***	***P = 0.2280***	***P = 0.0088***

[a] Fungicide treatments applied as a soil drench (118 ml per 15 cm pot) at transplant. Application was made 24 h before inoculation. E = etridiazole, C = cyazofamid, M = mefenoxam, F = fenamidone. Dashes (/) denote rotations while plus signs (+) denote tank mixes.

[b] Above-ground symptoms and root rot were rated individually on a 1 to 5 scale and ratings were averaged to create a disease severity score. Values represent data from two runs and three replicates per run averaged across three cultivars. Treatment least square means followed by the same letter are not different according to paired t-tests (α=0.05).

[c] MefS, FenS = mefenoxam sensitive, fenamidone sensitive isolate (PA1), MefR, FenS = mefenoxam insensitive, fenamidone sensitive isolate (PA2), MefS, FenR = mefenoxam sensitive, fenamidone insensitive (PA3), MefR, FenR = mefenoxam insensitive, fenamidone insensitive (PA4)

Table 3.7. Efficacy of six fungicide programs to suppress Pythium root rot in three poinsettia cultivars inoculated with isolates of *P. aphanidermatum* that are sensitive or insensitive to mefenoxam, fenamidone, or both fungicides

	Cultivars [b]			
Treatment [a]	Red Glitter	Prestige Red	Luv U Pink	**Treatment LSM**
No fungicide	4.6 ± 0.1 a	4.0 ± 0.2 a	2.4 ± 0.1 a	**3.7 ± 0.1 A**
E / C / E	1.3 ± 0.1 b	1.4 ± 0.2 b	1.1 ± 0.1 b	**1.3 ± 0.1 B**
C / M / C	1.0 ± 0.1 b	1.2 ± 0.2 b	1.1 ± 0.1 b	**1.1 ± 0.1 BC**
E / F	1.3 ± 0.1 b	1.1 ± 0.2 b	1.1 ± 0.1 b	**1.2 ± 0.1 BC**
E + M / C	1.2 ± 0.1 b	1.0 ± 0.2 b	1.0 ± 0.1 b	**1.1 ± 0.1 BC**
E + F / M	1.0 ± 0.1 b	1.2 ± 0.2 b	1.0 ± 0.1 b	**1.1 ± 0.1 BC**
E + M	1.0 ± 0.1 b	1.2 ± 0.2 b	1.0 ± 0.1 b	**1.1 ± 0.1 BC**
Non-inoculated	1.0 ± 0.1 b	1.0 ± 0.2 b	1.0 ± 0.1 b	**1.0 ± 0.1 C**
P > F	*P < 0.0001*	*P < 0.0001*	*P < 0.0001*	
Cultivars LSM	**1.6 ± 0.1 A**	**1.5 ± 0.1 A**	**1.2 ± 0.1 B**	

[a] Fungicide treatments applied as a soil drench (118 ml per 15 cm pot) at transplant. Application was made 24 h before inoculation. E = etridiazole, C = cyazofamid, M = mefenoxam, F = fenamidone. Dashes (/) denote rotations while plus signs (+) denote tank mixes

[b] Above-ground symptoms and root rot were rated individually on a 1 to 5 scale and ratings were averaged to create a disease severity score. Values represent data from two runs and three replicates per run averaged across four isolates. Treatment least square means followed by the same letter are not different according to paired t-tests (α=0.05).

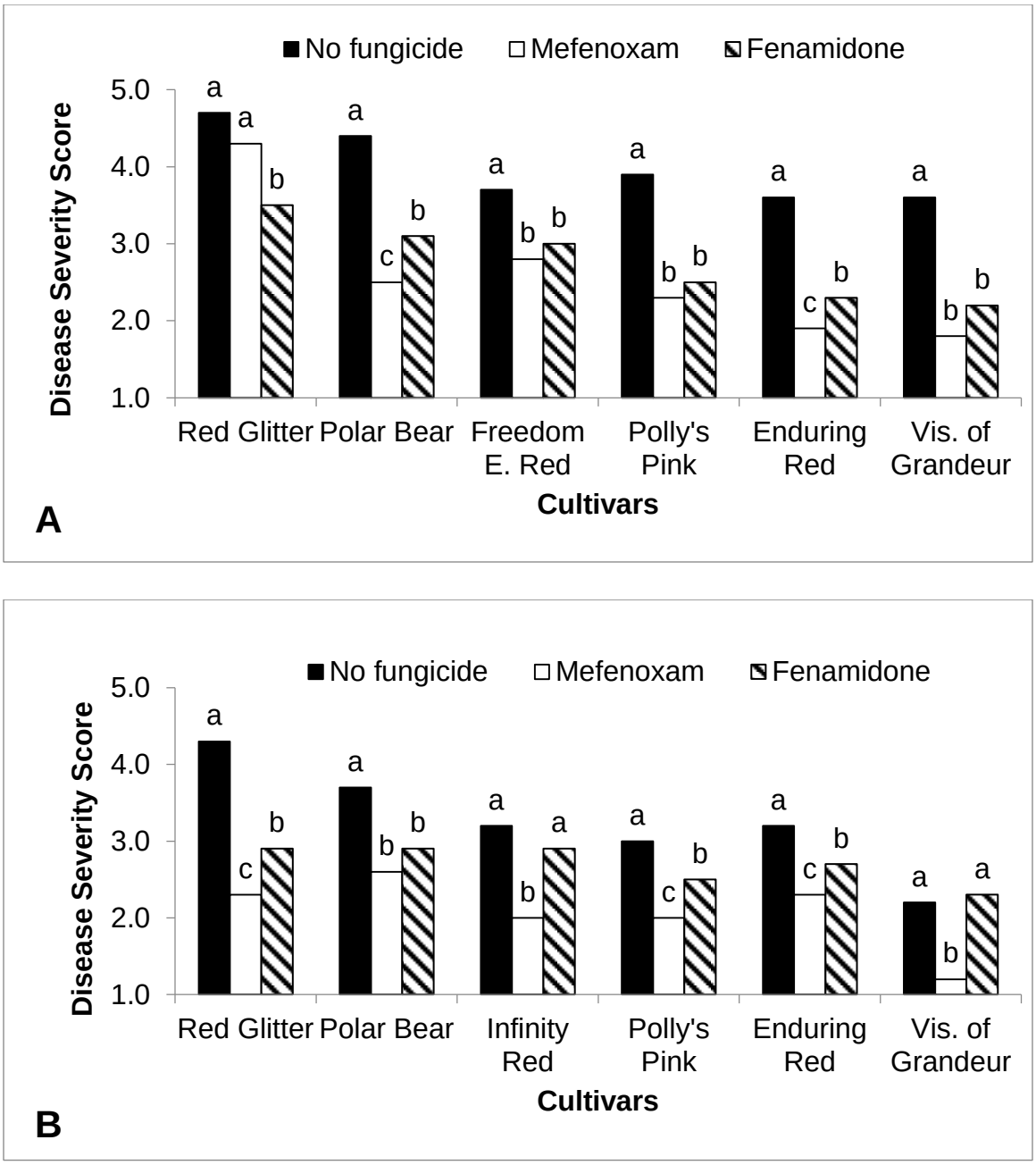

Figure 3.1. Pythium root rot disease severity scores in six cultivars of poinsettia treated with mefenoxam, fenamidone, or not treated with fungicide. Scores are averaged across four isolates of *P. aphanidermatum* and three replicates. Plants maintained vegetative state (**A**) throughout the trial or entered reproductive (bract color change) states (**B**). LSmeans followed by the same letter are not significantly different according to paired t-tests (α=0.05).

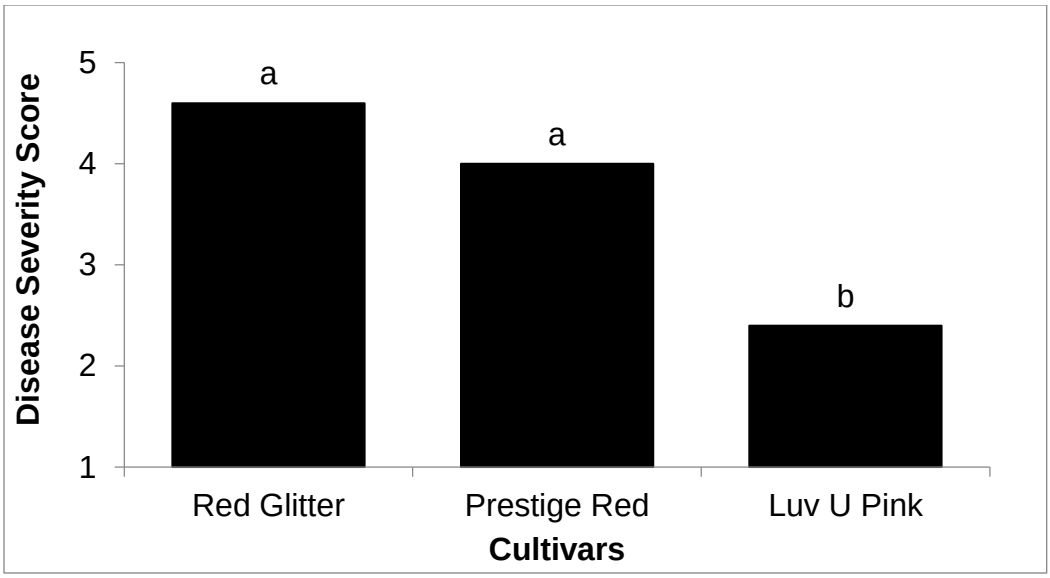

Figure 3.2. Pythium root rot disease severity scores of three cultivars of poinsettia not treated with fungicides. Scores represent data from two runs, three replicate plants per run, and averaged across four isolates. LSmeans followed by the same letter are not significantly different according to paired t-tests (α=0.05).

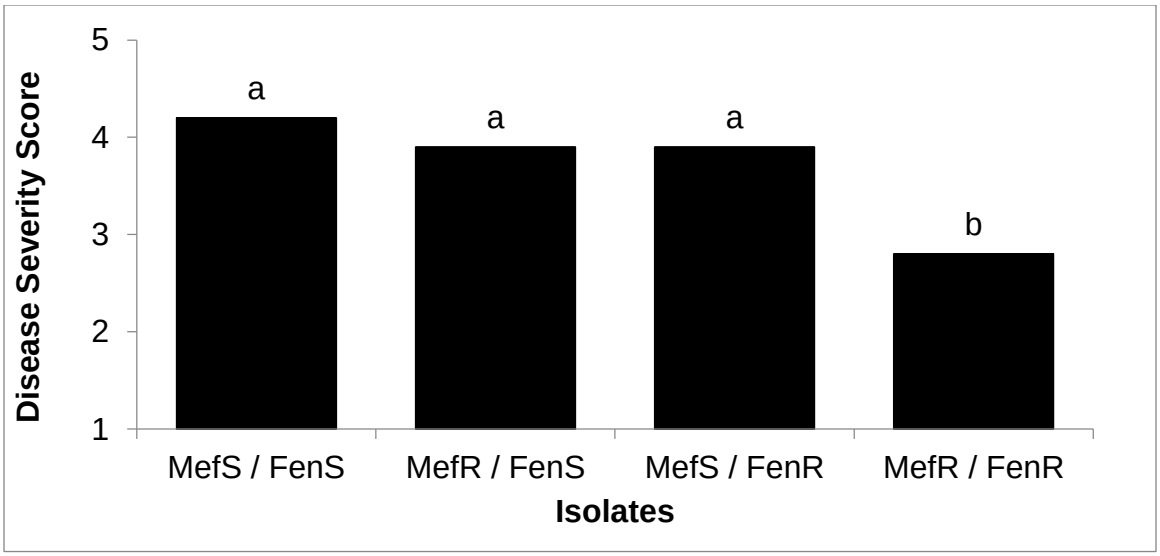

Figure 3.3. Pythium root rot disease severity scores of three cultivars of poinsettia not treated with fungicides. Scores represent data from two runs, three replicate plants per run, and averaged across three cultivars. MefS, FenS = mefenoxam sensitive, fenamidone sensitive isolate (PA1), MefR, FenS = mefenoxam insensitive, fenamidone sensitive isolate (PA2), MefS, FenR = mefenoxam sensitive, fenamidone insensitive (PA3), MefR, FenR = mefenoxam insensitive, fenamidone insensitive (PA4). LSmeans followed by the same letter are not significantly different according to paired t-tests (α=0.05).

CHAPTER 4

Fitness attributes of *Pythium aphanidermatum* isolates 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 NC. Resistance to mefenoxam is common in *P. aphanidermatum*, but resistance to fenamidone and other QoIs has only just been reported in greenhouse floriculture crops. *In-vitro* sensitivity or insensitivity to the label rate of mefenoxam (17.6 µl a.i/ml) and fenamidone (488 µl a.i./ml) was tested on 96 isolates of *P. aphanidermatum* and isolates were assigned to four fungicide resistance groups: mefenoxam-sensitive, fenamidone-sensitive (MefS, FenS) mefenoxam-insensitive, fenamidone-sensitive (MefR, FenS), mefenoxam-sensitive, fenamidone-insensitive (MefS, FenR), and mefenoxam-insensitive and fenamidone-insensitive (MefR, FenR). Fifty-eight percent of isolates were insensitive to one (MefR, FenS = 36%; 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, oospore production *in vitro*, and aggressiveness on poinsettia were evaluated to assess relative fitness of sensitive and insensitive isolates. Isolates that were insensitive to both mefenoxam and fenamidone had reduced vegetative growth at 30°C and produced fewer oospores *in vitro* than isolates that were sensitive to one or both fungicides, whereas isolates that were sensitive to both fungicides produced the most oospores *in vitro*. Aggressiveness on poinsettia varied by isolate and fungicide resistance profiles were not good predictors of *in vivo* aggressiveness. These results suggest that populations of *P. aphanidermatum* with dual resistance to mefenoxam and fenamidone may be a threat in poinsettia production but may also be less fit than sensitive populations under some conditions.

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 make this disease particularly difficult to control. Most commercially available poinsettia cultivars are very susceptible or only partially resistant to Pythium root rot. 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, an enantiomer of metalaxyl, is site specific, systemic, and inhibits r-RNA synthesis in oomycetes. Due to its site specificity, mefenoxam has a high intrinsic risk of resistance development in target organisms (Brent and Holloman 2007).

Resistance to metalaxyl was first reported in Pythium isolated from turfgrass in 1984, after only 3 years of use on the application site (Sanders 1984). Mefenoxam-insensitive strains or species of *Pythium* are now found in many locations and crops, including floriculture crops (Aegerter et al. 2002; Moorman and Kim 2004; Moorman et al. 2002; Lookabaugh et al. 2015; Múnera and Hausbeck 2016). 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 diseases caused by oomycetes. Recent research identified alternative fungicides for control of Pythium root rot caused by *P. aphanidermatum* on poinsettia (Lookabaugh and Shew 2017). Fenamidone provided excellent disease suppression; fenamidone and mefenoxam were equally effective in preventing Pythium root rot. Fenamidone belongs to the quinone outside-inhibiting (QoI)

class of fungicides which target the cytochrome b (CYTB) gene product located within the mitochondrial bc1 complex III and inhibit the transfer of electrons required for cellular respiration and subsequent ATP production (Anke et al. 1977; Bartlett et al. 2002; Gisi et al. 2002). Fenamidone and other strobilurins (FRAC group 11), including azoxystrobin and pyraclostrobin, are not commonly used for control of Pythium root rot but often are applied to control foliar diseases such as Botrytis blight and powdery mildew. Previously, *in vitro* insensitivity to fenamidone, azoxystrobin, and pyraclostrobin was identified in several isolates of *P. aphanidermatum* (Lookabaugh and Shew 2017). Additionally, one isolate was insensitive to both mefenoxam and fenamidone. It is unknown whether QoI-insensitive and dual insensitive strains are common in populations of *P. aphanidermatum*.

Implementation of anti-resistance strategies for mefenoxam has been difficult due to the general lack of inexpensive alternatives. If QoIs are to be used for Pythium root rot control and for resistance management, it is important to assess the level of insensitivity in current *P. aphanidermatum* populations to mitigate potential risks for resistance development before they are incorporated into fungicide programs.

In some instances, 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; Vanderplank 1982; Pringle and Taylor 2002). The evolution and establishment of fungicide resistance in pathogen populations is largely dependent on pathogen fitness and has important implications for disease

management (Peever and Milgroom 1994; Parnell et al. 2005). Assessing fitness of fungicide-insensitive isolates relative to sensitive isolates may help to predict whether insensitive isolates will persist in the absence of selection pressure (Crute and Harrison 1988; Chapara et al. 2011).

In general, limited information is available on potential fitness costs associated with fungicide insensitivity in oomycetes. Most studies have focused on phenylamide resistance in *Phytophthora* species, including *P. infestans*, *P. capsici*, *P. nicotianae*, and *P. erythroseptica*, and the downy mildew pathogens, *Bremia lactucae* and *Pseudoperonospora cubensis* (Cohen et al. 1983; Crute 1987; Café-Filho and Ristaino 2008; Chapara et al. 2011; Timmer et al. 1998; Hu et al. 2008; Ziogas et al. 2006; Day and Shattock 1997). Sensitive isolates of *P. aphanidermatum* are continually recovered in production facilities that report long histories of mefenoxam use and often are found concurrently with insensitive isolates (Lookabaugh et al. 2015). A continuous influx of sensitive isolates from propagative material (gene flow), or alternatively, an inability of insensitive isolates to compete with sensitive isolates could account for this observation (Williams and Gisi 1992; Dowley et al. 1995). The relative fitness of isolates of *P. aphanidermatum* insensitive to mefenoxam and fenamidone is unknown.

The objectives of this study were to 1) assess *in vitro* sensitivity to mefenoxam and fenamidone in isolates *P. aphanidermatum* obtained from infected hosts, 2) determine the genetic basis for QoI insensitivity in *P. aphanidermatum*, and 3) assess selected fitness attributes in fungicide-sensitive and insensitive isolates of *P. aphanidermatum*, including their aggressiveness on poinsettia.

Materials and Methods

In vitro assessment of sensitivity to mefenoxam and fenamidone in P. aphanidermatum.

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 microcentrifuge tubes containing sterile deionized water only or sterile water and hemp seeds for storage. Cultures were stored in the dark at 25°C. Previously collected isolates were recovered from storage by dispensing tube contents onto clarified V8 juice agar (CV8A; 50 ml clarified V8 juice, 15 g Bacto agar [Becton, Dickinson, and Co.]). Isolates were maintained on 2% water agar plates for the duration of the experiments. For sensitivity assessments, clarified V8 juice agar was amended with label rates of commercial formulations of fenamidone (Fenstop, 488 µl a.i./ml) or mefenoxam (Subdue MAXX, 17.6 µl a.i./ml) and dispensed in 100 x 15 mm Petri plates. A non-amended control for each isolate also was included. Salicylhydroxamic acid (SHAM, 50mg/ml) was added to media containing fenamidone. A cork borer was used to excise plugs 5-mm diameter plugs from the leading edge of a colony of each isolate. Plugs were placed mycelium-side down in the center of each Petri plate containing fungicide-amended agar. Cultures were incubated in the dark at 25°C with 3 replicate plates for each fungicide x isolate combination. Cultures were incubated for 27 h and percent mycelial inhibition (% inhib) was calculated by dividing the average radial growth of mycelia on fungicide-amended media by the average radial growth on non-amended media, 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 resistance groups: 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 detection of the G143A mutation in P. aphanidermatum isolates. A set of *cyt-b* gene-specific primers was developed using a publically available sequence (accession, AX577562.1) in GenBank (NCBI, USA). Primers PAcyt-F (5'-AATGATGGCAACAGCTTTCA -3') and PAcyt-R (5' TGGTGTTTTCATAGGATTTG CTT -3') were designed to amplify a 387 bp fragment of the cyt-b gene region using Primer 3 software.

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

PCR was carried out in a MyCycler Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA) with 2X Phire Plant Direct PCR Master Mix (25 µl), 0.5 µM of 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 duration 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, Cleveland, OH). Amplicons were submitted to ETON Bioscience (Research Triangle Park, NC) for custom sequencing. Nucleotide sequences were trimmed and contigs were assembled, aligned, and analyzed using CLC Workbench (Qiagen Bioinformatics, Redwood City, CA).

Experiments assessing fitness of P. aphanidermatum isolates with varying fungicide resistance profiles. A subset of 20 isolates of *P. aphanidermatum*, representing a range of fungicide resistance groups, was used to evaluate mycelial growth rate on non-amended media at three temperatures, oospore production *in-vitro*, and aggressiveness on poinsettia. Isolates were originally collected from poinsettia, garden mum, or tomato (Table 4.1). Four isolates, each representing a fungicide resistance profile, were used to assess aggressiveness in poinsettia: PA1 (MefS, FenS), PA2 (MefR, FenS), PA3 (MefS, FenR), and PA4 (MefR, FenR).

Effect of temperature on* in vitro *mycelial growth of P. aphanidermatum. An agar plug of each isolate was placed mycelium-side down in the center of a Petri plate containing non-amended V8 juice agar. Cultures were grown 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) x isolate (PA1 through PA20) combination. The experiment was conducted twice.

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

resistance group, temperature, and their interaction were treated as fixed effects while replicates and runs were treated as random effects. The fungicide resistance group x temperature interaction was significant; therefore, least squares means were calculated for the interactions and simple effects of groups were analyzed within treatments to determine significant differences. Least square means were separated using paired t-tests where appropriate.

Differences in* in vitro *oospore production by twenty isolates of P. aphanidermatum at 25°C. Agar plugs measuring 5-mm in diameter were excised with a cork borer from the leading edge of a colony of each isolate of *P. aphanidermatum* isolate. Plugs were placed mycelium-side down in the center of each 9-cm diameter Petri plate containing V8 juice agar, with 3 replicate plates for each isolate. After plates were incubated at 25°C for 96 h, three agar plugs (3-mm diameter) were removed, placed individually onto glass slides, and flattened with a cover slip. A light microscope was used to view the plugs at 200x 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.

Data from both runs were combined and analysis of variance was performed using PROC GLIMMIX procedures (SAS 9.4, SAS Institute). Fungicide resistance groups were treated as fixed effects while run and replicate were treated as random effects. Least-squares means were calculated for each fungicide resistance group to determine significant differences among groups ($P < 0.05$) and separated using paired t-tests where appropriate.

***Differences in aggressiveness of four isolates of P. aphanidermatum on poinsettia*.** 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 peat-based potting media. Once transplanted, the plants were fertilized with 5 g of Multicote 4 (14-14-16) sprinkled evenly on the pot surface and watered in.

Four isolates of *P. aphanidermatum*, representing each fungicide resistance group, 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 rice and 18 ml deionized water) and cultures were incubated for 5 days at 25°C (Holmes and Benson, 1994). 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; non-inoculated plants were included as controls. Three replicate plants per inoculation treatment were arranged in a randomized complete block design and the experiment was repeated, for a total of two runs. Plants were transplanted 21 Mar 2016, inoculated 27 Mar 2016, and harvested 6 May 2016 in run 1 and transplanted 14 Jun 2016, inoculated 20 Jun 2016, and harvested 27 Month 2016 in run 2.

At the end of the trials, shoot symptoms were evaluated on a disease severity scale of 1 = healthy plant, 2 = slightly stunted, 3 = chlorosis, moderate stunting and/or defoliation, 4 = wilting and/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 (Parker and Benson, 2013).

***Aggressiveness of twenty isolates of P. aphanidermatum on poinsettia*.** 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 4.1) was used to inoculate plants ca. 3 weeks after transplanting. Non-inoculated 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 Aug 2016, inoculated 15 Sept 2016, and harvested 14 Dec 2016 at both locations.

Statistical analysis. Each of the two inoculation experiments had two runs and data from these two 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 so averaged to obtain a disease severity score

(DSS) for each isolate. Least-square means were calculated for each isolate to determine significant differences among isolates ($P < 0.05$) and separated using paired t-tests where appropriate.

Results

In vitro assessment of sensitivity to mefenoxam and fenamidone in P. aphanidermatum. Isolates varied in response to mefenoxam and fenamidone. Isolates with mean percent inhibitions less than 50% were considered insensitive. Fifty-eight percent of isolates were insensitive to one (MefR, FenS = 36%, MefS, FenR = 16%) or both fungicides (MefR, FenR = 6%; Figure 4.1). 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 detection of the G143A mutation in P. aphanidermatum isolates. A single nucleotide mutation (G→C) in the codon 143 of cyt b (GGT→GCT) was present in all 21 of the fenamidone insensitive (FenR) isolates but not in the 75 fenamidone sensitive (FenS) isolates. Partial nucleotide sequence data of the *cyb b* gene for one fenamidone sensitive isolate and one fenamidone sensitive isolate studied in this work were deposited into the GenBank.

Effect of temperature on* in vitro *growth of P. aphanidermatum. Variation in colony diameter were significant for fungicide resistance group ($P = 0.0028$) and temperature ($P < 0.0001$). Colony diameter was greatest at 30°C. Averaged across three temperatures, mefenoxam insensitive, fenamidone sensitive (MefR, FenS) isolates had higher growth rates

than any of the other groups, while isolates insensitive to fenamidone or both fungicides (MefS, FenR; MefR, FenR) exhibited the least radial growth across the three temperatures tested (Table 4.2). Colony diameters in the resistance groups differed across temperatures as indicated by a significant group x temperature interaction ($P = 0.0455$). At 30°C, isolates that were insensitive to both mefenoxam and fenamidone (MefR, FenR) had the least radial growth compared to the other isolates ($P = 0.0025$, Table 4.2), whereas no differences in radial growth among groups were observed at 20 or 25°C.

Oospore production of P. aphanidermatum at 25°C. Oospore production varied among fungicide resistance groups ($P < 0.0001$). Isolates sensitive to both fungicides (MefS, FenS) produced significantly more oospores than isolates insensitive to one (MefR, FenS or MefS, FenR) or both fungicides (MefR, FenR; Figure 4.2). Isolates insensitive to both fungicides produced the fewest oospores *in vitro,* while no differences were observed between the isolates insensitive to only one fungicide.

Aggressiveness of four isolates of P. aphanidermatum on poinsettia. Aggressiveness varied by isolate ($P = 0.0005$). Plants inoculated with mefenoxam sensitive isolates (MefS, FenS and MefS, FenR) had higher disease severity scores than plants inoculated with mefenoxam insensitive (MefR, FenS and MefR, FenR) isolates (Figure 4.3). Among the plants inoculated with fenamidone insensitive isolates (MefS, FenR and MefR, FenR), numerically higher disease severity scores were observed on plants inoculated with MefS, FenR than on plants inoculated with the dual insensitive isolate (MefR, FenR).

Aggressiveness of twenty isolates of P. aphanidermatum on poinsettia. Isolates varied in aggressiveness on poinsettia ($P < 0.0001$) but the mean aggressiveness of fungicide

resistance groups did not differ (P = 0.4715; Table 4.3). Four mefenoxam sensitive 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 results of this study indicated that the frequency of insensitivity to the QoI fenamidone was surprisingly high in the population of *P. aphanidermatum* sampled from plants grown in commercial greenhouses in North Carolina. All isolates insensitive to fenamidone 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 (Lesniak et al. 2011; Fraaije et al. 2006; Kildea et al. 2010). This is the first report of QoI resistance conferred by the G143A mutation in *P. aphanidermatum* isolates collected from greenhouse crops.

Seventeen of the 21 QoI resistant isolates were collected from a single facility in North Carolina and were originally isolated from poinsettia and garden chrysanthemum. QoI-resistant isolates represented 71% of the isolates collected from that location. Intensive use of QoI fungicides for the control of Botrytis blight and powdery mildew on poinsettia and continued use of these products year-round on other hosts probably led to the selection and establishment of QoI-resistant populations in that facility. These isolates were collected over a period of five years, with sensitive isolates being collected concurrently during this period. The long-term stability of QoI resistance in greenhouse populations of *P. aphanidermatum* in the absence of selection pressure needs to be evaluated. Regardless, the use of fenamidone for Pythium root rot control should be limited to individual facilities where QoI resistance is not already present. Fenamidone and other QoIs should be used only in programs where fenamidone is mixed or rotated with another fungicide with a different mode of action to prevent selection of insensitive strains. 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, but the prevalence of insensitive strains in these facilities and in the industry generally remains unknown due to lack of intensive spatial and temporal sampling.

Rapid growth of mefenoxam-insensitive isolates compared to sensitive isolates has been reported in *Phythophthora infestans*, *P. nicotianae,* and *P. erythroseptica* (Kadish and Cohen 1988; Timmer et al. 1998; Chycoski and Punja 1996; Hu et al. 2008; Porter et al. 2006). However, other studies reported no differences between sensitive and insensitive isolates of *P. capsici.* Reduced fitness of mefenoxam-insensitive isolates was observed in isolates of *P. infestans* sampled from potato from Europe (Café-Filho and Ristaino 2008; Dowley et al. 2002; Day and Shattock 1997; Dowley and O'Sullivan 1985; Davidse et al. 1989). In the current studies, mefenoxam resistant, fenamidone sensitive isolates (MefR, FenS) had the average highest mean radial growth in the temperature studies, while isolates that were insensitive to fenamidone (MefS, FenR) or both fungicides (MefR, FenR) had the smallest mean radial growth. The ability of mefenoxam-insensitive isolates to grow vegetatively may provide a selective advantage during substrate colonization, but mefenoxam-insensitive isolates did not necessarily show increased aggressiveness on poinsettia.

Dual insensitivity to mefenoxam and QoI fungicides has not been reported previously in populations of *P. aphanidermatum* collected from commercial floriculture greenhouses. In this study, the incidence of dual insensitivity was higher than expected with 6% of the isolates tested belonging to this fungicide resistance group. Isolates with dual insensitivity had smaller mean radial growth than other isolates only at 30°C. Similarly, mefenoxam-insensitive isolates of *Plasmopora viticola* exhibited reduced fitness on grape leaves only at high temperatures of 30°C (Corio-Costet, 2012). These results suggest that isolates of *P. aphanidermatum* insensitive to both mefenoxam and QoIs may have reduced fitness at high

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 within the optimal temperature for 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 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 reproductive fitness in dually insensitive isolates. This may explain why sensitive isolates persist in facilities that regularly use mefenoxam and/or fenamidone since oospores are the primary survival structure produced by *P. aphanidermatum*. Similarly, a previous study observing sexual fitness of mefenoxam insensitive and mefenoxam sensitive isolates of *Phythophthora infestans* showed that oospore production was depressed when two mefenoxam resistant isolates were crossed, while oospore production was highest in crosses of two mefenoxam sensitive isolates (Hanson and Shattock, 1998). However, in another study, mefenoxam resistant isolates of *Phythophthora erythroseptica* produced 6 to 20 times more oospores than mefenoxam sensitive isolates, but sensitive isolates produced more zoospores (Porter et al. 2007). The effect of mefenoxam and fenamidone applied directly to oospores was not studied here. In earlier studies, commercial fungicides, including metalaxyl (an isomer of mefenoxam), reduced germination of active (thin-walled) oospores of *P. fragariae* and *P. ultimum*, but not

inactive (thick-walled) oospores. Thus, thick-walled oospores may provide a reservoir of propagules that, in the absence of fungicides, can later develop into thin-walled oospores and replenish the supply of germinable propagules, thereby creating genetic variation in the pathogen population (Duncan 1985; Stasz and Martin 1988). The high incidence of sensitive isolates in plants growing in facilities that regularly use fungicides suggests that oospores may not be subject to the same selection pressure as other pathogen propagules. Additionally, Groves and Ristaino (2000) demonstrated that low rates of mefenoxam induced normally heterothallic isolates of *P. infestans* to form oospores, including one mefenoxam-sensitive isolate. The role of oospores in the epidemiology of Pythium root rot is not well understood, but it is possible that the fungicides used to manage the disease could have non-target effects on the reproductive biology of the pathogen. While oospore production is a measure of isolate reproductive and survival ability, other traits such as sporangia production and zoospore formation may play important roles in the overall reproductive fitness of an isolate. Additional studies are needed to determine if insensitivity to one or more fungicides reduces asexual reproduction in *P. aphanidermatum*.

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 were evaluated in this study for their aggressiveness on poinsettia, plants inoculated with mefenoxam sensitive isolates had higher disease severity scores than plants inoculated with mefenoxam insensitive isolates, but no differences were observed between fenamidone sensitive and insensitive isolates. However, when the larger groups of twenty isolates were evaluated, fungicide sensitivity was not a clear predictor of aggressiveness. The different

outcomes of these studies could be attributed to the genetic background of individual isolates rather than the fungicide sensitivity groups. The mixed reproductive system of *P. aphanidermatum* coupled with a near continuous supply of susceptible host material, allows this pathogen to maintain large population sizes year round. Additionally, the movement of pathogen strains 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 forces may counteract the selection pressures imposed by fungicide use and 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 that were 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 can be well managed with sound fungicide programs. 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 to minimize losses due to fungicide insensitivity.

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Table 4.1. Isolates of *P. aphanidermatum* used in fitness experiments and their sensitivity to mefenoxam and fenamidone *in vitro*

Isolate	Host	Fungicide resistance group[a]
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

[a] Isolates were designated as sensitive (MefS) or insensitive (mefR) to label rates of mefenoxam (17.6 µl a.i./ml) and fenamidone (FenS and FenR; 488 µl a.i./ml) and assigned to one of four fungicide resistance groups.

Table 4.2. Colony diameter of twenty isolates of *P. aphanidermatum* grown on non-amended agar at 20, 25, or 30°C for 24 h

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

[a] Twenty isolates were assigned to fungicide resistance groups based on sensitivity (S) or insensitivity (R) to mefenoxam (Mef) or fenamidone (Fen).

[b] Values represent data from two runs averaged across three replicates per run. Least square means followed by the same letter are not significantly different based on paired t-tests ($\alpha = 0.05$)

Table 4.3. Differences in aggressiveness of isolates of *P. aphanidermatum* in different fungicide resistance groups when inoculated on poinsettia

Isolate	Host	Resistance Group	Disease Severity Score ± S.E.[a]
PA1	poinsettia	MefS, FenS	3.8 ± 0.6 abc
PA8	poinsettia	MefS, FenS	3.8 ± 0.6 abc
PA15	mum	MefS, FenS	1.2 ± 0.6 ef
PA16	mum	MefS, FenS	1.0 ± 0.6 f
PA17	tomato	MefS, FenS	4.5 ± 0.6 ab
PA18	tomato	MefS, FenS	3.8 ± 0.6 abc
PA19	tomato	MefS, FenS	4.5 ± 0.6 ab
PA20	tomato	MefS, FenS	3.6 ± 0.6 abc
		Mean	**3.3 ± 0.3**
PA2	poinsettia	MefR, FenS	3.8 ± 0.6 abc
PA9	poinsettia	MefR, FenS	2.7 ± 0.6 cde
PA10	poinsettia	MefR, FenS	2.8 ± 0.6 cd
		Mean	**3.1 ± 0.4**
PA3	poinsettia	MefS, FenR	4.5 ± 0.6 ab
PA11	mum	MefS, FenR	3.9 ± 0.6 abc
PA12	mum	MefS, FenR	1.0 ± 0.6 f
PA13	mum	MefS, FenR	4.2 ± 0.6 abc
PA14	mum	MefS, FenR	4.8 ± 0.6 a
		Mean	**3.7 ± 0.3**
PA4	poinsettia	MefR, FenR	3.2 ± 0.6 bcd
PA5	poinsettia	MefR, FenR	2.8 ± 0.6 cd
PA6	poinsettia	MefR, FenR	1.8 ± 0.6 def
PA7	poinsettia	MefR, FenR	4.1 ± 0.6 abc
		Mean	**3.0 ± 0.4**
		P > F for isolates	***< 0.0001***
		P > F for resistance groups	***= 0.4715***

[a] Values represent 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 based on paired t-tests ($\alpha = 0.05$)

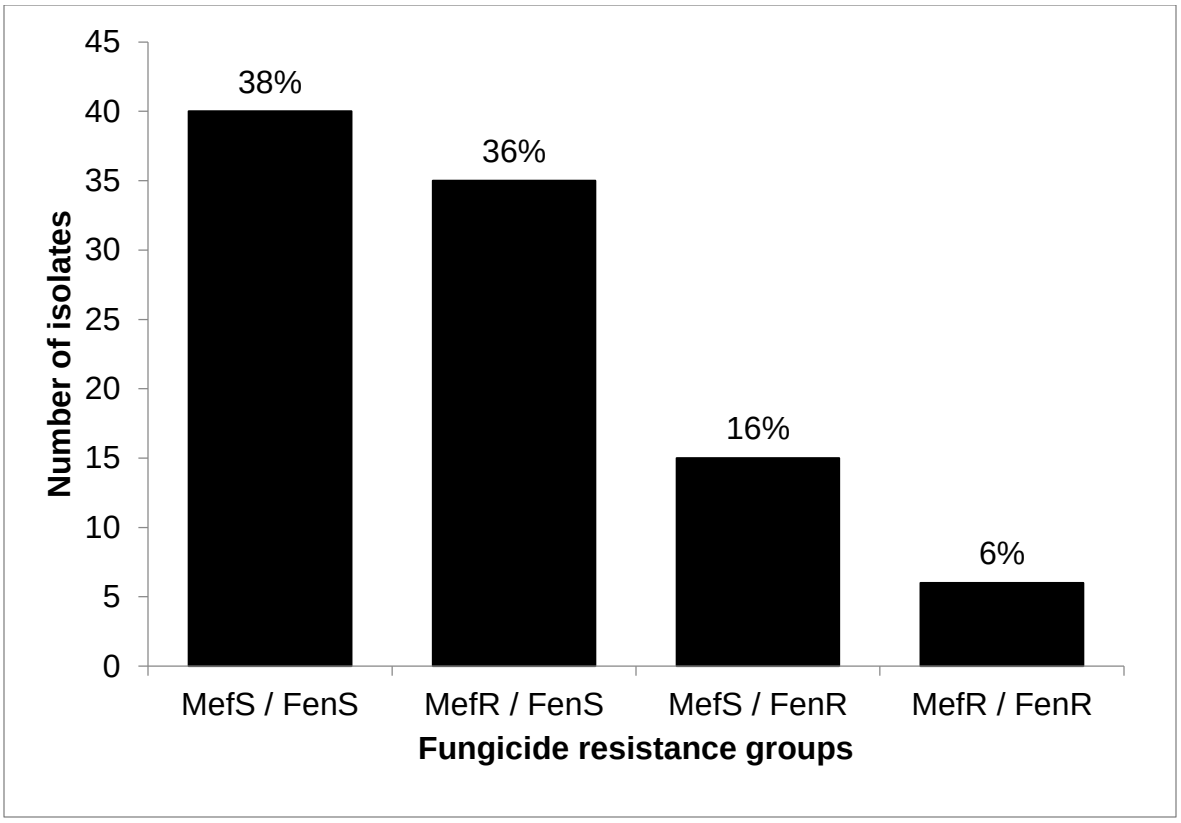

Figure 4.1. *In vitro* sensitivity of 96 isolates of *Pythium aphanidermatum* to mefenoxam (17.6 µl a.i./ml) and fenamidone (488 µl a.i./ml). Isolates having percent mycelial inhibitions less than 50% were considered insensitive. Isolates were assigned to fungicide resistance groups based on sensitivity (S) or insensitivity (R) to mefenoxam (Mef) or fenamidone (Fen).

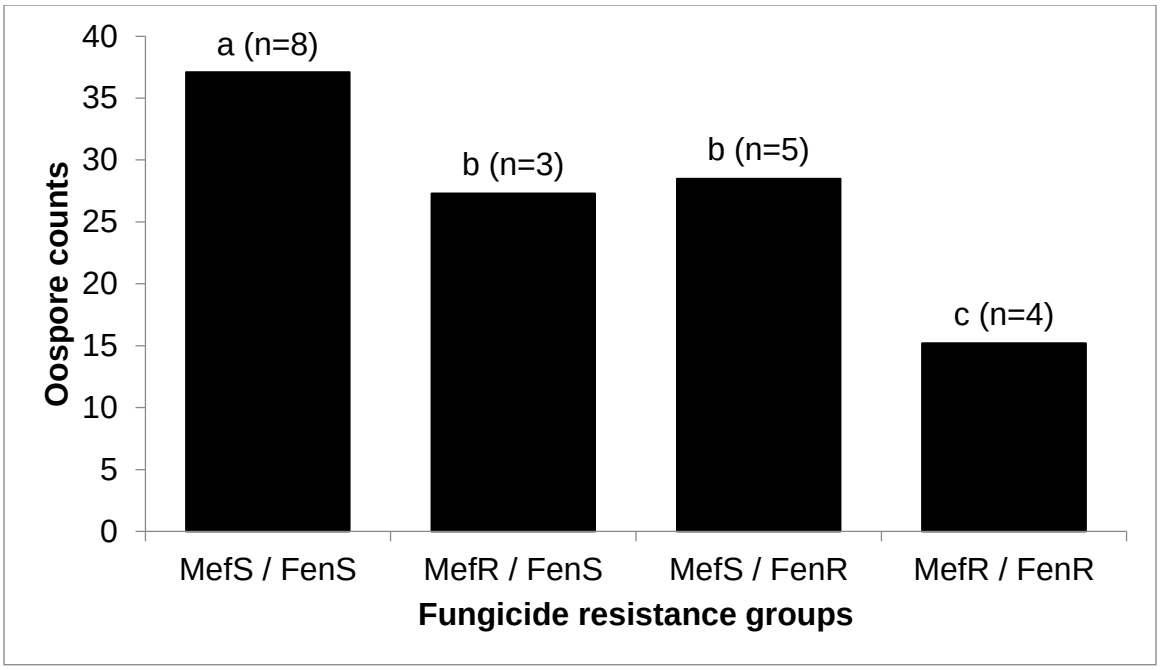

Figure 4.2. Differences in *in vitro* oospore production by twenty isolates of *P. aphanidermatum* belonging to four fungicide resistance groups. Isolates were assigned to fungicide resistance groups based on sensitivity (S) or insensitivity (R) to mefenoxam (Mef) or fenamidone (Fen). Scores were averaged across two runs and 3 replicates per run. Least square means with the same letter are not significantly different based on paired t-tests (α = 0.05).

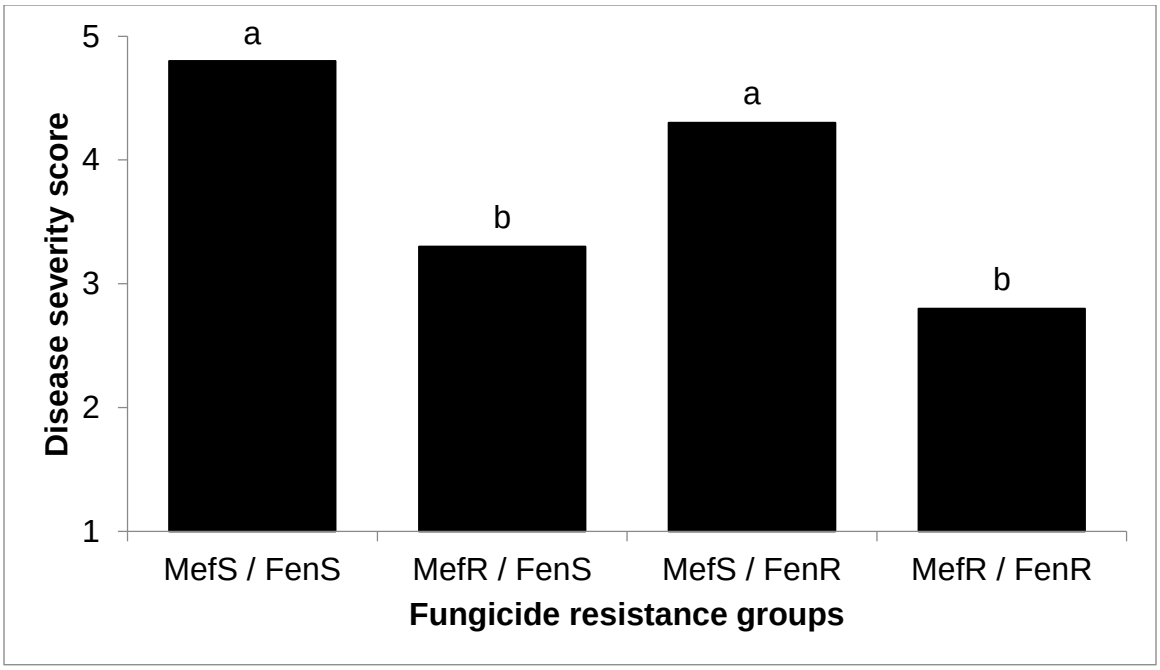

Figure 4.3. Differences in aggressiveness of four isolates of *P. aphanidermatum* with varying fungicide resistance profiles. Isolates were assigned to fungicide resistance groups based on sensitivity (S) or insensitivity (R) to mefenoxam (Mef) or fenamidone (Fen). Scores were averaged across two runs and 3 replicates per run. Least square means with the same letter are not significantly different based on paired t-tests ($\alpha = 0.05$).

APPENDICES

Appendix A

Susceptibility of commercial poinsettia cultivars inoculated with *P. aphanidermatum* at five different production ages

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Introduction

Pythium aphanidermatum is the predominant species causing Pythium root rot of commercially grown poinsettia in North Carolina. Long production seasons and common irrigation practices make this disease particularly difficult to control and Pythium root rot is an ongoing challenge for many growers.

Resistance of older plants to *Pythium* spp. has been reported in snapdragon, sorghum, sugarbeet, alfalfa, and maize (Mellano et al. 1970; Pratt and Janke 1980; Raftoyannis and Dick 2002). In many cases, seedlings infected with *Pythium* spp. are killed but older plants are not. Pythium root rot is commonly observed on mature poinsettia plants in commercial production systems and growers often report that symptoms intensify during periods of extreme heat or water stress. It these instances, it is not known when in the production cycle plants became infected with *Pythium* or how long after infection symptoms manifested. From a management perspective, the relationship between production age and susceptibility to Pythium root rot is especially important because it could influence fungicide application schedules and efficacy. The objective of this study was to determine the susceptibility of commercial poinsettia cultivars inoculated with *P. aphanidermatum* at five production ages.

Inoculum production

The isolate (PA1) used in this study was originally collected during a survey of North Carolina floriculture greenhouses and was identified as sensitive to mefenoxam (Lookabaugh et al. 2015). This isolate is aggressive on poinsettia and is sensitive to mefenoxam and fenamidone (Lookabaugh and Shew, 2017). 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 rice and 18 ml deionized water). Cultures were incubated for 5 days at 25°C and flasks were shaken twice daily to promote uniform colonization of rice grains and prevent clumping (Holmes and Benson, 1994).

Plant Culture

All planting materials were obtained from commercial sources (Dümmen Orange). Unrooted cuttings of the test cultivars were placed in Oasis Wedge growing media (Smithers-Oasis North America) and maintained under mist irrigation until roots began to grow out of the bottom of the foam wedge. Rooted plugs of poinsettia were transferred to new 15-cm plastic pots containing Fafard 4P peat-based potting mix. Once transplanted, the plants were fertilized with 5 g of Multicote 4 (14-14-16) sprinkled evenly on the pot surface and watered in. Plants were maintained under drip irrigation (2015) or hand-watered twice daily (2014) and treated with Marathon insecticide (imidacloprid; 1.4 g/15-cm pot) at transplanting to control whiteflies.

Assessing above-ground disease severity and root rot

Shoot symptoms were evaluated on an above-ground disease severity scale of 1 = healthy plant, 2 = slightly stunted, 3 = chlorosis, moderate stunting and/or defoliation, 4 = wilting and/or severe stunting, 5 = dead (Lookabaugh et al., 2015). Next, plants were carefully inverted, the pot was removed, and the 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 sloughs off easily, 4 =

75% root rot, brown dead roots evident throughout root system, root ball integrity severely compromised, very few white roots, 5= 100% brown dead roots, root ball has lost all integrity (Benson and Parker, 2013). In all trials, analysis with PROC CORR procedures (SAS 9.4, SAS Institute) indicated that root rot ratings and above-ground disease severity ratings were correlated (Spearman's $r \geq 0.4699$, $P < 0.0001$). These variables then were averaged for each observation to create disease severity scores (DSS) used in all subsequent analyses.

Statistical Analysis

Analysis of variance and correlation analysis were performed with PROC GLIMMIX and PROC CORR procedures (SAS 9.4, SAS Institute). Cultivar, treatment timing, and their interactions were treated as fixed effects while block was treated as a random effect. If interactions were significant, the least squares means were calculated for the interactions and the simple effects were analyzed to determine significant differences. Least square means were separated using paired t-tests where appropriate.

Susceptibility of three poinsettia cultivars inoculated with *P. aphanidermatum* at 1, 3, and 5 weeks after transplanting

This experiment was conducted at two greenhouses located approximately 2.5 km apart, for a total of two trials. Trials were conducted concurrently at both locations. Plants were transplanted 8 Nov 2014 and harvested 10 Mar 2015. Three cultivars were included in the experiment: 'Prestige Red', 'Advent Red', and 'Freedom Red'. Plants were rooted and transplanted as described above. Plants were inoculated at one of three treatment timings: 1, 3, and 5 weeks post-transplanting. Non-inoculated, non-treated plants were maintained as

controls but not included in the analysis. At the end of the trials, disease severity was evaluated as described previously. Plants entered reproductive states (bract color change) for trial 1 but light pollution caused plants to maintain vegetative states in trial 2. A randomized complete block design with five blocks was used in each trial and data from each trial was analyzed separately as described above.

Disease levels were moderate in both experiments and mortality was not observed. Disease severity scores were lower in the vegetative trial (disease severity scores < 3.0) than in the reproductive trial (disease severity scores < 4.0). Non-inoculated, non-treated plants did not exhibit disease symptoms and were not included in the data analysis. In the vegetative trial, disease severity scores did not differ across cultivar ($P = 0.6818$) or inoculation timings ($P = 0.1736$) and the cultivar x timing interaction was not significant ($P = 0.1256$). In the reproductive trial, disease severity scores differed across cultivars in response to inoculation timings as indicated by a significant cultivar x timing interaction ($P = 0.0137$). The main effect for inoculation timing was also significant ($P = 0.0035$). 'Prestige Red' plants inoculated one week after transplanting had higher disease severity scores than plants inoculated three or five weeks after transplanting (Table A.1). No significant differences were observed within 'Freedom Red' or 'Advent Red' inoculated at different timings.

Susceptibility of seven poinsettia cultivars inoculated with *P. aphanidermatum* at 3, 6, and 9 weeks after transplanting

This experiment was conducted during the fall 2015 growing season. Plants were transplanted 18 Aug 2015 and harvested 23 Nov 2015. The experiment included seven cultivars: 'Sparkling Punch', 'Enduring Red', 'Prestige Red', 'Luv U Pink', 'Jubilee White',

‘Jubilee Pink’, and ‘Ice Punch’. Plants were rooted and transplanted as described above. Plants were inoculated at one of three treatment timings: 3, 6, and 9 weeks post-transplanting. Non-inoculated, non-treated plants were maintained as controls but not included in the analysis. At the end of the trials, disease severity was evaluated as described previously. A randomized complete block design with three blocks was used in the experiment and data was analyzed as described above.

Disease severity and root rot scores were high for this experiment and mortality was observed in some plants. Non-inoculated, non-treated plants did not exhibit disease symptoms and were not included in the data analysis. Disease severity scores differed by cultivar ($P = 0.0003$) and inoculation timings ($P = 0.0004$) but the cultivar x timing interaction was not significant ($P = 0.2197$). Disease severity scores were highest in ‘Sparkling Punch,’ ‘Enduring Red’, and ‘Jubilee Pink’ with mortality occurring in some replicate plants (Figure A.1). ‘Luv U Pink’ had the lowest disease severity scores and exhibited only minor stunting symptoms. Disease severity scores were higher in plants inoculated three and six weeks after transplanting than plants inoculated nine weeks after transplanting (Figure A.2).

Conclusions

These findings provide evidence that Pythium root rot symptoms are more severe when plants are exposed to inoculum early in the production season, soon after transplanting. Disease severity differed across cultivars, with the most severe symptoms observed on ‘Sparkling Punch’, ‘Enduring Red’, and ‘Jubilee Pink’, while ‘Luv U Pink’ exhibited only minor stunting symptoms. These results are consistent with previous studies that identified

‘Luv U Pink’ as partially resistant to Pythium root rot (Lookabaugh and Shew 2017). On the susceptible cultivars ‘Sparkling Punch’ and ‘Enduring Red’, *P. aphanidermatum* caused moderately severe symptoms on mature plants inoculated nine weeks after transplanting. These results can be used to develop preventative fungicide programs to manage Pythium root rot. We suggest beginning preventative fungicide applications at transplant so that plants are protected when they are most susceptible to disease. Plants also were infected at later inoculation dates, indicating that additional fungicide applications may be necessary to provide adequate disease control of highly susceptible cultivars throughout the entire production season.

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Table A.1. Susceptibility of three poinsettia cultivars inoculated with *P. aphanidermatum* at 1, 3, and 5 weeks after transplanting

	Vegetative Trial[a]				**Reproductive Trial**			
	Cultivars[b]				**Cultivars**			
Timing of inoculation[c]	Freedom Red	Advent Red	Prestige Red	**Timing LSM**	Freedom Red	Advent Red	Prestige Red	**Timing LSM**
1 week	1.7 ± 0.15 a	1.9 ± 0.26 a	1.5 ± 0.13 a	**1.7 ± 0.11 A**	1.8 ± 0.27 a	2.2 ± 0.33 a	1.9 ± 0.20 b	**2.0 ± 0.16 A**
3 weeks	1.8 ± 0.15 a	1.6 ± 0.26 a	1.6 ± 0.13 a	**1.7 ± 0.11 A**	2.6 ± 0.27 a	2.7 ± 0.33 a	1.7 ± 0.20 b	**2.3 ± 0.16 AB**
5 weeks	1.6 ± 0.15 a	2.0 ± 0.26 a	2.2 ± 0.13 a	**1.9 ± 0.11 A**	2.3 ± 0.27 a	2.7 ± 0.33 a	3.3 ± 0.20 a	**2.8 ± 0.16 B**
Cultivar LSM	**1.7 ± 0.11 A**	**1.8 ± 0.11 A**	**1.8 ± 0.11 A**		**2.3 ± 0.16 A**	**2.5 ± 0.16 A**	**2.3 ± 0.16 A**	

[a] Plants entered reproductive states (bract color change) for trial 1. Plants were maintained in vegetative states in trial 2.

[b] Above-ground symptoms and root rot were rated individually on a 1 to 5 scale and ratings were averaged to create a disease severity score. Values represent data from five replicate plants. Treatment least square means followed by the same letter are not different according to paired t-tests (α=0.05).

[c] Plants were inoculated with *P. aphanidermatum* 1, 3, or 5 weeks after transplanting.

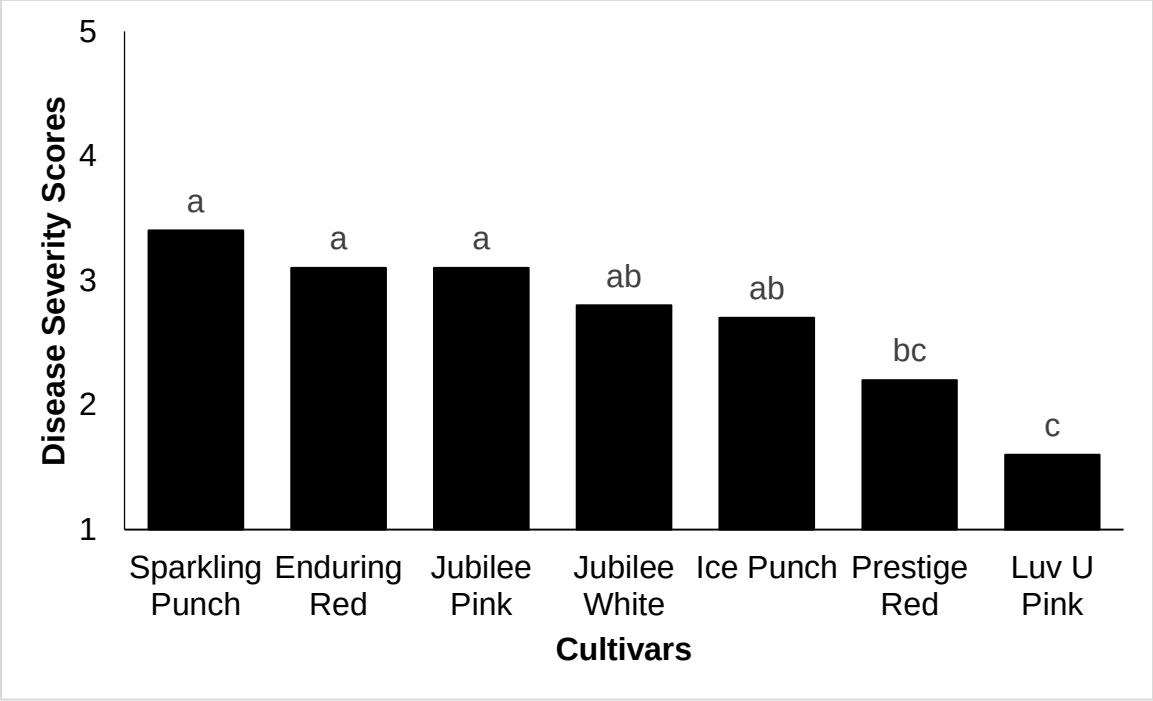

Figure A.1. Pythium root rot disease severity scores of seven cultivars of poinsettia inoculated with *P. aphanidermatum* at 3, 6, or 9 weeks after transplanting. Scores are averaged across three inoculation timings and three replicates. Lsmeans followed by the same letter are not significantly different according to paired t-tests (α=0.05).

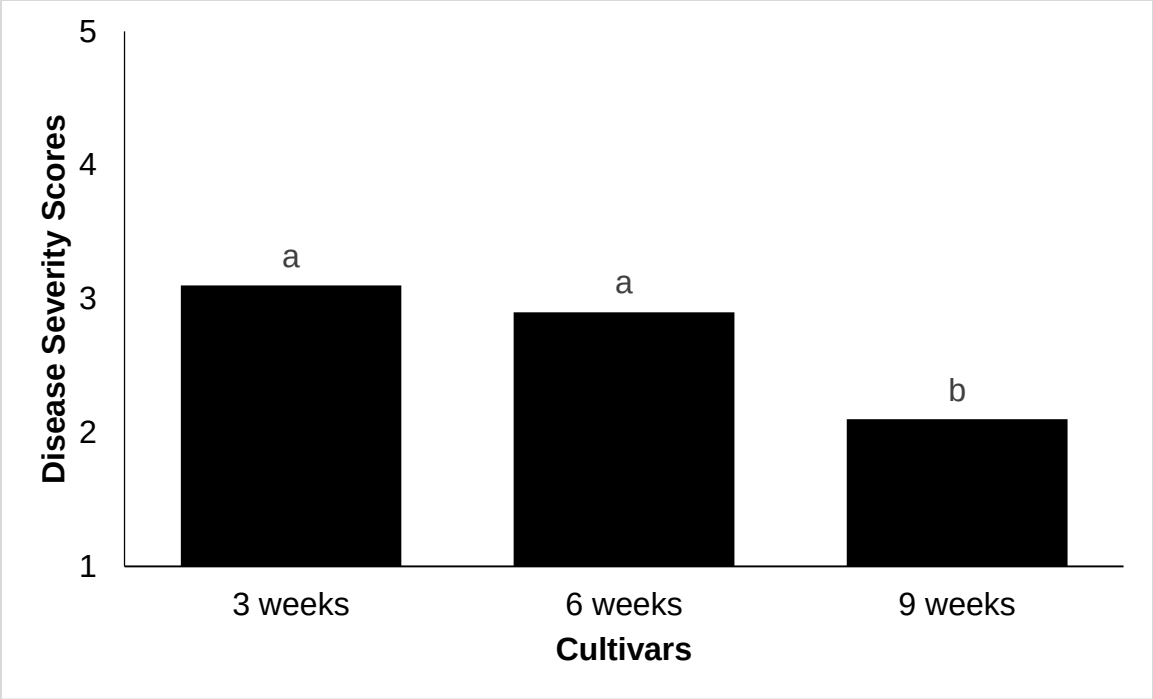

Figure A.2. Pythium root rot disease severity scores poinsettia inoculated with *P. aphanidermatum* at 3, 6, or 9 weeks after transplanting. Scores are averaged across seven cultivars and three replicates. Lsmeans followed by the same letter are not significantly different according to paired t-tests (α=0.05).

Appendix B

First report of Pythium root rot of stevia caused by *Pythium myriotylum*, *Pythium irregulare*, and *Pythium aphanidermatum* in North Carolina

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Stevia (*Stevia rebaudiana* (Bertoni) Bertoni) is an emerging perennial crop in the United States. In current production practices, stevia is started from seed in a greenhouse float tray system and 8 to 10 week old seedlings are transplanted into the field. Diseased stevia seedlings were observed in commercial float systems in Harnett County, North Carolina in June 2015 and 2016. Symptoms included stunting, leaf curl, wilting, root necrosis, and eventual total root loss. Necrotic root tissue was rinsed under running water, blotted dry, and cut into 1 cm pieces. Root pieces were plated on CMA-P5ARP (17 g/liter corn meal agar amended with pimaricin, ampicillin, rifampicin, and PCNB [Terraclor 75% WP, Southern Ag Insecticides, Inc.]) (Jeffers and Martin, 1986). Cultures were incubated at room temperature for 48 h and observed for colony morphology. Colonies resembling *Pythium sp.* were transferred to obtain pure cultures and pathogen identification was confirmed by sequencing the internal spacer (ITS) region of ribosomal DNA. DNA was extracted from mycelia with the PUREGENE DNA Isolation Kit for 10 to 20 mg of tissue (Qiagen, Valencia, CA) and subjected to a polymerase chain reaction using universal primers ITS 4,5. Isolates were identified as *Pythium myriotylum* (99% sequence identity with GenBank Accession No. HQ643704.1).

A second *Pythium* species was isolated from root lesions on overwintering crowns dug from a field planting of stevia in Rocky Mount, NC in March 2016. The isolate was identified as *Pythium irregulare* (98 % sequence identity with GenBank Accession No. HQ643622.1). A third *Pythium* species was isolated from wilting field plants with root lesions in Clinton, NC in June 2016. Pathogen identification was confirmed using universal primers ITS 4,5 and isolates were identified as *Pythium aphanidermatum* (99 % sequence identity with GenBank Accession No. AY598622.2). Koch's postulates were confirmed for each of the three species on 15 week old stevia plants, cv. G3, grown in 8-cm diameter pots. Rice grains colonized by the isolates of *P. myriotylum*, *P. irregulare*, or *P. aphanidermatum* served as the inoculum. Four rice grains were buried 1 cm deep approximately 2 cm from the base of the plant in six test pots for each species. Plants were observed over a three-week period for symptom development at which time root necrosis was evident on all inoculated plants. Non-inoculated plants did not develop symptoms. All *Pythium* species were re-isolated from respective symptomatic plants and species identifications were confirmed by sequencing and morphology (G. Abad, *unpublished*, van der Plaats-Niterink, 1981). To our knowledge, this is the first report of *P. myriotylum*, *P. irregulare*, and *P. aphanidermatum* causing root rot of *S. rebaudiana.* Float systems used to produce stevia are highly conducive to the development of Pythium root rot. At this time, no fungicides are registered for stevia so strict sanitation and cultural measures are necessary to prevent seedling losses in float systems.

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van der Plaats-Niterink, A. J. Stud. Mycol. 21:1, 1981.

Figure B.1: Symptoms of Pythium root rot caused by *P. myriotylum* on stevia in float tray production in North Carolina: (A) Stunting of float tray seedlings (B) Multiple plants exhibiting leaf curl symptoms (C) Necrosis and death of seedling roots (D) Root lesions

Appendix C

Three Pythium Species Isolated from Severely Stunted Wheat During an Outbreak in North Carolina

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Abstract

Large portions of eastern North Carolina experienced prolonged soil waterlogging in 2016. Severely stunted wheat plants from saturated fields were examined and *Pythium* consistently was associated with the symptoms observed. Three species of *Pythium* were identified among 15 isolates derived from wheat roots and crowns: *P. irregulare*, *P. spinosum*, and *P. vanterpoolii*. Each species was isolated from samples that came from between two and five counties. *P. vanterpoolii* and *P. spinosum* have not previously been reported as pathogens in U.S. wheat. All three species caused root rot when re-inoculated on wheat plants. These species are not opportunistic or mainly saprophytic on other hosts; therefore, it is likely that they contributed to the extreme stunting and yield loss observed in North Carolina wheat in 2016. The 15 isolates were tested for sensitivity to mefenoxam at 100ug/ml a.i. and none was insensitive. Prolonged hypoxia likely predisposed North Carolina wheat to unusual levels of Pythium root rot in 2016.

Introduction

Wheat can be severely or moderately stunted by a variety of root- and crown-infecting fungi and oomycetes. In the USA, most of the work on wheat root rots has been done under semi-arid conditions in the Pacific Northwest. In eastern Washington, *Pythium* spp. caused stunting, reduced tillering, and loss of feeder roots in wheat (Paulitz and Adams 2003).

Surveys in the Pacific Northwest have identified various *Pythium* species as prevalent in wheat roots. Chamswarng and Cook (1985) identified *P. aristosporum* and *P. volutum* as the *Pythium* species causing most damage to wheat in eastern Washington and northern

Idaho, where they cause seed decay, severe root rot, and root browning. Next most damaging were *P. ultimum*, *P. sylvaticum* complex, and *P. irregulare*.

In a survey of 80 wheat fields in eastern Washington state in the year 2000, Paulitz and Adams (2003), the most frequently isolated *Pythium* species (18-50%) were *P. abappressorium* sp. nov., *P. rostratum*, *P. debaryanum*, *P. heterothallicum*, *P. oligandrum*, an unidentified *Pythium* sp. (aff. *echinulatum*), and *P. ultimum*. In this survey, *P. irregulare* and several other *Pythium* species were found at lower frequencies. A later survey by Mavrodi et al (2012) identified at least 10 *Pythium* species that caused disease on wheat. Mavrodi et al (2012) indicated the most damaging *Pythium* strains causing damping off and root rot in eastern Washington wheat were *P. ultimum* Trow and *P. irregulare* group 1 Buisman.

Prolonged waterlogging can cause serious damage to wheat, principally because of hypoxia (low oxygen concentration). Seedling roots exposed to root-zone hypoxia for 10 to 19 days showed significant reductions in dry weight (Araki et al. 2012). If waterlogging is followed by a dry period, wheat root mass usually can recover to nearly normal levels. However, post-hypoxic injury is possible due to the generation of reactive oxygen radicals and toxic oxidative products such as acetaldehyde (Biemelt et al. 1998).

Little work on Pythium root rot of wheat has been done under the humid subtropical conditions that prevail in the southeastern USA. During the 2015-16 winter wheat growing season, portions of the U.S. Mid-Atlantic states experienced very heavy, persistent rainfall as the result of a strong El Nino weather pattern. Soils remained waterlogged for long periods. At around Feekes growth stage 30, wheat crops in many counties of eastern and southern

North Carolina began to exhibit unusual symptoms of stunting. The stunting often occurred in patches, with severely stunted plants adjacent to plants of normal size (Fig. 1).

Pythium was detected in the majority of wheat samples collected from affected areas, particularly in the most severely stunted plants. Fungi such as *Fusarium* and *Rhizoctonia* were also detected in some samples, but these fungi were not as frequent or as dominant in the wheat tissues as *Pythium*. The purpose of the present research was to determine which *Pythium* species were present in affected wheat plants in order to evaluate whether those species were responsible for the symptoms observed.

Diagnosis and identification

Dozens of plant samples were collected from wheat fields exhibiting patchy and/or widespread stunting and submitted to the NC State University Plant Disease & Insect Clinic in Raleigh, North Carolina. The plants displayed stunted shoots, rot and browning of lower plant parts, and stunted roots (Figs. 2 and 3). *Pythium* oospores ranging in diameter from 20 to 30 µm were observed in lower leaf sheaths of field samples (Fig. 4).

Isolations were performed from tissues of roots and lower crowns from 21 samples diagnosed as Pythium root rot. Symptomatic tissues were rinsed under running water, blotted dry and cut into pieces approximately 1 cm in length. Pieces were plated on sterile CMA-P5ARP agar (17 g/liter corn meal agar [Becton, Dickinson, and Company] amended with pimaricin, ampicillin, rifampicin [Sigma-Aldrich Co.], and PCNB [Terraclor 75% WP, Southern Ag Insecticides, Inc.]) (Jeffers and Martin 1986) dispensed in 100 x 15 mm Petri plates. Plates were incubated in the dark at room temperature for 24 to 72 h. Colonies with characteristic coenocytic hyphae typical of *Pythium* species were transferred to fresh CMA-

P5ARP. After 24 to 48 h, pure cultures were obtained by transferring actively growing hyphal tips onto fresh water agar (18 g/liter Bacto agar [Becton, Dickinson, and Company]) dispensed in 60 x 15 mm Petri plates. Fifteen isolates were recovered and identified using DNA-based and morphological identification methods.

Sequencing for identification to species

The internal transcribed spacer region (ITS1-5.8S-ITS2) of the rDNA gene was sequenced for DNA-based identification. Isolates were grown on potato dextrose agar (PDA; 35 g/liter PDA [Becton, Dickinson, and Company]) for 7 to 10 days at room temperature (20 to 25°C). Mycelium mats were stripped from the agar surface, placed in a 1.5-ml microcentrifuge tube, and stored at -20°C. Genomic DNA was extracted from mycelia with the PUREGENE DNA Isolation Kit for 10 to 20 mg of tissue (Qiagen, Valencia, CA) with a protocol optimized for *Pythium* and *Phytophthora* (K.L. Ivors, *unpublished.*).

PCR amplification of the ITS region was performed using ITS4 (5' TCCTCCGCTTATTGATATGC) and ITS5 (5' GGAAGTAAAAGTCGTAACAAGG) primers (White et al. 1990). Reactions were 25 µl in volume and composed of 5.0 µl of 5X GoTaq Colorless Flexi Buffer (Promega Corporation, Madison, WI), 1.5 µl of 25 mM $MgCl_2$, 2.5 µl of 40 mM dNTP mix (10mM of each dNTP; Promega Corporation), 0.25 µl of each 50 µM primer, 0.25 µl of GoTaq DNA polymerase (5U/µl; Promega Corporation), 14.25 µl nuclease-free water, and 1 µl of 1- ng/µl extracted DNA. Thermal cycling conditions consisted of an initial denaturation step of 95°C for 4 min; 35 cycles of 95°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. PCR

products were purified with USB ExoSAP-IT PCR Product Cleanup (Affymetrix) and submitted to ETON Bioscience, Inc. for sequencing.

DNA sequences were aligned and manually edited with CLC Main Workbench 7 (CLC Bio). Edited sequences of each isolate were compared with sequences in the non-redundant nucleotide database GenBank available through the National Center for Biotechnology Information using the BLAST algorithm (Altschul et al. 1997). The BLAST parameters for the sequences were e-values, maximum identity match, and query coverage. Identities were selected based on e-values of 0.0, maximum identity match of 98% or greater, and a query coverage of 98% or greater.

Morphological identification

Species identifications were confirmed morphologically using keys and descriptions of Plaats-Niterick (1981) and Abad et al. (*unpublished*). Isolates growing on water agar were transferred to Petri plates containing 0.5 ml 5% clarified V8 juice agar (CV8A; 50 ml clarified V8 juice, 15 g Bacto agar [Becton, Dickinson, and Co.]), 950 ml deionized water and grass-leaf cultures to observe colony morphology and induce production of sporangia, antheridia, oogonia, and oospores (Abad et al. 1994). Four 5-mm agar plugs were placed in a sterile 60 x 15 mm Petri plate containing 15 ml of sterile deionized water and 4-cm pieces of autoclaved tall fescue leaves. The grass-leaf cultures were incubated at room temperature under continuous fluorescent light for 3 to 5 days. The following species were identified: *P. irregulare*, *P. spinosum*, and *P. vanterpoolii*. Each was found in samples from more than one county (Table 1).

Confirmation and pathogenicity

To confirm pathogenicity using Koch's postulates, isolates of *P. irregulare*, *P. spinosum*, and *P. vanterpoolii* derived from the 2016 North Carolina wheat samples collected in Johnston, Perquimans, and Onslow counties, respectively, were used to inoculate wheat seedlings. Seedlings of cultivar Jagalene were grown for 14 days in a growth chamber (20°C, 12-hour light/dark cycle) in pots of standard growth medium (Fafard 2B Mix, Sun Gro Horticulture, Agawam, MA) before inoculation. Inoculum was prepared by transferring four 4-mm-diameter agar disks from an actively growing culture into 125-ml flasks of twice-autoclaved long-grain rice (25 g rice and 18 ml deionized water) and cultures were incubated for 5 days at 25°C (Holmes and Benson, 1994). Flasks were shaken twice daily to promote uniform colonization of rice grains and prevent clumping.

Plants were inoculated by placing six colonized rice grains in the potting media (1-cm deep and 2.5-cm from the stem) and observed over a four-week period. Pots were placed in plastic trays and flooded to simulate saturated conditions. Mild stunting, chlorosis, and root rot were observed. No symptoms were observed on the non-inoculated plants. Each species was re-isolated from symptomatic roots as described above. Identifications were confirmed morphologically.

Mefenoxam sensitivity

The 15 isolates were screened for mefenoxam sensitivity using an assay previously developed for *Phytophthora* spp. (S. N. Jeffers, Clemson University, personal communication) and modified for use with *Pythium* (Lookabaugh et al. 2015). The assay was conducted in 48-well microtiter plates (Olson et al. 2013). The wells were 10 mm in

diameter and arranged in eight columns × six rows (VWR International, Inc.). Three rows of wells contained 0.5 ml 5% CV8A and three rows contained CV8A amended with 100 µg a.i./ml mefenoxam (trade name Apron XL, Syngenta Crop Protection).

To obtain actively growing cultures, isolates were transferred to 60 × 15 mm Petri plates containing 5 ml 5% CV8A and incubated in the dark at 25°C for 24 h. Six 1-mm plugs were cut from the advancing edge of the actively growing colony with a sterilized glass Pasteur pipet. One plug was transferred into the center of six wells of a freshly prepared test plate with the aid of a flame-sterilized hypodermic needle. Plates were incubated in the dark at 25°C for 48 h or until mycelium in the control wells completely covered the agar surface. Each plate screened eight isolates (one isolate per column) with three observations in amended media and three observations in nonamended media.

Colonization was scored on a rating scale in which 0 = no growth, 1 = a few hyphae growing from plug but only visible microscopically, 2 = hyphae growing uniformly around plug but visible only microscopically, 3 = hyphae growing uniformly around plug and just visible macroscopically, 4 = hyphae visible macroscopically but not completely covering agar surface, and 5 = agar surface completely covered by mycelium and growth equal to that in nonamended wells. Isolates with mean sensitivity scores of 4 were considered resistant to mefenoxam. Each round of testing included a known mefenoxam-resistant isolate of *Pythium aphanidermatum* and a known mefenoxam-sensitive isolate of *P. aphanidermatum*. The entire procedure was completed twice for each isolate. All isolates tested were sensitive to mefenoxam at 100 µg a.i./ml.

Conclusions

Three *Pythium* species were consistently isolated from the samples in the North Carolina root rot and stunting outbreak, sometimes in association with other fungi, but often as the predominant organism. *Pythium* species were the predominant organisms isolated from stunted samples submitted from both Coastal Plain and Tidewater counties. To our knowledge, *Pythium vanterpoolii* and *P. spinosum* have not previously been reported as pathogens of wheat in the USA. This also is the first report of *P. irregulare* on wheat in North Carolina. *P. vanterpoolii* has been identified on common wheat in Canada, England, South Africa, the Netherlands and Greece. *P. vanterpoolii* is frequently recovered from turfgrass species with isolates ranging in aggressiveness from highly aggressive to nonpathogenic (Abad et al. 1994, Feng and Dernoeden 1999).

Pythium spinosum has been associated with root rot symptoms on lettuce in North Carolina and has been isolated from peanut and Stevia samples submitted through the Plant Disease and Insect Clinic (Lookabaugh, *unpublished*). *P. spinosum* was also reported causing damping off symptoms on cucumber in Oman and was most frequently isolated during cooler temperatures (Al-Sa'di et al. 2007). In addition to the 15 wheat samples discussed in this report, a sample of rye (*Secale cereale*) from Johnston County was evaluated during the same period for root rot, and *P. spinosum* was isolated and identified as the causal pathogen in the same manner as described above for the wheat samples (data not shown).

While some *Pythium* species are weak opportunists, others are aggressive plant pathogens that cause considerable damage to root systems with or without pre-existing plant

stress or injury. Wet or saturated soil conditions are favorable for disease and promote development of severe symptoms. For example, flooded soil conditions and longer periods of soil saturation resulted in increased frequency of *Pythium* species and more severe symptomology on soybean and maize (Kirkpatrick et al. 2006, Yanar et al. 1997). The species identified on wheat in the present report are not opportunistic or mainly saprophytic on other reported hosts (Abad et al. 1994, Al-Sa'di et al. 2007, Feng and Dernoeden 1999). Further observation will be needed to determine how frequently stunting in North Carolina wheat is associated with the three species of *Pythium* identified in this study.

It is common to find *Pythium* in combination with other diseases and abiotic problems. In the outbreak reported here, it was hard to determine the degree of damage caused by *Pythium* infection vs. waterlogging in the field samples. The two factors probably worked together to produce the extreme root damage and stunting. The close proximity of severely stunted and relatively unaffected plants that was observed in some fields (Fig. 1) suggested that critical levels of hypoxia could be very localized, and/or that *Pythium* infection was confined to pockets within such fields.

Economic losses due to wheat crown and root rot were severe in North Carolina during the 2015-16 growing season, although it was impossible to fully separate the yield impacts of *Pythium* and waterlogging from those of other less frequent root and crown rot pathogens, early development of leaf rust and powdery mildew, two spring freezes, and a multi-county outbreak of Fusarium head blight. Coastal Plain and Tidewater counties were especially severely affected with crown and root rot, and the worst cases of stunting were seen in low-lying fields and parts of fields. Thousands of acres of wheat were abandoned,

and thousands more had extremely low yields, resulting in hundreds of thousands of dollars of losses.

All 15 isolates tested were sensitive to mefenoxam at 100ug/ml a.i. Seed treatments with products containing mefenoxam should be effective in protecting from *Pythium* infection through emergence. However, when waterlogging occurs for several weeks or even months following emergence, the mefenoxam seed treatment would not be expected to provide sufficient ongoing protection.

Many North Carolina Tidewater agricultural fields are near sea level or in some cases even below it, making drainage a serious challenge. During El Nino episodes, heavy rainfall events are enhanced in frequency by 15%-30% in the Southeastern U.S., especially along the Eastern seaboard (Gershunov and Barnett 1998). Climate change is bringing more frequent heavy downpours to the Southeast, leading to an increase in the number of very wet periods, as well as periods of extreme drying (Carter et al. 2014). Coastal agricultural zones, in particular in the central and northern Tidewater regions of North Carolina, are considered vulnerable to sea-level rise and ground subsidence (Carter et al. 2014). Low-lying inland areas are at risk of increased flooding from rain because stormwater drainage systems may experience seawater inundation and slow draining. Given this picture, a reoccurrence of the conditions giving rise to the extreme stunting and crown/root rot of 2016 is a possibility in North Carolina.

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Table C.1. *Pythium* species isolated from wheat samples submitted to the Plant Disease and Insect Clinic in North Carolina

Pythium spp.	County (# isolates)
P. irregulare	Johnston (1), Onslow (1), Warren (1), Wayne (1), Beaufort (3)
P. spinosum	Pitt (1), Sampson (1), Perquimans (2), Wake (1), Wayne (1)
P. vanterpoolii	Onslow (1), Beaufort (1)

Figure C.1. Severely stunted wheat plants in a field in Sampson County, North Carolina, in April 2016; healthy plants immediately adjacent.

Figure C.2. Left, healthy wheat plants with normal root systems; right, severely stunted plants with minimal root systems found nearby. Beaufort County, April 2016.

Figure C.3. Severely stunted wheat seedlings infected with *Pythium* spp.; symptoms include poorly developed root systems, and rotten crowns and lower leaf sheaths.

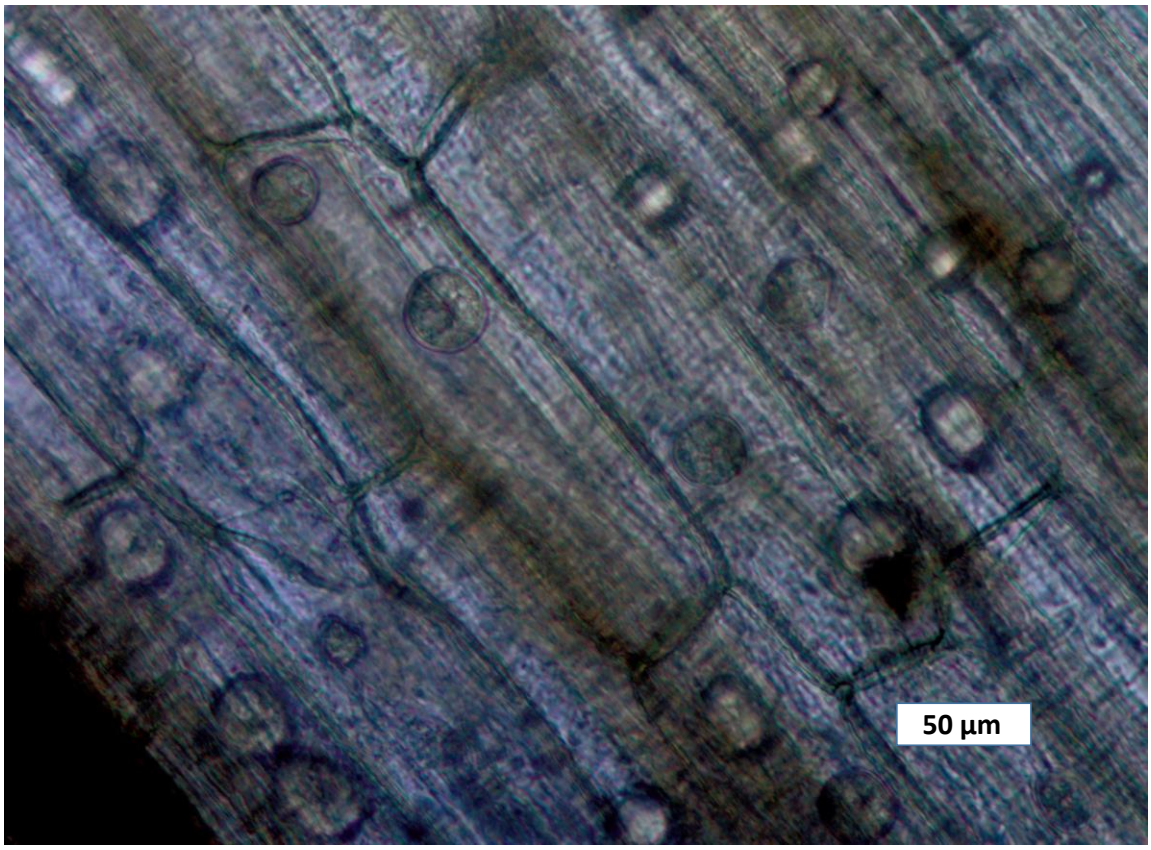

Figure C.4. Oospores of *Pythium* at 200X in wheat lower leaf sheath tissues from samples collected in a North Carolina wheat field in 2016.

Appendix D

First report of black leaf mold of tomato caused by *Psuedocercospora fuligena* in North Carolina

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Diseased tomato (*Solanum lycopersicum*) samples were submitted to the Plant Disease and Insect Clinic from a commercial greenhouse in Sampson County, North Carolina in January 2017. Pale yellow lesions were observed on the upper side of the leaves. Older lesions had brown centers surrounded by zones of pale-green to yellow. Lesions did not coalesce. Blackish-gray fungal sporulation was observed on the underside of the leaf tissue in association with the lesions. Severely affected leaves appeared twisted, with dense, black sporulation visible. Microscopic examination revealed *Cercospora*-like conidia. Conidia were cylindrical, 35.77 μm to 91.42 μm long by 4.21 to 5.00 μm wide, with 3 to 7 septations per conidium. Conidiophores were 33.42 μm to 51.05 μm by 4.30 μm to 4.99 μm wide. Conidia were streaked onto clarified V8 juice agar (CV8A) and single-conidia cultures were transferred to potato dextrose agar. Cultures were incubated at room temperature under continuous fluorescence lights for two weeks. DNA was extracted from mycelia with the PUREGENE DNA Isolation Kit for 10 to 20 mg of tissue (Qiagen, Valencia, CA) and the internal transcribed spacer (ITS) region of rDNA was amplified by polymerase chain reaction using universal primers ITS 4/5. The ITS sequence was 99% identical to those of GenBank accessions of *Pseudocercospora fuligena* from Korea (JX290079), Thailand (GU214675),

and Ohio (KF931141). To satisfy Koch's Postulates, conidia were harvested from 4-week-old cultures growing on CV8A media and four, 6-week-old tomato plants (cv. Better Boy hybrid) were sprayed with a suspension of 1 x 10^3 conidia/ml water. Four non-inoculated control plants were sprayed with sterilized water. Plants were maintained in a growth chamber at 27°C with a 12 hr day/night cycle. Yellow lesions appeared on inoculated plants approximately three to four weeks after inoculation and black sporulation was visible on the underside of the infected leaves. Infected leaves were dry, brittle and some defoliated. No symptoms were observed on non-inoculated plants. Conidia harvested from the symptomatic lesions were streaked onto CV8A media. Cultures were identified as *P. fuligena* based on morphology and sequence analysis as described above. A second diseased tomato sample was received from the same source in April 2017. *P. fuligena* was confirmed based on morphology and sequence analysis. These samples represent the first report of *P. fuligena* in North Carolina. This pathogen was first reported on tomatoes in Florida in 1974 and was recently reported on tomato plants (cvs. Geronimo, Rebelski, and Big Dena) submitted for diagnosis in Ohio (1, 2). The pathogen's emergence in Ohio coupled with the current report warrants improvement in crop sanitation practices in addition to other strategies to manage the disease.

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