Phytophthora nicotianae: Fungicide Sensitivity, Fitness, and Molecular Markers

By

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Phytophthora nicotianae: Fungicide Sensitivity, Fitness, and Molecular Markers

Jiahuai Hu

(ABSTRACT)

Mefenoxam has been a premier compound for Phytophthora disease control in the nursery industry for 30 years. The primary objectives of this research were to examine whether Phytophthora species have developed resistance to this compound and to investigate fungicide resistance management strategies. Phytophthora nicotianae, a destructive pathogen of numerous herbaceous and some woody ornamental plants, was used as a model system. P. cinnamomi, a major pathogen of a wide range of tree species and shrub plants, was also included for comparison.

Twenty-six isolates of P. nicotianae were highly resistant to mefenoxam with a mean EC$_{50}$ value of 326.5 µg/ml while the remaining 70 were sensitive with an EC$_{50}$ of <0.01 µg/ml (Label rate: 0.08µg/ml). All resistant isolates were recovered from herbaceous annuals and irrigation water in 3 Virginia nurseries. Resistant isolates were compared with sensitive ones using seedlings of Lupinus ‘Russell Hybrids’ in the absence of mefenoxam for relative competitive ability. Resistant isolates out-competed sensitive ones within 3 to 6 sporulation cycles. Resistant isolates exhibited higher infection rates and greater sporulation ability than sensitive ones.
No mefenoxam-resistant isolates were identified in *P. cinnamomi*. All 65 isolates of *P. cinnamomi* were sensitive to mefenoxam with an EC$_{50}$ of < 0.04 µg/ml. Attempts to generate mutants with high resistance to mefenoxam through UV mutagenesis and mycelial adaptation were not successful. However, there were significant reductions in sensitivity to mefenoxam; those slightly resistant mutants carried fitness penalties, which may explain why *P. cinnamomi* remains sensitive to mefenoxam.

The effect of propamocarb hydrochloride on different growth stages of *Phytophthora nicotianae* was evaluated in search for an alternative fungicide. Propamocarb greatly inhibited sporangium production, zoospore motility, germination and infection. However, it has little inhibition of mycelial growth and infections. Propamocarb can be used as an alternative fungicide to mefenoxam where mefenoxam resistance has become problematic. However, it must be used preventively; i.e., before infections occur.

The genetic inheritance of mefenoxam resistance in *P. nicotianae* was studied using *F$_1$* progenies of a cross between a resistant isolate and sensitive one. The *F$_1*$ progenies segregated for mefenoxam resistance in a ratio of 1R:1S, indicating that the mefenoxam resistance is controlled by a single dominant gene. One RAPD marker putatively linked to a resistant locus in the repulsion phase was obtained by bulked segregant analysis and was converted to the SCAR marker. This marker is capable of differentiating mefenoxam-resistant populations from sensitive populations included in this study.
DEDICATION

This dissertation is dedicated to my mother, Silan Chen, for her sacrifice, love, and support.
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Dr. Gary W. Moorman, Department of Plant Pathology, The Pennsylvania State University, University Park, PA. Dr. Moorman served on my committee. He critically read the manuscripts and made lots of specific suggestions to improve manuscripts.
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Chapter 1 Background: Fungicide resistance, *Phytophthora nicotianae* and *P. cinnamomi*

**Introduction**

The discoveries of Bordeaux mixture and organic fungicides like dithiocarbamates led to better control of crop diseases caused by fungi, the main pest microorganisms in agriculture (51). Repeated applications are required to protect new foliage from infection because all those fungicides are broad-spectrum protectant multisite inhibitors and cannot penetrate plant surfaces. Resistance was not a problem among those early fungicides (44, 57). In the 1960s, the narrow-spectrum but highly effective compounds such as benzimidazoles, phenylamides, and demethylation inhibitors (DMIs) started the era of systemic fungicides. These new fungicides have revolutionized crop protection. Since then, intensive use of fungicides has become an integral part of IPM programs for major crops worldwide (67).

Fungicide resistance became an increasingly serious worldwide problem since the late 1970s (31, 48, 57). In the last decades, fungicide resistance has been reported for more than 25 important plant pathogens (67). For example, in U.S.A., metalaxyl resistance has been detected in several *Phytophthora* species including *Phytophthora infestans* and *P. nicotianae* (53, 55, 80, 100, 146). Those phytopathogens usually have an asexual stage with high reproductive rates and thus are at great risk of resistance development. Numerous studies conducted by plant pathologists suggest that intensive use of site-specific fungicides for high levels of disease control and therefore strong selection pressures were responsible for problems of resistance (57, 59, 113). Fungicide Resistance
Action Committee (FRAC) implemented various anti-resistance measures to reduce the risk of resistance development. These strategies include the use of multiple-fungicide mixtures, lower application frequency, and close monitoring of resistance development (14, 59, 138).

**Fitness and genetics of fungicide resistance**

There is a fitness cost associated with resistance mutation in the majority of plant pathogenic fungi (113). The newly-resistant mutant microorganisms may have a reduced ecological fitness when growing in the absence of fungicides. The persistence of resistance will largely depend on whether natural selection can compensate for the fitness costs imposed by resistance (113). Information about the fitness of fungicide-resistant isolates is important in formulating effective disease management and anti-resistance strategies.

Fitness is defined as the expected genetic contribution of a given phenotype to subsequent generations (147). Theoretically, any traits contributing to successful competition and survival of a strain through the disease cycle could be considered as fitness indicators (59). In reality, the fitness of a microorganism is assessed as either predicted fitness or competitive fitness. Predicted fitness is estimated by measuring components of fitness, such as infection efficiency, lesion size, and sporulation ability in a single reproductive cycle (2). This approach assesses the fitness of an individual independent of others in the system. Competitive fitness is the assessment of an individual’s fitness in the context of competition with peers.

Plant pathogenic fungi attain resistance to fungicides by a single mutation or more in the nuclear genome. Mutations in a single gene confer a large and qualitative phenotypic decrease in sensitivity. This type of resistance mechanism dominates the majority of site-
specific fungicide groups, including phenylamides, carboxamides, and benzimidazoles. Several alleles of a single gene may confer different levels of resistance (98, 102, 150). Those genes showed either incomplete dominance or complete dominance in true diploid fungi-like Oomycetes (25, 49, 102, 127). The other type of resistance is quantitative and requires the additive interaction of several genes. The examples of quantitative resistance are relatively few and so far identified only in DMIs and morpholines. A total of ten different chromosomal loci in Aspergillus nidulans were involved in resistance to DMIs (46, 58).

**Detection of fungicide resistance**

Efficient diagnostic tools for rapidly detecting fungicide resistance are essential to monitor resistance development, to understand the spread of resistance within populations, and to manage anti-resistance strategies effectively. Three types of techniques are currently available to plant pathologists. These include *in vitro* screening, *in vivo* bioassay, and molecular techniques. Each method has its advantages and limitations.

Various *in vitro* screening techniques based on fungal mycelial growth and other stages of life cycle of a pathogen on fungicide-amended agar media have been used to detect or monitor fungicide-resistant phenotypes. The most common way to quantify fungicide sensitivity is using the EC$_{50}$, a concentration that inhibits 50% of the maximum response. The EC$_{50}$ value is determined by growing organisms on media containing various concentrations of the fungicide of interest. The second way to assay fungicide resistance is to measure the response of a microorganism in a medium containing a single discriminatory concentration of fungicide known to be correlated with resistance. Among stages of the life cycle of target pathogens, mycelial growth is the stage evaluated most
frequently for fungicide sensitivity (14, 15, 17, 31, 32, 34, 35, 38, 44, 57, 59, 93, 98, 102, 110, 120, 140, 153). The potential issue with in vitro tests is that they do not always predict the real sensitivity of a microorganism in plants treated with fungicide. This inconsistency may be due to strong fungicide/host interactions (63, 136).

In vivo bioassays assess fungicide resistance by inoculating intact plants or detached plant parts treated with fungicides. In vivo bioassays may give sufficient information, but in general are slow. In addition, those methods are labor-intensive and require isolating target pathogens first and subsequent evaluation of fungicide resistance.

DNA-based molecular techniques may offer the future direction of rapid resistance detection. Those molecular assays are highly sensitive, specific, and can be performed directly on diseased plant samples. Therefore, it is extremely useful for resistance monitoring. However, the development of molecular diagnostics initially requires pinpointing the DNA sequence associated with resistance; i.e., the DNA changes causing the resistance have to be identified. Currently, knowledge of the molecular mechanisms of resistance exists only for the benzimidazoles and strobilurins. The lack of such information has restricted the molecular techniques to the detection of resistance to benzimidazoles (104) and strobilurins (56). In general, allele-specific real-time PCR (56, 60), PCR-RFLP (105), and primer-introduced restriction analysis PCR (104, 140) have been successful in detecting the single nuclear DNA base pair change (SNPs) conferring resistance in benzimidazoles and strobilurins.

**Phytophthora nicotianae and P. cinnamomi**

In this study, the Oomycete pathogens *Phytophthora nicotianae* and *P. cinnamomi* are used as model microorganisms. Both species are agriculturally and horticulturally common
and destructive pathogens. They attack a broad range of ornamental plants and cause huge economic losses to growers each year. For example, *P. cinnamomi* attacks over 900 plant species (154) such as shade trees and ornamental shrubs. *P. nicotianae* attacks numerous annuals as well as some shrubs. Those two species occupy a wide ecological habitat, including water, soil, and plant materials. Such a wide spatial distribution makes disease management difficult and adds more demands to use of fungicides.

Both species are heterothallic (i.e., A1 and A2 mating types) and have sexual and asexual reproductive cycles. Sexual reproduction requires both mating types present to form oospores. The germination rate of oospores is relatively low. Most of life cycle exists in diploid nuclei and genetic inheritance is rather complicated, but a large portion of the nuclear genome follows Mendelian genetics (102, 108). The asexual stage repeatedly produces massive amounts of sporangia and zoospores in wet, favorable conditions, which make the Phytophthora diseases multicyclic. *P. cinnamomi* also produces numerous chlamydospores that can survive in soil for over 3 years (64, 154).

Chemical control is the primary approach for Phytophthora disease management. Fungicide control for Phytophthora diseases accounts for over 25% of the total annual global fungicide expenditure (48, 131). Major fungicides with different modes of action include: (i) mefenoxam (Subdue MAXX), (ii) propamocarb hydrochloride (Banol; Previcure Flex), (iii) fosetyl-al (Aliette), (iv) dimethomorph (Stature DM), and (v) etridiazole (Truban; Terrazole). In the nursery industry, mefenoxam and propamocarb are two premier compounds used for Phytophthora disease management. Therefore, mefenoxam and propamocarb were used in this study.
The fungicides mefenoxam and propamocarb

Mefenoxam is the R-enantiomer of metalaxyl and provides the same level of efficacy as metalaxyl at half its application rate. Mefenoxam was registered to replace metalaxyl in U.S.A. by EPA in 1996. Mefenoxam can be translocated upwards in xylem and is highly effective against most critical life stages of all Oomycete plant pathogens. It inhibits Oomycete ribosomal RNA polymerases (41). Specifically, it interrupts the normal functioning of ribosomal RNA polymerase I by preventing incorporation of uridine into rRNA (39-41, 58, 131). Metalaxyl-resistant isolates of P. infestans had a decreased binding affiliation between RNA polymerase and metalaxyl (40, 41). Resistance to mefenoxam was widely reported in agricultural species of Phytophthora (37, 61, 62, 100, 120) as well as related oomycete pathogens (17, 35, 114, 126, 129, 148). There is growing concern about the mefenoxam resistance problem in the nursery and floral industry.

Figure 1. Chemical structure of mefenoxam, N-(2, 6-dimethylphenyl)-N-(methoxyacetyl) alanine methyl ester

Propamocarb is a systemic carbamate fungicide against soilborne oomycetes. Since its introduction to the European market in 1978, propamocarb has been widely used for Phytophthora disease control on numerous crops including ornamentals, vegetables, and field crops (30, 119, 121, 124, 128). Propamocarb mainly has fungistatic activity against Phytophthora and Pythium species (31, 92, 119), while it occasionally exhibited fungicidal
activity against *Pseudoperonospora cubensis* on cucumbers (30). Propamocarb is acropetally translocated in plants and has a higher systemic activity when used as a soil-drench at high concentrations (30, 31). Propamocarb is a biosynthesis disruptor of the oomycete’s cell membrane (31, 119). Resistance was seldom reported in *Phytophthora cactorum* and *P. nicotianae* isolated from ornamentals in Germany (123). More recently, resistance to propamocarb was also detected in *Pythium* species obtained from a greenhouse in Pennsylvania (114).

![Chemical structure of propamocarb hydrochloride](image)

**Objectives**

The multibillion-dollar nursery industry mainly relies on mefenoxam for Phytophthora disease control. Mefenoxam resistance has been increasingly reported in this industry in the last decade (27, 53, 80, 83, 114) and poses a great threat to the future profitability and sustainability of the entire industry. A worse consequence would be that resistant pathogen populations may escape from production facilities via contaminated nursery stock plants and spread to the landscape. This is likely to cause losses of valuable vegetation. Growers in the nursery industry need to know how widespread the mefenoxam resistance problem is in Virginia and how likely the resistant phenotypes of the pathogen will replace the
sensitive ones and become established in production facilities. Given the widespread
distribution of mefenoxam-resistant strains, alternative fungicides against *Phytophthora*
are needed to address the problem of mefenoxam resistance. Finally, growers also need an
efficient diagnostic tool for rapidly detecting resistance and monitoring the spread of
resistant populations.

This dissertation was aimed to answer those important questions using *P. nicotianae* as
a model. This research also determined the likelihood of *P. cinnamomi* developing
resistance to mefenoxam under laboratory conditions. This dissertation will provide data
for developing molecular-resistance diagnostic protocols and recommendations for the
nursery industry to best manage Phytophthora diseases using fungicides.
Chapter 2 Mefenoxam Sensitivity and Fitness Analysis of *Phytophthora nicotianae* Isolates from Virginia Nurseries

**ABSTRACT**

Mefenoxam is one of the most commonly used compounds for managing Phytophthora diseases on ornamentals. The objective of this study was to determine whether *Phytophthora nicotianae*, a destructive pathogen to numerous herbaceous and some woody ornamental plants in nurseries, has developed resistance to mefenoxam and to evaluate the fitness of mefenoxam-resistant isolates. Ninety-five isolates of *P. nicotianae* were screened on 20% clarified V8 agar medium for sensitivity to mefenoxam at 100 a.i. µg/ml. Twenty-five isolates were found highly resistant to mefenoxam. The remaining isolates were sensitive to mefenoxam. Five resistant and six sensitive isolates were further assayed to determine their EC$_{50}$ values. Mean EC$_{50}$ values for resistant isolates ranged from 231.0 to 466.3 µg/ml. EC$_{50}$ values for sensitive isolates were less than 0.09 µg/ml. Nine resistant and seven sensitive isolates were tested for mefenoxam sensitivity on *Pelargonium × hortorum* cv. ‘White Orbit’. Mefenoxam provided good protection of geranium seedlings from colonization by sensitive isolates, but not by any resistant isolates. Two resistant and two sensitive isolates were compared for fitness components and their relative competitive ability on *Lupinus* ‘Russell Hybrids’ in the absence of mefenoxam. Resistant isolates were better competitors and out-competed sensitive ones after 3-6 sporulation cycles on lupine seedlings, regardless of their initial proportions in mixed zoospore inocula. Resistant isolates exhibited greater infection rate and higher sporulation ability than sensitive ones when they were applied separately onto lupines. These results suggest that fungicide
resistance may pose a serious challenge to the continued effectiveness of mefenoxam as a control option for nursery growers.

**Additional keywords:** fungicide resistance, IPM, ornamental crops, EC$_{50}$, competitive ability, fitness

**INTRODUCTION**

Since its first introduction in 1977, metalaxyl has been used widely and intensively to control oomycete diseases of numerous crops including ornamentals (31, 59, 133). Metalaxyl has strong inhibitory activities against both mycelium growth and sporulation, but little effect against either sporangium or zoospore germination (135, 139). The mode of action of metalaxyl is selective inhibition of ribosomal RNA synthesis by affecting the activity of the RNA polymerases (40, 41, 131). Due to its high site-specificity, metalaxyl has a relatively high intrinsic risk of resistance development in target pathogens (31, 59). Within 2 years of its introduction, several incidences of *Phytophthora infestans* resistance emerged in potato fields across Europe (42, 45, 115). Since then, metalaxyl resistance has been frequently detected in other *Phytophthora* species (37, 53, 61, 79, 84, 100, 146) and in other oomycetes as well (17, 35, 114, 126, 129).

Various studies using *P. infestans* as a model have been conducted both in the field and in the greenhouse to investigate the fitness of metalaxyl-resistant isolates and detect the association between metalaxyl resistance and enhanced pathogenic fitness. For example, resistant isolates were found to cause larger lesions (89-91) and resultant disease progressed more quickly than when sensitive isolates were involved (7, 90). Subsequently, the hypothesis of this linkage between those two attributes has been challenged and
disproved by two recent genetic analyses of oospore progeny (59, 102). Currently, there is little data on the fitness of metalaxyl resistance in other species of Phytophthora.

Root and crown rots caused by Phytophthora nicotianae Breda de Haan (syn: Phytophthora parasitica Dastur) are among the most destructive diseases of numerous herbaceous and some woody ornamental plant species grown in nurseries worldwide (11, 48, 51, 82). This species has been frequently recovered from diseased nursery plants (8, 72) and recycling irrigation water (20, 22, 70, 71, 122, 145). This pathogen can be easily spread to other production facilities and landscapes through movement of contaminated nursery stock (94). Thus, disease management can be difficult once plants and/or growing media are contaminated within a production facility or landscape.

Chemical control remains a primary approach for Phytophthora disease control on ornamental plants in nurseries. In USA, mefenoxam, metalaxyl-M (Subdue MAXX, Syngenta) is one of the major compounds registered to control root infections on ornamental crops (11, 83). This fungicide has been suspended from foliage use because of the high risk of resistance development in foliar pathosystems (141, 146). Due to the high effectiveness of mefenoxam, nursery growers continue to use this product, but resistance has been increasingly detected (53, 54, 80, 99, 100, 146). Information on the proportion of a pathogen resistant to fungicide and on the movement of resistance among nursery industries is critical to develop management strategies that can prevent development of resistance. However, little is known about the sensitivity of P. nicotianae to mefenoxam in Virginia. The objectives of this study were to determine (i) in vitro sensitivity of P. nicotianae isolates from Virginia nurseries to mefenoxam, (ii) EC$_{50}$ values of representative isolates to this compound, and (iii) efficacy of mefenoxam to protect
geranium seedlings against infection by *P. nicotianae*, and (iv) fitness components and competitive fitness of mefenoxam-resistant isolates interacting with lupine seedlings.

**MATERIALS AND METHODS**

**Isolate origin, identity confirmation, and mating type determination**

A total of 95 isolates of *P. nicotianae* were included in this study. The majority of these isolates were recovered from plant tissues of diverse nursery crops and irrigation water in Virginia. Several isolates from other crops and locations, kindly provided by Drs. James Adaskaveg (University of California, Riverside, California), Mannon Gallegly (West Virginia University, Morgantown, West Virginia), David Shew (North Carolina State University, Raleigh, North Carolina), and Sharon von Broembsen (Oklahoma State University, Stillwater, Oklahoma) were included for comparison. Most isolates were recovered since 2000; however, two isolates (23A8 and 23A9) were recovered in 1968, before mefenoxam was marketed for use on ornamental crops.

The identity of all isolates was verified using a single-strand conformation polymorphism analysis of the ribosomal DNA internal transcribed spacer 1 (95). Mating type was determined on 20% clarified V8 agar (CV8) for all isolates by pairing each isolate with tester isolates 23C4 (A1) and 23C6 (A2). Direct pairings were accomplished by placing two discs (5 mm in diameter) of agar culture one cm apart in the center of Petri dishes (9 cm diameter). Petri dishes were then sealed with Parafilm™ (American National Can, Menasha, Wisconsin) and incubated in darkness for 2 weeks at 25°C for oospore formation. Each pairing was conducted with two replicates. The mating type was determined microscopically based on the absence or presence of oospores at the interface between colonies of two isolates. A cross between the two testers of opposite mating type
and two selfings of the same mating type were included as positive control and negative control, respectively.

**Mefenoxam sensitivity**

Mefenoxam (Subdue MAXX; Syngenta, Greensboro, North Carolina, USA) was assessed at a concentration of 100 a.i. µg/ml. CV8 agar was used as a base medium. The fungicide was diluted in sterile distilled water then added to the autoclaved media at 50°C. Mycelial plugs (5-mm diameter) were cut from the margin of actively growing colonies of 5-day-old agar cultures. One plug was placed in the center of a 9-cm Petri dish with the mycelia in contact with the medium. Three replicate dishes per treatment were inoculated for each isolate. The inoculated dishes were then incubated at 23~25°C for 5 to 7 days in darkness. When colonies in non-amended control dishes had reached the edge, colony diameters were measured in two perpendicular directions for all treatments. The diameter of the mycelium plug was subtracted; the two measurements were averaged. The relative mycelial growth of an isolate on amended media was calculated by dividing colony diameter in amended dishes by that in the non-amended control dishes, and expressed as a percentage. Isolates were characterized as sensitive if the relative mycelial growth was less than 50%. Isolates were classified as resistant if the relative mycelial growth was not less than 50% (80).

**Effective concentration (EC\textsubscript{50})**

Five mefenoxam-resistant and six sensitive isolates from different host species/substrates were selected to determine their EC\textsubscript{50} values. These isolates were assessed using the same medium amended with mefenoxam at concentrations of 0.1, 1, 10, 50, 100, 500, 1000 a.i. µg/ml plus a non-treated control. Three replicate Petri dishes were
used for each treatment. The dishes were inoculated, incubated and colony growth was
determined as described above.

_in vivo tests with Pelargonium × hortorum cv. ‘White Orbit’_

Nine resistant isolates and seven mefenoxam-sensitive isolates were further tested for
mefenoxam sensitivity using seedlings of geranium (_Pelargonium × hortorum_ cv. ‘White
Orbit’). Seedlings were treated with mefenoxam then challenged with _P. nicotianae_ as
described previously (76). A plastic container (9 cm in diameter and 4.5 cm deep) was
used to hold a smaller Petri dish (6 cm in diameter) with a filter paper (Whatman #4) on its
top. Twenty-five ml of general purpose fertilizer (20% N, 20% P₂O₅, and 20% K₂O;
Scotts-Sierra Horticultural Products Co., Marysville, OH) in water at 1 mg mL⁻¹ was added
to each container to keep the filter paper moist. Five geranium seeds were placed on the
filter paper. The containers were placed inside a tray sealed with a transparent plastic cover
and incubated for five days in a growth chamber with fluorescent lights at 23~25°C.

Once the cotyledons were fully expanded, the fertilizer solution in each container was
replaced with the same amount of mefenoxam solution at 0.15 µl mL⁻¹ (twice the label
rate). Two days after replacement, both cotyledons of each seedling were inoculated with
mycelial plugs. Five-mm-diameter mycelial plugs were taken from the margin of a 3-day-
old culture. One plug was placed on the upper surface of each cotyledon after seedlings
were sprayed with sterile soil water extract (SSWE) to facilitate plug adhesion. All
inoculated plants were incubated in a moist chamber in the dark for 24 h and then grown
under fluorescent light at 23~25°C. Four to six days after inoculation, the number of
seedlings with blackened stem, leaf necrosis or wilted appearance was counted.
Fitness analysis

Two mefenoxam-resistant isolates (31D7 and 3A4) and two sensitive isolates (1E3 and 4J9) were included in fitness tests. Isolate 31D7 was recovered from petunia in 2004 and 3A4 from irrigation water in 2000. Isolate 4J9 was obtained from daphne in 2001 and 1E3 from irrigation water. Tests performed were: (i) fitness component analysis, and (ii) relative competitiveness comparison. All tests were done with 10-day-old seedlings of *Lupinus* ‘Russell Hybrids’ (28). Fitness components assessed included infection rates and sporulation ability by individual isolates. Competitive fitness tests involved three pairs of resistant/sensitive isolates (31D7+1E3, 3A4+4J9, and 31D7+4J9) at three initial ratios of 20, 50, and 80% of resistant populations in mixed zoospore inocula.

Lupine seeds were induced to germinate by soaking overnight in water and then placed on moistened paper towel with bottom heat of 35°C. Ten lupine seedlings were grown in vermiculite medium inside a plastic cup (15 cm ×10 cm) under white fluorescent light (10 h day/14 hr night) at 22~26°C. The plants were fertilized once a week with 50 ml of the same fertilizer as used for *in vivo* tests.

Zoospore suspensions of individual isolates were prepared separately following a standard procedure (Hong *et al.*, 2002). A block (3 cm×3 cm) of 1-week-old mycelial agar culture was cut and transferred to 6 cm Petri dishes and then evenly divided into 10 small pieces. Sporangial production was induced by overnight incubation with 10 ml of sterile soil water extract (SSWE). Zoospore release was triggered by temperature shock with 5 ml of pre-chilled (4°C) SSWE. Zoospore suspensions were adjusted to 3600 zoospores mL⁻¹ using a haemocytometer for inoculation experiments.
In fitness component tests, one 10-µl droplet of inoculum containing 36 zoospores of individual isolates was applied to the upper surface of each fully expanded cotyledon. Each treatment was replicated three times with 10 plants per replicate. Inoculated plants were maintained at 100% relative humidity in the dark for 24 h at 25°C to facilitate the infection process. Then the inoculated plants were transferred to grow under the conditions as described above. The number of both wilted leaves and wilted plants were recorded on a daily basis. A leaf or plant was scored as wilted if the entire leaf area or aboveground part was fully colonized and dehydrated. Four days after inoculation, sporulation ability was measured as follows: five diseased plants including the roots were collected from each treatment and surface disinfested with 10% bleach solution for 30 seconds, followed by copious rinses with sterile distilled water. Sporangium production was induced by incubating those sterilized plants in 6-cm Petri dishes containing SSWE under florescent light for 48 h. During incubation, the solution in each Petri dish was replaced with fresh SSWE at 24 h intervals. Zoospores release were triggered by using 5 ml of pre-chilled (4°C) SSWE. The number of zoospores was counted in eight fields with a haemocytometer.

To compare the relative competitive ability of resistant isolates to sensitive isolates, the inoculation was carried out with mixed inocula in the same way as described for fitness component tests. Mixed inocula containing 20, 50, and 80% of resistant zoospores were prepared by thoroughly mixing volumes of zoospore suspension of resistant isolates with sensitive isolates. Four days after inoculation, the number of wilted plants as described above was recorded. Zoospores were produced as previously described above. Zoospore suspensions obtained were used to (i) determine the percentage of resistant subpopulations,
and (ii) inoculate plants for the next cycle. Six successive sporulation cycles were conducted.

To determine the percentage of resistant subpopulations in mixed zoospore populations, 100 µl of zoospore suspension containing 36 zoospores was spread on PARP medium in Petri dishes (9 cm in diameter) to allow zoospore germination. After 36 hours of incubation at 25°C in darkness, sixty colonies per treatment were individually transferred to CV8 agar amended with mefenoxam at 100 µg/ml. A colony was scored as resistant to mefenoxam if it continued to grow on the 100 µg/ml mefenoxam medium and reach the edge of the dishes after 5 days of incubation at 25°C in darkness; a colony was identified as sensitive if no growth was observed. The percentage of mefenoxam-resistant zoospore populations was calculated by dividing the number of mefenoxam-resistant colonies by 0.6.

**Experiment design and data analysis**

Each experiment described above was conducted twice on different dates. Treatments within each repeating experiment were arranged in a completely randomized design. Data from two experiments were pooled according to homogeneity of variance. Linear regression lines of logit-transformed percentage against the log$_{10}$ of the mefenoxam concentration were fitted using Proc REG (SAS Institute, Cary, North Carolina, USA). The resulting equations were used to calculate EC$_{50}$ values. The Mann-Whitney $U$ test (Zuwaylif, 1979) was performed to determine the differences in mortality of geranium seedlings between the mefenoxam treatment and non-treated control for each isolate. One-way fixed effect analysis of variance using Proc GLM was performed. Duncan’s multiple
range test was used to determine statistical differences in fitness parameters among the isolates.

RESULTS

Isolate identity confirmation and mating type

All of the 95 isolates tested yielded ca. 300 bp amplicon and produced a SSCP banding pattern characteristic of *P. nicotianae* on 8% polyacrylamide gels. Seventy-one isolates were A2 mating type. Twenty four isolates were A1 mating type (Table 1). Abundant oospore formation was observed in the positive control, but not in the negative control.

Mefenoxam sensitivity

Approximately 26% of *P. nicotianae* isolates (25 out of 95) from different hosts/substrates and years were resistant to mefenoxam (Table 1, Figure 3). Seventy-six percent of isolates recovered from annual ornamental plants and 40% of isolates from irrigation water in Virginia were resistant to mefenoxam (Table 1). However, only one isolate was resistant to mefenoxam among all isolates recovered from perennials and woody ornamentals (Table 1). Among those 25 resistant isolates, nineteen were obtained from petunias in Virginia in 2004 and they had a greater mycelial growth in the presence of 100 µg/ml compared to that on non-amended controls; 3 were from annual vinca in 2001; 2 were from irrigation water, and the remaining one isolate was from lavender in 2002. The largest proportion of sensitive isolates had a relative growth on agar medium with mefenoxam at 100 µg/ml of less than 10% of non-amended control (Figure 3). None of the A1 mating type isolates were resistant to mefenoxam (Table 2). Fisher’s exact test revealed that there was an association between mating type and mefenoxam resistance among the 95 isolates tested in this study.
Effective concentration

The relative sensitivities of five resistant and sensitive isolates were shown in Figure 4. The EC$_{50}$ values for five resistant isolates ranged from 231.0 to 466.3 µg/ml with a mean EC$_{50}$ value of 326.5 µg/ml (Table 3). Most of sensitive isolates had over 50% inhibition of mycelial growth in the presence of 0.1 µg/ml and over 70% inhibition on the agar medium amended with mefenoxam at 1 µg/ml (Figure 4). The maximum EC$_{50}$ value for the five sensitive isolates tested was 0.04 µg/ml (Table 3).

In vivo tests with Pelargonium × hortorum cv. ‘White Orbit’

Colonization first took place at the inoculation site then rapidly spread either up to the growing tip or down the stem, causing stem rot (blackened stem). Eventually the plants collapsed. All sixteen isolates consistently infected, colonized, and killed the majority of non-treated control geranium seedlings (Table 4). Mefenoxam provided full protection of pretreated geranium seedlings against infection by sensitive isolates, but not resistant isolates (Table 4).
Table 1 Host, origin, mating type and sensitivity to mefenoxam of *Phytophthora nicotianae* isolates collected from ornamental crop nurseries in Virginia

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host/substrate</th>
<th>Nursery</th>
<th>Year</th>
<th>Mating type</th>
<th>% Growth</th>
<th>Supplier</th>
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<td>Year</td>
</tr>
<tr>
<td>------</td>
<td>-------------------------------</td>
<td>------------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>26F1</td>
<td><em>N. tabacum</em></td>
<td>...</td>
<td>2003</td>
<td>A2</td>
<td>1.4</td>
<td>DS (R1)</td>
</tr>
<tr>
<td>25J8</td>
<td>Irrigation water</td>
<td>E</td>
<td>2001</td>
<td>A2</td>
<td>0.6</td>
<td>CH</td>
</tr>
<tr>
<td>1E2</td>
<td>Irrigation water</td>
<td>F</td>
<td>...</td>
<td>A1</td>
<td>0.3</td>
<td>SV (II-16)</td>
</tr>
<tr>
<td>1B2</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>A2</td>
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<td>MH</td>
</tr>
<tr>
<td>2C10</td>
<td><em>B. sempervirens</em></td>
<td>D</td>
<td>2000</td>
<td>A1</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>2C7</td>
<td>Irrigation water</td>
<td>E</td>
<td>2000</td>
<td>A1</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>23A8</td>
<td>...</td>
<td>...</td>
<td>1968</td>
<td>A2</td>
<td>0.0</td>
<td>MG (P20)</td>
</tr>
<tr>
<td>25J9</td>
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<td>E</td>
<td>2000</td>
<td>A1</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>23B6</td>
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<td>...</td>
<td>...</td>
<td>A1</td>
<td>0.0</td>
<td>MG (P29)</td>
</tr>
<tr>
<td>2D1</td>
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<td>CH</td>
</tr>
<tr>
<td>2C11</td>
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<td>D</td>
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<td>A1</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>23C6</td>
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<td>...</td>
<td>...</td>
<td>A2</td>
<td>0.0</td>
<td>MG (P88)</td>
</tr>
<tr>
<td>1E3</td>
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<td>F</td>
<td>...</td>
<td>A1</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>26E7</td>
<td><em>Nicotiana tabacum</em></td>
<td>...</td>
<td>2003</td>
<td>A1</td>
<td>0.0</td>
<td>DS (R0)</td>
</tr>
<tr>
<td>31D6</td>
<td><em>Petunia</em> sp.</td>
<td>A</td>
<td>2004</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>23A9</td>
<td><em>Citrus limonium</em></td>
<td>...</td>
<td>1968</td>
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<td>0.0</td>
<td>MG (P21)</td>
</tr>
<tr>
<td>17H1</td>
<td><em>Forsythia</em> sp.</td>
<td>A</td>
<td>2001</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>23C3</td>
<td><em>Solanum tuberosum</em></td>
<td>...</td>
<td>1991</td>
<td>A1</td>
<td>0.0</td>
<td>MG (P77)</td>
</tr>
<tr>
<td>3A8</td>
<td><em>Lycopersicon esculentum</em></td>
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<td>2000</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>3A10</td>
<td><em>Berberis thunbergii</em></td>
<td>...</td>
<td>2000</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>1C8</td>
<td>...</td>
<td>...</td>
<td>2001</td>
<td>A1</td>
<td>0.0</td>
<td>MH</td>
</tr>
<tr>
<td>21J1</td>
<td><em>Citrus</em> sp.</td>
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<td>...</td>
<td>A2</td>
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<td>JA</td>
</tr>
<tr>
<td>18C4</td>
<td><em>C. roseus</em></td>
<td>A</td>
<td>2001</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>17H3</td>
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<td>A</td>
<td>2001</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>1B6</td>
<td><em>Rhododendron</em> sp.</td>
<td>...</td>
<td>2001</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>17H5</td>
<td><em>Forsythia</em> sp.</td>
<td>A</td>
<td>2001</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>49J</td>
<td><em>Daphne</em> sp.</td>
<td>A</td>
<td>2001</td>
<td>A1</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>18D1</td>
<td><em>C. roseus</em></td>
<td>A</td>
<td>2001</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>3A12</td>
<td><em>C. roseus</em></td>
<td>H</td>
<td>2000</td>
<td>A1</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>29F3</td>
<td><em>Forsythia</em> sp.</td>
<td>...</td>
<td>2003</td>
<td>A1</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>23C4</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>A1</td>
<td>0.0</td>
<td>MG (P86)</td>
</tr>
<tr>
<td>34A2</td>
<td><em>Veronica spicata</em></td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>34A3</td>
<td><em>V. spicata</em></td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>34A4</td>
<td><em>V. spicata</em></td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>34A5</td>
<td><em>V. spicata</em></td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>34A6</td>
<td><em>V. spicata</em></td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>34A7</td>
<td><em>Verbena</em> sp.</td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>31A8</td>
<td><em>Verbena</em> sp.</td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>31A9</td>
<td><em>Lavandula angustifolia</em></td>
<td>G</td>
<td>2006</td>
<td>A1</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>31B1</td>
<td><em>L. angustifolia</em></td>
<td>G</td>
<td>2006</td>
<td>A1</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>31B2</td>
<td><em>Scabiosa columbaria</em></td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>31B3</td>
<td><em>S. columbaria</em></td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>31B4</td>
<td><em>S. columbaria</em></td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>31B5</td>
<td><em>Penstemon mexicali</em></td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>31B6</td>
<td><em>P. mexicali</em></td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>31B7</td>
<td><em>P. mexicali</em></td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
<tr>
<td>31B8</td>
<td><em>P. mexicali</em></td>
<td>G</td>
<td>2006</td>
<td>A2</td>
<td>0.0</td>
<td>CH</td>
</tr>
</tbody>
</table>
the identity of each nursery where isolates were recovered was coded to protect these enterprises.

the mating type was determined by pairing all isolates with two tester isolates of *P. nicotianae* (23C4 and 23C6).

CH=Chuanxue Hong, Virginia Tech, Virginia Beach, Virginia; DS=David Shew, North Carolina State University, Raleigh, North Carolina; JA=James Adaskaveg, University of California, California, CA; MG=Mannon Gallegly, West Virginia University, Morgantown, West Virginia; MH=Mary Ann Hansen, Virginia Tech, Blacksburg, Virginia; and SV=Sharon von Broembsen, Oklahoma State University, Stillwater, Oklahoma.

…unknown.

Table 2 Fisher’s exact test of *Phytophthora nicotianae* isolates for linkage of mefenoxam sensitivity to mating type

<table>
<thead>
<tr>
<th>Mefenoxam sensitivity</th>
<th>Number of isolates</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1</td>
<td>A2</td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td>0</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Sensitive</td>
<td>24</td>
<td>46</td>
<td>70</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>71</td>
<td>95</td>
</tr>
</tbody>
</table>

| w |
Table 3 Effective concentration (EC$_{50}$) of mefenoxam for 50% inhibition in the mycelial growth of *Phytophthora nicotianae* isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host/substrate</th>
<th>Geographic origin</th>
<th>Year</th>
<th>Mating type</th>
<th>EC$_{50}$ (µg/ml) $^z$</th>
</tr>
</thead>
<tbody>
<tr>
<td>31A1</td>
<td><em>Petunia</em> sp.</td>
<td>Virginia</td>
<td>2004</td>
<td>A2</td>
<td>466.32</td>
</tr>
<tr>
<td>31D5</td>
<td><em>Petunia</em> sp.</td>
<td>Virginia</td>
<td>2004</td>
<td>A2</td>
<td>458.72</td>
</tr>
<tr>
<td>3A4</td>
<td>Irrigation water</td>
<td>Virginia</td>
<td>2000</td>
<td>A2</td>
<td>241.4</td>
</tr>
<tr>
<td>16C8</td>
<td>Irrigation water</td>
<td>Virginia</td>
<td>2000</td>
<td>A2</td>
<td>231.0</td>
</tr>
<tr>
<td>18C8</td>
<td><em>Catharanthus roseus</em></td>
<td>Virginia</td>
<td>2001</td>
<td>A2</td>
<td>235.24</td>
</tr>
<tr>
<td>25J9</td>
<td>Irrigation water</td>
<td>Virginia</td>
<td>2000</td>
<td>A1</td>
<td>0.04</td>
</tr>
<tr>
<td>18C6</td>
<td><em>C. roseus</em></td>
<td>Virginia</td>
<td>2001</td>
<td>A2</td>
<td>0.04</td>
</tr>
<tr>
<td>2C12</td>
<td><em>Buxus sempervirens</em></td>
<td>Virginia</td>
<td>2000</td>
<td>A1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>26E8</td>
<td><em>Nicotiana tabacum</em></td>
<td>North Carolina</td>
<td>2003</td>
<td>A2</td>
<td>0.001</td>
</tr>
<tr>
<td>23A9</td>
<td><em>Citrus limonum</em></td>
<td>California</td>
<td>1968</td>
<td>A1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>23A8</td>
<td><em>C. limonum</em></td>
<td>California</td>
<td>1968</td>
<td>A2</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

$^z$ EC$_{50}$ values were estimated by fitting a regression line of the logit-transformed inhibition against the log-transformed concentrations of the fungicides. These values are the mean of six replicates from two repeating tests.

Figure 3 Frequency distribution of 95 isolates of *Phytophthora nicotianae* in response to 100 µg/ml mefenoxam incorporated into 20% clarified V8 agar. The relative growth of each isolate is a mean value of six replicates from two tests.
Figure 4 Dose-response curves for the mycelial growth on clarified V8 agar of (a) five mefenoxam-resistant isolates and (b) five mefenoxam-sensitive isolates of *Phytophthora nicotianae*. The mycelial growth was expressed as percent growth on mefenoxam-amended medium compared to that of the control medium. Each number is a mean value of six replicates from two experiments.
Table 4 Efficacy of mefenoxam in protecting *Pelargonium × hortorum* cv. ‘White Orbit’ seedlings from infections by *Phytophthora nicotianae* isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Sensitivity</th>
<th>No. of diseased plants</th>
<th>Mefenoxam</th>
<th>$P^z$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>x</td>
<td>w</td>
<td></td>
</tr>
<tr>
<td>Non-treated control</td>
<td>Mefenoxam</td>
<td>$P^z$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30J5</td>
<td>R</td>
<td>5.0</td>
<td>4.5</td>
<td>0.5</td>
</tr>
<tr>
<td>30J7</td>
<td>R</td>
<td>4.5</td>
<td>4.5</td>
<td>1.0</td>
</tr>
<tr>
<td>31D7</td>
<td>R</td>
<td>4.0</td>
<td>3.0</td>
<td>0.17</td>
</tr>
<tr>
<td>31A8</td>
<td>R</td>
<td>4.0</td>
<td>3.5</td>
<td>0.5</td>
</tr>
<tr>
<td>3A4</td>
<td>R</td>
<td>5.0</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>31A5</td>
<td>R</td>
<td>5.0</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>31E2</td>
<td>R</td>
<td>4.5</td>
<td>4.5</td>
<td>1.0</td>
</tr>
<tr>
<td>18C8</td>
<td>R</td>
<td>5.0</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>16C8</td>
<td>R</td>
<td>5.0</td>
<td>4.5</td>
<td>0.5</td>
</tr>
<tr>
<td>26E8</td>
<td>S</td>
<td>4.5</td>
<td>0.0</td>
<td>0.001</td>
</tr>
<tr>
<td>23C7</td>
<td>S</td>
<td>4.5</td>
<td>0.5</td>
<td>0.001</td>
</tr>
<tr>
<td>25J9</td>
<td>S</td>
<td>5.0</td>
<td>0.0</td>
<td>0.001</td>
</tr>
<tr>
<td>1E3</td>
<td>S</td>
<td>5.0</td>
<td>0.0</td>
<td>0.001</td>
</tr>
<tr>
<td>23A9</td>
<td>S</td>
<td>5.0</td>
<td>0.0</td>
<td>0.001</td>
</tr>
<tr>
<td>23C3</td>
<td>S</td>
<td>5.0</td>
<td>0.0</td>
<td>0.001</td>
</tr>
<tr>
<td>18C4</td>
<td>S</td>
<td>5.0</td>
<td>0.0</td>
<td>0.001</td>
</tr>
</tbody>
</table>

$^w$ Mefenoxam sensitivity; R = resistant if the mycelial growth of an isolate on CV8 medium amended with 100 µg/ml mefenoxam was $\geq 50\%$ of that on the non-amended control, otherwise sensitive (=S).

$^x$ Five seeds of *Pelargonium × hortorum* cv. ‘White Orbit’ were germinated on filter paper moistened with 20 ml of fertilizer solution. Each seedling was inoculated by inverting a 5-mm-diameter mycelial plug onto upper surface of both fully expanded cotyledons. The Number of diseased plants is the mean value of 6 replicates from two repeating tests. The total Number of test plants per replicate was five.

$^y$ Seedlings treated with 0.15 µl mL$^{-1}$ mefenoxam fertilizer solutions (double the labeled rate)

$^z$ Man-Whitney $U$ test was performed to determine the differences in mortality of seedlings between mefenoxam-treated and non-treated control.
Fitness analysis

In the fitness component experiment, all four individual isolates were highly virulent to lupine seedlings, regardless of their sensitivity to mefenoxam. Zoospores successfully infected and colonized leaves within 24 h. Water-soaked symptoms rapidly spread from the inoculation site to the entire leaf and caused the leaf to wilt. In most cases, the pathogen continued to spread into stem and caused the entire aboveground plant parts to wilt and die within 5 days of inoculation.

Resistant isolates caused a significantly higher percentage of wilted leaves and plants than sensitive ones (Table 5). Significant differences in percentage of wilted plants also existed between two resistant isolates, but not between sensitive isolates. Resistant isolates produced significantly more zoospores per plant than sensitive isolates: 3A4 produced zoospores 4.1 and 2.5 times greater than sensitive isolates 4J9 and 1E3, respectively; 31D7 produced zoospores 2.2 and 1.3 times greater than sensitive isolates 4J9 and 1E3 (Table 5).

In the competitiveness experiment, resistant isolates were stronger competitors than sensitive ones in all pairings, regardless of their initial ratios. Resistant isolates dominate in the populations after 3-6 cycles of infection and colonization on lupine seedlings in the absence of mefenoxam (Figure 5). The number of sporulation cycles required by a resistant isolate to dominate the population was correlated with its initial frequency in inoculum mixtures. Regardless of pairings, if starting with 80% of initial resistant subpopulations, only three sporulation cycles were required for resistant isolates to reach 100% in the composition of zoospore populations. If starting with 50% of initial resistant subpopulations, five sporulation cycles were required. If starting with 20% of initial resistant subpopulations, six sporulation cycles were required (Figure 5).
Table 5 Fitness component analysis of mefenoxam-resistant and sensitive isolates of *Phytophthora nicotianae* on *Lupinus* ‘Russel's Hybrids’ Hort. \(^v\)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Sensitivity(^w)</th>
<th>% wilted leaves(^x)</th>
<th>% wilted plants(^y)</th>
<th>Sporulation capability ((\times 10^3 \text{ zoospores plant}^{-1}))^(^z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>31D7</td>
<td>R</td>
<td>95.0 a</td>
<td>90.0 a</td>
<td>33.4 b</td>
</tr>
<tr>
<td>3A4</td>
<td>R</td>
<td>87.5 b</td>
<td>73.3 b</td>
<td>62.4 a</td>
</tr>
<tr>
<td>1E3</td>
<td>S</td>
<td>62.5 d</td>
<td>41.7 c</td>
<td>24.1 c</td>
</tr>
<tr>
<td>4J9</td>
<td>S</td>
<td>70.0 c</td>
<td>46.7 c</td>
<td>15.1 d</td>
</tr>
</tbody>
</table>

\(^v\) All fitness tests were conducted using 10-day-old seedlings of Lupine. A 10 µl droplet containing 36 zoospores was applied onto cotyledons of each plant with a total of 60 plants inoculated for each isolate. Each isolate was replicated six times.

\(^w\) Mefenoxam sensitivity; R= resistant if the mycelial growth of an isolate on CV8 medium amended with 100 µg/ml mefenoxam was ≥ 50% of that on the non-amended control, otherwise sensitive (=S).

\(^x\) % wilted leaves is the percentage of leaves exhibiting symptom of being fully infected and dehydrated. Figures followed by different letters are significantly different \((P=0.05)\) according to Duncan’s multiple range test.

\(^y\) % wilted plants is the percentage of plants with both wilted leaves.

\(^z\) Five wilted lupines per replicate were selected to evaluate sporulation capability in 5 ml of sterile soil water extract. The Number of zoospores was microscopically counted 8 times with the aid of a haemocytometer.
Figure 5 Changes in percentage of the mefenoxam-resistant subpopulation in three pairs of isolate mixtures (a) 31D7+1E3, (b) 31D7+4J9, and (c) 3A4+4J9. Three initial ratios, 1R:4S, 1R:1S, 4R:1S, for each pair were applied onto lupine seedlings. The second sporulation cycle was initiated with resultant zoospores from the initial infection and subsequent sporulation cycles were conducted accordingly. Each point represents mean of six replicates.
DISCUSSION

This study investigated mefenoxam sensitivity in 95 isolates of *P. nicotianae* and the relative fitness of resistant isolates to sensitive ones. Our results demonstrate that 26% of isolates were highly resistant to mefenoxam and those isolates were much fitter than sensitive ones. This higher fitness is due partially to higher infection rate and enhanced sporulation ability by resistant isolates. These findings have several significant practical implications.

Mefenoxam resistance in *P. nicotianae* is widespread on herbaceous annuals in some ornamental crop nurseries. This contention is supported by our data on resistance screening (Tables 1 and 3) and *in vivo* assays (Table 4). Similar evidence was also documented for other geographic locations within the USA (54, 80). The use of mefenoxam should be discontinued in those herbaceous annuals in nurseries where resistant isolates have been detected. All of the resistant isolates in those studies including this study have a similar magnitude of resistance to mefenoxam with a mean EC$_{50}$ value much greater than the label rate for Phytophthora disease control (0.078 µl mL$^{-1}$, equivalent to 1 fl. oz. 100 gal$^{-1}$). This high level of mefenoxam resistance may be attributed to the intensive repeated exposure of the pathogen to mefenoxam in nursery production. Indeed, the majority of commercial nurseries grow several crops of different types of herbaceous annuals year-round. For example, annual vinca and petunia are grown in spring and summer, followed by begonia and pansy in fall. Many production facilities grow several crops of each individual plant species within each season. These annuals are highly susceptible to *P. nicotianae* and the disease incidence on those crops is usually high. Several applications of mefenoxam for each crop are required to effectively manage *Phytophthora* diseases. The repeated
application of mefenoxam subjects the pathogen to high selection pressure for resistance. There are significant differences in mefenoxam sensitivity of *P. nicotianae* isolates among the nurseries surveyed here. Thus, it is necessary to determine the mefenoxam sensitivity of *P. nicotianae* isolates before mefenoxam applications.

Unlike the situation found with annual crops, mefenoxam resistance was not found in isolates obtained from perennials and woody ornamentals in nearly all nurseries sampled. This result indicates that mefenoxam remains an effective control measure for *Phytophthora* on perennials and woody ornamentals. However, sensitivity of the pathogen on those crops should be closely monitored because resistant strains may be spread from herbaceous annuals to other ornamentals by recycling irrigation water (20, 71). *P. nicotianae* mainly causes root and crown rots on woody ornamentals (11, 48). Due to lower disease pressures on those crops, fewer applications of mefenoxam are needed to achieve good *Phytophthora* disease control. This hypothesis may help explain why resistance development was much slower on perennials and woody ornamentals than on herbaceous annuals.

Recycling irrigation water is an efficient means of spreading *Phytophthora* from crop to crop, from a single site to an entire nursery or farm and from one geographic location to other locations using the same water systems (71, 94). In this study, 40% of irrigation water isolates from Virginia were resistant to mefenoxam. Those resistant isolates in irrigation water most likely originated from herbaceous annuals. Furthermore, irrigation water may be a place where selection for resistance to mefenoxam occurs. Many *Phytophthora* species can survive in aquatic environments for a long period of time (20, 22, 71, 118). Such aquatic adaptation allows for the pathogen to have a prolonged period
of exposure to sublethal doses of the fungicide present in irrigation water and therefore increases the likelihood of resistance selection (43, 134).

Mefenoxam-resistant isolates are much fitter than sensitive isolates and can compete successfully with their partner sensitive ones (Table 5). The resistant isolates which predominated in the population at the last cycle of infection had a sporulation capacity higher than their respective partners. Therefore, sporulation capacity appears to have a direct impact on the final outcome of competition. The fact that the resistance recovery rate in nursery A increased from 25% in 2001 to 83% in 2004 (Table 1) may be an indication of enhanced pathogenic fitness. Mefenoxam is effective for disease control only when the percentage of resistant populations remains low (146). Thus, it is essential to monitor the sensitivity of the pathogen population to this compound.

This study found that in vitro mycelial growth on propamocarb-amended agar medium could not accurately predict the in vivo sensitivity of P. nicotianae to the fungicide (75). However, the in vitro assay of mycelial growth is a good measure of sensitivity of the pathogen to mefenoxam, and is correlated with the responses of P. nicotianae isolates to mefenoxam on plants. Therefore, in vitro mycelium growth on media amended with mefenoxam is an accurate predictor for mefenoxam sensitivity of P. nicotianae in plants. This also is supported by previous observations made in P. capsici (101).

There appeared an association between mefenoxam resistance and mating type among the isolates of P. nicotianae assessed in this study. However, this may be a reflection that resistant isolates resulted from single clonages rather than that a true linkage exists between these two biological traits. In the present study, all of the resistant isolates were A2 and recovered from nurseries A, B, and C, which are located geographically close to
each other in southeastern Virginia. It is common that pathogens move from one nursery to another through infested plant materials (80, 114). Investigation into whether these resistant isolates really were from single clonages is an interesting subject for further study.
Chapter 3 Effects of Propamocarb Hydrochloride on Mycelial Growth, Sporulation, and Infection by Phytophthora nicotiana
Isolates from Virginia Nurseries


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ABSTRACT

Propamocarb hydrochloride is a systemic fungicide used for control of Phytophthora diseases of nursery crops. Here we report on the effect of this compound on different growth stages of Phytophthora nicotiana, a major pathogen of numerous herbaceous and some woody ornamental plants. A total of 71 isolates were assayed for sensitivity to propamocarb at two concentrations of 1.8 mg/ml (label rate) and 10 mg/ml using clarified V8 agar as a base medium. All isolates grew at 10 mg/ml with the most sensitive isolate having 34.8% relative growth compared to growth on non-amended medium. Nine isolates were selected and further tested for mycelial growth at 0, 1, 10, 25, 50 and 100 mg/ml, as well as for sporangium production, zoospore motility and germination at 0, 5, 50, 500, 5,000 and 50,000 µg/ml. EC₅₀ values ranged from 2.2 to 90.1 mg/ml for mycelial growth, 133.8 to 481.3 µg/ml for sporangium production, 88.1 to 249.8 µg/ml for zoospore motility and 1.9 to 184.6 µg/ml for zoospore germination, respectively. In addition, seventeen selected isolates were evaluated for propamocarb sensitivity on Pelargonium × hortorum cv. ‘White Orbit’. Two days after seedlings were treated with propamocarb at 3.6 mg/ml,
they were inoculated by either inverting one 5-mm-diameter plug from a 3-day-old culture or applying a 10-µl drop containing 20 zoospores onto each cotyledon. Propamocarb hydrochloride provided good protection of geranium seedlings from zoospore infections but not from mycelial infections. These results suggest that this fungicide must be used preventively for Phytophthora disease management and that mycelial growth may not be the most reliable measurement to determine the development of fungicide resistance to this compound in *Phytophthora* species at production facilities and in the landscape.

Additional Keywords: fungicide resistance, IPM

INTRODUCTION

*Phytophthora* species are a group of common and destructive pathogens of a large number of agriculturally and ecologically important plants (48, 51). They cause rots of roots, crown, stems, leaves, and fruits and often result in severe plant losses. This is particularly significant for nursery crops (69, 70, 72, 80) because many ornamental plant species are grown close together and each may host several *Phytophthora* species. For example, rhododendron is susceptible to at least 17 different species of *Phytophthora* (66, 73, 130, 142, 149). *Phytophthora* species may produce chlamydospores that allow survival under harsh environments for a long period of time. They also produce flagellate zoospores that disseminate through irrigation systems (71). In addition, they can be easily spread to other production facilities and landscapes through movement of contaminated nursery stock (96). Thus, it is difficult to keep Phytophthora diseases under control once plants and/or growing media are contaminated within a production facility or landscape.
Chemical control is a current approach for management of Phytophthora diseases of nursery crops. Available compounds include mefenoxam, propamocarb hydrochloride (hereafter referred to as propamocarb), fosetyl aluminum, and etridiazole. Each of these fungicides has a unique mode of action and supposedly has good protective and curative activity. However, mefenoxam resistance has been documented in several Phytophthora species at nurseries in California (53) and North Carolina (80). Resistance to fosetyl-Al also was found in Bremia lactucae Regel, a close relative of Phytophthora species (15). There is a growing concern over whether Phytophthora species at nurseries and landscapes have developed resistance to other major compounds. Investigation into this question is of critical importance to formulate effective disease and fungicide resistance management programs locally and nationally.

Propamocarb was first introduced into European markets for control of oomycete pathogens in ornamental crops and certain vegetables in 1978 (121). It has since been widely used as a soil drench against Phytophthora and Pythium diseases of numerous crops (31, 47, 52, 121, 123, 124). Specifically, it has been used successfully to control potato late blight where metalaxyl-resistant populations presented an increasingly serious problem (5, 6). It has been speculated that the mode of action of propamocarb is through selective interference with the biosynthesis of oomycete membranes (119), although this has not been proven. Due to its lack of adverse effects on beneficial microorganisms such as mycorrhizae and Trichoderma species, propamocarb has been considered a good component of IPM programs (109, 151).

Propamocarb sensitivity has been assessed on a variety of Phytophthora species at several stages of their life cycles. It varied among species of Phytophthora and among
isolates of the same species (123). It also differed with growth stages of individual isolates. For example, EC$_{50}$ values for mycelial growth was 29.3 µg/ml for *P. cactorum* (Lebert & Cohn) J. Schrot. (144), 111 µg/ml for *P. palmivora* (E. J. Butler) E. J. Butler (143), >1000 µg/ml for *P. drechsleri* Tucker (26) and *P. nicotianae* Breda de Haan (syn: *Phytophthora parasitica* Dastur) (63), and 6,246 µg/ml for *P. infestans* (Mont.) de Bary (125). The EC$_{50}$ values for sporangium production had a similar magnitude of variation (25 to 4,000 µg/ml) among *P. infestans* (125), *P. palmivora* (24, 143), and *P. nicotianae* (63). Oospore formation by *P. infestans* was found to be very sensitive to propamocarb with complete inhibition at 100 µg/ml (3, 125).

In spite of varying in vitro activity, propamocarb often provided considerable protection of plants from infection by *Phytophthora* spp. For example, this fungicide provided good control of pepper blight caused by *P. capsici* Leonian (151), potato late blight by *P. infestans* (5, 6, 85, 125, 128), tobacco black shank by *P. parasitica* var. *nicotianae* (111, 124), and cucumber damping-off by *P. drechsleri* (26). This compound is used by the turf and ornamental industries in Pennsylvania and Virginia for control of oomycete diseases (68, 114), especially where mefenoxam resistance increasingly has become problematic (53, 54, 78, 80). Growers need to know how effective propamocarb is for control of Phytophthora diseases and whether similar fungicide resistance problems have arisen with this compound. However, little is known about propamocarb sensitivity of *Phytophthora* species in ornamental plants in the United States.

This study used *P. nicotianae* as a model system to determine its propamocarb sensitivity in media and geranium seedlings. *P. nicotianae* is one of the most common and damaging Phytophthora pathogens in numerous herbaceous annuals and perennials as well
as some woody ornamental plants (48, 51). We have frequently isolated this pathogen from an array of diseased nursery crops (69, 70, 72) and in irrigation water over the past 6 years (19, 21), and have accumulated a number of isolates. The objective of this study was to assess these isolates for their sensitivity to propamocarb to fill a major knowledge gap in fungicide resistance. In addition, we examined several growth stages in the life cycle of *P. nicotianae* and infection and colonization of geranium seedlings by two types of inoculum of selected isolates in an attempt to understand the inconsistencies between *in vitro* and *in vivo* efficacy data in the literature and to evaluate the reliability of using mycelial growth alone for propamocarb resistance screening.

**MATERIALS AND METHODS**

**Isolate origin and identity confirmation**

A total of 71 isolates of *P. nicotianae* were included in this study. The majority of these isolates were recovered from plant tissues of diverse nursery crops and irrigation water in Virginia. Several isolates from other crops and locations, kindly provided by Drs. James Adaskaveg (University of California, Riverside), Mannon Gallegly (West Virginia University), David Shew (North Carolina State University), and Sharon von Broembsen (Oklahoma State University) were included for comparison. Most isolates were recovered since 2000; however, two isolates (23A8 and 23A9) were recovered in 1968, before propamocarb was marketed for use on ornamental crops. The identity of all isolates was verified using a single-strand conformation polymorphism analysis of the ribosomal DNA internal transcribed spacer 1 (96).
Population sensitivity

Propamocarb (propyl [3-(dimethylamino) propyl] carbamate monohydrochloride; Bayer Environmental Science, Research Triangle Park, NC) was assessed at concentrations of 1.8 mg/ml (equivalent to label rate: 3 fl. oz./10 gal) and 10 mg/ml. Clarified V8 (CV8) agar (pH=6.3) was used as a base medium. The fungicide was diluted in sterile distilled water then added to the autoclaved medium at 50°C. Mycelial plugs (5-mm diameter) were cut from the margin of 4- to 5-day-old CV8 agar cultures. One plug was placed in the center of a 9-cm Petri dish with the mycelia in contact with the medium. Three replicate dishes per treatment were inoculated for each isolate. The inoculated dishes were then incubated at 23–26°C for 5 to 7 days in darkness. When colonies on non-amended control dishes had reached the edge, colony diameters were measured in two perpendicular directions for all treatments. The diameter of the mycelium plug was subtracted; the two measurements were averaged. Relative growth of an isolate on amended media was calculated by dividing colony diameter in amended dishes by that in the non-amended control dishes, and expressed as a percentage.

Effective concentration of propamocarb against mycelial growth

A subset of 9 isolates representing different levels of sensitivity to propamocarb based on the sensitivity assays described above was selected to determine their EC$_{50}$ values. These isolates were assessed using the same medium amended with propamocarb at 1, 10, 25, 50, and 100 mg/ml plus a non-treated control. Three replicate Petri dishes were used for each treatment. The dishes were inoculated, incubated and colony growth was determined as described above. The data for percentage inhibition of mycelial growth was transformed to probits based on a normal distribution.
Effective concentration against sporangium production

The same 9 isolates were tested at five concentrations of 5, 50, 500, 5,000 and 50,000 µg/ml plus a non-treated control. Sterile soil water extract (SSWE) was used as a medium to induce sporangia formation (48). SSWE was prepared by mixing 10 g (fresh weight) of a sandy loam soil in 1 liter of distilled water on a magnetic stirrer overnight at room temperature (23~25°C), settling for 8 h then filtering through a Whatman paper (#1). Propamocarb was added to SSWE at the designated concentrations. Four mycelial disks (5 mm diameter) were removed from the edge of 5-day-old CV8 agar cultures and placed in Petri dishes containing 8 ml of SSWE without or with propamocarb at a designated concentration. Three replicate dishes were used for each concentration per isolate. After incubation under florescent light for 10 h at 23~26°C, SSWE was removed and replaced with 5 ml of 10% bleach solution to stop formation of new sporangia. The number of sporangia along the margins of each mycelial disk was counted under a compound microscope. The percentage reduction in sporangium production was computed by dividing the average number of sporangia in a treatment by the corresponding number in the non-treated control.

Effective concentration against zoospore motility and germination

Sporangia were produced in SSWE as described above and zoospore release was triggered by replacing with pre-chilled sterile distilled water (at 4°C) and then warming for 20 min at room temperature. Zoospore concentrations were adjusted to about 1,800/ml. To assess zoospore motility, 5 ml of an adjusted zoospore suspension was added to each of three replicate Petri dishes containing equal volume of propamocarb solutions at 0, 10, 100, 1,000, 10,000, or 100,000 µg/ml to give a final concentrations of 0, 5, 50, 500, 5,000,
or 50,000 µg/ml. Zoospore suspensions were maintained at 25°C and observed microscopically to determine the time required for complete cessation of motility (zoospore movement time in minutes). Observations were made at 1-min intervals for the first 15 min, then at 15-min intervals for the next 45 min, and finally at 1-h intervals until no zoospore movement was detected. Relative motility was calculated by dividing zoospore movement time in a treatment by that in the non-treated control. To assess zoospore germination, 100 µl of an adjusted zoospore suspension was spread onto each of three replicate dishes of CV8 agar containing propamocarb at a concentration of 0, 5, 50, 500, 5,000, or 50,000 µg/ml. After a 2-day incubation at 25°C in the dark, the number of Phytophthora colonies appearing on each Petri dish was recorded. Relative germination was computed by dividing the number of colonies in a treatment by that in the non-treated control.

**In vivo efficacy tests**

Seventeen resistant isolates were further tested for propamocarb sensitivity using seedlings of geranium (*Pelargonium × hortorum* cv. ‘White Orbit’). Seedlings were treated with propamocarb then challenged with *P. nicotianae* as described by Moorman *et al.* (114) with some modifications. A plastic container (9 cm in diameter and 4.5 cm deep) was used to hold a smaller Petri dish (6 cm in diameter) with a filter paper (Whatman #4) on its top. Twenty-five ml of water-soluble fertilizer (N at 400 µg/ml; 20% N, 20% P₂O₅, 20% K₂O soluble fertilizer; Olympic Chemical Co., Mainland, PA) was added to each container to keep the filter paper moist. Five geranium seeds were placed on the filter paper. The containers were placed inside a tray sealed with a transparent plastic cover and incubated for 5 days in a growth chamber with florescent lights at 23~25°C. Once the
cotyledons were fully expanded, the fertilizer solution in each container was replaced with same amount of propamocarb solution at 3.6 mg/ml (twice the label rate). Two days after replacement, the plants were inoculated on both cotyledons with either mycelial plugs or with zoospores. Experiments with mycelial inoculum used 5 plants per replicate. Five-mm-diameter mycelial plugs were taken from the margin of a 3-day-old culture. One plug was placed on the upper surface in each leaf after seedlings were sprayed with SSWE to facilitate plug adhesion. The experiment with zoospore inoculum used 10 plants per replicate. Zoospore suspensions were prepared as described above. A 10-µl drop containing 20 zoospores was placed on the upper surface of each leaf. All inoculated plants were incubated in a moist chamber in the dark for 24 h and grown under fluorescent light at 23~25°C. Four to 6 days after inoculation, the number of seedlings with blackened stem, leaf necrosis or wilting appearance was counted.

**Experiment design and data analysis**

Each experiment described above was conducted twice on different dates. Treatments within each repeating experiment were arranged in a completely randomized design. Data from two experiments were pooled according to homogeneity of variance. Analysis of variance and multiple range tests were performed with SAS (Proc GLM, SAS Institute, Cary, NC). Linear regression lines of logit-transformed percentage against the log_{10} of the propamocarb concentration were fitted using Proc REG of SAS. The resulting equations were used to calculate EC_{50} values. The Mann-Whitney U test (157) was performed to determine the differences in mortality of geranium seedlings between the fungicide treatment and non-treated control for each isolate and inoculation experiment. Percent disease control was calculated by comparing the seedling mortality in propamocarb
treatment with that in non-treated control for individual isolates. Analysis of variance then was performed to determine the differences in disease control among the 17 isolates by type of inoculum.

RESULTS

Isolate identity confirmation

All isolates tested yielded ca. 300 bp amplicon and produced a SSCP banding pattern, characteristic of *P. nicotianae* on 8% polyacrylamide gels.

Population sensitivity

All isolates grew substantially in the presence of propamocarb at both concentrations (1.8 mg/ml and 10 mg/ml). Approximate 88% of isolates had over 50% relative growth of the non-amended controls at both concentrations. No isolates exhibited over 100% or less than 30% relative growth (Figure 6).

Effective concentration against mycelial growth

The relative sensitivity of five isolates of *P. nicotianae* to propamocarb was shown in (Figure 7). Estimated EC$_{50}$ values for nine isolates ranged from 2.2 to 90.1 mg/ml, depending on the host plant/substrate and geographic origin. Six isolates had EC$_{50}$ values between 2.2 and 10 mg/ml; one isolate had EC$_{50}$ values between 10 and 30 mg/ml (Table 6). Two isolates had EC$_{50}$ values of over 30 mg/ml; these two isolates were recovered from boxwood and nursery irrigation water. The overall mean of EC$_{50}$ value was 20.6 mg/ml.
Figure 6. Distribution of 71 isolates of *Phytophthora nicotianae* in response to 1.8 mg/ml and 10 mg/ml of propamocarb incorporated into 20% clarified V8 agar
Table 6. Effective concentration (EC$_{50}$) of propamocarb for 50% reduction in mycelial growth, sporangium production, zoospore germination, and duration of zoospore motility of *Phytophthora nicotianae* isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host/substrate</th>
<th>Geographic origin</th>
<th>Year</th>
<th>EC$_{50}$ (µg/ml)</th>
<th>Mycelial growth</th>
<th>Sporangium production</th>
<th>Zoospore germination</th>
<th>Zoospore motility</th>
</tr>
</thead>
<tbody>
<tr>
<td>25J8</td>
<td>Irrigation water</td>
<td>Virginia</td>
<td>2001</td>
<td>90,100</td>
<td>481.3</td>
<td>184.6</td>
<td>249.8</td>
<td></td>
</tr>
<tr>
<td>16C8</td>
<td>Irrigation water</td>
<td>Virginia</td>
<td>2000</td>
<td>3,500</td>
<td>218.2</td>
<td>1.9</td>
<td>88.1</td>
<td></td>
</tr>
<tr>
<td>1E3</td>
<td>Irrigation water</td>
<td>Oklahoma</td>
<td>...</td>
<td>3,200</td>
<td>198.4</td>
<td>1.9</td>
<td>104.5</td>
<td></td>
</tr>
<tr>
<td>2C10</td>
<td><em>Buxus sempervirens</em></td>
<td>Virginia</td>
<td>2000</td>
<td>80,100</td>
<td>442.1</td>
<td>131.4</td>
<td>215.6</td>
<td></td>
</tr>
<tr>
<td>31A1</td>
<td><em>Petunia sp.</em></td>
<td>Virginia</td>
<td>2004</td>
<td>5,500</td>
<td>204.2</td>
<td>6.9</td>
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<tr>
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<td><em>Petunia sp.</em></td>
<td>Virginia</td>
<td>2004</td>
<td>10,100</td>
<td>217.2</td>
<td>4.1</td>
<td>117.7</td>
<td></td>
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<tr>
<td>4J9</td>
<td><em>Daphne sp.</em></td>
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<td>5,400</td>
<td>133.8</td>
<td>8.8</td>
<td>100.7</td>
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<td>28B5</td>
<td><em>Lavandula angustifolia</em></td>
<td>Virginia</td>
<td>2002</td>
<td>6,900</td>
<td>200.6</td>
<td>39.1</td>
<td>134.1</td>
<td></td>
</tr>
<tr>
<td>26E7</td>
<td><em>Nicotiana tabacum</em></td>
<td>North Carolina</td>
<td>2003</td>
<td>2,200</td>
<td>162.0</td>
<td>30.8</td>
<td>98.8</td>
<td></td>
</tr>
</tbody>
</table>

$^{y}$ EC$_{50}$ values were estimated by fitting a regression line of the logit-transformed percent inhibition against the log-transformed concentration of propamocarb. These values are mean of six replicates from two repeating tests.

$^{z}$ unknown
Figure 7. Impact of propamocarb dose on mycelial growth (A) and sporangia production (B) of five representative isolates of *Phytophthora nicotianae*, expressed as relative growth compared to that of the control medium. Clarified V8 agar was used as a base medium for mycelial growth and sterile soil water extract for sporangium production assays. Isolate 26E7 was recovered from *Nicotiana tabacum* in North Carolina in 2003, 16C8 and 25J8 from irrigation water in central and southwestern Virginia (2000), 2C10 from *Buxus sempervirens* in central Virginia (2000), and 49J9 from *Daphne* sp. in eastern Virginia (2001). Each number is a mean of six replicates from two experiments.
Effective concentration against sporangium production

Nine isolates in non-treated control Petri dishes produced an average of 82 sporangia/agar disk (along the perimeter) after 10-h incubation in SSWE under florescent light. All isolates exhibited a similar pattern in response to propamocarb. Over 60% reduction in sporangium production was observed at 500 µg/ml for seven isolates and at 5 mg/ml for the two remaining isolates (Figure 7). No sporangia production was seen at 50,000 µg/ml. Little inhibitory effect on sporangium production was observed at 50 µg/ml or less. The EC$_{50}$ values of this compound against sporangium production by 9 isolates ranged from 133.8 to 481.3 µg/ml with a mean of 250.9 µg/ml (Table 6).

Effective concentration against zoospore motility and germination

All isolates responded to propamocarb similarly in terms of zoospore motility and germination (Figure 8). Zoospores retained their motility in SSWE for an average of 401 min in the absence of this fungicide. EC$_{50}$ values for reduction of zoospore motility ranged from 88.1 to 249.8 µg/ml among the nine isolates (Table 6). All isolates completely lost their motility within 10 min at a concentration of 5,000 µg/ml or above. Germination of zoospores was more sensitive to propamocarb than motility. On average, 32 Phytophthora colonies were recovered from the control Petri dish (without propamocarb). The EC$_{50}$ values for zoospore germination ranged from 1.9 to 184.6 µg/ml. Less than 20% of zoospores germinated at 500 µg/ml.
Figure 8. Impact of propamocarb dose on zoospore motility (A) and zoospore germination (B) of six isolates of *Phytophthora nicotianae*, expressed as relative zoospore motility or germination in comparison with that in the non-treated control. Sterile soil water extract was used to induce sporangium production then replaced with pre-chilled SSWE at 4°C to trigger zoospore release. Zoospore motility was determined by microscopic examination after mixing equal amounts of spore suspension and propamocarb solution and measured in period of time (min) required for complete cessation of all spores. Zoospore germination was determined by spreading aliquots of spore suspension in Petri dishes with clarified V8 agar amended with propamocarb then counting the emerging Phytophthora colonies. Isolate 25J8 and 16C8 were recovered in 2000 from irrigation water in southwestern and central Virginia, respectively, 2C10 from *Buxus sempervirens* in central Virginia (2000), 31A1 from *Petunia* sp. from eastern Virginia (2004), 26E7 from *Nicotiana tabacum* in North Carolina (2003), and 4J9 from *Daphne* sp. in eastern Virginia (2001). Each number is a mean of six replicates from two experiments.
**In vivo efficacy tests**

When the plants were inoculated with mycelial plugs, colonization first took place at the inoculation site then rapidly spread either up to the growing tip or down the stem, causing stem rot (blackened stem). Eventually the plants collapsed. The majority of isolates infected, colonized, and killed 80% or more of the geranium seedlings that had been pre-treated with propamocarb as well as the control plants (Table 7). When inoculated with zoospores, geranium seedlings developed similar symptoms but to a lesser degree. Hypersensitive lesions occasionally occurred in some inoculated leaves. Generally, longer periods of time were needed for symptom development and mortality was lower for seedlings challenged with zoospore inoculum than with mycelial plugs. The average mortality rate caused by zoospore inoculum was less than 50% in control plants. Propamocarb exhibited 60-100% protection of geranium seedlings against zoospore infections, depending on isolates (Table 7). There were significant differences in propamocarb protection of geranium seedlings from zoospore infection ($P<0.001$) but not from mycelial infection ($P=0.3239$) among the isolates tested.
Table 7. Efficacy of propamocarb in controlling mortality of *Pelargonium × hortorum* cv. ‘White Orbit’ seedlings caused by *Phytophthora nicotianae*

<table>
<thead>
<tr>
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<th>Mycelial plug</th>
<th>Zoospore</th>
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$x$ Symptoms began at the inoculation points with necrosis, then spread down to cause blackened stems and seedling collapse, or up to cause leaf curling and plant wilting. The number of infected plants is the mean value of 6 replicates from two repeating tests. The total number of test plants per replicate was 5 for inoculation tests with mycelial plugs and 10 for those with zoospores.

$y$ Seedlings treated with propamocarb at 3.6 mg/ml (equivalent to 6 fl. oz./10 gal, double the labeled rate) in water-soluble fertilizer

$z$ Mann-Whitney $U$ test was performed to determine the differences in mortality of geranium seedlings between the propamocarb treatment and non-treated control.
DISCUSSION

This study assessed the propamocarb sensitivity in 71 isolates of *P. nicotianae* at four different growth stages and its efficacy for reducing geranium mortality caused by two types of inoculum from the 17 selected isolates. This fungicide provided little or no inhibition of mycelial growth nor any control of mycelial infection. But it consistently suppressed all other growth stages assessed *in vitro* and reduced zoospore infection. These results have several significant practical implications.

Propamocarb remains an effective option for Phytophthora disease in a fungicide resistance management program but it must be used preventively and with extra caution. Contaminated irrigation water is a primary source of inoculum for Phytophthora diseases in nursery production (70, 71) where zoospores are the principal propagules (122, 137, 145). The fact that propamocarb effectively reduced seedling mortality by zoospore infection (Table 7) suggests that this fungicide can provide good protection of nursery crops if applied to plants prior to arrival of zoospore inoculum. This compound also can slow the disease progression by inhibiting sporangium production and zoospore motility (Table 6). Sporulation, zoospore motility, zoospore germination and infection all are important steps in the disease cycle. They are particularly significant for disease development of polycyclic pathogens like *P. nicotianae* by fueling secondary inoculum buildup. It must be pointed out that propamocarb had little effect on mycelial growth and infection (Tables 6 and 7). We performed additional *in vitro* assays with 10 other isolates and demonstrated that their mycelial growth had similar levels of propamocarb sensitivity. For example, isolates 23A8 and 23A9 had EC$_{50}$ values of 4.7 and 5.2 mg/ml. These observations suggest that once infection occurs, this fungicide may not prevent local
disease spread by mycelia. They also may be indicative that fungicide resistance to this compound in *P. nicotianae* is on the rise. Thus, repeated use of propamocarb should be avoided and resistance to this compound should be closely monitored.

Our results suggest that the inconsistency between *in vitro* and *in vivo* propamocarb efficacy data in the literature is a reflection of different growth stages differentially responding to this compound. Most previous *in vitro* assays were done with mycelial growth, suggesting insensitivity to this compound (109, 114, 156). In contrast, the vast majority of documented *in vivo* data were obtained from field trials (3, 47, 124), where zoospores were likely the major type of primary and secondary inoculum. As a result, this fungicide provided good protection of crops in field trials. Our contention is supported by the data of Reiter et al. (125) on the propamocarb sensitivity of mycelial growth vs. sporangium production, oospore formation and infection of tomato by *P. infestans*.

*In vitro* mycelium growth is a standard measurement for resistance screening to various fungicides including propamocarb (5, 31, 53, 63, 80, 100, 114, 144, 146). The contrasting efficacy data between mycelium vs. other growth stages in this study (Figures 6, 7 and 8) along with those in the literature suggest that this “standard” measurement is not the best one for screening of propamocarb resistance in *Phytophthora* species. An *in vitro* assay with mycelial growth to determine the status of propamocarb resistance is easy and fast, but solely relying on this measurement may overestimate the threat of resistance. In this case, additional growth stages must be tested in order to reliably determine whether isolates have developed resistance to this compound. An alternative approach would be to use a more sensitive growth stage such as zoospore germination (Table 6, Figure 8) in propamocarb resistance screening programs. This is because the objective of disease
control is to break the disease cycle rather than to control every step in the cycle. A long-
term solution would be to identify DNA markers tied to \textit{in vivo} propamocarb resistance.

Propamocarb sensitivity is a multigenic trait in \textit{P. nicotianae}. This hypothesis is
supported by both distinct sensitivities among the growth stages (Tables 6 and 7) and a
continuous distribution of mycelial growth among isolates assessed in the present study
(Figure 6). It was not clear whether propamocarb insensitivity of mycelial growth and
infection is a result of natural variation or selection pressure due to use of the fungicide
(114). The fact that the EC$_{50}$ values of isolates 23A8 and 23A9, recovered back in 1968,
were nearly three times the label rate points to the first possibility. However, there were
variations in propamocarb sensitivity among isolates from different nurseries and even
among those from the same nurseries. For example, three irrigation water isolates from one
nursery in southwestern Virginia had higher EC$_{50}$ values (ranging from 13.7 to 90.1
mg/ml) than those from other nurseries in central and eastern Virginia. The originating
nursery of these three isolates did not appear to have used propamocarb more frequently
than other locations; but we cannot be certain whether the three isolates had more exposure
to this compound as resistant isolates could have been brought into this production facility
in contaminated soil or on infected plants. Propamocarb sensitivity had no clear association
with mating type (data not shown). The genetic details of propamocarb ineffectiveness
against mycelial growth and infection process are yet to be determined. Identification of
linked molecular markers and quantitative trait loci are warranted to determine the number
of genes responsible for propamocarb sensitivity as well as the degree of contribution of
individual genes.
Chapter 4 Mefenoxam Sensitivity and Resistance Development Potential in *Phytophthora cinnamomi*

**ABSTRACT**

*Phytophthora cinnamomi* Rands is a destructive root rot pathogen of a wide range of ornamental plants. Mefenoxam has been extensively used to control root rotts caused by this pathogen. The first objective of this study was to investigate whether isolates of *Phytophthora cinnamomi* from nurseries have developed resistance to mefenoxam. Sixty-five isolates were assessed using clarified V8 juice agar amended with mefenoxam at 100 µg/ml. The mycelial growth of eight least sensitive isolates ranged from 10% to 18% compared to non-amended controls, while the majority of isolates were inhibited by over 90% in the presence of mefenoxam at 100 µg/ml. The EC$_{50}$ values of ten representative isolates narrowly ranged from 0.03 to 0.08 µg/ml. *In vivo* tests with seedlings of *Lupinus* ‘Russell Hybrids’ demonstrated that even isolates with the highest EC$_{50}$ values were not able to overcome the protection of plants provided by mefenoxam at the labeled rate. These results suggest that *P. cinnamomi* has not yet developed resistance to mefenoxam and remains highly sensitive to the compound. The second objective of this study is to test a hypothesis that repeated exposure to mefenoxam would result in reduced sensitivity. Mycelial adaptation experiments were conducted by weekly transferring mycelial plugs to agar medium amended with mefenoxam for 10 weeks. While significant changes in sensitivity to mefenoxam were observed in 3 of 5 tested isolates, resistance factors were relatively small (i.e. less than 5). This result indicated that the potential to increase resistance after repeated exposures is relatively low.
Additional Keywords: fungicide resistance, IPM, selection for resistance, mycelial adaptation.

INTRODUCTION

*Phytophthora cinnamomi* Rands is a destructive root rot pathogen that attacks a broad range of plant species worldwide (48, 64, 154). This species causes extensive economic losses to numerous shrubs and woody ornamentals including rhododendron, Fraser fir and camellia in USA (11, 97) and poses a major threat to natural ecosystems around the world (48, 64). The capability of *P. cinnamomi* to survive as chlamydospores in both soil and roots of symptomless plants makes this species extremely difficult to manage. The spread of *P. cinnamomi* in nursery production facilities is through contaminated irrigation water and nursery stock. Together, these characteristics place additional demands on fungicides to keep this destructive pathogen under control.

The principal approach for managing Phytophthora diseases on nursery crops is through the use of fungicides. Systemic mefenoxam and fosetyl aluminum are the two most effective fungicides against *P. cinnamomi*, while several new compounds have been developed for control of Oomycete pathogens (48, 106, 152). Fosetyl aluminum inhibits pathogen growth by inducing plant resistance (81) and has been widely used in natural ecosystems particularly to control *P. cinnamomi* (65). Mefenoxam blocks ribosomal RNA synthesis by affecting the RNA polymerase (40, 41, 131) and has been extensively used on both agricultural and ornamental crops for control of all species within the genus *Phytophthora*. Mefenoxam is highly effective against *Phytophthora* both *in vitro* (16,
and in vivo (9, 54). Growers continue to rely on this compound, but mefenoxam resistance has now developed in several Phytophthora species.

Field resistance to mefenoxam has been detected in most Phytophthora species (53, 59, 80, 100, 146). However, to date, no incidence of resistance has been documented in P. cinnamomi, although it is a widespread and destructive species with a wide range of host plant species. Various laboratory studies assessing the risk of resistance development have shown that resistance can be readily induced in four Phytophthora species, including P. infestans (152), P. nicotianae (23, 152), P. citricola (86), and P. capsici (13, 18, 152). However, no study has been conducted to determine the likelihood of resistance development in P. cinnamomi. The objectives of this study were to determine (i) in vitro and in vivo sensitivity of P. cinnamomi isolates to mefenoxam, (ii) EC_{50} values range of those isolates, and (iii) the potential of resistance development through artificial adaptation on mefenoxam-amended medium and UV mutagenesis.

**MATERIALS AND METHODS**

**Isolate origin and identity confirmation**

A total of 65 P. cinnamomi isolates were included in this study. Thirty-nine isolates were collected from various ornamentals in Virginia nurseries with 22 isolates from inkberry, 4 from southern wax myrtle, 3 from Japanese barberry, 2 from northern barberry, 3 from mountain laurel, 2 from American chestnut, 1 from heliamphora, 1 from rhododendron, and 1 from viburnum, respectively (Table 8); eight isolates were recovered from West Virginia with 6 from fraser fir, 1 from rhododendron, and 1 from pine, respectively (Table 8); the remaining 18 isolates from other crops and geographic locations...
were also included for comparison. Most of those isolates were recovered since 2000. The isolates were first identified based on the morphology of clean cultures and thereafter verified using a single-strand conformation polymorphism analysis of the ribosomal DNA internal transcribed spacer 1 (95).
Table 8. Origin and percent growth of *Phytophthora cinnamomi* isolates on clarified V8 juice agar amended with mefenoxam at 100 µg/ml compared to non-amended controls

<table>
<thead>
<tr>
<th>Isolate</th>
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<th>Geographic origin</th>
<th>Year</th>
<th>% Growth</th>
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<td>China</td>
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2 Unknown
Mefenoxam sensitivity and EC\textsubscript{50}

Mefenoxam (Subdue MAXX; Syngenta, Greensboro, North Carolina, USA) was assessed at concentrations of 100 a.i. µg/ml. CV8 agar was used as a base medium. The fungicide was diluted in sterile distilled water then added to the autoclaved media at 50°C. Mycelial plugs (5-mm diameter) were cut from the margin of actively growing colonies of 5-day-old agar cultures. One plug was placed in the center of a 9-cm Petri dish with the mycelia in contact with the medium. Three replicate dishes per treatment were inoculated for each isolate. The inoculated dishes were then incubated at 23~25°C for 5 to 7 days in darkness. When colonies in non-amended control dishes had reached the edge, colony diameters were measured in two perpendicular directions for all treatments. The diameter of the mycelium plug was subtracted; the two measurements were averaged. The relative mycelial growth of an isolate on amended media was calculated by dividing colony diameter in amended dishes by that in the non-amended control dishes, and expressed as a percentage.

Four isolates which had the most mycelial growth in the presence of mefenoxam at 100 µg/ml and six isolates with the least growth were selected to estimate their EC\textsubscript{50} values (effective concentration that inhibited mycelial growth by 50%). These isolates were assessed using the same medium amended with mefenoxam at concentrations of 0.0, 0.1, 1, 10, and 100 µg/ml. Three replicate Petri dishes were used for each treatment. The dishes were inoculated, incubated and colony growth was determined as described above. The EC\textsubscript{50} value was estimated for each isolate from a regression of probits transformed from relative mycelial growth versus the logarithm of mefenoxam concentration.
In vivo tests with *Lupinus* ‘Russell Hybrids’

Twelve isolates were further tested for mefenoxam sensitivity using seedlings of lupine (*Lupinus* ‘Russell Hybrids’). Lupine is highly susceptible to a number of *Phytophthora* species including *P. cinnamomi* (28). Seedlings were treated with mefenoxam then challenged with *P. cinnamomi* as described previously (76). Lupine seeds were induced to germinate by soaking overnight in water and then placed on moistened paper towel with bottom heat of 35°C. Ten lupine seedlings were grown in vermiculite medium inside a plastic cup (15 cm ×10 cm) under white fluorescent light (10 h day/14 hr night) at 23~25°C. The plants were fertilized once a week with 50 ml of 1mg/ml general purpose fertilizer (20% N, 20% P₂O₅, and 20% K₂O; Scotts-Sierra Horticultural Products Co., Marysville, OH).

Seedlings with fully expanded cotyledons were treated by drenching with 25 ml of either mefenoxam at 0.078 µl/ml (equivalent to 1 fl. oz./100 gal) or distilled water 2 days prior to inoculations. Two days after treatment, both cotyledons of each seedling were inoculated with 5-mm mycelial discs. Mycelial discs were taken from the margin of a 3-day-old culture. One plug was placed on the upper surface of each cotyledon after seedlings were sprayed with sterile soil water extract (SWWE) to facilitate disc adhesion. All inoculated plants were incubated in a moist chamber in the dark for 24 h and then grown under fluorescent light at 23~25 °C. Four days after inoculation, the number of seedlings that had been infected or killed was counted. Each isolate was replicated with three cups containing 10 seedlings a piece. The experiment was conducted twice on different dates.
Selection of resistant mutants by either mycelial adaptation on mefenoxam-amended medium or UV mutagenesis

Five isolates of *P. cinnamomi* (28H5, 15D8, 30E5, 28D8, and 28E7) were selected to test the hypothesis that repeated exposure to fungicide would lead to the development of significant levels of resistance. A mycelial adaptation experiment was carried out by weekly transferring a 5-mm mycelial disc from the actively growing edge of the mycelial colony to 20% clarified V8 medium amended with mefenoxam. All five test isolates were initially transferred to the CV8 medium amended with mefenoxam at 0.1 µg/ml. At the subsequent transfers, mefenoxam concentration was gradually increased, e.g. to 0.5 µg/ml between the forth and seventh transfers, and then to 1µg/ml for the remaining three transfers. In order to test viability of mycelial growth, a similar disc was also transferred to 20% clarified V8 medium without mefenoxam. Each isolate was replicated with 5 Petri dishes. A total of 10 transfers were conducted for each isolate. At the end of final transfer, the EC$_{50}$ value was determined using the same procedure as described previously.

UV mutagenesis was conducted with a UV lamp (FG30T8, 10W, Japan) at a wavelength of 254 nm by irradiating a 3-day old culture grown on CV8 agar in open 90-mm-diameter Petri dishes for 20 min at a distance of 50 cm. After irradiation, 5-mm mycelial plugs were cut from the margins and inverted on 20% CV8 agar medium containing mefenoxam at 5 µg/ml. The Petri dishes were then incubated in the dark at 25 °C for 3 weeks. The resultant resistant mutants were subsequently maintained on CV8 agar medium containing mefenoxam at 5 µg/ml. The EC$_{50}$ values of all resistant mutants were determined using the same procedure as described above.
RESULTS

Isolate identity confirmation

All isolates tested yielded *ca.* 300 bp amplicon and produced a SSCP banding pattern, characteristic of *P. cinnamomi* on 8% polyacrylamide gels.

Mefenoxam sensitivity and EC\textsubscript{50}

Mycelial growth of 51 isolates was inhibited by an average of 95.3% at 100 µg mefenoxam/ml compared with the non-amended controls, while the other 14 isolates were completely inhibited (Table 8). Eight of 65 isolates grew on mefenoxam-amended medium with the relative growth ranging from 10% to 18% compared to non-amended controls. The level of sensitivity was unrelated to geographical location and host origin.

The relative sensitivity of five isolates to mefenoxam was shown in Figure 9. Average growth of all test isolates on amended media with 1µg mefenoxam/ml was inhibited more than 90% when compared with the non-amended control (Figure 9). Estimated EC\textsubscript{50} values ranged from 0.01 to 0.08 µg/ml and the mean EC\textsubscript{50} value was 0.04 µg/ml (Table 9). There were no large variations in sensitivity to mefenoxam among all test isolates.
Table 9. Effective concentration (EC\textsubscript{50}) of mefenoxam for ten isolates of \textit{Phytophthora cinnamomi} on clarified V8 agar

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host/substrate</th>
<th>Geographic origin</th>
<th>Year</th>
<th>EC\textsubscript{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28H5</td>
<td>\textit{Taxus} sp.</td>
<td>Massachusetts</td>
<td>1992</td>
<td>0.08</td>
</tr>
<tr>
<td>30E5</td>
<td>forest soil</td>
<td>China</td>
<td>2004</td>
<td>0.07</td>
</tr>
<tr>
<td>23B2</td>
<td>\textit{Persea americana}</td>
<td>Puerto Rico</td>
<td>1960</td>
<td>0.04</td>
</tr>
<tr>
<td>1C9</td>
<td>…</td>
<td>…</td>
<td>2000</td>
<td>0.04</td>
</tr>
<tr>
<td>15D8</td>
<td>\textit{Ginkgo biloba}</td>
<td>South Carolina</td>
<td>1997</td>
<td>0.03</td>
</tr>
<tr>
<td>31B9</td>
<td>\textit{Ilex glabra}</td>
<td>Virginia</td>
<td>2004</td>
<td>0.03</td>
</tr>
<tr>
<td>28E7</td>
<td>\textit{Castanea dentata}</td>
<td>Virginia</td>
<td>1990</td>
<td>0.02</td>
</tr>
<tr>
<td>1A3</td>
<td>\textit{Camellia japonica}</td>
<td>California</td>
<td>2000</td>
<td>0.02</td>
</tr>
<tr>
<td>32B3</td>
<td>\textit{Ilex glabra}</td>
<td>Virginia</td>
<td>2004</td>
<td>0.02</td>
</tr>
<tr>
<td>28D8</td>
<td>\textit{Pinus} sp.</td>
<td>West Virginia</td>
<td>2003</td>
<td>0.01</td>
</tr>
</tbody>
</table>

\*Unknown

Figure 9. Dose-response curves for the mycelial growth on clarified V8 agar of five isolates of \textit{P. cinnamomi}. The mycelial growth was expressed as percent growth on mefenoxam-amended medium compared to that of the control medium. Each number is a mean value of six replicates from two experiments.
**In vivo tests with Lupinus ‘Russell Hybrids’**

All test isolates were highly pathogenic and virulent to lupine seedlings. Three days after inoculation, water-soaked lesions occurred at inoculation sites, then aggressively spread to the stem and caused damping-off of the seedlings within two days. Eventually, the whole plant collapsed. All twelve isolates consistently colonized and killed over 92% of non-treated control lupine seedlings (Table 10). No test isolates were able to overcome the protection provided by mefenoxam at the label rate. While the majority of mefenoxam-treated seedlings that were inoculated with *P. cinnamomi* isolates had no visible disease symptoms on both cotyledons, one of seedlings per isolate occasionally was infected, colonized, and exhibited the water-soaked lesions on their cotyledons. There were significant differences in the ability of those isolates to infect non-treated control and mefenoxam-treated plants (Table 10).

**Selection of resistant mutants by either mycelial adaptation on mefenoxam-amended medium or UV mutagenesis**

Three isolates (i.e. 15D8, 28H5, and 28D8) exhibited significant changes in sensitivity to mefenoxam after continuous exposure to the fungicide for 10 weeks (Table 11). However, relatively little shift in sensitivity was detected in isolates 28E7 and 30E5. In the absence of mefenoxam, the mycelial growth rate of each mutant derived from the same parental isolate appeared to be negatively correlated to resistance levels (Figure 10), but with a positive correlation pattern in the presence of mefenoxam (Figure 10). There were no changes in mycelial growth rate during continuous transfers on CV8 medium without mefenoxam, indicating no vitality loss (Data not shown). The levels of resistance in UV-induced mutants were similar to those of the adaptation mutants (Table 11).
Table 10. *In vivo* mefenoxam sensitivity test of *Phytophthora cinnamomi* isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Growth (%) (x)</th>
<th>Mean number of infected plants (y)</th>
<th>Non-treated</th>
<th>Mefenoxam-treated (z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30E5</td>
<td>15.6</td>
<td>9.7 (a)</td>
<td>0.3 (b)</td>
<td></td>
</tr>
<tr>
<td>15D8</td>
<td>14.7</td>
<td>10.0 (a)</td>
<td>0.5 (b)</td>
<td></td>
</tr>
<tr>
<td>28E7</td>
<td>12.2</td>
<td>9.2 (a)</td>
<td>0.5 (b)</td>
<td></td>
</tr>
<tr>
<td>31C1</td>
<td>9.0</td>
<td>10.0 (a)</td>
<td>0.7 (b)</td>
<td></td>
</tr>
<tr>
<td>28D8</td>
<td>8.8</td>
<td>9.5 (a)</td>
<td>0.7 (b)</td>
<td></td>
</tr>
<tr>
<td>31B8</td>
<td>8.8</td>
<td>10.0 (a)</td>
<td>0.6 (b)</td>
<td></td>
</tr>
<tr>
<td>31H2</td>
<td>4.4</td>
<td>9.8 (a)</td>
<td>0.4 (b)</td>
<td></td>
</tr>
<tr>
<td>31H8</td>
<td>0.8</td>
<td>10.0 (a)</td>
<td>0.0 (b)</td>
<td></td>
</tr>
<tr>
<td>28H5</td>
<td>0.3</td>
<td>9.3 (a)</td>
<td>0.7 (b)</td>
<td></td>
</tr>
<tr>
<td>32D3</td>
<td>0.0</td>
<td>10.0 (a)</td>
<td>0.0 (b)</td>
<td></td>
</tr>
<tr>
<td>32B3</td>
<td>0.0</td>
<td>9.5 (a)</td>
<td>0.0 (b)</td>
<td></td>
</tr>
<tr>
<td>32C3</td>
<td>0.0</td>
<td>10.0 (a)</td>
<td>0.0 (b)</td>
<td></td>
</tr>
</tbody>
</table>

\(x\) Percent growth on 20% CV8 medium amended with mefenoxam at 100 µg/ml compared with that on non-amended control.

\(y\) Ten seedlings of *Lupinus ‘Russell Hybrids’* were grown in a plastic cup and inoculated by inverting a 5-mm plug of colonized CV8 agar onto the leaves of each seedling.

\(z\) Seedlings were treated with 25 ml of mefenoxam solutions per cup at labeled rate of 0.078 µl/ml. Figures within a row followed by different letters are significantly different (\(P=0.01\)) according to Duncan’s multiple range test.

Table 11 Effective concentration (EC\(_{50}\)) values of *P. cinnamomi* mutants generated by either mycelial adaptation on mefenoxam-amended CV8 medium or UV irradiation

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Treatment</th>
<th>EC(_{50}) values (z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15D8</td>
<td>Wild type</td>
<td>0.031 (a)</td>
</tr>
<tr>
<td></td>
<td>UV</td>
<td>0.041 (a)</td>
</tr>
<tr>
<td></td>
<td>Adaptation</td>
<td>0.153 (b)</td>
</tr>
<tr>
<td>28H5</td>
<td>Wild type</td>
<td>0.076 (a)</td>
</tr>
<tr>
<td></td>
<td>UV</td>
<td>0.158 (b)</td>
</tr>
<tr>
<td></td>
<td>Adaptation</td>
<td>0.178 (b)</td>
</tr>
<tr>
<td>30E5</td>
<td>Wild type</td>
<td>0.070 (a)</td>
</tr>
<tr>
<td></td>
<td>UV</td>
<td>0.110 (a)</td>
</tr>
<tr>
<td></td>
<td>Adaptation</td>
<td>0.083 (a)</td>
</tr>
<tr>
<td>28D8</td>
<td>Wild type</td>
<td>0.010 (a)</td>
</tr>
<tr>
<td></td>
<td>Adaptation</td>
<td>0.073 (b)</td>
</tr>
<tr>
<td>28E7</td>
<td>Wild type</td>
<td>0.018 (a)</td>
</tr>
<tr>
<td></td>
<td>Adaptation</td>
<td>0.018 (a)</td>
</tr>
</tbody>
</table>

\(z\) For each isolate, figures followed by the same letter within the column are not statistically different.
Figure 10. Relative mycelial growth on CV8 agar medium without or with mefenoxam at 1 µg/ml of individual isolates after they were subcultured weekly on the mefenoxam-amended medium for 2, 4, 6, 8, and 10 weeks. Each number is a mean value of six replicates from two experiments.
DISCUSSION

This study evaluated mefenoxam sensitivity in 65 isolates of *P. cinnamomi* and investigated the relative ability of resistance development after continuous exposure to the fungicide. Our results show that the oomycete *P. cinnamomi* has a relatively narrow range of sensitivities to mefenoxam with EC$_{50}$ values ranging from 0.01 to 0.08 µg/ml. This indicates that *P. cinnamomi* is still highly sensitive to mefenoxam. The mycelial adaptation on mefenoxam-amended medium experiments demonstrated that there is relatively low potential for those sensitive isolates to develop a high level of resistance even under repeated exposure to mefenoxam at a partial inhibitory dose. These findings have several significant practical implications.

One hypothesis that may help explain why resistance to mefenoxam has not yet been detected in *P. cinnamomi* is that mefenoxam resistance in this species carries a fitness cost by reducing the mycelial growth. The situation for other Oomycetes is entirely different. Occurrence of field mefenoxam-resistant isolates has been frequently detected in several *Phytophthora* species (37, 53, 61, 79, 84, 100, 146) as well as other Oomycetes (17, 35, 114, 126, 129). Moreover, enhanced fitness was found to be associated with those resistant isolates (33, 77, 90). However, there was a fitness cost associated with a higher level of resistance in *P. cinnamomi*. Our mycelial adaptation experiment demonstrated that the resistance level in *P. cinnamomi* increased with a longer time of exposure to mefenoxam. This higher resistance level exhibited as greater mycelial growth in the presence of mefenoxam (Figure 10). Mutations causing mefenoxam resistance have large negative side effects on mycelial growth rate in the absence of mefenoxam (Figure 10). This reduced mycelial growth of resistant mutants is likely to be caused by the development of
resistance, instead of decreased vitality due to continuous subculturing on CV8 medium, because there were no significant changes in mycelial growth after continuous transfers on agar without mefenoxam (data not shown). During ten continuous weekly transfers on mefenoxam-amended medium, no compensatory mutations occurred at a second site to reduce the resistance-associated fitness. The consequence of fitness costs imposed by development of resistance would be that resistant populations may be gradually replaced by sensitive populations when little selection pressure is presented. This implication has important applications in terms of resistance management. The best strategy to reduce resistant populations in fields would be to withdraw the use of mefenoxam from where moderately resistant strains were found and instead use other fungicides as alternatives. Further research on comparative competitiveness between resistant mutants and wild type sensitive isolates in mixed populations is needed to test this hypothesis.

The *P. cinnamomi* isolates tested in this study exhibited low variability in mefenoxam sensitivity, although its host range spectrum is quite broad (48, 64, 154). In the present study, Virginia isolates exhibited a similar range of sensitivity to mefenoxam as other isolates from several different host species originating from different geographical locations spread over three continents (Table 8). The sensitivity of all test isolates fell within a narrow range with EC$_{50}$ values between 0.01 and 0.08 µg/ml (Table 9). *In vivo* tests with lupine demonstrated that those isolates were not able to overcome the protection provided by mefenoxam at the label rate (Table 10). Therefore, *P. cinnamomi* has not developed resistance to mefenoxam and remains highly sensitive to this chemical. Mefenoxam continues to be a valuable disease management tool for Phytophthora root rots. Several previous studies evaluating baseline sensitivity of *P. cinnamomi* isolates to
metalaxyl also reported a similar narrow range of EC$_{50}$ values between 0.07 to 0.14 µg/ml (9, 10, 29). One of those studies conducted before official release of metalaxyl into the commercial market detected an isolate of $P. cinnamomi$ whose EC$_{50}$ value was 0.11µg/ml (9). Years later, there was no directional shift in sensitivity to mefenoxam detected in this species (10). Such a low variability in sensitivity to mefenoxam detected in all those studies may reflect the natural sensitivity range of $P. cinnamomi$. Therefore, mefenoxam might result in less selection for resistance in $P. cinnamomi$ even though growers may be using mefenoxam two or three times a year for control of Phytophthora root rot in nursery productions.

We were not able to generate mutants of $P. cinnamomi$ with high levels of resistance to mefenoxam by either mycelial adaptation on mefenoxam-amended medium or UV irradiation. Instead, both approaches yielded some moderately resistance mutants (Table 1). A major effect chromosomal locus genetically confers mefenoxam resistance, but may be further modified by several additional minor effect loci (49, 87, 88, 102). While resistance to mefenoxam due to mutations in major effect locus may not occur readily in $P. cinnamomi$, the moderate resistance observed in this study could be due to possible alterations in those additional loci. Those additional loci may encode proteins or regulators responsible for detoxification and reduced uptake.
Chapter 5 Genetic Analysis of Resistance to Mefenoxam and Identification of DNA Markers Linked to Mefenoxam Resistance in Phytophthora nicotianae

ABSTRACT

Three crosses involving nursery isolates of *P. nicotianae* with differing mefenoxam sensitivity were established to study the inheritance of mefenoxam resistance. Mefenoxam sensitivity was determined by comparing the mycelial growth on mefenoxam-amended CV8 agar to that on non-amended agar. When both parents had the same phenotype (both were resistant or both were sensitive), all *F*₁ progeny had the parental phenotype and no segregation was observed for the major-effect gene. However, mefenoxam resistance appears to be affected by the segregation of minor-effect genes. When the cross involving one resistant and one sensitive parent, the *F*₁ progeny segregated for mefenoxam resistance in a ratio of 1:1 (resistant: sensitive), indicating that mefenoxam resistance is controlled by a single dominant gene. One RAPD marker linked to a resistant locus in the repulsion phase was obtained by bulked segregant analysis using RAPD markers and was converted to a sequence characterized amplified region marker (SCAR). The SCAR marker identified in this study was validated on 120 isolates of *P. nicotianae* isolates recovered from nurseries in Virginia.

INTRODUCTION

Phenylamide fungicides are ribosomal RNA synthesis inhibitors with specific target site in Oomycetes. Their mode of action is to interfere with the activity of the RNA polymerase
Mefenoxam is a commercially important fungicide of this group. This compound is widely used for control of root, crown, and fruit rots, and foliar diseases caused by *Phytophthora* spp. in a variety of crops. However, mefenoxam was predicted to have a relatively high risk for resistance development in foliar pathosystems (141, 146). Subsequently, the rapid development of resistance to mefenoxam has compromised the effectiveness of this compound in chemical management of those diseases (59). To date, there are numerous reports of mefenoxam resistance in Oomycete pathogens (17, 36, 37, 53, 61, 79, 84, 100, 114, 126, 146).

Genetic control of mefenoxam resistance varies among oomycete species. An incompletely dominant major locus confers resistance in the majority of oomycetes including *P. capsici* (100, 103), *P. erythroseptica* (1, 61), *P. infestans* (49, 87, 132, 133), *P. sojae* (12), *Albugo candida* (107), *Bremia lactucae* (36), and *Peronospora parasitica* (116). However, metalaxyl resistance in *P. nicotianae* (23, 25) was genetically controlled by a single dominant gene. In addition to a major gene effect, several minor-effect genes were speculated to be involved in conferring certain degrees of resistance to metalaxyl (49, 88, 102, 133).

DNA markers are useful for predicting resistance development and monitor the spread of resistant populations. Several linked RAPD markers have been identified for metalaxyl resistance in *P. infestans* (49, 87), but those RAPD markers have a problem with low reproducibility. In order to increase the reproducibility and reliability of PCR assays, specific primers called sequence characterized amplified regions (SCARs) need to be developed from the sequences of RAPD fragments. Furthermore, DNA markers tightly linked to the gene (<10 centimorgans) or the gene itself should be more practical for the
above uses. The objectives of this study were to: (i) characterize the inheritance of mefenoxam-resistance in *P. nicotianae*, (ii) identify the RAPD marker associated with the mefenoxam resistance gene in *P. nicotianae* by bulked segregant analysis, (iii) clone and sequence RAPD maker MR25J8, and (iv) convert the RAPD marker to a specific SCAR marker.

**MATERIALS AND METHODS**

**Isolate origins and single-zoospore cultures**

According to their geographic and phenotypic diversity, four mefenoxam-resistant and 4 sensitive isolates of *P. nicotianae* from various hosts/substrates were selected as parental isolates to investigate the inheritance pattern of the mefenoxam-resistance locus (Table 12). All 8 isolates were individually cultured on 20% CV8 media at 25°C in darkness. To establish single-zoospore derivatives, 5 discs (5 mm in diameter) of the 4 day-old agar culture were incubated with 8 ml of soil water extract (SSWE) overnight under fluorescent light at 25°C for sporangium production. The desired concentration of zoospores was obtained by flooding the plates with pre-chilled sterile distilled water (5°C). One ml of zoospore suspension was transferred into a 1.5 ml PCR tube. Prior to counting, zoospores were forced to encyst by agitating the tube for 45 seconds with a vortex mixer. The concentration of encyst suspension was adjusted to approximately 350 spores/ml with 0.1% agar solution. One hundred µl of the spore suspension was spread on PARP agar medium plates (4). Within 12-15 hours of incubation at 25°C in darkness, each plate was examined for the presence of germinating spores under a dissecting microscope. The bottom of plates containing single germinating spores (Figure 11) was circled with a sharp
marker (Sharpie) and the developing colony in those circles was immediately transferred to PARP agar. After incubation on PARP for 4 days, the single-zoospore isolates were transferred onto CV8 agar medium for routine maintenance. Approximately 2-5 single-zoospore derivatives were established for each isolate.

Table 12 Phenotypic characteristics of parental isolates used in crosses

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Geographic origin</th>
<th>Original host/substrate</th>
<th>Mating type &lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mefenoxam resistance &lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A4</td>
<td>Virginia</td>
<td>Irrigation water</td>
<td>A2</td>
<td>R</td>
</tr>
<tr>
<td>18C9</td>
<td>Virginia</td>
<td><em>Catharanthus roseus</em></td>
<td>A2</td>
<td>R</td>
</tr>
<tr>
<td>30J5</td>
<td>Virginia</td>
<td><em>Petunia</em> sp.</td>
<td>A2</td>
<td>R</td>
</tr>
<tr>
<td>31A8</td>
<td>Virginia</td>
<td><em>Petunia</em> sp.</td>
<td>A1</td>
<td>R</td>
</tr>
<tr>
<td>23C7</td>
<td>Virginia</td>
<td>Irrigation water</td>
<td>A1</td>
<td>S</td>
</tr>
<tr>
<td>4J9</td>
<td>Virginia</td>
<td><em>Daphne</em> sp.</td>
<td>A1</td>
<td>S</td>
</tr>
<tr>
<td>2C11</td>
<td>Virginia</td>
<td><em>Buxus sempervirens</em></td>
<td>A1</td>
<td>S</td>
</tr>
<tr>
<td>26E8</td>
<td>North Carolina</td>
<td><em>Nicotiana tabacum</em></td>
<td>A2</td>
<td>S</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by pairing with known tester isolates A1 and A2 on 20% CV8 agar; oospore production within 1 week of incubation.

<sup>b</sup> R=resistant; S=sensitive. Mefenoxam sensitivity was determined by growing isolates on 20% CV8 agar amended with mefenoxam at 100 µg/ml. An isolate was classified as resistant if its percent growth at 100 µg/ml was > 40% of non-amended control; as sensitive if percent growth < 40%.
Figure 11. A single zoospore of *P. nicotianae* isolate (31D7) germinating on PARP plates after a 12-hour incubation in darkness at 25°C

**Mefenoxam sensitivity assays and mating type determination**

Mating type and sensitivity to mefenoxam were characterized for all parental isolates and the *F*\textsubscript{1} progeny. Mating type was determined by pairing unknown isolates with testers (A1 and A2) on CV8 agar medium and noting the presence or absence of oospores at the interface of two colonies after a 2-week period of incubation at 25°C in the dark. The sensitivity test was conducted by placing mycelial plugs (5 mm diameter) on CV8 agar plates containing 0, 10, and 100µg a.i./ml of mefenoxam. Those inoculated plates were incubated at 25°C in the dark for 5-10 days. Radial growth of each colony was measured twice at perpendicular angles for each plate. Relative growth of an isolate on amended media was calculated by dividing the colony diameter in amended plates by that of the non-amended control plates. Isolates were classified as resistant if their percent growth of
the control at 100µg/ml was more than 40% or as sensitive if the percent growth of the control at 100µg/ml was less than 40%. Statistical analysis was performed using the SAS program (Proc GLM, SAS Institute, Cary, NC).

**In vitro establishment of sexual crosses**

*In vitro* crosses were carried out by pairing 2 isolates with different mating types on CV8 agar plates as described before. Each individual cross was repeated in 4 plates. A total of 15 crosses were made, 9 of which were performed between 3 mefenoxam-resistant and 3 mefenoxam-sensitive isolates; three of which were made among mefenoxam-resistant isolates; and the remaining 3 crosses were performed among mefenoxam-sensitive isolates. In order to stimulate maturation of oospores, all plates were incubated at 25°C in darkness for two weeks and then placed under florescent light for another 2 weeks (25). Oospores were harvested by removing the agar zone containing the oospores and homogenizing it in a cooled mixer at full speed for 5 min. The mixtures were filtered through a mesh (pore size 75 µm), and followed by overnight incubation of 1 ml filtrate with 1.7% Glucanex (Novo Nordisk, A/S, Bagsværd, Denmark). To remove the enzyme Glucanex, the samples were centrifuged three times with sterile water for 3 min each (3000 g). After each centrifugation the supernatant was removed and the pellet resuspended in 1 mL sterile water. Oospores were counted microscopically twice with a haemocytometer. One hundred µl of final preparations were pipetted onto 1.5% of water agar plates, and allowed to germinate under blue light (wavelength: 475 nm) at 25°C until germination was observed. Germinating oospores were counted microscopically after a incubation of 10 days. To establish $F_1$ progeny populations, germinating single oospores were transferred
individually to PARP. $F_1$ progeny isolates were established for 3 crosses and maintained routinely on CV8 agar medium at 25°C in the dark.

**Genomic DNA extraction**

Genomic DNA was extracted using Qiagen DNeasy Plant Mini Kits (Qiagen, Valencia CA) following the manufacturer’s instructions. Isolates were routinely maintained on solid CV8 agar medium at 25°C. For DNA isolation, isolates were grown in test tubes containing 10 ml of 20% CV8 liquid media at 25°C for 5-7 days. Mycelium was harvested by scraping from CV8 agar plugs, then rinsing with distilled water and filtering through microcloth. The mycelium was ground in liquid nitrogen and the resulting fine powder was incubated in 1.5-ml microcentrifuge tubes containing lysis buffer with RNAase. The remaining steps were carried out by following manufacturer’s instructions. For each sample, DNA concentration and purity were measured using a Beckman Coulter Du 800 spectrophotometer (Beckman Coulter Inc., CA, USA) at a wavelength of 260 nm versus 280 nm.

**Bulked segregant analysis**

Molecular markers putatively linked to a mefenoxam-resistance gene were identified by using bulked segregant analysis (BSA) (112). Resistant and sensitive DNA bulks were constructed by pooling equal amounts of DNA (0.5 µg) from randomly selected resistant and sensitive isolates in separate bulks, respectively. Since a resistant phenotype could be either homozygous or heterozygous for resistance, smaller bulks with genomic DNA from 5 individuals with extreme phenotypes were used. DNA samples of resistant isolates (18C7) and sensitive isolates (2C11) along with those two bulks were screened for polymorphism with RAPD and microsatellite markers.
RAPD and random amplified microsatellite (RAMS) screening

A total of 22 random 10-nucleotide primers (Operon Technologies Inc., Alameda, CA) were screened on the two bulks. Those decamer primers were selected based on their ability to detect polymorphisms in *P. nicotianae* isolates from tobacco (155) and demonstrated linkage to metalaxyl resistance in *P. infestans* (49, 87, 155). The PCR reactions were carried out in 25 µl volumes each containing 25 ng of genomic template DNA, 0.2 µM of the particular primer, 100 µM of each dNTP, 2.5 µl of 10X PCR buffer, 0.5 units of Taq™ DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) and 1.5 mM MgCl$_2$. PCR amplification was performed on a Master Cycler Gradient (Eppendorf, Germany) under the following conditions for RAPD analysis: initial denaturing at 94°C for 2 min; followed by 40 cycles of denaturing at 94°C for 30 s, annealing at 35°C for 30 s, extension at 72°C for 60 s, and final extension at 72°C for 2 min. RAMS PCR used the same cycling conditions as described above except annealing at 42°C. After amplification, 8 µl of amplification products of 25 µl reactions were electrophoresed on 1.5% agarose gels at 50 constant voltage for 3 hour in pre-chilled TBE buffer (Tris-borate 89 mM, 2mM EDTA, pH 8.0). The gel was stained in ethidium bromide (1ug/ml) for 10 min and visualized under UV light. Images were captured using the BioImaging & Chemi System (UVP Lab, Inc, Upland, CA).

One hundred random microsatellite primers available from University of British Columbia (primer set #9, http://www.michaelsmith.ubc.ca/services/NAPS/Primer_Sets/, Vancouver, Canada) were screened against two DNA bulks. The PCR reactions with the volume size of 25 µl were carried out according to recommended protocol from the manufacturer as follows: 20 ng of template DNA, 40 ng of primer, 2.5 µl of 10 × PCR
buffer, 2 µl of 2.5 mM dNTPs, 0.1 µl (5 U/µl) of Taq™ DNA polymerase ((TaKaRa Bio Inc., Shiga, Japan) and 13.4 µl of sterilized nanopure water. PCR was programmed with an initial denaturing step at 95°C for 2 min, followed by 35 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 10 min. Amplification products were separated on 1.5% agarose gel, stained with ethidium bromide and photographed on the BioImaging & Chemi System (UVP Lab, Inc, Upland, CA).

Development of SCAR marker

Polymorphic fragments amplified by closely linked RAPD markers were excised from the gel and purified using Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI). The purified DNA was cloned into the pGEM®-T Easy vector (Promega, Madison, WI). The resulting white colonies on X gal-IPTG-LA plates containing 50 µg/ml ampicillin were removed and grown in Luria broth and plasmid DNA was isolated using the QIAprep® Spin Miniprep kit (Qiagen, Valencia, CA). The cloned fragment was sequenced from both ends using universal primers at the DNA sequencing facility of VBI of Virginia Tech University.

Based on the sequence information, several pairs of oligonucleotide primers were designed and synthesized for specific amplification of the loci identified by each RAPD marker (Table 12). PCR amplification was performed in a total volume of 25 µl containing 20 ng of template DNA, 20 ng of each SCAR primer, 2.5 µl of 10 × PCR buffer, 2 µl of 2.5 mM dNTPs, 0.1 µl (5 U/µl) of Taq™ DNA polymerase ((TaKaRa Bio Inc., Shiga, Japan) and 13.4 µl of sterilized nanopure water. PCR was programmed with an initial denaturing step at 95°C for 2 min, followed by 35 cycles of 94°C for 1 min, 63.8°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 10 min. Amplification
products were separated on 1.5% agarose gel, stained with ethidium bromide and photographed on the BioImaging & Chemi System (UVP Lab, Inc, Upland, CA).

RESULTS

Oospore production and germination in $F_1$ progeny

All 16 crosses were successful and began producing oospores after 7 days of incubation in darkness. The number of oospores harvested varied greatly among crosses, ranging from $3.3 \times 10^4$ to $9.2 \times 10^5$ per plate (Table 13). The highest number of oospores was produced by cross 30J5×31C8, followed by 3A4×2C11 and 3A4×4J9. Oospores started germinating on water agar within 2 days under blue light, and continued to germinate for 2-3 weeks. Oospores germinated by formation of germ tubes and long branching hyphae without producing sporangia (Figure 12). Germination rate of oospores was variable among crosses, ranging from 9.1% to 32.1%.
Table 13 Oospore formation on 20% CV8 agar plates (after 4 weeks) and oospore germination on 1.5% water agar for crosses of *Phytophthora nicotianae* isolates

<table>
<thead>
<tr>
<th>Cross</th>
<th>Mating type and mefenoxam sensitivity</th>
<th>No. of oospores ($\times 10^3$) per plate</th>
<th>Germination of oospore (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A4×2C11</td>
<td>A2R×A1S</td>
<td>865 ± 17</td>
<td>25.5 ± 1.8</td>
</tr>
<tr>
<td>3A4×4J9</td>
<td>A2R×A1S</td>
<td>733 ± 10</td>
<td>24 ± 0.7</td>
</tr>
<tr>
<td>3A4×23C7</td>
<td>A2R×A1S</td>
<td>33 ± 2</td>
<td>21.1 ± 0.7</td>
</tr>
<tr>
<td>18C9×2C11</td>
<td>A2R×A1S</td>
<td>86 ± 3</td>
<td>14.3 ± 0.6</td>
</tr>
<tr>
<td>18C9×4J9</td>
<td>A2R×A1S</td>
<td>372 ± 28</td>
<td>9.1 ± 1.2</td>
</tr>
<tr>
<td>18C9×23C7</td>
<td>A2R×A1S</td>
<td>475 ± 13</td>
<td>12.9 ± 0.5</td>
</tr>
<tr>
<td>30J5×2C11</td>
<td>A2R×A1S</td>
<td>621 ± 13</td>
<td>24 ± 0.8</td>
</tr>
<tr>
<td>30J5×4J9</td>
<td>A2R×A1S</td>
<td>697 ± 3.6</td>
<td>32.1 ± 1.1</td>
</tr>
<tr>
<td>30J5×23C7</td>
<td>A2R×A1S</td>
<td>397 ± 5</td>
<td>17.1 ± 0.8</td>
</tr>
<tr>
<td>3A4×31A8</td>
<td>A2R×A1R</td>
<td>102 ± 2</td>
<td>9.8 ± 0.5</td>
</tr>
<tr>
<td>18C9×31A8</td>
<td>A2R×A1R</td>
<td>290 ± 12</td>
<td>29.9 ± 1.5</td>
</tr>
<tr>
<td>30J5×31A8</td>
<td>A2R×A1R</td>
<td>918 ± 6</td>
<td>14.5 ± 1.7</td>
</tr>
<tr>
<td>2C11×26E8</td>
<td>A1S×A2S</td>
<td>385 ± 14</td>
<td>25.5 ± 1</td>
</tr>
<tr>
<td>4J9×26E8</td>
<td>A1S×A2S</td>
<td>432 ± 27</td>
<td>20 ± 0.5</td>
</tr>
<tr>
<td>23C7×26E8</td>
<td>A1S×A2S</td>
<td>253 ± 25</td>
<td>28 ± 0.9</td>
</tr>
</tbody>
</table>

$^y$ R=resistant; S=sensitive

$^z$ Mean values of four replicates with standard deviation.

Table 14 Chi-square analysis of *Phytophthora nicotianae* crosses for segregation of mefenoxam resistance and linkage of mating type and mefenoxam resistance

<table>
<thead>
<tr>
<th>Type of cross</th>
<th>Parents</th>
<th>Number of progeny</th>
<th>Segregation in progeny</th>
<th>$\chi^2 (P)$</th>
<th>Mating type and mefenoxam resistance linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>mef S × mef R</td>
<td>2C11 3A4</td>
<td>161</td>
<td>79:82</td>
<td>0.06 (0.81)</td>
<td>59:11:68:20(R/A2:R/A1:S/A2:S/A1)</td>
</tr>
</tbody>
</table>
Figure 12 Oospores of *P. nicotianae* isolate (31D7) germinating on PARP plates by producing branching hyphae
Segregation of mefenoxam sensitivity in $F_1$ progeny populations

The distribution of percent growth of progeny from each cross exhibited a continuous pattern in the range at both 10µg/ml and 100µg/ml of mefenoxam (Figure 13). A total of 161 single oospore $F_1$ progeny isolates were successfully established from cross 3A4×2C11 (Mef S × Mef R), of which 128 belong to mating type A2, 31 to A1. Two isolates produced oospores in the absence of any opposite mating type (self-fertile). The progeny of cross 3A4×2C11 segregated for mefenoxam resistance. Segregation of sensitive and resistant phenotypes was observed among the progeny of this cross and their continuous distribution was also observed at both concentrations of mefenoxam (Figure 13). However, as expected, the percentage growth of the control at 100µg/ml of mefenoxam was generally less than that at 10µg/ml. Most isolates gave an intermediate response to mefenoxam at both concentrations tested (Figure 13). The progeny of this cross also fit a 1:1 segregation (79:82 at 100µg/ml) of sensitive and resistant phenotype ($\chi^2 = 0.06; P=0.81$). The segregation of sensitive progeny in a ratio of 1:1 indicates that the resistant parent (3A4) is heterozygous for resistance to mefenoxam. chi-square test for linkage between mefenoxam resistance and mating type in the progeny of cross 3A4×2C11 showed that there was no linkage between those two traits (Table 14).
Figure 13 Distribution of mefenoxam resistance among progeny from 3 *in vitro* crosses. Mef S=mefenoxam sensitive; Mef R=mefenoxam resistant. A line is drawn at 40% growth of the control which divides the progeny into sensitive and resistant at 100µg/ml. The percent growth of each progeny was a mean value of three replicates.
No segregation for mefenoxam resistance was observed in two crosses: 2C11×26E8 (Mef S × Mef S) and 3A4 × 31A8 (Mef R × Mef R). All 25 progeny from cross 2C11×26E8 were sensitive with percentage growth of control ranging from 0% to 30.8% at 10µg/ml of mefenoxam, and from 0.0% to 17.3% at 100µg/ml of mefenoxam, respectively (Figure 13). In cross 3A4 × 31A8, all 25 progeny were resistant to mefenoxam (Table 14); their percentage growth of control ranged from 65.2% to 111.2% at 10µg/ml of mefenoxam, and from 56.0% to 117.0% at 100µg/ml of mefenoxam, respectively (Figure 13).

Identification of DNA markers

Of 22 RAPD primers screened, 16 primers generated polymorphic bands between resistant isolate (18C7) and sensitive isolate (2C11) varying in size from 300 bp to 2,500 bp. The number of fragments produced by each primer ranged from three to nine. Of 100 RAMS primers, 24 primers were able to detect polymorphisms. On average, one primer generated one to five bands varying in size from 600 bp to 2,500 bp.

All 40 primers that produced polymorphisms between those two isolates were tested three times for reproducibility and only those primers were subsequently used to detect polymorphisms between the two DNA bulks. In the BSA, only one RAPD primer (primer sequence 5’-3’: AGCGCCATTG) screened detected polymorphism between resistant bulk and sensitive bulk, indicating their association with the mefenoxam-resistance gene. This RAPD marker amplified the polymorphic fragment only in the sensitive isolates and thus was associated in a repulsion phase linkage with the mefenoxam-resistance gene (Figure 14). This marker was further validated on 128 isolates of P. nicotianae from a variety of crops in different geographic locations.
Development of SCAR marker

Based on the sequence information, five putative SCAR primer pairs were designed using Primer3 software (http://seqtool.sdsc.edu/CGI/BW.cgi#) (Table 15). These primers were used to amplify ten mefenoxam-resistant and 10 sensitive isolates under optimal PCR conditions. The SCAR primer pairs 1 were found to be polymorphic and gave unique band amplification with the expected size in sensitive isolates. However, the other four SCAR primer pairs resulted in one identical size band and did not produce any polymorphisms between resistant and sensitive isolates. This SCAR marker was also tested for its specificity on 128 isolates of P. nicotianae as described above. Amplification occurred with DNA from all 92 sensitive isolates and yielded a specific fragment of 1206 bp linked in repulsion to the resistance locus. No amplification occurred with DNA from 35 resistant isolates. However, one resistant isolate (3A4) also produced the specific fragment.
Table 15 Sequence size of original RAPD markers and characteristics of SCAR marker derived from RAPD markers MR25J8 linked to mefenoxam-resistance gene in repulsion phase in *Phytophthora nicotianae*

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>5’-3’ sequence</th>
<th>Annealing temp (°C)</th>
<th>Product Size (bp)</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F: AGCGCCATTGGGTATGAAA</td>
<td>65.3</td>
<td>1206</td>
<td>polymorphic</td>
</tr>
<tr>
<td></td>
<td>R: AGCGCCATTGAGCTGAACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F: CACGCGAATTTCAAGCA</td>
<td>60.0</td>
<td>153</td>
<td>monomorphic</td>
</tr>
<tr>
<td></td>
<td>R: AAGTCAGTGCAGGCTTCTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F: CATTCCGATTCCAGCTAAA</td>
<td>63.8</td>
<td>269</td>
<td>monomorphic</td>
</tr>
<tr>
<td></td>
<td>R: GAAAAACCTTCAGGACATA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>F: GCTGGCAAGTCCATCAATT</td>
<td>66.5</td>
<td>324</td>
<td>monomorphic</td>
</tr>
<tr>
<td></td>
<td>R: TCATTACAGAGGCGGACACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F: AAGCGGGGTACAGTTGTTGG</td>
<td>67.5</td>
<td>289</td>
<td>monomorphic</td>
</tr>
<tr>
<td></td>
<td>R: TGGGAATCGGAATGCATTG</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**DISCUSSION**

This study analyzed the segregation pattern of mefenoxam resistance in *F₁* progeny for 3 crosses and identified one SCAR marker putatively linked to mefenoxam resistance. Our results are significant for several reasons. First, our data clearly support a hypothesis that mefenoxam resistance in *P. nicotianae* is controlled by a single gene with complete dominance. In this study, sensitive isolates are homozygous recessive for mefenoxam resistance but resistant isolates may be homozygous and heterozygous. The heterozygosis of resistant isolate 3A4 enables us to predict the homozygosis of resistant isolate 31A8 if we assume there is only one allele for resistance. Because all the progeny in cross 3A4 × 31A8 (Mef R × Mef R) were resistant (Figure 13), at least one parent must have been homozygous for resistance, otherwise there would have been segregation for sensitive progeny in this cross 3A4 × 31A8. Therefore, if the above assumption that only a single resistant allele is responsible for mefenoxam resistance was true, a similar degree of resistance to mefenoxam can be conferred by either homozygous or heterozygous isolates.
In addition to a major gene effect, genes with minor effects may be involved in conferring a certain degree of resistance to mefenoxam. While all progeny of cross 2C11×26E8 (Mef S × Mef S) are homozygous sensitive and all progeny of cross 3A4 × 31A8 (Mef R × Mef R) exhibited resistance to mefenoxam, there was a range of growth rates among the progeny in both crosses (Figure 13). This result demonstrated that there are minor genes segregating in those crosses. Other researchers also found minor-gene effects involved in metalaxyl genetics of *P. infestans* (49, 88, 102, 133). Those minor genes act collectively to modify the phenotype of resistance to mefenoxam.

Our work has identified a SCAR marker that is capable of distinguishing between mefenoxam-resistant populations and sensitive populations of *P. nicotianae*. The SCAR marker linked to mefenoxam resistance may provide a valuable tool to study the development and spread of resistance through populations and to develop assays to predict resistance to mefenoxam. The single SCAR marker developed in this study was polymorphic and amplified a 1206-bp long band linked in repulsion phase to the resistance locus. However, a non-specific monomorphic fragment of approximate 1.1k-bp was also amplified. Various PCR protocol changes attempted did not eliminate this non-specific band. This however did not interfere with the scoring for a polymorphic fragment of 1206 bp linked to the resistance locus and thus, the marker can still be suited to the above uses. Presence of non-specific amplification with SCAR markers has also been reported in other studies (74, 117). This has been attributed to the SCAR primers containing ubiquitous sequences in multiple genomic regions that may result in mismatched primer annealing during PCR.
The successful validation of the SCAR marker over a set of 128 isolates of *P. nicotianae* suggested that this marker is highly specific to the mefenoxam resistance locus. However, both SCAR markers and their progenitor RAPD markers followed a dominant inheritance mode, as indicated by the presence or absence of a specific band. Consequently, these markers cannot distinguish between homozygous and heterozygous genotypes. The dominant nature of the SCAR marker will likely limit the application of this marker in predicting sensitivity to mefenoxam. In the present study, only one resistant isolate (3A4) was found to be a heterozygote for the mefenoxam resistance locus. The SCAR marker identified in this study amplified the specific fragment of 1206-bp which would be predicted to be sensitive isolates. One of the crucial steps in positional cloning is the identification of DNA markers flanking the gene of interest. The marker discovered in this study will provide the basis for physical mapping and eventual positional cloning of the resistance gene.
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Table 16. Binary data set generated from RAPD analysis for *Phytophthora nicotianae* isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host/substrate</th>
<th>Mefenoxam sensitivity</th>
<th>Primers</th>
<th>Fragments</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A4</td>
<td>Irrigation water</td>
<td>R</td>
<td>OPW-04</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>2C11</td>
<td><em>Buxus sempervirens</em></td>
<td>S</td>
<td>OPW-04</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>30J7</td>
<td><em>Petunia</em> sp.</td>
<td>R</td>
<td>OPW-04</td>
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<td>1</td>
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<td>1</td>
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<td>28B5</td>
<td><em>Lavandula angustifolia</em></td>
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<td>OPW-04</td>
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<tr>
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<td>2D1</td>
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<tr>
<td>18C4</td>
<td><em>Catharanthus roseus</em></td>
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<td>OPW-04</td>
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1 denotes presence of fragment and 0 denotes absence of fragment; F1 represents largest amplification products (topmost bands in the gel), followed by F2 etc. down to the bottom of the gel. The band location and size with the same letter (i.e. F1) varied with different primers.
Table 17. The mating type and mefenoxam sensitivity of F1 progenies from cross 3A4×2C11

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<sup>*</sup> Crosses, mating type, and mefenoxam sensitivity were conducted on 20% CV8 agar medium.
Figure 15. Photo gallery from *in vivo* tests

Geranium seedlings (*Pelargonium × hortorum* cv. ‘White Orbit’)

Lupine seedlings (*Lupinus* ‘Russell Hybrids’)

Inoculation (10 µl of zoospore suspension containing 20 spores)
Diseases caused by *P. cinnamomi* isolates on lupine (Without mefenoxam treatment)

No infections by *P. cinnamomi* isolates on mefenoxam-treated lupine (at label rate)
Mycelial infections by *P. nicotianae* (propamocarb at label rate, 1.8mg/ml)
Figure 16. Blind test of RAPD marker D11 on *Phytophthora nicotianae* isolates from North Carolina and South Carolina
Appendix 1 Media and Solutions

A.1.1 Clarified V8 juice

Add 1 g CaCO₃ per 100 ml of V8 juice. Stir at room temperature for 30 min with a magnetic bar. Centrifuge at 4 °C for 10 min at 10,000 rpm. Transfer supernatant into storage bottles.

A.1.2 20% CV8 agar medium

Mix in a 1 liter flask: 400 ml dH₂O, 100 ml clarified V8 juice as described above, 7.5 g Difco Bacto™ agar (Becton Dickinson; Sparks, MD). Autoclave and cool to 50 °C.

A.1.3 PARP medium

Mix in a 1 liter flask: 475 ml dH₂O, 25 ml clarified V8 juice as described above, 7.5 g Difco Bacto™ agar (Becton Dickinson; Sparks, MD). Autoclave and cool to 50 °C. Add 1 ml of stock solution A and 0.5 ml of stock solution B:

To prepare 50 ml of stock solution A: 50 ml of 70% ethanol, 0.25 g of rifamycin sv sodium (ICN Biomedicals, Inc.; Aurora, OH), and 0.5 g of 50% benomyl

To prepare 50 ml of stock solution B: 30 ml of sterile dH₂O, 1.25 g of ampicillin sodium salt (Fisher Scientific; Fairlawn, N.J.), 20 ml of 2.5% pimaricin (Sigma Chemical Co., St. Louis, MO), 0.67 g of 70% PCNB, and 0.25 g of 100% hymexazol (Tachigaren™).

A.1.4 Sterile Soil Water Extract (SSWE)

To prepare 1% SSWE, add 10 g of fresh sandy loam soil to 1 liter dH₂O. Stir at room temperature overnight and then allow the soil particles to settle for 6 hr. Filter the aqueous portion through a Whatman paper No 1. Autoclave the filtrate.
Appendix 2 DNA sequence of RAPD marker MR25J8

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ATTGCACCAGACAGAAGGTATTGCTCTCATAATGGTCAGCTACAAATGGGCGT
VITA

Jiahuai Hu was born to Silan Chen and Fangde Hu in Kuze village, Qiaojia County, Yunnan, China on December 8, 1977 (Chinese lunar calendar). He grew up with his younger brother on his family’s farm and enjoyed a happy childhood on the Jinsha River. In 1995 he graduated from Zhaotong # 1 high school and then continued his college education at Hefei Economic and Technological institute. Jiahuai received his Bachelor of Agriculture degree in Plant Protection in June 1999. In 2003, he received a Master of Agriculture degree in Plant Pathology from the Department of Plant Pathology at China Agricultural University, Beijing, China. Upon graduation from China Agricultural University, Jiahuai continued his Doctorate training in Plant Pathology at Virginia Polytechnic institute and State University in August 2003. His main area of research involved fungicide resistance in Phytophthora species and molecular marker identification. In spring 2007, he was awarded a Doctor of Philosophy degree from the Department of Plant Pathology, Physiology, and Weed Science at Virginia Tech.