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Mefenoxam sensitivity and fitness analysis of *Phytophthora nicotianae* isolates from nurseries in Virginia, USA

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Mefenoxam is one of the most commonly used fungicides for managing diseases caused by *Phytophthora* spp. on ornamentals. The objectives of this study were to determine whether *Phytophthora nicotianae*, a destructive pathogen of numerous herbaceous annual and perennial plant species in nurseries, has developed resistance to mefenoxam, and to evaluate the fitness of mefenoxam-resistant isolates. Ninety-five isolates of *P. nicotianae* were screened for sensitivity to mefenoxam on 20% clarified V8 agar at 100 a.i. μg mL^{-1}. Twenty-five isolates were highly resistant to this compound with EC_{50} values ranging from 235·2 to 466·3 μg mL^{-1} and four were intermediately resistant with EC_{50} values ranging from 1·6 to 2·9 μg mL^{-1}. Sixty-six isolates were sensitive with EC_{50} values less than 0·04 μg mL^{-1}. Nine resistant and seven sensitive isolates were tested for mefenoxam sensitivity on *Pelargonium* × *hortorum* cv. White Orbit. Mefenoxam provided good protection of pelargonium seedlings from colonization by sensitive isolates, but not by any resistant isolates. Four resistant and four sensitive isolates were compared for fitness components and their relative competitive ability on *Lupinus* Russell Hybrids in the absence of mefenoxam. Resistant isolates outcompeted sensitive ones within 3 to 6 sporulation cycles on lupin seedlings, regardless of their initial proportions in mixed zoospore inoculum. Resistant isolates exhibited greater infection rate and higher sporulation ability than sensitive ones when they were applied separately onto lupins. These results suggest that fungicide resistance may pose a serious challenge to the continued effectiveness of mefenoxam as a control option for nursery growers.

Keywords: fungicide resistance, geranium, IPM, *Lupinus*, ornamental crops, phenylamide, *Pelargonium* × *hortorum*

Introduction

Since its first introduction in 1977, metalaxyl and its more recent isomer mefenoxam have been used widely and intensively to control oomycete diseases of numerous crops including ornamentals (Cohen & Coffey, 1986; Erwin & Ribeiro, 1996). Mefenoxam has strong inhibitory activities against both mycelium growth and sporulation (Staub & Young, 1980). The mode of action of mefenoxam is selective inhibition of ribosomal RNA synthesis by affecting the activity of the RNA polymerases (Davidse *et al*., 1983). Due to its site-specificity, mefenoxam has a relatively high intrinsic risk of resistance development in target pathogens. Within 2 years of its introduction, several incidences of *Phytophthora infestans* resistance emerged in potato fields across Europe (Gisi & Cohen, 1996). Since then, metalaxyl/mefenoxam resistance has been frequently detected in other *Phytophthora* species and oomycetes (Timmer *et al*., 1998; Moorman & Kim, 2004; Hwang & Benson, 2005).

Fitness is defined as an organism's ability to contribute to the subsequent gene pool (Vanderplank, 1982), which is measured as a function of its ability to grow, reproduce and survive through repeated life cycles. It is estimated by either measuring components of fitness such as reproductive ability and pathogenicity or assessing the actual pathogen population after competing with each other on a susceptible host. Information on fitness of mefenoxam-resistant and -sensitive populations in *P. nicotianae* is particularly important because of its central role in population dynamics and making anti-resistance strategies. Previous studies reported that metalaxyl-resistant isolates of *P. infestans* caused larger lesions than sensitive ones, but they did not differ significantly from each other in sporulation capacity (Kadish & Cohen, 1988a,b; Kadish *et al*., 1990). Fitness testing indicated that resistant isolates of *P. erythroseptica* had greater mycelial growth rate and oospore formation than sensitive ones (Porter

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et al., 2006). Competition experiments using *P. nicotianae* from citrus and *P. infestans* indicated that metalaxyl-resistant isolates were better competitors than were sensitive isolates (Kadish & Cohen, 1988a,c; Timmer *et al*., 1998).

Phytophthora nicotianae (syn: *Phytophthora parasitica*) is a destructive pathogen on numerous herbaceous annuals and perennial plant species (Erwin & Ribeiro, 1996; Benson & von Broembsen, 2001). *Phytophthora nicotianae* causes foliar diseases on herbaceous annual plants and is disseminated to neighbouring plants by splash of sporangia (Benson & von Broembsen, 2001). Due to repeated growing of herbaceous annuals in nurseries, *P. nicotianae* may complete several disease cycles on annuals within a growing season. However, this pathogen causes root and crown rots on perennial plants and a single cycle of disease is needed on those hosts. The pathogen has been frequently recovered from diseased nursery plants and recycled irrigation water (Hong & Moorman, 2005), and can be easily spread to other production facilities and landscapes through movement of contaminated nursery stock. 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 on ornamental plant nurseries. In the USA, mefenoxam, metalaxyl-M (Subdue MAXX, Syngenta) is one of the major compounds registered as a soil drench to control root infections on ornamental crops (Benson & von Broembsen, 2001; Jeffers & Miller, 2001). Due to the high effectiveness of mefenoxam, nursery growers repeatedly used this product. As a result, mefenoxam resistance has been increasingly detected in *Phytophthora* spp. recovered from nurseries (Ferrin & Rohde, 1992; Jeffers *et al*., 2004; Hwang & Benson, 2005). Information on fungicide resistance and on the movement of resistant populations among nursery production facilities is critical to develop management strategies that can prevent a further rise of resistance. However, little is known about the sensitivity of *P. nicotianae* to mefenoxam in Virginia, USA. The objectives of this study were to determine (i) *in vitro* sensitivity to mefenoxam of *P. nicotianae* isolates from state-wide nurseries, (ii) EC_{50} values of representative isolates, (iii) efficacy of mefenoxam to protect geranium seedlings against infection by *P. nicotianae*, and (iv) fitness and competitiveness of mefenoxam-resistant and -sensitive isolates in lupin seedlings.

Materials and methods

Culture collection and maintenance

A total of 95 isolates of *P. nicotianae* were included in this study. The majority of these isolates originated from plant tissues of diverse nursery crops and irrigation water in Virginia since 2000 (Table 1). Several isolates from other crops and locations were included for comparison. All isolates were maintained in sterile distilled water on sterilized hemp seed at 15°C. The species identity of all isolates was verified using a single-strand conformation polymorphism analysis of the ribosomal DNA internal transcribed spacer 1 (Kong *et al*., 2003b).

Mefenoxam sensitivity and EC_{50}

Mefenoxam (Subdue MAXX; Syngenta) was assessed at 100 a.i. μg mL^{-1}. CV8 agar (20% clarified V8 juice, 0·4% $CaCO_3$, and 1·5% agar in distilled water) 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 using a flamed cork borer. One plug was placed in the centre of a 9-cm Petri dish with mycelia in contact with the medium. Three replicate dishes per treatment were inoculated for each isolate. The inoculated dishes were 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. After the diameter of the mycelium plug was subtracted, two measurements were averaged. Relative mycelial growth of an isolate on fungicide-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 (i) sensitive if mycelial growth was little or none, (ii) intermediately resistant if mycelial growth was less than 40% of that on control dish, or (iii) resistant if mycelial growth was greater than 40% when compared to growth on control dish.

EC_{50} values were determined for representative isolates of resistant (n = 5), intermediately resistant (n = 4) and sensitive (n = 5) groups, respectively. Isolates were purposely selected to represent the greatest number of host/substrates for each sensitivity group. 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^{-1} 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. Linear regression lines of logit-transformed percentage against the $\log_{10}$ of the mefenoxam concentration were fitted using Procedure GLM of SAS (SAS Institute Inc.). The resulting equations were used to calculate EC_{50} values.

In vivo tests with *Pelargonium* × *hortorum* cv. White Orbit (geranium)

Nine resistant and seven 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 (Hu *et al*., 2007). 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 millilitres of general purpose fertilizer (20%

Table 1 Sensitivity to mefenoxam and EC_{50} values of *Phytophthora nicotianae* isolates from nurseries in Virginia, USA

Host/substrate	No. of isolates	Year	Geographic origin	Sensitivity[c]	EC_{50} of representative isolates (μg mL^{-1})[d]
Annuals/water					
Petunia spp.*[a]	20	2004	Virginia	Resistant	466·3
Petunia sp.	2	2004	Virginia	Intermediate	2·9
*Catharanthus roseus**	3	2001	Virginia	Resistant	235·2
Catharanthus roseus	2	2001	Virginia	Intermediate	1·6
Catharanthus roseus	3	2001	Virginia	Sensitive	< 0·01
Irrigation water*	2	2000	Virginia	Resistant	236·2
Irrigation water*	3	2000	Virginia	Sensitive	0·04
Irrigation water	2	2000	Oklahoma	Sensitive	ND
Perennials					
Armeria maritima	2	2006	Virginia	Sensitive	< 0·01
Delosperma cooperi	1	2006	Virginia	Sensitive	ND
Malephora crocea	5	2006	Virginia	Sensitive	ND
Penstemon mexicali	4	2006	Virginia	Sensitive	ND
Scabiosa columbaria	3	2006	Virginia	Sensitive	ND
*Veronica spicata**	5	2006	Virginia	Sensitive	< 0·01
*Lavandula angustifolia**	2	2006	Virginia	Sensitive	ND
*Lavandula angustifolia**	1	2002	Virginia	Resistant	ND
Hemerocallis sp.	1	2003	Virginia	Sensitive	ND
Abies fraseri	1	1986	West Virginia	Sensitive	ND
Berberis thunbergii	1	2000	Virginia	Sensitive	ND
Buxus sempervirens	4	2000	Virginia	Sensitive	< 0·01
Daphne sp.*	1	2001	Virginia	Sensitive	< 0·01
Forsythia sp.	1	2001	Virginia	Sensitive	ND
Mandevilla sp.	1	2006	Virginia	Sensitive	ND
Rhododendron sp.	1	2001	Virginia	Sensitive	ND
Other crops					
Solanum tuberosum	1	1991	North Carolina	Sensitive	ND
Solanum lycopersicum	1	2000	Virginia	Sensitive	ND
Nicotiana tabacum	4	2003	North Carolina	Sensitive	ND
Citrus sp.	3	...	California	Sensitive	ND
Citrus limonium	2	1968	California	Sensitive	ND
...[b]	13	...	...	Sensitive	ND

[a]Isolates with asterisk were selected for fitness analysis.

[b]Unknown.

[c]Sensitivity tests were conducted on CV8 agar amended with mefenoxam at 100 μg mL^{-1}. Isolates were characterized as (i) sensitive if mycelial growth was little or none, (ii) intermediate if mycelial growth was less than 40% of that on control dishes, or (iii) resistant if mycelial growth was greater than 40% of that on control dishes.

[d]EC_{50} is the effective concentration of mefenoxam at which mycelial growth is inhibited by 50% when compared to growth on control dish. These values are mean of six replicates from two repeating tests. ND = not determined.

N, 20% P_2O_5 and 20% K_2O; Scotts-Sierra Horticultural Products Co.) in water at 1 mg mL^{-1} was added to each container to keep the filter paper moist. Five pelargonium 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 fluorescent lights at 23–25°C.

Once the cotyledons were fully expanded, plants were treated either with water as non-treated control or with mefenoxam at 0·15 μL mL^{-1} (twice the label rate). Twenty-five millilitres of mefenoxam at the above specified concentration, or water in the case of the control, were used to replace the fertilizer solution in containers. Two days after treatment, both cotyledons of each seedling were inoculated with mycelial plugs. Five millimetre-diameter mycelial plugs were taken from the margin of a 3-day-old culture of *P. nicotianae*. 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. SSWE was prepared by mixing 10 g (fresh weight) of a sandy loam soil in 1 L 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), and autoclaving the extract for 20 min. 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 6 days after inoculation, the number of seedlings with blackened stems, leaf necrosis or wilting was counted. The Mann-Whitney *U* test was performed to determine the differences in mortality of geranium seedlings between the mefenoxam treatment and non-treated control for each isolate.

Selection of isolates for fitness analysis

Four mefenoxam-resistant (MR) and four mefenoxam-sensitive (MS) isolates (see selected isolates with an asterisk in Table 1) were selected to assess mycelial growth rate in CV8 agar and infection rate in *Lupinus* Russell Hybrids. Zoospore production was also determined both *in vitro* and *in vivo*. These selected isolates were recovered from six plant species or substrates in geographically different nurseries and therefore represented the potential diversity between and among MR and MS isolates. A subset of two MR and two MS isolates was selected from among the eight isolates for further assessment of their relative competitive ability using lupin seedlings.

Mycelial growth, sporangium and zoospore production and infection rate

Mycelial growth rate was assessed on unamended CV8 agar. Mycelial plugs (5-mm diameter) were taken from the margin of actively growing colonies of 5-day-old agar cultures. One plug was placed in the centre of a 9-cm Petri dish with the mycelia in contact with the medium. The inoculated dishes were then incubated at 25°C in darkness. Five replicate dishes per treatment were inoculated for each isolate. After 5 days of growth, mean colony diameter was measured as previously described.

Sporangium production was assessed using SSWE as a medium to induce sporangium formation. Ten mycelial discs (5 mm diameter) were removed from the edge of 5-day-old cultures on CV8 agar and placed in Petri dishes containing 10 mL of SSWE. Five replicate dishes were used for each isolate. After incubation under fluorescent light for 10 h at 25°C, the number of sporangia along the peripheral margins of each agar disc was counted under a compound microscope.

Zoospore production from agar discs was also assessed using SSWE. Sporangia were produced as described above. Five replicate dishes were used for each isolate. After incubation under fluorescent light for 10 h at 25°C, the entire set of Petri dishes was placed in a refrigerator at 4°C for 1·5 h and then placed back at 25°C for 45 min to complete the process of zoospore release. Zoospore suspensions were collected in 10-mL test tubes and three 10-μL aliquots were removed from each test tube to estimate the concentration of zoospores using a haemocytometer. Subsequently the number of zoospores produced per agar disc was determined.

Infection rate was determined by inoculating the upper surface of fully expanded cotyledons of 10-day-old lupin seedlings with one 10-μL droplet of inoculum containing ~20 zoospores. Zoospore inoculum was prepared as described previously. Lupin seeds were induced to germinate by soaking overnight in water and then placed on moistened paper towel with bottom heat of 35°C. Ten lupin seedlings were grown in vermiculite medium inside a plastic cup (15 cm × 10 cm) under white fluorescent light (10 h day/14 h night) at 24–26°C. Plants were fertilized once a week with 50 mL of the same fertilizer as used for *in vivo* tests. 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 infection. Then the inoculated plants were transferred to grow under the conditions as described above. A leaf or plant was scored as wilted if the entire leaf area or above ground part was fully colonized and dehydrated. The number of wilted leaves and wilted plants were recorded daily.

Four days after inoculation, zoospore production from diseased plants was measured as follows: five entire diseased plants including the roots were collected from each treatment and surface sterilized with 10% $NaHClO_3$ for 30 s, followed by copious rinses with sterile distilled water. Sporangium production was induced by incubating surface sterilized plants in 6-cm Petri dishes containing SSWE under fluorescent light for 48 h. During incubation, the solution in each Petri dish was replaced with an equal volume of fresh SSWE every 24 h. Zoospores were triggered to release by using 5 mL of pre-chilled (4°C) SSWE. The number of zoospores was counted in eight fields with a haemocytometer to determine the number of zoospores produced per plant.

Competitive ability of mefenoxam resistant (MR) and sensitive (MS) isolates on lupin

The relative competitiveness of three pairs of resistant/sensitive isolates (31D7 (MR) + 1E3 (MS), 3A4 (MR) + 4J9 (MS) and 31D7 (MR) + 4J9 (MS)) was compared at three initial MR:MS ratios of zoospore concentrations, 1:4, 1:1, and 4:1. Zoospore suspension of each isolate was prepared as described previously. Mixed inocula were generated by thoroughly mixing appropriate volumes of zoospores to obtain the desired MR:MS ratios. Inoculation was carried out with mixed inocula the same way as described for infection rate tests. Four days after inoculation, five entire diseased plants including the roots were collected from each treatment to produce zoospores as previously described. 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 resultant zoospore suspensions, 100 μL of zoospore suspension containing ~36 zoospores was spread on PARP medium in 9-cm diameter dishes to allow zoospore germination (Hu *et al*., 2007). After 36 h of incubation at 25°C in darkness, sixty colonies were selected arbitrarily from each treatment and subcultured on CV8 agar amended with mefenoxam at 100 μg mL^{-1}. A colony was scored as resistant to mefenoxam if it continued to grow on the mefenoxam-amended medium and reached the edge of the dishes after 5-day incubation at 25°C in darkness. A colony was scored as sensitive if no growth was observed. The percentage of mefenoxam-resistant zoospore populations was calculated.

Each experiment described above was conducted twice on different dates. Treatments within each repeating

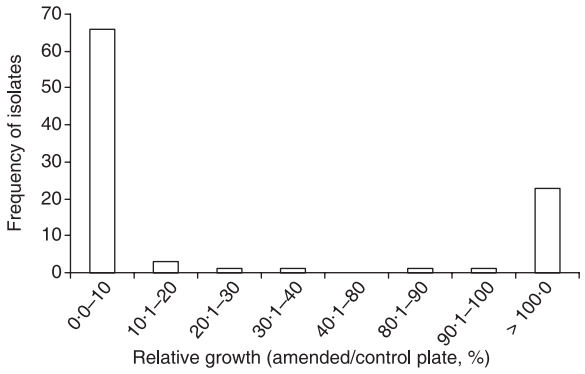

Figure 1 Frequency distribution of 95 isolates of *Phytophthora nicotianae* in response to 100 μg mL^{-1} mefenoxam incorporated into 20% clarified V8 agar. The relative growth of each isolate is a mean value of six replicates from two tests.

experiment were arranged in a completely randomized design. Data from two experiments were pooled together according to homogeneity of variance using the GLM procedure of SAS to determine homogeneity of variance among repeated experiments. One-way fixed effect analysis of variance was performed using the Procedure GLM of SAS to determine statistical differences in fitness parameters between resistant and sensitive isolates.

Results

Mefenoxam sensitivity and EC_{50}

Approximately 26% of *P. nicotianae* isolates were highly resistant to mefenoxam. Four isolates were intermediately resistant (Table 1; Fig. 1). The majority of isolates recovered from annual ornamentals were either resistant or intermediately resistant and 40% of nursery isolates from irrigation water in Virginia were resistant (Table 1).

The EC_{50} values for resistant isolates ranged from 235·2 to 466·3 μg mL^{-1} (Table 1); intermediately resistant isolates had EC_{50} values ranging from 1·6 to 2·9 μg mL^{-1}. In contrast, all isolates from perennials, except one isolate from *Lavandula*, were sensitive to mefenoxam (Table 1). Most sensitive isolates had over 50% inhibition of mycelial growth in the presence of 0·1 μg mL^{-1} mefenoxam and over 70% on the agar medium amended with 1 μg mL^{-1} mefenoxam (Fig. 2b).

In vivo tests on geranium

Colonization of geranium plants took place at the inoculation site then rapidly spread either up to the growing tip or down the stem, causing stem rot (blackened). Eventually infected plants collapsed and died. All 16 isolates consistently infected and killed the majority of non-treated control seedlings (Table 2). Mefenoxam provided full protection of geranium seedlings against infection and colonization by sensitive isolates, but not resistant isolates (Table 2).

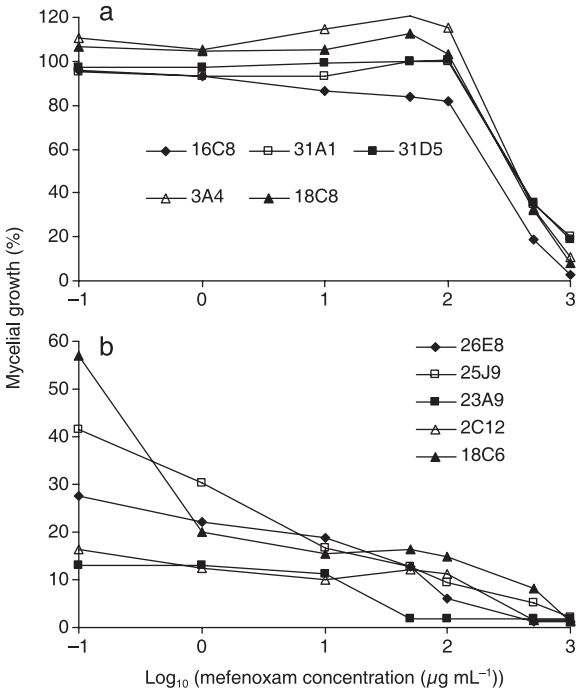

Figure 2 Dose-response curves for the mycelial growth on clarified V8 agar of (a) five mefenoxam-resistant isolates and (b) five mefenoxam-sensitive isolates of *Phytophthora nicotianae*. The mycelial growth was expressed as percent growth on mefenoxam-amended medium compared to that of the control medium. Isolate 16C8, 3A4 and 25J9 were recovered from irrigation water in central and southwestern Virginia, USA (2000), 2C12 from *Buxus sempervirens* in central Virginia (2000), 18C6 and 18C8 from *Catharanthus roseus* in eastern Virginia (2001), 31A1 and 31D5 from *Petunia* sp. in eastern Virginia (2004), 26E8 from *Nicotiana tabacum* in North Carolina (2003), and 23A9 from *Citrus limonium* (1968). Each number is a mean value of six replicates from two experiments.

Mycelial growth, sporangium and zoospore production and infection rate

MR isolates did not differ significantly from MS isolates in mycelial growth on CV8 agar (Table 3). However, the MR isolates produced significantly more sporangia and zoospores (P = 0·001 and 0·009 respectively) than the MS isolates (Table 3). In infection rate tests, all test isolates were highly aggressive to lupin seedlings, regardless of their sensitivity to mefenoxam. Water-soaked symptoms rapidly spread from the inoculation site to the entire leaf and caused leaf wilting within 3 days of inoculation. However, resistant isolates caused a significantly (P = 0·001) higher percentage of plant death than sensitive ones (Table 3).

Competitive ability of MR and MS isolates on lupin

Resistant isolates were better competitors than sensitive ones in all pairings, regardless of their initial ratios. Resistant isolates dominated in the populations after 3–6 cycles of infection on lupin seedlings in the absence of

Table 2 Efficacy of mefenoxam in protecting *Pelargonium* × *hortorum* cv. White Orbit seedlings from infection by *Phytophthora nicotianae* isolates

		No. of diseased plants[b]		
Isolate	Sensitivity[a]	Non-treated control	Mefenoxam[c]	P[d]
30J5	R	5·0	4·5	0·5
30J7	R	4·5	4·5	1·0
31D7	R	4·0	3·0	0·17
31A8	R	4·0	3·5	0·5
3A4	R	5·0	5·0	1·0
31A5	R	5·0	5·0	1·0
31E2	R	4·5	4·5	1·0
18C8	R	5·0	5·0	1·0
16C8	R	5·0	4·5	0·5
26E8	S	4·5	0·0	0·001
23C7	S	4·5	0·5	0·001
25J9	S	5·0	0·0	0·001
1E3	S	5·0	0·0	0·001
23A9	S	5·0	0·0	0·001
23C3	S	5·0	0·0	0·001
18C4	S	5·0	0·0	0·001

[a]Sensitivity tests were conducted on CV8 agar amended with mefenoxam at 100 μg mL^{-1}. Isolates were characterized as (i) sensitive if mycelial growth was little or none, (ii) intermediate if mycelial growth was less than 40% of that on control dishes, or (iii) resistant if mycelial growth was greater than 40% of that on control dishes.
[b]Five seeds of *Pelargonium* × *hortorum* cv. White Orbit per replicate were used in each test. No. of diseased plants is the mean value of six replicates from two repeated tests.
[c]Seedlings treated with 0·15 μL mL^{-1} mefenoxam fertilizer solutions (double the labelled rate).
[d]Mann-Whitney U test was performed to determine the significance of differences in mortality of seedlings between treatment and non-treated control (P = probability).

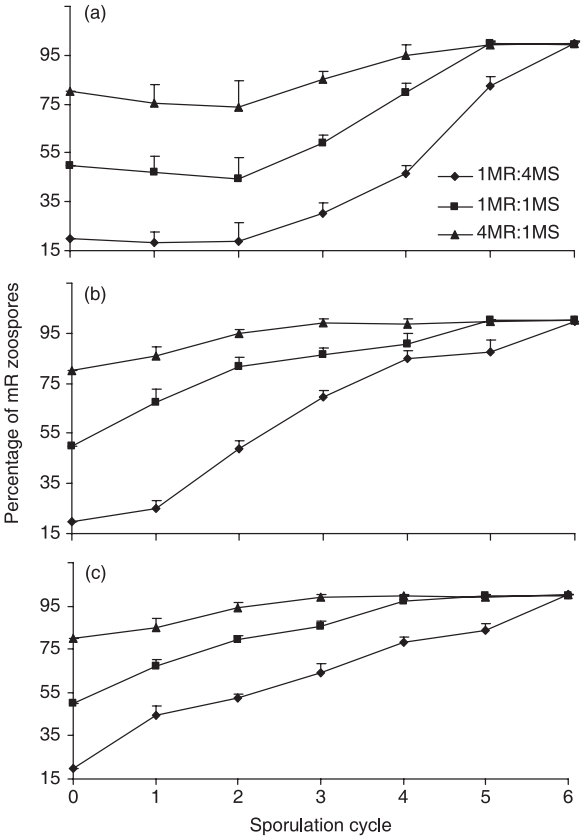

Figure 3 Changes in percentage of mefenoxam-resistant (MR) subpopulation of *Phytophthora nicotianae* in three pairs of isolate mixtures (a) 31D7 + 1E3, (b) 31D7 + 4J9 and (c) 3A4 + 4J9. Three initial ratios, 1R:4S, 1R:1S, 4R:1S, for each pair were applied onto lupin seedlings. The second sporulation cycle was initiated with resultant zoospores from the initial infection and subsequent sporulation cycles were conducted accordingly. Each point is a mean of six replicates.

mefenoxam (Fig. 3). The number of sporulation cycles required by a resistant isolate to dominate the populations was correlated with its initial frequency in inoculum mixtures. Regardless of pairings, only three sporulation cycles were required for resistant isolates to reach 100% of the zoospore population with a starting ratio of MR:MS of 4:1 whereas six sporulation cycles were required when the initial proportion was MR:MS 1:4 (Fig. 3).

Discussion

This study shows that mefenoxam-resistance in *P. nicotianae* occurs predominantly on herbaceous annuals, but rarely on perennials. These results support previous studies conducted elsewhere in the USA (Ferrin & Rohde, 1992; Hwang & Benson, 2005). The difference in sensitivity level between isolates from annuals and perennials may be due to the pathogen's varying degrees of exposure to the fungicide and distinct nature of resultant diseases on those two dissimilar categories of hosts. It is common for commercial nurseries in Virginia to grow several crops of different types of herbaceous annuals year-round. For example, annual vinca, begonia and petunia are grown in spring and summer, followed by pansy in the autumn. Many production facilities grow several crops of each individual plant species within each season. *Phytophthora nicotianae* is largely a foliar pathogen on these annuals and disease incidences on those crops are usually high. Several applications of mefenoxam for each crop are required to manage phytophthora diseases. This results in the repeated exposure of *P. nicotianae* to mefenoxam on a regular basis. However, fewer applications of mefenoxam are needed on perennials because *P. nicotianae* mainly causes root and crown rots on those crops (Erwin & Ribeiro, 1996; Benson & von Broembsen, 2001). Therefore, selection pressures for resistance development in *P. nicotianae* are much greater on annual than perennial plants suggesting mefenoxam should be discontinued on herbaceous annuals in nurseries where resistant isolates have been detected.

Mefenoxam-resistant isolates are likely to spread from annual crops onto perennials through irrigation water. This study found that 40% of irrigation water isolates from Virginia were resistant to mefenoxam (Table 1).

Table 3 Fitness component analysis of mefenoxam-resistant and -sensitive isolates of *Phytophthora nicotianae*

Fitness component/Phenotype[a]	Value[g]	LSD[h]	*P*-value
Mycelial growth (mm)[b]			
Resistant	78·1	3·8	0·68
Sensitive	76·2		
Infection rate (% wilted plants)[c]			
Resistant	90·3*	2·4	0·001
Sensitive	84·6		
Sporangium production (No. sporangia per disc)[d]			
Resistant	94·0*	4·2	0·001
Sensitive	81·0		
Zoospore production (No. zoospores per disc)[e]			
Resistant	29640·0*	5892·5	0·009
Sensitive	22758·0		
Zoospore production (No. zoospores per plant)[f]		4587·6	0·003
Resistant	16463·0*		
Sensitive	10191·0		

[a]Phenotype = sensitivity to mefenoxam. Four resistant and four sensitive isolates were tested. Each isolate was replicated five times.
[b]Mean colony diameter (mm) after 5 days of growth in CV8 agar at 25°C in the darkness.
[c]Percentage of wilted lupin seedlings exhibiting symptom of fully infected and dehydrated after 4 days of inoculation.
[d]Number of sporangia produced by each agar disk along peripheral margins after 10 h incubation in SSWE under fluorescent light at 25°C.
[e]Number of zoospores produced per agar disc.
[f]Number of zoospores produced per diseased plant.
[g]*represents a significant difference ($P < 0·05$) between resistant and sensitive isolates.
[h]LSD = least squares difference between resistant and sensitive isolates for a given fitness component.

Those resistant isolates in irrigation water most likely originated from herbaceous annuals. Recycling irrigation water is an efficient means of spreading inoculation of *Phytophthora* spp. from crop to crop, from a single site to entire nursery or farm and from one geographic location to other locations using the same water systems (Kong *et al*., 2003a; Hong & Moorman, 2005). Therefore, sensitivity of the pathogen on perennials should be closely monitored. Many *Phytophthora* species can survive in aquatic environments for a long period of time (Oudemans, 1999; Bush *et al*., 2003, 2006; Hong & Moorman, 2005). Such aquatic scenario allows for the pathogen to have a prolonged period of exposure to sublethal doses of fungicide present in irrigation water and therefore increase the likelihood of resistance development (Davis & Dennis, 1981).

This study demonstrated that *in vitro* mefenoxam screening gives a good indication of the sensitivity of *P. nicotianae* to mefenoxam in ornamental plants. Data obtained through *in vitro* assays corresponded well to that collected from *in vivo* tests (Table 2). *In vitro* screening of isolates using a single concentration of mefenoxam has been the major approach for sensitivity evaluation in *Phytophthora* species (Ferrin & Kabashima, 1991; Timmer *et al*., 1998; Parra & Ristaino, 2001; Taylor *et al*., 2002; Jeffers *et al*., 2004; Hwang & Benson, 2005). However, it has been known that *in vitro* screening of oomycete isolates may not accurately predict the sensitivity to chemicals under *in vivo* conditions (Bruck *et al*., 1980; Joseph & Coffey, 1984; Moorman & Kim, 2004), including a previous study with propamocarb (Hu *et al*., 2007). The inconsistency between *in vitro* and *in vivo* tests may be caused by various factors such as plant-fungicide interactions, aggressiveness of isolates, cultivar susceptibility and methods of fungicide application.

EC_{50} values of mefenoxam-resistant isolates in this study were on average over 1000 times greater in resistant than in sensitive isolates and the label rate (0·078 μL mL^{-1}) for *Phytophthora* disease control. The magnitude of EC_{50} values of resistant isolates in this study were consistent with previously reported values of resistant isolates of *P. nicotianae* from California (Ferrin & Rohde, 1992), North Carolina (Hwang & Benson, 2005) and Florida (Timmer *et al*., 1998). Moderately resistant isolates accounted for 4% of isolates screened (Table 1). Due to the fact that moderately resistant isolates were rare, this study focused on isolates that were either sensitive or highly resistant. However, a recent study using *P. erythroseptica* demonstrated that field application of mefenoxam could not control moderately resistant isolates with EC_{50} values as low as 1·1 μg mL^{-1} (Taylor *et al*., 2006). The biological significance of moderately resistant isolates identified in this study should be addressed in future work.

Mefenoxam-resistant isolates of *P. nicotianae* were more fit than sensitive isolates. Nuclear DNA analysis using 20 RAPD markers and 100 random amplified microsatellite markers revealed that there were many genetic variations between isolates from herbaceous annuals and those from perennials, as well as within each group (unpublished data). Further detailed genetic profiling of these isolates

could give a better picture of the potential population substructuring based on host type (perennial vs. annual), and thereby enhance the interpretation of the results. While sporangium and zoospore productions are measures of an isolate's reproductive ability, other fitness parameters such as chlamydospore and oospore formation ability may also play important roles in the overall fitness of an isolate. The relative degree of each factor's contribution to overall fitness may vary greatly with the particular nature of different disease cycles and pathogen life histories. The usefulness of these results of fitness on lupin seedlings may be limited to the scenario of herbaceous annuals because of similar disease cycle and pathogen life history on those hosts. The results may not be applicable to other hosts, particularly those with root and crown rots in nurseries, because other fitness parameters may be involved in perennials. Due to the selective advantage of resistant isolates, the competition between sensitive and resistant isolates cannot produce an ideal equilibrium where mefenoxam-resistant isolates consist of only a small fraction of the population. Therefore, the use of mefenoxam should be discontinued in nurseries where mefenoxam resistance has been detected. Fungicide application may temporarily prevent infected plants from showing disease symptoms in production facilities, but diseases could develop soon after plants leave nursery lots. It is not uncommon for growers to purchase these latently infected plants which are likely lost shortly after being planted in the landscape. Fungicide resistant populations are also spread around by this process. Therefore, a more robust approach such as an integrated control strategy including using sterile potting mix, nursery hygiene and chlorination of irrigation water is urgently required to substitute the use of mefenoxam for a better disease control in production nurseries.

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