Sensitivity of *Pythium irregulare*, *P. sylvaticum*, and *P. ultimum* from Forest Nurseries to Mefenoxam and Fosetyl-Al, and Control of Pythium Damping-off

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Abstract

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Mefenoxam and fosetyl-Al are common fungicides used to supplement disease control of Pythium damping-off and root rot in forest nurseries of the western United States. However, it is unknown whether fungicide-resistant *Pythium* isolates are present or whether new fungicide and biological treatments might also provide supplemental disease control. Isolates of *Pythium irregulare*, *P. sylvaticum*, and *P. ultimum* from three forest nurseries were evaluated for in vitro sensitivity to mefenoxam and fosetyl-Al. A greenhouse study was also conducted to assess efficacy of fungicide and biological treatments in Douglas-fir (*Pseudotsuga menziesii*) seedlings inoculated with *Pythium dissotocum*, *P. irregulare*, and *P. 'vipa'*. *P. irregulare* was approximately three times less sensitive to mefenoxam (0.20 µg/ml) than *P. sylvaticum*

(0.06 µg/ml) and *P. ultimum* (0.06 µg/ml), and two resistant isolates of *P. ultimum* were identified (≥311 µg/ml). All three *Pythium* spp. were similarly sensitive to fosetyl-Al (1,256 to 1,508 µg/ml) and no resistant isolates were found. In the disease control efficacy trial, both fosetyl-Al and phosphorous acid consistently provided good protection against damping-off caused by *P. dissotocum*, *P. irregulare*, and *P. 'vipa'*. Other treatments, including mefenoxam, also provided good or intermediate protection but efficacy depended upon which *Pythium* sp. was used. Growers should consider rotating mefenoxam use with other fungicide chemistries or biological treatments to prevent further development and spread of mefenoxam-resistant isolates.

Pythium spp. are regarded as one of the most important soil-borne pathogens affecting tree seedling production in bareroot forest nurseries (6). Numerous *Pythium* spp., including *Pythium dissotocum*, *P. irregulare*, *P. mamillatum*, *P. sylvaticum*, and *P. ultimum*, have been isolated from forest nursery soils and seedlings (12,17,35) and are associated with root lesions, damping-off, and root rot (31,36). Disease control is primarily achieved via soil fumigation with a mixture of methyl bromide and chloropicrin or with dazomet (38). However, the fungicide mefenoxam is also frequently applied for supplemental disease control (J. Weiland, unpublished data). Occasionally, phosphonates such as fosetyl-Al (aluminum tris-O-ethyl phosphonate) or phosphorous acid are used (J. Weiland, unpublished data).

Mefenoxam, a phenylamide fungicide and the active R-isomer component of metalaxyl, specifically inhibits ribosomal RNA synthesis in oomycetes (5), whereas phosphonates, usually composed of the active ingredients fosetyl-Al or potassium phosphite, are thought to generally and nonspecifically affect oomycete species by toxicity to multiple nonspecific sites, and also are thought to stimulate certain plant host defenses (11). Mefenoxam resistance has been documented in several *Pythium* spp., including *P. irregulare* and *P. ultimum*, particularly in situations where use of the fungicide is common (24,27,32). However, resistance to metalaxyl has also been found in *Pythium* isolates from fields where metalaxyl use was absent or infrequent (3); therefore, a similar occurrence might arise with the R-isomer mefenoxam. In contrast, comparatively little research has been conducted regarding the presence

of phosphonate resistance in species of *Pythium*. Sanders et al. (29), for example, did not observe mycelial growth inhibition among 25 *Pythium* isolates, including *P. dissotocum* and *P. irregulare*, treated with fosetyl-Al at up to 100 µg/ml. Similarly, Martinez et al. (21) characterized 14 isolates of *P. macrosporum*, *P. sulcatum*, and *P. sylvaticum* as generally insensitive to the fungicide at concentrations ≤100 µg/ml.

Because of the prevalence of soil fumigation in the forest nursery industry (14,17,38), comparatively little research has been focused on the efficacy of fungicides or biological treatments for the control of *Pythium* spp. affecting tree seedling production. Biological treatments (e.g., *Trichoderma*, *Streptomyces*, and *Bacillus* spp.) have been largely unsuccessful or inconsistent (14,20,38). On the other hand, fungicides such as etridiazole or etridiazole plus thiophanate-methyl, mefenoxam, or metalaxyl and the phosphonates have proven effective (16,20), while results with thiram and captan have been mixed (7,20,34). However, most fungicide efficacy trials were conducted prior to the 1980s, and there is little information about the efficacy of newer fungicide chemistries (e.g., the strobilurins) against the *Pythium* spp. that affect forest tree seedlings (20). As the frequency of methyl bromide fumigation continues to decline due to the Montreal Protocol and restrictions for soil fumigation increase, fungicides and other alternative disease control treatments (e.g., biological control, soil amendments, and so on) will likely play an increasingly important role in mitigating tree seedling damage caused by *Pythium* spp. (38).

Despite the widespread use of mefenoxam in forest nurseries, nothing is known about the range of mefenoxam sensitivity or whether mefenoxam resistance is present among *Pythium* spp. and isolates that are associated with tree seedling production. The same information is also lacking for the phosphonate fungicides. The effectiveness of mefenoxam and fosetyl-Al for supplemental control of tree seedling damping-off and root rot might be compromised if resistance to these commonly used fungicides develops. There may also be risk for the movement of fungicide-resistant isolates to new locations because nursery stock and equipment are frequently moved among nurseries. Therefore, the objectives of

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this research were to (i) determine the range of sensitivity among forest nursery isolates of *P. irregulare*, *P. sylvaticum*, and *P. ultimum* to mefenoxam and fosetyl-Al; (ii) determine whether species and locational differences in mefenoxam and fosetyl-Al sensitivity exist among *P. irregulare*, *P. sylvaticum*, and *P. ultimum*; (iii) determine whether there are isolates of *P. irregulare*, *P. sylvaticum*, and *P. ultimum* that are resistant to mefenoxam and fosetyl-Al; and, (iv) compare the efficacy of mefenoxam to other fungicide chemistries and biological treatments for the potential to reduce damping-off severity of Douglas-fir (*Pseudotsuga menziesii*) seedlings.

Materials and Methods

Pythium isolates. *Pythium* isolates were originally obtained from the soil at three forest nurseries in Oregon and Washington in 2008 (35). Nursery A was located in southwestern Washington while nurseries B and C were located in northwestern Oregon. Isolates were obtained by dilution plating a soil solution onto PARP agar, a semiselective medium for Pythiaceae species (19), or by baiting submerged soil with rhododendron leaf disks and Douglas-fir needles (35). The isolates were then identified to species using the DNA sequence from the internal transcribed spacer (ITS) region and by morphology, according to methods described previously (35). All isolates were stored on colonized corn or water agar plugs in sterile water at 20°C and grown as active cultures on plates containing 20 ml of potato dextrose agar (PDA).

Fungicide sensitivity assay. In all, 117 isolates of *Pythium irregulare*, *P. sylvaticum*, and *P. ultimum* were selected as representatives of three highly virulent (aggressive) pathogens of Douglas-fir, the most common tree species grown in forest nurseries of Oregon and Washington (36). Virulence, as used here, refers to the quantitative, relative ability of each isolate to cause disease. *P. irregulare* was isolated from all three forest nurseries (nursery A = 20 isolates, nursery B = 18 isolates, and nursery C = 11 isolates) whereas *P. sylvaticum* (nursery B = 17 isolates and nursery C = 20 isolates) and *P. ultimum* (nursery B = 12 isolates and nursery C = 19 isolates) were only isolated from two nurseries (Table 1). In addition, the three *Pythium* spp. used in the fungicide and biological treatment efficacy assay (one isolate each of *P. dissotocum*, *P. irregulare*, and *P. 'vipa'*, below) were also assessed for their sensitivity to mefenoxam and fosetyl-Al.

The sensitivity of each isolate was evaluated in 84-mm-diameter petri plates containing 16 ml of V8 juice agar amended with either mefenoxam (Subdue MAXX, 22% a.i.; Syngenta Crop Protection: 0.1, 1.0, 5.0, 10.0, and 100 µg a.i./ml) or fosetyl-Al (Aliette WDG, 80% a.i.; Bayer CropScience LP: 1, 500, 1,000, 1,500, 2,000, and

2,500 µg a.i./ml). The recommended label dose for Subdue MAXX was 19 to 38 µg a.i./ml for foliar application or 758 to 1,516 µg a.i./ml for soil surface application. For Aliette WDG, the recommended dose was 2,400 to 4,800 µg a.i./ml for foliar application or 384 to 768 µg a.i./ml for soil drenches. Plates containing nonamended V8 juice agar were used as negative controls. V8 juice agar was prepared with either 12 g of V8 juice broth (VWR International) and 15 g of agar/liter of distilled water or with 3.4 g of CaCO₃ per 340 ml of V8 filtered through eight layers of cheesecloth, diluted 1:4 with distilled water and 17 g of agar/liter. For each isolate-treatment combination, a single 5-mm-diameter colonized agar plug was cut from the margin of a 4- to 6-day-old culture and then placed into the center of each replicate treatment plate. After 2 or 3 days of incubation (for mefenoxam or fosetyl-Al, respectively) at 22 ± 1°C, the diameter of each culture was measured in two perpendicular directions to the nearest mm. Then, after 7 days of incubation at 22 ± 1°C, a 5-mm-diameter colonized agar plug was removed from each replicate plate of each isolate and transferred to a nonamended plate of V8 juice agar to evaluate whether mortality had occurred at each fungicide concentration level. Isolates that did not resume growth from the transferred, colonized agar plug within 2 weeks were rated as dead. All treatments, including the negative control, were replicated three times, and the entire experiment was repeated.

Fungicide and biological treatment efficacy assay. In order to address concerns about the management of the most commonly isolated *Pythium* spp. at each of the three forest nurseries (35), one isolate each of *P. dissotocum* (isolate 41-08, nursery C), *P. irregulare* (isolate 45-08, nursery A), and *P. 'vipa'* (isolate 09-08, nursery B) were evaluated for efficacy in control by specific fungicide or biological treatments. The isolate designated as *P. 'vipa'* had an ITS sequence with 99% maximum sequence identity to the ITS region of isolate 96-224 (DQ528743.1) in GenBank, which was originally assigned the name *P. 'vipa'* (13). Inoculum for each isolate was produced using a method modified from previous experiments (36,37) by placing 3 liters of dry coarse vermiculite and 2.1 liters of dilute, clarified V8 juice (6.8 g of CaCO₃ per 340 ml of V8 juice, filtered through eight layers of cheesecloth, then mixing clarified V8 juice at 75 ml/liter of distilled water) into fungal spawn bags (Fungi Perfecti). Bags were then autoclaved three times at 48-h intervals, and a single *Pythium* isolate was placed into each bag by cutting an entire plate containing a 1-week-old culture grown on 20 ml of V8 juice agar into approximately 1.5-cm² pieces. Spawn bags were heat sealed and then incubated at 20°C in the dark for 60 days with weekly mixing by hand to main-

Table 1. Fungicide sensitivity of *Pythium irregulare*, *P. sylvaticum*, and *P. ultimum* to mefenoxam and fosetyl-Al

Species, nursery ^y	Number of isolates	EC ₅₀ µg/ml ± SE (range) ^x	
		Mefenoxam	Fosetyl-Al
<i>P. irregulare</i>			
A	20	0.19 ± 0.012 (0.07–0.51) a	1,239 ± 11.5 (1,082–1372) a
B	18	0.25 ± 0.016 (0.07–0.56) a	1,257 ± 12.3 (990–1378) a
C	11	0.13 ± 0.016 (0.05–0.44) a	1,285 ± 13.0 (1,211–1412) a
All three nurseries	49	0.20 ± 0.009 (0.05–0.56) A	1,256 ± 7.2 (990–1412) A
<i>P. sylvaticum</i>			
B	17	0.07 ± 0.006 (0.05–0.17) a	1,288 ± 9.0 (1,172–1376) a
C	20	0.06 ± 0.001 (0.05–0.07) a	1,335 ± 11.9 (1,180–1567) a
Both nurseries	37	0.06 ± 0.003 (0.05–0.17) B	1,314 ± 7.8 (1,172–1567) A
<i>P. ultimum</i> ^z			
B	11 (12)	0.05 ± 0.001 (0.05–0.06) a	1,525 ± 16.4 (1,406–1649) a
C	18 (19)	0.07 ± 0.007 (0.05–0.37) a	1,498 ± 9.1 (1,407–1652) a
Both nurseries	29 (31)	0.06 ± 0.05 (0.05–0.37) B	1,508 ± 8.4 (1,406–1652) A

^x EC₅₀ is the concentration of fungicide required to reduce the culture diameter by half as compared with the nonamended control plate; *n* = six replicate plates/isolate. Means followed by the same letter are not significantly different according to Tukey's test at *P* = 0.05 among locations within each species separately (lowercase letters) or among species (uppercase letters).

^y Nursery A is located in southwestern Washington and nurseries B and C are located in northwestern Oregon.

^z Means and mean separation tests for mefenoxam do not include two *P. ultimum* isolates (one each from nursery B and C) that were identified as outliers based on Dixon's test. These two isolates were not resistant to fosetyl-Al and, therefore, were included in means and means separation tests for fosetyl-Al. The number of isolates used to calculate mean *P. ultimum* EC₅₀ values are indicated in front of the parentheses for mefenoxam and within the parentheses for fosetyl-Al.

tain adequate aeration. Inoculum was removed from the bags, air dried for 2 days, and stored in resealable polyethylene bags at 20°C until used to infest soilless potting media.

In all, 11 fungicide or biological treatments and 3 control treatments were evaluated (Table 2) using methods adapted from Linderman et al. (20). The three control treatments were (i) a nontreated, inoculated control for each *Pythium* sp. (no fungicide or biological treatment to establish baseline seedling mortality associated with each *Pythium* spp.); (ii) a nontreated, noninoculated control (healthy check to establish average seed germination in the absence of disease); and (iii) a treated, noninoculated control (to evaluate each fungicide or biological treatment for potential phytotoxicity in the absence of disease). For the fungicide treatments, soilless potting media (OBC Northwest Seedling Mix number 1; OBC Northwest Inc.) was infested with each respective *Pythium* isolate (10% [vol/vol] vermiculite inoculum/potting media), distributed into eight replicate 450-cm³ polystyrene pots per treatment, then moistened slightly with water and allowed to rest for 5 days. Final population counts for each *Pythium* sp. in the infested soil were approximately 3,000 propagules per gram (ppg) of dry potting media. Fungicide treatments were then applied according to the rates in Table 2. For the two biological control treatments only (*Trichoderma* and *Trichoderma* + phosphorous acid), the potting media was first drenched with the respective biological control agent (*Trichoderma harzianum* strain T-22 and *T. virens* strain G-41) at the recommended rate 3 days prior to infesting the soilless media with the respective *Pythium* isolate (Table 2). Infested potting media was then distributed into eight replicate 450-cm³ polystyrene pots per treatment. At this time, 10 cold-stratified Douglas-fir seeds were planted into each pot and the entire experiment was arranged according to a randomized complete block design with eight replicate blocks. Finally, phosphorous acid was applied to the *Trichoderma* + phosphorous acid treatment 14 days later, and select fungicides were reapplied after 14 to 28 days according to the schedule in Table 2. The entire experiment was repeated once. A preliminary report of the first experimental repeat has been published (9).

Data analyses. For the in vitro fungicide sensitivity experiment, the concentration of mefenoxam or fosetyl-Al required to reduce the culture diameter of each isolate by 50% (EC₅₀ value) was calculated from plots of the culture diameters at each fungicide concentration. Isolates with mefenoxam EC₅₀ values ≥100 µg/ml are considered resistant to that fungicide (24). A similar value has not been established for fosetyl-Al with *Pythium* spp., although many

are considered insensitive to the fungicide with EC₅₀ values of 100 to ≥500 µg/ml (21, 29, 33). EC₅₀ values were analyzed by three-way analysis of variance (ANOVA) for effects of trial, species, isolate, and factor interactions using trial and isolate (nested within species) as random factors and species as a fixed factor. Because two isolates of *P. ultimum* (isolates Ult A2-2 and Ult C2-7) tested as outliers for resistance to mefenoxam (Dixon's test) and significantly skewed ANOVA results, these two isolates were omitted from further ANOVA tests involving mefenoxam sensitivity (30). For each *Pythium* sp. separately, isolates were further evaluated for an effect of location on fungicide sensitivity by including nursery as a fixed factor in the ANOVA model. Treatment means for all analyses were separated using Tukey's test at *P* = 0.05.

For the in vivo fungicide efficacy experiment, the ability of each fungicide to reduce the incidence of damping-off was assessed by recording the number of healthy seedlings remaining in each replicate pot for each treatment 5 weeks after planting. These data were then converted into proportions (number of remaining healthy seedlings in each fungicide or biological treatment per average number of seedlings in the nontreated noninoculated control treatment) and then transformed by the arcsine of the square root to homogenize variances. Transformed data were analyzed for effects of trial, fungicide treatment, and interaction using two-way ANOVA, with trial as a random factor and fungicide treatment as a fixed factor. Fungicide and biological control treatments were also rated as providing good, intermediate, or poor protection against each *Pythium* isolate based on whether the percentage of surviving seedlings was statistically similar (*P* ≥ 0.05) to the nontreated, noninoculated control (good protection); similar to the nontreated, inoculated control (poor protection); or was significantly different (*P* < 0.05) from either control treatment (intermediate protection). Treatment means were separated using Tukey's test at *P* = 0.05. All analyses were conducted with Minitab Statistical Software (release 15; Minitab Inc.) or SAS 9.2 (SAS Institute Inc.).

Results

Fungicide sensitivity assay. As a species, *P. irregulare* was approximately three times less sensitive (EC₅₀ = 0.20 µg/ml) to mefenoxam than either *P. sylvaticum* or *P. ultimum* (EC₅₀ = 0.06 µg/ml for both species, (*P* < 0.001; Table 1). However, there was considerable variation in sensitivity among *P. irregulare* isolates (*P* < 0.001), with 16/49 (33%) of the *P. irregulare* isolates exhibiting the same low EC₅₀ values (0.05 to 0.09 µg/ml) that were found among the majority of *P. sylvaticum* and *P. ultimum* isolates; the

Table 2. Effect of fungicide and biological treatments on survival of Douglas-fir seedlings grown in soilless potting media infested with one of three *Pythium* spp.

Active ingredients	Trade name	Rate/liter	Reapplied (days) ^z	Surviving seedlings (%) (SE) ^y		
				<i>P. dissotocum</i> isolate 41-08	<i>P. irregulare</i> isolate 45-08	<i>P. 'vipa'</i> isolate 09-08
Noninoculated control	...	...	...	100 (7.5) a	100 (7.5) a	100 (7.5) a
Phosphorous acid	Alude	1.00 ml	14	71 (4.4) ab	67 (8.2) ab	79 (6.9) ab
Fosetyl-Al	Aliette WP	0.72 g	...	58 (7.3) abc	69 (8.8) ab	55 (7.1) abc
Mefenoxam	Subdue MAXX	0.08 ml	21	61 (5.7) abc	60 (8.8) abc	47 (6.9) bc
<i>Trichoderma virens</i> G-41	RootShield Plus	0.45 g	...	35 (6.7) cd	63 (8.6) ab	44 (8.6) bc
<i>T. harzianum</i> T-22	...	...	...	...	...	...
Azoxystrobin	Heritage	0.07 g	21	57 (7.4) bc	36 (6.5) bcd	37 (5.3) bcd
Fluopicolide	Adorn 4FL	0.16 ml	14, 28	21 (4.6) d	26 (6.2) cde	46 (8.6) bc
<i>T. virens</i> G-41	RootShield Plus	0.45 g	...	39 (5.6) bcd	33 (7.5) bcde	31 (6.8) cd
<i>T. harzianum</i> T-22 + phosphorous acid	+ Alude	+1.00 ml	...	...	...	...
Fluoxastrobin	Disarm 480SC	0.03 ml	14, 28	30 (4.3) cd	15 (4.7) de	24 (4.9) cd
Caprylic acid	Cg100	0.75 ml	...	31 (5.9) cd	16 (4.0) de	21 (3.7) cd
Pyraclostrobin + boscalid	Pageant 38WG	0.90 g	14, 28	16 (5.0) d	18 (5.2) de	16 (4.3) cd
Fenamidone	Fenstop	0.78 ml	28	14 (4.3) d	19 (5.3) de	14 (3.7) cd
Inoculated control	...	...	...	10 (2.9) d	6 (2.8) e	4 (1.7) d

^y Mean percent surviving seedlings as a function of the nontreated, noninoculated control; *n* = 10 Douglas-fir seed in each of 16 replicate pots; SE = standard error. Seed were planted into pots containing soil infested with one of three *Pythium* spp. and treated with 1 of 11 fungicide or biological treatments. A nontreated, noninoculated control (Noninoculated control) and a nontreated, inoculated control (Inoculated control) were also included. Means within each column that share a letter are not significantly different according to Tukey's test at *P* = 0.05.

^z Select fungicides were reapplied at 14, 21, or 28 days after initial treatment.

remaining *P. irregulare* EC₅₀ values ranged up to 0.56 µg/ml. In comparison, there was no variation in sensitivity among isolates of *P. sylvaticum* ($P = 0.310$) and little variation among isolates of *P. ultimum* ($P < 0.001$). Most *P. sylvaticum* isolates (28/31 isolates or 90%) and *P. ultimum* isolates (35/37 or 95%) had EC₅₀ values between 0.05 and 0.09 µg/ml. However, two isolates of *P. ultimum*, isolate Ult A2-2 from nursery B and isolate Ult C2-7 from nursery C, were approximately 5,000 to 6,000× more resistant to mefenoxam (EC₅₀ values of 375 and 311 µg/ml, respectively) than all other *P. ultimum* isolates except isolate Ult C2-9 from nursery C, with an EC value of 0.37 µg/ml. Within each species separately, there was no evidence that isolate sensitivity to mefenoxam differed by location ($P \geq 0.218$). Though culture diameter growth was inhibited completely with mefenoxam at 100 µg/ml for all *Pythium* isolates except Ult A2-2 and Ult C2-7, all isolates resumed growth when transferred to plates containing nonamended media. Therefore, none of the isolates were killed when exposed to mefenoxam at 100 µg/ml. No trial effect ($P = 0.583$) or trial-species interaction ($P = 0.916$) was observed in the analyses. A small trial-isolate interaction was observed ($P < 0.001$) but results were similar when each trial was analyzed separately (*data not shown*).

In contrast to the mefenoxam sensitivity assay, all three *Pythium* spp. were similarly sensitive to fosetyl-Al ($P = 0.060$; Table 1). There was relatively little variation in fosetyl-Al sensitivity observed among isolates of *P. irregulare* and *P. sylvaticum* ($P \leq 0.010$) and no variation among isolates of *P. ultimum* ($P = 0.440$). No resistant isolates were found for any of the three *Pythium* spp. and the two *P. ultimum* isolates that were highly resistant to mefenoxam, Ult A2-2 and Ult C2-7, were sensitive to fosetyl-Al. The EC₅₀ values for these two isolates (1,513 and 1,390 µg/ml, respectively) were similar to those of all other *P. ultimum* isolates tested on fosetyl-Al (1,406 to 1,652 µg/ml). Within each species separately, there was no evidence that isolate sensitivity to fosetyl-Al differed by location ($P \geq 0.125$). Culture diameter growth was inhibited completely with fosetyl-Al at 2,500 µg/ml for 47/49 (96%) of *P. irregulare* isolates and 35/37 (95%) of *P. sylvaticum* isolates. All except three *P. irregulare* isolates resumed growth when transferred to plates containing nonamended media. In contrast, the culture diameter growth of only 9/31 (29%) of *P. ultimum* isolates was inhibited completely with fosetyl-Al at 2,500 µg/ml. All except one isolate of *P. ultimum* resumed growth when transferred to plates containing nonamended media. Therefore, only four *Pythium* isolates were killed when exposed to fosetyl-Al at 2,500 µg/ml. No trial effect was observed ($P = 0.419$). Although small trial-species and trial-isolate interactions were observed ($P < 0.001$), results were similar when each trial was analyzed separately (*data not shown*).

Fungicide and biological treatment efficacy assay. The sensitivity of each of the three *Pythium* isolates used in the fungicide and biological treatment assay (*P. dissotocum* 41-08, *P. irregulare* 45-08, and *P. 'vipa'* 09-08) to mefenoxam and fosetyl-Al was comparable with the EC₅₀ values found for *P. irregulare*, *P. sylvaticum*, and *P. ultimum* isolates in the fungicide sensitivity assay. *P. irregulare* isolate 45-08 (nursery A) was similar in sensitivity to mefenoxam and fosetyl-Al (0.19 ± 0.05 and $1,097 \pm 9.7$ µg/ml, respectively) compared with all other *P. irregulare* isolates from nursery A (Table 1). In contrast, *P. dissotocum* isolate 41-08 and *P. 'vipa'* isolate 09-08 were more sensitive to mefenoxam (0.009 ± 0.0002 and 0.007 ± 0.0001 µg/ml, respectively) than all other isolates tested, regardless of species. However, these two isolates were just as sensitive to fosetyl-Al ($1,162 \pm 10.8$ and $1,119 \pm 9.7$ µg/ml, respectively) as all other *Pythium* isolates tested, regardless of species.

P. dissotocum, *P. irregulare*, and *P. 'vipa'* caused similar amounts of damping-off on Douglas-fir seed planted in the nontreated, inoculated control pots. *P. dissotocum* reduced seedling survival by 90%, *P. irregulare* by 94%, and *P. 'vipa'* by 96%. Two fungicide treatments, phosphorous acid and fosetyl-Al, consistently provided good protection against damping-off, regardless of which *Pythium* spp. was used, and seedling survival in both treatments

was similar to that of the nontreated, noninoculated control ($P \geq 0.050$; Table 2). A similar level of protection was also provided by mefenoxam against *P. dissotocum* and *P. irregulare* but not against *P. 'vipa'*. Likewise, the *Trichoderma* biological treatment (consisting of *T. harzianum* and *T. virens*) provided good protection against *P. irregulare* but not against *P. dissotocum* or *P. 'vipa'*. Intermediate levels of protection were provided by mefenoxam against *P. 'vipa'*, by the *Trichoderma* biological treatment against *P. 'vipa'*, by fluopicolide against *P. 'vipa'*, and by azoxystrobin against both *P. dissotocum* and *P. irregulare*. All other fungicide or biological control treatments were no different than the nontreated, inoculated control pots and, therefore, provided poor protection against damping-off by these three *Pythium* spp. No effects of reduced germination or phytotoxicity were observed for any of the 11 fungicide or biological treatments in the treated noninoculated controls (*data not shown*). Although trial effects were observed ($P < 0.001$), the differences were due to increased germination in trial 2 compared with trial 1 and results were consistent within each trial separately (*data not shown*). No trial-treatment interactions were observed ($P \geq 0.088$).

Discussion

The range of mefenoxam sensitivity observed among *Pythium* spp. and isolates of forest nurseries was consistent with those reported from mefenoxam studies in other agricultural systems. In Idaho, Oregon, and Washington, the mefenoxam sensitivity of 68 *P. ultimum* isolates obtained from infected potato tubers ranged from <0.05 to >100 µg/ml, with 64 isolates having EC₅₀ values <1.0 µg/ml (32). Wheeler et al. (39) found that *Pythium* spp. from peanut ranged in sensitivity to mefenoxam from 0.001 to 0.270 µg/ml, and isolates of *P. irregulare* were less sensitive to mefenoxam (0.049 to 0.173 µg/ml) than those of *P. ultimum* (0.005 and 0.006 µg/ml), which was similar to our observations. However, the number of isolates evaluated for each species was extremely limited (two and five isolates, respectively) and another study found no differences in sensitivity between *P. irregulare*, *P. sylvaticum*, and *P. ultimum* from corn and soybean (4). Similar mefenoxam EC₅₀ values have been reported from *Phytophthora* spp., including *Phytophthora capsici* (0.12 to 863 µg/ml), *P. drechsleri* (initially reported as *P. cryptogea*, 0.1 to 549.5 µg/ml), and *P. erythrosepica* (<1.0 to 205 µg/ml) (15,25,26,32).

Less information is available about the range of fosetyl-Al sensitivity among *Phytophthora* and *Pythium* spp. For carrot cavity spot, Martinez et al. (21) found that the sensitivity of three *Pythium* spp. (*Pythium macrosporum*, *P. sulcatum*, and *P. sylvaticum*) to fosetyl-Al ranged from 97.6 to >500 µg/ml. All three *P. sylvaticum* isolates from that study had EC₅₀ values >500 µg/ml and were considered insensitive to the fungicide. In turfgrass, eight *Pythium* spp., including *P. dissotocum* and *P. ultimum*, were not inhibited by concentrations of fosetyl-Al up to 100 µg/ml (29). However, higher concentrations of the fungicide were not evaluated and final EC₅₀ values were not reported. Therefore, it is not known how the sensitivity of those isolates compares with *P. ultimum* isolates in our study. Similar fosetyl-Al EC₅₀ values have been reported for *Phytophthora* spp., including *Phytophthora citrophthora* (24 to 285 µg/ml), *P. infestans* (62 to 1,021 µg/ml), and *P. parasitica* (1,146 µg/ml) (2,8,22).

The presence of two mefenoxam-resistant isolates of *Pythium ultimum*, one at each Oregon nursery, indicates that there is risk that the fungicide may fail to provide adequate disease control in the future, especially if mefenoxam continues to be the primary fungicide used for supplemental disease control in forest nurseries. In other crops, mefenoxam (or metalaxyl) resistance has been discovered in many of the *Pythium* spp. that are also known to occur in forest nurseries, including *P. dissotocum*, *P. irregulare*, *P. sylvaticum*, and *P. ultimum* (1,21,24,32). Moorman et al. (24), for example, found that 36% (21/59 isolates) of the *P. irregulare* isolates collected from greenhouse floral crops in Pennsylvania were resistant to mefenoxam as well as a single isolate each of *P. dissotocum* and *P. ultimum*. In contrast, Taylor et al. (32) rarely found

resistant *P. ultimum* isolates in their survey of infected potato tubers. Only 3% (2/68 isolates) of the isolates from Idaho, Oregon, and Washington were characterized as mefenoxam resistant. Previous work on the related oomycete genus *Phytophthora* also established that resistance to metalaxyl could result in detectable selection for fungicide resistance within a single growing season (10). These prior results support our concern for risk in continued use of mefenoxam in seedling nurseries for control of *Pythium* spp.

There is little equivalent data for fosetyl-Al resistance in *Pythium* spp. Although Martinez et al. (21) characterized *P. sylvaticum* isolates as not sensitive based on EC₅₀ values that were >500 µg/ml, it is not known how well these values correspond to actual disease control. Sanders et al. (29), however, reported that fosetyl-Al soil drenches were ineffective at reducing *Pythium* blight severity on perennial ryegrass inoculated with *P. aphanidermatum* isolates that were insensitive to the fungicide at 100 µg/ml (29). On the other hand, foliar sprays with fosetyl-Al were somewhat effective at reducing disease severity, which may have been indicative of the fungicide's ability to stimulate host defenses instead of being directly toxic to the pathogen. Similar reports of reduced sensitivity to fosetyl-Al have also been reported from *Pythium* spp. on turfgrass in Italy (33). Finally, resistance to fosetyl-Al was induced in a field isolate of *P. aphanidermatum* via chemical mutagenesis with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (28). The resultant mutant isolates had EC₅₀ values of 3,000 to 4,700 µg/ml for phosphorous acid and were capable of causing disease on creeping bentgrass treated with fosetyl-Al. In our study, all EC₅₀ values for fosetyl-Al were ≥990 µg/ml but the fosetyl-Al soil drench was still one of the most effective treatments to control Douglas-fir damping-off in the fungicide and biological treatment efficacy assay.

Our results from the fungicide and biological treatment efficacy assay confirm that the phosphonates and mefenoxam continue to be effective for reducing Douglas-fir damping-off caused by *Pythium* spp. (16,20). In contrast to Linderman et al. (20), we found that azoxystrobin provided intermediate protection against *P. irregulare* (as well as *P. dissotocum*). Fluopicolide also provided intermediate protection but only against *P. 'vipa'*. To our knowledge, this is the first time that this fungicide has been tested against *Pythium* spp. from forest nurseries. Finally, we found that the biological treatment consisting of *T. virens* strain G-41 and *T. harzianum* strain T-22 provided good protection against *P. irregulare*, intermediate protection against *P. 'vipa'*, but no protection against *P. dissotocum*. However, *T. harzianum* strain T-22 alone was ineffective against damping-off caused by a different isolate of *P. irregulare* in the Linderman et al. (20) study. These differences in efficacy among *Pythium* spp. and isolates might partially explain why disease control with *T. harzianum* has been lacking in the field where multiple *Pythium* spp. and isolates are present (18). The addition of *T. virens* strain G-41 to *T. harzianum* strain T-22 might also have improved efficacy against *P. irregulare* specifically. However, further studies are needed to elucidate how *Pythium* spp. and isolate diversity affects biological control.

In contrast to the results from our previous pathogenicity research, where *P. dissotocum* isolate 41 and *P. 'vipa'* isolate 9 caused little to no Douglas-fir seedling mortality (36), over 90% seedling mortality was observed with both isolates in the present study. *P. irregulare* also caused much greater seedling mortality in this study (90%) than was observed previously (38%). This was likely due to the much greater inoculum pressure obtained in the present study (approximately 3,000 ppg with the 10% inoculum amendment rate) than was used in our preceding study (100 ppg), and suggests that both *P. dissotocum* and *P. 'vipa'* may act as aggressive pathogens when high inoculum densities are present. The inoculum amendment rate used in this study was the same as that used by Linderman et al. (20) to evaluate fungicide and biological treatments for efficacy in controlling damping-off of Douglas-fir seedlings with a different isolate of *P. irregulare*. In that study, seedling survival was reduced up to 45% in infested soil without a fungicide or biological treatment as compared with noninoculated controls. However, the final count of *Pythium* propagules in in-

festes soil was not reported; therefore, a direct comparison of seedling mortality as a function of inoculum density is not possible. Although the inoculum density in this study was greater than that usually found in the field (<300 ppg), *Pythium* populations can increase dramatically after incorporating organic matter into the soil. For example, populations of up to 1,100 ppg were observed in field soils following a pea cover crop at a forest nursery in Washington (12), and populations of up to 4,486 ppg were noted in apple orchard soils amended with *Brassica napus* seed meal (23). Nevertheless, the efficacy of mefenoxam, fosetyl-Al, and phosphorous acid in reducing damping-off under high inoculum pressure in this study is consistent with the efficacy observed from other forest nursery fungicide studies (16,20). It is likely that these fungicides will continue to perform satisfactorily against the lower inoculum levels that are usually found in the field as long as a substantial proportion of the *Pythium* community at each nursery does not develop resistance to each fungicide.

The mefenoxam and fosetyl-Al data presented here establish the baseline range of sensitivity for three highly virulent *Pythium* spp.: *P. irregulare*, *P. sylvaticum*, and *P. ultimum*. This information will be useful in monitoring for changes in fungicide sensitivity in forest nursery *Pythium* communities and will allow growers to determine whether the proportion of resistant isolates increases over time. This study also provides alternative fungicide and biological treatment options for the control of *Pythium* spp. If mefenoxam resistance becomes more prevalent in the forest nursery industry, these treatments may help provide supplemental control. However, none of the alternative treatments tested in our study provided as much protection as either mefenoxam or the phosphonates. Therefore, growers should focus on rotating fungicides with different modes of action to help reduce the further development of fungicide-resistant isolates.

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