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PYTHIUM SPECIES ASSOCIATED WITH FOREST TREE NURSERIES OF OREGON AND WASHINGTON

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ABSTRACT

Pythium species are one of several pathogen genera responsible for damping-off of conifer seedlings in forest tree nurseries. Field trials were established in 2008 at three nurseries (2 in OR, 1 in WA) to: 1) evaluate the impact of lower application rates of fumigants on *Pythium* soil populations; and 2) identify *Pythium* species associated with damping-off. Six fumigant treatments (including a conventional methyl bromide treatment and a nonfumigated control) were applied according to a randomized complete block design with four blocks at each nursery. Soil samples were collected before and after fumigation and *Pythium* populations were assessed by baiting with rhododendron leaves and Douglas-fir needles and by dilution plating onto PARP, a semiselective medium for pythiaceous species. Isolates were identified based on their ITS sequence. Prior to fumigation, populations averaged 40-45 cfu/g soil at nurseries A and B and 19 cfu/g soil at nursery C. All fumigant treatments reduced soil populations by at least 86% and populations were similar 7 months after fumigation. Of the 450 isolates identified to date, 42% are *P. irregulare*, 27% are *P. dissotocum*, 10% are *P. macrosporum*, and the remaining 21% are composed of 12 different *Pythium* species.

INTRODUCTION

Forest tree nurseries of the Pacific Northwest (Idaho, Oregon, and Washington) produce approximately 200 million conifer seedlings annually. Most of the conifer seedlings grown and sold are two-year-old Douglas-fir transplants (*Pseudotsuga menziesii*). In the absence of fumigation, production can be severely limited by several root pathogens, including *Pythium* species, which cause damping-off and root rot. These pathogens infect at or below the soil line and kill young, succulent tissues of seedlings.

Traditionally, management of soilborne pathogens has been accomplished by fumigation with 392 kg/ha of methyl bromide/chloropicrin 67:33 under a critical use exemption permit. However, methyl bromide use will eventually cease as stocks become depleted under the Montreal Protocol and fumigants are becoming increasingly regulated due to safety concerns. A field study was conducted to compare disease control efficacy of lower rates of alternative fumigants to the traditional application of methyl bromide. The objectives of this portion of the study are to assess: 1) the impact of lower rates of fumigants on *Pythium* soil populations; and 2) identify the *Pythium* species associated with damping-off of Douglas-fir seedlings.

METHODS

Field trials were established at two forest nurseries in Oregon and one forest nursery in Washington. Six fumigant treatments (including a conventional methyl bromide/chloropicrin treatment and a nonfumigated control) were applied in a randomized complete block design with four replicate blocks at each nursery in early August 2008 (Table 1). Each treatment plot was approximately 12 × 46 m (nonfumigated control plots 12 × 30 m). Soil samples were collected before (1 week) and after fumigation (1 and 7 months) by taking 20 soil cores in a randomized pattern to a depth of 30 cm from each treatment plot within a block. Soil samples were bulked within each treatment by block and nursery to create composite samples.

Table 1. Fumigant treatments at each of three forest tree nurseries.

Fumigant	Application Rate	Plastic Film Type
Methyl Bromide/Chloropicrin	392 kg/ha(67:33)	High Density Polyethylene
Methyl Iodide/Chloropicrin	274 kg/ha (50:50)	High Density Polyethylene
Methyl Iodide/Chloropicrin	274 kg/ha (50:50)	Virtually Impermeable Film
Metam Sodium/Chloropicrin	467 l/ha + 137 kg/ha	Virtually Impermeable Film
Dimethyl Disulfide/Chloropicrin	561 l/ha + 135 kg/ha	Virtually Impermeable Film
Untreated	none	none

To sample for *Pythium* species, ten grams from each composite sample were added to 90 ml of 0.2% water agar and shaken for 45 minutes at 1500 rpm. Aliquots of the suspension (0.5 ml) were then spread on 10 petri plates containing PARP, a semiselective medium for Pythiaceous species (Erwin and Ribiero 1996). Plates were incubated at room temperature for two days and the number of *Pythium* isolates per plate was counted. The assay was conducted twice (two trials) for each of the three time periods of soil collection (1 week before and 1 and 7 months after fumigation).

Pythium species were also assayed using the double-cup leaf disk baiting method from Linderman and Zeitoun (1977). Briefly, 15 ml of each composite soil sample were placed in a 150-ml wax paper cup. A second wax paper cup with its bottom replaced by a double layer of cheesecloth was positioned firmly on top of the sample and 50 ml of distilled water were added. Leaves of *Rhododendron* 'Nova Zembla' and needles of Douglas-fir were then used to bait for *Pythium* species. Leaves and needles were initially rinsed in running tap water for 10 minutes and then surface disinfested by immersing in 0.06% sodium hypochlorite for 10 minutes. After air drying, 10 5-mm-diameter *Rhododendron* leaf disks or 10 split Douglas-fir needles were floated on the water surface in each cup at room temperature. After 48 hours, disks and split needles were removed from the cups with sterile forceps, blotted dry on clean paper towels, and plated on PARP. Plates were incubated at room temperature for two days and the number of *Pythium* isolates per plate was counted. The assay was conducted twice (two trials) for each of the three time periods of soil collection.

A subset of isolates (up to three isolates per composite soil sample, when available) was identified on the basis of the internal transcribed spacer (ITS) region. Genomic DNA was extracted by using a procedure modified from Martin and Semer (1997). Briefly, cultures of each *Pythium* isolate were grown on 20 ml of 10% clarified V8 juice agar for 3 days (1 g $CaCO_3$ per 100 ml V8 juice strained through eight layers of cheesecloth. Mix 100 ml clarified V8 juice in 900 ml distilled water and 17 g agar). A small amount of hyphae (< 1

mm^3) was then removed from each culture with a sterile toothpick and incubated at 95.9 C for 5 minutes. Ten microliters of the DNA extract was then added to a 40 µl PCR reaction mixture containing 20 µl 2.5x 5 Prime HotMasterMix (5 Prime Inc., Gaithersburg, MD, USA), 18 µl sterile water, and 1 µl each of 10mM primers ITS1 and ITS4 (White and others 1991). Amplification was performed in a Veriti Thermal Cycler (Applied Biosystems Inc, Foster City, CA, USA) with the following temperature profile: one cycle of 1 minute at 95°C; 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C; and 10 min at 72°C. PCR products were separated by electrophoresis on a 0.7% agarose gel in 1x TAE buffer. Gels were stained with ethidium bromide and photographed under UV light.

RESULTS

Average prefumigation counts by dilution plating of *Pythium* for each nursery (all results reported are from trial 1 only) were greatest at nursery B (45 cfu/g dry soil) and A (40 cfu/g dry soil), and least at nursery C (19 cfu/g dry soil). Dilution plate counts of *Pythium* in all fumigant treated plots were reduced by at least 86% one month after fumigation, or by at least 95% seven months after fumigation (Fig. 1). Analyses of variance on the counts of *Pythium* indicated an effect of treatment one month after fumigation ($P = 0.036$), but not after seven months ($P = 0.313$). Only nonfumigated control treatments had significantly less reduction in *Pythium* counts than those counted from plots treated with any of the five fumigant treatments. However, nonfumigated control plots also experienced a reduction in *Pythium* counts by 60-92% one month after fumigation or by 68-98% seven months after fumigation. No fumigant treatment was significantly different from the conventional application of methyl bromide/chloropicrin. No difference in efficacy was observed between HDPE and VIF of the methyl iodide/chloropicrin treatments.

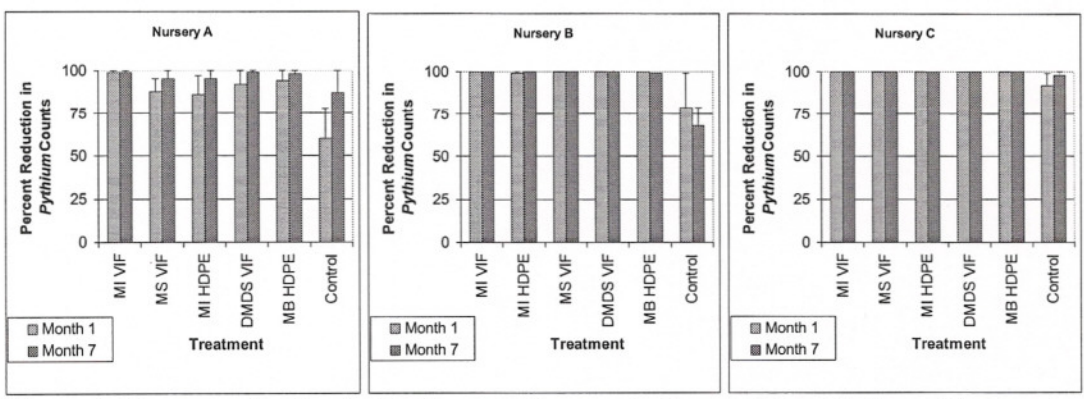

Figure 1. Percent reduction in *Pythium* species counts from dilution plating of fumigant-treated soil at three forest tree nurseries. MI = methyl iodide, MS = metam sodium, DMDS = dimethyl disulfide, MB = methyl bromide, VIF = virtually impermeable film, HDPE = high density polyethylene.

Four hundred fifty isolates of *Pythium* from leaf baits of the three nurseries have been identified on the basis of ITS sequence to date. One hundred eighty nine isolates (42%) were identified as *P. irregulare*, 122 as *P. dissotocum* (27%), 45 as *P. macrosporum* (10%), and

27 as *P. spiculum* (6%). The remaining 15% of the isolates were identified as one of 11 separate species including: *P. cylindrosporum, P. folliculosum, P. mamillatum, P. middletonii, P. monospermum, P. pachycaule, P. rostratfingens, P. rostratum, P. sylvaticum, P. torulosum,* and *P. ultimum.*

DISCUSSION

Sequencing of isolates from soil dilution plating and baiting is in progress. Several species such as *P. irregulare, P. macrosporum,* and *P. dissotocum* may more properly be described as species complexes that include several distinct ITS sequences in the nucleotide database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). These species complexes consist of several species that differ genetically but appear similar morphologically. *Pythium irregulare*, for example, was recently divided into two separate species (*P. irregulare* and *P. cryptoirregulare*) on the basis of AFLP markers and ITS and cox II gene sequences and likely consists of several other species that have yet to be elucidated (Garzon and others 2007). As new information on these species complexes becomes available, further analyses of the isolates from the present study will be conducted.

In May 2009, two-year-old transplants of Douglas-fir were transplanted into each treatment plot. A subset of transplants was assayed for *Pythium* colonization by plating 1-cm-length root pieces on PARP. Most transplants were apparently free of *Pythium* colonization. However, *Pythium* was isolated from transplants from two seedling sources, and these isolates are currently being identified to species. Transplant harvest will occur in October-November 2009, and a final soil and root assay for *Pythium* species will occur at that time.

Other components of this research that are in progress include: 1) comparison of *Pythium* species isolation frequency as a function of isolation method; and 2) pathogenicity tests. Preliminary evidence suggests that recovery frequency of certain *Pythium* species is dependent on the method (dilution plating versus baiting) and on the baits used (rhododendron versus Douglas-fir) used. In addition, greenhouse assays of Douglas-fir germination in soil infested with single spore *Pythium* isolates (from the present study) found that at least 11 species are pathogenic.

This study addresses several key components of *Pythium* damping-off in forest tree nurseries. In addition to evaluating the efficacy of lower doses of alternative fumigants for management of *Pythium*, we are also analyzing *Pythium* species frequency and diversity within three nurseries. Knowledge of *Pythium* species identity is critical for disease management. Some species may not be pathogenic to conifer seedlings, and those that are pathogenic may vary in the amount of damage that they cause. In addition, the relative abundance of each species may play an important role in evaluating economic thresholds. Results from this study are expected to detail the efficacy of lower doses of alternative fumigants for management of *Pythium* species and to elucidate the etiology of *Pythium* species associated with damping-off in forest tree nurseries.

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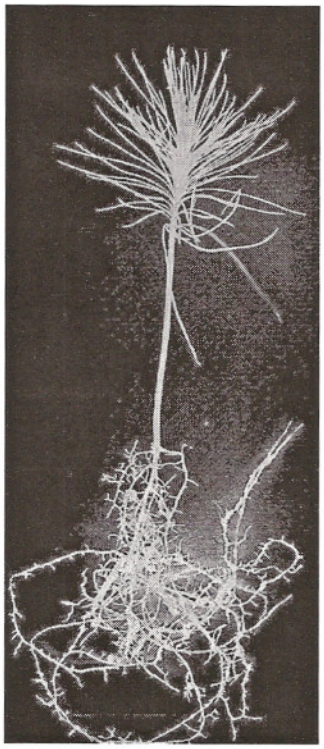

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