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Methods to control ectomycorrhizal colonization: effectiveness of chemical and physical barriers

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Abstract We conducted greenhouse experiments using Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings where chemical methods (fungicides) were used to prevent ectomycorrhizal colonization of single seedlings or physical methods (mesh barriers) were used to prevent formation of mycorrhizal connections between neighboring seedlings. These methods were chosen for their ease of application in the field. We applied the fungicides, Topas® (nonspecific) and Senator® (ascomycete specific), separately and in combination at different concentrations and application frequencies to seedlings grown in unsterilized forest soils. Additionally, we assessed the ability of hyphae to penetrate mesh barriers of various pore sizes (0.2, 1, 20, and 500 μm) to form mycorrhizas on roots of neighboring seedlings. Ectomycorrhizal colonization was reduced by approximately 55% with the application of Topas® at 0.5 g l^{-1}. Meshes with pore sizes of 0.2 and 1 μm were effective in preventing the formation of mycorrhizas via hyphal growth across the mesh barriers. Hence, meshes in this range of pore sizes could also be used to prevent the formation of common mycorrhizal networks in the field. Depending on the ecological question of interest, Topas® or the employment of mesh with pore sizes <1 μm are suitable for restricting mycorrhization in the field.

Keywords Ectomycorrhizal colonization · Common mycorrhizal networks · Hyphal restriction · Fungicides · Mesh barriers

Introduction

In mycorrhizal research, evaluation of mycorrhizal effects on plant performance often requires comparisons between mycorrhizal and non-mycorrhizal plants. Creating effective, yet feasible methods to control mycorrhizal colonization in the field has become of utmost importance as there has been a recent demand to increase the ecological relevance of mycorrhizal research (Read 2002). This requires moving away from laboratory-based work to experiments conducted in natural environments.

Currently, most studies have obtained non-mycorrhizal plants by employing one of three methods: substrate sterilization (via autoclaving, steam sterilization, or gamma irradiation), the creation of mutant plants unable to form mycorrhizas, or the use of fungicides applied to soil around plant roots. Sterilizing soil can result in substantial changes in its chemical and physical properties (Lensi et al. 1991; Chambers and Attiwill 1994; Sheremata et al. 1997; Shaw et al. 1999); moreover, its application in the field is futile because contamination is certain. The development of defective plants that lack the ability to form mycorrhizas has been limited to a few plant species associating with arbuscular mycorrhizal fungi (AMF) (Marsh and Schultze 2001). More research is also required to determine whether the functioning of mutants is otherwise identical to

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nonmutant plants (Kahiluoto et al. 2000). Of the fungicides, benomyl has been effectively used to reduce arbuscular mycorrhizal colonization of plants in the field by as much as 80% (Hartnett and Wilson 1999; Wilson et al. 2001; Callaway et al. 2004; Dhillion and Gardsjord 2004). Benomyl, no longer licensed for use in some countries and relatively ineffective against basidiomycetes, is however, not an option to control ectomycorrhizal (EM) fungi. Fungicides have generally not been employed in EM systems (but see Page-Dumroese et al. 1996; Manninen et al. 1998). Ectomycorrhizal fungal communities are more taxonomically diverse than arbuscular mycorrhizal fungal communities, thus, requiring a broad spectrum fungicide to adequately decrease EM colonization. Of the three methods currently employed to control mycorrhization, the use of fungicides appears to be the most feasible for field research in EM systems.

Two fungicides, Topas® and Senator®, have been suggested by greenhouse managers for control of EM hyphal growth. Propiconazole, the active ingredient in Topas® (25% a.i.), interferes with ergosterol biosynthesis, which is critical to the formation of fungal cell membranes (Kendrick 2000). The lack of normal sterol production slows or stops the growth of the fungus, effectively preventing further infection and/or invasion of host tissues (Kendrick 2000). Propiconazole incorporated into agar media at 1 ppm or higher inhibited growth of many EM fungal strains (Zambonelli and Iotti 2001; Laatikainen and Heinonen-Tanski 2002). Colonization of *Pinus sylvestris* roots by EM fungi decreased by approximately 20%, with some morphotypes affected more than others, when propiconazole was applied for two consecutive years in the field at a rate of 250 g l^{-1} every 2 weeks (Manninen et al. 1998). Thiophanate-methyl, the active ingredient in Senator® (70% a.i.), interferes with the functioning of microtubules, so that treated cells cannot divide. Thiophanate-methyl targets the cells of ascomycetes (Kendrick 2000), but to our knowledge has not been used to control EM fungi.

Studies of common mycorrhizal networks (CMNs) in plant communities form a unique subset of studies on mycorrhizal effectiveness (Simard and Durall 2004). They require comparisons between plants that are linked with those that are not linked by a CMN (Simard et al. 1997; Booth 2004). In these studies, control plants may be mycorrhizal, but hyphal linkages between plants must be absent. While non-mycorrhizal or non-linked controls are easily established in the laboratory using substrate sterilization techniques, this is more problematic in the field where seedlings are grown in native soils. Mesh barriers constructed of either steel or nylon have been used to prevent formation of ectomycorrhizal connections between plants (e.g., Francis and Read 1984; Schüepp et al. 1992; Booth 2004; Kranabetter 2005) or provide root-free compartments where mycorrhizal hyphae can explore and grow. To allow movement of soil solution while restricting penetration of roots and hyphae, mesh with pores 1 μm or smaller has been used (Robinson and Fitter 1999; Johnson et al. 2001; Zabinski et al. 2002; Cardoso et al. 2004), even though it is unknown whether it prevents formation of mycorrhizal connections between plants. Furthermore, given that hyphal width varies (from 1.5 to 9 μm), a mesh with pore sizes larger than 1 μm may restrict penetration of some mycorrhizal fungal species, but not others. Consequently, the mesh pore size could alter the EM fungal community composition. Ectomycorrhizal fungi vary in their ability to absorb and transport nutrients and water (Simard and Durall 2004); therefore, any alteration of the community may affect transport within the CMN.

The objective of this study was to examine the effectiveness of chemical and physical methods at controlling formation of ectomycorrhizas on Douglas-fir seedlings. We tested the effectiveness of the fungicides, Topas® and Senator®, at various concentrations and application frequencies. We predicted that both fungicides would reduce EM colonization, however, we expected that colonization of ascomycete fungi would be particularly reduced with the application of Senator®. Thus, the composition of the EM fungal community would be altered compared to untreated controls. In addition, we tested the effectiveness of nylon mesh with various pore sizes at preventing hyphal penetration and its effects on EM community composition of neighboring seedlings. We predicted that percent colonization and similarity of EM communities between seedlings on opposite sides of the mesh barrier would decrease with decreasing mesh pore size.

Materials and methods

Field soil collection

On August 27–28 of 2003, we collected 600 l of soil from the Black Pines variable retention cut (also known as a green-tree retention cut where some trees are not harvested) and adjacent forest approximately 50 km northwest of Kamloops, British Columbia (120°26′ W, 50°42′ N). The Black Pines variable retention cut occurs in the dry cool subzone of the Interior Douglas-fir (IDFdk) biogeoclimatic zone (Meidinger and Pojar 1991). It has an elevation of 1,180 m above seal level (asl) and loamy Gray Luvisolic soil (Krzic et al. 2004). The plant community is dominated by residual Douglas-fir [*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco] and subalpine fir [*Abies lasiocarpa* (Hook.) Nutt.] trees and advanced regeneration (saplings),

with shrub and herbaceous layers dominated by soopolallie [*Sherpherdia canadensis* (L.) Nutt.] and pinegrass (*Calamagrostis rubescens* Buckley), respectively.

We collected forest floor (30 cm × 30 cm) together with mineral soil (to 40 cm depth) from 15 random locations in 1 ha of the Black Pines forest. This soil was used for both experiments. The 15 samples were combined and thoroughly mixed, then, stored at room temperature until needed (see below).

Plant material

Interior Douglas-fir seedlings (seedlot #48520, British Columbia Ministry of Forest Tree Seed Center, Surrey, British Columbia, Canada) were grown at the University of British Columbia (Vancouver, Canada) greenhouse (temperature minimum, 20°C; temperature maximum, 24°C; average humidity, 60%). Seeds were moist-stratified at 4°C for 21 days. Seeds were then sterilized in constantly mixed 3% H_2O_2 for 2 h. Styroblock™ 512B trays (Beaver Plastics, Edmonton, Alberta, Canada) were cut in half horizontally and filled with autoclaved peat and sawdust (3:1, v:v). Three seeds were sown in each cavity and 4 weeks later were thinned to one seedling per cavity. The trays were placed under a mist tent for 12 days and then moved to a greenhouse bench for the remaining time. To improve seedling vigor and discourage mycorrhizal colonization, we applied 1.9 g l^{-1} water soluble Rose Plant Food (Miracle-Gro, Scotts Canada, Mississauga, Ontario, Canada) (18:24:16 N:P:K) once per week for 4 weeks following germination. Afterwards, we fertilized with 4 ml l^{-1} Peter's solution (Plant-Prod®, Plant Products, Brampton, Ontario, Canada) (20:20:20 N:P:K) once per week until the seedlings were transplanted into the treatment pots. For the duration of the two concurrent experiments (5 months), natural daylight in the greenhouse was supplemented by 400 W high pressure sodium lamps to maintain an 18-h photoperiod.

Fungicide experiment

Experimental design and treatments

On September 16, 2003, 14-week-old seedlings were transplanted into 3.2-l pots (175 mm × 180 mm) (Listo Products, Surrey, British Columbia, Canada) with drainage holes. The pots contained field soil mixed with perlite (3:1, v:v). A 3 × 3 × 3 factorial set of treatments with a separate control group was replicated ten times in a completely randomized design, where the factors were fungicide type, rate of application, and frequency of application (270 seedlings+10 controls=280 total). The three fungicide types were Senator®, Topas®, and a combination of the two fungicides (both from Engage Agro, Guelph, Ontario, Canada). The three rates of application were: 0.5, 1, or 1.5 ml l^{-1} of Senator®; and 0.5, 1, or 1.5 g l^{-1} of Topas®. Recommended concentrations of Senator® and Topas® are 0.5 ml l^{-1} and 0.5 g l^{-1}, respectively. To our knowledge, this is the only study assessing the effect of these fungicides on EM fungi; thus, we decided, as a starting point, to use the above rates. The fungicide was mixed with water and added at a constant volume of 600 ml pot^{-1}; therefore, seedlings that were treated with Senator® and Topas® in combination received 300 ml of each fungicide–water mixture. The three frequencies of application were: once at the beginning of the experiment, every 2 months (three applications total), or every month (five applications in total). For each fungicide application, we drenched the soil around the seedlings, avoiding contact with foliage. Additionally, ten control seedlings were grown in pots to which only water was applied. On September 30, 2003, initial height was recorded for all seedlings. The seedlings were watered as necessary and their locations re-randomized monthly.

Seedling measurements

On February 10, 2004, the height of all surviving seedlings was measured. Shoots were removed, dried at 65°C for 48 h and weighed. The roots and intact soil of up to seven replicates were stored at 4°C for 45 days before processing. Each root system was soaked in tap water, rinsed clean of soil, and cut into 1-cm fragments. The sample was then divided approximately in half, and one-half was dried and weighed. We used this measurement to estimate dry weight of the remaining roots, which were weighed wet, and then, cleared and stained following the methodology of Phillips and Hayman (1970) to assess percent EM colonization. For a given seedling, percent EM colonization was calculated as:

$$\text{Percent EM Colonization} = \frac{\text{Active EM root tips}}{\text{Active EM root tips} + \text{Active nonEM root tips}} \times 100$$

A root tip surrounded by a mantle was classified as mycorrhizal.

In addition to assessing percent colonization, we recorded the abundance and richness of EM morphotypes in each of the treatments. Root systems of the remaining three replicates from each of the ten treatments were carefully washed under running tap water and then cut into approximately 1-cm pieces. All root fragments were placed in a baking dish containing water and thoroughly mixed. We randomly subsampled and counted up to 100 EM or

100 non-EM root tips, whichever came first. Generally, EM tips were turgid and smooth, had emanating hyphae or rhizomorphs (Harvey et al. 1976), and had a Hartig net. A root tip that was dark and wrinkled, or was somewhat hollow and fragmented under minimal pressure was classified as "dead." Gross morphology of EM roots and rhizomorphs were described using a stereomicroscope, while the mantle, cystidia, and emanating hyphae were described using a compound microscope under ×400 or ×1,000 magnification. When possible, mantles were peeled by separating the fungal tissue from the root with forceps and micro-scalpels, and then, described. Morphological descriptions were made with reference to Agerer (1987–1998), Ingleby et al. (1990), Goodman et al. (1996), and Hagerman et al. (2001). Morphotyped roots were then dried and weighed.

Mesh barrier experiment

Experimental design and treatments

To test the effect of pore size on penetration by EM fungi, we grew seedlings in 3.2-l pots divided vertically by nylon mesh barriers with different pore sizes. The pore sizes of the four meshes were: 0.2 μm (catalogue number 25007, polyamide type 250 membrane, Sartorius AG, Goettingen, Germany), 1 μm (catalogue number 03-1/1 Nitex, Sefar America, Depew, NY, USA), 20 μm (catalogue number 03-20/14 Nitex), and 500 μm (catalogue number 06-500/47 Nitex). Control pots were divided by an impermeable acetate sheet to test for EM contamination through insufficient sterilization or water and airborne EM propagules. Each of the five barrier treatments was replicated 12 times in a completely randomized design. The pots were first sterilized in a 20% bleach solution for at least 1 h, cut in half vertically, and then, reassembled using non-toxic adhesive silicone sealant (catalogue number 3145-Grey-RTV; mil-A-46146, Dow Corning Midland, MI, USA) to attach the mesh and hold the two halves of the pot together. Each pot had two compartments. On August 30, 2003 one compartment was filled with field soil mixed with perlite (3:1, v:v), watered, and planted with 14-week-old seedlings (see Plant material for growth conditions). Three weeks after the seedlings were transplanted into the unsterilized soil, the second compartments were filled with sterilized field soil. Uncolonized 17-week-old seedlings were then transplanted into the sterilized soil and watered. The purpose of transplanting seedlings into the unsterilized field soil, 3 weeks before the introduction of seedlings into the other half of the pot, was to insure that the seedlings were already colonized by EM fungi when the experiment was started. We refer to the initially transplanted seedlings as "source seedlings." If hyphae from the source seedlings were able to penetrate a mesh of a given pore size, we expected to see mycorrhizal root tips on "recipient" seedlings grown in sterilized field soil (Fig. 1).

Once all source and recipient seedlings had been transplanted into the pots, the seedlings were watered as necessary. Just before transplanting, we destructively subsampled 15 source seedlings to quantify EM colonization following the methodology of Phillips and Hayman (1970). Afterwards, pot location on the greenhouse bench was re-randomized monthly. Initial shoot height was measured shortly after transplanting on September 30, 2003.

Seedling measurements

At harvest, January 11, 2004, shoot height and biomass (dried at 65°C for 48 h) were measured. During the harvest,

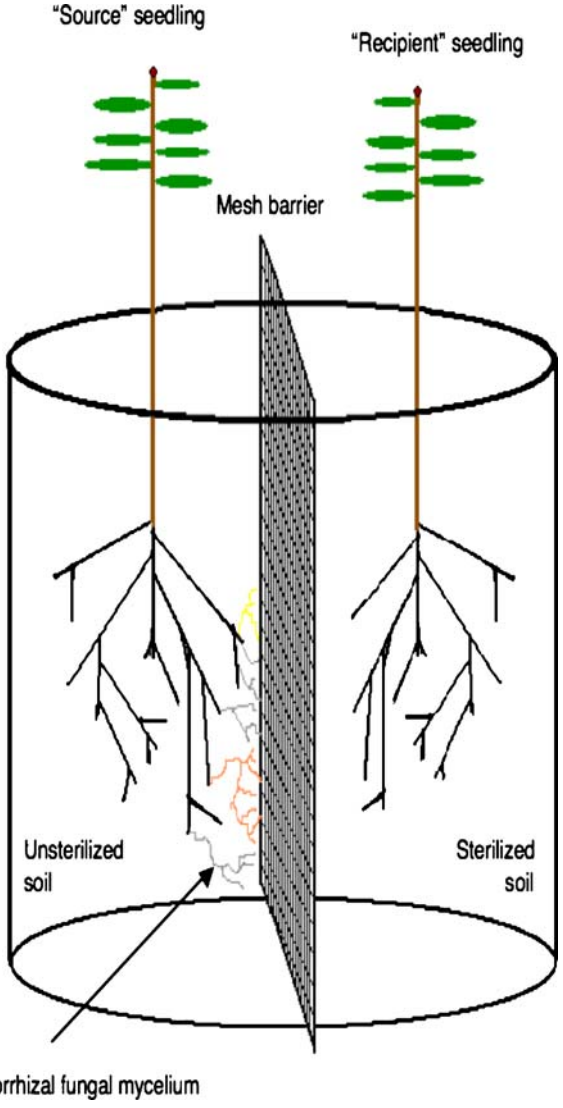

Fig. 1 Schematic diagram of the pot design used to test hyphal penetration of mesh barriers

we also inspected mesh barriers for signs of hyphal penetration using a stereomicroscope. We chose to randomly select ten replicates per mesh barrier treatment for morphotyping using similar methods outlined above (5 treatments × 2 seedlings per pot × 10 replicates = 100 seedlings). Three replicate sets of one root tip per morphotype from different seedlings were lyophilized before storage for subsequent molecular analysis. On average, 3% of the total roots tips per morphotype examined were sent for molecular analysis. The remainder of the morphotyped roots were dried and weighed with the remainder of the root sample.

Molecular confirmation of EM fungal species identification

Total genomic DNA was extracted from single EM tips by pulverizing them for 45 s at a speed of 5.0 U using a Bio101 Systems Fast Prep FP120 high frequency shaker (Q-biogene, Carlsbad, CA, USA). DNA was isolated using the procedure of Baldwin and Egger (1996). The final DNA pellet was dried using a speed vacuum concentrator and then resuspended in 50 µl ethylenediaminetetraacetic acid (EDTA)–Tris–EDTA (TE) buffer.

Following DNA extraction and isolation, the internal transcribed spacer (ITS) region of the fungal nuclear rDNA was specifically amplified by the primers NSI1 and NLC2 (Martin and Rygiewicz 2005). Polymerase chain reactions (PCRs) typically included 1 µl template DNA, 18.6 µl sterile purified water (Barnested Nanopure Diamond water purifier), 0.2 mM deoxyribonucleoties (dNTPs), 2.5 µl 10× PCR buffer, 1.5 mM $MgCl_2$, 0.48 mM each primer, 1.6 mg ml^{-1} bovine serum albumin (BSA), and 0.25 U μl^{-1} AmpliTaq Gold™ (Applied Biosystems, Foster City, CA, USA). Samples were amplified using a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA). A 10-min hot start was followed by PCR cycling as follows: 45 s at 94°C followed by 34 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 45 s, ramping 72°C for 1 min with a 1-s extension after each cycle, and extension at 72°C for 10 min, and then, the temperature was held at 4°C. The PCR products were visualized on 1.5% agarose gels using a Gel Logics 440 (Kodak Instruments, Rochester, NY, USA). The PCR product was cleaned using the QIAquick PCR Purification kit (Qiagen, Valencia, CA, USA). Before sequencing, the large ITS fragment produced above, was re-amplified in a nested PCR reaction using the primers ITS 1 and ITS 4 (White et al. 1990). PCR products were quantified and then sequenced using a 3730 DNA Capillary Sequencer (Applied Biosystems) at the University of British Columbia Nucleic Acid and Protein Services Unit. All unique morphotypes were sequenced and then aligned using Sequencher software (Gene Codes, Ann Arbor, MI, USA). Taxonomic matches were based on BLAST results with ≥98% sequence similarity.

Statistical analysis

The fungicide experiment examined a 3 × 3 × 3 factorial set with a separate control group of treatments (i.e., separate from the factorial but combined in the layout) in a completely randomized design (Bergerud 1989). We used percent colonization data obtained from the cleared and stained roots and normalized the data with a square root transformation for analysis of variance (ANOVA). We analyzed EM community data (richness and diversity, relative abundance of morphotypes with >5% of EM root tips), seedling growth, and square root of percent colonization, first, by using the GLM procedure in SAS (SAS Institute Inc. 1999). We then ran a second GLM procedure with a contrast statement to compare the control treatment against all other treatment combinations. Analyses on data collected from cleared and stained roots and morphotyped root tips were done separately and consequently graphed separately. ANOVA tables were constructed manually to obtain the proper experimental error terms and degrees of freedom. When significant main treatment effects occurred, we separated means using the Bonferroni multiple comparison test.

For the mesh barrier experiment, the percent colonization and EM richness for both seedlings per pot were used to calculate the Steinhaus index of EM community similarity (Legendre and Legendre 1998) and to calculate the difference in morphotype richness (integral of the number of morphotypes on the donor root system minus the number on the receiver root system). The effects of mesh pore size on EM community data (richness difference and Steinhaus index of similarity), percent EM colonization, and seedling growth (shoot height, biomass, and root biomass) were detected with a one-way ANOVA using the GLM procedure in SAS (SAS Institute Inc. 1999). For both percent EM colonization and seedling growth, the difference in the response variable between source and recipient seedlings within a pot was calculated and used in the analysis. Differences were considered significant at $\alpha = 0.05$. Where significant mesh barrier treatment effects occurred, we separated means using the Bonferroni multiple comparison test. Effects of sterilization on seedling growth and total percent EM colonization were analyzed using the TTEST procedure for each mesh size (SAS Institute Inc. 1999).

Results

Fungicide treatments

Approximately 30% of the roots of control seedlings (i.e., seedlings receiving only water) were colonized after 21 weeks in the treatment pots. Application of fungicide reduced EM colonization by up to 50%, depending on

Table 1 Analysis of variance for effect of fungicide type (*F*), concentration (*C*), and application frequency (*A*) on square root percent ectomycorrhizal colonization ($\sqrt{\text{PEC}}$) and size of Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings after 5 months

Source of variation	*df*	$\sqrt{\text{PEC}}$			Height			Shoot biomass			Root biomass		
		MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Control vs all others	1	13.55	9.67	<0.0001	0.03	0.00	0.9756	0.01	0.01	0.9038	0.03	0.32	0.5700
Fungicide type	2	9.70	6.92	<0.0001	69.40	2.06	0.1316	0.29	0.60	0.5487	0.08	1.01	0.3670
Concentration	2	0.23	0.16	0.8500	16.10	0.48	0.6313	0.62	1.29	0.2786	0.04	0.48	0.6210
Application frequency	2	22.81	16.28	<0.0001	46.00	1.37	0.2587	0.74	1.55	0.2163	0.11	1.47	0.2346
FxC	4	1.56	1.11	0.3526	22.90	0.68	0.6068	0.36	0.75	0.5625	0.06	0.77	0.5455
FxA	4	1.99	1.42	0.2304	47.90	1.42	0.2302	0.15	0.32	0.8660	0.05	0.68	0.6059
CxA	4	0.25	0.18	0.9480	8.32	0.25	0.9111	0.73	1.52	0.1997	0.16	2.11	0.0835
FxCxA	8	1.44	1.03	0.4181	17.90	0.53	0.8306	0.43	0.90	0.5208	0.02	0.29	0.9677
Error	135	1.40			33.70		0.48	0.48			0.08		

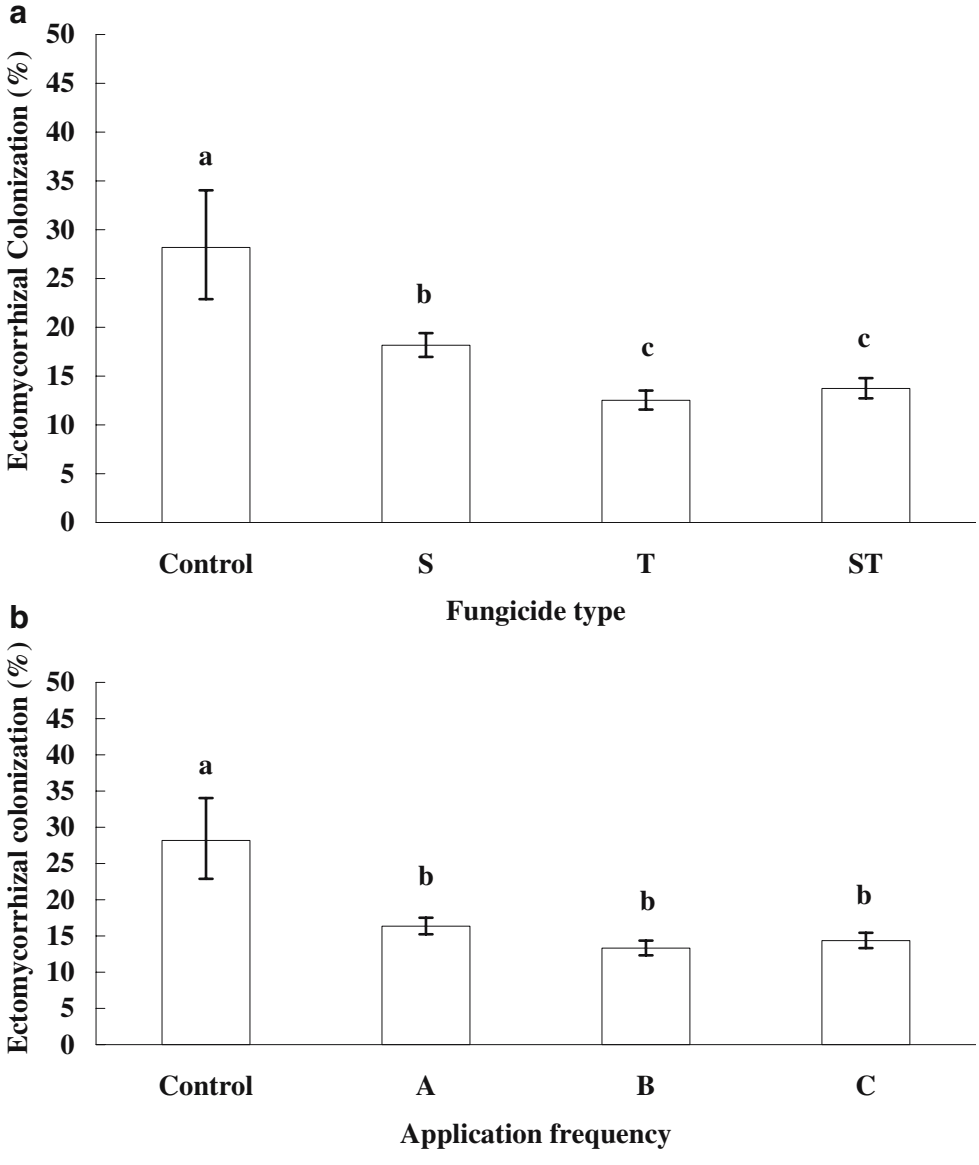

Fig. 2 Effect of **a** fungicide type and **b** application frequency on percent ectomycorrhizal colonization (determined by clearing and staining root tips) of Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings. Fungicide abbreviations: *S* Senator® and *T* Topas®. Frequency abbreviations: *A* Once upon commencement of the experiment, *B* every 2 months, and *C* once a month. Statistically significant fungicide treatment effects detected by a Bonferroni multiple comparison test are designated by *different letters* ($P<0.05$). *Error bars* are one standard error of the mean

Table 2 Description of morphological characteristics of ectomycorrhizas observed on Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) grown in the fungicide (F) and mesh (M) study

Study	Morphotype and blast match	Macroscopic description	Mante type(s)	Emanating hyphae	Rhizomorphs	Cystidia
F and M	*Rhizopogon/Suillus*-type (R/S)	Unbranched to subtuberculate silvery white mycorrhiza with rough texture	Outer: felt prosenchyma, hyphae 3–4 μm smooth, and thick-walled; inner: net synenchyma, thin, hyphae 2 μm	3 μm wide; no clamps, crystalline ornamentation, and elbow-like bends	Compact brown with crystalline ornamentation and elbow-like bends	Absent
F and M	*Thelophora*-type (T) blasted to *Thelephora terrestris*, accession No. U83486, 619/627 base pairs = 99%	Unbranched or irregular bright orange to brown (sometimes whitish) mycorrhiza with smooth reflective texture	Outer: net synenchyma, hyphae 3 μm wide; inner: incomplete interlocking irregular synenchyma, hyphae 4–5 μm wide	Rare, 3 μm wide; clamps, smooth with occasional enlarged hyphal junctions	Absent	Common, 40–50 μm long and 3 μm wide with basal clamp
F and M	*Cenococcum geophilum* (Cg)	Unbranched, black mycorrhiza with rough hairy texture	Outer: net synenchyma in a stellate pattern, hyphae 6 μm wide; inner: net synenchyma	5–6 μm wide black, straight	Absent	Absent
F and M	*Wilcoxina*-type (W) blasted to *Wilcoxina rehmii*; accession No. DQ069001, 510/519 base pairs = 98%	Irregular dark brown to orangish mycorrhiza, often wrinkled, also called Estrain	Outer: not seen; inner: patchy and incomplete net prosenchyma, hyphae 2 μm wide	Absent	Absent	Absent
F and M	*Mycelium radicis atrovirens*type (MRA)	Unbranched black to brown mycorrhiza with curled hairy or very rough texture	Outer: felt prosenchyma, hyphae 3 μm wide; inner: net synenchyma, hyphae 2–3 μm wide	Rare, 5–7 μm wide, no clamps, smooth but becoming progressively more verrucose away from the mantle	Absent	Absent
F and M	Undifferentiated (Undif)	Young orange mycorrhiza with no distinct characters	Barely visible net synenchyma readily turning into Hartig net	Absent	Absent	Absent
F	*Tomentella*-type (Tom)	Swollen dark-brown sandy textured mycorrhiza	Outer: squarish incomplete interlocking irregular synenchyma with thick-walled hyphae; inner: net synenchyma	Absent	Absent	Absent
F	*Piloderma*-type (P)	Yellow coarsely felty mycorrhiza with abundant rhizomorphs	Not determined	Absent	Finely verrucose, septa common, not clamped, approximately 3 μm wide	Absent

fungicide type ($P<0.0001$) but not application concentration ($P=0.8500$) (Table 1). The most effective treatment regime was Topas® applied alone or in combination with Senator® (Fig. 2a). Senator® alone was less effective at decreasing EM colonization, with only a 36% reduction compared with 56% reduction using Topas®. There were no differences associated with different application frequencies (Fig. 2b) and there were no significant interactions among any combination of the three treatment factors ($P>0.05$, Table 1). None of the fungicides applied at any concentration or application frequency affected seedling height or shoot or root biomass (Table 1).

A total of eight morphotypes were identified and described (Table 2). Two had >98% sequence matches of

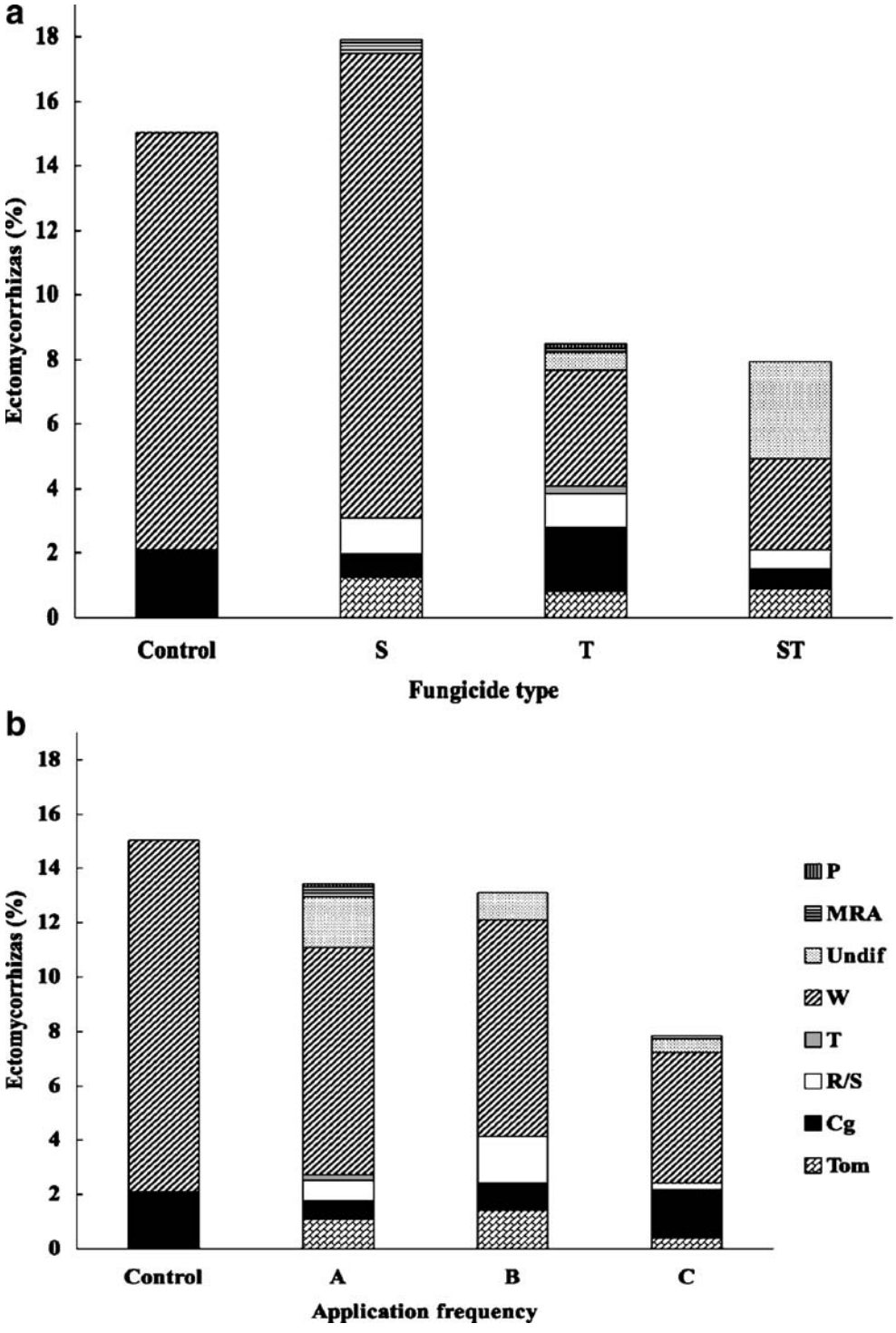

Fig. 3 Relative abundance of morphotypes: *Tomentella*-type (*Tom*) *Thelephora terrestris* (*T*); *Mycelium radicis atrovirens*-type (*MRA*); *Wilcoxinarehmii* (*W*); *Cenococcum geophilum* (*Cg*); *Rhizopogon/Suillus*-type (*R/S*); *Piloderma*-type (*P*) and Undifferentiated (*Undif*) found on morphotyped Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) root systems grown in soil treated with different **a** fungicide types and **b** application frequency. Fungicide abbreviations: *S* Senator® and *T* Topas®. Frequency abbreviations: *A* Once upon commencement of the experiment, *B* every 2 months, and *C* once a month

their ITS sequences to *Wilcoxina rehmi* and *Thelephora terrestris* accessions in Genbank. DNA from the other six morphotypes either did not amplify or had less than 98% sequence homology with genotypes in Genbank. One morphotype was not identifiable and was classified as undifferentiated. Only the *Rhizopogon/Suillus*-type formed rhizomorphs; the remainder had relatively smooth mantles (Table 2).

On average, we observed more morphotypes on seedlings that were subject to fungicides than those that were not (Fig. 3). However, neither EM community richness (P=0.2198) nor diversity (P=0.3100) was significantly affected by the fungicides. The relative abundance of *W. rehmii* mycorrhizas (the most common ectomycorrhiza) as a percentage of all root tips examined was reduced by Topas® applied alone or in combination with Senator®,

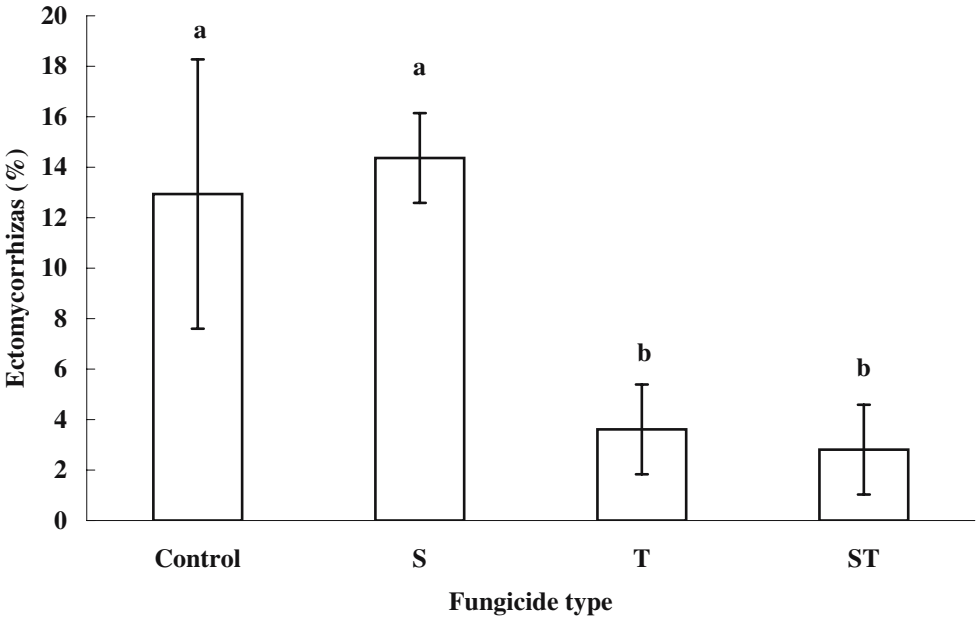

Fig. 4 Relative abundance of *Wilcoxina rehmii* ectomycorrhizas, as a percentage of all root tips examined on Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) grown in soil treated with fungicides. Fungicide abbreviations: *S* Senator® and *T* Topas®. Statistically significant fungicide type treatment effects detected by a Bonferroni multiple comparison test are designated by *different letters* ($P<0.05$). *Error bars* are one standard error of the mean

when compared to Senator® alone or the control (Fig. 4). The abundance of *Cenococcum geophilum*, the other dominant ascomycetous mycorrhiza, was not affected by application of fungicides ($P=0.5861$, data not shown). Similarly, the abundances of *Rhizopogon/Suillus*- and *Tomentella*-type mycorrhizas, the most abundant basidiomycetes, were also not affected by fungicide treatment ($P=0.6673$, $P=0.7924$, respectively, data not shown).

Mesh barrier treatments

Source seedlings had greater shoot height, shoot biomass, root biomass, and EM colonization than recipient seedlings across all mesh treatments except the 20-μm pore size (Table 3), and mesh size did not affect the magnitude of these differences (Table 4). Across all mesh sizes, on average, 50 and 21% of roots of source and recipient seedlings were colonized by EM fungi, respectively. These colonization levels contrast early measurements where colonization of source seedlings were less than 1%.

We found six distinct morphotypes on source seedlings, (Table 2). Most of the six morphotypes were represented in all mesh treatments (Fig. 5). *W. rehmii* ectomycorrhizas comprised >85% of the community on source and recipient seedlings separate with mesh barriers of 1 μm or larger (>80%). By contrast, both the 0.2- and 1-μm pore-sized meshes blocked the formation of *Rhizopogon/Suillus*-type mycorrhizas on recipient seedlings (Fig. 5). This type formed approximately 5% of the mycorrhizas on source seedlings. MRA-type morphotypes were found on all source seedlings, but were absent from recipient seedlings of all mesh treatments. *T. terrestris* ectomycorrhizas formed an increasingly high proportion of the community on recipient seedlings as mesh size decreased, whereas, they were not found on source seedlings. The abundance of *C. geophilum* mycorrhizas was too low to be useful in detecting mesh effects.

Ectomycorrhizal community similarity, which takes into account richness and relative abundance, between recipient seedlings vs source seedlings increased with mesh pore sizes greater than 0.2 μm ($P<0.0001$) (Fig. 6a). The EM communities separated by the full barrier (control) or by mesh of a pore size of 0.2 μm were significantly dissimilar from those separated by mesh with pore sizes 1 μm and larger (Fig. 6a). The difference in morphotype richness between source and recipient seedlings was large in the full barrier treatment and generally decreased as mesh size increased ($P=0.0924$) (Fig. 6b). When examined under the microscope, we observed hyphae penetrating pore sizes of 1 μm and larger and root penetrating only 500 μm pores. Three of the mesh barriers were torn in pots of the 0.2-μm mesh treatment; these replicates were omitted from the analyses.

Discussion

Fungicide effects on EM colonization

This study suggests that fungicides can be used to significantly reduce EM colonization in controlled experiments. Topas® was more effective than Senator® at reducing EM colonization levels. The manufacturer's recommended concentration was effective in reducing colonization, and there was no advantage to applying Topas® repeatedly during the course of the experiment. In

Table 3 Effect of sterilization on growth and EM colonization of Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings

Mesh (μm)	Soil	*n*	Height increment (cm)	SEM[a]	*P*	n	Shoot gain (g)	SEM	*P*	n	Root gain (g)	SEM	*P*	n	Root: Shoot ratio gain	SEM	*P*	n	Percent EM colonization (%)	SEM	*P*
	U	10	15.2	2.6		10	0.906	0.137		9	0.316	0.056		9	0.330	0.1559		9	50	7	
Control					0.0294				0.0061				0.6633				0.0075				<0.0001
	S	12	9.7	2.6		12	0.420	0.137		10	0.290	0.056		9	0.880	0.155		9	3	7	
	U	12	13.9	0.8		12	0.967	0.084		10	0.442	0.064		10	0.440	0.056		7	43	12	
0.2					<0.0001				<0.0001				0.0055				0.0675				0.0195
	S	12	9.0	0.8		12	0.341	0.084		12	0.193	0.064		10	0.557	0.056		9	11	12	
	U	11	20.2	2.4		11	1.555	0.196		8	0.606	0.123		7	0.492	0.145		9	57	8	
1					0.0195				<0.0001				0.0360				0.6007				0.0113
	S	11	14.5	2.4		11	0.703	0.196		9	0.307	0.123		7	0.412	0.145		9	33	8	
	U	12	17.8	3.6		12	1.229	0.379		10	0.477	0.204		5	0.423	0.077		10	47	9	
20					0.3541				0.4363				0.4843				0.5329				0.1308
	S	8	12.5	3.6		8	0.858	0.379		5	0.295	0.204		5	0.371	0.077		5	29	9	
	U	12	20.6	4.1		12	1.511	0.319		11	0.502	0.074		9	0.347	0.083		11	51	10	
500					0.0588				0.0333				0.0400				0.0427				0.0434
	S	9	10.8	4.1		9	0.606	0.319		9	0.295	0.074		8	0.566	0.083		9	28	10	

A series of *t* tests were used to determine differences between grown in unsterilized (U) and sterilized (S) soils for each mesh barrier treatment. Seedling growth is expressed as height and biomass measured after 5 months.

[a] *SEM* Standard error of the mean

Table 4 Effect of mesh treatment on growth and ectomycorrhizal colonization of Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings

Mesh (μm)	*n*	Height increment difference (cm)	SEM[a]	*n*	Shoot gain difference (g)	SEM	*n*	Root gain difference (g)	SEM	*n*	Root:shoot gain ratio difference (g)	SEM	*n*	EM colonization difference (%)	SEM
Control	10	5.7	*a*±2.6	10	0.486	*a*± 0.220	9	0.025	*a*± 0.088	9	−0.550	*a*±0.105	9	47	*a*±8
0.2	12	4.9	*a*± 2.4	12	0.627	*a*± 0.201	10	0.231	*a*± 0.084	10	−0.117	*ab*± 0.100	7	30	*a*±9
1	10	6.8	*a*±2.6	10	0.900	*a*± 0.220	7	0.330	*a*± 0.100	7	0.080	*b*±0.120	8	26	*a*±9
20	8	3.6	*a*±2.9	8	0.313	*a*± 0.247	5	0.157	*a*± 0.118	5	0.052	*b*±0.142	5	6	*a*± 11
500	9	9.0	*a*±2.7	9	0.818	*a*± 0.232	9	0.181	*a*± 0.088	8	−0.205	*ab*± 0.112	9	29	*a*±8

Response differences between source and recipient seedlings were calculated for each pot. This single number was used in the ANOVA for each response variable. Statistically significant mesh treatment effects detected by a Bonferroni multiple comparison test are designated by different letters ($P<0.05$).

[a] *SEM* Standard error of the mean

our study, EM colonization decreased by as much as 56% compared with the control. Douglas-fir control seedlings in this experiment had relatively low levels of colonization (approximately 30%), but these levels are typical for greenhouse-grown interior Douglas-fir (5–42%) (Hagerman and Durall 2004; Teste et al. 2004). Our results are consistent with another study using propiconazole. Manninen et al. (1998) found that 0.15 g of propiconazole applied to seedlings in the field (vs 9.6 g at the highest application frequency in our study) caused a decrease in EM colonization of almost 33% (from 67 to 45% colonization) 2 years after 2-year-old nursery grown *P. sylvestris* seedlings were outplanted.

Although the fungicides did not eliminate EM colonization altogether, we propose that Topas® reduces colonization to an extent to be useful for field studies. Similar decreases in arbuscular mycorrhizal colonization following benomyl application have resulted in substantial changes in structure of the plant community. For example, reductions in arbuscular mycorrhizal colonization of 60% have changed plant nitrogen and phosphorus concentrations and aboveground community productivity in Boreal grassland communities (Dhillion and Gardsjord 2004). Hartnett and Wilson (1999) found that a 75% decrease in arbuscular mycorrhizal colonization coincided with biomass decreases of dominant C_4 grasses. Callaway et al. (2004) reported that interactions between native grassland species and the invasive *Centaurea maculosa* were substantially altered when experimental plots were treated with benomyl; the fungicide decreased arbuscular mycorrhizal colonization by >80%, resulting in a *C. maculosa* biomass decrease when mixed with *Koeleria cristata* or *Festuca idahoensia*. Assuming reductions in arbuscular and EM colonization result in similar functional responses in plant communities, we expect that Topas® applied at the recommended rate once every 5 months will reduce EM colonization sufficiently to affect seedling performance in the field.

The specificity of the fungicides for ascomycetes and basidomycetes differed from that expected. Senator® is reported to be more effective against ascomycetes than basidiomycetes, and yet, it appeared to have no effect on *W. rehmii*, a dominant ascomycete in this study. Manninen et al. (1998) reported that propiconazole was also more effective at inhibiting ascomycete than basidiomycete symbionts, and this is confirmed by Laatikainen and Heinonen-Tanski (2002). The latter found that low concentrations of propiconazole (0.1 ppm) increased growth of *Suillus bovinus* and *Suillus variegatus* strains grown in vitro and that these fungi were tolerant of concentrations up to 1 ppm. In our study, the effectiveness of propiconazole (Topas®) could not be predicted strictly by taxonomic status. For example, it caused a substantial reduction in colonization by *W. rehmii*, but not by *C. geophilum*, another important

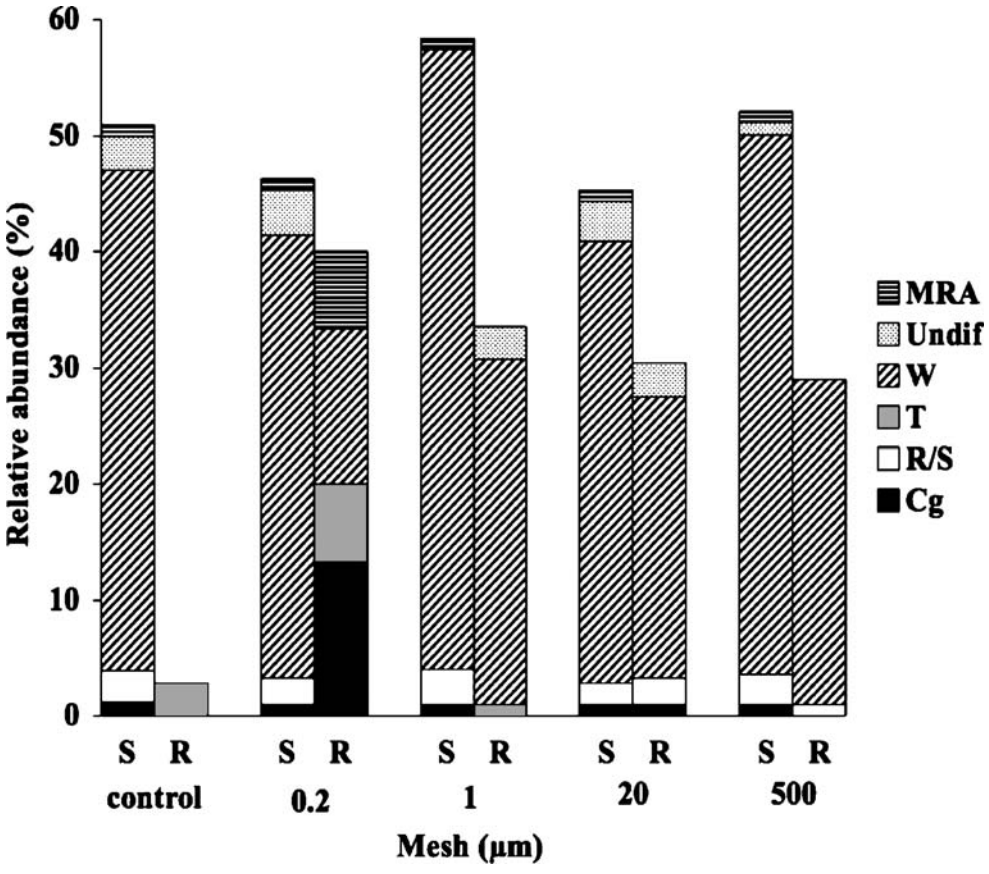

Fig. 5 Relative abundance of morphotypes: *Thelephora terrestris* (*T*); *Mycelium radicis atrovirens*-type (*MRA*); *Wilcoxina rehmii* (*W*); *Cenococcum geophilum* (*Cg*); *Rhizopogon/Suillus*-type (*R/S*); and Undifferentiated (*Undif*), as a percentage of all root tips examined on source (*S*) and recipient (*R*) Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) separated by a mesh barrier

ascomycete. Colonization by the basidiomycetes forming *T. terrestris*, *Tomentella*-type, and *Rhizopogon/Suillus*-type mycorrhizas either increased or was not affected by either fungicide, however. In our study, Topas® targeted the most abundant EM fungi, *W. rehmii*, so that the additional application of Senator® provided no further advantage.

Other fungicides have had variable effects on EM colonization. O'Neill and Mitchell (2000) applied benomyl to *Picea sitchensis* seedlings and found that colonization was reduced from 60 to 20%; however, only one morphotype, *Wilcoxina mikolae*, was observed on the nursery grown seedlings. In another study, the percent of roots colonized by *T. terrestris* or *Laccaria laccata* decreased when 0.3% Dithane M-45 was applied to *Pinus patula* seedlings grown in pouches, and similar reductions in hyphal dry weight occurred when the fungicide was applied to in vitro cultures (Reddy and Natarajan 1995). A wide range of responses were exhibited by 64 strains of EM fungi grown in vitro and exposed to relatively low concentrations (<10 ppm) of five fungicides (benomyl, chorothalonil, copper oxychloride, maneb, and propiconazole) (Laatikainen and Heinonen-Tanski 2002). Conversely, in some other laboratory studies, fungicides have increased EM colonization (Pawuk et al. 1980; Marx and Rowan 1981; de la Bastide and Kendrick 1990). This effect is likely due to the selective inhibition of fungi that are competitive towards EM fungi (Summerbell 1988). In our study, interactions among EM fungi could have resulted in the increase of basidomycetes observed. *W. rehmii*, a rapid colonizer of nursery seedlings (Mikola 1988) was suppressed by the application of Topas®. Removal of this rapid colonizer could have allowed other EM fungi to colonize seedling root tips. Surveys of the entire fungal community on a large number of replicate seedlings is required to investigate this possibility.

Our results suggest that Topas® should be effective at reducing morphotypes commonly found in greenhouse bioassays of field soils, but there are two caveats. First, we could not assess the effects of fungicides on rare EM fungal species or those that do not colonize seedlings in greenhouses. Second, Topas® may affect seedling physiology and/or other soil biota. These impacts are more difficult to identify and quantify by short-term experiments in a greenhouse setting. Propiconazole has been shown to have growth-regulator effects on plants in the Solanacaeae family (Kendrick 2000), and it has also been shown to affect soil fauna, such as flagellates (Ekelund et al. 2000) and soil respiration (Elmholt 1992). Topas® is recommended for prevention of a variety of foliar fungal diseases, and its mode of action by preventing ergosterol synthesis makes it likely to also affect non-target saprotrophic and parasitic soil fungi. A change in this community would alter

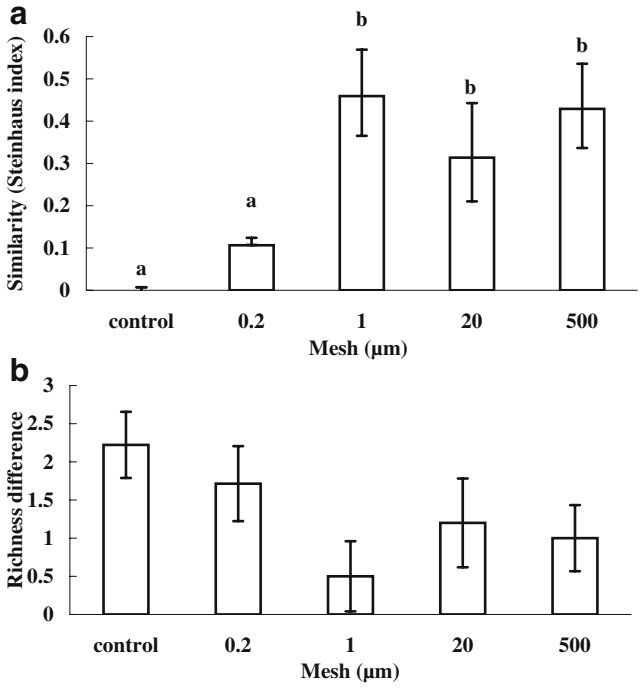

Fig. 6 Ectomycorrhizal community differences. **a** Steinhaus similarity index for ectomycorrhizal communities observed on source and recipient seedlings separated by a mesh barrier. **b** Richness difference = number of morphotypes observed on source Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) root systems minus morphotypes present on recipient Douglas-fir separated by a mesh barrier. Statistically significant mesh treatment effects detected by a Bonferroni multiple comparison test are designated by *different letters* ($P<0.05$). *Error bars* are one standard error of the mean

potential food substrates of soil fauna. In experiments where EM reduction is of primary concern and side-effects on the soil biota is unimportant, then applications of Topas® can be an effective treatment regime. Given that the active ingredient in Topas® is fungistatic, repeated applications may be required where there is high hyphal turnover, as would happen over a temperate growing season, or where there is high fungal propagule pressure; both of these conditions occur in field situations.

Mesh barrier effects on hyphal penetration

Our study indicates that mesh with pore size of 0.2 μm is effective at reducing hyphal penetration and mycorrhizal colonization of neighboring seedlings. However, we conclude that the threshold for restricting EM hyphal penetration lies between 0.2 and 1 μm. Ectomycorrhizal richness tended to increase in sterilized compartments where mesh size equaled or exceeded 1 μm, suggesting that hyphae from the source seedlings compartment penetrated the mesh and colonized the recipient seedlings growing in the sterilized compartment. Of even greater significance, EM community similarity between source and recipient seedlings greatly increased in meshes ≥1 μm. If the recipient seedlings were mycorrhiza-free, differences in richness alone should have indicated mesh effectiveness at restricting hyphal penetration, regardless of abundance, but the small number of morphotypes may have rendered richness as a measure with little resolving power.

The EM community observed in our study was typical for interior Douglas-fir seedlings inoculated with field soil and grown in the greenhouse (Jones et al. 1997; Simard et al. 1997; Hagerman and Durall 2004, Teste et al. 2004). The six morphotypes formed on the source seedlings also represented a broad range of mantle types (texture and thickness), width of emanating hyphal forms (width and extension 3 to 7 μm), and the presence or absence of rhizomorphs. They allowed us to test the effectiveness of the pore sizes at preventing hyphal penetration by different EM fungi. We might predict, for example, that a mesh with a smaller pore size would be required to prevent penetration of single hyphae, compared to the size required to stop penetration of rhizomorphs. Our findings support this prediction as we found that the rhizomorph-forming *Rhizopogon/Suillus*-type morphotype was restricted by a mesh size between 1 to 20 μm. We propose that meshes with pore sizes smaller than 1 μm would be adequate in field situations.

Although the mesh with 0.2 μm pores was the most effective at reducing hyphal penetration, it was very fragile. This characteristic of nylon mesh with pore sizes smaller than 1 μm has been noted previously (Tarafdar and Marschner 1994). Our results suggest that field experiments requiring fine mesh (0.2 μm) should use more durable nylon (i.e., mesh thickness >115 μm) or metal-based mesh.

Our finding that mesh with pore sizes between 0.2 and 1 μm are most effective at inhibiting EM colonization must be interpreted cautiously because some EM were found in sterilized soils with a 0.2-μm mesh barrier. Within the sterilized compartment of these pots, the EM community was reduced but not eliminated. For example, *W. rehmii* was on the recipient seedlings, regardless of the mesh barrier type, but was not observed in control pots, suggesting that hyphal penetration or spore dispersal may have occurred. We are uncertain why *W. rehmii* was not found in the sterilized compartment of the control pots. Further research is warranted on *W. rehmii* propagating strategies in nurseries (e.g., hyphal and spore) and morphological plasticity. We also found that *T. terrestris* had colonized root tips in one seedling of the control treatment (i.e., sterilized soil with a full barrier), confirming previous studies that it is a common greenhouse contaminant. Statistical analyses were run without *T. terrestris* (data not shown); however, results were similar, and did not change our conclusions about the hyphal restriction properties of the mesh treatments. MRA-type mycorrhizas

were also only observed on source seedlings across all mesh treatments, suggesting that chemical changes induced by autoclaving may have inhibited this particular EM fungus. Rhizomorphs were completely excluded from sterilized compartments separated by 1 or 0.2 μm mesh.

The use of mesh barriers vs fungicides for controlling EM colonization depends on the ecological processes that must be maintained and those that can be compromised in the experiment. Future CMN research can benefit from the use of mesh barriers. Mesh barriers with a gradient of pore sizes have the potential to tease out carbon and nutrient pathways (soil-only, hyphal-only, rhizomorph-only, etc.) in resource sharing CMN studies. However, installing mesh barriers will disrupt soil structure and potentially reduce water flow through small pore sizes. We are currently addressing this issue in research programs on CMNs in the field (F. Teste, S.W. Simard, and D.M. Durall, unpublished data). If the purpose of the mesh is to exclude mycorrhizal hyphae and maintain non-mycorrhizal status of the enclosed host, the soil contained in the mesh barrier compartment will require sterilization. Mesh with pore sizes <1 μm appear to reduce hyphal penetration; however, care will be required to exclude fungal propagules arriving via air or water pathways. We suggest that mesh barriers, apart from their disruptive installment, are a more promising method than fungicides to completely exclude fungi.

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