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ARTICLE

Influence of tree species and salvaged soils on the recovery of ectomycorrhizal fungi in upland boreal forest restoration after surface mining

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Abstract: Surface mining in the Canadian boreal forest involves the removal of vegetation and soils, resulting in the local loss of biodiversity and ecosystem functioning. Ectomycorrhizal (EM) fungi are critical to ecosystem processes; however, their recovery following reclamation is not well understood. This study investigated the importance of reclamation cover soils (forest floor material, peat, and subsoil) and tree seedling species (*Populus tremuloides* Michx., *Pinus banksiana* Lamb., and *Picea glauca* (Moench) Voss) in structuring the community composition of EM fungi. We used 1-year-old seedlings to assay cover soils in the field for 3 months, and grew seedlings in each of the cover soils in a growth chamber assay for 5 months. Nonmetric multidimensional scaling indicated host identity structured the community composition of EM fungi in the field, while both host identity and cover soil influenced the composition of EM fungi in the growth chamber. However, pre-colonization of seedlings by nursery fungi complicates interpretation of field results. The rate of EM fungus colonization of seedlings collected across both assays was relatively low, approximately 23%. Our results indicate cover soils used in reclamation of surface-mined landscapes retain propagules of EM fungi, and using a wide variety of tree species in upland boreal forest restoration may increase the diversity of EM fungi recovered.

Key words: Populus tremuloides, *Pinus banksiana*, *Picea glauca*, ITS rDNA, Sanger sequencing, reclamation, plant–soil links.

Résumé : L'exploitation minière de surface dans la forêt boréale canadienne implique l'ablation de la végétation et des sols résultant en une perte locale de la biodiversité et du fonctionnement de l'écosystème. Les champignons ectomycorhizes (EM) sont critiques aux processus de l'écosystème ; cependant leur rétablissement à la suite de la réhabilitation du terrain n'est pas bien compris. Cette étude s'est penchée sur l'importance de la réhabilitation des sols de surface (matière du tapis forestier, tourbe et sous-sol) et des espèces de semis d'arbres (*Populus tremuloides* Michx., *Pinus banksiana* Lamb. et *Picea glauca* (Moench) Voss) dans la structuration de la composition de la communauté des champignons EM. Les auteurs ont utilisé des semis âgés d'un an pour tester les sols de surface sur le terrain pendant 3 mois, et ils ont cultivé les semis sur chacun des sols de surface lors d'un test en chambre de croissance pendant 5 mois. Une mise à l'échelle multidimensionnelle non métrique a indiqué que l'identité de l'hôte structurait la composition des champignons EM sur le terrain, alors que l'identité de l'hôte et le sol de surface influençaient la composition des champignons EM dans les chambres de croissance. Cependant, la pré-colonisation des semis par des champignons de pépinière complique l'interprétation des résultats sur le terrain. Le taux de colonisation des semis par les champignons EM récoltés à travers les deux tests était relativement faible, soit approximativement 23 %. Les résultats des auteurs indiquent que les sols de couverture utilisés en réhabilitation des terrains miniers de surface conservent des propagules de champignons EM, et que l'utilisation d'une grande variété d'espèces d'arbres pour la restauration de la forêt boréale située en hauteur peut accroître la diversité des champignons EM récupérés. [Traduit par la Rédaction]

Mots-clés : Populus tremuloides, *Pinus banksiana*, *Picea glauca*, ADNr de l'espaceur transcrit interne, séquençage de Sanger, réhabilitation, liens végétal–sol.

Introduction

Surface mining, a relatively new disturbance in the Canadian boreal forest, involves the removal of vegetation and soils, resulting in the local loss of biodiversity and ecosystem functioning. Prior to accessing the target resources, the organic and upper mineral soil horizons are selectively salvaged and either stored in stockpiles or directly transferred to areas ready to be reclaimed. In Alberta, Canada, mine operators on public lands are required to reclaim land to be self-sustaining and of equivalent capability (i.e., land-use activities) as what existed previously (Alberta Environment 1999). Towards this requirement, restoration of ecosystem processes presents a unique challenge because many components (e.g., hydrology, soils, and vegetation) must be considered. In particular, restoring the biological linkages between aboveground vegetation and belowground soil organisms is important for re-establishing plant–soil feedbacks that are critical to the functioning of ecosystems as a whole (Kardol and Wardle 2010).

In boreal forests, symbioses are common between ectomycorrhizal (EM) fungi and roots of most trees species, which facilitate nutrient and water uptake from the soil at the tree level and contribute to nutrient and carbon cycling at the ecosystem level (Read et al. 2004; van der Heijden et al. 2008; Courty et al. 2010). Restoring these symbioses may promote tree survival and growth,

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as these long-lived plants are likely to encounter stress during their lifetime; for example, inoculation of tree seedlings with EM fungi has been performed since the 1970s in mine reclamation to improve seedling growth and survival (Marx 1975). However, the need for inoculation may be avoidable if extra effort is made to protect soils and the propagules within. Although the ectomycorrhizas formed between trees and fungi are lost through vegetation removal with mine disturbance, resistant propagules (EM inoculum) may survive in salvaged soils, depending on the time since disturbance and the handling techniques of soils. There are four factors likely to affect the species and abundance of fungi comprising EM inoculum present in reclaimed soils: (*i*) initial differences in the species of fungi present in salvaged soils, (*ii*) vegetation selected for re-establishment, (*iii*) abiotic conditions of reclaimed sites, and (*iv*) EM fungus immigration. Both initial stand composition and soils supporting those stands (i.e., the origin of the EM fungus inoculum) will influence the inoculum potential of salvaged soils (Hagerman and Durall 2004; O'Brien et al. 2011). The species of tree seedlings selected for afforestation of reclaimed areas may also affect the composition of the EM community; some EM fungi exhibit strong host preference (Molina et al. 1992; Massicotte et al. 1999; Izzo et al. 2006; Ishida et al. 2007; Tedersoo et al. 2008). Moreover, interactions between soils and host may be indicative of the importance of particular combinations of each, giving rise to variation in inoculum potential. Once salvaged soils have been placed, environmental stress often associated with reclaimed areas may also influence EM fungus inoculum potential. Soils of reclaimed areas may have low moisture and nutrient availability, high acidity, and high salinity (Bussler et al. 1984; Andersen et al. 1989; Cassleman et al. 2006), and species of EM fungi have been shown to vary in their sensitivity to such variables (Gehring et al. 1998; Swaty et al. 1998; O'Dell et al. 1999; Kjøller and Clemmensen 2009; Jones et al. 2012). Following soil placement, immigration of propagules may also influence the composition of EM communities. Surrounding intact boreal forest may be a source of EM fungus spores capable of air (Peay et al. 2012) or animal dispersal (Ashkannejhad and Horton 2006; Frank et al. 2006).

Of the factors influencing EM fungus inoculum potential of reclaimed areas, two stand out as being most easily controlled by restoration ecologists: the selection of hosts and cover soils. Selecting appropriate host – cover soil combinations may capture a range of EM fungus species that will be important for promoting seedling establishment and survival in reclaimed areas. Towards this goal, the Aurora Soil Capping Study was constructed in 2012 to test the effects of cover soil type, configuration, and depth on seedling performance. The study is a replicated field experiment, located in the Athabasca oil sands region of northern Alberta, designed to test various reclamation protocols at an operational scale. In the current study, we tested the inoculum potential of three locally salvaged materials used as cover soils (peat, forest floor material (FFM), and subsoil) over one growing season in both field and growth chamber assays using seedlings of three common boreal tree species: *Populus tremuloides* Michx., *Pinus banksiana* Lamb., and *Picea glauca* (Moench) Voss. Across the field and growth chamber assays, we asked: (*i*) Are EM fungi present and viable in each of the cover soils? (*ii*) Do EM communities exhibit structure that suggest the presence of host-specific taxa and, similarly, do cover soils influence EM communities in ways that suggest taxa with preferential affinities?

Methods

Site description

The Aurora Soil Capping Study (denoted "capping study" herein) is located within the Aurora North mine (57°19′20″N, 111°30′24″W) on the lease of Syncrude Canada Ltd., approximately 75 km north of Fort McMurray, Alberta. Oil sands surface mining in northern Alberta falls within the Central Mixedwood subregion of the Canadian boreal forest (Natural Regions Committee 2006). The general landscape of this region is undulating, with forests occurring in uplands, and bogs and fens in the lowlands and depressions. Upland forests in this region consist predominantly of *Picea glauca*, *Pinus banksiana*, and *Populus tremuloides*, and soils tend to be of the Luvisolic or Brunisolic orders, which typically contain a thin eluvial A horizon, a well-defined Bt or Bm horizon, and an underlying C horizon. Bogs are dominated by *Picea mariana* Britton, Sterns & Poggenb., and fens are dominated by *Picea mariana* and *Larix laricina* (Du Roi) K.Koch. The soils are poorly to very poorly drained and accumulate organic matter (peat). Salvaged peat is often used as an organic amendment in reclamation due to its abundance on this landscape.

The capping study (Fig. A1) covers an area of 36 hectares and was designed to test different cover soil configurations and depths to cap an overburden (material below the soil profile and above the ore deposit) disposal area. All cover soils were salvaged from within the Aurora North mine lease; specifically the upland material (forest floor material and subsoil) were salvaged from forests dominated by *Pinus banksiana*, and the peat was salvaged from lowland forests dominated by *Picea mariana*. All cover soils were salvaged and directly placed (e.g., no stockpiling or storage of materials) onto the research site during the winter prior to tree planting, which occurred in the spring of 2012 (described below). The directly placed soils were moved during the winter to minimize disturbance to soil structure and biota, such as EM fungus and plant propagules. Cover soils included peat salvage (surface to approximately 200 cm), upland FFM salvage (surface to depth of approximately 15 cm), and a blended mineral subsoil that included B and C soil horizons (approximately 50–100 cm soil depth; North Wind Land Resources Inc. 2013). Textural and chemical characteristics were independently assessed by North Wind Land Resources Inc. by subsampling each material immediately following placement. Peat material (n = 84) was free of sand, silt, and clay, and had a mean pH of 7.4 (min. 5.0, max. 7.8) and a mean electric conductivity (EC) of 1.2 $dS \cdot m^{-1}$ (min. 0.4, max. 2.3). FFM (n = 48; 92% sand, 4% silt, 4% clay) had a mean pH of 5.6 (min. 4.9, max. 7.1) and a mean EC of 0.2 $dS \cdot m^{-1}$ (min. 0.1, max. 0.6). Blended B–C subsoil material (n = 84; 95% sand, 2% silt, 5% clay) had a mean pH of 7.2 (min. 6.2, max. 7.9) and a mean EC of 0.2 $dS \cdot m^{-1}$ (min. 0.1, max. 0.7) (North Wind Land Resources Inc. 2013). Each cover soil treatment (1 ha each) was replicated three times; each containing three 25 m × 25 m single-species tree plots. In May 2012, single-species plots were planted with 1-year-old commercially grown container seedling stock of *Populus tremuloides* with a plug diameter of 6 cm and depth of 15 cm, *Picea glauca* (plug diameter 6 cm, depth 15 cm), or *Pinus banksiana* (plug diameter 4 cm, depth 12 cm). Seedlings were grown at Smoky Lake Forest Nursery (Smoky Lake, Alberta) from a mixture of open-pollinated seed collected from several populations in the Fort McMurray region. Based on a subsample of 20 seedlings, the initial mean (±SE), seedling height prior to outplanting was 30 ± 1.9 cm, 18 ± 0.6 cm, and 29 ± 1.2 cm for *Populus tremuloides*, *Pinus banksiana,* and *Picea glauca,* respectively. All tree plots were planted with 1 m spacing (10 000 stems per hectare). Daily air temperatures at the capping study for 2012, the year of sampling, ranged between 34.6 °C and 6.3 °C for the growing season (June to August; high to low; data collected by O'Kane Consultants). Cumulative precipitation for the 2012 growing season (June–August) at the capping study was 90.1 mm, the majority of which occurred throughout June and the beginning of July (data collected by O'Kane Consultants).

Field assay of EM fungi

To characterize the initial recovery of EM fungi in soils following reclamation, we surveyed roots of planted seedlings for EM fungus associations in two stages. First, at the time of planting, we randomly selected 20 seedlings of each tree species to assess EM

fungi present on roots formed during nursery production. Second, in late August 2012, after a full growing season, we harvested a total of 10 seedlings per species ranging from three to four subsamples from the single-species plots within each replicate of three selected cover soil materials (peat, FFM, and subsoil, $n = 3$). Seedlings were chosen systematically to ensure an even distribution in terms of visual size and health status. Once harvested, roots were separated from shoots and kept on ice until arrival at the University of Alberta where roots were stored at –20 °C until processing.

Growth chamber assay of EM fungi

To test the EM inoculum potential of the cover soils, we germinated seeds of the three host tree species in the cover soils in a growth chamber assay. We collected approximately 140 L of each cover soil from the same tree plots used in the field assay. These soils were immediately transported to the University of Alberta where they were pooled and homogenized. Samples of each cover soil were distributed onto a tarp and mixed manually for approximately 10–15 min using shovels and rakes. Homogenized cover soils were then stored at 4 °C for approximately 2 months until the start of the experiment. There were a total of 180 pots in this study. We filled 30 2L pots with each of the cover soils (peat, FFM, subsoil) for a total of 90 pots. To assess potential growth chamber contamination, we filled an additional 30 pots each with previously sterilized cover soils. Sterilization was achieved by heating cover soils to 121 °C at 103.4 kPa twice for 2 h, with a 24 h rest in between. Prior to potting, all pots were sterilized with Kleengrow disinfectant (Pace Chemicals Inc., Burnaby, British Columbia) and lined with window screen to prevent substrate loss during watering.

For the growth chamber assay, we used seeds of the same host species (*Picea glauca*, *Pinus banksiana*, and *Populus tremuloides*) and the same seed source which had been used to grow the seedling stock for the field assay of the capping study. Seeds of *Pinus banksiana* were stratified by soaking in water for 24 h at 4 °C, and afterwards placed on moist filter paper at 4 °C for 3 weeks. Seeds of *Picea glauca* were stratified by soaking in water for 24 h at 4 °C prior to planting, while seeds of *Populus tremuloides* were directly seeded. All species were initially germinated in sterilized generic potting soil (Sunshine Professional Growing Mix; Sun Gro Horticulture Canada Ltd., Vancouver, British Columbia). Single *Populus tremuloides* seedlings were transplanted to the experimental pots 2 weeks after germination, while *Pinus banksiana* and *Picea glauca* seedlings were transplanted in the same manner approximately 3–4 weeks after germination. Seedlings were arranged into five blocks rotated throughout the growth chamber every 3 weeks to expose each block to the range of air temperatures we knew to exist in the growth chamber. There were 36 pots in each block, in which each cover soil – tree species combination was represented twice. We randomly assigned the location of each seedling within a block (using a random number generator), and the locations were re-randomized with each rotation. All seedlings were fertilized (including those grown in sterilized cover soils) at low rates to promote seedling survival while minimizing negative effects on EM development. Fertilization occurred once immediately after transplanting with 1 $g \cdot L^{-1}$ of 10:52:10 (N:P:K) and thereafter monthly with 1 $g \cdot L^{-1}$ of 15:30:15 (N:P:K). Air temperature in the growth chamber was kept between 17 and 21 °C throughout the experiment and photoperiod was set at 16 h with a light intensity of 350 μmol photons$\cdot m^{-2} \cdot s^{-1}$ (photosynthetic active radiation). Dormancy was induced after 5 months by reducing air temperature to 10–12 °C and light to 8 h for an additional 2 weeks before seedlings were harvested (seedlings were approximately 22 weeks old at this time). At harvest, roots were stored at –20 °C before they were assessed for ectomycorrhizas (see below).

Description of ectomycorrhizas — morphotyping

All roots were thawed overnight at 4 °C. Adhering soil and debris was gently washed with tap water over a 1.2 mm sieve. Cleaned roots were cut into approximately 1 cm fragments, placed into a container (10 cm × 10 cm × 5 cm) filled with water, and homogenized using forceps for approximately 45 s. After homogenization, we used forceps to subsample from three different locations within the container and placed the roots onto a large petri dish filled with water for morphotyping. Entire root systems were subsampled from seedlings grown in the growth chamber assay; however, only lateral roots present outside the originally planted root plug were sampled from field seedlings, to target those ectomycorrhizas formed after planting. To ascertain the number of EM root tips required to adequately characterize the diversity of EM fungi colonizing roots of seedlings, we did a preliminary survey on a subset of seedlings harvested from the field. We generated accumulation curves based on the observation of 750 randomly selected root tips for three seedlings per species per treatment. Across all species, we generally found morphotype richness to plateau before examining 200 root tips, therefore we used this value for morphotyping the remaining seedlings (Fig. A2).

Colonized root tips were morphotyped under a dissecting microscope on the basis of root tip texture and colour, followed by further examination under a compound microscope for variation in hyphal and mantle formations at 100× magnification (Agerer 2001). If available, samples of four to five root tips from each morphotype per seedling were collected for DNA extraction.

Description of ectomycorrhizas — molecular analysis

Genomic DNA from two root tips representing each morphotype per seedling was individually extracted using Sigma Extraction Solution and Neutralization Solution B following the manufacturer's protocol (Sigma, Gillingham, Dorset, UK). Nested polymerase chain reaction (nested-PCR) amplification was performed using the fungus specific primer combinations NLC2–NSA3 and NSI1–NLB4 (Martin and Rygiewicz 2005). An aliquot of 1.0 μL of extracted DNA was combined with PCR reactants (8.0 μL of Red Taq (Sigma–Aldrich, St. Louis, Missouri, USA), 5.4 μL sterile MilliQ H_20, and 1.6 μL of 10 $mmol \cdot L^{-1}$ primers), making up a 16 μL reaction. The first round of amplifications (NLC2–NSA3) were performed with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1.5 min, 67 °C for 1 min, and 72 °C for 1.5 min, with a final extension of 72 °C for 10 min. The second round of amplifications (NSI1–NLB4) were performed with an initial denaturation at 95 °C for 5 min, followed by 27 cycles of 95 °C for 1.5 min, 55 °C at 1 min, and 72 °C at 1.5 min, with a final extension of 72 °C for 10 min. Gel electrophoresis using a 1% agarose gel was used to visualize PCR products. Successful PCR products (55% success rate) were purified using ExoSAP-IT (USB, Cleveland, Ohio, USA). Cycle sequencing was conducted using BIGDYE version 3.1 (Applied Biosystems, Foster City, California, USA) with NSI1 and NLB4 primers, and the resulting products were precipitated following the manufacturer's instructions for EDTA/ethanol. Bi-directional sequences were analyzed on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems). Sequences were edited with Geneious (Biomatters, Auckland, New Zealand). Nucleotides were converted to Ns if they had phred scores below 15. The ends of sequences were trimmed using an error probability of 3%. In Geneious (Biomatters), sequences across both experiments were aligned using MUSCLE (alignment software) into operational taxonomic units (OTUs). Consensus sequences were queried against the GenBank database using nBLAST. Sequences of OTUs identified were then submitted to GenBank for accession number assignment (Table 1). Most matches came back as "uncultured

Table 1. Closest nucleotide BLAST and UNITE species hypothesis of consensus sequences submitted to GenBank.

Genbank accession No.	BLAST closest match	Hit accession No.	Maximum score	Query cover	Percent identity	Query length	UNITE species hypothesis	UNITE species hypothesis number	Distance to closest species hypothesis
KJ938030	Uncultured ectomycorrhiza (*Amphinema*)	EF218741.1	1474	99%	99% (829/837)	843	*Amphinema byssoides*	SH133496.06FU	1%
KJ938039	Uncultured fungus	KC96595.1	1148	93%	99% (646/651)	695	*Cenococcum* 1	SH196545.06FU	1.5%
KJ938033	Uncultured *Rhizopogon* clone	FJ554251.1	1303	86%	98% (755/772)	896	*Rhizopogon rubescens* var. *pallidimaculatus*	SH086837.06FU	1%
KJ938031	Uncultured ectomycorrhiza (*Thelephora*)	EF218819.1	1566	95%	99% (872/876)	921	Thelephoraceae 1	SH195956.06FU	1.5%
KJ938040	Uncultured fungus clone	KC965209.1	1389	92%	99% (779/785)	852	*Tuber* 1	SH204354.06FU	1.5%
KJ938035	*Wilcoxina mikolae* voucher	GQ267499.1	1126	71%	99% (632/636)	889	*Wilcoxina mikolae*	SH227976.06FU	1.5%
KJ938032	Uncultured fungus clone	KF296921.1	1398	94%	98% (802/818)	864	*Hebeloma hiemale*	SH200120.06FU	1.5%
KJ938037	Uncultured fungus clone	KC966038.1	1234	96%	95% (747/787)	811	Thelephoraceae 2	SH220161.06FU	1.5%

EM fungus", therefore sequences of fungus OTUs were queried against the UNITE database and given identities based on distance to closest species hypothesis match given a 97%–99% similarity (Table 1; Kõljalg et al. 2013).

Data analysis

In the field assay, seedlings collected within each of the single-species plots (three to four subsamples per cover soil) were pooled ($n = 3$). Rates of EM fungus colonization and abundances were each averaged. The growth chamber assay had a randomized complete block design with 10 replicates. The experiment contained five blocks of seedlings which were rotated throughout the growth chamber every 3 weeks, removing the effect of blocking. There was no effect of blocking when added to the model for all analyses, therefore all analyses were run without blocking as a factor. All statistical analyses were performed using R (R Development Core Team 2008). The effects of host species and cover soil on total EM fungus percent colonization per seedling (i.e., the proportion of root tips colonized of the 200 observed × 100) and abundance of each OTU per seedling (i.e,. the proportion of root tips colonized by each OTU of the 200 observed root tips × 100), were tested using analysis of variance (ANOVA) following a general linear model approach when assumptions were met, and a permutation ANOVA when assumptions were not met (Wheeler 2010). If main effects were significant, post-hoc tests were performed using Tukey's honestly significant difference test. To visualize the community composition of EM fungi in relationship with seedling host and (or) cover soil, we used nonmetric multidimensional scaling (NMDS) using the metaMDS procedure from the vegan package in R (Oksanen 2013), with a random starting configuration, a stability criterion of 0.0005, the Bray–Curtis distance measure, and two default standardizations: a square root transformation to deal with the large data counts, and the Wisconsin-style double standardization to normalize data into maximum percent abundance. The field data set required five runs, while the growth chamber data set required three. Graphs were made using the first two dimensions with species vectors added at a maximum p value of 0.001. For inclusion of uncolonized seedlings in the NMDS, all EM fungus species abundances were transformed by the addition of a randomly selected positive value (1.5) maintaining distance relationships among treatments.

Results

Host preference of EM fungi recovered from the field assay

Presence of EM fungi on nursery seedlings prior to outplanting

Fifteen percent of *Populus tremuloides*, 15% of *Pinus banksiana*, and 40% of *Picea glauca* seedlings subsampled from the nursery stock were colonized by EM fungi. Two OTUs were identified from two observed morphotypes: *Amphinema byssoides* (Pers.) J. Erikss. and one species belonging to Thelephoraceae (Thelephoraceae 1). Thelephoraceae 1 occurred on roots of *Populus tremuloides* and *Pinus banksiana* seedlings, while *A. byssoides* occurred on roots of *Picea glauca* seedlings (Tables 1 and 2).

Presence of EM fungi across cover soils

After seedlings grew for one season at the capping study, six OTUs were identified from seven observed morphotypes (two of which matched those found prior to outplanting; see Table 2): *A. byssoides*, *Hebeloma hiemale* (uncertain naming status), *Rhizopogon rubescens* var. *pallidimaculatus* A.H. Sm., Thelephoraceae 1, and *Wilcoxina mikolae* (Chin S. Yang & H.E. Wilcox) Chin S. Yang & Korf. One morphotype was identified as belonging to multiple species, typically *Cenococcum* spp. and a species of *Meliniomyces*; therefore, it was referred to as "*Cenococcum*-like fungus" for this assay. Across all hosts and cover soils, *A. byssoides* was the most abundant EM fungus occurring on 100% of *Picea glauca* seedlings. Thelephoraceae 1 and *Rhizopogon rubescens* var. *pallidimaculatus* was found

Table 2. Occurrence of ectomycorrhizal (EM) fungi (i.e., percentage of seedlings colonized) on nursery grown seedlings of *Populus tremuloides*, *Pinus banksiana*, and *Picea glauca* before (n = 10) and after outplanting in three reclamation cover soils (forest floor material (FFM), peat, subsoil) in a field assay (n = 3).

		Populus tremuloides			*Pinus banksiana*			*Picea glauca*		
EM taxon	Sample period	FFM	Peat	Subsoil	FFM	Peat	Subsoil	FFM	Peat	Subsoil
Amphinema byssoides	Before	0%	0%	0%	0%	0%	0%	40%	0%	0%
	After	0%	0%	0%	0%	0%	0%	50%	60%	50%
Rhizopogen rubescens	Before	0%	0%	0%	0%	0%	0%	0%	0%	0%
var. *pallidimaculatus*	After	0%	0%	0%	60%	20%	100%	0%	0%	0%
Thelephoraceae 1	Before	15%	0%	0%	15%	0%	0%	0%	0%	0%
	After	0%	0%	0%	40%	40%	20%	0%	0%	0%
Wilcoxina mikolae	Before	0%	0%	0%	0%	0%	0%	0%	0%	0%
	After	0%	0%	0%	0%	10%	20%	10%	20%	0%
Hebeloma hiemale	Before	0%	0%	0%	0%	0%	0%	0%	0%	0%
	After	90%	100%	80%	0%	0%	0%	0%	0%	0%
Cenococcum-like fungus	Before	0%	0%	0%	0%	0%	0%	0%	0%	0%
	After	7%	1%	2%	4%	0%	4%	6%	1%	0%

on 78% and 67% of *Pinus banksiana* seedlings, respectively. *Hebeloma hiemale* occurred on roots of all *Populus tremuloides* seedlings. *Cenococcum*-like fungus occurred on 100% of *Populus tremuloides* seedlings and 78% of *Pinus banksiana* and *Picea glauca* seedlings. *Wilcoxina mikolae* was found on 44% of *Pinus banksiana* seedlings and 33% of *Picea glauca* seedlings. Mean (±SE) EM fungus colonization per seedling was 23% ±16.9% and was not influenced by host, cover soil, or their interaction (minimum p = 0.16).

Nonmetric multidimensional scaling indicated that abundances of EM fungi were influenced by host species rather than cover soil (Fig. 1*a*). Host identity drove the abundance of *A. byssoides*, Thelephoraceae 1, *R. rubescens* var. *pallidimaculatus*, and *H. hiemale*, while cover soil type influenced the abundance of *Cenococcum*-like fungus (Table 3; Table A1). Most fungi were specific to hosts. For instance, *A. byssoides* was found exclusively on *Picea glauca*, Thelephoraceae 1 and *R. rubescens* var. *pallidimaculatus* on *Pinus banksiana*, and *H. hiemale* on *Populus tremuloides*. *Cenococcum*-like fungus was more abundant on seedlings grown in FFM compared with peat or subsoil regardless of species (Table 3). There was no effect of host or cover soil on the abundance of *W. mikolae* (Table A1).

Host and cover soil preference of EM fungi recovered from the growth chamber assay

Sterilization substantially reduced the presence of EM fungi; after 22 weeks of growth in the growth chamber assay, only 5% of seedlings grown in sterilized cover soils were colonized compared with 76% of the seedlings grown in unsterilized cover soils (p < 0.01). Colonization was less than 1% for the few EM seedlings growing in sterilized cover soils compared with a mean of 23% for seedlings grown in unsterilized cover soils. EM fungi colonizing seedlings grown in sterilized cover soils were identified as one species belonging to the Thelephoraceae, which was different from the species that colonized the seedlings in the field assay.

A total of five OTUs were identified from seven observed morphotypes found on seedlings grown in unsterilized cover soils: *H. hiemale*, Thelephoraceae 2, *A. byssoides*, *Tuber* 1, and *Cenococcum* 1 (Table 4). Two rare morphotypes (occurring on <2% of seedlings and in low abundance) were not identifiable and therefore disregarded from further analysis. Across all hosts and cover soils, *H. hiemale* was the most prevalent EM fungus occurring on 20% of *Populus tremuloides*, 20% of *Pinus banksiana*, and 57% of *Picea glauca* seedlings. The second most prevalent OTU was Thelephoraceae 2 occurring on 73% of *Populus tremuloides* and 37% of *Pinus banksiana* seedlings. *Amphinema byssoides* occurred on roots of 10% of *Picea glauca* seedlings, and *Tuber* 1 occurred on 27% of *Populus tremuloides*, and 7% of *Pinus banksiana* seedlings. *Cenococcum* 1 was found on 67% of *Populus tremuloides* and 3% of *Picea glauca* seedlings. EM fungus colonization was not affected by host, cover soil, or their interaction (23 ± 2.7%; minimum p = 0.24).

Nonmetric multidimensional scaling indicated abundances of EM fungi were driven by host species and cover soil depending on the OTU (Fig. 1*b*). Cover soil affected the abundance of *Tuber* 1 (Table A2); host and cover soil affected the abundance of *H. hiemale,* and Thelephoraceae 2, (Table A2), and the abundance of *Cenococcum* 1 was influenced by an interaction between host and cover soil (Table A2). More specifically, *Tuber* 1 and *H. hiemale* were more abundant on seedlings grown in peat than in FFM and subsoil (Table 4), while Thelephoraceae 2 and *Cenococcum* 1 were more abundant on seedlings grown in FFM than in peat (Table 4). *Cenococcum* 1 was found only on *Picea glauca* seedlings grown in FFM and was also more abundant on seedlings grown in FFM than in subsoil (Table 4). *Hebeloma hiemale* was more abundant on *Picea glauca* than on *Pinus banksiana* or *Populus tremuloides* seedlings (Table 4). The extent of colonization of Thelephoraceae 2 was similar for *Populus tremuloides* and *Pinus banksiana* seedlings.

Discussion

We investigated the EM inoculum potential of three directly placed, locally salvaged cover soils used in upland boreal forest restoration following oil sands mining. We show that EM fungi are present and viable in salvaged, directly placed FFM, peat, and subsoil based on their ability to colonize three species of tree seedlings common to the region: *Populus tremuloides*, *Pinus banksiana*, and *Picea glauca*. Because seedlings used in the field study were colonized by EM fungi before planting, interpretation of our results is somewhat difficult. The presence of fungi formed in the nursery may have affected the inoculum potential of the cover soils, and new symbioses formed between seedlings and EM fungi. However, given this caveat, EM fungi displayed a preference for specific hosts, the extent of which depended on the type of assay performed.

Community composition of EM fungi in reclaimed soils

From both field and growth chamber inoculum potential assays, we identified EM fungi common to disturbed areas. *Rhizopogon* spp., *Wilcoxina* spp., also known as “E-strain fungi”, and species belonging to Thelephoraceae have been found in early successional forests (Visser 1995; Jones et al. 1997; Twieg et al. 2007), i.e., those with natural regeneration or planted within 1–6 years after disturbance by clearcut or fire. These types of EM fungi are considered “pioneer” fungi (with the exception of *Rhizopogon* spp., which is better characterized as a “multi-stage”

Fig. 1. Nonmetric multidimensional scaling ordination of ectomycorrhizal fungus species abundances measured as a percentage of root tip colonization per seedling for three host species (*Populus tremuloides*, *Pinus banksiana*, and *Picea glauca*) grown in three reclamation cover soils (forest floor material (FFM), peat, subsoil (SS)) in a (*a*) field (stress = 0.120) assay and (*b*) growth chamber (stress = 0.100) assay. Vectors are ectomycorrhizal fungal species; their direction and length represent their influence on the ectomycorrhizal community composition. Ellipses were generated using the SD of point scores to group seedlings by host species.

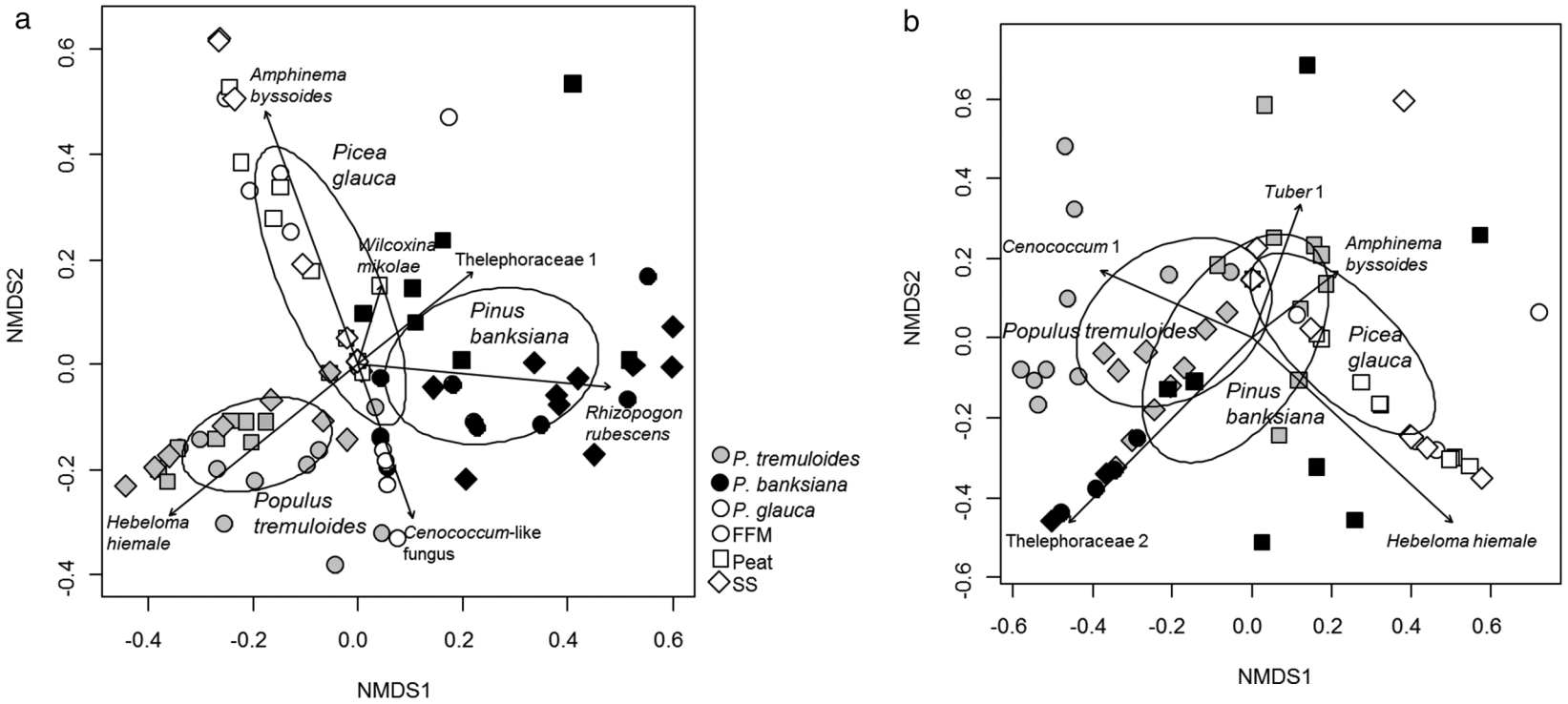

Table 3. Mean (±SE) ectomycorrhizal (EM) fungus abundance measured as a percentage of root tip colonization by each fungus operational taxonomic unit for *Populus tremuloides*, *Pinus banksiana*, and *Picea glauca* grown in three reclamation cover soils (forest floor material (FFM), peat, subsoil) in a field assay (n = 3).

	Populus tremuloides			*Pinus banksiana*			*Picea glauca*		
EM fungus	FFM	Peat	Subsoil	FFM	Peat	Subsoil	FFM	Peat	Subsoil
Amphinema byssoides	0±0.0a	0±0.0a	0±0.0a	0±0.0a	0±0.0a	0±0.0a	21±6.9a	24±17.6a	30±18.9a
Rhizopogon rubescens var. *pallidimaculatus*	0±0.0b	0±0.0b	0±0.0b	12±6.8b	6±5.8b	35±7.3a	0±0.0b	0±0.0b	0±0.0b
Thelephoraceae 1	0±0.0a	0±0.0a	0±0.0a	6±3.7a	12±9.1a	1±0.9a	0±0.0a	0±0.0a	0±0.0a
Wilcoxina mikolae	0±0.0a	0±0.0a	0±0.0a	0±0.0a	1±0.6a	1±0.5a	6±5.7a	2±1.4a	0±0.0a
Hebeloma hiemale	10±1.9a	12±1.5a	8±1.1a	0±0.0b	0±0.0b	0±0.0b	0±0.0b	0±0.0b	0±0.0b
Cenococcum-like fungus	7±3.1a	1±0.2b	2±1.0ab	4±0.6ab	0±0.5b	4±0.8ab	6±0.9ab	1±0.7ab	0±0.3b

Note: Differences in lettering represents statistical significance ($p < 0.05$) for each EM fungi across all hosts and cover soils.

Table 4. Mean (±SE) ectomycorrhizal (EM) fungus abundance as a percentage of root tip colonization of *Populus tremuloides*, *Pinus banksiana*, and *Picea glauca* grown in three reclamation cover soils (forest floor material (FFM), peat, subsoil) in a growth chamber assay (n = 10).

	Populus tremuloides			*Pinus banksiana*			*Picea glauca*		
EM fungus	FFM	Peat	Subsoil	FFM	Peat	Subsoil	FFM	Peat	Subsoil
Hebeloma hiemale	0±0.0b	5±2.0a	0±0.0b	0±0.0b	17±6.6a	0±0.0b	13±6.6ab	28±8.7a	18±8.3ab
Thelephoraceae 2	27±8.3a	2±1.3b	16±3.8ab	21±9.4ab	9±4.2ab	15±10.0ab	0±0.0b	0±0.0b	0±0.0b
Amphinema byssoides	0±0.0a	0±0.0a	0±0.0a	0±0.0a	0±0.0a	0±0.0a	4±3.5a	0±0.0a	3±2.9a
Tuber 1	0±0.2a	5±2.6a	0±0.1a	0±0.0a	6±3.9a	0±0.0a	0±0.0a	0±0.0a	0±0.0a
Cenococcum 1	15±3.2a	2±0.5b	1±0.8b	0±0.0b	0±0.0b	0±0.0b	0±0.0b	0±0.0b	0±0.0b

Note: Values here are from unsterilized cover soils only. Differences in lettering represents statistical significance ($p < 0.05$) for each EM fungi across all hosts and cover soils.

fungus) because of prolific spore dispersal and fast root colonization (Newton 1992). Additionally, *A. byssoides*, species of Thelephoraceae, E-strain fungi, *Cenococcum* spp., and *Tuber* spp. have all been documented on nursery grown seedlings (Hunt 1991; Smaill and Walbert 2013) indicating their ubiquitous dispersal and colonization abilities. We found two taxa of Thelephoraceae; one on seedlings grown in sterilized cover soils in the growth chamber, potentially a growth chamber contaminant, and the other found on nursery seedlings before and after outplanting in the field.

Colonization of seedlings was lower in our study than that reported from studies of seedlings (hosts ranging from 8 weeks old, and root samples from mature forests) growing in early-to-late successional areas, which generally report values greater than 50% (Visser 1995; Taylor and Bruns 1999; Nara et al. 2003; Bois et al. 2005; Pennanen et al. 2005). Relatively low values of EM colonization in both field and growth chamber assays suggests that directly placed reclamation cover soils have less inoculum potential than early successional, such as young clearcuts, and late successional forests. Levels of colonization may be lower in reclaimed soils because of the complete disruption (i.e., salvage, hauling, placement, and spreading) of the surface soil materials and the dilution of propagules in addition to the loss of the predisturbance vegetation. In our experiment, cover soils were directly placed, without stockpiling, an activity which can even further reduce the viability of EM fungus propagules (Reddell and Milnes 1992). Further, the capping study is young (<3 years old) and isolated; the distance to undisturbed forest edges at the capping study exceeds those found for average clearcuts (>1 km), thus, dispersal from undisturbed forests may have been limited for many EM fungi (Peay et al. 2012). However, over time, EM fungus immigration, germination of dormant spores, and host establishment may occur; all of which could increase colonization of plants occurring on reclaimed areas.

Host preference expressed by EM fungi

Composition of EM communities in the field assay was structured by host identity regardless of cover soil, while the trend was less clear in the growth chamber assay. Of notable interest is the difference in host preference of *H. hiemale* between field and growth chamber assays. In the field assay, *H. hiemale* only associated with *Populus tremuloides* seedlings; while in the growth chamber, it associated with *Picea glauca* in greater abundance than *Populus tremuloides*. This difference contributes to, and complicates, our conclusions on host preference. In each assay, *H. hiemale* was found primarily on one host (which supports our conclusions on host preference); however, the host species was different between the two assays (complicating this conclusion). Because the EM fungal species found in our study tend to be classified as "pioneer" and "multi-stage" fungi, we do not expect to see strong host specificity; however, in different circumstances these types of fungi may exhibit host preference (as shown in this study), but the species of host an EM fungus shows preference towards may differ. General discrepancies in host and cover soil preference of EM fungi between the assays could be due to (*i*) differences in abiotic conditions, (*ii*) the size and age of seedlings, and (or) (*iii*) the mycorrhizal condition at the start of the experiment. For instance, the growth chamber seedlings were initially lightly fertilized and watered consistently throughout the experiment to ensure survival, while the field seedlings relied on nutrients and moisture available in the cover soils, which likely varied throughout the season. Air temperatures in the growth chamber were held constant throughout the experiment while seedlings in the field were exposed to variation in air and soil temperatures due to naturally occurring seasonal and diurnal fluctuations. By design, the Aurora Soil Capping Study removed many sources of natural variation, such as topography and cover soil depth; however, completely eliminating all variation in large-scale soil placement operations is difficult. This means variation within the single 1 ha treatments may have precluded a consistent cover soil signal. Field studies of early successional systems have also found that EM fungi are host-specific and that cover soil has little influence on shaping the fungus community. A recent study by Walker and Jones (2013) found EM fungus communities to differ between intact and clearcut forests while homogeneous across microhabitats. Twieg et al. (2007) found a dominance of host-specific EM fungi in young forest stands (~5 years) compared with older stands in mixed temperate forests in interior British Columbia.

In addition to general differences in abiotic variables between the two assays, seedlings were also of different sizes. The growth chamber seedlings germinated from seed and developed their root system in the cover soil, whereas the field seedlings were nursery-grown and had been outplanted into the field. Growth chamber seedlings had lower biomass than those grown in the field (data not shown), and combined with the relatively low light levels in the growth chamber; field seedlings may have had greater potential to allocate carbon to supporting ectomycorrhizas. Consequently, potential carbon limitation in growth chamber seedlings may have hindered host preference among EM fungi. Low host specificity among EM fungi has also emerged in studies from stressful environments at ecosystem scales such as the arctic tree line (Botnen et al. 2014). Host specificity may be too costly for EM fungi under such extreme abiotic conditions.

Aside from abiotic and seedling size and age differences between the two assays, initial EM status of seedlings prior to the start of both assays was different. Field seedlings prior to outplanting were colonized by either *A. byssoides* or Thelephoraceae 1, while the growth chamber seedlings had zero initial colonization because they were germinated by seed first into a sterilized potting soil and later transplanted directly into cover soils. For the field seedlings, it is unclear whether the cover soils or the nursery was the source of Thelephoraceae 1 and *A. byssoides* (see Table 2). Because some of the seedlings grown in sterilized cover soils in the growth chamber were also colonized by Thelephoraceae 1, this EM fungus may therefore be a growth chamber contaminant. *Thelephora terrestris* (Thelephoraceae family) and *A. byssoides* have often been found to colonize seedlings grown in nurseries (Hunt 1991), and *T. terrestris* is able to colonize roots from spore or small amounts of fungus mycelia (Visser 1995; Jones et al. 1997; Kranabetter and Friesen 2002), suggesting that these fungi are ubiquitous.

Conclusions

We investigated two factors likely to influence the species of fungi comprising EM inoculum in reclaimed sites: (*i*) initial differences in the species present in salvaged soils, and (*ii*) host species selected for vegetation re-establishment. We tested for differences in EM fungus inoculum potential based on initial differences caused by cover soil origin and host-mediated effects. Of these, the primary factor found to influence the community composition of EM fungi in the field assay was the host species selected for vegetation re-establishment, while in the growth chamber assay, both the type of salvaged soil and the host species influenced the EM composition. The drivers of EM community composition between the two assays could be due to differences in abiotic conditions, the size and age of seedlings, and mycorrhizal condition at the start of the experiment. In addition, differences in belowground carbon allocation between seedlings from the two assays may have also affected the composition of EM fungi. Despite these differences between the two assays, because both experiments were relatively short (3 and 5 months for the field and growth chamber assays, respectively), the similarities in the outcomes of the two experiments likely tell us more than the differences. The extent of host preference of pioneer or multistage EM fungi in early successional ecosystems has important implications for ecosystem restoration. Planting a range of plant species on upland boreal forest reclamation areas may be a strategy for recovering and potentially maintaining a greater diversity of EM fungi. In addition to retaining EM fungi, cover soil type may also be critical for seedling survival based on adequate moisture retention, nutrient availability, and soil temperature. The recovery of EM fungi in different reclamation scenarios should be continually monitored over time to better understand how different host – cover soil combinations govern future community dynamics in combination with seedling performance.

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Appendix A

Appendix A begins on the next page.

Fig. A1. The Aurora Soil Capping Study manipulates type, configuration, and depth of cover soils. Map shows directly placed cover soils used in this study (n = 3): Peat, 30 cm cover of peat; FFM, 20 cm cover of forest floor material; and subsoil, 150 cm cover of blended B–C subsoil soil horizons. Within each replicate, seedlings of *Populus tremuloides*, *Pinus banksiana*, and *Picea glauca* were planted in single-species 25 m × 25 m vegetation plots at 10 000 stems per hectare. Vegetation plots are labeled as "A" (*Populus tremuloides*), "S" (*Picea glauca*), or "P" (*Pinus banksiana*).

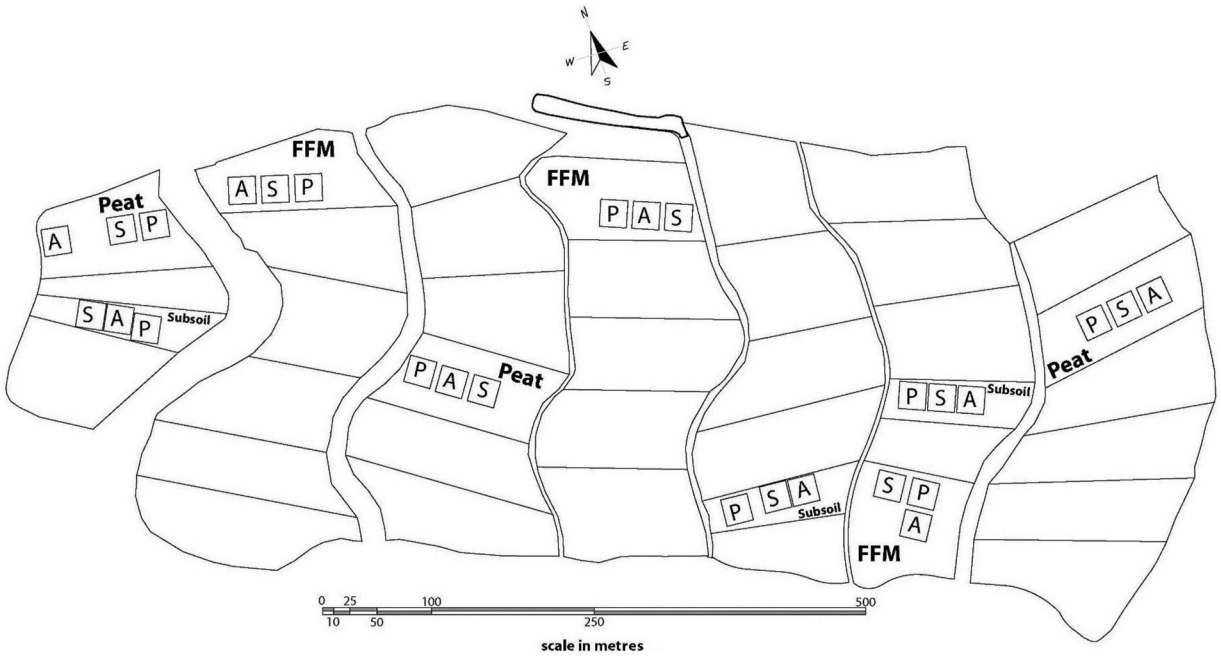

Table A1. Results of permutation ANOVAs from a field assay testing effects of host species and cover soil used in forest restoration following oil sands mining on rates of ectomycorrhizal fungus colonization.

	Statistics	df	SS	MS	Iter	p
Amphinema byssoides	Host	2	3789.9	1895.0	5000	**0.008**
	Cover soil	2	43.4	21.7	51	1.000
	Host × cover soil	4	86.8	2107	60	1.000
	Residuals	18	4293.1	238.5		
Thelephoraceae 1	Host	2	235.3	117.6	5000	**0.008**
	Cover soil	2	57.2	28.6	196	0.403
	Host × cover soil	4	114.4	28.6	1220	0.471
	Residuals	18	579.3	32.2		
Hebeloma hiemale	Host	2	582.7	291.4	5000	**<0.001**
	Cover soil	2	8.5	4.2	454	0.260
	Host × cover soil	4	17.0	4.2	1494	0.214
	Residuals	18	57.4	3.2		
Rhizopogon rubescens var. *pallidimaculatus*	Host	2	1769.9	884.9	5000	**0.001**
	Cover soil	2	421.3	210.7	4720	0.165
	Host × cover soil	4	966.9	241.7	5000	0.092
	Residuals	18	818.2	45.5		
Wilcoxina mikolae	Host	2	29.6	14.8	1029	0.295
	Cover soil	2	13.3	6.6	269	0.933
	Host × cover soil	4	39.0	9.8	339	0.679
	Residuals	18	207.6	11.5		
Cenococcum-like fungus	Host	2	1.7	0.8	121	0.686
	Cover soil	2	120.0	60.0	5000	**<0.001**
	Host × cover soil	4	28.8	7.2	4019	0.156
	Residuals	18	78.5	4.4		

Note: Values in boldface type are statistically significant at $\alpha < 0.05$. df, degrees of freedom; SS, sums of squares; MS, mean square error; Iter, number of iterations.

Table A2. Results of permutation ANOVAs from a growth chamber assay testing effect of host species and cover soil used in forest restoration following oil sands mining on rates of ectomycorrhizal fungus colonization per host seedling.

	Statistics	df	SS	MS	Iter	p
Tuber 1	Host	2	78.4	39.2	1339	0.185
	Cover soil	2	264.1	132.0	5000	**<0.001**
	Host × cover soil	4	136.8	34.2	1266	0.179
	Residuals	81	1982.6	24.5		
Amphinema byssoides	Host	2	96.3	48.1	69	0.594
	Cover soil	2	24.1	12.0	51	1.000
	Host × cover soil	4	47.5	11.9	69	0.841
	Residuals	81	1948.6	24.1		
Cenococcum 1	Host	2	722.5	361.2	5000	**<0.001**
	Cover soil	2	405.8	202.9	5000	**<0.001**
	Host × cover soil	4	802.3	200.6	5000	**<0.001**
	Residuals	81	1006.0	12.4		
Thelephoraceae 2	Host	2	4682.3	2341.1	5000	**0.002**
	Cover soil	2	2242.2	1121.1	4369	**0.048**
	Host × cover soil	4	1593.6	398.4	5000	0.231
	Residuals	81	25284.8	312.2		
Hebeloma hiemale	Host	2	5357.4	2678.7	5000	**0.001**
	Cover soil	2	2705.0	1352.5	5000	**0.007**
	Host × cover soil	4	519.2	129.8	409	0.731
	Residuals	81	21866.4	267.0		

Note: Values in boldface type are statistically significant at $\alpha < 0.05$. df, degrees of freedom; SS, sums of squares; MS, mean square error; Iter, number of iterations.

Fig. A2. Accumulation curves of number of (#) ectomycorrhizal morphotypes per number of root tips counted for (A) *Populus tremuloides*, (B) *Pinus banksiana*, and (C) *Picea glauca* grown in forest floor material (FFM), peat, and subsoil.