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Parsing the roles of abiotic and biotic factors in Douglas-fir seedling growth☆

Justine Karst *, Jason D. Hoeksema, Melanie D. Jones, Roy Turkington

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

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SUMMARY

Many studies have confirmed the potential importance of mycorrhizas to plant growth and community structure. Needed now are studies that focus on the relative ecological importance of mycorrhizas, specifically how the effect of mycorrhizas compares with other environmental factors present in soil that may affect plant growth. In a greenhouse, we grew Douglas-fir seedlings in soil collected from 6 sites in a dry region in British Columbia, Canada, predicting that these soils would vary in the ectomycorrhizal fungi present, and in several physical characteristics. In addition, we imposed a gradient of soil moisture. Using model selection based on information theory and multimodel inference, we ranked the relative importance of several abiotic (watering level, pH, C:N) and biotic (ectomycorrhizal richness and % colonization by ectomycorrhizal fungi) variables in models predicting seedling biomass. Variation in abiotic factors, namely watering level and pH, tempered by the effects of ectomycorrhizal richness, contributed most to variation in seedling growth. For young Douglas-fir seedlings growing in disturbed environments, small shifts in the species richness of ectomycorrhizal fungi bear importance, yet overall remain subordinate to abiotic gradients. By utilizing natural variability in soil abiotic properties and mycorrhizal community composition along with experimental manipulations of additional factors, the experimental approach utilized here helps to move us beyond simply testing for the significance of mycorrhizas for plants to understanding the relative importance of mycorrhizas in comparison with other influential ecological factors.

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Introduction

The potential influence of mycorrhizas on host growth and plant communities has been demonstrated repeatedly (Van der Heijden et al. 1998; Hartnett and Wilson 1999; Vogelsang et al. 2006; Karst et al. 2008; Hoeksema et al. 2010). The relative influence of mycorrhizas compared with other environmental variables is still poorly understood, however (Klironomos et al. 2011). In particular, the relative importance of mycorrhizal fungal presence and composition for plant community composition has rarely been tested, and only in systems supporting arbuscular mycorrhizal fungi (see Table 1 in Klironomos et al. 2011). Even studies of plant growth, however, have rarely quantified the relative importance of ectomycorrhizas compared to other factors. Typically, such studies may cross single-species ectomycorrhizal inoculation with one or two other factors, and yield yes/no answers of significance for each factor rather than quantifying the relative contribution of each factor to plant growth.

The paucity of studies testing the relative importance of presence and composition of ectomycorrhizal fungi versus other factors is in part due to methodological challenges; manipulations of ectomycorrhizal fungal composition and/or abundance are difficult and typically require sterile laboratory environments. The confounding variables present in field-based observational studies makes causation difficult to ascertain. Though each approach has problems, a combined approach may serve as a compromise between reductionist laboratory experiments and the ecological realism present in the field. For example, surveys in the field show that the location of a site can have a pronounced effect on the composition and diversity of ectomycorrhizal communities (Dahlberg 2001; Kjøller and Bruns 2003; Izzo et al. 2005; but see Bidartondo et al. 2001) and steep species accumulation curves indicate that new species are encountered over relatively short distances (Horton and Bruns 2001; Lilleskov et al. 2004). With distance, however, come changes in soil abiotic conditions, such that there are correlated changes in ectomycorrhizal fungal community composition and environmental variables. If those correlations are imperfect, we may be able to use spatial variability of ectomycorrhizal fungal community composition and environmental variables as a natural experiment testing their relative effects on plants; however, to most effectively decouple fungal composition and soil environmental variables, experimental manipulation may also be required.

We used the spatial variability in ectomycorrhizal community richness and soil properties as a natural experiment to indirectly test the effects of those factors on a host plant by growing plants in soils collected from different locations. In addition to this natural variability, we manipulated a putatively important ecological variable to host growth, soil moisture. In western North America, the ectomycorrhizal host species interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca* [Beissn.] Franco) is a major component of dry interior forests (Hermann 1985). Soil moisture and fertility are well known modifiers of height and biomass of Douglas-fir in these habitats (Burns and Honkala 1990). The southern interior of British Columbia, Canada, is characterized by dry, hot summers and moisture is an important limiting resource for plants. From this region, we collected soils adjacent to mature Douglas-fir trees at six sites. We then planted Douglas-fir seedlings into the soils in a greenhouse and applied and maintained three different soil moisture regimes. After confirming the role of site in variation of seedling growth, we then used multi-factor statistical models to simultaneously estimate the effects on plant growth of multiple variables relating to the abiotic soil conditions and ectomycorrhizal richness and abundance of those sites. We used likelihood-based information-theoretic methods (Burnham and Anderson 2002) to define relative importance of the different ecological factors included in the candidate models and to generate quantitative estimates of the effects of those factors. This approach can take us beyond testing for the significance of host response to the presence/absence of mycorrhizas to understanding the importance of that response in comparison with other influential ecological factors.

Materials and methods

Origin and collection of soils

Soil samples were collected from the Thompson and Okanagan Valleys in the southern interior plateau of British Columbia, Canada. This area has a continental climate, with warm, dry summers and cool winters. In valley bottoms, the average daily minimum temperature for the winter months (November–February) is −5 °C; for the summer months (May–August), the average daily maximum is 25 °C (Environment Canada 2004). There is a strong elevational gradient in annual precipitation ranging from 300 mm at lower elevations (300–800 m above sea level (a.s.l.)) to greater than 1000 mm at subalpine (1200–1400 m a.s.l.) elevations (Meidinger and Pojar 1991). Open forests of Douglas-fir mixed with Ponderosa pine (*Pinus ponderosa* Dougl. Ex P. & C. Lawa.) and several species of grasses (*Koeleria macrantha* [Ledeb.] J. A. Schultes f., *Poa pratensis* L. and *Calamagrostis rubescens* Buckl.) occur at lower elevations, whereas at higher elevations, Douglas-fir grades into hybrid spruce (*Picea engelmanni* Parry ex Engelm. × *Picea glauca* [Moench] Voss) and lodgepole pine (*Pinus contorta* Dougl. Ex. Loud. var. *latifolia* Engelm.) (Meidinger and Pojar 1991).

We collected soil samples in October 2003 from six sites, each approximately 400 m². Sampling locations were chosen on the basis of 3 criteria: (1) several mature (cone-bearing; > 10 m in height) Douglas-fir trees were present, (2) sites were separated by at least 5 km, and (3) the location of sites spanned the elevational gradient described above. Sites were distributed over a distance of 140 km and ranged in elevation from 360 to 1396 m a.s.l. (Table S1). After removing loose litter or moss, we collected 50 cm × 50 cm × 10 cm deep volumes of soil from within the rooting zones of 2–3 Douglas-fir trees at each of the six sites. No attempt was made to control the relative inputs of organic and mineral horizons to the collected soils. We sieved the soil through a 2.5 cm² mesh in the field to remove woody debris and stones and afterwards refrigerated the soil at 4 °C in plastic tubs. A subsample of mixed soil from each of the six sites was analyzed (Soilcon Laboratories Ltd., Richmond, British Columbia, Canada) for pH, % organic matter measured by loss on ignition, total organic C, ammonium N, nitrate and nitrite N, total N, available P, and estimated C:N, using standard procedures described in Carter (1993) and McKeague (1978).

Plant material

In mid-November 2003, non-mycorrhizal Douglas-fir seedlings were grown in a greenhouse at the University of British Columbia, Vancouver, from a mixture of open-pollinated seeds (seedlot #48520, collected at 850–950 m a.s.l. obtained from the BC Ministry of Forest Tree Seed Center (Surrey, British Columbia, Canada). Seeds were moist stratified at 4 °C for 21 days, then sterilized in 3% H_2O_2 while being stirred constantly for 2 h. We sowed the seeds into #1206 bedding inserts (Kora Products, Bramalea, Ontario, Canada) filled with an autoclaved 3:1 (v:v) mixture of peat and perlite. Two seeds were placed into each cavity and covered with 0.5 cm of sterilized sand. The trays were misted each day for six weeks, after which seedlings were transplanted into sterilized pots. Just prior to transplanting, a random subsample of twenty seedlings was harvested to determine the initial average mass of seedlings. We cleared and stained roots from fifteen additional seedlings to confirm their non-mycorrhizal status (none were mycorrhizal). Throughout the experiment, natural daylight in the greenhouse was supplemented by 400 W high pressure sodium lamps for 18 h daily. Greenhouse temperature ranged from 20 to 24 °C while the relative humidity was maintained at 60%.

Maintenance of soil moisture

In December 2003, the field soil was removed from cold storage and mixed with perlite (3:1 v:v). Three watering treatments, low medium and high, corresponding to 10% (low), 20% (medium), and 30% (high) volumetric soil moisture, were applied to soils from each sampling site. The range in watering regimes was based on the range of field measurements of soil moisture at 450 m a.s.l. and 1200 m a.s.l. taken throughout one week in July 2003. In total, 180 1.2 l pots were prepared into which single seedlings were transplanted (6 soil sites × 3 watering regimes × 10 replicates = 180). Dry soil was determined to be equivalent to 3% soil moisture using a CS620 Hydrosense soil moisture probe (Campbell Scientific, Inc., Utah, USA). The mass of a pot required to obtain the designated soil moisture levels was then calculated based on this initial measurement. The seedlings were transplanted on January 6, 2004, and over the following 8 months, we regularly weighed pots and added enough water to bring the pot mass up to the appropriate mass for the watering treatment imposed. It was impractical to maintain the pots at constant soil moisture so pots were allowed to dry to 10% below their designated soil moisture before adding water. Consequently, the seedlings were watered every three days at the beginning of the experiment and each day by the end of the experiment. At the end of the experiment, no seedling weighed greater than 1% of the total pot mass; therefore, we did not adjust the amount of water added to pots to compensate for increasing seedling mass. The location of all pots was randomized monthly.

Final harvest

Seedlings were harvested on August 18, 2004. Shoots were dried at 65 °C for 48 h and weighed. Roots were bagged along with their attached soil and refrigerated at 4 °C. For processing, entire root systems were carefully washed under gently running tap water and cut into 1 cm pieces. All root fragments were placed in a dish containing tap water and a random subsample was then distributed in a Petri

plate. When possible, we inspected at least 100 root tips per individual seedling. In cases where seedlings had less than 100 root tips, all tips were counted. Generally, ectomycorrhizal tips were turgid and smooth, had emanating hyphae or rhizomorphs, and 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 ectomycorrhizal roots and rhizomorphs was determined under a stereomicroscope while Hartig net, mantle, emanating hyphae, and other such features were observed using a compound microscope under 400 or 1000× magnification. When possible, mantle peels were made by separating the fungal tissue from the root with fine forceps and micro-scalpels. Morphological descriptions were made with reference primarily to Ingleby et al. (1990) and Goodman et al. (1996).

Once processed, roots were dried at 65 °C for 48 h and weighed. Two root tips representing each morphotype were lyophilized, and total genomic DNA was extracted from single ectomycorrhizal tips by pulverizing them for 45 s at a speed of 5.0 units 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 re-suspended in 50 µl 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). PCR reactions typically included 1 µL template DNA, 18.6 µL sterile purified water (Barnested Nanopure Diamond water purifier), 0.2 mM deoxyribonucleotides (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^{TM} (Applied Biosystems, Foster City, CA, USA). Samples were amplified using a PTC-200 thermal cycler (MJ Research Inc., 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 Inc., Valencia, CA, USA). Prior to 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 Corporation, Ann Arbor, MI, USA). Taxonomic matches were based on GenBank BLAST results with ≥98% sequence similarity.

Statistical analyses

Testing effects of site and watering regime on seedling growth and ectomycorrhizal properties.

Using ANOVA, we tested the effect of site and watering regime on total biomass (dry mass of root and shoot together) and root:shoot of seedlings. Due to seedling mortality, not all treatment combinations were present (see note in Fig. 1) and consequently we were unable to test the interaction between site and watering regime. We also tested the main effects of site and watering on % colonization (the percentage of live root tips forming ectomycorrhizas for each seedling) and morphotype richness to confirm that these biotic properties of the soils varied across sites and to determine whether watering affected biotic properties. Neither % colonization nor morphotype richness conformed to assumptions of normality; consequently we used randomization tests (with
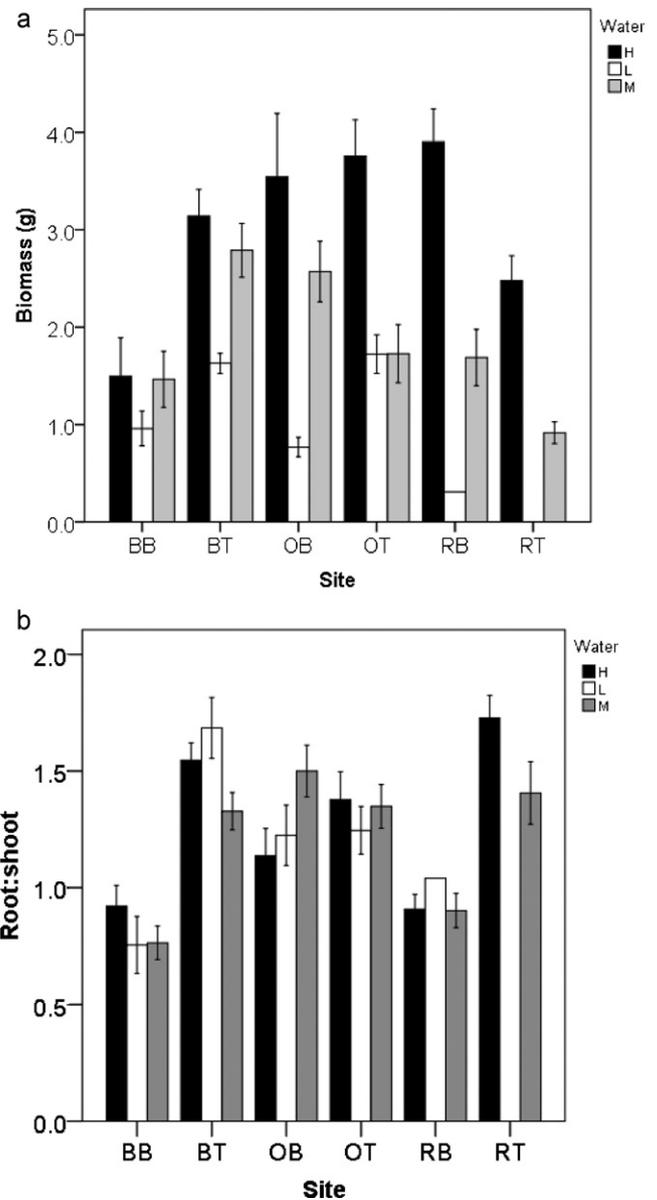
Fig. 1. (a) Mean biomass (g) (±1 SE) and (b) mean root:shoot (±1 SE) of Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings grown in different soils collected from southwestern British Columbia and under different watering regimes. The three watering levels, high, medium and low correspond to 30%, 20% and 10% soil moisture, respectively. Note that there is no bar for "RT" soils because no seedlings survived, and no error estimate for "RB" soils because only one seedling survived.

10,000 iterations) to calculate probability values for each of the main effects. Effects were considered significant at $\alpha = 0.05$. We did not test the effect of watering on abiotic properties of the soils because they were measured before watering treatments were applied.

Estimating the relative importance of biotic and abiotic variables for seedling growth

Both the effects of site and watering on plant growth (total biomass and root:shoot) were significant (see Table 1), so we continued to explore factors of the sites that might account for this result, as well as the relative importance of those factors compared to watering. In addition to watering, we used pH, C:N, P, morphotype richness and % colonization as fixed "explanatory" variables. These factors were chosen based on the assumption

Table 1
Summary of ANOVA for the effects of soil site and watering level on total biomass and root:shoot of Douglas-fir (*Pseudotsuga menziesii* var. *glauca*).

Response	Source	df	SS	F ratio	P > F
Total biomass (g)	Site	1, 5	35.42	7.111	<0.0001
	Watering level	1, 2	91.86	46.114	<0.0001
Root:shoot	Site	1, 5	11.13	20.44	<0.0001
	Watering level	1, 2	0.095	0.44	0.65

that soil moisture and C:N mediate nutrient availability, whereas pH affects nearly all soil properties (Brady and Weil 1996). The uptake of P, a generally immobile macronutrient, can be one of the primary benefits of ectomycorrhizal fungi to mycorrhizal plants (Jones et al. 1990; Smith and Read 2008). We also hypothesized that seedling growth may be modified by factors such as morphotype richness and amount of colonization by ectomycorrhizal fungi because seedlings may gain physiological versatility by being associated with a high diversity of ectomycorrhizal fungi (Perry et al. 1989; Jones et al. 1997; Simard et al. 1997) and the relative number of ectomycorrhizal root tips has been reported to be positively correlated with seedling biomass, though this relationship is far from consistent (Karst et al. 2008). Both morphotype richness and colonization were designated as "explanatory"; however, we recognize that assigning direct causality between seedling growth and these factors is difficult in this context. We used this analysis to explore the relative importance of a variety of ecological factors, rather than determine explicit causation. We also tested statistical interactions between morphotype richness or % colonization and each of the selected abiotic variables, but only considered statistical models containing one of these interactions at a time.

Variables associated with site characteristics are observational and, as such, are likely to show collinearity. If used to analyze such a dataset, a stepwise multiple regression approach for selecting a single statistical model based on *P*-values associated with individual factors would likely result in substantial errors in model selection and parameter estimation (Chatfield 1995, Burnham and Anderson 2002; Whittingham et al. 2006). Thus, we chose to use information-theoretic criteria to rank candidate multiple regression models having different combinations of explanatory variables and to rank the relative importance of the variables in those models. We also focused on estimating the parameters associated with important explanatory variables, rather than testing their statistical significance (Burnham and Anderson 2002). Overall, our analytical approach allows for inference from multiple models, focuses inference on the weight of evidence in the data for different models and factors and on the size and direction of effects, and avoids testing trivial null hypotheses about individual factors that may or may not belong in the best model or models.

For seedling biomass and root:shoot separately, we quantified the relative importance of the different fixed factors by analyzing a series of multiple regression models, including models containing all of the main effects of the six factors, models containing a single interaction between a biotic factor (% colonization or morphotype richness) and one of the four abiotic factors, and models with all possible subsets of these main effects and interactions, omitting models with interactions that did not include as main effects both of the variables comprising the interaction. To ensure that we tested variation in plant growth due to variation in morphotype richness and % colonization among sites, rather than due to experimental error variation in those factors within sites, we included site and the interactions between site and morphotype richness and % colonization as random effects in each model. This approach allows us to include all seedlings while avoiding an exaggerated estimate of variation. This approach was not necessary for the remaining observational soil variables (pH, C:N, and P) because they did not

vary within a site due to experimental error, having been measured in the soils before the experiment.

For each multifactor model, we recorded the information-theoretic criterion AIC_C (Akaike's Information Criterion corrected for small samples, which converges on AIC for large samples), which was then compared among alternative models to provide a relative measure of support in the data for those alternative models (Burnham and Anderson 2002). Within each analysis (seedling biomass and root:shoot), we ranked all statistical models by their AIC_C scores, including the global model. Based on these rankings and the magnitude of differences in AIC_C scores between models, we calculated an Akaike weight (w_i) for each model i, which represents the weight of evidence in favor of model i being the best model in the set of candidate models, given the data (Burnham and Anderson 2002). The Akaike weights sum to 1 and may be interpreted as the probability that model i is the actual expected best model, given the data. We then defined a 95% confidence set of models for inference by summing w_i progressively from the best model to the worst, until the sum of w_i exceeded 95%, eliminating models not included in that 95% confidence set. Finally, we calculated the relative importance of the multiple fixed factors considered in the candidate models by summing the w_i of the models containing each factor. This sum is an estimate of relative variable importance, because a variable appearing more often in the better models will have a higher w_i sum. We present each variable appearing in the 95% confidence set of models; however, we limit our inference to those with $w_i > 0.5$ (Burnham and Anderson 2002). For these particular variables, we present estimates of slope (or least-squares means) and standard errors from the best model (i.e., the model with the lowest AIC_C). To assess the degree to which the best model represented an improvement in explanatory power over the null model estimating only the residual variance, we calculated a pseudo-R^2 statistic as $1 - (var_{best}/var_{null})$ where var_{best} and var_{null} are the residual variance estimates from the best model and null model respectively. All multiple regression analyses were performed with the Mixed procedure in SAS (SAS v. 9.1, SAS Institute, Inc., Cary, North Carolina). Collinearity among pH, C:N, P, % colonization and morphotype richness in our dataset was explored with Spearman's Rho correlation coefficients using a Bonferroni adjustment for multiple comparisons.

Results

Effects of site and watering on seedling growth

The site of soil collection significantly affected seedling mass and root:shoot (Table 1; Fig. 1a and b). Seedlings increased in mass with watering; however, root:shoot did not respond to watering (mean = 1.26 ± 0.42 SD).

Ectomycorrhizas present and their coincidence with site and watering

Across all seedlings, seven morphotypes were identified (Fig. 2). Two *Wilcoxina* morphotypes, followed by *Cenococcum geophilum,* were the most frequent ectomycorrhizal fungi to colonize root tips of individual seedlings (Fig. 2). Fungal DNA from *Wilcoxina* mycorrhizas with abundant, smooth emanating hyphae matched that of *Wilcoxina mikolae* in a BLAST search of Genbank (99% match over 585 bp to accession number DQ069000). We were not successful in amplifying fungal DNA from other morphotypes, and thus we relied on morphological characteristics to differentiate among ectomycorrhizal types. A second type of *Wilcoxina* mycorrhiza, which we refer to as *Wilcoxina* II, was clearly distinguishable from the first because it had few, roughly verrucose emanating hyphae. Those
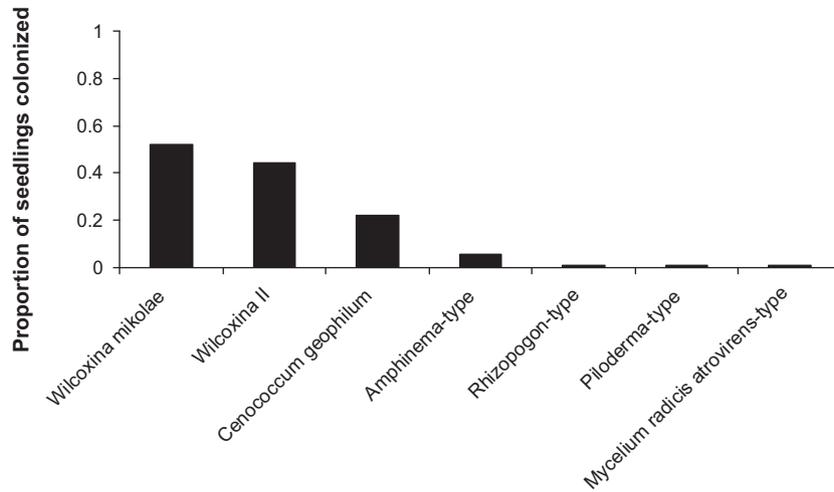
Fig. 2. Frequency of ectomycorrhizal morphotypes observed on seedlings of Douglas-fir (*Pseudotsuga menziesii* var. *glauca)* grown under different watering regimes in soils collected from southwestern British Columbia.

matching descriptions of mycorrhizas formed by *Rhizopogon* spp., *Amphinema* spp., and *Piloderma* spp., as well as *Mycelium radicis atrovirens* (MRA) – type mycorrhizas, as described by Jones et al. (1997) and Hagerman et al. (1999), were less frequent. On roots of individual seedlings, morphotype richness ranged from 0 to 3 with a mean of 0.8. Both morphotype richness and % colonization varied across sites ($P < 0.0001$ and 0.005, respectively). Morphotype richness was lowest at high watering levels and did not differ between medium and low watering levels ($P = 0.0001$; Fig. 3a). Percent colonization decreased with watering level ($P = 0.0315$; Fig. 3b).

Estimating the relative importance of biotic and abiotic variables for seedling growth

In addition to variation in morphotype richness and % colonization, there was substantial variation in soil fertility across the 6 sites (Table 2). Notably, pH varied by nearly 3 units, available P ranged from 26 to 100 mg/kg, and C:N values ranged from 14 to 44. None of the abiotic factors included in model selection exhibited significant collinearity with each other, or with either of the biotic factors; however, morphotype richness significantly positively covaried with % colonization (Table 3).

Model selection resulted in a set of 17 models (out of 191 considered) used for inference on the factors underlying total seedling biomass (Table S2). Among the seven factors under consideration, abiotic factors were found to be most important in explaining variation in seedling biomass. Watering regime in particular was the most important, appearing in all 17 of the best models (Table 4). pH was negatively related to seedling growth (Table 4) and was included in 10 of the 17 best models (Table S3). Morphotype richness appeared in 11 of the 17 best models and was positively related with seedling mass (Table 4). Percent colonization, although included in some of the models used for inference, was relatively unimportant to seedling mass (Table 4). The order
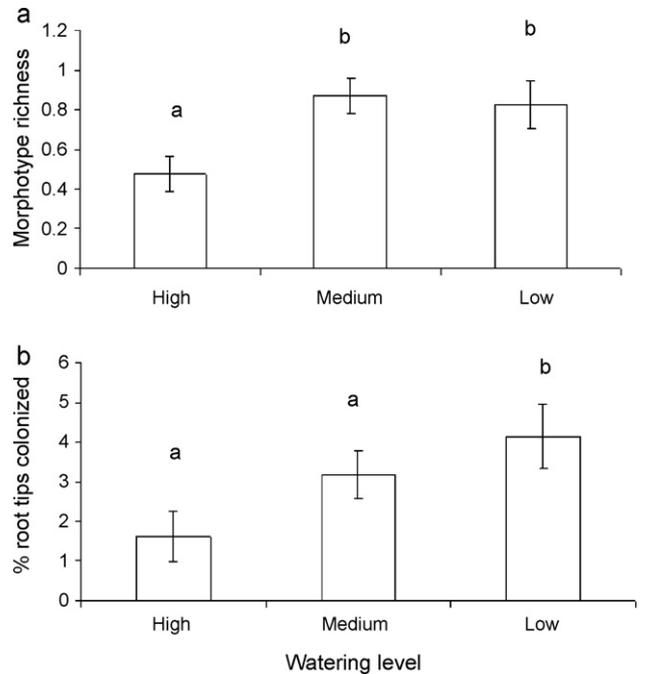
Fig. 3. (a) Mean ectomycorrhizal morphotype richness (± 1 SE), and (b) mean percent colonization (± 1 SE) of root tips of Douglas-fir (*Pseudotsuga menziesii* var. *glauca)* seedlings grown at three watering regimes, high, medium and low corresponding to 30%, 20% and 10% soil moisture, respectively. Bars not denoted by same letter are significantly different ($\alpha = 0.05$) based on Tukey HSD test.

of relative variable importance in the models was: watering (sum $w_i = 0.94$) > pH (sum $w_i = 0.65$) > morphotype richness (sum $w_i = 0.62$) > morphotype richness * watering (sum $w_i = 0.30$) > C:N (sum $w_i = 0.25$ > morphotype richness * pH (sum $w_i = 0.07$) > % colo-

Table 2
Fertility characteristics of soils collected from six sites in the Thompson–Okanagan region of British Columbia. Values are from a composite of 6 samples per site.

Site	pH	Organic matter (LOI) (%)	Organic C (%)	NH_4^+ (mg/kg)	NO_3^- or NO_2^- (mg/kg)	Total N (%)	P (Bray-P1) (mg/kg)	C:N
BB	7.3	8.4	4.2	10	6	0.30	44.3	14
BT	6.0	11.7	5.8	9	0.1	0.15	25.9	40
OB	5.0	16.8	8.4	20	0.1	0.22	86.9	38
OT	4.7	18.2	9.1	19	0.1	0.21	51.9	44
RB	6.2	11.0	5.5	22	16.5	0.19	100.0	29
RT	5.6	9.8	4.9	9	0.5	0.14	57.7	35

Table 3
Spearman rho correlation coefficient matrix for variables pertaining to soils collected from different sites in southwestern British Columbia. Value in bold is significant at $\alpha = 0.005$ (α adjusted for multiple comparisons).

Ectomycorrhizal morphotype richness	% Root tips colonized by ectomycorrhizal fungi	pH	P	C:N
1	**0.95**	−0.70	−0.40	0.90
	1	−0.54	−0.52	0.82
		1	−0.21	−0.90
			1	−0.041
				1

Table 5
Ranked relative importance of variables associated with root:shoot of Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings, showing model estimates of slope and variance. Slope and variance estimates not provided for variables with $\Sigma w_i < 0.5$. Estimates of importance, Σw_i, made across all models in the set where variable j occurred.

Explanatory variable	Σw_i	Slope estimate	Variance estimate
pH	0.73	−0.19998	0.128158
C:N	0.21	–	–
Morphotype richness	0.13	–	–
Water level	0.04	–	–
P	0.016	–	–
Morphotype richness * pH	0.01	–	–
% Colonization	0.02	–	–

nization (sum $w_i = 0.02$) (Table 4). The pseudo-R^2 for the best model was 0.35.

Model selection resulted in a set of 12 models used for inference on the factors underlying root:shoot (Table S3). Root:shoot was best explained by abiotic factors; pH was the most important factor appearing in 7 of the 12 best models (Table S3). Seedlings allocated more biomass to roots with decreasing pH (Table 5). The order of relative variable importance in the models was: pH (sum $w_i = 0.73$) > C:N (sum $w_i = 0.21$) > morphotype richness (sum $w_i = 0.13$) > watering (sum $w_i = 0.04$) > P (sum $w_i = 0.01$) > morphotype richness * pH (sum $w_i = 0.01$) > % colonization (sum $w_i = 0.02$) (Table 5). The pseudo-R^2 for the best model was 0.39.

Discussion

A dominant theme of mycorrhizal research has been to explore the contributions of mycorrhizas to the performance of individual plants (Smith and Read 1997, 2008). A primary objective of this work has been to determine whether colonization by mycorrhizal fungi has an effect on some aspect of the growth of host plant seedlings. With recognition that individual plants host more than one species of mycorrhizal fungi, variation in fungal identity or diversity has also been included in more recent experiments (Hoeksema 2005; Maherali and Klironomos 2007; Jansa et al. 2008). We and others (Klironomos et al. 2011) argue that the next step in mycorrhizal research should not only evaluate the effect of variation in mycorrhizal communities on host plants, but should also do so relative to variation in other ecologically important factors, especially soil conditions. Ideally, to understand the relative contributions of mycorrhizal fungal attributes (such as richness and community composition) and other factors, multifactorial manipulative experiments are needed, as are statistical analytical approaches that quantify the relative importance of factors rather than simply testing the significance of each. While results from variants of this type of experiment have addressed the relative importance of plant and soil conditions to mycorrhizal fungal community composition (e.g. Johnson et al. 1992; Högberg et al., 2007), and the interaction between soil type and lineages

of plant and ectomycorrhizal fungi to host outcome (Piculell et al. 2008), those that address the relative importance of mycorrhizas and soil conditions to host response are sparse. Manipulating and maintaining the composition of ectomycorrhizal fungal communities in the field independent of soil conditions has, to date, proven difficult (Kranabetter 2004), and although some level of understanding may be obtained by studying parallel factors driving host and mycorrhizal communities (Kernaghan 2005), ultimately our knowledge on how various community attributes of ectomycorrhizal fungi affect host plants is extremely poor. To this end, the results presented here represent a unique contribution to understanding the relative roles of soil conditions (abiotic factors) and ectomycorrhizal fungal richness and colonization levels (biotic factors) on growth responses of host seedlings.

We suggest that in disturbed environments where seedlings are exposed only to resistant propagules of ectomycorrhizal fungi, variation in abiotic factors may supersede that of biotic factors in affecting growth of young seedlings. In our greenhouse study, we found that watering regime and pH were the most important factors in explaining variation in seedling biomass and root:shoot, respectively. The only biotic variables we measured were ectomycorrhizal properties; however, it must be acknowledged that organisms other than ectomycorrhizal fungi (e.g. animals and other microbes) were present, and likely varied among soils and affected seedling growth. Though our focus was on the relative effects of ectomycorrhizas on seedling growth, we cannot dismiss the potential importance of these other organisms, which may have contributed to some of the unexplained variance in plant performance. Of the ectomycorrhizal properties we considered, morphotype richness, though low, was an important determinant of seedling biomass. Although this variable was correlated with watering, it still ranked relatively high in importance and appeared with watering regime in some of the best multi-factor models, suggesting that the importance of morphotype richness is not completely explained by its correlation with soil moisture. Percent colonization, though partially collinear with morphotype richness

Table 4
Ranked relative importance of variables associated with total biomass of Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings, showing best model estimates of group (high, medium and low watering level) mean, slope and variance. Slope and variance estimates not provided for variables with $\Sigma w_i < 0.5$. Estimates of importance, Σw_i, made across all models in the set where variable j occurred.

Explanatory variable	Σw_i	Group mean/slope estimate	Variance estimate
Water level	0.94	High: 3.13 Medium: 1.87 Low: 1.15	High: 0.24 Medium: 0.24 Low: 0.27
pH	0.65	−0.28296	0.252338
Morphotype richness	0.62	0.121224	0.166388
Morphotype richness * water level	0.30	–	–
C:N	0.25	–	–
Morphotype richness * pH	0.08	–	–
% Colonization	0.02	–	–

and dependent on watering regime, did not emerge as an important singular or interactive explanatory variable.

A strength of these results is that they go beyond simply testing for statistical effects of the presence/absence of mycorrhizas on hosts, to weighing the relative importance of these factors against other gradients that seedlings are likely to encounter in nature. Moreover, the gradients to which seedlings were subjected in this experiment were ecologically realistic, as watering regimes mimicked the range of soil moisture conditions found in the summer in ecosystems from which the soils were collected. The number and types of morphotypes found on individual seedlings were typical for Douglas-fir seedlings of this approximate age growing in disturbed conditions (Jones et al. 1997; Teste et al. 2004; Cline et al. 2005), and a recent meta-analysis showed that plant responses to mycorrhizal fungi are often substantially higher when inoculations use more than one fungal species rather than just a single species (Hoeksema et al. 2010). According to our results, for young Douglas-fir seedlings, small shifts in the species richness of ectomycorrhizal fungi bear importance, yet overall remain subordinate to abiotic gradients.

For both seedling biomass and root:shoot, richness of morphotypes ranked above % colonization in relative importance, suggesting that the composition of ectomycorrhizas, rather than their abundance on roots, elicits a stronger response in host seedlings. Though colonization levels were low, a recent meta-analysis reported that the presence of ectomycorrhizal fungi on roots tips was more important than colonization levels in explaining seedling responses to mycorrhizal inoculation (Karst et al. 2008).

Composition of ectomycorrhizas, in particular the presence of *Wilcoxina* sp., found on seedlings of *Picea engelmanni* was more important in the accumulation of ^{15}N versus extent of colonization (Jones et al. 2009). *Wilcoxina* spp., the most abundant morphotypes present in our study, are dominant members in fungal communities of ecosystems following disturbance (Yu et al. 2001), and to some degree are also found in mature forests (Tedersoo et al. 2006). They colonize a wide range of hosts including those from the genera of *Pinus*, *Larix*, *Pseudotsuga*, *Abies*, *Picea*, *Lithocarpus*, *Arbutus* and *Betula* (see review by Yu et al. 2001), and their thick-walled chlamydospores enable them to survive disturbances in which hosts are removed, such as fires (Torres and Honrubia 1997) and clearcutting (Hagerman et al. 1999; Mah et al. 2001). These two disturbances are common in Douglas-fir forests in the interior of British Columbia. Given that we severed all hyphal connections with mature trees when we collected the soils, our results are most applicable to seedlings regenerating after a disturbance, when few mature trees are present. Species of fungi available would be those from the resistant propagule pool, similar to the species that seedlings interacted with in our study.

Our results supported our prediction that soil moisture would be important in determining seedling growth. Watering regime, however, was a poor predictor of root:shoot biomass partitioning by seedlings. Optimal partitioning models suggest that plants preferentially allocate resources to the organs responsible for acquiring the most limiting resource (Thornley 1972). In this framework our results are somewhat counterintuitive because soil moisture is important, not only for supplying water, but also because it mediates the availability of nutrients; nevertheless, moisture did not influence root:shoot. That root:shoot did not vary with soil moisture may be because (1) differences in water availability are regulated through stomatal responses rather than biomass allocation or (2) shoot and root responses are equally affected. The pseudo-R^2 values calculated for the best models in each analysis indicate that substantial variation in seedling growth and biomass allocation is unexplained, suggesting there may be unmeasured variables contributing to seedling growth. Alternatively, our measurements of soil abiotic factors may have been coarser than the level of heterogeneity to which seedlings respond.

Roots of seedlings growing in low watering levels (~10% soil moisture) had higher levels of ectomycorrhizal colonization than those growing in medium and high watering levels (~20–30% soil moisture). A relationship between ectomycorrhizal colonization and soil moisture has been previously reported but the direction of the response varies (Lodge 1989; Gehring and Whitham 1994; Runion et al. 1997; Nilsen et al. 1998; Swaty et al. 1998; Valdes et al. 2006). The portion of the soil moisture gradient studied clearly influences the response of ectomycorrhizas to soil moisture. Additionally, morphotype richness increased with colonization levels, exhibiting a relationship that may be akin to that between area (number of individuals) and species richness. In particular, as the number of root tips colonized increases, the likelihood of colonization by an additional species of ectomycorrhizal fungi increases. Comparisons of fungal richness on roots versus that present in the soil would help resolve the role of host/fungal selection in the composition of mycorrhizas found on a given root system.

Given the myriad of factors a seedling encounters in addition to those associated with mycorrhizal conditions, it is imperative to consider the entire ecological context when assessing host outcome in response to mycorrhizas. Using multimodel selection and inference, we were able to quantify the relative importance of several abiotic factors versus potentially important biotic factors associated with the ectomycorrhizal symbiosis. However, the interactions between abiotic and biotic factors are complex and may be site dependent. Future work should expand the scope of factors considered, including non-mycorrhizal biotic factors, the contribution of variation in ectomycorrhizal fungal species present via common mycelium networks, and additional abiotic factors such as available light.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pedobi.2011.05.002.

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