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Justin Trowbridge · Ari Jumpponen

Fungal colonization of shrub willow roots at the forefront of a receding glacier

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Abstract Shrub willows (*Salix* spp.) form associations with arbuscular mycorrhizal (AM), ectomycorrhizal (EM) and dark septate endophytic (DSE) fungi. Willow root colonization by these three types of fungi was studied on a deglaciated forefront of Lyman Glacier, Washington, USA. Root colonization was low; less than 1% of the root length was colonized by AM and 25.6% by DSE. EM colonized 25% of the root tips and 19.4% of the root length. AM and DSE colonization were not related to distance from the present glacier terminus or to canopy cover. EM colonization increased with distance from the glacier terminus based on gridline intercept data but not on root tip frequency data. Availability of propagules in the substrate was low, but numbers of propagules increased with distance from the glacier terminus. The EM communities were dominated by three ascomycetes showing affinity to Sordariaceae in BLAST analyses. Other frequent taxa on the glacier forefront included species of Cortinariaceae, Pezizaceae, Russulaceae, Thelephoraceae and Tricholomataceae. When occurrence of individual taxa was used as a response variable to canopy cover, distance from the glacier terminus, and their interaction, four different fungal guilds were identified: 1) fungi that did not respond to these environmental variables; 2) fungi that occurred mainly in intercanopy areas and decreased with distance from the glacier terminus; 3) fungi that were insensitive to canopy cover but increased with distance from the glacier terminus; 4) fungi that occurred mainly under willow canopies and increased with distance from the glacier terminus. We suggest that fungal colonization is mainly limited by fungal propagule availability. Environmental conditions may also limit successful establishment of plant-fungus associations. We propose that the four EM guilds partly explain successional dynamics. The initial EM community comprises fungi that tolerate low organic matter and nitrogen environment (first and second guilds above). During later community development, these fungi are replaced by those that benefit from an increased organic matter and nitrogen environment (third and fourth guilds above).

Keywords Dark septate endophytes · Glacier forefront · Mycorrhiza · Primary succession · *Salix*

J. Trowbridge · A. Jumpponen (✉)
Division of Biology, Kansas State University,
Manhattan, KS, 66506, USA
e-mail: ari@ksu.edu
Tel.: +1-785-5326751
Fax: +1-785-5326653

Introduction

Primary successional ecosystems, such as those on the forefronts of receding glaciers or sites of volcanic outflow, provide unique environments for studying mechanisms of revegetation and fungus re-establishment. Pickett et al. (1987) proposed an hierarchical approach for succession that incorporated availability of open sites for establishment, availability of establishing species and differential performance of species at the available sites. Similar principles can be applied to fungal succession. For ectomycorrhizal (EM) fungi, open sites for colonization can be interpreted as vacant root space. Additionally, host specificity among root-colonizing fungi (Molina et al. 1992) affects the susceptibility of hosts to colonization and, therefore, may be an important factor in determining successful fungal colonization of uncolonized roots (Jumpponen et al. 1999a, 2002). Availability of establishing species depends on dispersal ability and availability of fungal propagules, which initially determine the range of possible fungus-plant associations (Allen 1988; Jumpponen et al. 2002). Finally, differential performance of the components of a fungal community is determined at the level of an ecosystem or microhabitat and probably selects fungi capable of persisting under the prevailing environmental conditions (Jumpponen et al. 1999a; Termorshuizen and Schaffers 1989; Trappe and Luoma 1992; Wöllecke 2001).

Succession of EM fungi is driven by a variety mechanisms, many of which may interact or change over

extended periods of time. The processes of EM succession often have been viewed in the framework of the physiology of aging hosts or characteristics of stands modified by host trees (see Deacon and Fleming 1992; Dighton et al. 1986; Gibson and Deacon 1988, 1990; Last et al. 1987). Indeed, that mycorrhizal fungi differ at different stages of plant succession has been observed after drastic disturbances such as wildfires and glacier retreats (Helm et al. 1996; Jumpponen et al. 2002; Visser 1995). The shifts observed in fungus communities often correlate with the successional dynamics of the plant communities (Alfredsen and Høiland 2001; Jumpponen et al. 2002; Termorshuizen and Schaffers 1989). In contrast to community dynamics in secondary successional environments, where the primary source of fungus inoculum may be a resident propagule bank (Baar et al. 1999; Horton et al. 1998), early-establishing mycorrhizal fungi in primary succession rely on long-distance dispersal by wind and animal vectors or stochastic events depositing debris and inoculum from established adjacent vegetation (Cázares 1992; Jumpponen et al. 2002; Trappe and Luoma 1992). Successful colonizers are selected by their compatibility with available host plants and prevailing environmental conditions. Environmental and edaphic conditions are continuously modified by the changing plant communities and increasing vegetative cover (Matthews 1992; Ohtonen et al. 1999), resulting in changes in fungus communities both above and below ground (Helm et al. 1996; Jumpponen et al. 2002; van der Heijden et al. 1999). Using coastal dune ecosystems as a model, Read (1989, 1992) proposed that non-mycorrhizal plants establish first in dynamic successional systems, followed by facultative and obligate arbuscular mycorrhizal (AM) hosts and their root-associated fungi. Once edaphic conditions (pH, limiting nutrients, soil organic matter content) are adequately modified by established vegetation, EM hosts and their symbiotic fungi would establish. Read (1989, 1992) argued that soil conditions were pivotal in determining the dominant fungi colonizing plant roots.

In addition to AM and EM fungi, roots of various plants are often colonized by other (possibly non-mycorrhizal) fungi characterized by melanized hyphae, intra- and intercellular colonization, and frequent formation of coils of inflated cells termed microsclerotia (Jumpponen and Trappe 1998; Yu et al. 2001). The function and taxonomic affinities of these fungi are poorly understood, and they are collectively called dark, septate endophytes (DSE) to emphasize the melanized hyphae and affinity to higher fungi, likely ascomycetes (Jumpponen 2001). Cázares (1992) and Jumpponen (1999) observed frequent root colonization by DSE, and Jumpponen (1999) isolated DSE fungi from various host plants on the forefront of a receding glacier.

The studies reported here complement prior research on a primary successional glacier forefront in the North Cascade Mountains of Washington state, USA. We aimed to: 1) enumerate infective propagules of AM, EM (see Jumpponen et al. 2002) and DSE at different distances from the glacier terminus; 2) estimate the extent of shrub willow root colonization by these three types of root-associated fungi; 3) test whether or not shrub willow root colonization by the three types was related to distance from the glacier terminus (correlated with time since deglaciation; Jumpponen et al. 1998) or willow canopy cover; 4) identify dominant EM fungi colonizing shrub willows; 5) test whether or not shrub willow root colonization by different EM fungi was related to distance from the glacier terminus or willow canopy cover.

Materials and methods

Study site

Lyman Glacier is located in the Glacier Peak wilderness area in the North Cascade Mountains (48° 10′ 52″N, 120° 53′ 87″W) of Washington, USA. The elevation of the present glacier terminus is about 1,800 m a.s.l. The deglaciated forefront is approximately 1,000 m long over an elevation drop of only 60 m with no recessional moraines (Cázares 1992; Jumpponen et al. 1998). The glacier has receded steadily since the 1890s, opening the forefront to pioneering plants. Photographs and snow survey data have allowed estimation of the retreat rate over the last century (Jumpponen et al. 1998).

Most probable number of propagules

The most probable number (MPN) method was employed to estimate the number of infective AM and DSE propagules over the deglaciated forefront (Alexander 1965; Daniels and Skipper 1982). Enumeration of EM propagules in these samples has been reported previously (Jumpponen et al. 2002). Ten non-vegetated patches were selected at each of four sites located approximately 250, 450, 650, and 850 m from the present glacier terminus. Ten sampling locations were selected on the east and west sides (five on each side) of the central transect, and the topsoil (0–5 cm) was sampled. Soil samples were sieved through a 6-mm soil sieve, the large fraction discarded, and approximately 200 ml of the fine fraction pooled into one composite sample representing east and west sides at each of the four distances. Samples were stored on ice until processed for the MPN assay.

Within 2 weeks of collection, a 10-fold dilution series (undiluted, 10^{-1}, 10^{-2}, and 10^{-3}) was prepared by mixing the soil samples with pasteurized Willamette Valley sandy loam. Five replicate tubes (Ray Leach, Corvallis, Ore.) were prepared for each dilution. Contamination was monitored by 20 tubes containing only the pasteurized substrate. These were free of fungus colonization at harvest.

Each tube was watered and sown with sudan grass seed [*Sorghum sudanense* (Piper) Stapf.] that had been surface sterilized and scarified by soaking in 30% H_2O_2 for 5 min. The substrate surface was covered with a ca. 1-cm layer of sterilized quartz sand after sowing. The seedlings were grown in a growth chamber at constant 25°C with a 16/8-h day/night cycle and a light intensity of approximately 270 μmol s^{-1} m^{-2}. Plants were watered every third day. Seedlings were harvested 10 weeks after seed germination, thoroughly washed, cleared with 10% KOH, stained with Trypan blue (Phillips and Hayman 1970) and assessed for the presence of AM and DSE colonization under a dissecting microscope.

Sample collection

We selected shrub willows (*Salix commutata* and *Salix planifolia*) for this study because they form associations with AM, EM and DSE (Cázares 1992; Harley and Harley 1987; Jumpponen et al.

1999a; Read 1989) and this allowed simultaneous quantification of colonization by the three types of fungi.

Thirty samples were collected in August 2001. Three willow shrubs of approximately equal size were selected at distances of 300, 450, 600, 750 and 900 m from the glacier terminus. One 200-ml sample of soil containing *Salix* fine roots was taken under the canopy of each shrub. An additional sample was taken from a non-vegetated patch 3 m outside the canopy of each plant. A primary root of each shrub was traced from the willow to the non-vegetated patch to ensure collection of willow roots. Samples were stored on ice until processed.

Roots were handpicked from soil, rinsed with tap water and cut into 1-cm pieces. One half was randomly selected for morphotyping of EM fungi and the other used for estimation of colonization by the magnified gridline intercept method. Feeder rootlets were separated from the thicker perennial roots prior to microscopic examination.

Estimation of colonized root length

Two samples lacking adequate material for observation were omitted from further analyses. Roots were destained by boiling in KOH for 60 min, neutralized for 1 min in 1% HCl, stained with Trypan blue (Phillips and Hayman 1970) and stored in acidic glycerol. Ten 1-cm-long sections of small-diameter feeder rootlets were randomly selected and examined under a compound microscope at ×400 magnification. In a total of 100 intercepts, the frequencies of DSE hyphae, microsclerotia, hyaline septate hyphae, hyphae with clamp connections, hartig net, pseudoparenchymatous mantle, non-septate hyphae, arbuscules and vesicles were recorded. Roots were considered EM if intercepts contained mantle or hartig net, colonized by DSE if inter/intracellular melanized hyphae or microsclerotia were encountered, or colonized by AM if vesicles, arbuscules, or aseptate hyphae were present.

Ectomycorrhiza morphotyping

Root samples were examined under a dissecting microscope and the numbers of EM and non-mycorrhizal root tips recorded. Roots with a well-developed fungal mantle were divided into morphotypes based on mantle color and/or shape, the presence or absence of emanating hyphae, and the presence or absence of cystidia. Representatives of each morphotype from each of the samples were stored frozen in CTAB extraction buffer for later molecular analysis. All tips of morphotypes with less than 10 tips per sample were collected as were ca. 10 of the frequent types. We also saved representatives of types with no mantle if abundant emanating hyphae were present or if root tips showed altered morphology.

Molecular characterization of ectomycorrhizal fungi

DNA was extracted from individual root tips as described by Gardes and Bruns (1996). The internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene was amplified by polymerase chain reaction (PCR) with ITS1F and ITS4 primers, as described by Gardes and Bruns (1996) with the following alterations: *Taq* DNA polymerase and the manufacturer's 10× buffer (Promega; Madison, Wis.) were used in a 25-μl reaction containing 2 μl of undiluted template. Aliquots (5 μl) of the amplicon were visualized on a 1.5% agarose gel, and amplicon size determined using an AlphaImager (Alpha Innotech Corp. San Leandro, Calif.) image analysis system. Aliquots (8 μl) of the PCR product were digested with *Alu*I and *Hinf*I as described by Gardes and Bruns (1996). RFLP pattern/fragment lengths were visualized on a 3% agarose gel (Gardes and Bruns 1996) and fragment sizes estimated using AlphaImager. PCR amplifications and RFLP digests were also performed on a selection of fruiting bodies collected at the glacier site. The sporocarp DNA templates failed to amplify and produced no visible amplicons. As a result, sequences were obtained from one representative of each ITS-RFLP type using fluorescent dideoxy-terminators (ABI Prism BigDye; Applied Biosystems, Foster City, Calif.) and an automated ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, Calif.) at the DNA Sequencing and Genotyping Facility at Kansas State University. Prior to sequencing, ITS amplicons were purified with a Qiaquick PCR Purification Kit (QIAGEN, Valencia, Calif.). The ITS1 region was sequenced in both directions using primers ITS1F and ITS2 and the ITS2 region was sequenced using primers ITS3 and ITS4. Sequences are available at the GenBank (accession numbers AY187593–AY187618). Putative taxon affinities were determined by BLAST analyses (Altschul et al. 1997).

The number of EM species (species richness) was estimated as number of EM PCR-RFLP phenotypes per sample. Species diversity and evenness were estimated using Simpson's indices as described by Helm et al. (1996).

Statistical analysis

Estimates of the percent root tips and root length colonized were $\log_{10}$ transformed because of increasing variances with distance from the glacier terminus. The species richness, diversity, and evenness estimates were also $\log_{10}$ transformed prior to analysis. The transformed data were analyzed by analysis of variance in a general linear models procedure (PROC GLM in SAS 1989). The responses to linear (distance from the glacier terminus) and categorical (canopy vs. inter-canopy) main effects as well as their interaction were tested with a Type III error term at $\alpha<0.05$. Occurrence data for the EM morphotypes were analyzed by logistic regression (PROC GENMOD in SAS 1997) using binary response (presence/absence) of a target species to the explanatory model. Such analysis is preferable to proportion or species frequency data because binary data do not overdisperse and the test variables do not require rescaling. The binary response data are also more robust and less prone to error than proportion or species frequency data.

For the occurrence of ascomycetous and basidiomycetous ectomycorrhizas, the relative proportions of mycorrhizal types were estimated in two ways: 1) mycorrhizal tips only, or 2) total number of tips (including non-mycorrhizal). This was necessary because all samples contained either or both types of mycorrhizas. These data were corrected for overdispersion by a scaling parameter estimated during the logistic regression analysis (option DSCALE in SAS 1997).

Results

MPN of propagules

Overall, the number of AM and DSE propagules was low. Number of DSE propagules slightly increased with increasing distance from the glacier terminus (Fig. 1). This increasing trend was similar to that observed earlier for EM (Jumpponen et al. 2002).

Fungus colonization of shrub willow roots

All samples contained EM based on morphotyping and gridline intercept microscopy. On average, 25.0±14.9% (mean±SD) of all *Salix* root tips were EM. Based on the magnified gridline intercept method, estimated colonization was slightly lower, averaging 19.4±15.6% of root length. EM colonization increased with distance from the glacier terminus based on gridline intercept data but not on EM morphotyping data (Table 1).

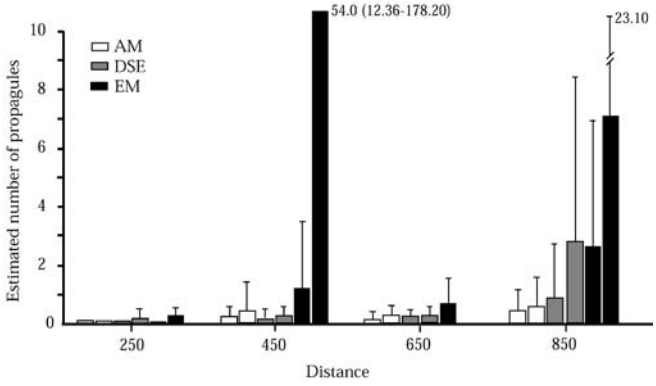

Fig. 1 Most probable number of arbuscular mycorrhizal (AM) and dark septate endophyte (DSE) propagules (mean+SE) on the forefront of the receding Lyman glacier, Washington state, USA. Propagule numbers for ectomycorrhizal fungi (EM) from Jumpponen et al. (2002) are shown for comparison

Of the 28 samples analyzed for DSE and AM colonization, 89% (25/28) were colonized by DSE but only 29% (8/28) had AM structures. On average, 25.6±26.3% of the root length was colonized by DSE (inter- and intracellular hyphae and microsclerotia) and <1% by AM. Microscopic observations corresponded well with our MPN assay data for AM and DSE. AM colonization was too low to warrant further analyses. The variation in DSE colonization was substantial: 0–80% of root length was colonized by DSE. In contrast, EM colonization ranged from 0 to 40% of root length. Although our model using only distance and canopy cover did not explain observed patterns of DSE colonization (Table 1), DSE colonization was strongly positively correlated with EM colonization (Spearman's rank correlation coefficient 0.610; P=0.0006).

Table 1 ANOVA tables of multiple regression models characterizing fungal colonization of *Salix* spp. on receding Lyman Glacier forefront in North Cascades mountain range in Washington state. Colonization by arbuscular mycorrhizal (AM) fungi was on average <1% and thus excluded from analyses. Significant effects are shown in italics (*DSE* dark septate endophyte, *EM* ectomycorrhizal)

Parameter	DF	F	P
EM colonization (gridline intersection)			
Canopy	1	0.11	0.744
Distance from glacier	*1*	*4.44*	*0.046*
Interaction	1	0.16	0.689
EM colonization (morphotyping)			
Canopy	1	0.27	0.608
Distance from glacier	1	1.84	0.187
Interaction	1	0.37	0.551
DSE colonization (gridline intersection)			
Canopy	1	0.01	0.938
Distance from glacier	1	2.22	0.150
Interaction	1	0.03	0.855

Identification of EM fungi colonizing willow roots

PCR amplification was attempted from 308 root tips; 135 (44%) produced a visible amplicon. RFLP analysis revealed that 20 of the 135 successful reactions comprised multiple fragments and these were omitted from further analysis. *Alu*I and *Hinf*I digests of the ITS region yielded fragment sizes as shown in Table 2. All RFLP types were putatively identified by sequencing and subsequent BLAST analyses (Table 2). Several unknown PCR-RFLP phenotypes strongly resembled existing GenBank sequences including Russulaceae species 1 (97% similarity to *Russula fragilis*; GenBank accession AF335443), Tricholomataceae species 1 (97% similarity to *Laccaria laccata*; GenBank accession AF204814), Pezizaceae species 2 (99% similarity to *Peziza alaskana*; GenBank accession U40473) and Cortinariaceae species (96–98% similarity to unidentified *Cortinarius* species; GenBank accession AY097038, AF495455). Others showed only low similarity to existing sequence data in the GenBank, including the three dominant ascomycetous Sordariaceae species (87–89% similarity to *Porosphaerella cordanophora*; GenBank accession AF178563) and Pezizaceae species 1 (88% similarity to unknown Pezizales; GenBank accession AF266709). The samples without a mantle but with plentiful emanating hyphae or altered root morphology either produced no visible PCR amplification or, if successful, the sequenced products were similar to *Sebacina vermifera* (Auriculariales) (94–100% partial similarity; GenBank accession AF202728).

Table 2 Observed EM fungi and their ITS-RFLP patterns at Lyman Glacier forefront. Accession numbers refer to most similar ITS1 sequences (ITS1 and ITS2 for Pezizaceae species 1 and Sordariaceae species) in GenBank which were used to infer the phylogenetic affinities of the unknown EM root tips. PCR amplicons generated with primers ITS1F and ITS4 were digested with *Alu*I and *Hinf*I (*sp.* species)

Morphotype	Accession no.	BLAST match (%)	*Alu*I digest	*Hinf*I digest
Cortinariaceae sp. 1	AY097038	96	508; 77	372; 161; 90
Cortinariaceae sp. 2	AF495455	98	653	446; 152; 81
Cortinariaceae sp. 3	AF477000	93	386; 228; 185	455; 300; 80
Pezizaceae sp. 1	AF266709	88	502; 208	712
Pezizaceae sp. 2	U40473	99	484; 264	256; 223; 91
Russulaceae sp.	AF335443	97	592; 257	427; 330
Sordariaceae sp. 1	AF178563	88	230; 186; 178	468; 54
Sordariaceae sp. 2	AF178563	87	536	370; 175
Sordariaceae sp. 3	AF178563	89	489; 110	318; 175; 90
Thelephoraceae sp. 1	AF184742	94	488; 82	190; 155
Thelephoraceae sp. 2	U83475	93	600; 146	227; 190
Tricholomataceae sp.	AF204814	97	300; 121; 88	460; 362

Occurrence of EM taxa on the glacier forefront chronosequence

The number of species per sample (species richness) varied from one to nine taxa per sample but did not vary with distance from the glacier terminus (P=0.1036) or between canopy and open areas (P=0.2491). Species diversity as measured by the Simpson diversity index was not affected by canopy (P=0.8511) or distance from the glacier terminus (P=0.8233). Like species diversity, evenness was not affected by canopy cover (P=0.2556) but varied significantly with distance (P=0.0316). Although the evenness differed significantly among the different distances, no linear trend was detected (P=0.3275 for H_o: $\beta_{Distance}$=0, indicating that the estimated slope for regression was not different from 0).

Despite the absence of significant differences in species richness or diversity, analyses of the occurrence of ascomycetous and basidiomycetous EM as well as analyses of various individual taxa revealed interesting patterns. It is essential to note that these results are based on a limited number of samples and should, therefore, be viewed with caution. Significant interaction between distance from the glacier terminus and canopy cover (Table 3) indicated that ascomycetous EM colonization responded to distance from glacier terminus differently in canopy and intercanopy areas. In areas beyond the *Salix* canopies, ascomycetous EM increased in frequency with increasing distance from the glacier terminus (Fig. 2a, Fig. 2b). Such an increase was not observed in areas under the willow canopies. These trends were detected when only mycorrhizal tips were included in the analysis and when EM tips were analyzed as a proportion of all *Salix* tips. Like ascomycete EM, basidiomycete EM were evenly distributed along the chronosequence in areas under the willow canopy, but basidiomycete occurrence decreased significantly in areas beyond the canopies, when estimated as a proportion of mycorrhizal tips (Table 3). This is opposite to analysis of the ascomycetous EM colonization, when only mycorrhizal tips were considered. In contrast to the occurrence of the ascomycetous EM, basidiomycetous EM frequency decreased when taken as a proportion of all *Salix* roots and the interaction between canopy cover and distance from the glacier terminus was not significant (Table 3; Fig. 2c).

Of the 12 mycorrhizal taxa detected in the samples collected at the Lyman Glacier, three ascomycetous taxa putatively identified as members of Sordariaceae (Sordariaceae species 1–3) occurred most frequently (Fig. 3). Russulaceae species 1, Thelephoraceae species 1, Tricholomataceae species 1, and two species of Cortinariaceae (Cortinariaceae species 2 and 3) occurred at comparable frequencies. Only two taxa (Cortinariaceae species 1 and Thelephoraceae species 2) were detected in two or fewer samples.

Occurrence of four of the 12 putatively identified EM taxa in our study could be explained to some degree by one or both of the two tested variables (Table 3). In addition to those EM fungi that did not respond to either

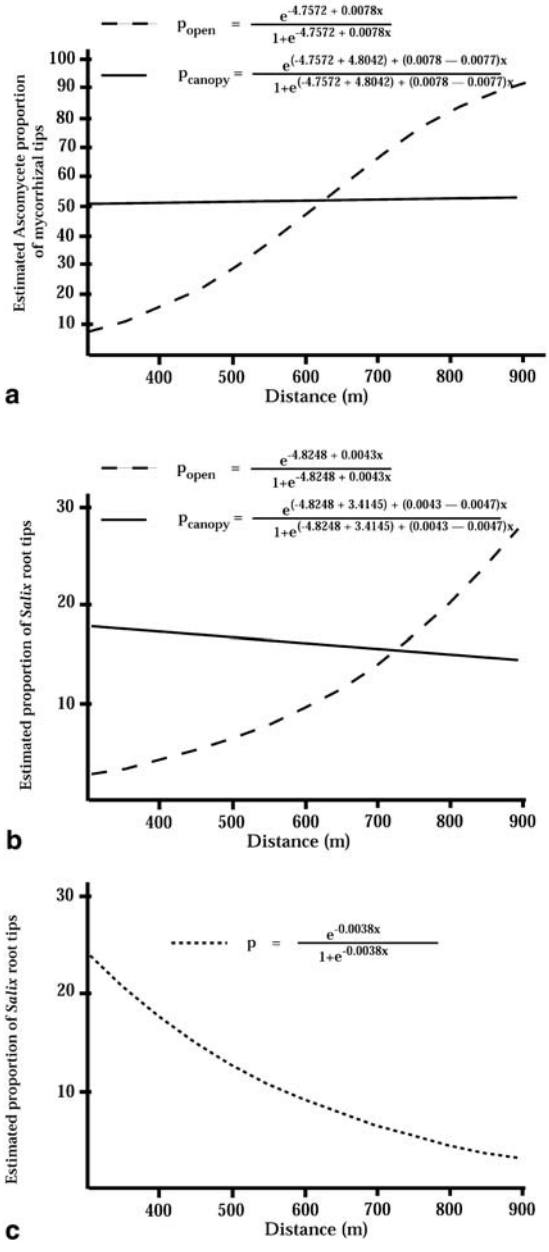

Fig. 2 Estimated probabilities of ascomycete and basidiomycete occurrence on the receding forefront of Lyman Glacier. **a** Ascomycete occurrence estimated as a proportion of mycorrhizal tips; note that the basidiomycete occurrence is the inverse of these functions (Table 3). **b** Ascomycete occurrence estimated as a proportion of all root tips. **c** Basidiomycete occurrence estimated as a proportion of all *Salix* root tips; note that the interaction term is not significant and that occurrence is explained as a function of distance alone (*short dashes*). For parameter estimates, see Table 3; parameters significant at α<0.05 were used. Probability of occurrence was solved from the odds ratios: 1) for intercanopy (*long dashes*) and, if the interaction term was not significant in Table 3, all samples (*short dashes*) $\ln[p\times(1-p)^{-1}]=\alpha_{Intercept}+\beta_{Distance}\times X$; 2) for canopy (*solid line*) samples if canopy and/or the interaction term was significant in Table 3 $\ln[p\times(1-p)^{-1}]=\alpha_{Intercept}+\beta_{Canopy}+(\beta_{Distance}+\beta_{Interaction})\times X$

Table 3 Parameter estimates for the occurrence of EM taxa on the receding forefront of Lyman Glacier. Parameters indicate the odds ratio for taxon occurrence. $\alpha_{Intercept}$ is the intercept of the estimated occurrence function, β_{Canopy} indicates the difference in the intercept between the canopy and intercanopy samples, $\beta_{Distance}$ indicates the linear change in taxon occurrence as a function of distance from the glacier terminus, $\beta_{Interaction}$ indicates the difference in $\beta_{Distance}$ between the canopy and intercanopy samples. Note that occurrence functions for Ascomycetes and Basidiomycetes as a proportion of mycorrhizal tips are inverse. Significant effects are shown in italics. Cortinariaceae sp. 1and Thelephoraceae sp. 2 appeared in fewer than three samples and were omitted from this analysis (*ns* not significant, *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$)

Taxon	$\alpha_{Intercept}$	β_{Canopy}	$\beta_{Distance}$	$\beta_{Interaction}$
Cortinariaceae sp. 1	–	–	–	–
Cortinariaceae sp. 2	*−3.36±1.86**	−0.70±2.31^{ns}	−0.0011±0.0029^{ns}	0.0019±0.0035^{ns}
Cortinariaceae sp. 3	−1.90±1.60^{ns}	−2.36±2.33^{ns}	−0.0027±0.0028^{ns}	0.0037±0.0037^{ns}
Pezizaceae sp. 1	1.43±2.13^{ns}	*−4.45±2.52**	*−0.0118±0.0058***	0.0098±0.0062^{ns}
Pezizaceae sp. 2	*−4.74±1.96**	−1.80±4.78^{ns}	0.0018±0.0027^{ns}	−0.0004±0.0065^{ns}
Russulaceae sp.	−1.70±1.62^{ns}	−1.88±2.50^{ns}	−0.0005±0.0024^{ns}	0.0021±0.0036^{ns}
Sordariaceae sp. 1	*−4.58±2.66**	3.16±3.01^{ns}	0.0026±0.0035^{ns}	−0.0045±0.0042^{ns}
Sordariaceae sp. 2	*−4.52±1.72***	*3.31±1.99**	*0.0048±0.0023**	*−0.0048±0.0027**
Sordariaceae sp. 3	*−5.93±1.91***	3.33±2.16^{ns}	*0.0062±0.0024***	−0.0045±0.0028^{ns}
Thelephoraceae sp. 1	*2.36±1.41**	*−6.55±2.46***	*−0.0067±0.0027* *	*0.0091±0.0038**
Thelephoraceae sp. 2	–	–	–	–
Tricholomataceae sp.	*−2.62±1.51**	−1.23±2.02^{ns}	−0.0018±0.0025^{ns}	0.0021±0.0032^{ns}
As a proportion of mycorrhizal tips only				
Ascomycetous EM	*−4.76±2.26**	*4.80±2.55**	*0.0078±0.0034**	*−0.0077±0.0039**
Basidiomycetous EM	*4.76±2.26**	*−4.80±2.55**	*−0.0078±0.0034**	*0.0077±0.0039**
As a proportion of all *Salix* tips				
Ascomycetous EM	*−4.82±1.47**	*3.41±1.71**	*0.0043±0.0019**	*−0.0047±0.0023**
Basidiomycetous EM	0.23±1.06^{ns}	−1.35±1.45^{ns}	*−0.0038±0.0019**	0.0028±0.0024^{ns}

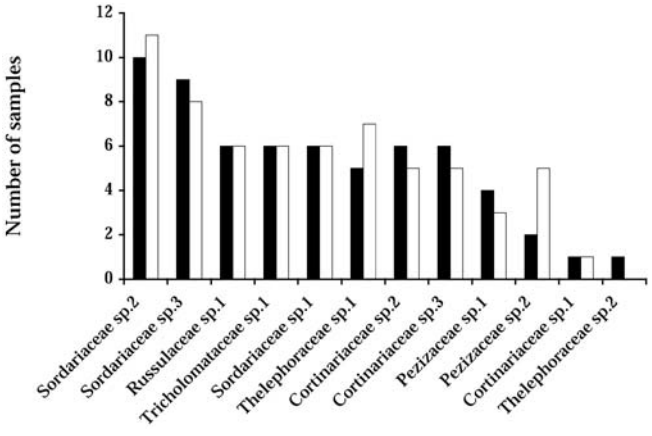

Fig. 3 Rank order of the 12 detected EM taxa colonizing willow roots on the forefront of the receding Lyman glacier. Number of samples with an EM taxon on the *y*-axis (*Filled bars* canopy samples, *open bars* intercanopy samples)

variable, three clear patterns are evident in our data. First, the occurrences of Pezizaceae species 1 and Thelephoraceae species 1 decreased with increasing distance from the glacier terminus in the open areas and they were infrequent within the canopy (Fig. 4a, Fig. 4b). Second, the occurrence of Sordariaceae species 2 increased with distance in open areas but its occurrence was not related to distance under willow canopies (Fig. 4c). Finally, the occurrence of Sordariaceae species 3 increased with distance from the terminus both in open and canopy areas (Fig. 4d).

Discussion

Our MPN data and results from previous studies (Jumpponen et al. 2002) indicate that overall the number of root-colonizing fungus propagules is low in this primary successional ecosystem. Furthermore, the MPN results indicate that EM and DSE propagule numbers increase with increasing distance from the glacier forefront, whereas the number of AM propagules is low throughout the glacier forefront. It is important to note that the means of the propagule number estimates varied substantially. This suggests that some microsites are more likely to accumulate large numbers of viable propagules and thus favor higher rates of fungal colonization of host roots. Jumpponen et al. (1999b) proposed that plant establishment is partially determined by the anchoring of the emerging seedling in a protected, safe microsite. Similarly, deposition of fungal propagules at this glacier forefront may be governed by contour and surface characteristics of the microsites as well as by microclimatic characteristics such as wind speed and turbulence.

The increasing propagule numbers observed here corroborate other studies on microbial biomass and its dynamics on glacier forelands (Ohtonen et al. 1999; Sigler and Zeyer 2002). Studying the very same forefront of Lyman Glacier, Ohtonen et al. (1999) estimated microbial biomass by direct microscopic counts, substrate-induced respiration, and phospholipid fatty acid analyses. All three methods indicated increasing microbial biomass with increasing distance from the glacier forefront. Furthermore, when bacterial and fungal biomass were separated, the fungal biomass increased more than bacterial biomass.

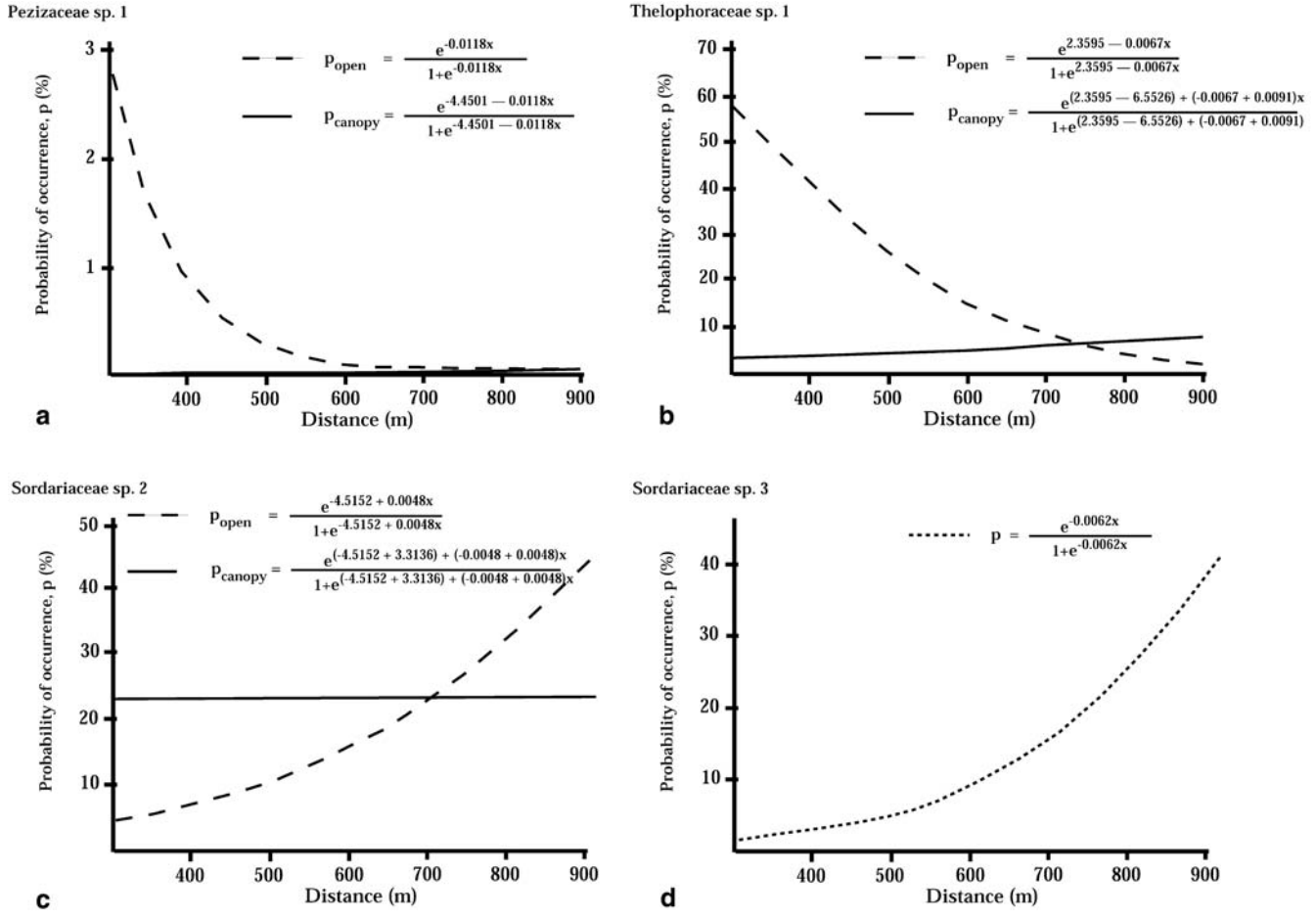

Fig. 4 Estimated probabilities of selected EM fungus occurrences on the receding forefront of Lyman Glacier. **a** Pezizaceae species 1. **b** Thelephoraceae species 1. **c** Sordariaceae species 2. **d** Sordariaceae species 3; note that the interaction term was not significant and occurrence was explained as a function of distance alone. See Table 3 for parameter estimates and the legend of Fig. 2 for the probability of taxon occurrence estimation

Fungal communities can be affected by various soil characteristics (Tyler 1989). A well-developed plant litter layer affects EM species composition as well as diversity and abundance of EM fungi (Baar 1996). In that study, effects of litter were largely attributed to pH and nitrogen concentrations. Several additional studies have focused on the effects of nitrogen availability on EM fungi, in particular their fruiting and community composition (Wallenda and Kottke 1998). Nitrogen amendment often modifies the fruiting EM community (e.g. Lilleskov et al. 2002b) and recent studies confirm that these effects are also visible in belowground EM communities (Lilleskov et al. 2002a; Peter et al. 2001). Previous studies at the Lyman Glacier forefront have shown that soil organic matter and nitrogen are generally higher under established canopies than in intercanopy spaces, and that they increase with distance from the glacier forefront (Jumpponen et al. 1998; Ohtonen et al. 1999). We chose canopy cover and distance from the glacier terminus for our modeling of EM fungus occurrence because they are likely to be strongly correlated with edaphic, physical, chemical and microclimatic parameters, which are difficult to measure at this remote site.

Fungal colonization of willow roots was consistently low throughout the glacier forefront. EM colonization was comparable to *Salix alaskensis* colonization reported by Helm et al. (1996) during the poorly vegetated phases on an Alaskan glacier forefront. Our study and that of Helm et al. (1996) report lower EM colonization of willow roots than in successional dune systems (van der Heijden and Vosatka 1999). Similarly, AM colonization in our study was substantially lower than that reported by van der Heijden and Vosatka (1999). It is not known whether the low colonization rate is due to limiting soil properties or unavailability of adequate inoculum. The glacier forefront site represents a harsh environment characterized by drastic variation in daily temperature, exposure to radiation and general low availability of nitrogen. At the same time, the propagule availability is low, as indicated by this and prior studies (Jumpponen et al. 2002).

It is possible that many of the fungi detected by the MPN assay are not compatible with willows. Because of

the unreliable germination and limited availability of *Salix* seed, we used *Pinus* for EM enumeration (Jumpponen et al. 2002) and *Sorghum* for DSE and AM enumeration in the MPN assays. The poor AM colonization most likely results from limited propagule dispersal. Read (1989) postulated that EM become important in later successional stages. Based on our gridline intercept microscopy, EM colonization indeed increased over time since deglaciation. However, such a trend was not evident when EM colonization was estimated as a proportion of all root tips or for DSE colonization. These results corroborate previous studies by van der Heijden and Vosatka (1999), who concluded that the age of a site is not significantly correlated with EM colonization in coastal dune ecosystems. Although Helm et al. (1996) reported an increase in EM colonization with successional time, other edaphic or microscale variables may better characterize EM colonization than time since deglaciation or canopy cover (see Allen et al. 1987; Allen 1988). Increasing diversity of host species likely contributes towards increasing root colonization as the numbers of EM fungi increases (Jumpponen et al. 1999a, 2002). Our colonization rates resemble those observed by Helm et al. (1996) in the early successional stages. When only *Salix alaskensis* was considered, overall colonization did not change with increasing time since deglaciation.

The positive correlation between EM and DSE colonization is interesting as a negative correlation between the two types of root colonizers has been reported (Wurzburger et al. 2001). Similarly, AM and EM have been suggested to behave antagonistically (Lodge and Wentworth 1990; Neville et al. 2002). We found no evidence for antagonistic interactions among the different types of root-colonizing fungi. With the low overall colonization rates, the willow root systems are likely to provide adequate non-colonized root space to allow simultaneous colonization by various types of fungi. The positive association between EM and DSE fungi may thus indicate either similar environmental tolerances or similar requirements for dispersal and establishment.

A large proportion of willow root tips remained free of visible EM colonization. Some of these, nonetheless, hosted fungus colonization, although Hartig net and mantle were not observed. When PCR amplifiable, these roots appeared most similar to *Sebacina vermifera* in BLAST analyses. Although fungi belonging to Sebacinaceae have been shown to form ectomycorrhizae in vitro (Warcup 1988) and in vivo (Glen et al. 2002; Selosse et al. 2002), it is unclear whether *Sebacina vermifera* or its close relatives are mycorrhizal with Salicaceae. Because of a very poorly developed mantle and absence of the Hartig net, we decided to omit *Sebacina vermifera* from further analyses.

PCR-RFLP and sequencing indicated 12 putative EM taxa representing six families on the Lyman Glacier forefront. Sporocarps of 13 taxa were observed in the course of 17 expeditions over 12 years (Jumpponen et al. 2002). Unfortunately, our sporocarp material proved unamplifiable and no EM could be RFLP-matched to sporocarps from the site. In spite of numerous attempts, the sporocarps produced no visible PCR amplicons. This is likely to be the result of preservation of these samples by drying with a propane-fueled field dryer, which allowed little or no control of the drying temperature. Nonetheless, many taxa observed in the below ground EM community are likely to be conspecific to those observed in an earlier survey of ectomycorrhizal sporocarps on the glacier forefront (Jumpponen et al. 2002). The prolifically fruiting *Cortinarius* spp., *Inocybe lacera* and *Laccaria* sp., which are likely to be represented by unidentified members of Cortinariaceae (Cortinariaceae species 1–3) and Tricholomataceae species 1, were among the six most frequently detected EM. In addition, Russulaceae species 1, which was one of the three most common taxa, was possibly represented by a single *Russula fragilis* collection in the sporocarp survey. Our earlier sporocarp surveys failed to detect many of the most frequent EM fungi in the belowground survey. These include ascomycetes (identified putatively as members of Pezizaceae and Sordariaceae) and basidiomycetes with likely resupinate sporocarps (identified putatively as Thelephoraceae). Several species of basidiomycetes observed in the sporocarp surveys were not represented in the direct belowground EM assessment, including species potentially associated with *Salix* sp. (*Hymenogaster glacialis* and *Lactarius* spp.). Some taxa not represented by the molecular data are specific to hosts other than *Salix*, such as *Larix*-specific species of *Suillus*. Although there is some congruence between sporocarp and belowground glacier forefront fungi, our data show incongruences similar to other systems (Gardes and Bruns 1996).

Three ascomycetes putatively identified as members of Sordariaceae (Sordariaceae species 1–3) comprised a significant component of the EM community throughout the glacier forefront. Two additional ascomycetes, probably Pezizaceae, were also present. Baar et al. (1999) observed ascomycetes among the dominant EM fungi in field-collected and bioassayed *Pinus muricata* seedlings after a wildfire. Many ascomycetes evidently establish from propagules that have been deposited into the resistant propagule bank in soil (see also Jumpponen 2003).

Our data indicate increasing proportions of ascomycetous EM and decreasing proportions of basidiomycetous EM with increasing distance from the glacier terminus in the intercanopy areas. It is important to bear in mind that our data are limited and the results from analyses of only 30 samples should be viewed with caution. In secondary successional forests, colonization of roots by EM fungi occurs largely from resident mycelium and propagules (Baar et al. 1999; Bruns et al. 2002; Horton et al. 1998). As established by our MPN assay, propagule numbers are low in the glacier forefront site, so EM colonization may depend heavily on transient airborne propagules rather than on established mycelial inoculum. Many asco- and basidiomycetes are wind dispersed and can establish short-lived colonization of host plants (Danielson 1991; Deacon and Fleming 1992;

Gryta et al. 1997). Prolific fruiting and ability to colonize roots may be the fungus traits that succeed in primary successional ecosystems (see Deacon and Fleming 1992). However, the fungal environmental tolerances, relative competitive abilities, and limitations set by the site characteristics are also important. We attribute the increasing ascomycete abundance over distance from the glacier terminus in the intercanopy areas at the Lyman site to the relatively young successional ecosystem and associated environmental conditions. In contrast to intercanopy areas, areas under the canopy exhibited nearly uniform ascomycetous and basidiomycetous EM occurrence over the entire chronosequence. The mechanisms resulting in the observed differences between ascomycetes and basidiomycetes remain unclear.

Species richness and diversity were not affected by distance from the glacier terminus or presence of canopy cover. Evenness varies at different distances from the glacier terminus, but no clear trend is evident. Our results contrast with an earlier study of a glacier forefront site, in which number of EM morphotypes increased with increasing distance from a glacier terminus (Helm et al. 1996). In that study, several host genera and species were included, whereas we focused on a single host genus. Our young primary successional system and limited host diversity likely explain the absence of trends in species richness and diversity. Our estimates of evenness seemed to be stochastic along the transect. The observed differences are most likely due to vast variation in number of species and the variation in abundance of the component species and suggest patchiness in propagule abundance, diversity, and distribution.

Although fungus colonization and species richness did not vary predictably in this study, examination of individual taxa and their occurrence yielded interesting insights into successional dynamics among EM fungi. A majority of the detected taxa could not be categorized by simple models using only two main factors. This emphasizes the complexity of environmental parameters or purely stochastic biotic and abiotic factors that may control fungal occurrence in natural environments. Nonetheless, we propose and speculate on four guilds defined by observed environmental tolerances that may explain fungal occurrence in primary successional ecosystems.

The first guild is characterized by relative insensitivity to the environmental parameters chosen for this study. Occurrence of these taxa was not affected by canopy cover or distance from the glacier terminus. The environmental tolerances of these taxa are probably determined by other parameters.

The second guild is characterized by infrequent occurrence under the willow canopies and a decrease with distance from glacier terminus. We suggest that these fungi are adapted to low organic matter or low nitrogen environments. They are probably ruderal and their propagules are abundant and readily dispersed. These species may be poor competitors in more favorable environments, explaining their decrease with distance from the glacier terminus and their limited occurrence under the canopies where organic nutrients accumulate. This group is exemplified by Thelephoraceae species 1, which may behave like the ubiquitous *Thelephora terrestris*, an abundant fungus colonizing young seedlings in forest nurseries with suspected ability to colonize young plants by wind-dispersed spores (Trappe and Strand 1969).

The third guild is characterized by an increase with distance from the glacier terminus. We speculate that these fungi are favored by ecosystem parameters that uniformly change with time since glaciation (e.g. increasing nitrogen availability resulting in allochthonous nitrogen deposition). They are apparently insensitive to canopy/intercanopy differences and, accordingly, are not affected by canopy cover. This group is exemplified by Sordariaceae species 3 in our study.

The fourth guild is characterized by an increase with distance from the glacier terminus but a consistent occurrence under willow canopies. These fungi are likely to depend on soil organic legacies built up by established vegetation. They may colonize open areas but only after the organic matter content of the soil has increased as a result of leaf litter or root turnover from adjacent vegetation. This group is exemplified by Sordariaceae species 2 in our study.

We propose that these EM guilds partly explain fungus successional dynamics. The initial EM community comprises fungi that tolerate the harsh environment and are superior competitors in a low organic matter and nitrogen environment. Later during community development, these fungi are replaced by those that are superior competitors in soils with elevated organic matter and nitrogen.

In plant succession, the universal conditions that define successional dynamics have been proposed to include availability of open sites, availability of colonizing species, and differential performance among the species at the site (Pickett et al. 1987). The results of our study focusing on succession of fungi colonizing willow shrubs in a primary successional ecosystem suggest that availability of the open sites (vacant, uncolonized willow roots) may not limit entry of root-colonizing fungi because only a relatively small proportion of the roots was colonized. However, the species availability and performance among the available root-colonizing species appear to be major factors. Our data suggest that root colonization is strongly limited by inoculum availability. This is especially the case for AM fungi. Furthermore, the environmental tolerances or competitive dynamics among the available species seem to control fungal occurrence in canopy versus intercanopy locations and substrates of different ages along the glacier forefront chronosequence. Further experimental studies are necessary to separate the different mechanisms driving the fungal community composition in successional ecosystems.

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