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Moth Outbreaks Alter Root-Associated Fungal Communities in Subarctic Mountain Birch Forests

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Abstract Climate change has important implications on the abundance and range of insect pests in forest ecosystems. We studied responses of root-associated fungal communities to defoliation of mountain birch hosts by a massive geometrid moth outbreak through 454 pyrosequencing of tagged amplicons of the ITS2 rDNA region. We compared fungal diversity and community composition at three levels of moth defoliation (intact control, full defoliation in one season, full defoliation in two or more seasons), replicated in three localities. Defoliation caused dramatic shifts in functional and taxonomic community composition of root-associated fungi. Differentially defoliated mountain birch roots harbored distinct fungal communities, which correlated with increasing soil nutrients and decreasing amount of host trees with green foliar mass. Ectomycorrhizal fungi (EMF) abundance and richness declined by 70–80 % with increasing defoliation intensity, while saprotrophic and endophytic fungi seemed to benefit from defoliation. Moth herbivory also reduced dominance of Basidiomycota in the roots due to loss of basidiomycete EMF and increases in functionally unknown Ascomycota. Our results demonstrate the top-down control of belowground fungal communities by aboveground herbivory and suggest a marked reduction in the carbon flow from plants to soil fungi following defoliation. These results are among the first to provide evidence on cascading effects of natural herbivory on tree root-associated fungi at an ecosystem scale.

Keywords Fungal community · Defoliation · ITS2 · Root-associated fungi · Subarctic ecosystem

Introduction

Global warming has been predicted to increase the abundance and range of forest insect pests [1]. Recent large-scale insect outbreaks in forests across the northern hemisphere, such as those caused by bark beetles in North America and Central Europe and geometrid moth outbreaks in northern Fennoscandia, have been linked to warming climate [2–4]. Extensive damage of the canopy reduces carbon allocation below ground, with severe consequences on soil microbiota such as symbiotic root fungi.

Ectomycorrhizal fungi (EMF) colonizing the fine roots of all boreal forest trees have a pivotal role in tree nutrition due to their ability to mobilize organic nutrients [5, 6]. As a result, mycorrhizal fungi are responsible for the majority of plant nitrogen acquisition [7]. Although EMF contribute to organic matter decomposition, they are generally considered to have a limited ability for autonomous saprotrophic lifestyles [8, 9].

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Instead, EMF largely depend on tree-derived carbohydrates, especially on recently fixed photosynthates, which are transported from the host to the EMF with high temporal resolution [7, 10]. As roots and root-associated fungi constitute a major part of soil carbon storage and sequestration of recently fixed carbon below ground [11, 12], information on responses of root fungi to defoliation is essential for estimating ecosystem level effects of moth defoliation.

As a result of reduced carbon assimilation and consequent decline in carbon availability belowground, foliar herbivory usually has a negative impact on EMF. Hence, insect or simulated herbivory of trees reduces EMF sporocarp yields [13, 14], fungal biomass in the fine roots [15] and, in some cases, EMF colonization [16–18]. Boreal trees tend to maintain high EMF colonization despite foliage loss [13, 19, 20], possibly due to obligatory host adaption to fungal uptake of organic nutrients. In addition to quantitative reductions in fungal biomass and reproductive structures after herbivory, changes in diversity and composition of EMF communities have been detected after defoliation indicating that EMF species may respond differentially to changes in photosynthate availability [19–22]. Especially vulnerable to herbivore damage are "high biomass EMF" that produce large amounts of fungal mycelia, presumably due to higher carbon demand from the host [15, 20–22]. Apart from recent reports on beetle outbreaks [14, 23] information on ecosystem scale herbivore impacts on EMF communities is lacking.

In this paper, we report the effects of extensive moth defoliation on root fungal communities of the subarctic mountain birch (*Betula pubescens* ssp. *czerepanovii* (Orlova (Hämet-Ahti)), which forms a transition ecotone between coniferous boreal forests and treeless tundra in the mountainous Fennoscandia and northwestern Russia. Mountain birch forests are regularly attacked by autumnal (*Epirrita autumnata* (Bkh.)) and winter (*Operophtera brumata* L.) geometrid moths, on ca. 10-year intervals with occasional high population densities leading to forest defoliation across large regional scales [24, 25]. The range of the winter moth is predicted to expand in response to global warming [25]. In the most recent outbreak from 2000 to 2008, moth larvae defoliated 10,000 km^2 of mountain birch stands, representing 1/3 of birch forests in northern Fennoscandia [26]. Extensive moth outbreaks have profound effects on carbon balance of northern birch forests causing a dramatic decrease in carbon sink strength [27]. We analyzed root-associated fungal communities in mountain birch stands after the large-scale natural defoliation by autumnal and winter moths. We hypothesized that the moth defoliation would lead to decline in the EMF symbiont communities and their partial replacement by dominantly saprobic fungal communities. Specifically, we expected the defoliation effects to be most detrimental to the high biomass EMF colonizing roots [15].

Methods

Site Description and Sampling

We selected three mountain birch sites in northernmost Finland, i.e., Njuorggan (70°0′N, 27°4′E; 127 m a.s.l.), Veahcat (69°6′N, 27°2′E; 216 m a.s.l.), and Mierasluoppal (69°4′N, 27°2′E; 255 m a.s.l.), which were subjected to successive defoliations by autumnal (*E. autumnata*) and winter moth (*Operophtera brumata*) larvae (see Kaukonen et al. [28] for details). Sites were situated ca. 60 km apart. At each site, three distinct mountain birch stands with different moth damage levels were selected. Defoliation levels of birch stands were: (1) a control with no visible moth feeding, (2) a 100 % foliage loss during one growing season (year 2008) only, and (3) 100 % foliage loss on two or more growing seasons (during years 2004–2008; Electronic Supplementary Material (ESM) Fig. 1). Within each site and defoliation level, we established three ~25-m^2 plots at a distance of 50–200 m from each other with three mountain birches within each plot, resulting in a total of 27 study plots.

Defoliation by geometrid moths typically occurs in May–June and does not appear to show particular selection with respect to host trees [29, 30]. Similarly, at each of our study sites the moth defoliation was systemic with large areas (hectares to square kilometers) affected, and study plots were placed in the core of homogeneously defoliated or intact landscape units. All sites were originally low productivity mountain birch heaths with similar crowberry-dominated mountain heath vegetation typically occurring on acidic bedrock with no eutrophy-indicating species [31]. The soil is podzol with rather shallow (ca. 5 cm) organic humus layer. Mountain birch typically compensates for lost foliage by producing basal sprouts after death of the main stem [32, 33]. Proportion of mountain birches with green foliage either in canopy or in basal sprouts was recorded two seasons after outbreak collapse (summer of 2010) from experimental plots and along a 50 m × 6 m transect in each study area.

Samples for identifying fungal communities within mountain birch roots were collected in August 2010. Twenty soil cores were sampled randomly around the three mountain birches in each plot at a distance of ca. 1 m from the trunk. Soil cores of 3 cm in diameter and ca. 5 cm in depth were taken from the organic layer, which is the biologically most active part of the soil profile and contains most of the fine root biomass in boreal and subarctic soils [34, 35]. Samples were stored in −20 °C until further preparation to inhibit growth of opportunistic fungi. Mountain birch fine roots (<1 mm in diameter) were extracted from thawed soil samples in the laboratory. Roots were rinsed on a sieve (mesh size 1 mm) under running tap water and picked onto a petri dish with water for further cleaning and examination under a stereomicroscope, where soil particles were removed and non-degraded roots

collected. Viable roots were identified by peeling of the bark from long roots: fragments with light colored cortex were selected, whereas dark, degrading roots were omitted. We collected all viable root material from 5 to 14 soil cores per plot to get ca. 250 mg of fresh roots. The roots were pooled into one composite sample within the plot. We failed to extract enough root material from one plot (Veachat, once defoliated), making the total number of samples 26. The samples were frozen in liquid nitrogen and stored in −80 °C until DNA extraction.

In addition to the soil sampled for the present study, we used earlier data on soil nutrients and other environmental parameters collected from the same study sites in 2009. We used soluble calcium (Ca), magnesium (Mg), potassium (K), phosphorus (P), and ammonium (NH_4) in humus, and nitrogen/carbon (C/N) and fungi/bacteria ratios as a background data for our analyses (see Kaukonen et al., 2013 for details).

Molecular Analyses

Root samples of mountain birch were freeze-dried for 48 h and ground with a mortar and pestle in liquid nitrogen. DNA was extracted using MoBio Power Soil® DNA isolation kit according to manufacturer's instructions, with an exception that samples were incubated in 70 °C for 10 min after adding solution C1 to facilitate cell lysis and final DNA was eluded into 50 μl of C6 buffer instead of 100 μl to concentrate DNA. In each sample, DNA concentration was adjusted to 5 ng/μl. The Internal Transcribed Spacer 2 (ITS2) region of the ribosomal RNA gene was amplified for 454 pyrosequencing using fungal specific primer set 58A2F [36] and ITS4 [37]. Fusion primers for 454 sequencing were constructed by adding adapter A and a 10-base-pair multiplex identifier (MID) to the primer ITS4 and adapter B to the primer 58A2F. MID barcodes were designed with at least 2 bp difference for post-sequencing sample recognition. Amplicons were produced in triplicate 20-μl reactions with 10 ng of DNA template, 2 μM of each fusion primer, 0.5 mM dNTP's, 0.4 U Phusion High-Fidelity polymerase and 5× High-Fidelity buffer with 1.5 mM $MgCl_2$ (Thermo Scientific) in an Eppendorf MasterCycler with an initial denaturation for 3 min at 95 °C; followed by 26 cycles of 15 s at 95 °C, 30 s at 50 °C, 1 min at 72 °C, and a final extension for 7 min at 72 °C. The amplicons were cleaned with AMPure XP (Beckman Coulter Inc.) and quantified with PicoGreen (Invitrogen, Carlsbad, CA). The triplicate samples were equimolarly pooled and further gel-cleaned and size-selected on 1 % agarose gel. Fragments of expected size targets were extracted from gels with Minelute gel extraction kit (Qiagen) and purified twice with AMPure XP (Beckman Coulter Inc.). Amplicons were pyrosequenced with 454 GS Junior (Roche) in Bioser Oulu, Department of Biology, University of Oulu.

Bioinformatics and Statistical Analyses

Sequences were processed with MOTHUR pipeline, version 1.30.2 [38]. Data were demultiplexed and denoised using flowgrams to reduce the sequencing error [39]. Sequences were trimmed to 200 bp, matching the minimum ITS2 length in these data. Chimeric sequences (n=126) were identified using UCHIME [40] implemented in MOTHUR and removed. Sequences were clustered into Operational Taxonomic Units using average neighbor method at 97 % similarity in a pairwise distance matrix. A random representative sequence from each OTU was queried against UNITE + NCBI databases using massBLASTer tool implemented in PlutoF workbench [41]. The taxonomically most informative hit was selected with≥97 % sequence similarity and>90 % query coverage. However, some non-singleton fungal sequences (817 sequences representing 84 OTUs) had lower (≥80 %) similarity, but were included in the data and may be potentially undescribed fungi. All non-fungal sequences (273 sequences and 25 OTUs), OTUs that could not be identified to a phylum (n=392 in 56 OTUs), and singleton OTUs (412) were manually removed [42]. Species hypothesis groups based on phylogenetic analyses [43] were used for those OTUs (n=46) that had best match with unidentified or uncultured fungi to improve their taxonomic assignment. Finally, identified OTUs were classified into following functional groups: EMF, saprotrophic, dark septate endophytic (DSE), and ericoid mycorrhizal (ERM) [44–46]. The raw sequence data were deposited in National Center for Biotechnology Information (NCBI) in Sequence Read Archive under accession nos. SRS585984, SRS586032, and SRS588719-SRS588742. Pyrosequencing typically produces variable library sizes [47], ranging in our data from 727 to 1390 sequences per plot. To account for uneven sequence yields, we rarefied the number of sequences in each plot to the minimum number of sequences observed [47]. A total of 727 reads were randomly sampled without replacement from each plot and subjected to square root double Wisconsin transformation, where the square root of sequences in each OTU is first divided by the maximum number for each OTU, then divided by the sum of values in each plot [48]. The resulting values measure the abundance of each OTU in each plot and were used to calculate the Bray–Curtis dissimilarities between plots. The dissimilarity matrix was used to calculate a two-dimensional solution to a non-metric multidimensional scaling (NMDS) using the metric version of this analysis as the initial starting point of the iterative solution process. All analyses were conducted using Matlab 8.0 (The Mathworks Inc.), unless stated otherwise.

The rarefying transformation and ordination process was replicated 1,000 times for different random selection of sequences. We suggest this method is useful in the correction of other sampling effects than sequence yield and also with other analyses of community composition than ordination.

The first of the 1,000 NMDS solutions was used as a template where other results were fitted with a procrustean analysis. Variation in the ordination results is represented by fitting a confidence ellipse of 1 SD around each plot. Although rarefication does not change the species composition of the plot with lowest sequence yield (Veachat, several times defoliated, VS1), it is represented by a confidence ellipse because its Bray–Curtis dissimilarity to other plots changes as the rarefying process affects all other plots. We fitted soil background data [28], tree survival (proportion of trees with green foliage), EMF, and saprotroph variables to the mean ordination results with the envfit-function of community ecology package 'vegan' [49] running under R-software version 2.11.0. Only the variables with statistically significant ($p<0.05$) correlation with the ordination are displayed.

We tested effects of defoliation and site on root fungal community composition using PERMANOVA (function "adonis" of the "vegan" package; [49]) with 1,000 permutations. Indicator species analysis for fungal OTUs was performed using the "multipatt" function of the R package "indicspecies" [50]. Also, we used factorial ANOVA for individual tree and fungal variables with defoliation and site as fixed factors (IBM SPSS Statistics; SPSS Inc., release 20.0.0). Changes in proportion of trees with green foliage in controls and once-defoliated plots were examined with one-way ANOVA. Tukey's HSD test was used post hoc to determine significant differences between defoliation groups. Square-root and log10 transformations were used for response variables prior to analysis as required to meet the assumptions of variance homogeneity. Results are expressed as mean ± SE of proportions of fungal taxonomic and functional groups of sequences (abundance) and OTUs (richness) per sample. Results from relative proportion data were uniform to those obtained from numeric sequence and OTU data due to the rarefying procedure.

Results

Data and Taxonomic Characteristics

Pyrosequencing with GS Junior resulted in a total of 31 471 raw sequences. After quality filtering with MOTHUR and removing singletons, non-fungal sequences, and OTUs unidentified at phylum level, the dataset consisted of 24,336 sequences distributed across 338 OTUs at 97 % sequence threshold. Rarefying the number of sequences to minimum sample size further reduced the read number to 18,902, but did not affect the number of OTUs.

The most commonly encountered phyla in the data were Ascomycota (43 % of OTUs, 54 % of reads) and Basidiomycota (34 % of OTUs, 41 % of reads; Table 1). Unidentified fungi with the best match to fungi sequenced from environmental soil samples formed fairly large proportion of fungal richness (ca. 17 % of OTUs) but were not abundant (only ca. 2 % of reads). Other fungal phyla (e.g., Glomeromycota and basal clades representing the former Zygomycota) were detected but rare. Common ascomycete orders were Helotiales and Chaetothyriales, while Agaricales, Russulales, and Thelephorales dominated in Basidiomycota (Table 1). The most common OTU was assigned to *Leotiomyceta* sp. (19 % of all data), followed by EMF species *Pseudotomentella tristis*, *Cortinarius armillatus*, and *Leccinum scabrum*.

Table 1 Taxonomic distribution of the data showing proportion of operational taxonomic units (OTUs) and reads for different phyla and orders

	Total	
	OTU %	Read %
Basidiomycota	33.73	40.65
Agaricales	12.13	14.95
Russulales	5.03	8.67
Thelephorales	2.66	9.44
Sebacinales	2.37	0.69
Boletales	1.78	2.34
Auriculariales	0.30	2.69
Basidiomycota rest[a]	5.33	1.00
Unidentified Basidiomycota	4.14	0.86
Ascomycota	43.19	54.39
Helotiales	22.49	18.51
Chaetothyriales	6.80	5.18
Verrucariales	1.48	8.45
Ascomycota rest[b]	5.62	1.88
Unidentified Ascomycota	6.80	20.37
Basal lineages	5.92	2.84
Mortierellales	5.03	2.49
Mucorales	0.89	0.35
Glomeromycota	0.6	0.03
Glomerales	0.30	0.012
Archaeosporales	0.30	0.013
Unidentified	16.57	2.10

[a] Includes orders Amylocorticiales, Atheliales, Cantharellales, Corticiales, Filobasidiales, Hymenochaetales, Leucosporidiales, Polyporales, Sporidiobolales, Trechisporales, and Tremellales

[b] Includes orders Hypocreales, Ostropales, Pezizales, Rhytismatales, Saccharomycetales, and Thelebolales

Top-Down Impacts of Defoliation on Host Tree and Root-Associated Fungi

Moth herbivory decreased the proportion of mountain birch trees bearing photosynthesizing foliage (in canopy or at the basal sprouts) ($F_{2,27}=102.7$, $p<0.001$; Fig. 1a, ESM Table 1). No birch stems survived in our study plots after recurring defoliation; however, ca. 10 % of mountain birches in the surrounding area were alive as indicated by our transect surveys (Fig. 1a). The

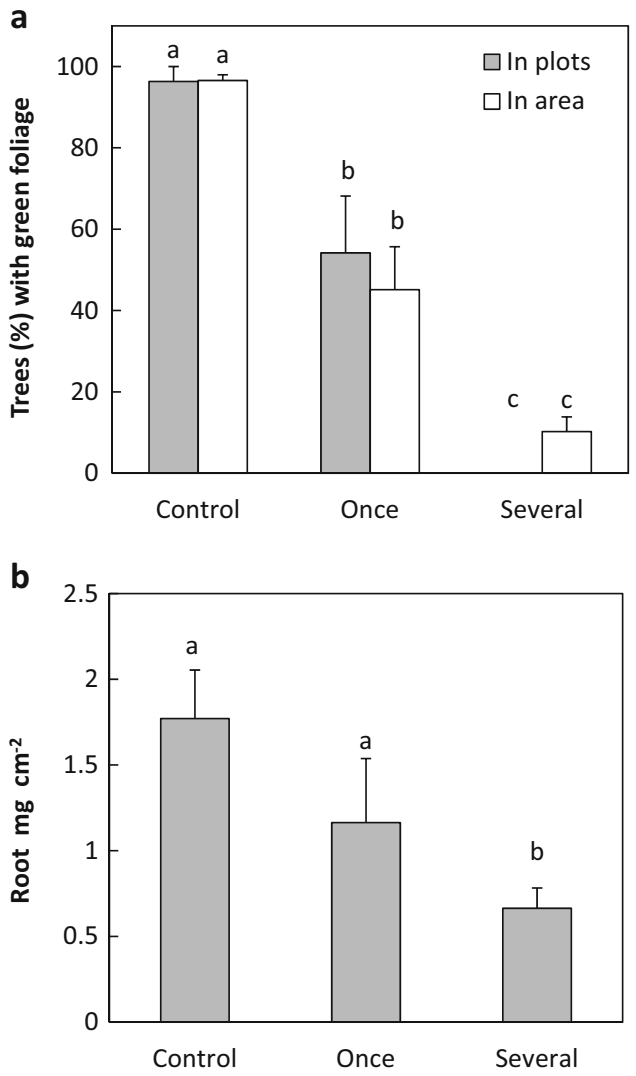

Fig. 1 **a** Proportion of mountain birch trees with green foliage (in canopy or in basal sprouts) after one or several moth defoliations in studied plots and in the area in general; **b** amount of fine roots in plots shown in milligram of root dry weight per square centimeter (mean ± SE). *Letters* indicate statistically significant differences (Tukey's HSD) between defoliation levels in each parameter

number of viable or recently dead roots also declined with defoliation intensity ($F_{2,26}$=6.93, p<0.006; Fig. 1b).

Fungal OTU richness in mountain birch roots increased after moth defoliation from average of 51 in intact controls to 57 in once defoliated and further to 76 in most severely defoliated plot ($F_{2,26}$=9.65, p=0.002; ESM Table 2). However, herbivory differentially affected the relative richness in functional and taxonomic fungal groups. EMF OTUs declined with increasing defoliation intensity ($F_{2,26}$=40.81, p<0.001; ESM Fig. 2), while DSE (representing *Phialocephala* spp.) and saprotrophic OTUs increased with several defoliations ($F_{2,26}$=8.66, p=0.003 and $F_{2,26}$=3.08, p=0.072, respectively; ESM Tables 2 and 3). Similarly to fungal richness, EMF sequences in birch roots decreased from a dominance of 53 % in controls to only about 15 and 9 % in once and repeatedly defoliated plots ($F_{2,26}$=13.47, p<0.001; Fig. 2a), suggesting 70–80 % loss in EMF abundance. Decrease in EMF abundance was a result of reduction in Agaricoid (mainly *Cortinarius* spp.), Boletoid (*Suillus*, *Leccinum* spp.), and Athelioid (*Piloderma* spp.) clades, while Russuloid (mainly *Lactarius tabidus* and *Russula decolorans*) and Tomentelloid (mainly *Pseudotomentella tristis* and *Tomentella* sp.) fungi persisted after moth defoliation (Fig. 2a). Saprotroph sequences increased gradually from ca. 6 % in controls to 21 % in repeatedly defoliated plots ($F_{2,26}$=6.88, p=0.006), a result of an increase in Agaricoid (mainly *Mycena* spp.) and Mortierelloid (*Mortierella* spp.) clades (Fig. 2b). Also, DSE abundance increased from ca. 5 % in controls to 13 % in our repeatedly defoliated plots ($F_{2,26}$=4.01, p<0.036; ESM Tables 2 and 3). Fungi with undefined function were common and increased in abundance ($F_{2,26}$=5.68, p=0.013) and marginally also in richness ($F_{2,26}$=3.38, p=0.058) as a result of defoliation (ESM Tables 2 and 3). A shift in taxonomic fungal community was

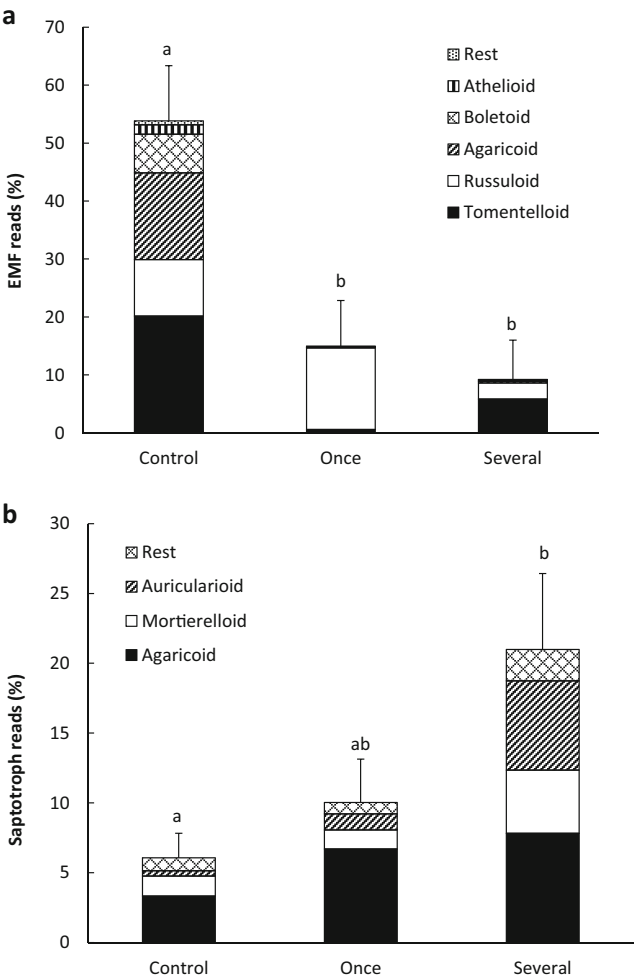

Fig. 2 Proportion of ectomycorrhizal (**a**) and saprophytic (**b**) sequences (mean ± SE) and related clades within them in undefoliated control, once-defoliated, and several-times-defoliated plots. *Letters* indicate statistically significant differences (Tukey's HSD) in fungal functional groups between defoliation levels

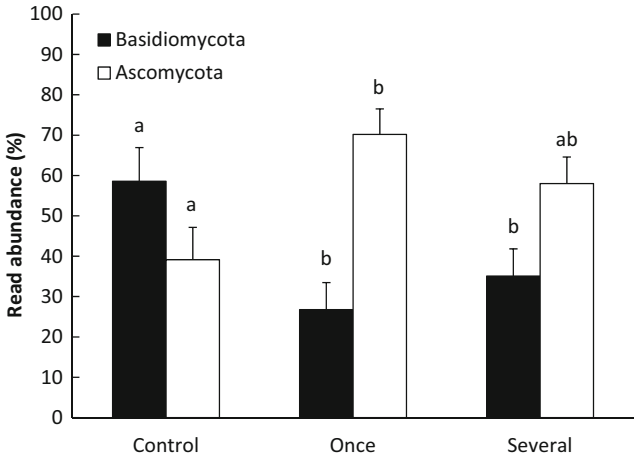

Fig. 3 Proportion of Basidio- and Ascomycota from all fungal sequences (mean ± SE) in undefoliated control, once-defoliated, and several-times-defoliated plots. *Letters* indicate statistically significant differences (Tukey's HSD) between defoliation levels and within fungal taxa

detected from dominance of Basidiomycota in controls to dominance of Ascomycota in the roots defoliated birches (Fig. 3; ESM Table 2).

Bootstrapped NMDS ordination on rarified data suggested distinct root fungal communities in controls and in once or repeatedly defoliated mountain birch stands (Fig. 4; ESM Table 4; ESM Fig. 3). Many environmental background variables, tree survival, and fungal functional groups in roots correlated significantly in our ordination analyses. EMF richness and abundance, tree survival, fungi/bacteria, and C/N ratios correlated with intact control plots, while NH_4 increased in once-defoliated plots; and saprotroph richness and abundance, Ca and P in repeatedly defoliated plots (Fig. 4). Altogether, we identified a total of 36 indicator species typical for different defoliation levels in indicator species analyses; 14 most abundant (represented by >50 sequences across all experimental units) of these are listed in Table 2. In controls, all six indicator species were EMF, whereas in once and repeatedly defoliated plots, OTUs representing saprobes, DSE, or other functional groups had significant indicator value. Listed indicator taxa represented ~26 % of our sequences.

Discussion

Moth defoliation of mountain birch led to distinct compositional and functional shifts in the root-associated fungal community. Increasing intensity of moth defoliation shifted the

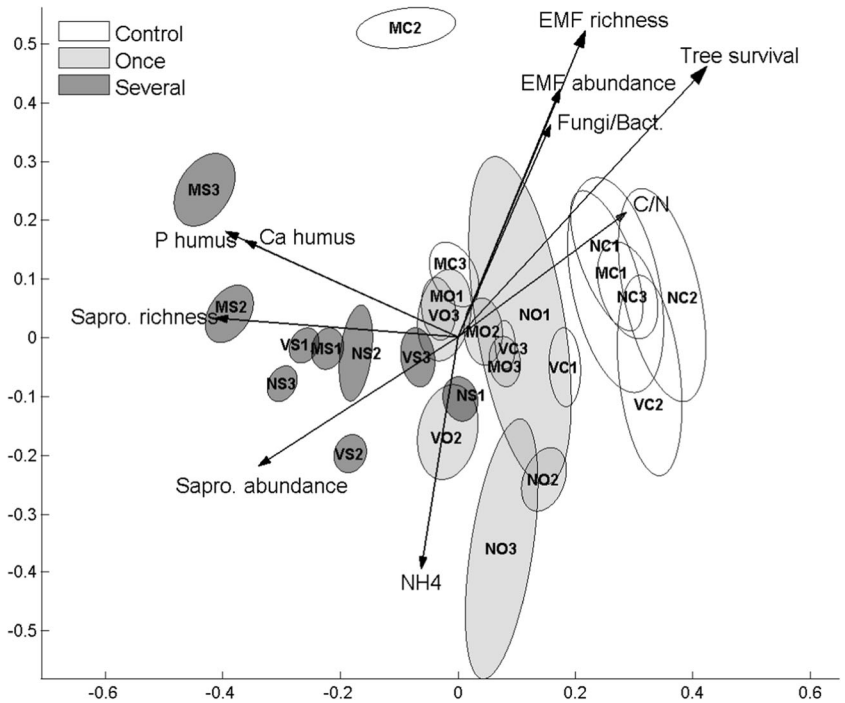

Fig. 4 NMDS ordination for mountain birch root associated fungal OTU communities across different sites and moth herbivory intensities (*white to gray fill*). The ellipses denote sampling plots where the *first letter* indicates site (*N*=Njuorggan, *V* = Veahcat, *M* = Mierasluoppal), the *second letter* indicates herbivory level (*C* = intact control, *O* = once defoliated, *S* = several times defoliated). The *numbers* refer to replicate plots within site and treatment. The ellipses show 1 SD of variation across 1,000 ordinations of random OTU assemblages resulting from the rarefaction of sequences (see Methods for details). The vectors indicate the direction of increase in the environmental variables that have significant ($p<0.05$) correlation with the ordination. *EMF* and *Sapro. richness* denote number of ectomycorrhizal and saprotrophic fungal OTUs or, respectively; *EMF* and *Sapro. abundance* denote proportion of sequences in the these fungal groups; *Fungi/Bact.* show fungi/bacteria ratio based on PFLAs [28]; *C/N* gives total carbon/nitrogen ration in humus; *Ca humus*, *P humus*, and *NH_4* give soluble calcium, phosphorus and ammonium contents (in milligram per gram of soil dw) in humus. Details of soil parameters in Kaukonen et al. [28]

Table 2 List of indicator fungal taxa (OTUs) for undefoliated control, once-defoliated, and several-times-defoliated mountain birch plots

Defoliation	Taxon	IndVal	Ecology	Accession	Sum of sequences				OTU in *n* plots
					Control	Once	Several	Total	
Control	*Pseudotomentella tristis*	0.834***	EMF	FN565319	950	31	228	1209	13
	Lactarius vietus	0.779**	EMF	JQ888186	339	71	78	488	12
	Cortinarius uliginosus	0.816**	EMF	GQ159785	327	0	0	327	6
	Piloderma sphaerosporum	0.804**	EMF	JQ711930	107	0	3	110	7
	Tomentellopsis sp.	0.662*	EMF	GU998686	69	0	1	70	5
Once	Chaetothyriales	0.764*	Unknown	FM997928	151	265	61	478	24
	Galerina sp.	0.720*	Sapro	JQ272382	14	47	22	83	16
Several	*Phialocephala sphaeroides*	0.912**	DSE	HQ157892	162	210	585	957	26
	Agaricales	0.883***	unknown	FJ475676	2	132	442	575	17
	Helotiales	0.862**	Unknown	HQ157833	5	9	139	152	11
	Verticillium sp.	0.864**	Sapro	FN565379	16	26	121	163	19
	Mortierella	0.845***	Sapro	HQ211894	16	15	59	90	22
	Phialocephala helvetica	0.839**	DSE	AY078136	12	20	43	76	14
	Mortierella alliacea	0.833**	Sapro	JX976019	8	10	42	60	20

Accession shows codes for best matching sequences deposited in UNITE or NCBI. Sum of sequences per indicator taxon are given separately for each of the defoliation levels and combined. OTU in n plots refers to number of plots in which taxon is present (maximum 26). Indicator OTUs with <50 sequences in the data are not shown

*$p<0.05$. ** $p<0.01$. *** $p<0.001$; IndVal gives indicator value with related statistical significance, ecology refers to fungal functional groups

EMF ectomycorrhizal fungus, *sapro* saprotroph, *DSE* dark septate endophyte, *unknown* functionally unknown fungus

dominance of the fungal community from Basidiomycota to Ascomycota and from mutualistic EMF to saprotrophs and putatively endophytic fungi, implying differences in carbon nutrition of these fungal functional groups. Severing carbon flow from host trees to belowground microbial community has been shown to cause rapid and persistent shifts from EMF to saprotrophic and opportunistic free-living fungi in coniferous forests [51, 52]. Our findings are congruent with Štursová et al.[23], who reported very similar functional and taxonomic responses in forest soil and litter fungal communities after a bark beetle attack in Norway spruce.

The growth and reproduction of EMF depend largely on recently fixed carbohydrates derived from a live host [7, 8, 11, 13, 53, 54]. Thus, lower foliar and root biomass in defoliated birch forests lead to a decline in EMF and a consequent reduction in Basidiomycota. We found strikingly similar response in EMF richness and abundance after only 1 year of defoliation compared to repeated defoliation. This implies severe and long-lasting reductions in belowground carbon allocation even after one severe insect attack. Declining EMF clades which declined in abundance in defoliated mountain birches were Agaricoid (mostly *Cortinarius* spp.), Boletoid, and Athelioid, all of which represent so called "distance exploration types" of EMF and produce large amounts of vegetative mycelial biomass in soil [55]. These high biomass EMF are assumed to require more carbon for growth and maintenance metabolism, and are likely the most susceptible to decline when carbon flow from the host is impaired [15, 20–22, 54, 56]. Those EMF which persisted even in the most severely defoliated plots with no live stems were members of Russuloid and Tomentelloid clades, representing low biomass symbionts. It is possible that root systems of mountain birch trees remain viable for years after foliage loss, since damaged trees possess high capacity for rejuvenation via basal sprouting [33]. In addition, some EMF in the most severely defoliated plots may also originate from trees (10 %) surviving outside the study plots [57] or even include metabolically inactive or even dead tissues. Our findings are similar to those in managed forests, where active EMF roots declined but were still detected 3 years after clear cutting [58].

Along with the decline in EMF, we found simultaneous increase in saprotrophs and putative endophytes (*Phialocephala* spp.) in defoliated plots. Though the latter taxa contributed to the increase in Ascomycota after defoliation, the majority of Ascomycota remained taxonomically and, therefore, functionally poorly identified. Systemic moth defoliation has likely induced a massive substrate input below ground in the form of dying plant and fungal material, favoring especially growth of litter decomposers, such as *Mycena*. Also some *Phialocephala* spp. may grow actively on woody debris for several years [59] and also respond positively to defoliation [60], which implies at least a facultative free-living lifestyle. Dead fungal mycelium also provides a high-quality nutrient source for other microbes [61]. A recent

isotope study revealed that EMF necromass is rapidly degraded and incorporated in saprotrophic fungal mycelium [62]. Moreover, other co-acting drivers may contribute to changes in the root-associated fungal community. We previously found elevated soil nitrogen levels especially after single moth defoliation, most likely due to fertilization effect of larval frass and decaying of nitrogen-enriched mycelia [28]. Since many EMF are sensitive to nitrogen increment [63, 64] and saprotrophs, on the other hand, may be limited by low nitrogen availability [65, 66], decoupling the effects of simultaneous increase in soil nitrogen and a decrease in carbon availability is difficult.

Fungal community analyses corroborate the above results: root-associated fungal assemblages showed a consistent relationship to the degree of herbivory, as also indicated by significant defoliation term in PERMANOVA, and a correlation with proportion of host trees with green foliar mass. Fungal community composition was related to soil NH_4 in once-defoliated plots and to P and Ca in several-times-defoliated plots, indicating that sequential nutrient flushes following herbivory are important drivers of fungal assemblages in birch roots. On the other hand, fungal communities in control plots correlated with C/N and fungi/bacteria ratios, implying relatively low nitrogen availability and fungal-based energy channel in intact mountain birch forests [28]. It is also noteworthy that only basidiomycete EMF were identified in undefoliated controls by the indicator species analysis, while saprotrophs and DSE fungi from Ascomycota and basal lineages were indicators in defoliated plots. However, the fact that a large part of fungal diversity remained poorly identified especially in defoliated plots constrains the interpretation of our results, albeit high number of unidentified taxa seems to be characteristic for next-generation sequencing data [67]. Communities were also different within each defoliation level, which appears as a significant site term in our analyses.

Rarefying has recently gained criticism especially in the context of normalizing uneven sample sizes of microbial metagenomic datasets [68, 69]. We attempt to control the effect of rarefying by our novel use of repeated rarefying and procrustean fitting of ordination patterns to each other provided a confidence region for the ordination space occupied by each plot. The method helps to evaluate whether the apparent difference in the placement of plots in the ordination space is genuine or if it could be a result of varying sampling intensities. A comparison of NMDS ordination between rarefied and non-rarefied data suggests that rarefying has only a modest effect on the results and will not change the general interpretation (ESM Fig. 3). Only one plot (NO1, Nuorgam, once defoliated) was markedly affected by the rarefying procedure. The sensitivity of this plot to rarefying is represented as a large and partially overlapping confidence ellipse in rarefied ordination graph (Fig. 4), suggesting that the community may not be distinguishable from some of the control and once-defoliated plots. The lack of overlap with severely defoliated plots suggests that despite the variation introduced by rarefying, the community is markedly dissimilar from the severely defoliated plots.

We report impacts of natural large-scale herbivory on fungal community composition for the first time assessed directly from host tree roots. We found a substantial decline of over 70 % in EMF abundance and richness even only after 1 year of moth defoliation. This might have consequences on carbon flow belowground via fungal mycelia. Impacts of severe moth defoliation are especially detrimental in low productivity mountain birch forests, which may require decades for full regrowth of lost foliage [30, 32]. In such systems where nutrient turnover is highly fungal-mediated [70], loss of EMF is likely to impair the forest recovery, e.g., due to the loss of shared EMF networks supporting seedling establishment [71]. Consequently, moth outbreak caused a large-scale top-down shift in fungal communities, which is likely to weaken bottom-up influences of belowground fungal communities on aboveground primary producers and negatively impact consumers.

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