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Effects of Early, Small-Scale Nitrogen Addition on Germination and Early Growth of Scots Pine *(Pinus sylvestris)* Seedlings and on the Recruitment of the Root-Associated Fungal Community

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Abstract: Scots pine (*Pinus sylvestris* L.) is one of the most economically important species to the Swedish forest industry, and cost-efficient planting methods are needed to ensure successful reestablishment after harvesting forest stands. While the majority of clear-cuts are replanted with pre-grown seedlings, direct seeding can be a viable option on poorer sites. Organic fertilizer has been shown to improve planted seedling establishment, but the effect on direct seeding is less well known. Therefore, at a scarified (disc trencher harrowed) clear-cut site in northern Sweden, we evaluated the effect of early, small-scale nitrogen addition on establishment and early recruitment of fungi from the disturbed soil community by site-planted Scots pine seeds. Individual seeds were planted using a moisture retaining germination matrix containing 10 mg nitrogen in the form of either arginine phosphate or ammonium nitrate. After one growing season, we collected seedlings and assessed the fungal community of seedling roots and the surrounding soil. Our results demonstrate that early, small-scale N addition increases seedling survival and needle carbon content, that there is rapid recruitment of ectomycorrhizal fungi to the roots and rhizosphere of the young seedlings and that this rapid recruitment was modified but not prevented by N addition.

Keywords: boreal forest; clear-cut; fungal community composition; ectomycorrhiza; Scots pine; nitrogen addition; mycobiome

1. Introduction

Swedish forested land makes up part of the wider boreal forest area, which is the largest terrestrial biome covering more than 10% of the land surface, extending across Eurasia and North America between 45° to 70° northern latitude [1–3]. It is characterized by nitrogen (N) limitation, slow regeneration, low plant community species richness and one of the largest carbon (C) pools in the world [1,3–5]. Plants growing in the boreal forest form symbioses with ectomycorrhizal fungi (ECM), one of the most diverse groups of soil organisms that establish mutualistic interactions with plants [6,7]. ECM are generally considered to enhance nutrient and water uptake by plants, seedling establishment and survival, and increased plant resilience against environmental stressors [8]. However, the ecological role of ECM can vary from mutualistic to parasitic [9–11], and in situations such as strong N limitation, mycorrhizal fungi can immobilize available N and aggravate plant N limitation [12–14].

In Sweden, forested land accounts for approximately 60% of the country [15], and forestry is one of the most important export industries [2], with the conifers Norway spruce

(*Picea abies* (L.) H. Karst.) and Scots pine (*Pinus sylvestris* L.) being the ecologically dominant and economically most important species [16,17]. The current Swedish silvicultural strategy, which has been used since the mid 20th century, focuses on clear-cutting mature Norway spruce and Scots pine forests that are re-planted within a few years of harvesting [18,19]. Using this method, approximately 50,000 to 70,000 individual clear-cuts, covering between 150,000–300,000 ha, are created each year [18]. The majority of clear-cuts are replanted with pre-grown seedlings, but direct seeding can be a viable alternative for low-nutrient sites with adequate moisture, limited competing vegetation, and where *Hylobius abietis* attacks are minimal [20,21]. Prior to any type of re-planting, site soils are scarified to improve soil properties (e.g., soil temperature and porosity) [22]. In addition, soil scarification increases natural regeneration [23] and seedling survival [24] by reducing above- and belowground competition with older trees [25,26]. Despite the benefits of soil scarification, it also increases nutrient leaching and site water loss [24,27,28]. Moreover, clear-cutting these N-limited boreal forests leaves the root-associated ECM fungi without photosynthetically fixed C, leading to a rapid decrease in biomass within the soil community [29,30]. In addition, scarification disturbs the upper, organic layer of the soil to expose the deeper mineral horizon [31,32], further disrupting the mycorrhizal network and increasing fungal necromass [30,33]. Scarification also exposes dead fine roots and complex C, increasing available carbon sources for opportunistic saprotrophs, which have been shown to increase in abundance in soil communities after both clear-cutting and forest fires [34–37]. Climate change will continue to drive increases in air and soil temperatures and the frequency and severity of drought in boreal forests [38]. These ongoing changes to the boreal environment also have the potential to alter plant–ECM interactions, with higher soil temperatures known to reduce ECM formation [39,40] at a time when the establishment of ECM symbiosis has the potential to enhance seedling drought tolerance [41,42].

In order to alleviate N limitation in the Swedish boreal forest soils, fertilization with inorganic N has been one of the most used strategies to improve tree growth and forest yield when used in the final 10 years prior to harvest [43–45]. Inorganic N addition has also been used extensively to produce high-quality seedlings in nurseries, but this often results in a reduction of the seedling root to shoot ratio [46,47]. Recently, it has been found that the production of seedlings using an organic N fertilizer produces high-quality seedlings with a well-developed root system, which improves out-planting performance [46,48]. However, it is less well known whether direct, small-scale N addition to site-planted seeds can improve early establishment and survival, and how this impacts early ECM fungal recruitment. Here, we used orchard-produced seeds supplied with controlled addition of inorganic and organic N sources supplied in a moisture-retaining and biodegradable germination matrix (seedPAD^{TM}; Figure S1), to investigate the role of early, small-scale N addition on seed germination and seedling establishment and fungal recruitment in situ on a scarified clear-felling site.

2. Materials and Methods

2.1. Site Description

The field site is located at 63°28′ N, 17°29′ E in the Svanatjärn area in northern Sweden (Figure S2A) and was part of a production forest belonging to Holmen Skog AB that was harvested in 2014, followed by scarification during the autumn of 2016. Prior to harvest, the forest was a mix of Scots pine and Norway spruce, and the trees were between 90 to 110 years old, growing on a podzol soil. The topography of the site is relatively flat with a slight slope toward the Svanatjärn lake shore, where water saturates the soil. Monthly mean temperatures at the site during the growing season range from 14 °C in July to 4 °C in October. In 2017, the mean temperature in summer was approximately 0.5 °C higher than normal, while in autumn it was approximately 0.7 °C lower. Precipitation at the site ranges from 56 mm per month in June, increasing to 78 mm in July and decreasing to 45 mm in October (Figure S3). The observed precipitation in 2017 was 30% lower in June,

approximately 20% higher during July and August, and around 50% lower in September (Figure S3).

2.2. Planting, Treatment and Sampling

In September 2014, an area of approximately 2.4 ha was harvested and subsequently scarified using a disc trencher to prepare for manual planting. From the site, a smaller area of about 0.9 ha was selected and on 15 June 2017, 300 seedPADs (seedPAD^TM, Arevo AB, Umeå, Sweden), each containing one Scots pine (*Pinus sylvestris*) seed, were placed on top of the exposed mineral soil. These seedPADs represented three different treatments, with 100 seedPADs containing 10 mg nitrogen (N) in the form of arginine phosphate, 100 seedPADs containing 10 mg N in the form of ammonium nitrate, and 100 controls containing no added N (Figure S1). They were distributed across three different sub-plots within the selected planting area (Figure S2B). At the end of the first growing season, on 20 October 2017, all surviving seedlings were collected without damaging the root system. The roots were washed with distilled water and separated from the shoot, and both root and shoot were weighed. These were then stored separately at $-80\,^\circ$C until further processing. The soil surrounding the root system (from now on referred to as the 'ectorhizosphere') was also collected and stored separately at $-80\,^\circ$C. In parallel, soil samples from the scarified site were collected to assess the effect of clear-cutting and scarification on the fungal community (hereafter 'scarified soil'). Soil was sampled from the upper 10 cm of the soil profile, and three cores in a triangle of around 15 cm side length were pooled for one sample.

2.3. Carbon and Nitrogen Content

Needle samples were ground to a fine powder in liquid nitrogen and air-dried at $70\,^\circ$C until constant weight. Soil samples were air-dried at $70\,^\circ$C until constant weight and sieved (<2 mm). The C and N content of 5 mg of the dried needle and soil samples were determined by conversion to CO_2 and N_2 by combustion and measured with an isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific, Bremen, Germany), following the method of Werner et al. [49].

2.4. Genomic DNA Extraction

Genomic DNA from the rinsed roots was extracted using a cetyl trimethylammonium bromide (CTAB) based method [50]. Briefly, mortar-ground samples were homogenized and washed for cleaning with chloroform:isoamyl alcohol (24:1). After cleaning, the genomic DNA was precipitated with cold isopropanol and cleaned further with 80% ethanol before being resuspended in 10 mM TRIS buffer (pH 8.5). Co-extracted RNA was eliminated with RNase A (10 mg/mL). The ectorhizosphere and scarified soil samples were freeze-dried prior to DNA extraction. Soil DNA was extracted using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN, Hulsterweg, The Netherlands) following the manufacturer's instructions. Purity was assessed using a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and the concentration was quantified using a QuBit 2.0 fluorometer (ThermoFisher Scientific). Before PCR amplification, DNA was diluted to a concentration of 5 ng/μL.

2.5. PCR Amplicon and Sequencing

The amplification protocol was based on the procedure proposed by Beckers et al. [51]. Briefly, PCR amplification was performed in a two-step PCR; in the first step, the primers gITS7 and ITS4 (Table S1) targeting for the internal transcribed spacer (ITS) region were used to amplify the fungal ITS2 region [52,53]. The PCR reactions were performed using HotStar HiFidelity Kit (QIAGEN, The Netherlands) according to the manufacturer's instructions, and 0.5 ng/μL of bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) in a final volume of 25 μL. The PCR amplification was run in triplicate for each sample. Five min at $95\,^\circ$C were used for initial activation of the polymerase, then 15 s at $94\,^\circ$C for denaturation, 1 min at $55\,^\circ$C for annealing, and 45 s at $72\,^\circ$C for extension, repeated 35 times;

for final extension, 10 min at 72 °C were used. Triplicate PCR products were then pooled, and PCR amplification success was confirmed using a 1.2% agarose gel. Twenty microliters of each sample was used for clean-up using AMPure XP magnetic beads (Beckman Coulter Genomics, Danvers, MA, USA) following the manufacturer's instructions, and 2.5 µL of the clean product was used for the second PCR step, where each sample was assigned a unique pair of barcodes (Table S2). The PCR amplification program used was the same as before, except 20 cycles were used instead of 35. Five microliters of each sample was pooled into one library, and the resulting pool was cleaned with AMPure XP magnetic beads. Library concentration was assessed with Qubit 2.0 and further purified to the desired fragment length (350–800 bp, accounting for variability of the ITS region) using BluePippin (Sage Science, Beverly, MA, USA) following the manufacturer's instructions. Final concentration was measured with QuBit 2.0, and the library was diluted to 10 nM. A fungal mock community, described in Haas et al. [54], was used as a positive control, and water samples as negative controls; both mock and water were treated as samples through the amplification and sequencing process.

The library pools were sequenced at the Science for Life laboratory in Stockholm, using an Illumina MiSeq and 600 cycles, yielding paired end reads of 300 bp length. Raw data were demultiplexed and quality filtered at the sequencing facility prior to delivery.

2.6. Sequence Analysis

The Illumina data were processed using QIIME2 [55] version 2019.1. Raw sequence data were imported using the q2-import plugin using the setting for Illumina fastq files, followed by denoising with Dada2 [56] via q2-dada2 denoise-paired, setting truncation at 301 bp for forward read and 300 for reverse. Taxonomy was assigned with q2-feature classifier [57] plugin using the UNITE database [58] (version 8.0) at a 97% similarity and dynamic level. Amplicon sequence variants (ASVs) were aligned with MAFFT [59] and used to infer a phylogenetic tree using FastTree [60], both processes via q2-phylogeny pipeline align-to-tree-mafft-fasttree using default settings.

To assign fungal functional guilds, we used the FUNGuild [61] Python script Guilds_v1.1.py (available at https://github.com/UMNFuN/FUNGuild, accessed on 17 November 2021) using the author's default settings. Taxa not classified by FUNGuild were completed manually through literature searches, wherever possible.

2.7. Statistics

The "kruskal" function of the agricolae package [62] was used to assess the effect of arginine phosphate or ammonium nitrate fertilization on C and N content of the soil, ectorhizosphere and needles (C or N content ~ Fertilization type) and seedling growth (root/shoot mass ~Fertilization type). The function provides Fisher's least significant difference (LSD) as post hoc analysis and statistical grouping based on Bonferroni correction when significant differences are detected in the Kruskal–Wallis test ($\alpha = 0.05$). Statistical differences in the survival rate were tested using a Cox proportional hazards regression model using the "coxph" function from the survival package [63,64] to assess the hazard ratio of the arginine phosphate- or ammonium nitrate-treated seedlings compared to the control.

Sequence analysis outputs were performed in R (Version 3.5.3) [65] and analyzed with the phyloseq package [66]. Prior to any analysis, ASVs with lower than 10 sequences were removed from the dataset using the "filterfun" function from the genefilter package [67]. Additionally, ASVs with an abundance lower than 0.005% per sample type were also removed. By doing this, 1214 low abundant ASVs were removed from the dataset. Finally, one sample with a library size lower than 10,000 filtered reads was removed from the dataset.

After filtering, species richness was estimated using the "pd" function of the picante package [68] after rarefaction of the samples to 10,894 reads, which was the minimum number of reads of a sample in the dataset. The Shannon diversity index was estimated

from raw, non-rarefied samples using the "diversity" function from the vegan package [69]. The "kruskal" function [62] was used to perform the Kruskal–Wallis rank sum test to assess the effect of arginine phosphate or ammonium nitrate fertilization on the fungal diversity (Shannon Index ~ Fertilization type). At the same time, the effect of the different sample types on the fungal diversity was tested (Shannon Index ~ Sample type).

Beta diversity was tested using the "ordinate" function from the phyloseq package, using Bray–Curtis to build the distance matrix with the rarefied samples and principal coordinate analysis (PCoA) for visualization. Changes in the community composition were tested using PERMANOVA with the vegan package [69], testing the effect of sample type and fertilization. Kruskal–Wallis was used to test any further differences between the different levels. Statistical groups were assigned based on Fisher's least significant difference (LSD) test.

To test for significant differences in abundance of the ASVs, we used the DESeq function of DESeq2 package [70] to estimate a sequencing library size factor and variance dispersion prior to fitting a generalized linear model (GLM) using the non-normalized data. Results of pairwise comparison of sample types with Log2fold change > 0.5 and adjusted *p-value* < 0.01 were used for visualization.

3. Results

3.1. Site Description

To characterize the mineral soil exposed by scarification, soil carbon (C) and nitrogen (N) content were measured at three locations within the experimental site covering the areas where the different seedPAD treatments were placed. The C and N content were highly variable in the scarified soil samples, indicative of mixing of the organic and mineral soils during scarification (Figure 1D,E). To assess whether N supplementation from the seedPADs altered soil N or C, the soil surrounding the seedling root systems (i.e., ectorhizosphere) was measured. The ectorhizosphere soil was less variable than the more mixed scarified soil but was not statistically different from it (Figure 1A–C; Kruskal–Wallis *p-value* > 0.05). Furthermore, there were no significant differences between the ectorhizosphere soil from the different treatment subsites (Figure 1D–F; Kruskal–Wallis *p-value* > 0.05). Thus, the soil across the different subsites was generally similar in N and C content and there was no evidence of significant N enrichment from the SeedPADs to the ectorhizosphere 4 months after planting.

3.2. Nitrogen Addition Increases Seedling Survival but Not Early Biomass

Seed germination and seedling survival increased 1.5-fold relative to the control when seeds were supplemented with either arginine phosphate or ammonium nitrate (Cox proportional hazard *p-value* < 0.05; Figure 2A). Furthermore, supplementation with either arginine phosphate or ammonium nitrate increased total needle C content (Kruskal–Wallis *p-value* > 0.05; Figure 2B). Although the higher survival and increase in needle C of N-enriched seedlings was not reflected in statistically greater overall shoot or root biomass, supplementation with arginine phosphate and ammonium nitrate did tend to increase shoot biomass compared to the control (Figure 2E,F).
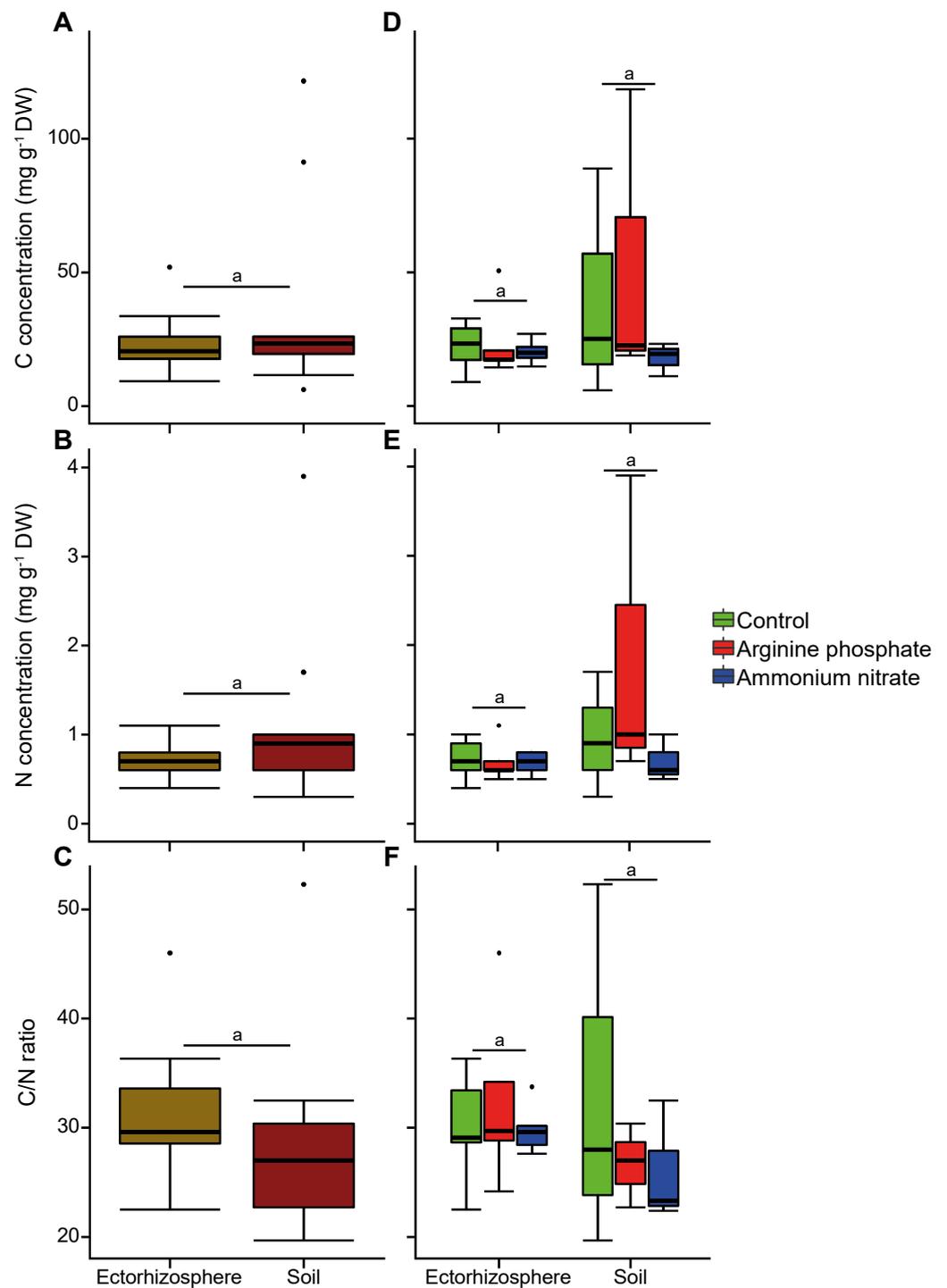
Figure 1. Boxplots of the mass fraction of carbon (**A**,**D**), nitrogen (**B**,**E**) and C to N ratio (**C**,**F**) in ectorhizosphere and scarified soil samples. Panels (**A**–**C**) depict the nutrient content per sample, and panels (**D**–**F**) depict the same data split by fertilization treatment. The lowercase letters in the upper part of the boxplots represent Fisher's least significant difference (LSD) statistical grouping testing sample type effect. Whiskers represent 1.5 × inter-quartile range (IQR).
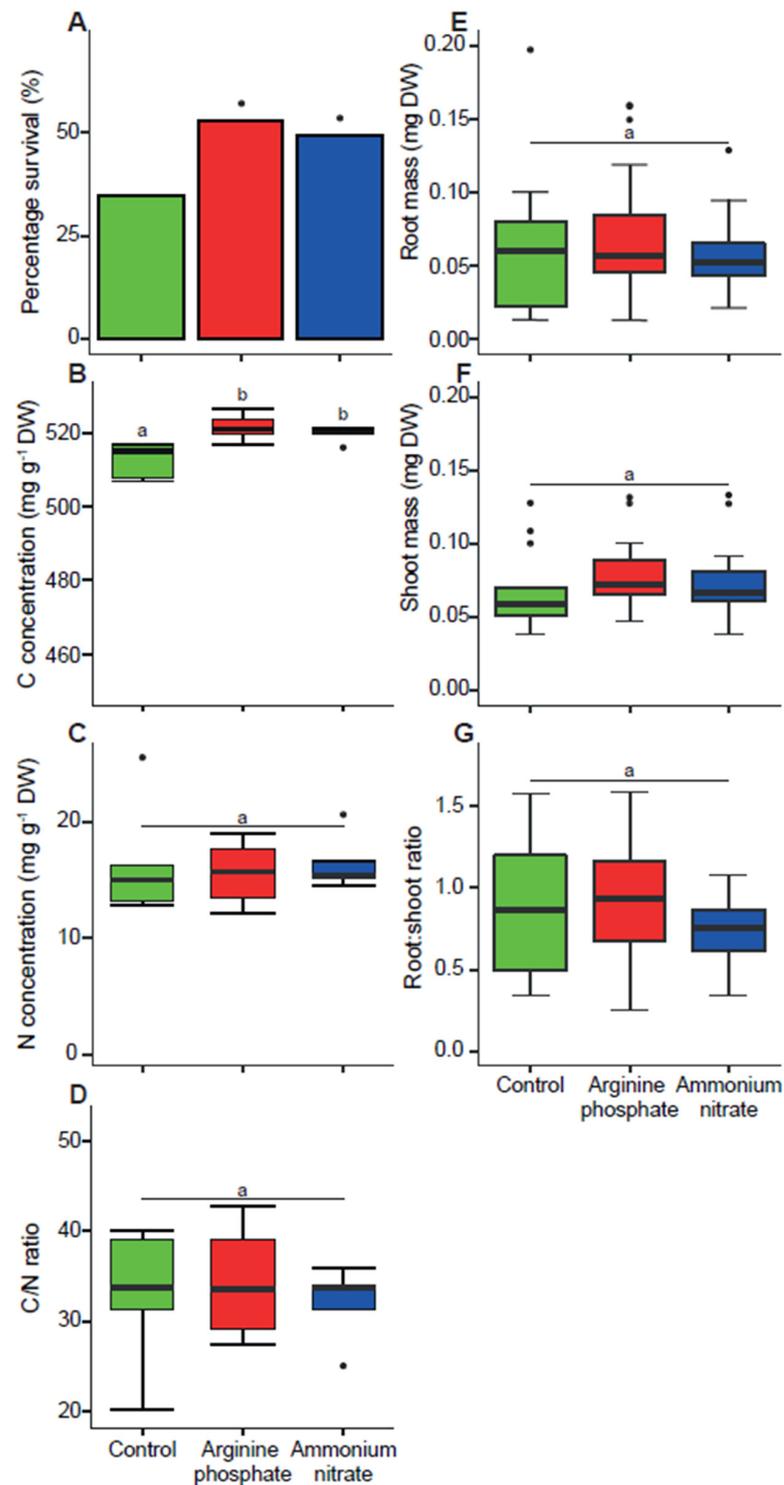
Figure 2. Barplot of the survival rate (**A**) and boxplots of the mass fraction of needle carbon (**B**), nitrogen (**C**), C-to-N ratio (**D**), root weight (**E**), shoot weight (**F**) and root-to-shoot ratio (**G**) of SeedPAD-planted Scots pine seedlings growing in scarified soil or supplied with either arginine phosphate or ammonium nitrate prior to germination. Asterisks represent statistical differences of Cox proportional hazard regression for *n* = 100. Lowercase letters in the upper part of the boxplot represent Fisher's least significant difference (LSD) statistical grouping testing treatment effect. Whiskers represents 1.5 × inter-quartile range (IQR). *n* = 22 (arginine phosphate), 18 (ammonium nitrate) and 13 (control).

3.3. Early Established Seedlings Are Colonized by Fungi Regardless of Nitrogen Supplementation

ITS2 amplicon sequence analysis was used to evaluate the fungal communities of the scarified soil, ectorhizosphere and washed roots. In control samples, the scarified and ectorhizosphere soils had, on average, two- and three-times higher richness compared to the roots (Kruskal–Wallis *p-value* < 0.001; Figure 3A). Diversity indicators showed that both scarified and ectorhizosphere soils had similarly structured fungal communities both in terms of evenness (Kruskal–Wallis *p-value* > 0.05; Figure 3B) and diversity (Kruskal–Wallis *p-value* > 0.05; Figure 3C). Both soil types had more even (Kruskal–Wallis *p-value* < 0.05) and more diverse (Kruskal–Wallis *p-value* < 0.05) communities than the roots, which had a community dominated by fewer ASVs.
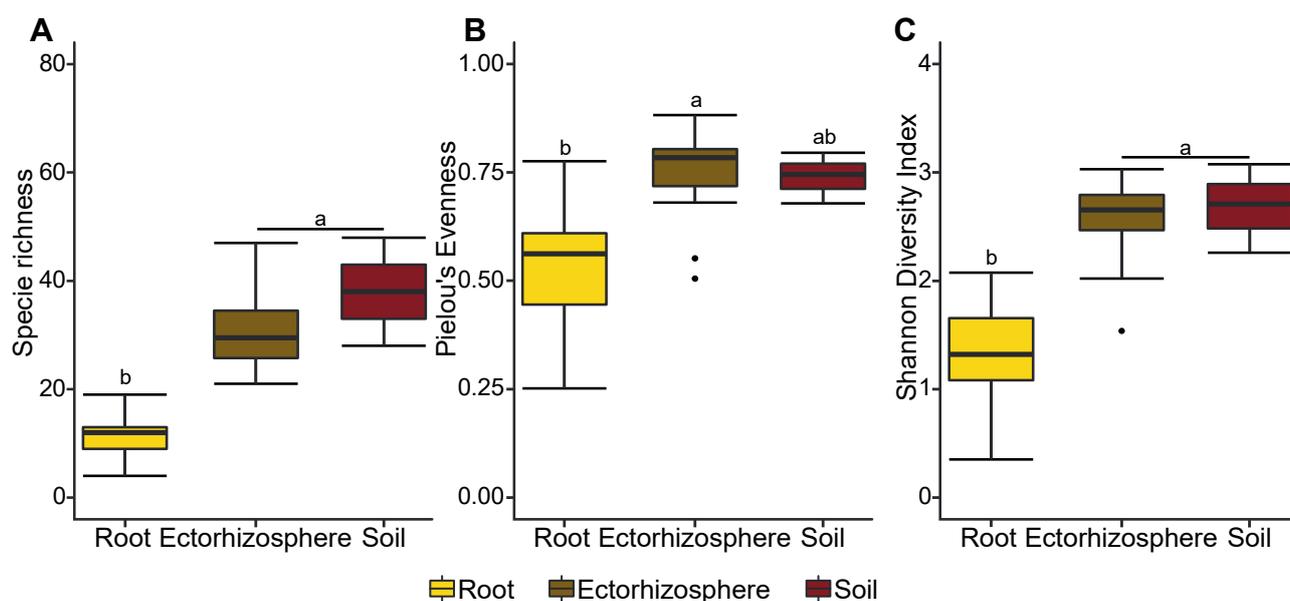
Figure 3. Boxplots of (**A**) species richness, (**B**) Pielou's evenness, and (**C**) Shannon diversity of control samples of roots, ectorhizosphere and scarified soils. Lowercase letters in the upper part of the boxplot represent Fisher's least significant difference (LSD) statistical grouping testing scheme 1.5 × inter-quartile range (IQR).

While species richness, Pielou's evenness and Shannon diversity all provide broad information about community structure, they do not provide information about community composition. An analysis of the community composition indicated that while the scarified soil and ectorhizosphere fungal communities were similar (PERMANOVA; adjusted *p-value* > 0.05), they harbored different dominant ASVs (Figure 4). For example, within the scarified soil community, the most dominant ASV was identified as belonging to the opportunistic taxon *Helotiales* sp., while in the ectorhizosphere *Leotiomycetes* sp., together with ASVs assigned to known saprotrophic taxa such as *Penicillium*, *Umbelopsis*, *Mortierella* and *Mycena* along with the ericoid mycorrhiza *Oidiodendron*, were dominant (Figure 4). In contrast, the community composition of the roots was significantly different from both the scarified soil (PERMANOVA adjusted *p-value* < 0.005) and the ectorhizosphere (PER-MANOVA adjusted *p-value* < 0.005), with the root-associated fungal community strongly enriched for ectomycorrhizal symbiotrophs such as *Russula, Piloderma* and *Suillus*, but depleted in saprotrophic fungi (Figure 4).
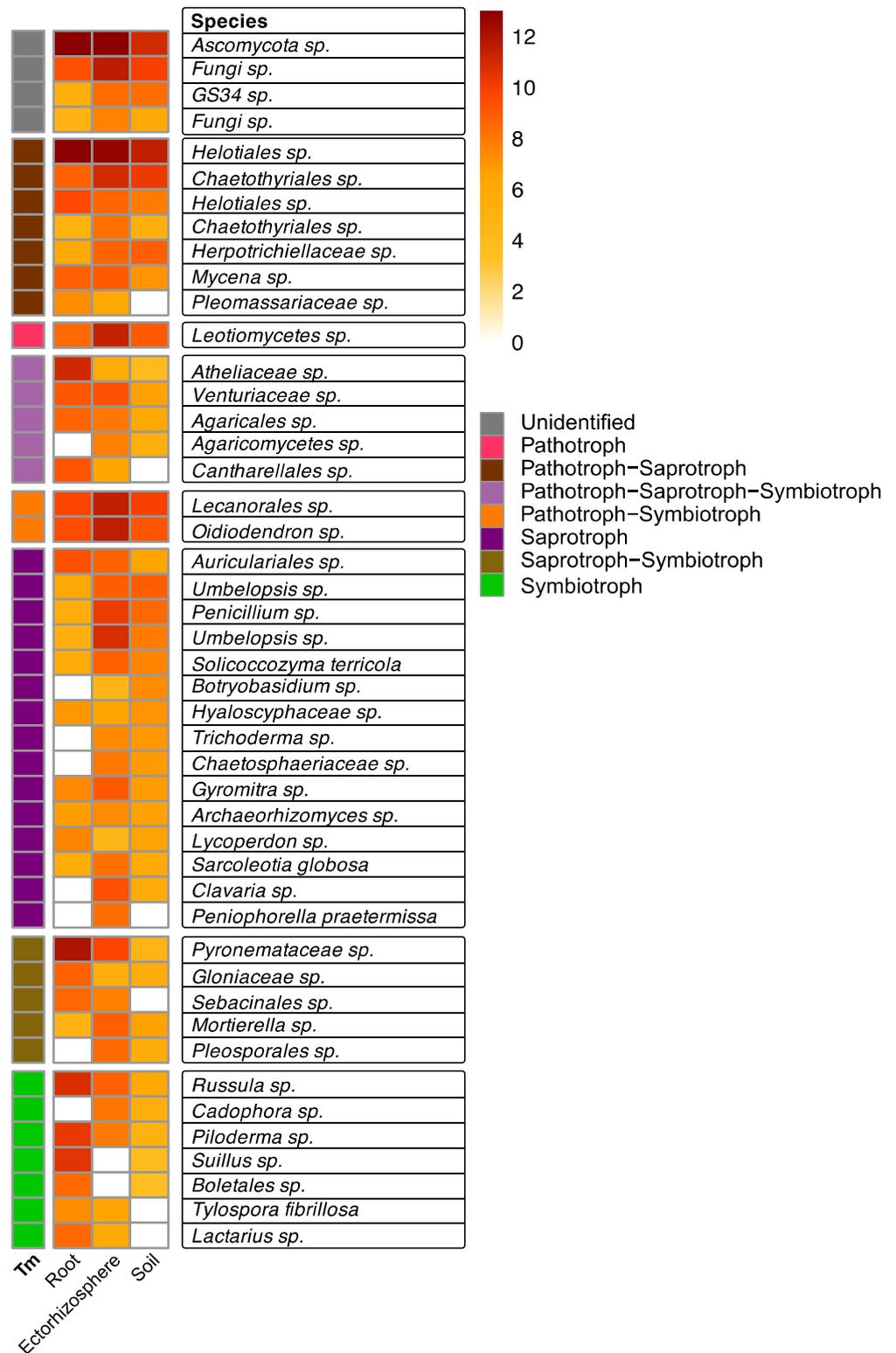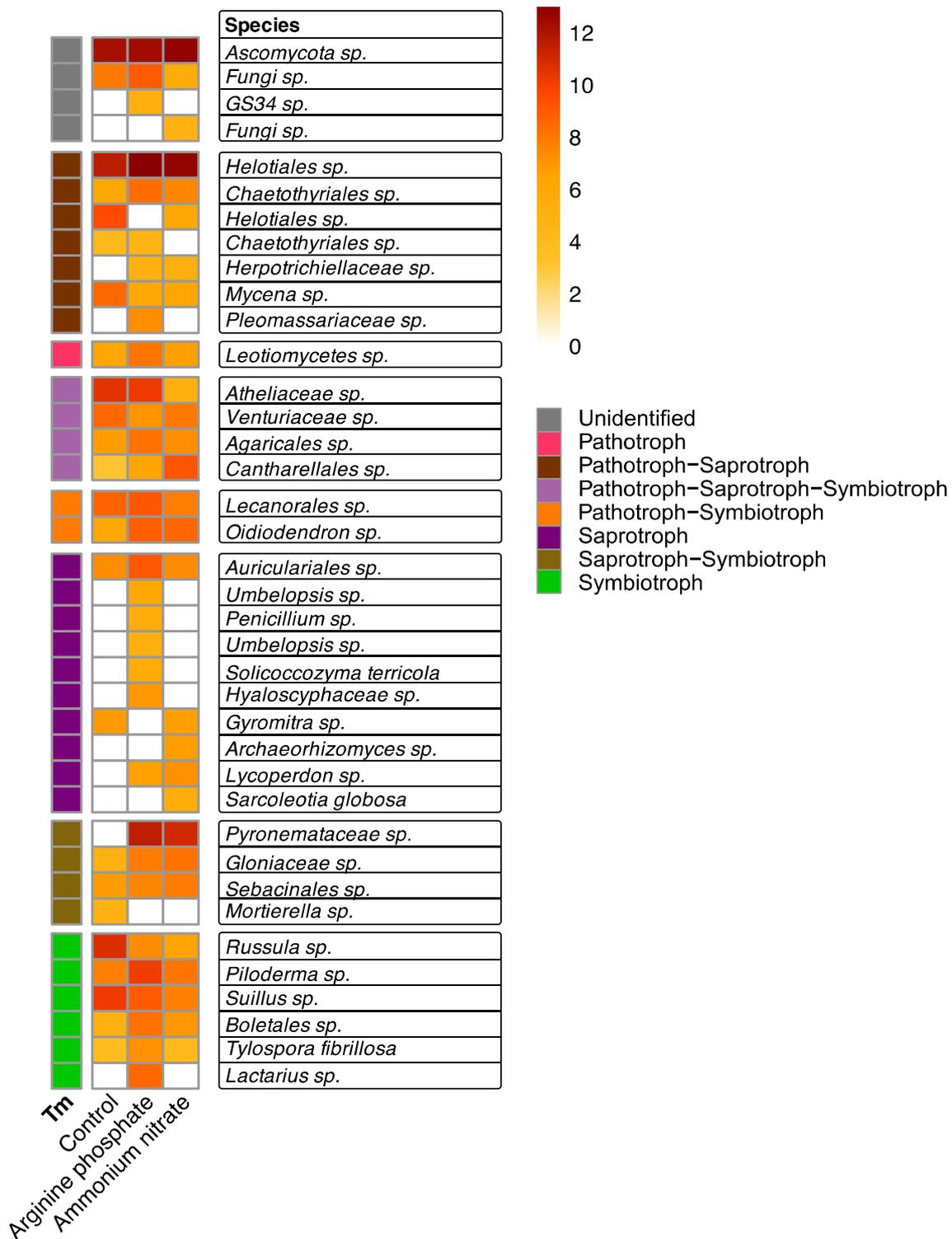
Figure 4. Heatmap depicting the most abundant amplicon sequence variants (ASVs) adding up to a total abundance of 50% of the total community of roots, ectorhizosphere or scarified soils. Left column colored by trophic modes annotated on FUNGuild V1.1. Scale is $\log_{10}$ of the sequencing reads. The complete list of ASVs can be found in Table S3.

When looking closer at the abundances of ECM fungal ASVs in roots, ectorhizosphere and bulk soil, we observed three differing patterns of occurrence (Figures 4 and S4).

(i) Several ectomycorrhizal ASVs in both types of soil samples, belonging to *Russula*, *Piloderma*, *Suillus* and *Cortinariaceae*, were enriched in root samples (Symbiotrophs in Figure S4), indicating their presence across the site as well as a rapid colonization of seedling roots. (ii) Two ASVs identified as *Cortinarius* sp. and *Russula densifolia* were present in bulk soil samples but never close to or on the seedling roots, which might indicate a preference for other host species or older trees. (iii) Several ectomycorrhizal ASVs (*Inocybe soluta*, *Tomentellopsis echinospora*, *Tylospora fibrillosa*, *Amphinema byssoides*, *Lactarius* sp.) were found only on roots and the rhizosphere, potentially indicating that these fungi very specifically grew to colonize the seedlings, with dormant spores in bulk soil staying below the detection threshold (Figures 4 and S4).

PCoA analysis of the N-supplemented samples indicated the presence of a core community that was persistent regardless of N-supplementation treatments. This community included ASVs identified as symbiotrophs, such as *Tylospora* and *Amphinema*, as well as ASVs identified as 'pathotroph-symbiotrophs' (e.g., *Lecanorales* and *Oidiodendron*) and a large number of unidentified Ascomycota and opportunistic *Helotiales* and saprotrophs (e.g., *Umbelopsis* and *Penicillium*) (Figure S4). Supplementation with arginine phosphate increased the number of unique ASVs in the root-associated community, accounting for 28.5% of the root-associated community (Figure S5). Despite this increase, no significant differences were found in diversity indexes (Figure S6; Kruskal–Wallis *p-value* > 0.05) nor at compositional level (PERMANOVA adjusted *p-value* > 0.05).

To further test the effect of N supplementation on the root-associated fungal community recruited by the roots of newly germinated pine seedlings, ectomycorrhiza fungi abundance was estimated based on the ASV counts and statistical significance (Figure 5). From the results, ASVs of the family *Pyronemataceae* were positively increased by supplementation with either arginine phosphate or ammonium nitrate (Figure 5, Table S4). Another ASV assigned to the genus *Russula*, which contains both nitrophilic and nitrophobic species [71], showed a significant negative response to N supplementation (Figure 5; Table S4). An ASV assigned to the mostly N-tolerant genus *Lactarius* was present only in the arginine phosphate-treated samples (Figure 5). ASVs assigned to the nitrophobic taxon *Piloderma* showed an increase in abundance in the presence of arginine phosphate, while *Suillus* showed reduced abundance in response to both N-supplementation treatments (Figure 5). Interestingly, a member of the *Cortinariaceae* family, which is known to be nitrophobic [71], was only present on roots of fertilized seedlings (Figure S7).

Figure 5. Heatmap depicting the most abundant amplicon sequencing variants (ASVs) with a cumulative presence of 50% of the total community on roots. Left column colored by trophic modes annotated on FUNGuild V1.1. Scale is $\log_{10}$ of the sequencing reads.

4. Discussion

In the Swedish boreal forest, soil scarification is the current practice for site preparation after clear-cut timber harvesting. Clear-cutting stops the transport of current photosynthate to roots and the associated rhizospheric community, which leads to a drastic shift in soil

fungal communities, reducing ECM abundance [30]. The subsequent soil scarification further disrupts the ECM, leaving only fragments of the previous ECM networks. These scarified clear-cuts are typically replanted with nursery-produced seedlings 2 years after harvesting or, on specific sites, directly sown with orchard-produced seeds. The boreal forest is generally characterized by nutrient-poor soils, with N being the most limiting nutrient [15], and nursery-produced seedlings are deliberately loaded with nutrients during production to improve initial seedling survival and growth [72–74]. However, it is not known whether the same positive effect of N supplementation can be achieved upon direct seeding. Our results show that a small addition of either organic or inorganic N did increase seedling survival (*p-value* < 0.05) and carbon accumulation aboveground (*p-value* < 0.05) (Figure 2). Furthermore, we provide evidence for a strong early effect on fungal recruitment by the root, with an enrichment of ECM fungi in the saprobe-dominated, disturbed soil after only a few months (Figure 4).

Reduction in recent plant-sourced C in the soil caused by site preparation (i.e., tree removal and soil scarification) reduces ECM dominance, favoring saprobes [30,75]. Kohout et al. [29] found a progressive shift from an ECM- to saprotrophic-dominated soil community in a spruce forest over a timeframe of around 20 months after harvesting, which corresponds to the time that usually passes between harvest and replanting. Our data are consistent with this finding, showing that saprotrophs were the most dominant trophic mode in the scarified soil and ectorhizosphere (Figure 4; Table S3), accounting for about 15% of the whole community. However, while greatly diminished, ECM were not completely removed from the scarified soil community of our study site, accounting for about 5% of the community (Figure S4). The persistence of these ECMs might be explained by hyphae, sclerotia or spores remaining in the soil [76]. These different types of propagules are believed to remain dormant and can be induced to restart growth or germinate, respectively, by the presence of closely growing roots [76,77]. Our results clearly show this stimulating effect of the exploring root (Figure 4; Figure S4), with ECMs associated with roots being twice as abundant relative to either the ectorhizosphere or scarified clear-cut soils. Similar results were found by Kyaschenko et al. [78] in a study performed in an age-gradient Scots pine stand, where 1 year after site preparation the soils were saprobe-dominated, while the abundance of ECM was generally low, with a dominance of species such as *Piloderma* and *Tylospora.* They proposed that these ECM fungi from the *Atheliaceae* family are favored by the high levels of soil inorganic N and host-derived C in the soils of the younger stand [78]. We also found that *Atheliaceae* such as *Piloderma, Tylospora* and *Amphinema* were among the dominant ECM families in the root samples (about 25%; Figure S7), supporting the view that they are among the first ECM colonizers of Scots pine seedlings germinating in disturbed soils, and that recruitment by these seedlings occurs very rapidly despite the saprobe-dominated community of the disturbed scarified soil.

Growing in association with already-established ECM is often positive for seedlings, and established ECM fungi can enhance survival at the plant community level [79]. However, it has been observed that under some environments, such as nutrient-poor soils, belowground interactions limit seedling growth due to their low competitive strength against adult trees [14,25,80,81]. Thus, although seedlings that germinate in undisturbed boreal forest soils have access to established mycorrhiza, from which they may gain valuable nutrients [82], seedling establishment in such undisturbed forests is often poor, and these seedlings are generally smaller compared to those that establish in scarified soils [25]. This was illustrated by Axelsson et al., [25] who performed an experiment in a Scots pine stand where they observed that the seedlings successfully established and grew larger, both when C flux belowground was eliminated by girdling the adult trees, or in a clear-cut ca. 10 m away from the forest edge [25]. Similar results were found by Pasanen et al., [26] in artificial canopy gaps with soil preparation in a Scots pine-dominated forest, where most of the establishing seedlings were found in the disturbed soil plots [26]. Thus, in highly disturbed soils, such as a scarified clear-cut site, where mycorrhizal networks and plant-derived C flux have been heavily disrupted, initial seedling establishment is enhanced and

resistant propagules (i.e., sclerotia and spores) become the dominant mycorrhizal inoculum source [76,83,84]. This might explain why we found some ECM fungi such as *Inocybe soluta*, *Tomentellopsis echinospora* or *Amphinema byssoides* at relatively high abundances in root and rhizosphere samples, while being absent from bulk soil samples (Figure S4). Their apparent absence in the bulk soil might be due to these propagules falling below the detection threshold of our profiling method, while other ectomycorrhizal ASVs belonging to *Russula* and *Piloderma* were still present in the disturbed soil with relatively higher biomass abundance, possibly due to higher resilience to disturbance or higher abundances before clear-cutting.

The addition of a small amount of N to the germinating seedling did not significantly alter the already-strong selective pressure of the roots on the fungal community (Table S4). However, we did find evidence of initial changes in the abundance of specific fungal ASVs, particularly in *Pyronemataceae* sp. and *Russula* (adjusted *p-value* < 0.01; log2Fold > 2). Although the specific nature of most *Pyronemataceae* is unknown, different members of the family have been characterized as saprobes or ectomycorrhizal [85,86], while *Russula* is a large ECM genus with both nitrophobic and nitrophilic species, and is typically dominant in older stands [71,78,87]. Within the other ASVs recruited by the roots, the nitrophobic *Suillus* was negatively affected by both arginine phosphate and ammonium nitrate (Figure 5) while *Piloderma*, which has the ability to take up, use and deliver amino acids to Scots pine [88], responded positively to the addition of arginine phosphate but not ammonium nitrate (Figure 5). While previous large-scale studies have shown that short-term N addition to undisturbed boreal forest soils had no significant influence on root-associated fungal communities [54,89], our study showed that small, localized applications of either organic or inorganic N in disturbed soils both increases seedling survival and facilitates the rapid recruitment of some taxa of ECM fungi, such as members of *Pyronemataceae*, *Cortinariaceae* and *Piloderma*.

5. Conclusions

Traditional forest management includes clear-cutting of mature trees and subsequent soil scarification for new seedling plantations. While this increases seedling establishment success and growth, its collateral effect is the local disruption of the soil, altering nutrient equilibrium and microbial composition. Further complicating future management decisions, the impacts of climate change-related increases in air and soil temperatures and increasing drought on the plant–soil community interaction are uncertain. While increases in soil temperature are expected to enhance seed germination and survival in the northern hemisphere [90–92], higher soil temperatures are also known to reduce ECM formation [39,40], potentially leaving the roots more vulnerable to pathogen attacks [93] or drought. Our study provides evidence that small-scale N addition to onsite-planted seeds can be used to enhance seedling establishment without N leakage into the soil, and that such small-scale N addition does not significantly alter the strong selective pressure of the growing root in rapidly reshaping the root-associated fungal community from the opportunistic dominated community of the scarified soil into an ECM-dominated community.

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