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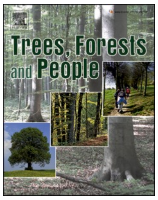

Effects of small-scale outplanting fertilization on conifer seedling growth and fungal community establishment

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ABSTRACT

Forestry in Sweden largely relies on planting genetically improved seedlings after clear-cutting, and high survival and early growth of planted seedlings is vital for stand establishment, economic viability, and carbon sequestration. Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) are the two most important tree species in Swedish forest stands and both are associated with a variety of ectomycorrhizal fungi. While seedlings are generally not fertilized at outplanting, previous results have shown that fertilization with arginine phosphate can increase root growth and seedling survival. However, it is not clear how this affects fungal community composition on the roots of growing seedlings. In a planting experiment sampled after one and two growing seasons, we found that planting position had the largest effects both on seedling performance and on fungal community composition and provide insight into the early stages of fungal community succession on planted Norway spruce and Scots pine seedlings. Fungal taxa present on seedlings before planting persisted on seedling roots, while some degree of novel colonization by site indigenous taxa was observed. Fertilization modified the relative abundance of some fungal taxa but did not lead to significant changes in overall community composition. In terms of seedling performance, ammonium nitrate led to increased mortality while arginine phosphate improved root growth.

1. Introduction

Forestry in Sweden is currently largely based on clear-felling followed by replanting the harvested area with genetically selected nursery grown seedlings. This requires a large investment in nurseries to produce these seedlings (Nilsson et al., 2010), and places high importance on maximizing seedling survival after outplanting, with higher survival of these “plus” trees also entailing higher levels of aboveground carbon (C) sequestration (Ahtikoski et al., 2020). Thus, to ensure successful seedling establishment, the best possible initial growth conditions need to be provided and combined with optimized site preparation practices to maximize survival and early growth. The two conifers Norway spruce (*Picea abies* (L.) H. Karst.) and Scots pine (*Pinus sylvestris* L.) are the ecologically and economically most important forest tree species in Sweden and comprise the majority of standing wood in the Fennoscandian boreal forest. Tree growth in Sweden is limited by the relatively low nitrogen (N) availability in soils of the boreal and nemo-boreal zones, and in southern Sweden is additionally limited by water availability during the growing season (Bergh et al., 1999; Högberg et al., 2017). Soil microbial communities play a crucial role in C and N cycling in global soil ecosystems (Bahram et al., 2018). In the N limited boreal forest soils, ectomycorrhizal (ECM) fungi are especially important, forming symbiosis with the trees and colonizing over 90 % of tree root tips (Taylor et al., 2000), while dominating soil layers below the upper organic horizon, including mineral soil (Clemmensen et al., 2013; Rosling et al., 2003). The ECM fungi exchange N that they obtain through their mycelial networks in the soil for photosynthetic C from the trees, which has important implications for tree health and C cycling processes. The exact implications of the ECM symbiosis for tree N supply under different conditions is under debate, with some previous studies

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stressing the advantages for the trees in the form of improved drought tolerance and nutrient uptake (Gehring et al., 2017; Taylor and Peterson, 2005; Velmala et al., 2014; Wallander, 2000; Yin et al., 2018), and others suggesting that the relationship between tree and fungus is more competitive in N limited environments (Alberton et al., 2007; Henriksson et al., 2021; Näsholm et al., 2013).

Before replanting, the soil in clear-cut areas is usually prepared by scarification. Previous studies have shown that survival, growth, and even ECM root colonization of planted Norway spruce and Scots pine seedlings is improved by site preparation when compared to unprepared sites (Hébert et al., 2014; Hille and den Ouden, 2004; Johansson et al., 2006; Örlander and Nilsson, 1999; Pennanen et al., 2005). Site preparation can be performed by either mounding or disc trenching, depending on site characteristics, resulting in spots or a trench with exposed, bare mineral soil, and a so-called capped mound next to it. Ideally, the capped mound consists of a double layer of inverted organic soil, covered by a layer of mineral soil (Häggström et al., 2021). This capped mound is the recommended planting position as it has a smaller risk for frost damage due to the elevated position and high nutrient availability from the turned over organic soil below. However, seedlings planted in capped mounds are more susceptible to drought, and in dry springs the recommended planting position in the south of Sweden is high up in the exposed mineral soil (Skogsstyrelsen, 2023). In line with this, the survival of planted seedlings has been shown to depend, in large part, on the amount of precipitation in the first month after planting, and in dry weather survival is usually better in mineral soil (Häggström et al., 2021). Apart from these clear advantages for forest regeneration, clear-cutting and subsequent soil scarification disrupts the organic layer of the soil and the residing fungal communities, leading to a shift from mostly ECM fungi to a dominance of saprotrophic and opportunistic fungi (Kohout et al., 2018; Lindahl et al., 2010). Furthermore, water and nutrient retention capacities of the soil are temporarily reduced (Pennock and van Kessel, 1997; Piirainen et al., 2007) and local C emissions temporarily increased (Peichl et al., 2023; Vestin et al., 2020).

Due to the N-limited tree growth in the boreal forest, there have been a range of experiments to use fertilizer to improve seedling growth. Swedish experiments in the early 20th century showed a negative effect of inorganic N fertilization on seedling survival and growth when applied to naturally generating seedlings, despite initially good performance. Romell and Malmström (1945) attributed these negative effects to the resulting lush above-ground growth without corresponding root growth, which is concordant with low root:shoot ratios being associated with higher susceptibility to drought and other stresses in different types of plants (Grossnickle, 2005; McDowell et al., 2008; Xu et al., 2015). Similarly, in experiments using Douglas fir, larger doses of inorganic fertilizer applied after outplanting led to poorer root development and decreased drought tolerance (Jacobs et al., 2004). As a result of such negative reported impacts, the current consensus in Sweden is to not fertilize seedlings upon outplanting. However, most nursery grown seedlings are fertilized in the nursery to increase growth rates, which traditionally has been done using conventional inorganic fertilizer. Additionally, seedlings are fertilized after growth cessation in autumn to induce additional N storage in the seedlings, which has been termed 'nutrient loading', and leads to improved performance in the field when seedlings are planted in spring (Heiskanen et al., 2009; Luoranen and Rikala, 2011). Boreal forest trees take up a significant portion of their N in the form of amino acids (Näsholm et al., 2009), and using organic N sources in the nursery instead of conventional fertilizer has been shown to increase root growth and the proportion of mycorrhizal root tips, as well as overall field performance after outplanting (Gruffman et al., 2012; Vaario et al., 2009). Some Swedish nurseries have switched to organic fertilizer for this reason. Enhanced root growth is especially beneficial since initial root growth is believed to be an important factor determining stress resilience of outplanted seedlings (Davis and Jacobs, 2005; Grossnickle, 2005). Large-scale Swedish studies have shown that Scots pine seedlings are more likely to survive when a small dose of arginine phosphate is supplied upon outplanting (Häggström et al., 2023, 2021), and in a Spanish study, Scots pine seedlings showed improved P uptake capabilities and higher chlorophyll concentration after fertilization with an amino acid mix (Sigala et al., 2021). An additional advantage of arginine phosphate specifically is that it prevents leaching of N into the environment (Öhlund and Näsholm, 2002), most likely due to the positively charged molecules binding to soil particles (Inselsbacher et al., 2011).

Given these new findings on changes to root growth following seedling fertilization with organic N, and the ability to study microbial communities using high-throughput sequencing methods, we aimed to test how seedling establishment is affected by different N sources, and how the fungal communities associated with seedling roots develop in the early stages after outplanting under these conditions. To address these questions, we investigated the effects of planting position in combination with small doses of inorganic or an arginine-based organic fertilizer on outplanted Norway spruce and Scots pine seedlings and associated fungal communities at a clear-cut site in southern Sweden. Our specific aims were to determine: i) whether planting position or small-scale fertilization (organic or inorganic) at outplanting influence seedling performance; and ii) whether planting position or small-scale fertilization at outplanting affect fungal community development on seedling roots.

2. Materials and methods

2.1. Sampling site description

The clearcut study site is situated in the boreo-nemoral zone close to Norrköping, Sweden (58°43′42.1″N; 16°02′56.3″E). The site is located at an elevation of 100–110 m, with an annual mean temperature of 7.3 °C and a mean annual precipitation of 700 mm, between 1997 and 2016. The average daily temperature in the growing season 2017 was 0.3 °C lower than the previous 20 year average, while the growing season 2018 was 2.6 °C warmer. In terms of total yearly precipitation, 2017 was a close to average year (660 mm), while 2018 was drier than average (490 mm), with both years having exceptionally dry May and July months. Precipitation in the first month after planting (May 2017) was around 45 % lower than the mean of the previous 20 years for the same month. Weather data was sourced from the Swedish Institute for Meteorology and Hydrology (SMHI) weather stations Hult D (precipitation, 10 km from the site) and Kålmorden-Strömsfors (temperature, 25 km from the site).

The topsoil on the site consists of glacial till with partly exposed bedrock in the northern part of the site, with a south-facing, slight slope leading into a nutrient-rich peat marsh in the southern section, in close proximity to a pair of small lakes (Geological Survey of Sweden, 2023, Fig 1A). The varied soil profile on the clear-cut, sloping from a stony and sandy glacial till in the northern section, to a nutrient-rich peat soil in the southern section (Fig 1A), was reflected in significantly higher carbon and nitrogen contents of the soil from the southern edge compared to the other three edges ($p < 0.001$, Fig 1B).

Before harvest, the forest on the site consisted of mainly Scots pine with an approximate age of 60 years that were harvested in September 2015. The clear-cut has a total area of around 9 ha. The site was scarified using an attached mounder in the early spring of 2017, creating patches of bare, exposed mineral soil and adjacent capped mounds, consisting of a mound of organic soil capped with a layer of mineral soil (Häggström et al., 2021). The site is overall fertile, with a site index of 30 for Norway spruce, meaning that Norway spruce trees will reach a maximum average height of 30 m at 100 years. An area of around 0.5 ha was set aside for the planting experiment, situated in the northern half of the clearcut (Fig 1A). As described above, this part of the site is characterized by glacial till and thinly covered bedrock, and, expectedly, the sampling blocks showed relatively low variation in C and N content and C:N ratio compared to the clearcut edges, with comparatively minor

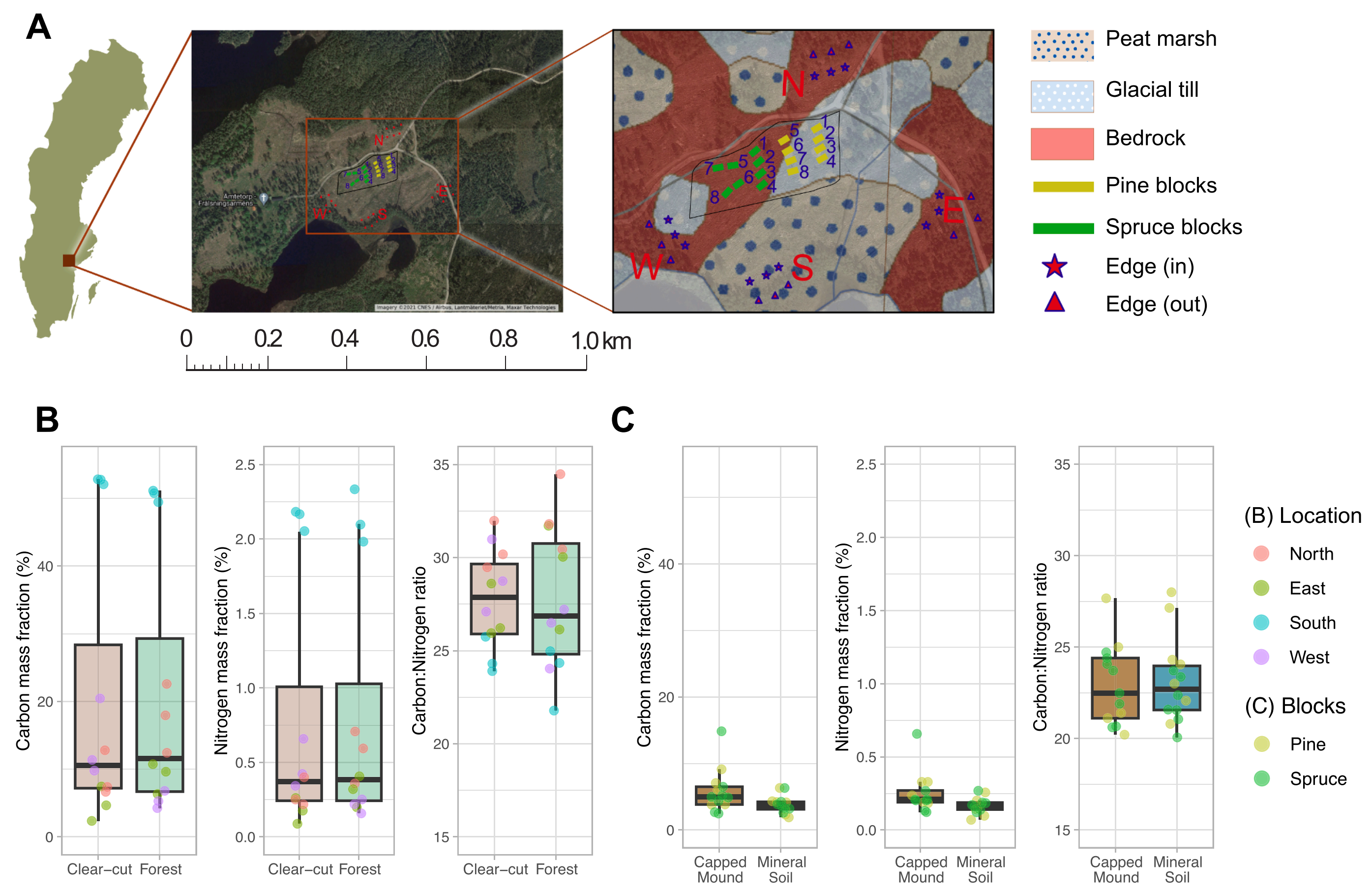

Fig. 1. Site overview and carbon (C) and nitrogen (N) contents of clearcut soil, surrounding forest soil, and sampling block soils. **A.** Map showing location of site (left panel), colored by ground type 0.5 m below the surface (right panel) and with sampling blocks indicated in green (Norway spruce) and yellow (Scots pine). Red symbols with blue margins indicate sampling locations for edge samples from within the clearcut (stars) and from the surrounding forest (triangles). **B.** Boxplots showing C content (first panel from the left), N content (second panel) and C:N ratio (third panel) of clear-cut (light brown boxes) and surrounding forest (light green boxes) soil samples. Samples are plotted as points additionally, colored by sampling locations (NESW). **C.** Boxplots showing C content (first panel from the left), N content (second panel) and C:N ratio (third panel) of control samples from sampling blocks in the clearcut. Boxes are split and colored by planting positions, color legend to the far right. Samples are plotted as points additionally, colored by block (Norway spruce or Scots pine, see locations in **A**). To facilitate comparison with B, y axes are kept on the same scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

variation between blocks (Fig 1C). C:N ratios in the sampling area ranged from 21 to 25. Comparing the planting positions, the capped mounds generally had slightly, but not significantly, higher C (p = 0.066) and N (p = 0.056) content than the soil sampled from the bare mineral soil (Fig 1C). The soil sampling procedure is described below.

2.2. Plant material, planting, treatment, and sampling

Seedlings were sourced from the Holmen Skog AB Friggesund nursery, where they received weekly to biweekly fertilization treatment with the fertilizer products arGrow MIX (growing season) and Argrow Complete (autumn) (Arevo AB, Umeå, Sweden). arGrow Complete contains nitrogen in the form of l-arginine in combination with macro and micronutrients, while arGrow Mix, in addition to l-arginine also contains l-lysine and nitrate as nitrogen sources. Seedlings were treated with a variety of commonly used fungicides and herbicides in the nursery (Table S1), with the same substances being used for both species. Scots pine seedlings were additionally treated with the product Binap t Skogsplanta, containing *Trichoderma* spp. inoculates (Binap, Helsingborg, Sweden). Norway spruce seeds originated from the Gälltofta-2 seed orchard (latitude 55°58′), harvested in 2006 and were pre-grown in 120 ml Starpot cassettes (Holmen Skog AB, Stockholm, Sweden). Scots pine seedlings were grown from seeds from the Gottardsberg seed orchard (latitude 58°56′), harvested in 2011 and grown in 90 ml Starpot cassettes. The seedlings were grown in block peat harvested from Svenarum in Småland, Sweden. The autumn before planting Norway spruce seedlings had an average height of 24 cm with a needle N content of 2.4 %, and Scots pine seedlings an average height of 30 cm and needle N content of 2.0 %.

In May 2017, a total of 1850 Norway spruce and 1997 Scots pine seedlings were planted by first filling the sampling area, in separate sections for Norway spruce and Scots pine (Fig 1A). All of these were used for survival statistics after the first growing season. A total of 480 seedlings of each species (960 in total) were used to establish the factorial treatment combinations between two planting positions and three fertilization treatments in every block (Fig 1A), explained in the following: Seedlings were planted on two different planting positions, either in the scarified bare mineral soil or in the adjacent capped mound consisting of turned-over soil. During planting, seedlings were fertilized with either 40 mg of nitrogen in the form of arginine phosphate granulat (arGrow® Granulat, Arevo AB, Umeå, Sweden) or 40 mg of nitrogen as ammonium nitrate slow release granules (Osmocote exact mini Release 15–9–11; Scotts Miracle-Gro, Ohio, United States) per plant. A planting tube with automatic dosage (Pottiputki, Savonlinna, Finland) was used to apply fertilizer into the bottom of the planting hole for every treated seedling. The control treatment was left unfertilized. We established eight blocks for both species, and each block consisted of three parallel scarification rows, one row per treatment and with two planting positions within each row, bare mineral soil and capped mounds. Within one block, each row contained ten seedlings in each planting position (Fig 1). Remaining seedlings were planted to fill the clearcut and to further assess survival, and additional treated seedlings were planted to have spare seedlings in case of low survival. A few seedlings of both species were set aside and frozen to be used as nursery reference.

After the first growing season, in early November 2017, a first set of samples were taken. Five seedlings were sampled from every treatment in blocks 1–4 from both species, and the whole root ball was excavated. Additionally, bulk soil samples were taken in close proximity to the seedlings, within the designated blocks, from now on referred to as "clearcut soil" (Table 1). For all bulk soil samples, three pairs of soil cores taken about 20 cm apart were pooled. Litter and moss were removed, and the upper 10 cm of the soil cores were sampled. The seedlings were stored at 4 °C in individual bags during transport and then processed upon arrival about 24 h later. Shoot length, pine needle length and stem base diameter were measured first. Then, five separate fine roots (<2 mm diameter, around 5 cm each) from around the root mass were cut off and washed with distilled water. Soil shaken off from fine roots was sampled as rhizosphere soil. Soil and root samples designated for DNA extraction were stored at −70 °C before subsequent freeze drying. In order to distinguish root growth happening in the field from roots that were formed before planting, we only used what we call 'field roots', i.e. the roots growing outside of the nursery peat ball for root growth measurements. For the aboveground biomass measurement we used the whole shoot of the plants. The above- and below-ground portions of the seedlings were separated and dried for 48 h at 70 °C before weighing dry 'field root' and shoot mass.

A second round of sampling was performed in November 2018, but without excavating the entire root. Seedlings were dug up from one side using a shovel, and fine roots and rhizosphere soil were sampled destructively. Shoot length and diameter were measured on site, before sampling fine roots and rhizosphere soil from five seedlings of all treatments in blocks 5–8, respectively. Additionally, bulk soil samples from all blocks and treatments were taken (block soils, Table 1), as well as soil samples from the edges of the clearcut, both within the clearcut (clearcut edge samples) and outside in the surrounding forest (forest edge samples; Table 1, Fig 1). For all bulk soil samples, three cores were taken with about 15 cm distance, litter was removed from the top and the top 10 cm of the underlying soil was sampled.

All root and soil samples were freeze dried and subsequently stored at −70 °C before further processing.

2.3. Soil measurements

For measurement of carbon and nitrogen contents of soil samples, freeze dried sample material was sieved and subsequently oven dried at 70 °C for 18 h to determine dry mass, then converted to CO_2 and N_2 by combustion, and quantities measured using the Elemental Analyzer (Flash EA 2000, Thermo Fisher Scientific, Bremen, Germany) and isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific, Bremen, Germany), using the method outlined in (Werner et al., 1999). Results were corrected for drift and sample size effect (non-linearity).

Phospholipid fatty acid (PLFA) concentrations in soil samples were measured using Gas Chromatography-Mass Spectrometry (GC–MS). The following machines were used specifically: TriPlus RSH (Autosampler), Trace CG Ultra (Gas Chromatograph), ISQ LT (Single Quadropole Mass Spectrometer) (All from Thermo Fisher Scientific, Bremen, Germany). Samples were extracted with an adjusted method based on (Bligh and Dyer, 1959) and (White et al., 1979). Samples were injected using splitless injection and separated on a 30 m x 25 mm x 0.25 μm DB-5 column (Agilent Technologies, Santa Clara, CA, USA) and measured on a single quadropole mass spectrometer. Concentrations were calculated against the internal standard (19:0, 74208 Sigma-Aldrich) and based on TIC signal (mz 35–450). We used the PLFA 18:2ω6,9 as an indicator for fungal biomass (Frostegård and Bååth, 1996; Näsholm et al., 2013).

Table 1
Overview of soil sample types included in this study, with sample names used throughout the text (first column) and specification of exact sampling locations and subgroups within each sample type.

Soil sample name	Location	Subgroups
Disturbed clearcut soil	Sampling blocks, between seedlings	Tree species, planting positions, treatments
Block soil	Sampling blocks, next to seedling	Tree species, planting positions, treatments
Rhizosphere soil	Sampling blocks, around seedling roots	Tree species, planting positions, treatments
Nursery peat	Nursery seedling peat plugs (pre-planting)	Tree species
Clearcut edge soil	Clearcut edge, inside clearcut	Location (NESW)
Forest edge soil	Clearcut edge, outside clearcut	Location (NESW)

2.4. DNA extraction, library preparation and sequencing

DNA extractions and library preparations from freeze dried soil samples were performed according to (Castro et al., 2021). In brief, DNA was extracted from soil samples using the DNeasy PowerLyzer Powersoil Kit (Qiagen, Hilden, Germany), and from plant root samples using a modified version of a Cetyl TrimethylAmmonium Bromide (CTAB) based extraction protocol from (Hanania et al., 2004). Primers gITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990) were used to amplify the fungal ITS2 region from the extracted DNA in a nested PCR setup based on (Beckers et al., 2016). Cleaned, size selected and multiplexed ITS libraries were sequenced on MiSeq (MSC 2.5.0.5/RTA 1.18.54) with a 2×301 setup using 'Version3′ chemistry at the Science for Life Laboratory in Stockholm, Sweden.

2.5. Sequence processing

2.5.1. Preprocessing, denoising and clustering

The raw ITS reads were cut using the PCR primer sequences and cutadapt (v 3.4) (Martin, 2011). All following steps were completed using dada2 (v 1.16) ((Callahan et al., 2016), processing each sequencing library separately. The cut reads were filtered and trimmed with the parameters 'maxN = 0, maxEE = c(6,6), truncQ = 2, minLen = 50, rm.phix = TRUE'. This filtered set of reads was dereplicated and used to learn errors. Denoising was performed, and forward and reverse reads were merged with a maximum allowed mismatch of 2 bases. Chimeras were removed using the 'consensus' method.

The ASV count tables from all sequencing libraries were merged, resulting in one large matrix containing all fungal ASVs from all samples. The R package decontam (v 1.12) was used to infer likely contaminants using the negative control samples that were added before DNA extraction, and before the PCR steps (Davis et al., 2018). We used a probability threshold of 0.05 to identify statistically significant contaminants. After removing contaminants, we merged within-block replicates by adding the counts per ASV from samples within one block. Recent studies have shown that intraspecific variation can be a factor for ITS sequences, hence further clustering beyond the ASV stage has been recommended (Estensmo et al., 2021). For this, swarm (v 3.0) was used with the parameter d set to −3, to perform single linkage clustering on the ASV sequences (Mahé et al., 2015), resulting in swarm clusters, hereafter referred to as operational taxonomic units (OTUs). Subsequently, ITSx (v 1.1.3) was used to remove non-fungal OTUs from the dataset, with the parameters '–preserve T –partial 50 –minlen 50 –detailed_results T' (Bengtsson-Palme et al., 2013). To eliminate low abundant noise, OTUs with less than 0.005 % abundance or less than three reads in at least two replicates of one "treatment group" (species, sample type, treatment, planting position) were removed before further analysis. To assign taxonomy to OTUs we ran the constax tool (v 2.0.15), using vsearch, RDP classifier and blast, with a confidence threshold of 0.8 (Liber et al., 2021). Fungal guilds were determined using FUNGuild (Nguyen et al., 2016), and completed or modified with additional references, wherever possible or deemed necessary by the authors (Table S2). Additionally, based on these annotations we classified all fungi into “Not ECM” – most likely or definitely not ectomycorrhizal, “Putative ECM” – Members of this taxon can be ECM, “ECM” – all members of this taxon are ECM fungi, “Unknown” – trophic mode and/or ecological guild cannot be determined due to lack of literature or lacking taxonomic annotation.

2.5.2. Data analysis and statistics

All statistical analyses were run in R (v 4.1.2), unless specified otherwise (R Core Team, 2019). Plots were created using ggplot2 (v 3.3.5) (Wickham, 2016), unless specified otherwise. Statistical differences in survival were tested using a Cox proportional hazards regression model with the ‘coxph’ function from the survival package (v 3.2) (Therneau and Lumley, 2013) to assess the hazard ratio of the control seedlings planted in mineral and capped mound soils, and the effect of arginine phosphate and ammonium nitrate treated seedlings compared to the control. Error bars for 95 % confidence intervals were calculated with survival curves, using the ‘survfit’ function from the same package. The ‘kruskal’ function from the agricolae package (v 1.3.5) (de Mendiburu, 2019) was used to test the statistical differences between plant species (growth measurement ∼ Plant Species), the effect of the planting position on seedling growth (growth measurements ∼ Planting position) and the effect of arginine phosphate and ammonium nitrate treated seedlings compared to the control (growth measurements ∼ Fertilization type). The function provides Fisher’s least significant difference (LSD) as Post-hoc analysis and statistical grouping based on false discovery rate (fdr) correction when significant differences were detected in the Kruskal–Wallis test (alpha = 0.05). Statistical differences between fungal biomass measurements (PLFA 18:2ω6,9 in nmol g^{-1} dry weight of soil) were also calculated using the kruskal function.

To test for significant differences in abundance of the OTUs, we used the ‘DESeq’ function of DESeq2 package (Love et al., 2014) 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 considered differentially abundant (DA) and used for visualization. Beta diversity was tested using the ‘ordinate’ function from the phyloseq package (v 1.38) (McMurdie and Holmes, 2013), using Bray–Curtis to build the distance matrix with the rarefied samples and principal coordinate analysis (PCoA) for visualization. Group centroids were calculated using the ‘gg_ordiplot’ function from ggordiplots package (v 0.4.1) (Quensen, 2020). Changes in the community composition were tested using PerMANOVA with adonis2 function from vegan package (v 2.5) (Oksanen et al., 2020), further statistical differences were tested with pairwise.adonis2 function from the pairwiseAdonis package (Martinez Arbizu, 2020). 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. Shannon diversity index values were calculated using the diversity function from the vegan package.

3. Results

Additional results and discussion can be found in the supplementary materials (Appendix C).

3.1. The effect of planting position on survival, growth and fungal communities of seedlings

3.1.1. Growth and survival of control seedlings

After one growing season, we assessed survival of Norway spruce and Scots pine seedlings planted into bare mineral soil or capped mounds. From 889 Norway spruce and 958 Scots pine seedlings planted in the control treatment, 841 and 678 seedlings, respectively, survived, with Scots pine having significantly higher mortality than Norway spruce in both planting positions ($p < 0.001$, Fig 2A). Moreover, both Norway spruce and Scots pine seedlings planted in capped mound soil (survival rates 90.1 % and 65 %, respectively) had a significantly ($p < 0.001$) higher mortality than when planted in exposed mineral soil (survival rates 98.7 % and 76.4 %, respectively).

To determine control seedling root and shoot growth after the first growing season, we weighed the ‘field roots’ growing outside of the nursery peat ball, as well as the total aboveground biomass. Norway spruce seedlings had significantly increased field root and shoot biomass (Fig 2B), field root:shoot ratio (Fig 2C), height (Fig 2D) and diameter (Fig S1A) when growing in bare mineral soil compared to the capped mounds. Scots pine showed no significant differences in these growth parameters between planting positions, but had a tendency for increased biomass when growing in capped mounds (Fig 2B,D).

After the second growing season, we again measured the height and diameter of the seedlings before taking root and soil samples for DNA extraction. We did not see any significant differences in absolute height or diameter between planting positions for either species in the second year (Fig S1), indicating that Norway spruce seedlings planted on capped mounds had caught up in growth compared to the first year. There was a non-significant tendency for more aboveground biomass in the exposed mineral soil in both species after the second growing season (Fig S1E, G). While there were no significant differences in overall height or diameter between planting positions at the end of the second growing season for Scots pine, the relative diameter increments from first to second year were significantly greater in the mineral soil compared to capped mounds (Fig S1F).

3.1.2. Fungal communities in site soils and on roots of nursery and control seedlings

Overall, we found the site soils to be relatively poor in ECM fungi (average relative abundance in surrounding forest soil: 1.3 %; clearcut soil: 4.6 %; average relative abundance on seedling roots 2017: 53.2 % and 2018: 53.1 %). The relative abundance of all types of mycorrhizal and putative mycorrhizal fungi was higher in the surrounding forest (49.6 % in surrounding forest vs 35.1 % in clearcut soil samples), with the majority being putative ericoid mycorrhizal fungi. Most ECM found in the clearcut and the forest edge soils were species adapted to disturbed and nutrient rich soils, such as the genera *Tylospora, Tomentella* and *Amphinema* from the *Atheliales* order, or the very abundant *Thelephora terrestris*. The site overall was dominated by saprotrophic and putative ericoid mycorrhizal genera including *Oidiodendron, Trichoderma, Meliniomyces* and *Penicillium*.

Before outplanting, we found largely the same OTUs occurring on Norway spruce and Scots pine seedling roots (Fig S2), which were both dominated by the Basidiomycete ECM *Thelephora terrestris* and various root associated Ascomycetes (*Oidiodendron*, Helotiales) to different degrees. Subsequently, we compared root samples at the end of the first growing season to the nursery root samples. Both Norway spruce and Scots pine showed a significant change in community composition (Fig 3A/B; $p < 0.05$), fungal richness (Fig S3A+*B;* $p < 0.05$), and Shannon diversity index (Fig S3C+D; $p < 0.05$), suggesting an active recruitment of fungi to field roots in the clearcut in both planting positions. Within this newly restructured community, the majority of fungi present in the nursery were also present on field roots after one growing season. Looking at differentially abundant (DA) fungi between the nursery seedlings and the field roots, the OTU annotated as Helotiales (OTU 4), which was the most abundant OTU on Norway spruce seedling roots before planting (Fig S2), displayed significantly lower abundance (Fig 3C). On the other hand, the majority of DA fungi in Norway spruce roots were newly recruited ones, not detected in nursery samples. Among these were several ECM fungi (*Tylospora, Amphinema, Piloderma,*

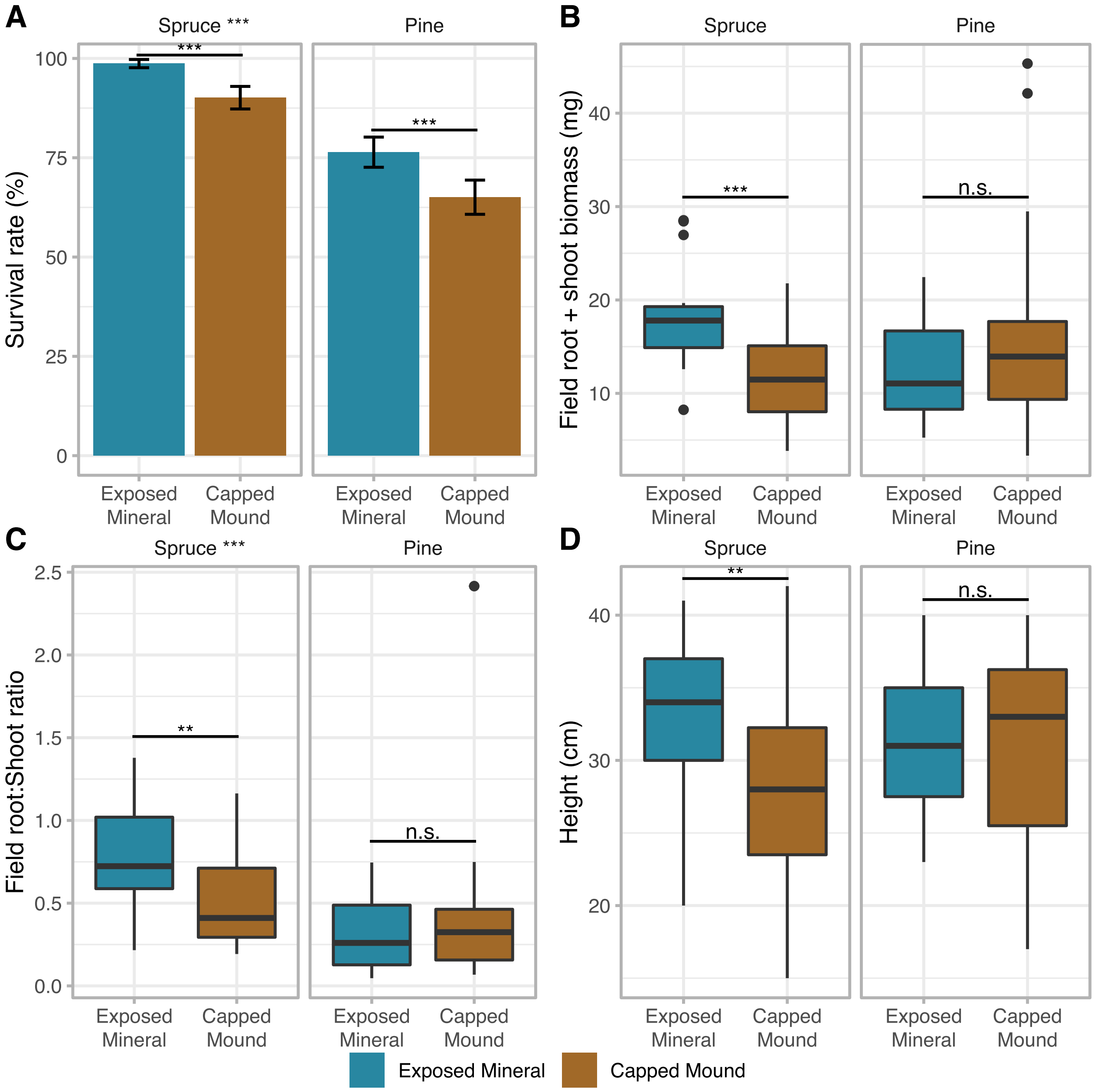

Fig. 2. Survival and growth statistics of Norway spruce and Scots pine control seedlings growing in exposed mineral or capped mound soils after one growing season. Asterisks represent *p*-value ranges: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. n.s.: $p \geq 0.05$. **A.** Barplot of survival rates. Stars represent statistical differences of Cox proportional hazard regression. Error bars represent 95 % confidence intervals calculated after survival curve. **B.** Boxplots of combined biomass of shoot and field roots. **C.** Boxplots of field root:shoot ratio. **D.** Boxplots of seedling height. For (**BCD**) asterisks in the upper part of the boxplot represent statistical differences of Kruskal-Wallis Rank Sum test, and whiskers represent 1.5 × inter-quartile range (IQR).

Clavulina) alongside a large number of opportunists and saprotrophs (*Helotiales*, Aspergillaceae, *Oidiodendron*). In Scots pine, the majority of the most abundant OTUs from the nursery were still present after one growing season, with only six OTUs (unclassified members of Helotiales and Mollisiaceae) having significantly lower abundance (Fig 3D). The unclassified Helotiales (OTU 4) that was dominant on both Norway spruce and Scots pine roots in the nursery also had significantly lower abundance in Scots pine field roots. Despite the overall higher number of detected taxa on Norway spruce roots, there were more taxa on Scots pine roots that significantly increased in abundance after the first growing season, indicating a more consistent colonization by fewer species for Scots pine roots. Among these DA novel colonizers were, similar to Norway spruce, a number of ECM fungi (*Tylospora, Amphinema, Hydnotrya, Inocybe, Piloderma*) and a larger number of saprotrophs, opportunists, and potential ericoid mycorrhizal fungi such as *Oidiodendron, Penicillium*, and *Lophodermium*. Comparing the two growing seasons, we observed a higher differentiation in Norway spruce root samples in both planting positions between the first and second growing season (Fig 3A) than in Scots pine (Fig 3B).

Comparing the communities between planting positions, we observed significantly higher root-associated fungal richness and Shannon diversity index in Norway spruce growing in capped mounds compared to the bare mineral soil ($p < 0.05$), suggesting a richer and more diverse community in Norway spruce roots in the capped mound soils. Similarly, significant differences were found in the community composition in Norway spruce growing in the mineral soil compared to

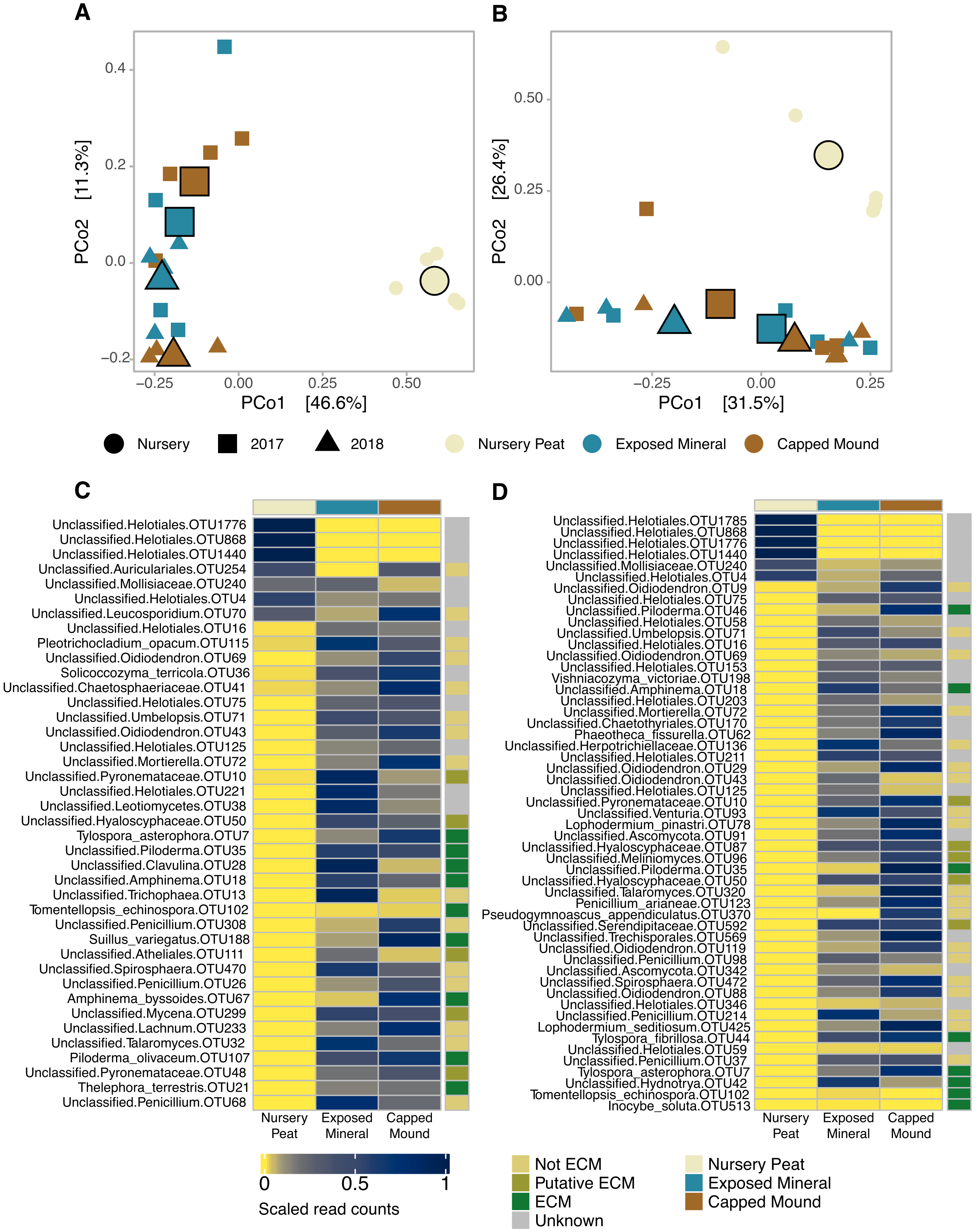

Fig. 3. Principal coordinate analysis (PCoA) showing community composition on nursery and control root samples of (**A**) Norway spruce and (**B**) Scots pine after one and two growing seasons (indicated by shape) of growth in exposed mineral or capped mound soil (indicated by color). Enlarged, black-bordered shapes correspond to group centroid. Heatmaps of differentially abundant OTUs of control root samples of Norway spruce (**C**) and Scots pine (**D**) contrasting both planting positions against nursery roots. OTUs with a Log2fold change > 0.5 and adjusted *p*-value < 0.01 were used for visualization. Values represented by the color scale correspond to normalized OTUs read counts and are scaled by quantile for visualization purposes. Right column based on the fungal guild annotated with FUNGuild v.1.1. Heatmap rows are ordered by log2FoldChange. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

the capped mounds ($p < 0.05$), further suggesting that Norway spruce recruited different fungi depending on the planting position. Four DA OTUs were identified in Norway spruce roots between planting positions, with all four being higher abundant in the capped mounds (Fig S4). From these, *Tausonia pullulans* (OTU 405) was the most dominant. Other significant OTUs were assigned as unclassified Auriculariales and Trechisporales (OTUs 254 and 569), and *Pseudogymnoascus appendiculatus* (OTU 370). In contrast, Scots pine had no statistical differences in richness ($p > 0.05$), alpha ($p > 0.05$) or beta ($p > 0.05$) diversities between planting positions, suggesting that Scots pine recruited similar fungi in terms of quantity (richness) and composition (alpha and beta diversity) regardless of planting position. Scots pine had two DA OTUs between planting positions, with one being in common with Norway spruce (*Pseudogymnoascus appendiculatus,* OTU 370).

From the most abundant nursery OTUs (Fig S2) some were very successful at continued colonization of newly grown field roots, and even after the second growing season the roots were still dominated by several of the fungi that were prevailing, or at least present, on the seedlings leaving the nursery (Fig 4), such as *Thelephora terrestris, Oidiodendron* and *Penicillium*. The majority of highly abundant new colonizers were ECM fungi such as *Piloderma, Amphinema, Suillus*, or *Clavulina. Thelephora terrestris* was the most abundant species on both tree species, and also colonized new Norway spruce roots to the highest degree, despite not being as highly abundant on Norway spruce nursery seedlings. Several phylotypes of *T. terrestris* were present in sampled soils across the site, including the one also highly abundant in the nursery samples.

3.2. *Fertilization effect on growth, survival and fungal communities*

3.2.1. *Growth and survival*

After one growing season, ammonium nitrate significantly decreased survival of both Norway spruce (−6.9 % and −6.4 % for bare mineral soil and capped mound soil, respectively) and Scots pine (−8.4 % and −11.2 % for bare mineral and capped mound soil, respectively) growing at both planting positions ($p < 0.05$; Fig 5). Significantly lower survival was also observed for arginine phosphate addition, but only for Norway spruce seedlings planted in bare mineral soil (−2.7 %; $p < 0.05$). In terms of growth, Norway spruce showed significant differences in N treatment, planting position and their interaction, with the combination mineral soil:arginine phosphate having the most positive impact on growth ($p < 0.05$; Fig 5), despite the slight negative effect on survival. Ammonium nitrate induced increased shoot growth in Norway spruce (Fig S5C), which without corresponding root growth resulted in a lower field root:shoot ratio (Fig 5C). Scots pine, on the other hand, showed no significant growth effects of N treatment or planting positions alone ($p > 0.05$) but a weak interaction effect, with seedlings growing in bare mineral soil showing a positive effect of arginine phosphate on diameter, root and shoot biomass, root:shoot ratio and needle length values ($p < 0.05$; Fig 5, Fig S5), and an improved root:shoot ratio of arginine phosphate fertilized seedlings growing in capped mounds. In Scots pine, ammonium nitrate did not have any significant growth effects compared to the control (Fig 5, Fig S5).

After the second growing season we observed significantly greater diameter in both Norway spruce and Scots pine seedlings fertilized with ammonium nitrate and growing in capped mounds ($p < 0.05$), compared to control seedlings growing in capped mounds (Fig S5). We detected no differences in absolute height between any of the treatment factors (Fig S5). However, Scots pine seedlings growing in capped mounds additionally had significantly greater height and diameter increases between the two growing seasons when fertilized with ammonium nitrate.

3.2.2. *Fungal communities*

The increase in fungal richness and diversity observed from the nursery after one growing season for control plants was not affected by either arginine phosphate or ammonium nitrate in Norway spruce ($p > 0.05$) or Scots pine ($p > 0.05$) roots, despite the positive impact of the former on root biomass and root:shoot ratio in both plant species. After the second growing season, the overall community composition differences between the fertilization treatments were reduced even further

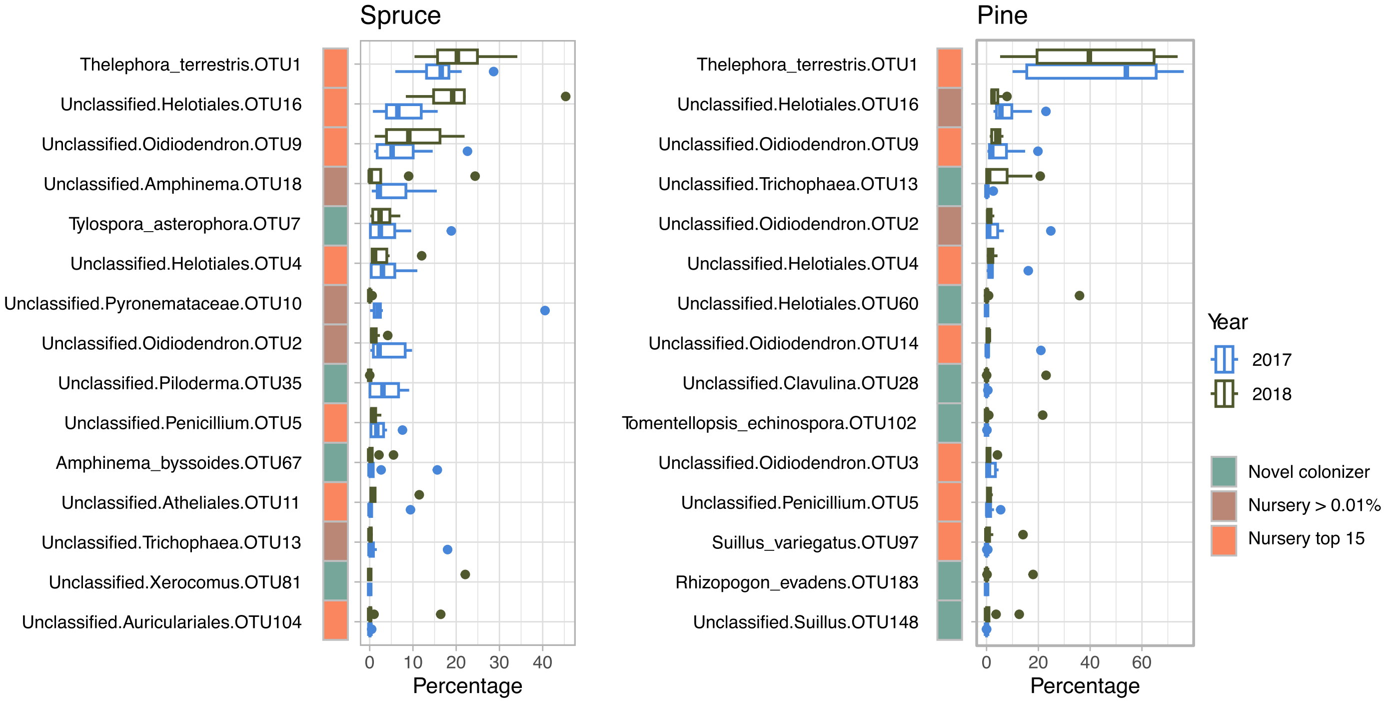

Fig. 4. The 15 most abundant operational taxonomic units (OTUs) in Norway spruce (left panel) and Scots pine (right panel) seedling root samples after planting. Boxplots show relative abundances split by year. Colors indicate presence on nursery root samples: Novel colonizers are colored green, OTUs present in nursery root samples (>0.01 % average rel. ab.) are colored brown, and OTUs that were among the 15 most abundant in nursery root samples are colored orange. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

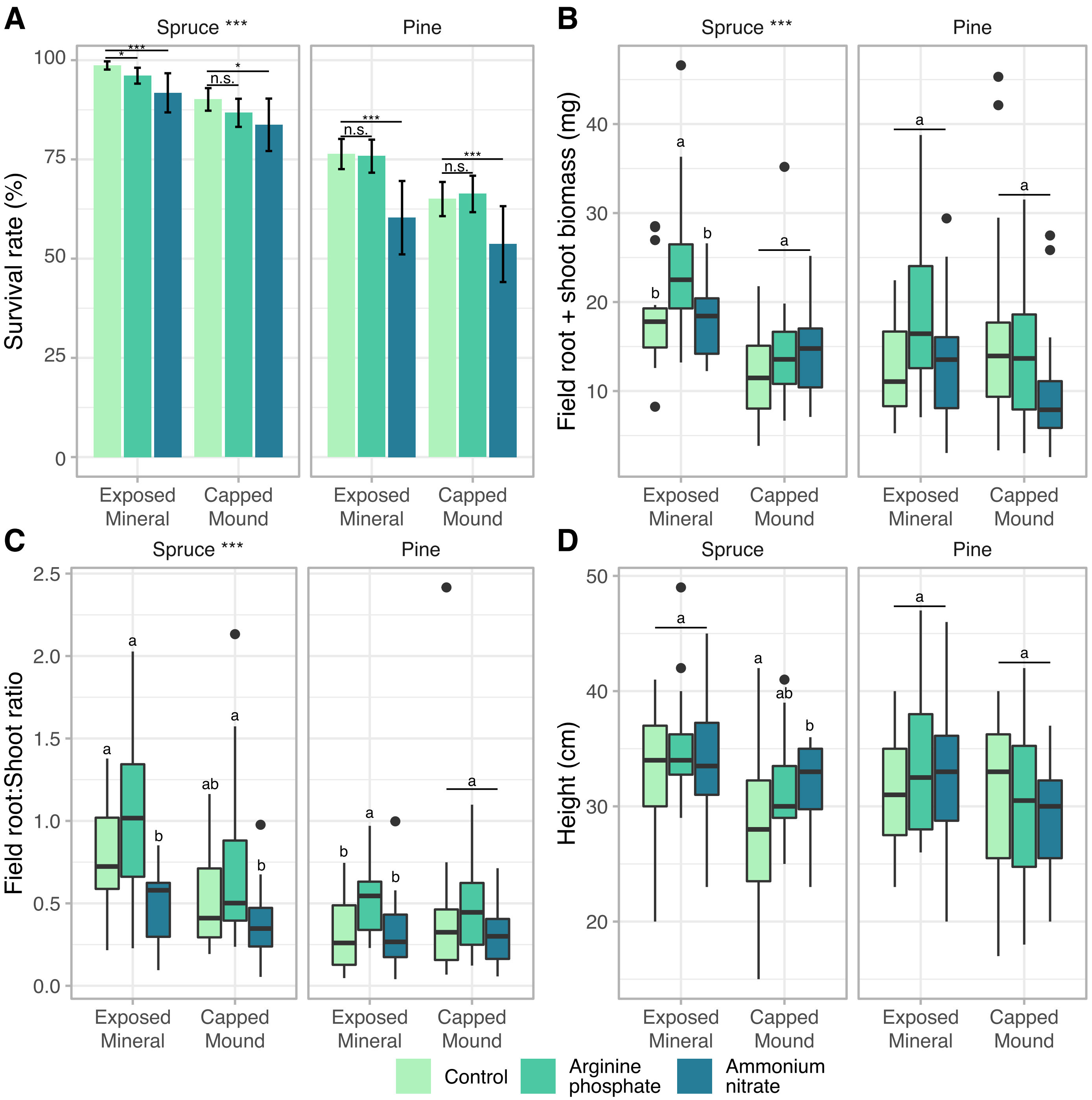

Fig. 5. Survival and growth statistics of Norway spruce and Scots pine control seedlings colored by treatment, growing in exposed mineral or capped mound soils after one growing season. Number of stars (**A**) indicate *p*-value: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. n.s.: $p \geq 0.05$. Letters above boxplots (**BCD**) represent statistical differences resulting from Kruskal-Wallis Rank Sum test, and whiskers represent 1.5 × inter-quartile range (IQR). **A.** Barplot of survival rates. Stars represent statistical differences of Cox proportional hazard regression. Error bars represent 95 % confidence intervals calculated after survival curve. **B.** Boxplots of combined biomass of shoot and field roots. **C.** Boxplots of field root:shoot ratio. **D.** Boxplots of seedling height. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

(Fig 6). Nevertheless, a number of OTUs found on seedling roots were DA between fertilization treatments (Fig S6). Only few fungal OTUs (annotated as *Inocybe soluta, Tylospora fibrillosa*, Leotiomycetes, *Sebacina* sp., *Mortierella* sp.) were negatively affected by the fertilization treatments, but the majority of DA OTUs were positively affected by fertilization and showed higher abundance on roots of fertilized seedlings (Fig S6). Some examples of DA ECM fungi were annotated as *Thelephora* sp., *Amphinema, Suillus variegatus, Russula* sp. and *Cortinarius gentilis* (Fig S6), but also many saprotrophic species (e.g. *Mycena* sp., *Luellia* sp., *Penicillium* spp., *Capronia* sp.) profited from the small fertilizer addition. We did not see significant differences in fungal biomass (PLFA marker 18:2ω6,9) in the seedling rhizosphere between the fertilization treatments in the two years. Only Scots pine seedlings growing in bare mineral soil showed a significant decrease in fungal biomass for the arginine phosphate treatment (Fig S7), but only after the first growing season.

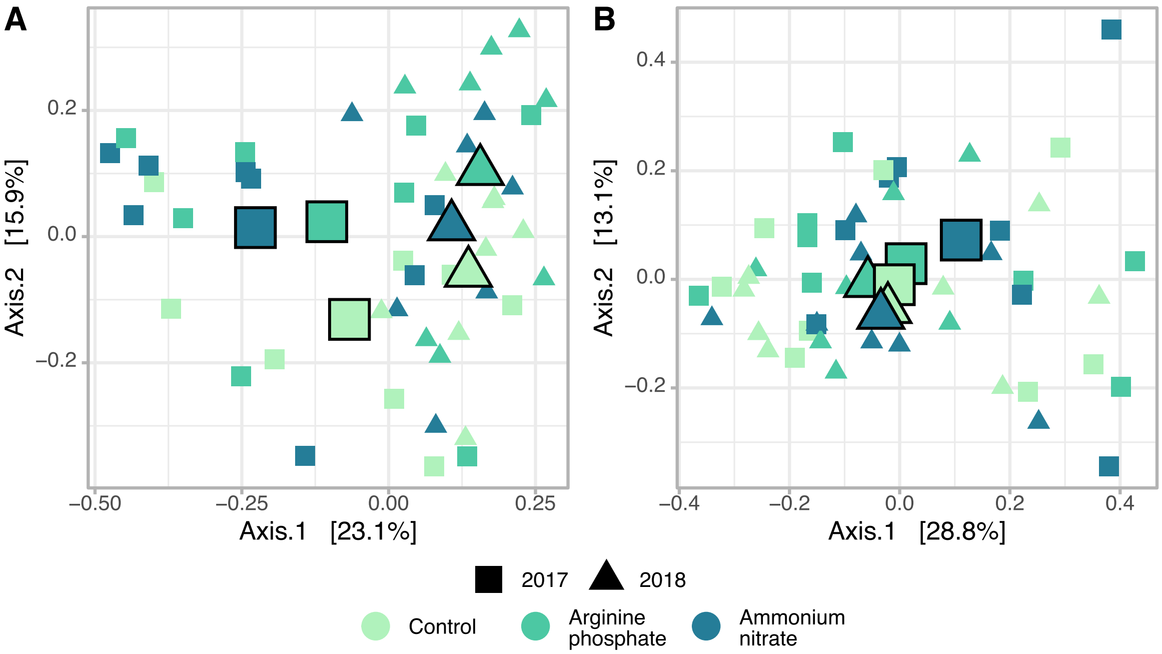

Fig. 6. Principal coordinate analyses (PCoA) of fungal communities in Norway spruce (**A**) and Scots pine (**B**) root samples, with colors indicating treatment (control, arginine phosphate, ammonium nitrate) and shapes indicating years (2017: first growing season; 2018: second growing season). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

4. Discussion

4.1. Seedling survival and growth after outplanting

Seedling survival after planting is influenced by many factors, for instance environmental stresses, herbivores or diseases after planting, as well as seedling quality before outplanting (Grossnickle, 2012). At our site, the first month after outplanting was relatively dry (precipitation 45 % lower than usual), which can account for the increased survival of both Norway spruce and Scots pine seedlings planted in the bare mineral soil compared to the capped mound (Fig 2A), as water availability is usually higher in the bare mineral soil planting position (Häggström et al., 2021; Örlander, 1986). Scots pine had overall significantly lower survival than Norway spruce after the first growing season in both planting positions, which was not expected given the site characteristics, and Scots pine reportedly being more drought tolerant (Krakau et al., 2013). While Norway spruce typically prefers more nutrient rich and moist sites than Scots pine, recent studies suggest that Norway spruce might have a slightly higher physiological drought tolerance than Scots pine (Ivanov et al., 2019; Kunert, 2020). At the seedling stage this higher physiological tolerance of Norway spruce might be more relevant and important in determining seedling establishment and survival while the drought tolerance that Scots pine acquires through deeper roots or lower surface transpiration may become more relevant in mature trees. Additionally, notes from the nursery indicate that Scots pine seedlings were in part affected by grey mould (*Botrytis* sp.), which may have contributed to lowered survival after outplanting. The addition of 40 mg nitrogen as ammonium nitrate strongly negatively affected survival of both species in both planting positions, but more so for Scots pine. Also arginine phosphate had a small, but significant, negative effect on survival but this effect occurred only for Norway spruce seedlings growing in bare mineral soil (Fig 5A). A recent study identified positive survival effects of arginine phosphate on Scots pine in southern Sweden (Häggström et al., 2023). Other studies of different plants have shown that organic N leads to more root growth and more branched fine root structure in seedlings than inorganic N (Franklin et al., 2017; Gruffman et al., 2012), which could be crucial for seedling survival in dry conditions. However, this does not completely explain the patterns in our data, where seedlings fertilized with ammonium nitrate had significantly lower survival than unfertilized control seedlings, without any noticeable changes in field root biomass (Fig S5). Only Norway spruce reacted to ammonium nitrate with increased aboveground growth and decreased field root:shoot ratio, but the decrease in Scots pine survival after ammonium nitrate fertilization was not correlated with observed changes in any growth parameters. However, our results only consider total field root mass, and we cannot use this data to determine changes to root morphology and structure that may have been caused by either treatment. From another viewpoint, Lim et al. (2022) showed that fertilization with inorganic and organic N lead to different patterns in N availability. Inorganic N leads to a short-term spike of high N availability, while organic fertilizer makes the same of amount of N available at lower rates over a longer time span. Since the seedlings were grown exclusively on organic N fertilizer in the nursery, the sudden strong increase in available inorganic N for seedlings fertilized with ammonium nitrate may have factored in as an additional stressor, especially combined with the drought stress shortly after planting. Unfertilized control seedlings would be exposed to the clearcut soil as a main nitrogen source, in which the available N pool is likely to have a high proportion of organic N (Inselsbacher and Näsholm, 2012) similar to the nursery environment, thus not contributing to additional stress and seedling mortality to the same extent after outplanting.

Norway spruce grew significantly better in the bare mineral planting position during the first growing season, but the capped mound seedlings caught up in growth after the second year (Fig S1). While the second year was even drier then the first, a combination of root growth from the first year (Fig S1, Fig S5) and compaction of capped mound soil after the first winter in the field (Häggström et al., 2021) might have increased water availability, which in combination with the slightly higher nutrient content in the capped mounds would lead to increased growth in the long term. While Scots pine did not have any significant differences in absolute trait values after either growing season, there was a significant diameter increase from the first to second year for seedlings growing in capped mounds. Stem diameter is considered to be one of the key measures for seedling vitality, giving an indication for root system size and survival chances (Hines and Long, 1986; Mexal and Landis, 1990; South et al., 2005), potentially indicating that Scots pine seedlings started to establish better in the second year after the considerably worse performance in the first growing season. Except for Scots pine seedlings growing in capped mounds, arginine phosphate addition significantly increased field root:shoot ratios after the first growing season (Fig 5B), concordant with previous studies (Franklin et al., 2017; Gruffman et al., 2012). Moreover, we show that arginine phosphate improved growth, especially in bare mineral soil for Scots pine. Häggström et al. (2021) showed that this positive effect on root growth is more noticeable in sites with longer growing seasons, with all the sites in that study being in Swedish regions with shorter growing seasons than this site.

4.2. *Fungal community succession on seedling roots*

Before outplanting, we found largely the same OTUs occurring on Norway spruce and Scots pine (Fig S2), which were both dominated by the Basidiomycete ECM *Thelephora terrestris* and various root associated Ascomycetes (*Oidiodendron*, Helotiales). Swedish and Finnish nursery seedlings have previously been shown to be colonized by a mix of nitrogen and stress tolerant ECM fungi including *Thelephora terrestris, Amphinema byssoides*, and opportunists such as *Penicillium* spp. (Flykt et al., 2008; Kļaviņa et al., 2015; Menkis et al., 2005; Stenström et al., 2014), which coincides well with the fungal taxa we identified on nursery seedling roots and the surrounding peat ball (Fig S2).

After outplanting we found relatively few DA OTUs that decreased in abundance, indicating continued root colonization by the same fungi. Even after two growing seasons in the field, the seedling fungal communities remained largely dominated by the fungi that were present on nursery seedlings. However, we also observed a rapid recruitment of new OTUs on seedling roots after the first growing season. While many of the novel colonizers were OTUs from either the same or very closely related taxa as the nursery fungi, we also detected successful novel colonization by several new ECM taxa, including *Clavulina, Tomentellopsis, Xerocomus* and *Piloderma* that were not present on nursery seedling roots. One of the prominent community members on our nursery seedlings, *Thelephora terrestris*, has also previously been shown to persist on inoculated Scots pine seedlings one and two years after outplanting (Hilszczańska and Sierota, 2013). A Finnish study also found nursery ECM fungi to persist on outplanted seedlings, especially in prepared soil (Pennanen et al., 2005). Concordantly with our data, an early Swedish study (Dahlberg, 1990) found that while the majority of outplanted *Pinus sylvestris* and *P. contorta* seedlings had to some degree been colonized by site indigenous ECM after one growing season, the majority of newly formed ECM root tips were still colonized by fungi that came from the nursery, with a very similar result reported in an additional study (Dahlberg and Stenström, 1991). A study from Canada also found that new ECM roots on establishing seedlings were, to a large part, colonized by the fungi coming from the nursery (Jones et al., 2002). This phenomenon has more generally been described as “priority effects”, meaning that already established ECM fungi have an advantage in colonizing new roots over new colonizers (Kennedy et al., 2009). While the pre-colonization in the nursery is very likely to be beneficial for the seedlings (Lehto, 1992; Pennanen et al., 2005), studies using sterile outplanted seedlings as controls would help to clearly distinguish further colonization of nursery fungi from novel colonization. An interesting additional experimental setup would be to study colonization of germinating seeds in more detail using an approach as recently employed to test direct seeding of Scots pine with and without fertilization using organic or inorganic N (Castro et al., 2021).

The relative differences in community composition between Norway spruce and Scots pine decreased after the first growing season in the field, indicating that the differences we found between the nursery seedlings from both species were not likely to be due to a strong tree-species effect. After the first growing season, Scots pine seedlings had a higher number of statistically significant novel colonizers than Norway spruce. Since mostly Scots pine was growing on the site before the previous harvest, it is likely that there were remaining propagules, such as spores, mycelium or sclerotia (Jones et al., 2003), that preferentially colonized Scots pine seedlings. Seedling root exudates have been shown to trigger germination of dormant ECM spores in previous experiments (Ali and Jackson, 1988; Fries, 1984; Fries et al., 1985), however, to our knowledge, there have not been any studies showing differential colonization of Norway spruce and Scots pine when growing at the same site. In both Norway spruce and Scots pine seedlings, the effects of the fertilization treatments on fungal community diversity and composition were modest, and smaller than the effects of planting position, tree species and growing season. Apparently, the previously discussed increases in root growth after arginine phosphate addition were not associated with any larger changes in root community composition. However, there were several OTUs that did significantly respond to the fertilization treatments (Fig S6). Several of the positively affected OTUs were assigned to ECM taxa, but we did not detect any major patterns among the DA OTUs. Previous studies also suggest that larger amounts of N over longer timeframes are needed to induce significant changes in fungal community composition (Hasselquist and Högberg, 2014; Marupakula et al., 2021).

5. Conclusions

The outplanting of forest tree seedlings is a critical stage in Swedish forestry, which is likely to suffer increased pressure from drought and rising temperatures in the future. Maximizing survival and early growth is thus of great importance, and all possible factors should be taken into consideration. The choice of site preparation techniques, planting position and tree species can be adapted to site characteristics to maximize survival and early growth after outplanting, but less is known about the effects of early fertilization on fungal community dynamics. In this study we applied 40 mg nitrogen as arginine phosphate or ammonium nitrate, to test if this would improve survival and early growth and whether it would induce changes in the fungal communities growing on seedling roots and the surrounding soil. Taken together we conclude that planting position was the most important factor for early establishment, and that the use of inorganic N at outplanting was not beneficial due to the negative effect on survival of both Norway spruce and Scots pine seedlings. The use of organic N had no clear beneficial or detrimental effects on survival and aboveground growth, but improved root growth in the bare mineral soil in both species after the first growing season. Fungi already present in the nursery dominated seedling roots even after the second growing season, but we also found high levels of novel colonization by site indigenous ECM taxa on both Norway spruce and Scots pine seedlings, with N treatment having no significant influence on novel colonization. While further experiments on sites with different nutrient status and climatic conditions are needed to further determine the factors that shape the mycobiomes of establishing seedlings after outplanting, this study sheds light on fungal communities in early regeneration stages and how they are influenced by priority effects and succession dynamics.

CRediT authorship contribution statement

Andreas N. Schneider: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation. **David Castro:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis. **Mattias Holmlund:** Writing – review & editing, Project administration, Methodology, Investigation. **Torgny Näsholm:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Vaughan Hurry:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Nathaniel R. Street:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Mattias Holmlund reports a relationship with Arevo AB that includes: employment, stock ownership. Torgny Näsholm reports a relationship with Arevo AB that includes: employment, stock ownership. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw sequencing data has been deposited in the European Nucleotide Archive (ENA) under the accession PRJEB74806 (Schneider, 2024). Scripts and code for pre-processing and analysis of data has been made publicly available on Github (Schneider and Castro, 2023).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tfp.2024.100568.

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