Fungal Effects on Tree Growth in a Primary Succession

The fungi community development in a primary succession and its effect on newly planted *Pinus sylvestris*

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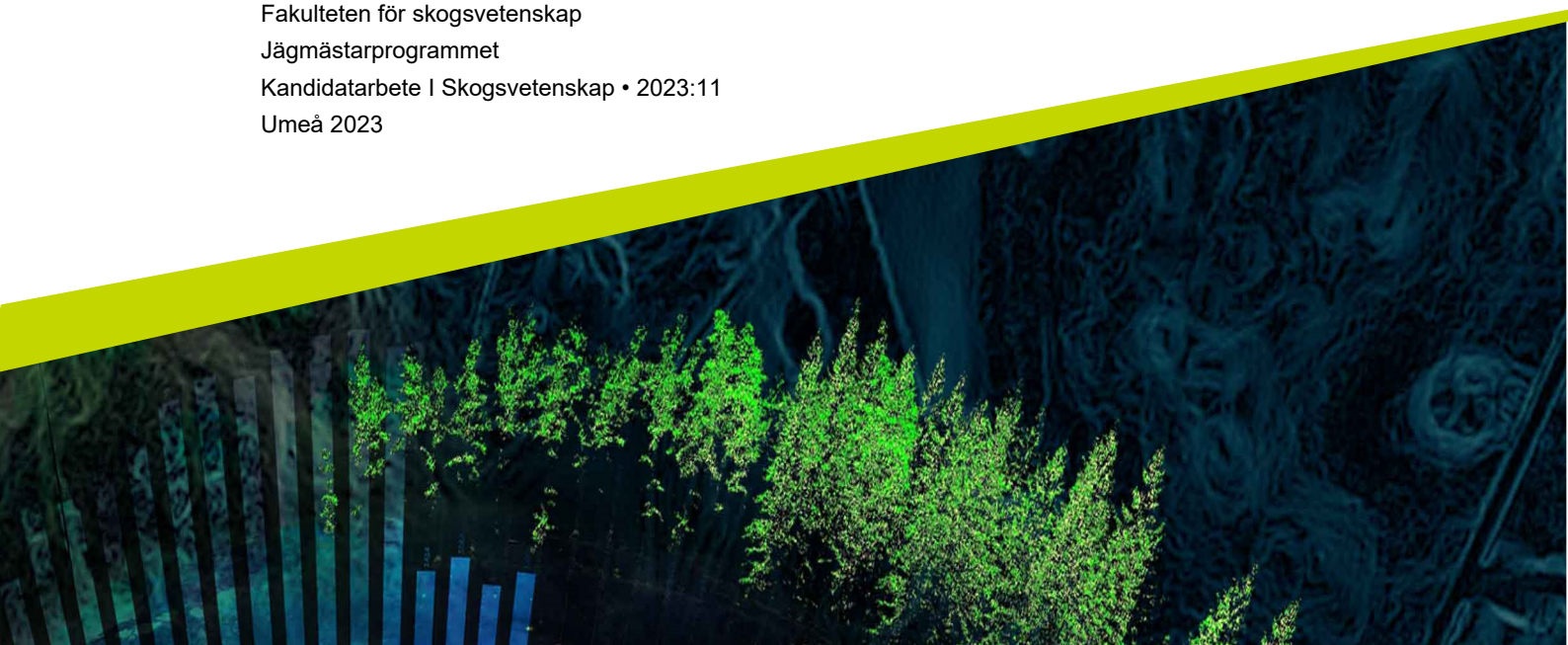
Kandidatarbete • 15 hp
Sveriges Lantbruksuniversitet, SLU
Fakulteten för skogsvetenskap
Jägmästarprogrammet
Kandidatarbete I Skogsvetenskap • 2023:11
Umeå 2023



Fungal Effects on Tree Growth in a Primary Succession. The fungi community development in a primary succession and its effect on newly planted *Pinus sylvestris*

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Credits:	15 credits
Level:	First cycle, G2E
Course title:	Självständigt kandidatarbete i skogsvetenskap
Course code:	EX0911
Programme/education:	Jägmästarprogrammet
Course coordinating dept:	Department of Forest Ecology and Management
Place of publication:	Umeå
Year of publication:	2023
Copyright:	All featured images are used with permission from the copyright owner.
Title of series:	Kandidatarbeten I Skogsvetenskap
Part number:	2023:11

Keywords: primary succession, succession, ectomycorrhiza, saprotroph, tree growth, Pinus sylvestris, soil fungi

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Abstract

In the Boreal zone, ectomycorrhiza is the most common mycorrhiza found among trees and helps with nitrogen and nutrient uptake. Most research on mycorrhiza, its effect, and community development are done on secondary succession (succession in an ecosystem after a disturbance) such as after a clearcut.

This case study focuses on primary succession (succession in a previous lifeless ecosystem with no organic matter) of 15- and 30-year-old Scots pine (*Pinus sylvestris*) in an old gravel pit outside Uppsala, Sweden. The aim of the study is to find a connection between tree growth and its associated soil fungal community, especially with the ectomycorrhizal community. To do this, two hypotheses are proposed:

(i) Difference in tree growth depends on which fungal guild dominates around that tree; trees with lower growth are dominated by free-living saprotrophs, and trees with higher growth are dominated by symbiotic ectomycorrhizal fungi.

(ii) Species diversity of ectomycorrhizal fungi increases with higher tree growth, and there is a gradient from pioneer species to N-immobilizers, to N-miners as tree height increases.

The categorization relies on how the genus obtains nitrogen and when they first colonize a habitat. A few selected genera of ectomycorrhiza were categorized as either pioneers (first colonizers), N-immobilizers (later colonizers), or N-miners (late colonizers).

Overall, the results support the two hypotheses. What was found regarding the first hypothesis was that as tree height increased, the abundance of saprotrophs decreased while ectomycorrhiza or other root associated fungi increased. For the second hypothesis, the number of observed ectomycorrhiza increased with tree height, and the hypothesized gradient was observed.

Keywords: primary succession, succession, ectomycorrhiza, saprotroph, tree growth, Pinus sylvestris, soil fungi.

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Abbreviations

Asc	Ascomycetes
Bas	Basidiomycetes
ECM	Ectomycorrhiza
N	Nitrogen
OM	Organic matter
ROOT	Root associate
SAP	Saprotroph

1. Introduction

1.1 Succession

Ecological succession describes how species composition changes over time. After a disturbance such as a forest wildfire, storm, or clearcutting a major shift in species and the local environment follows. In secondary succession, some organisms survive the disturbance and OM (organic matter) is still present. The succession proceeds with pioneer species that are physically able to inhabit the area, characterizing the first step. The pattern follows with intermediate species moving in, and over time a climax community (steady equilibrium state) emerges (Britannica 2019). The progression of secondary succession is determined by the intensity and magnitude of the disturbance and the surrounding landscape that provides the inhibiting species to immigrate.

A primary succession on the other hand occurs during the colonization of a previously lifeless habitat with no OM. For example, it can be caused by a major disturbance such as a volcanic eruption or mining which eradicates all organisms and OM remains. The first inhabitants are communities of microbes who can fixate carbon and nitrogen from the atmosphere (Schmidt et. al 2013).

Earlier there was no effective way of studying and understanding the progression of the first microbial communities colonizing a lifeless habitat in detail. Thanks to high-throughput DNA sequencing technologies it is now possible to study the assembly of microorganisms over time and place (Schmidt et. al 2013; Kwon & Ricke 2011).

Most studies regarding primary succession focus on newly exposed land masses from e.g., glacier pulling back or lake dunes. This case study, on the other hand, will explore succession in a recently planted gravel pit with an adjacent forest to the East. This gives an intriguing opportunity to study how primary succession progresses in this environment. From a barren plot of land to forest production. To see which organisms reside in this new habitat, forming communities and how they interact with each other and the trees. This study focuses mainly on how communities of different fungi guilds affect tree growth and how the progression of different competing guilds play out.

1.2 Guilds

> "A guild is defined as a group of species that exploit the same class of environmental resources in a similar way. This term groups together species without regard to taxonomic position, that overlap significantly in their niche requirements" (Simberloff & Dayan 1991).

Concerning fungi there are many guilds e.g., saprotrophs (SAPs), ectomycorrhizal fungi (ECMs), plant pathogens and yeasts to name a few (Nguyen et al 2016). This paper will mainly focus on SAPs, ECMs, and Root associated (ROOTs) fungi given that they are the most abundant and of influence for the trees in this case study.

SAPs account for the largest group of fungi in the world and bear a central role in carbon cycling thanks to their ability to decompose complex wood compounds such as lignocellulose with extracellular enzymes (Várnai et al, 2014). These fungi are the principal decomposer of the litter layer on the forest floor, with the capability to efficiently reallocate its N and mycelia growth from well degraded litter to newer litter of high quality (Boberg. et al. 2014). This gives SAPs a major influence on the carbon and nitrogen pools in the boreal ecosystem (ibid).

1.3 ECM symbionts

In ECM symbioses, fungi form a mantel around the root tips of their host plants, and a net of hyphae between the root cortical cells (the "Hartig net"). This contrasts with endomycorrhiza, for example the widespread arbuscular mycorrhiza, where the involved fungi make various structures inside of the host root cells, and do not form mantles. ECM and endomycorrhiza can have vastly different structures, yet there are two traits that they share: the association between fungi and plant roots, and the extramatrical mycelium foraging the forest floor for nutrients (Johnson & Gehring 2007). ECMs are a common symbiont to most of the trees growing in the boreal forests. The mutualistic connection occurs between certain families of gymnosperms and angiosperms, most common in the boreal forest is Pinaceae and Betulaceae (ibid).

In an experiment by Högberg & Högberg (2002), the biomass of extramatrical ECM mycelium was calculated to constitute at least 32% of the microbial biomass in the boreal forest floor. This makes ECMs an important part of the carbon and nitrogen pools in forests. ECMs are a crucial symbiont for trees in the process of acquiring organic nitrogen (Abuzinadah et al., 1986 see Lindahl & Clemmensen 2017). Especially in harsher conditions with limited nutrient supply and unfavorable climate, trees invest a high amount of carbon in their symbionts to secure a sufficient amount of nitrogen for survival (c.f. Northup et al., 1995 see Lindahl & Clemmensen 2017). In an experiment, Karlsen-Ayala et al. (2022) showed that ECM symbionts were crucial for pine seedlings to survive and thrive

in the harsh Florida rock lands. Native symbionts were revealed to be more successful than commercial inoculants and the pines grew better with higher amounts of mycorrhizal associations. In another experiment, Sim & Eom (2006) showed similar results on *Pinus densiflora* (Japanese red pine) seedlings. Seedlings inoculated with different ECM fungi resulted in varying growth responses. Seedlings inoculated with five symbionts had significantly higher biomass and grew better compared with the ones with a single symbiont and the control. These two experiments are great examples of the mutualistic relation between fungi and trees, to better understand the role fungi play.

1.4 *Ascomycota* vs *Basidiomycota*

1.4.1 Overall phyla distribution

Evidence from molecular research suggests that some ECMs arose from SAPs, and some evidence suggests that ECM fungi evolved from plant endophytes (Lewis J.D 2016). It is estimated that 80 lineages of ECM fungi have evolved independently, which explains their diversity (Tedersoo & Smith, 2013).

In 2011 there were approximately 100,000 described species of an estimated 800,000 to 5,100,000 of the world's fungi species (Blackwell 2011). Thanks to the history of research in boreal forests, there is a good overall knowledge of the soil microbe, compared to the subtropical and tropical ecosystems (Tedersoo et al., 2014). In a study by Tedersoo et al. (2014) 365 soil samples from all over the world were analyzed and revealed that 55,7% of the species belonged to the phylum basidiomycetes and 31,3% to the division ascomycetes. These two phyla constitute a clear majority of the fungal kingdom and contains fungi from many different guilds (Nguyen et al., 2016; Tedersoo et al. 2014; Tedersoo & Smith, 2013) Different ecologies are often associated with the two phyla in the boreal ecosystem (Boddy & Hiscox 2017; Clemmensen et al., 2014; Boberg et al., 2014)

1.4.2 Separated by competition

The top layer (Litter layer) in soils is dominated by litter degrading SAPs of both ascomycetes and basidiomycetes, regardless of the age of the forest stand (Kyaschenko et al. 2017: Clemmensen et al., 2014). In the uppermost layer of litter, species of ascomycetes generally dominate and in the lower, more degraded litter is basidiomycetes dominant (Clemmensen et al., 2014). This pattern is largely due to the fact that ascomycetes lack the ability to produce the enzymes manganese peroxidase which allows them to access nutrients that are locked up in recalcitrant OM, which makes them a weak decomposer (Lindahl et al., 2021). This type of

vertical separation gradient is widespread in ECM dominated ecosystems. With saprotrophic communities largely confined to the litter layer and root associated communities dominating the deeper layers (Kyaschenko et al. 2017: Clemmensen et al., 2014).

Root-associated fungi from both ascomycetes and basidiomycetes display general traits that characterize them. Most root associated ascomycetes in boreal forests have short-ranging mycelia with sturdy melanized cell walls to withstand harsh conditions such as low pH, and drought (Butler & Day, 1998; Robinson, 2001; Greletet al., 2010; Fernandez & Koide, 2014). Melanin is a sturdy polymer that provides the fungi protection, it has been described as `fungal armor´ (Gómez & Nosanchuk, 2003). These slow growing hyphae of ascomycetes have a long lifespan that persist after death (Koide et al., 2014). Given that ascomycetes are somewhat poor degraders (lack of extracellular peroxidase) with long lived necromass, they contribute to the accumulation of OM by restricting nutrient cycling and thus reducing productivity in the forest they dominate (Lindahl & Clemmensen, 2016). This can explain why ascomycetes usually dominate in less fertile soils and basidiomycetes dominate in more fertile soils in both the litter layer and humus layer (Sterkenburg et al., 2015).

Additionally, ascomycetes lack the ability to form mycelia cords, unlike basidiomycetes. These cords are long-lived and maintain connectivity in the mycelia network while high turn-over hyphae forage the surroundings after suitable substrates (ibid.). Basidiomycetes can produce a range of degrading enzymes (including manganese peroxidase) which make them efficient decomposers who can degrade litter that ascomycete fungi are unable to degrade and therefore leave behind (ibid.). Some basidiomycetes fungi can form large well developed mycelia networks with their mycelia cords. This enables them to spatially reallocate resources in the heterogeneous nitrogen limited boreal forest (Boberg 2014). All this makes root-associated basidiomycetes excellent competitors, who can even compete with SAPs for resources during nutrient limited conditions. Nutrients can become so scarce that fungi depend on resources that the tree invests in these symbionts (Lindahl et al., 2021; Baskaran et al., 2017; Lindahl & Tunlid, 2015).

1.5 Fungal strategies

Constant successions due to forestry

The boreal forests of Sweden are defined by a history of forestry that is still going strong to this day. Clearcutting has a major effect on the belowground microbial communities, which regulates nutrient cycles for OM production and carbon storage (Kyaschenko et al 2017). Fungal communities and the progression of their succession is correlated with the age of the forest, revealed to be most noticeable in

the deepest humus layer (ibid). Considering Sweden's intense forestry, it is possible to imagine how common and widespread these fungal secondary successions are over the forests. As the forest is exposed to disturbances such as thinning and clearcutting, new successions embark in the soil. Primary successions are less common but may help to understand how tree establishment and growth depend on different guilds and ECM fungi, without any effects of remaining organisms from a previous forest phase.

Categorization due to varying strategies

There are several ways of categorizing ECM depending on how they appear in a succession, assist in nutrient uptake and compete within a community. ECMs have different exploration types, foraging strategies, and life history e.g., S-, R-, and C-selected (stress, ruderal and competition) (Boddy & Hiscox 2017).

ECMs can also be categorized according to their role in acquiring nitrogen and their order of appearance in a succession. These categories are pioneers (first colonizers), N-immobilizers (second colonizers) and N-miners (late colonizers) (Ishida et al., 2008). Why these species colonize at different times in a primary succession is less researched, but the germination rate and infectivity of their spores play a key role (ibid). The effectiveness of late colonizers spores could be related to hormones released by older trees which catalyzes the spore germination and infectivity (ibid). Other factors such as the presence of certain bacteria and fungi also plays a role in the colonization of later species (Ali & Jackson,1989; Iwase 1992). These three categories vary in their way of nitrogen uptake. Although it is debated how correct these generalizations are and whether other factors affect it. It is said that pioneer species are more prone to inorganic nitrogen uptake, and there is a gradient towards species that decompose OM and rely on uptake of organic nitrogen for the late colonizers (Velmala et al. 2014; Phillips et al. 2014; Kyaschenko et al 2017; Karst et al. 2021).

1.6 Aim of study

This case study relies on newly established forest stands which are 15 and 30 years old. The stands were old gravel pits which had been planted with Scots Pine (*Pinus sylvestris*). The forest owner noticed that the tree growth varied significantly without any apparent reason. The Department of Forest Mycology and Pathology from SLU based in Uppsala organized a study to see if there was any connection to the fungal community present in the soil.

Compared to other studies done on succession of fungal communities, this study is done on a new forest which was planted on `dead soil´. Other studies are often done on clearcuts where there are already microbial, fungal and vegetative

communities in place and the soil contains OM containing nitrogen and other nutrients. The soil after the gravel pit contains practically no OM and the same regarding lifeforms inhabiting the area.

The aim of this study is to examine if there is a connection between tree growth and the soil fungal community: to see if different ratios of fungal guilds affect tree growth. In theory, if the soil around the tree is dominated by free-living SAPs the growth will be limited, but if the soil is dominated by ECMs growth will be boosted. Although SAPs might lower C/N and release nutrients (needing to break down OM to acquire energy and carbon) which in itself benefits the tree, its effect shouldn't be as strong as the presence and aid of ECMs which efficiently compete for inorganic N and mobilize N from OM.

The study also aims to see if there is a difference in species diversity within the ECM community and if there is a gradient from pioneers through N-immobilizers to N-miners as tree growth increases. The idea is that tree growth is favored by a larger number of ECMs present and as time passes there is a succession of different species.

1.7 Hypotheses

(i) Difference in tree growth depends on which fungal guild dominates around that tree; trees with lower growth are dominated by free-living saprotrophs, and trees with higher growth are dominated by symbiotic ectomycorrhizal fungi.

(ii) Species diversity of ectomycorrhizal fungi increases with higher tree growth, and there is a gradient from pioneer species to N-immobilizers, to N-miners as tree height increases.

2. Materials & method

2.1 Gravel pit data

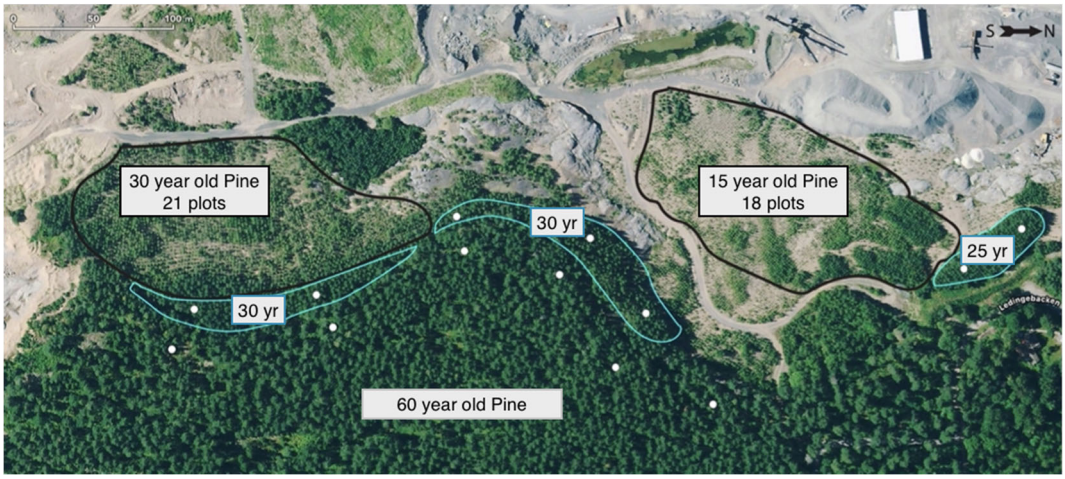

Figure 1. Map overview of the stands where the data was captured.

The basis of this case study relies on data collected at the gravel pit near Rimbo outside Uppsala in 2016 and 2017. As the gravel mining has been terminated in different parts of the pit, pine seedlings have been planted, resulting in areas with differently aged pine trees. However, within each area with trees of the same age, the growth of single trees has been very different resulting in a mosaic of pines of height, as seen on the air photo (figure 1). The idea with this study was to capture as wide a range as possible across the planted areas inside of the old gravel pit area, to relate pine growth with soil development and fungal communities. A total of 53 sample plots were randomly selected across the different stands. 18 plots in a 15-year stand and 21 plots in a 30-year stand marked in black in figure 1. These are so-called internal plots. In adjacent 30- and 25-year-old stands (marked in blue), there are an additional seven sample points, but they have been deemed to have been influenced by the older adjacent 60-year-old forest and have been taken into account in the analyses. These are so-called border plots. In addition to these sample points, five more were taken in the 60-year stand and two in a nearby 150-year stand for reference. For the most part, only internal plots are used in this study's

analyses, but the border and reference plots are sometimes used to see a bigger picture.

Each sample plot has a pine tree in its center, where its height was measured. Three 5 cm diameter bore cores were made under the crown of the sample trees, and the core samples were divided into four different soil layers: litter (L), fermentation-humus layer (FH), 0-10 cm mineral soil (M1), and 10-20 cm mineral soil (M2). In these layers, the fungal biomass was measured with µg ergosterol per g OM and µg ergosterol per m^2.

Ergosterol is a sterol that fungi produce abundantly (Rodrigues 2018). Measuring ergosterol content is often used to assess the amount of fungi present in an area.

2.2 DNA samples and data

Soil samples were taken for DNA sequencing to identify fungal species and their guild. This was done by freezing soil samples until preparation in the lab. The frozen samples were freeze-dried, grinded down to a fine powder and DNA was extracted with solvents and centrifuges (NucleoSpin soil kit, Macherey-Nagel). Using the PCR-method, the primers connected to a small region called ITS2 were activated and the fungal ITS2 region was amplified. ITS2 is a region of rDNA (ribosomal DNA, its genes are relevant and associated to the creation and regulation of ribosomes) which is often called the “fungal barcode”. The DNA samples were sent to SciLife in Uppsala for PacBio sequencing. The results are inputted into SCATA (Sequence Clustering and Analysis of Tagged Amplicons, a program created by SLU) where quality assurance is done. In SCATA, DNA sequences with a similarity of 98,5% or above are clustered together into the same species. For more information about the lab method, see Clemmensen et al. (2016). For more information about the DNA sequencing technique, see Lindahl et al. (2013).

These clusters were given to us where we proceeded to identify the 400 most abundant DNA sequences into species with the help of UNITE massBLASTer (BLAST v2.13.0). For more information about UNITE, see Abarenkov et al. (2010).

The fungal guilds were determined with the aid of Fungal Traits (Polme et al. 2020), descriptions in UNITE, a list of ECM composed by Tedersoo & Smith (2013), and our mentor Karina Clemmensen. The species were categorized into the fungal guilds:

A) Saprotrophs

1) Ascomycetes (SAPasc), and whether it is a yeast (SAPasc-yeast)
2) Basidiomycetes (SAPbas), and whether it is a yeast (SAPbas-yeast)
3) Mucoromycetes (SAPmuc)

B) Ectomycorrhizas

1) Ascomycetes (ECMasc)
2) Basidiomycetes (ECMbas)

C) Root associated, meaning ericoid mycorrhizal or potential for several mycorrhizal associations (for example both ecto- and endomycorrhiza) or general root associated without known function

1) Ascomycetes (ROOTasc)
2) Basidiomycetes (ROOTbas)

D) Lichens, ascomycetes (LICasc)
E) Unknown or uncertain guild/ecology (UNK)

2.3 ECMs categorization

The ECM genera *Inocybe* and *Wilcoxina* were further categorized as pioneers, *Piloderma*, *Amphinema* and *Tylospora* as inorganic "N-immobilizers", and *Cortinarius* and *Russula* as organic "N-miners". The categorization was done with the help of other research papers such as Ishida et al. (2008), Kyaschenko et al. (2017) and our mentor.

2.4 Analyses

The statistical significance analyses done during the study were made using Pearson's correlation coefficient brought forward in Excel. The value can vary between -1 and 1. A value with -1 means there is a perfect negative correlation and +1 means there is a perfect positive correlation. A significant level of 10% is marked with *, 5% with ** and 1% with ***.

Multivariable analysis was done with de-trended correspondence analysis, DCA, in CANOCO. A DCA visualizes the largest differences in fungal communities across all samples and produces sample- and species- plots based on one analysis. Sample and species plots can be overlain to interpret which fungal species are associated with which samples. To aid interpretations, we also correlated the following factors to the two first DCA axes: tree height, stand age, plot type (internal, border or reference), and soil layer.

The genus gradient graphs (figure 9 and 10) were made using the polynomial trendline in Excel, the number of degrees was chosen to best fit the average and overall abundance of that genus. The graphs are made to best represent the data visually. No statistical analyses were done for these graphs.

3. Results

3.1 General results

Out of the complete DNA data set, the 400 most common species were assigned a guild and most of them identified down to the genus level. These 400 species represented 95,2 % of all the data collected. After removing plants and other species which are thought to have contaminated the samples, the remaining data covers 91,1 % of the total data. The remaining 91,1 % are used in calculations and analyses.

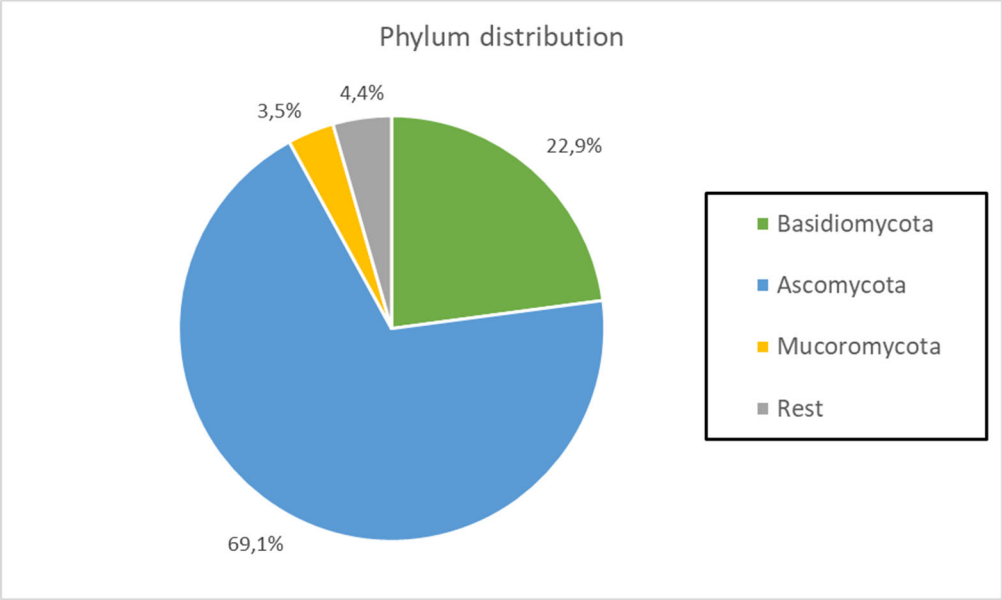

Figure 2. Overall phylum distribution. "Rest" consists of other phylums such as zoopagomycota and rozellomycota and the species categorized as UNK. The two phylums were not assigned any proper guild due to unknown ecology.

Figure 2 shows that ascomycota is a major phylum with 69,1% coverage and basidiomycota is the second biggest with 22,9%.

Table 1: Sum of fungal biomass across the four soil layers measured in µg ergosterol per m^2 in internal sample plots and its percentage.

	L	FH	M1	M2
Percentage	22,7	50,7	18,8	7,8
Sum (µg ergosterol/m^2)	460 818	1 031 102	382 361	159 444

Table 1 shows that the FH soil layer contains the majority (50,7%) of all fungal biomass with 1 031 102 µg ergosterol/m^2.

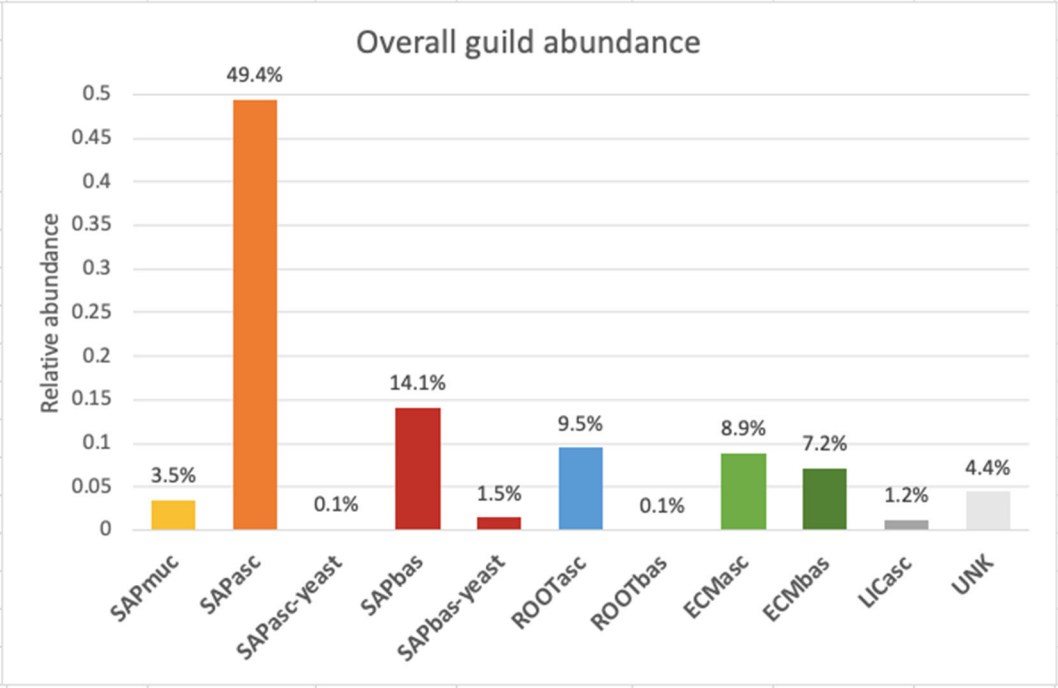

Figure 3. Guild abundance across the data set.

SAPasc accounts for nearly half of the coverage with 49,4%, next is SAPbas with 14,1%. Combined, SAPs take up 68,6%, ROOTs 9,6%, and ECMs 16,1% (figure 3).

3.2 Guilds in soil layers

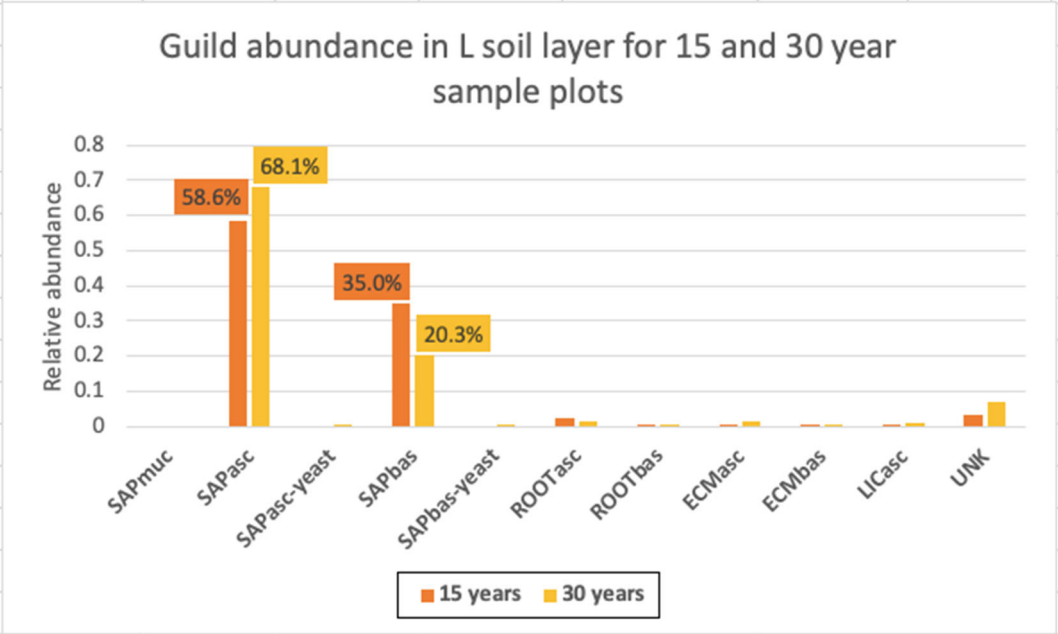

Figure 4. Guild abundance in L soil layer for the internal 15- and 30-year-old pine stands.

Figure 4 shows that SAPasc and SAPbas are the main guilds in the L soil layer. Combined, SAPs cover 93,6% and 88,4% in the 15- and 30-year-old stands, respectively. For both stands, SAPasc is the bigger guild.

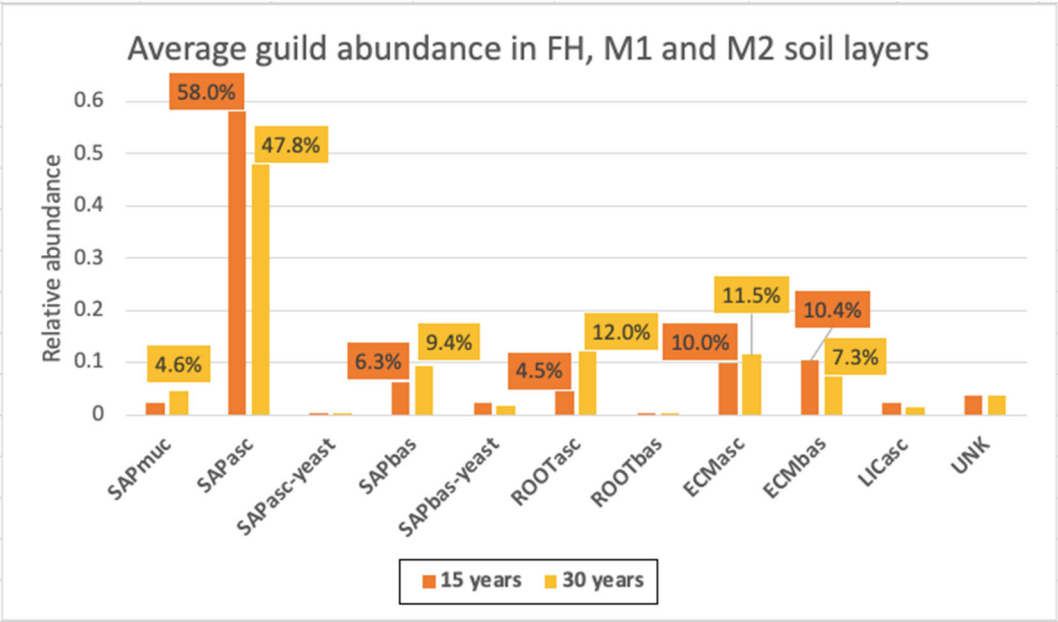

Figure 5. Average guild abundance in FH, M1 and M2 for 15- and 30-year-old sample plots.

In the remaining soil layers (figure 5), SAPasc is less dominant and SAPbas has decreased to below 10%. ROOTs and ECMs have appeared: ROOTasc is taking up on average 8,2%, ECMasc 10,8% and ECMbas 14%.

3.3 Hypothesis 1

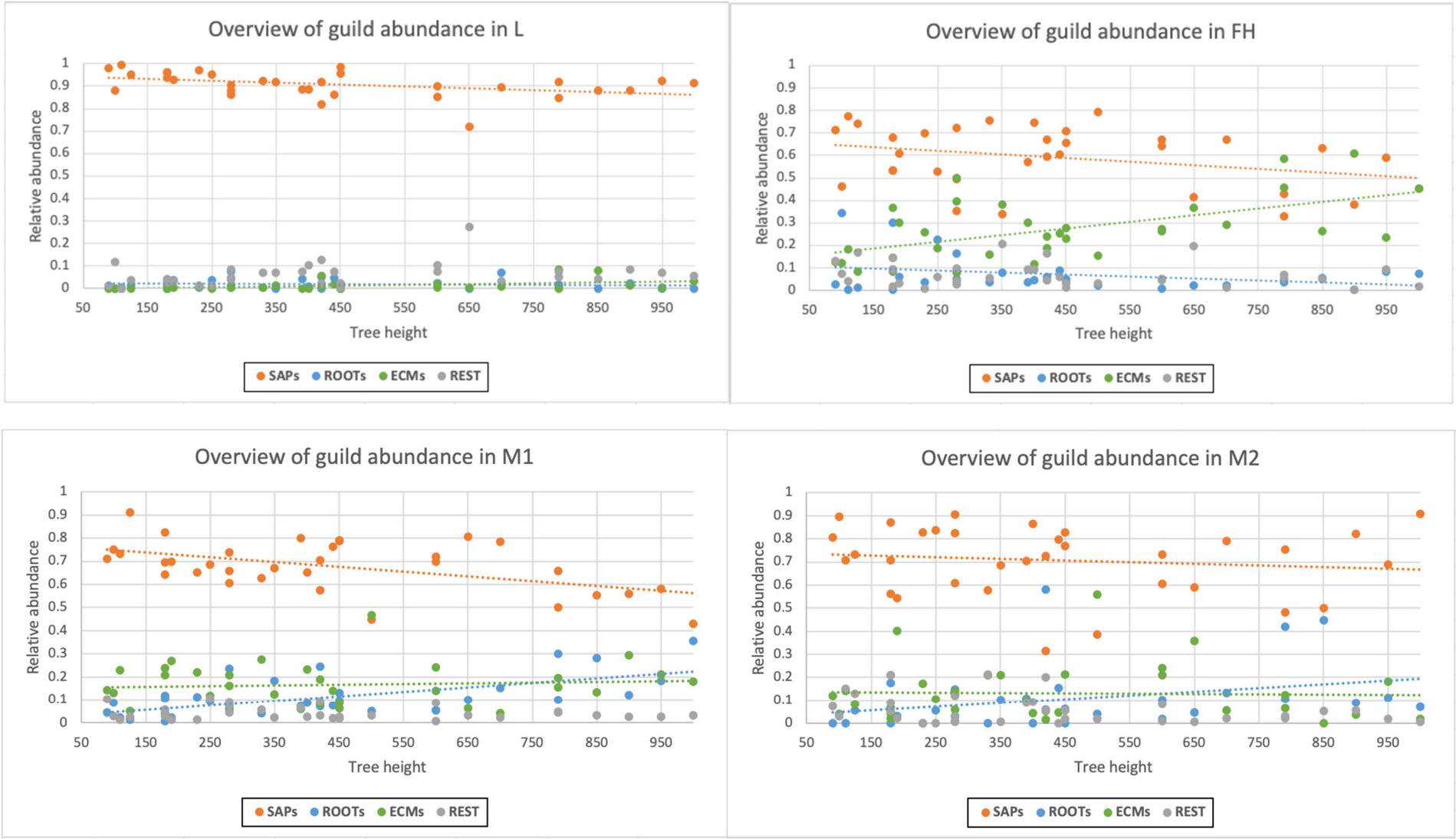

Figure 6a, b, c, d. Guild abundance in each soil layer (L: 6a, FH: 6b, M1: 6c, M2: 6d) for internal sample plots and their linear trendlines.

Overall, the abundance of SAPs appears to decrease with tree height in all soil layers. In FH, ECMs have the biggest increase with tree height (figure 6). In M1 and M2, ECMs seem stable while ROOTs appear to increase.

3.3.1 ECM phylum shift

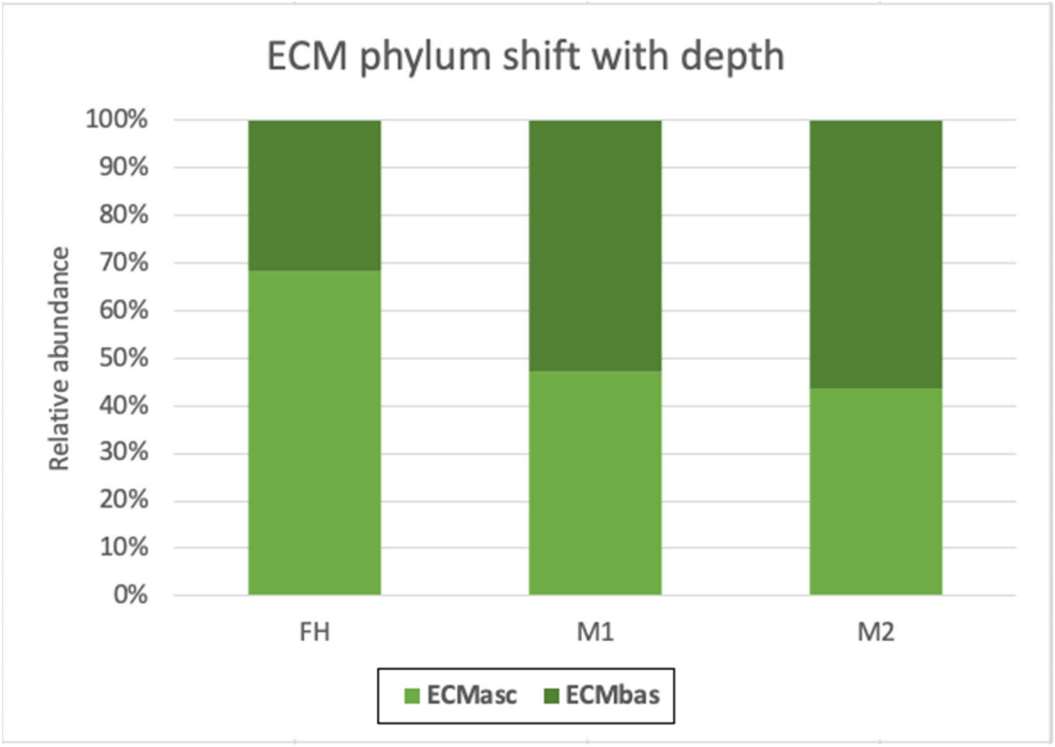

Figure 7. Percentage of ECM phylum in different soil layers for internal sample plots.

As the depth increases, ECMs shift from ascomycetes dominance to basidiomycetes dominance. Starting at nearly 70% ECMasc in FH, it drops to just below 50% in M1 and in M2 it drops slightly to around 45% (figure 7).

3.3.2 Correlation analyses 1

Table 2a. Pearson's correlation coefficient between ECM abundance in each soil layer and tree height for the 15- and 30-year-old stands separately and combined. The values highlighted in bold are significant. The sample sizes in each soil layer are n=15 for the 15s, n=18 for 30s, and n=33 for combined. Except for the L soil layer where it is n=14 for the 15s, n=18 for 30s, and n=32 for combined.

	15s	**30s**	**Combined**
	Pearson's correlation coefficient	Pearson's correlation coefficient	Pearson's correlation coefficient
L	0,405	**0,415 ***	**0,497 *****
FH	0,180	**0,605 *****	**0,564 *****
M1	0,102	0,313	0,088
M2	0,181	0,045	-0,026

There was a significant positive correlation in L and FH for 30-year-old plots and for the combined (Table 2a). The trendlines, significant or not, were always positive except in M2 for the combined plots.

Table 2b. Pearson's correlation coefficient between ROOTs abundance in each soil layer and tree height for the 15- and 30-year-old stands separately and combined. The values highlighted in bold are significant. The sample sizes in each soil layer are n=15 for the 15s, n=18 for 30s, and n=33 for combined. Except for the L soil layer where it is n=14 for the 15s, n=18 for 30s, and n=32 for combined.

	15s	**30s**	**Combined**
	Pearson's correlation coefficient	Pearson's correlation coefficient	Pearson's correlation coefficient
L	**0,428 ***	-0,189	-0,109
FH	0,003	**-0,599 *****	**-0,297 ***
M1	**0,437 ***	**0,458 ****	**0,599 *****
M2	**0,641 *****	0,119	**0,324 ***

There is a significant positive correlation in M1 and M2 for all ages (except for 30s in M2) (table 2b). There is also a significant positive correlation in L for 15s. A significant negative correlation is seen in FH for 30s and combined.

Table 2c. Pearson's correlation coefficient between SAPs abundance in each soil layer and tree height for the 15- and 30-year-old stands separately and combined. The values highlighted in bold are significant. The sample sizes in each soil layer are n=15 for the 15s, n=18 for 30s, and n=33 for combined. Except for the L soil layer where it is n=14 for the 15s, n=18 for 30s, and n=32 for combined.

	15s	**30s**	**Combined**
	Pearson's correlation coefficient	Pearson's correlation coefficient	Pearson's correlation coefficient
L	**-0,612 ****	-0,135	**-0,409 ****
FH	0,036	-0,192	**-0,321 ***
M1	-0,260	**-0,529 ****	**-0,504 *****
M2	-0,373	-0,014	-0,126

A significant negative correlation is seen for the combined plots except in M2 (table 2c). It is also seen in the L soil layer for 15s and in the M1 soil layer for 30s. Overall, the trendlines are negative except for FH in 15s.

3.4 Hypothesis 2

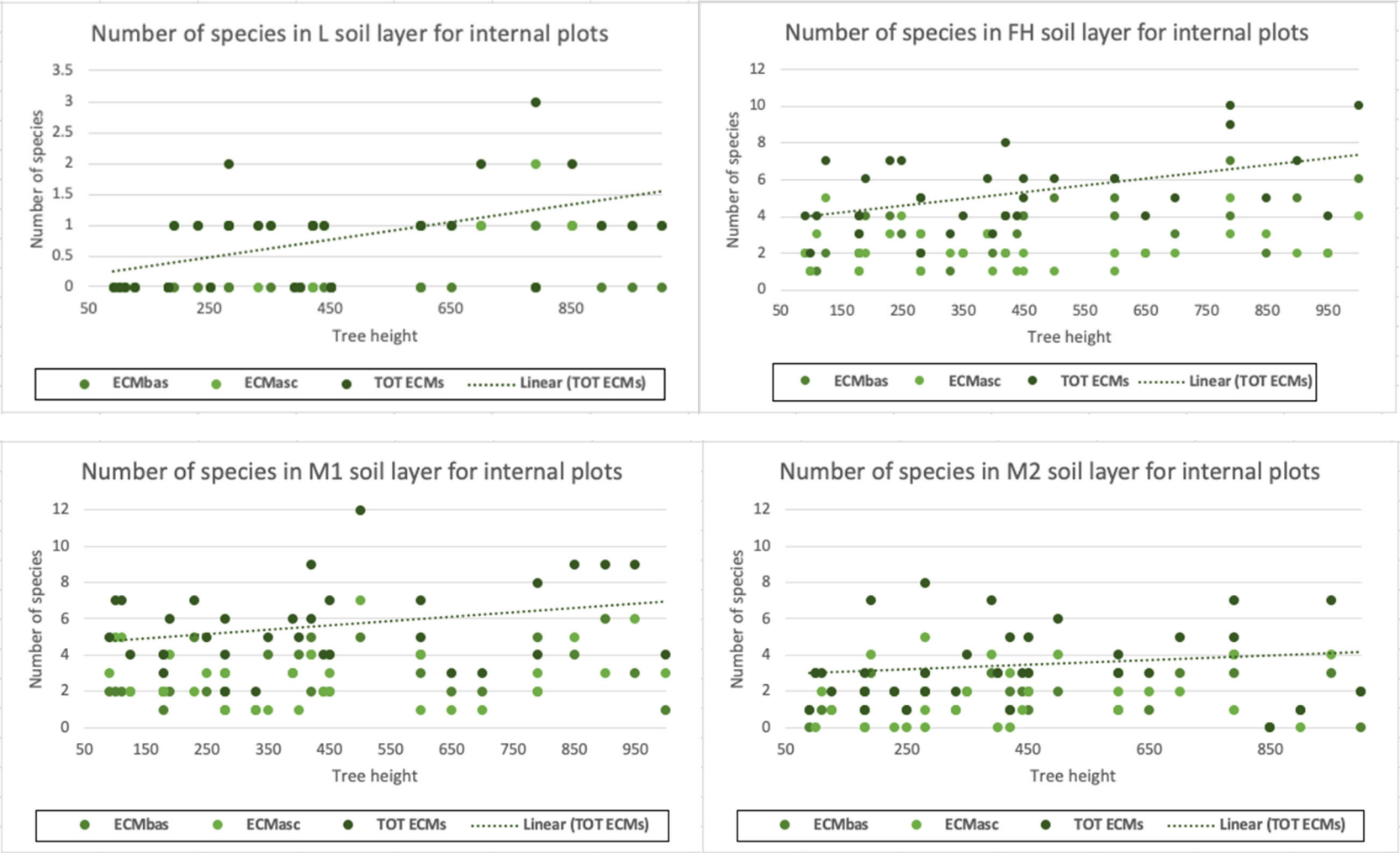

Figure 8a, b, c, d. Number of ECM species observed in each soil layer (L: 8a, FH: 8b, M1: 8c, M2: 8d) for internal plots and the linear trendline for the total number of ECM species. Note that in 8a (top left), the y-axis has a different scale.

A positive trendline for the total numbers of ECMs species is observed in each layer with increasing tree height (figure 8).

3.4.1 Correlation analyses 2

Table 3. Pearson's correlation coefficient between the total number of ECM species in each soil layer and tree height for the 15- and 30-year-old stands separately and combined. The values highlighted in bold are significant. The values highlighted in bold are significant. The sample sizes in each soil layer are n=15 for the 15s, n=18 for 30s, and n=33 for combined. Except for the L soil layer where it is n=14 for the 15s, n=18 for 30s, and n=32 for combined.

	15s	**30s**	**Combined**
	Pearson's correlation coefficient	Pearson's correlation coefficient	Pearson's correlation coefficient
L	**0,443 ***	**0,422 ***	**0,514 *****
FH	0,257	**0,666 *****	**0,450 *****
M1	0,293	0,356	0,277
M2	0,310	0,172	0,215

Table 3 shows that the number of ECMs have a significant positive correlation in the L soil layer for all ages, and in FH for 30s and combined. The rest have an observed positive trend.

3.4.2 Genus gradient

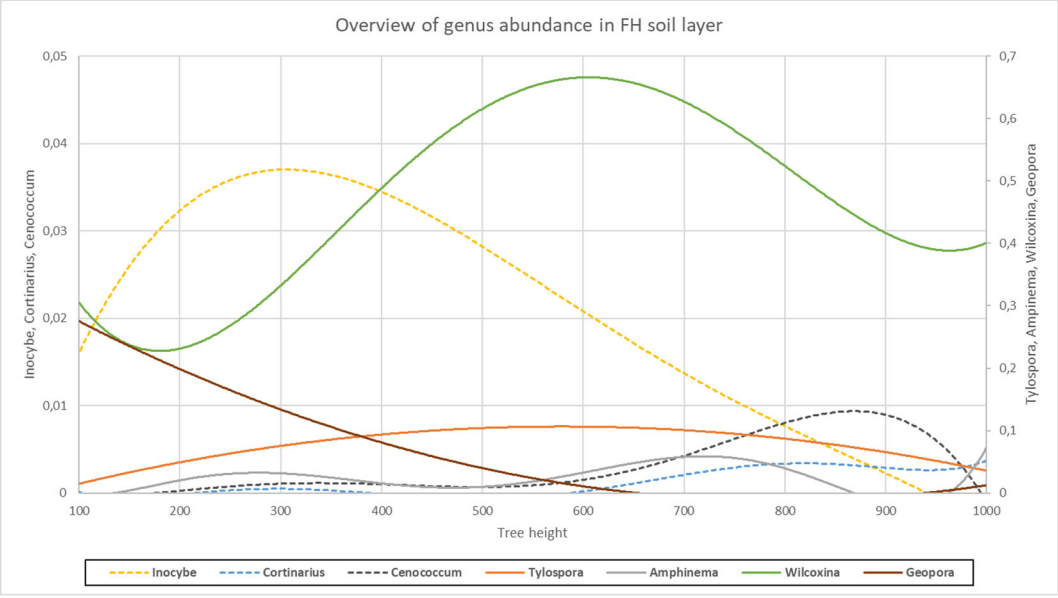

Figure 9. Overview of certain genus abundance in the FH soil layer for internal plots. Note that there are different amounts of species in each genus. There is one species for Tylospora and

Cortinarius, two species for Amphinema, Wilcoxina, Geopora, and Cenococcum and four species for Inocybe. Relative genus abundance is shown on the y-axes.

The abundance of the selected genera in the FH layer is spread differently depending on tree height (figure 9). *Inocybe* and *Geopora* are mostly abundant in smaller trees, *Cenococcum* and *Cortinarius* are most abundant on the taller trees, *Tylospora* and *Wilcoxina* are found across all tree heights with a peak in the middle-sized trees. *Amphinema* is seen around most tree heights but with a lean towards the bigger trees.

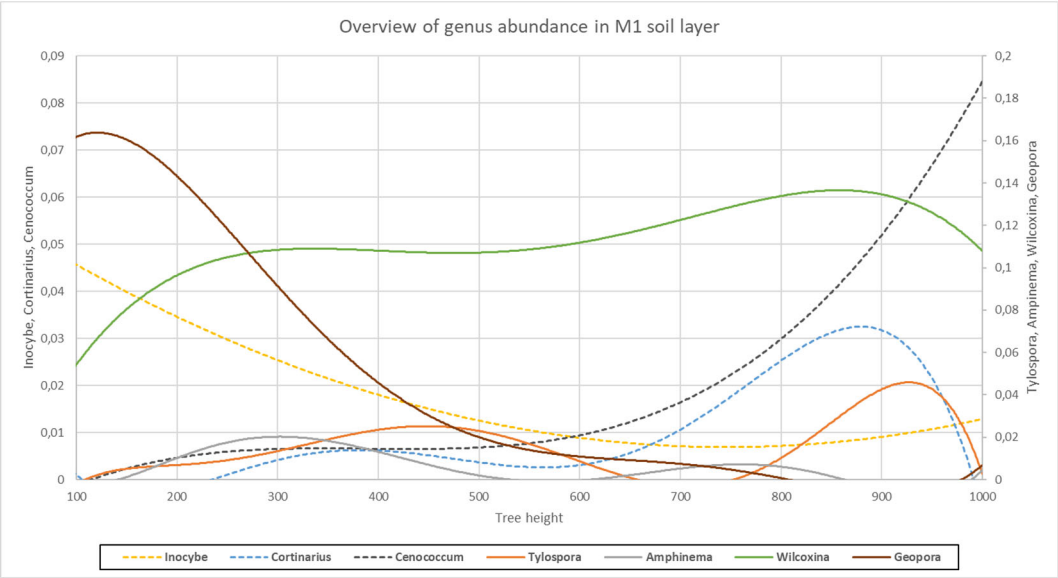

Figure 10. Overview of certain genus abundance in the M1 soil layer for internal plots. Note that there are different amounts of species in each genus. There is one species for Tylospora, two species for Cortinarius, Amphinema, Wilcoxina, Geopora, and Cenococcum, and four species for Inocybe. Relative genus abundance is shown on the y-axes.

In the M1 layer, the trends for each genus appear the same as in FH but with different abundances (figure 10).

The more abundant genus in FH such as *Tylospora*, *Amphinema*, *Wilcoxina* and *Geopora* are less abundant in M1 while the opposite is observed for the less abundant genera. Overall, there is less of a gap between the most and the least abundant genus in M1 compared to FH.

There is no genus abundance graph for the L soil layer because there are so few ECM species present. There is neither a graph for M2 since there is such little fungal biomass (7,8% from table 1).

3.5 DCA analyses

The variable “fungi reads”, which is how many DNA sequences there are for each species, does not explain much of the variation within the data and is removed from the analyses.

Two outliers, sample plot 46 and 50 in M2, are removed to better the resolution on the remaining data.

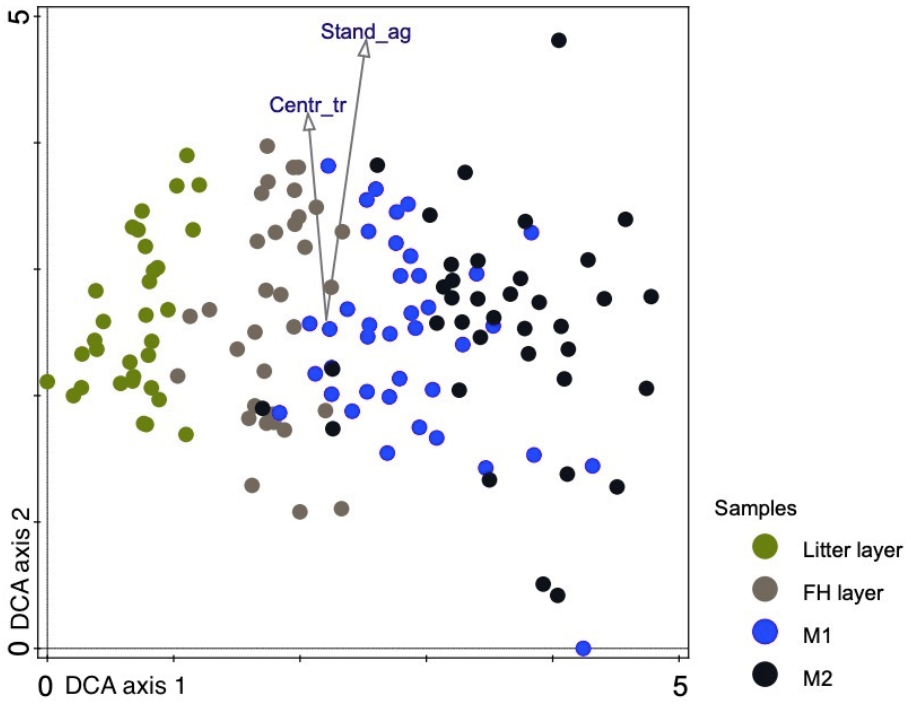

Figure 11. DCA over all internal fungi sample plots.

Figure 11 shows the DCA analysis of total fungal communities in the internal plots. Each dot represents the entire fungal community (SAPs, ECMs, ROOTs, and other) for that sample. The closer the dots are to each other, the more similar they are. The figure shows a clear separation of the soil layers (different colors) along the first axis (similar to the x-axis). The difference in fungal communities along the second axis was correlated with both stand age (Stand_ag) and tree height (Centr_tr). Which is seen within the colors where the dots are spread on the same axes as age and height. The total variation (or Eigenvalues) of the data is 12,7. The first axis has an explained variation (or cumulative variation) of 4,52% and the second axis of 3,30%.

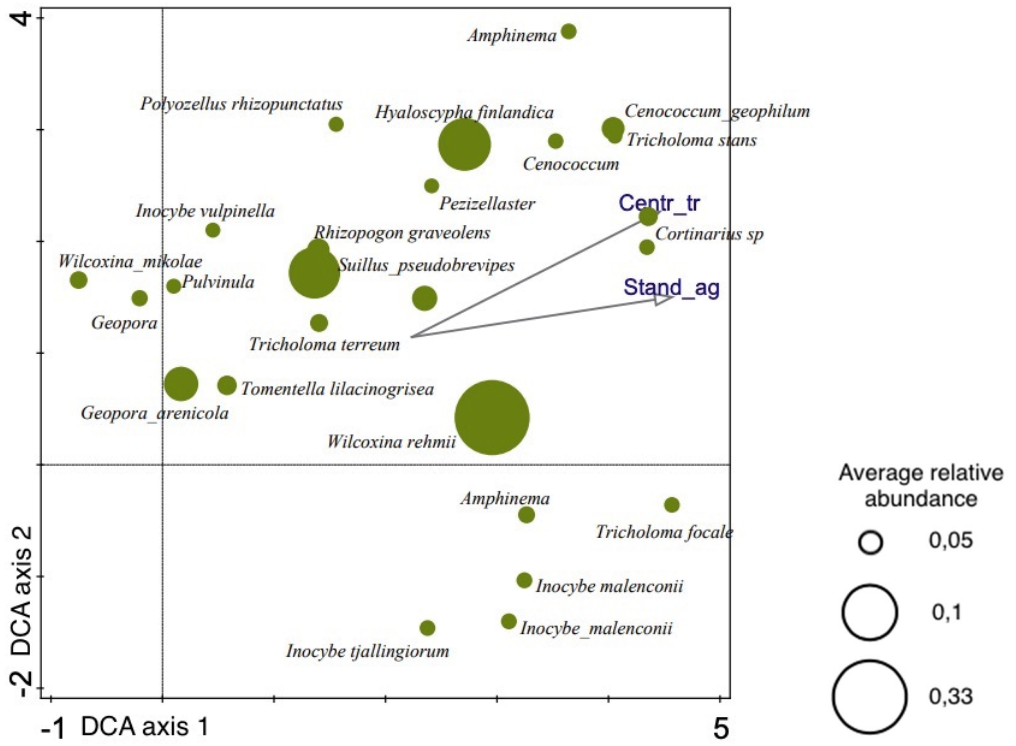

Figure 12. DCA over ECMs in the internal sample plots in the FH soil layer.

The DCA analysis above (figure 12) of the ECM communities in the internal plots in the FH soil layer shows a gradient along the first axis correlated with tree height and age. The spread seen on the second axis is due to an unknown variable. Bigger plots have a relatively higher abundance. The total variation of the data is 2,95. The explained variation of the first axis is 20,62% and the second axis is 10,95%.

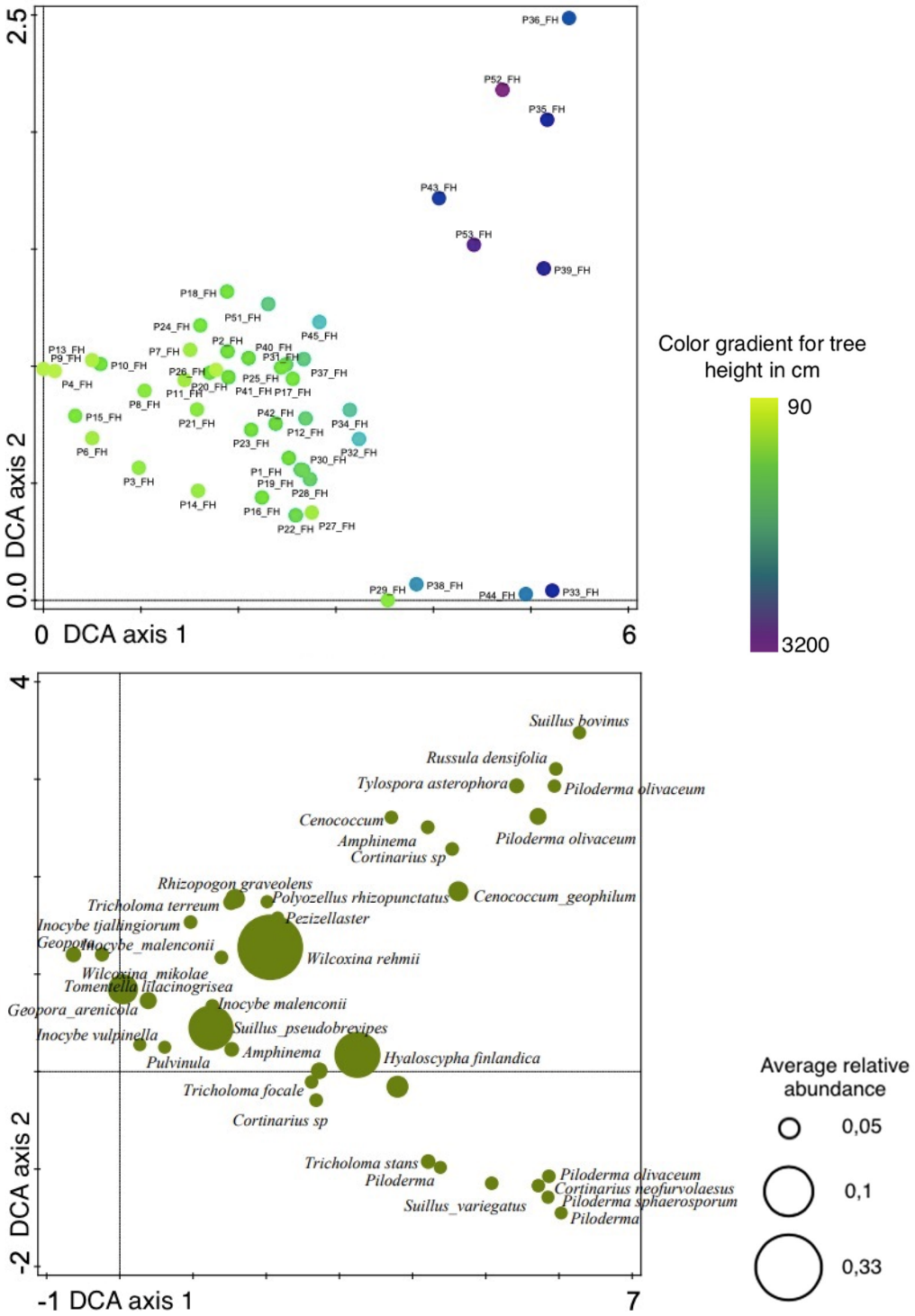

Figure 13a, 13b. DCA over ECMs in all sample plots in the FH soil layer either colored according to a tree height gradient (upper figure 13a) or with different sized dots according to species abundance average (lower figure 13b). All sample plots include internal, border and reference plots. Both a and b are the same analysis and can be overlaid.

The DCA analysis of ECM communities in all sample plots in the FH soil layer shows a height gradient (from green to purple) along the first axis (figure 13a). Since the two figures are from the same analysis, they can be overlaid. The ECMs

(figure 13b) move into two branches as the plots correlate positively with height as seen in figure 13a. The spread seen on the second axis is due to an unknown variable. The total variation of the data is 4,97. The explained variation of the first axis is 14,32% and the second axis is 7,20%.

4. Discussion

4.1 General

4.1.1 Overall fungal community

Overall, ascomycetes are the most common in the data set (figure 2) which points towards that the soil is less productive and infertile (Sterkenburg et al., 2015). This is reasonable since the samples were taken in a primary succession from an old gravel pit. Perhaps there is a higher abundance of basidiomycetes in the adjacent forest where there is more OM and nutrients in circulation with a more diverse and rich fauna and flora.

Only 4,4% of the data is identified as "Rest" (figure 2). Which means that 95,6% of all the other species were assigned a guild and there is not much left to discover. A high identification rate also means less unexplained variation within the data set.

The FH soil layer contains about 50% of all fungal biomass (table 1), It also has the biggest ECMs abundance (figure 7b) of all soil layers. It suggests that FH is where most of the roots and ECM activity is present and is the most important soil layer to study in this case.

The next biggest soil layer regarding fungal biomass is L. But it is heavily dominated by SAPs across all ages (figure 4) because L consists of recently shed pine litter that contains easily available carbon (such as cellulose). ECMs get their carbon in the form of sugars directly from the plants, so they don't need to decompose litter. Instead, ECMs are more competitive among the decomposed OM deeper in the soil, where the roots are also abundant and where most of the nutrient uptake takes place. The remaining soil layers, M1 and M2, contain 18,8% and 7,8% (table 1) of the fungal biomass respectively and mostly follow the same patterns and trends as FH. Since they contain less fungal biomass (especially M2) they are of less importance to this study and the results from the layers are less reliable.

Figure 8 shows a dominance shift from ECMasc to ECMbas with soil depth. FH is dominated by ECMasc, and in M1 and M2 it is dominated by ECMbas. This shift might be because basidiomycetes can be regarded as a superior degrader of recalcitrant OM and has a longer reach. Why ascomycetes dominate the FH layer

might be because of other variables (e.g., pH and poor fertility) and the litter being of the right quality.

4.1.2 DNA samples and sequencing

A difficulty with DNA amplification is to choose a suitable primer. If the primer is too unspecific and general, non-fungi sequences, like plants or bacteria, might also be amplified and will need to be removed later in the process. If the primer is too specific, then some fungi might not be included.

The quality assurance in SCATA removed sequences which were missing primers, were too short, or if the overall quality was too poor. Somewhere along the process of collecting samples, to manipulating the DNA sequences, to processing and identifying the data, there was an error which resulted in discarding that sample. This means that for some samples, there was no fungi data. It could also be that there wasn't enough DNA data for there to be any usable results.

Some genera can have more than one ecology/guild. Meaning that while a genus' primary guild is an ECM, it can still be SAP if it hasn't found any hosts. These categorizations change over time which is why it's important to check the latest information. The same goes for a species categorization in family or order. The taxonomy for species changes with time and must be kept in mind when working to avoid confusion and errors.

4.2 Hypothesis 1

There is only a significant correlation between ECMs and tree height in L and FH for the 30-year-old plots and for the combined plots (table 2a). There could be several reasons for why there is no significant correlation in the younger stand. Tree growth could be more correlated to total ECM biomass rather than ECM relative abundance, or to which ECM genus and species are present could play a significant role, or that there simply has not passed enough time for there to develop a significant correlation.

It could also be a purely mathematical issue. Either there are not enough sample plots, or the tree height min and max are too similar for there to be an observed difference.

In the mineral soil (M1 and M2), there is generally very little ECM correlation regardless of age (table 2a). Instead, there is a significant positive correlation for ROOTs, except in M2 for 30s (table 2b). So, while it might not necessarily be ECMs that increase with tree height, at least other root associates do.

The first hypothesis is somewhat correct. It states that there is a shift in which guild dominates around a tree when it goes from lower growth to a higher growth, from SAPs to ECMs.

Although there might not be a complete shift in abundance dominance from SAPs to ECMs, there is still a positive correlation between ECM abundance and tree height in the L and FH soil layer which is where most of the tree's roots are present and there is most fungal activity (fungal biomass) (table 2a). At the same time, SAPs have a negative correlation for the combined plots (table 2c).

The shift in dominance can be seen in multiple ways. SAPs have a lesser effect on trees than ECMs do, so while ECMs are not dominant in abundance, a slight change in ECM abundance can have a big effect on their host. It is also important to note that as ECMs relative abundance increases, total fungal biomass increases at the same time. So, there is a double effect from ECMs: not only do they become more abundant, relatively, but they also increase in overall biomass.

The data relies on the quantity of DNA sequences per species. Different species can have different DNA amounts per biomass which isn't considered in this study (Mohanta & Bae 2015). So, although a species might appear more abundant than another, it might not actually be more abundant in reality. Its biomass might be smaller or its effect on its environment and trees might not be as big.

The findings from this study are similar to those found on fungal community development after a clearcut. After a clearcut, the litter layer remains SAP dominant regardless of age (figure 2a from Kyaschenko et al. 2017), similar to this study's results (figure 6a). In the remaining fragmented litter and humus (in this study it would compare to upper FH and lower FH), ECMs and ROOTs become more abundant with age (figure 2b and 2c from Kyaschenko et al. 2017) and ROOTs are more abundant in the lower soil depths. Both these findings correspond with this study's findings (figure 6c and table 2b). It is worth noting that in a primary succession (such as this study) the ROOT guild is not as predominant as in the rest of the boreal forest since there is not much vegetation established yet. As time passes, more and more bushes such as *Calluna vulgaris*, *Empetrum nigrum*, and *Vaccinium myrtillus* appear which are often associated with ericoid mycorrhiza (ROOTs) (Johnson & Gehring 2007).

4.3 Hypothesis 2

4.3.1 Increased species diversity with tree height

In figure 8, a trend is seen where the number of ECMs species increases with tree height. Table 3 shows that there is a significant correlation in L and FH (except for 15s in FH).

This supports the first part of the second hypothesis which states that species diversity of ECMs increases with tree height.

Although there are multiple ways of measuring diversity. This study found an increase in species richness and not necessarily an increase in species evenness. Shannon's diversity index can be used to get a better grasp on how ECMs diversity develops.

ECM abundance only had a significant increase in L and FH (table 2a). Once again, there is only a significant correlation in the two first soil layers (table 3), indicating that those are the most important soil layers to study in relation to tree growth.

4.3.2 ECM gradient

ECM recategorization and shifts

Table 4. Categorization of the used genera. Genera in bold are either added or moved from the initial categorization, genera crossed out are removed from the analyses due to no or few observations.

Pioneers	N-immobilizers	N-miners
Wilcoxina >>	***Wilcoxina***	***Cenococcum***
Geopora	*Tylospora*	*Cortinarius*
Inocybe	*Amphinema*	~~*Russula*~~
	~~*Piloderma*~~	

Regarding the initial categorization of ECM species, *Russula* and *Piloderma* were not used because there were very few or no observations in the internal plots. Instead, *Cenococcum* and *Geopora* were introduced and studied.

Inocybe, which was put as a pioneer, is indeed abundant with the smaller trees across all soil layers. *Geopora*, which follows a similar trend to *Inocybe*, can also be categorized as a pioneer (figure 9 and 10).

Wilcoxina, also classed as pioneer, is abundant across a wide range of tree heights with a slight lean towards bigger sized trees. Perhaps it would be best suited for it to be classed as an N-immobilizer.

As for N-immobilizers, *Tylospora* and *Amphinema* are most common around the middle-sized trees.

Cenococcum follows a similar trend as *Cortinarius*, an N-miner. Both are most abundant on bigger trees.

These findings support the second part of the second hypothesis. The results show that as tree height increases, there is a gradient from pioneers to N-immobilizers to N-miners. Their abundance varies a little depending on the soil layers, but the trend stays concise.

DCAs

As to be expected, when the sample sizes increase, the total variation of the data increases at the same time as the explained variation decreases. An explained variation of 4,52% and 3,3% (figure 11) might not seem much but it's enough to make out trends such as the layer separation. As for figure 12 and 13, the explained variation is very high, with its highest value of 20,62% (figure 12) and lowest 7,2% (figure 13), and so the observed trends and conclusions draw from the analyses are well-grounded.

Figure 11 confirms that looking at soil layers separately and doing analyses within each soil layer was a good choice since there is a clear layer separation.

Kyaschenko et al. (2017) also noticed this in their research on fungal successions after a clearcut. The fungal community is divided vertically where SAPs are pushed and limited to the litter layer and ECMs are abundant in FH.

When comparing the DCAs of ECMs in the internal plots in FH (figure 12) with the overview of species abundance across tree height (figure 9), there are similarities. For example, *Geopora* is found on the left in figure 16, which correlates negatively with tree height and is found most abundant on smaller trees in figure 9. *Inocybe* is mostly found scattered on the bottom right (figure 12). Some unknown variables set it aside, but it correlates negatively rather than positively but less so than *Geopora*.

Cenococcum and *Cortinarius* are found in the top right (figure 12) correlating positively with tree height and are most abundant among taller trees in figure 9.

For the N-immobilizers, *Amphinema* and *Wilcoxina*, they are placed around the origin of the tree height and age arrows (figure 12), (except for one outlier *Amphinema* placed at the very top) signifying they don't correlate much with tree height. Figure 9 also illustrates this, where the N-immobilizers are placed around the middle-sized trees with a spread towards smaller and taller trees.

So, for all three categories, the DCAs and genus shift graphs are in accordance.

When adding the remaining sample plots (border and reference), it shows that they complete and continue the trends found in the internal plots (figure 13). The pioneers, *Inocybe* and *Geopora*, are found on the left correlating negatively with height, the N-miners, *Cenococcum* and *Cortinarius*, are found on the right correlating positively with tree height. It seems that the fungal succession in a primary succession found in the gravel pit goes towards the fungal succession of the forest outside it. While adding border and reference plots to the analysis no longer suits a study done on primary succession, it still helps to reinforce the categorization done of the selected genera.

The results from the genera shift graphs and DCAs support the second part of the second hypothesis. Some recategorization had to be made (table 4) to fit the data as certain genus were not usable and other uncategorized genera followed the

trend of an already categorized genus and were introduced. In the end, the expected genus shift trends and succession were visible.

4.4 Finishing thoughts

The 400 most abundant fungal species in the data set accounted for about 95% of the total DNA sequences in the data set which is why the number of species identified is probably not a limiting factor for the analyses. While identifying more would increase certitude, it probably wouldn't change the end results. To better the analyses, more sample plots would need to be taken.

Surprisingly, there was never any significant correlation between ECM abundance and tree height in M1 and M2, and that there was a significant correlation in L (table 2a). The FH soil layer had the highest correlation coefficient regardless of analyses, meaning it is the most important soil layer to study in a primary succession.

If there were more sample plots and more ages included in the data, perhaps there would be a higher correlation coefficient in the remaining M1 and M2 soil layers.

Figure 3 shows that ECMs take up a small portion of the overall fungal community with 8,9% ECMasc and 7,2% ECMbas. This points towards the fact that while ECMs are relatively scarce, they have a disproportionally big impact on the ecosystem.

For the DCAs, perhaps more variables should have been involved in the analyses. Adding pH, inorganic N availability, and C/N ratios would have given more information and variables to find correlations with.

The fact that there is a significant correlation between ECMs and ROOTs with tree height and that there is a genus succession does not mean that there is causality. It reminds of the chicken or the egg dilemma. Do more ECMs, N-immobilizers and -miners increase tree growth, or do bigger trees mean more room for ECMs and a more suitable habitat for N-immobilizers and -miners?

Trees need to be big enough with enough needles and small branches to fall as litter for there to be enough OM for N-miners to colonize and get nutrients from. The growth boost could have come before the introduction of later colonizers such as N-immobilizers and -miners, perhaps it is more relevant to look at when the first ECMs were introduced to the tree. Early introduction of ECM pioneers could be the first step in boosting tree growth.

It could be a question of a positive feedback loop. The early introduction of ECMs boosts tree growth, which in turn creates more habitat for ECMs (with more OM and roots to colonize), which invites more ECMs to better the situation for the host tree.

5. Conclusion

Hypothesis (i): Difference in tree growth depends on which fungal guild dominates around that tree; trees with lower growth are dominated by free-living saprotrophs, and trees with higher growth are dominated by symbiotic ectomycorrhizal fungi.

While the study's results can't say that tree growth depends on the dominating guild surrounding it, the results can show a significant correlation. Table 2a shows that in L and FH for 30s and combined, ECMs have a significant positive correlation with tree height. Table 2b shows that in M1 and M2, for all ages (except 30s in M2), ROOTs have a significant positive correlation with tree height. All the while SAPs seem to decrease with tree height with a significant negative correlation for the combined plots, except M2, (table 2c).

There might not be a dominance shift but there is an ECM or ROOT increase as SAPs decrease (table 2).

Hypothesis (ii): Species diversity of ectomycorrhizal fungi increases with higher tree growth, and there is a gradient from pioneer species to N-immobilizers, to N-miners as tree height increases.

As tree height increases, the number of observed ECMs seem to increase in all soil layers (figure 8). The positive correlation is only significant in L and FH for all ages (except 15s in FH).

Figures 9 and 10 illustrate how the selected genus for each ECM category changes in abundance with tree height for FH and M1. After recategorizing some species and adding others, the ECM types follow a pattern. Pioneers such as *Inocybe* and *Geopora* are most abundant in the smaller trees, N-immobilizers such as *Tylospora*, *Amphinema*, and *Wilcoxina* are most abundant around the medium sized trees, and N-miners such as *Cortinarius* and *Cenococcum* are found around the bigger trees. These trends are similar in all three soil layers with some change in abundance and different leans towards either bigger or smaller trees.

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Acknowledgements

We would like to thank and give our gratitude to our energetic, experienced, and most helpful mentor Karina Clemmensen.

We would also like to thank our friends and classmates for their support, feedback, and constructive criticism.

Without them this work would not be what it is.

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