Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Forest Sciences

Biological control of fungal diseases in a forest nursery: Impact on the fungal community, species abundance and some fungal pathogens

Elin Persson

Degree project [30] credits

Forest Science - Master's program

Department of Forest Mycology and Plant Pathology

Umeå 2020

Biological control of fungal diseases in a forest nursery: Impact on the fungal community, species abundance and some fungal pathogens

Biologisk kontroll av svampsjukdomar i en skogsträds-plantskola: Påverkan på svampsamhället på, artabundans och några skadesvampar

Elin Persson

Supervisor:	Åke Olson, Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology
Assistant supervisor:	Audrius Menkis, Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology
Examiner:	Malin Elfstrand, Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology

Credits:	[30] credits
Level:	A2E/Advanced
Course title:	
Course code:	EX0972/10135
Program/education:	Forest Science- Master's Program
Course coordinating department:	Department of Forest Mycology and Plant Pathology
Place of publication:	Umeå
Year of publication:	2020
Cover picture:	Elin Persson
Keywords:	Biological control, forest nursery, fungal communities, pathogens, metabarcoding.

Swedish University of Agricultural Sciences
Faculty of Forest Sciences
Department of Forest Mycology and Plant Pathology

Abstract

There is a large production of regeneration material in Sweden, where modernization and new technologies have made it possible to produce a large number of high-quality seedlings. To provide the whole forestry sector with seedlings for regeneration, forest nurseries must as well make sure that the management generate healthy seedlings. Fungal diseases have for a long time been a constant problem for nurseries, but kept under control using chemical fungicides. As chemicals are gradually reduced in the Swedish forestry sector, new methods of disease management must be found and tested. The environment in forest nurseries are suitable for many pathogens, and controlling and changing the climate to disfavor them seems like an impossible task, not economically feasible.

However, pathogens like *Botrytis* sp., *Phoma*. sp. and *Sydowia* sp. must be controlled in some way, where one alternative to chemical fungicides are biological control agents. In this study, the four biological control products *Binab*, *Serenade*, *Prestop* and *Mikroferm* are tested in a forest nursery with pine seedlings. A negative control, which included seedlings sprayed with water, was included in the study. DNA from needles as well as the growth substrate, was extracted and by using sequencing of the ITS2 region it was possible to identify the presence of fungi on seedlings. *Phoma* sp. was the most commonly identified fungal species among all samples (12 % of all sequences) and *Botrytis cinerea* and *Sydowia polyspora* were among the five most common species in needle samples. The fungal community composition differed a lot between needle and peat samples. However, when the fungal community composition of the samples belonging to each treatment were compared, there were no significant differences shown. The fungal community composition changed during time, and significant differences of the fungal community composition was shown when data from four different timepoints were compared.

In conclusion: the biological control products did not have a significant effect on the fungal community structure, or certain pathogens studied. Stronger were the effects of time, where certain fungal species increase and decrease during time. In general, the management in connection to this trial generated healthy seedlings.

Keywords: Biological control, forest nursery, fungal communities, pathogens, metabarcoding.

Sammanfattning

Produktionen av föryngringsmaterial till skogsbruket är stor i Sverige och modernisering med ny teknologi har gjort det möjligt att producera stora mängder plantor av hög kvalitet. För att fortsättningsvis kunna försörja hela skogssektorn med plantor för föryngring, måste plantskolorna dessutom se till att deras skötsel och produktion genererar friska plantor. Svampsjukdomar har länge varit ett ständigt problem för plantskolor, men det hela har hållits under kontroll med kemiska bekämpningsmedel. Eftersom kemikalier gradvis fasas ut i den svenska skogsbrukssektorn, måste nya metoder för sjukdomshantering hittas och testas. Ur skötselsynpunkt, är det inte ekonomiskt möjligt att bekämpa svampsjukdomar endast genom att göra miljön i plantskolan ogynnsam för dem.

Skadegörare som exempelvis *Botrytis* sp., *Phoma*. sp. och *Sydowia* sp. måste ändå kontrolleras på något sätt och ett alternativ till kemikalier är biologiska bekämpningsmedel. I denna studie testas de fyra biologiska bekämpningsmedlen *Binab*, *Serenade*, *Prestop* och *Mikroferm* i en plantskola på tallplantor. Ytterligare var en negativ kontroll med i studien, vilken bestod av plantor som endast blev behandlade med vatten. DNA från barr och tillväxtunderlaget, torv, extraherades och genom sekvensering av ITS2 regionen var det möjligt att identifiera svampar närvarande på plantorna. *Phoma* sp. var den vanligast identifierade svamparten bland alla prover (motsvarande 12 % av alla sekvenser), *Botrytis cinerea* och *Sydowia polyspora* var bland de fem vanligaste arterna bland barrproverna. Svampsamhället i barrprover och torvprover skilde sig åt. Däremot visades ingen signifikant skillnad på svampsamhällets sammansättning när prover från varje behandling jämfördes. Svampsamhällets sammansättning förändrades över tid och en signifikant skillnad på samhällets komposition visades när data från fyra olika tidpunkter jämfördes.

Sammanfattningsvis: de biologiska bekämpningsmedlen hade inte någon signifikant skillnad i påverkan på plantornas tillväxt, sjukdomsförekomst, svampars samhällsstruktur eller skadesvamparna i sig, när behandlingarna jämfördes. Starkare var istället effekterna av tiden där vi kan se svamparter öka och minska med tiden. Skötseln av plantor till försöket generade friska plantor.

Nyckelord: Biologiska bekämpningsmedel, plantskola, svampsamhällen, skadegörare, metabarcoding.

Table of Contents

Dictionary

Term/ abbreviation	Explanation
Amplicon	A DNA segment that undergoes amplification, contains replicated genetic material.
Amplification	Production of multiple copies of a DNA sequence.
Biological control	A method for controlling pathogens, harmful animals, and plants using other organisms that can inhibit them, e.g. bacteria, insects, nematodes or fungi.
DNA extraction	Procedure to get access to DNA from the sample, so that it can be analyzed.
Fungicide	Chemical product used for plant protection, controls fungal diseases by inhibiting or killing the fungus.
Global clusters	A grouping of similar sequences, representing one Operational Taxonomic Unit.
Global singletons	Sequences that were unique and only found once.
Metabarcoding	A DNA barcoding method to identify multiple species (barcodes) from mixed samples.
Operational taxonomic unit (OTU)	Definition used to group individuals by similarity.
Polymerase Chain Reaction (PCR)	A widely used method for DNA amplification.
Relative abundance	Is the proportion (in %) of an organism, relative to the total number of all organisms. The proportion is measured by the number of DNA sequences that contributes to each species.
Sequencing depth	Defines the number of the unique number that a sequence has been read, giving information about the resampling of samples.

Introduction

Swedish Seedling Production

The conventional clear-cut forestry method starts with a regeneration phase, where the future possibilities for the stand's development are set. In Sweden, this often entails planting of seedlings, and as much as 84 % of all harvested areas were replanted with the use of seedlings between the year 2017-2019. The rest of the documented practices are natural regeneration (which is used in 11 % of all harvested areas) or sowing (which is used in 3 % of all harvested areas). Consequently, the demand for adequate plant material is great, and in 2018 about 380 million seedlings were produced in Sweden (Skogsstyrelsen, 2018). To ensure maximum utilization of the site production capacity, plants with the crucial characteristics for survival and establishment should be planted. An appropriate balance of aboveground biomass and root system, together with a large diameter in relation to the height, are desired qualities of a seedling. The industry has not agreed on any official limits of these ratios, instead "fitness for purpose" is what can define the quality of a seedling (Hallsby, 2013). Both the forestry sector and the private forest owners have different demands regarding seedling properties, and therefore, the forest nurseries deliver seedlings of different type, different species, different size and provenance (Johansson et al., 2014). Forest nurseries in Sweden are growing businesses built upon advanced processes, experience and competence, that enables the delivery of a large number of high-quality plant material to the whole country (Svenska Skogsplantor, 2019).

The production of seedlings in the forest nurseries is homogeneous in many ways. At greenhouse level, the seedlings are all of the same age, of the same species and the cassettes are put in a regular pattern. This has its advantages like high production but also some drawbacks. These drawbacks mainly concern the vulnerability of the seedlings and the creation of an environment suitable for fungal pathogens. Plants with symptoms of infection cannot be delivered and the loss of seedlings will mean an economic loss. For this reason, disease control and limitation of pathogen spread has become an important part of the nursery's management (Lilja et al., 2010).

Fungal Communities

In boreal forests, most fungi are decomposers of organic matter and they are also the main component behind nutrient cycling in these ecosystems. However, when fungi attacks living trees in our managed forests and causes a disease, it is classified as a pathogen rather than an organism completing its life cycle by colonization and reproduction in living plant tissues. This is what makes fungi important for dynamics in unmanaged forest stands, but often undesirable in production forests (Bödeker et al., 2016). In the same way, pathogens can be really devastating in forest nurseries, as

nurseries lack the normally occurring dynamic health processes of a "natural" forest. This control instead becomes a task for staff at the nursery (Bloomberg, 1985; Oliva et al., 2013). For a disease to occur, a pathogen, a host and the right environmental conditions are needed. In addition, virulence, inoculum type, dispersal potential, host range and the possibility to alternate between hosts will affect the outcome and severity of the disease. The effects on attacked plants can be different; pathogens gaining nutrients from living cells or by killing cell tissue can cause severe symptoms of the host. On the other hand, pathogenic fungi can be present even though there are no signs of infection, this because of an incubation period where it remains latent until preferable environmental conditions to come up (Oliva et al., 2013).

Pathogenic Fungi in a Forest Nursery

Below, four pathogenic fungi occurring in forest nurseries, (either permanent or periodic) are described. Species of the genus *Botrytis* that causes grey mold are of high economic importance for nurseries (Elad et al., 2007). Early symptoms of infection are discolored spots on needles and later, grey mycelia can be present together with spores that looks like cotton. Once fungal spores are present in the forest nursery, they can be spread in many ways; by humans, insects, wind and greenhouse ventilation, and this makes it a frequent disease in forest nurseries (Lilja et al., 2010).

Another fungal pathogen in forest nurseries are species of the genus *Phoma*. These fungi are important plant pathogens as they can infect through already existing wounds, moreover, weather and other organisms may affect development of *Phoma* sp. and the conidia can easily be spread by water-splash, birds and insects (Aveskamp et al., 2008).

Thirdly, *Sydowia polyspora* is a fungus that can cause shoot and tip dieback and necrosis on seedlings, it is often isolated as an endophyte but has shown to function as a foliar pathogen due to, for example, the ability to reduce development of *Pinus ponderosa* seedlings (Ridout et al., 2018).

Fusarium sp. has since the 1990's been a problem in nurseries where it causes root rot on seedlings. The fungi penetrate the root epidermal cell wall and grow intracellularly while decomposing cellular material and *Fusarium* sp. is nowadays causing problems in nurseries in many European countries (Won et al., 2018; Hassan Dar, 2011).

Detecting Fungi

One traditional way of categorizing fungi is by examining the fruit bodies, but when they are not visible for the naked eye, there is a need for complementary methods.

The former most common method, isolation, has come to be replaced by new molecular methods. Furthermore, molecular methods to get access to DNA, have developed fast and for fungi there is now one approved barcode of the DNA named the Internal Transcribed Spacer 2 (ITS2) region. This region can be used to sequence whole fungal communities by meta-barcoding, extracted DNA from large number of organisms can quickly be analyzed against databases, and the closest match to phylum, class or even the full species name be determined (Schoch et al., 2012).

Disease Management

Generally, diseases are easier to prevent than to cure, and the control and prevention of nursery diseases requires a good hygiene in all processes, from sowing to packaging. Management practices that can be applied to lower the risk of pathogen development, is to ensure proper irrigation, ventilation, fertilization, and seedling densities. Fungal pathogens are often dependent on the right environmental conditions to complete their life cycles, and that is a factor hard to control in a forest nursery. Because of this, either biological or chemical control is used in forest nurseries and often seen as a must (Lilja & Poteri, 2013).

Fungicides

Ever since its introduction, chemical control, has been widely used. Agricultural and industrial enterprises account for a large part of the use and release of chemicals in the environment. Even though fungicides in forestry are mostly used in the seedling phase, they may affect the environment around the nursery, and the future stand negatively. Another less attractive side of chemical control is the fact that chemicals have a slow degradation that makes them stay for a very long period. Society has therefore started to question its presence since chemical compounds can be toxic to living creatures and have serious consequences for health, even at small concentrations (Robbins & Sharp, 2008). Besides, in a forest nursery perspective, fungicides may have an impact on the fungal community composition in terms of decreasing the number of useful organisms in the peat that are the growth substrate around the seedlings, for example (Paungfoo-Lonhienne et al., 2015).

Regulations have come up forcing companies to act and search through their business. For instance, a fungicide must be approved by the *Swedish Chemical Agency* to be used, and they regularly check and update what applies when it comes to the usage of fungicides. This means that chemicals that are currently in use can be prohibited at a relatively short notice. Many forest companies and private forest owners are certified in Sweden either to Forest Stewardship Council (FSC) or Programme for the Endorsement of Forest Certification (PEFC). The FSC policy states that an FSC certified organization shall avoid or aim at eliminating the use of chemical pesticides, and that chemicals prohibited by FSC should not be used at all

(FSC, 2010) while PEFC clearly states that chemicals should be used minimally (PEFC, 2015). All EU countries should implement Integrated Pest Management (IPM), an approach of pest management with main principles to apply in the practice. One principle is to use products for plant protection at a level that reduces risks to both the environment and the human health (European Commission, 2020).

Alternatives to Fungicides

Knowledge about pathogens, other fungi, and endophytes' ability of forming communities as invaders or colonizers can be used to control disease. It can be assumed by looking at the complex system of a forest nursery, that a prohibition of some of the most used fungicides will come with a need for other options. Four examples of biological control products available at the market are *Binab*, *Mikroferm*, *Prestop* and *Serenade,* which consist of either fungi or bacteria that can reduce the risk of infection by several different actions. *Binab* is a biological control product based on *Trichoderma polysporum* and *Trichoderma atrovirdie.* It works in many of different ways, but mainly by changing the surrounding of the plant and facilitate the plant's life (Woo et al., 2014). One example of features making it a good biological control agent are the good survival in many ecological niches which is possible because of the life stage as chlamydospores, that can be dried and remain dormant until they are wetted (Widmer, 2014). Microbial control of *Botrytis* sp. has shown good results were *Binab* are sprayed in a preventative manner and successfully have restricted available nutrients. *Botrytis* sp. is sensitive to competition, and if the microclimate is changed, the pathogen has a harder time infecting the plant (Elad et al., 2007). Capieau et al. (2004) tested four treatments on seedlings of *Pinus sylvestris* in growth room assays. *Binab* could prevent infections by reducing incidence in needles by 94 %, but Binab and the other treatments in the study, suppressed the infections between 16-57 % compared to the water control, in seedlings in the field experiments. Finally, the simplicity of producing the agent and the good storing ability are some properties of *Trichoderma* sp. making it a suitable biological agent (Woo et al., 2014).

Bacillus subtilis is a bacterium of the genus *Bacillus*, that are used in industry for microbiology purposes and have shown several positive effects on plant pathogens. It is the main ingredient of the biological control agent *Serenade* and can colonize the root system of seedlings. The colonization becomes a barrier against potential pathogens, and the bacteria can produce metabolites that can reduce disease occurrence. The treatment can be used to minimize occurrence of mildew in forest nurseries and works mainly by substances which destroy the cell membrane of the pathogen and prevents further growth (Bayer, 2017). In addition, it is harmless to other living organisms. It can enhance seedling growth and total yield by making the plant produce growth hormones auxin, gibberellin and increase nitrogen fixation (Debabov, 1982). Won et al. (2018) conducted a study and tried to control *Fusarium*

root rot in a Coastal pine nursery by using *Bacillus lichenformis*. In this case, the agent showed potential of controlling the pathogen. By producing enzymes with the ability to impair the fungal cell walls, reduction of growth appeared (Won et al., 2018). Another study by Edwards and Seddon (1992), showed that application of biological control of *Bacillus brevis* on Chinese cabbage, resulted in a shorter wet period in the seedling canopy and therefore a shorter period suitable for *B. cinerea* infection appeared as a secondary benefit from the biological product.

Clonostachys roseae (*Gliocladium catenulatum*) are a part of the biological control agent *Prestop*. The treatment can reduce the risk of infection by *Phytophthora* sp. by colonizing both roots and other foliar parts, and in that way prevent attack of the pathogen (Verdera, 2019). The fungus produces volatile organic compounds that can kill or inhibit pathogens and showed a killing effect on *Pythium ultimum* (Stinson et al., 2003). The biological control is effective in the growth substrate and has shown ability to increase plant weight of seedlings even though they were infested with *Phytophthora cactorum* (Smith, 1990).

Yeast fungi, for example *Saccharomyecs cerevisiae*, can be beneficial for seedlings by increasing nutrient uptake ability (Shalaby & El-Nady, 2008). The biological agent *Mikroferm* consists of *Saccharomyecs cerevisiae* probiotic soil microbes naturally occurring in the nature photosynthesizing bacteria *(Rhodopseudomonas palustris)* and *lactic bacteria Lactobacillus plantarum* and *L. casei*. The agent works in the soil zone and produce substance which strengthen the underground ecosystem (Agriton Sverige AB, 2019) and a study done in a sugar-beet plantation showed that the yeast has growth promoting properties, can enhance emergence of seedlings, and increase leaf area (Shalaby & El-Nady, 2008).

Lack of Knowledge

There are knowledge gaps regarding the actual effect of biological control when used under production conditions, in nurseries. Profound experiments done under lab conditions can surely give guidance in how fungi and bacteria (both pathogens and components of biological control agents) develop and react, directly in that certain environment. Since the environmental conditions in greenhouses, outside greenhouses (under drape or not), cannot be fully controlled or predicted, it is not possible to have disease management with a focus on making environments not suitable for pathogens. As mentioned, it is an impossible task not economically possible. Forest nursery need some other disease management and biological control agents based on several modes of action need a clarification regarding efficiency and population ecology under field conditions (Elad et al. 2007). In addition, climate changes may lead to weather and environmental conditions become even more difficult to predict, and will therefore in addition make it hard to predict development and spread of pathogens at both large, and a small scale (Sturrock et al., 2011).

Aim of the Study

Biological control agents must be tested in field conditions, here nurseries, if we are to assess the opportunity to have them replacing chemicals. The study tested four commercially available biological control products *Binab*, *Serenade*, *Prestop* and *Mikroferm* were studied in a forest nursery with pine seedlings. The study will help answering the following questions:

- Will mean height and diameter increment differ between the four treatments? When measured in the autumn, 18 weeks after first measurement -To see if the growth promoting effects of biological treatment have an impact on mean height and diameter increment of the seedlings.

- How will the fungal communities change during the first time for sampling, to the last time for sampling? The study period is nine weeks and sampling occur at four times, with three-weeks interval. -To understand when pathogens are expected and see if spraying with biological control agents have any impact on them.

- Will the biological control agents change the species abundance in the fungal community? -To understand if the biological control agents affect the whole fungal community or certain pathogens.

- For how long time after spraying, will the biological control agents persist on needles and peat? -To evaluate for how long time the effect of the biological control agents remains on the seedlings.

- In addition, the study will especially focus on identifying fungi in needle and peat samples, through metabarcoding and amplification of the ITS2 region. -To further on answer the question of relative species abundance and the occurrence of pathogens.

Material and Method

Sampling and Treatment

Sampling and treatment of seedlings took place at the forest nursery of Svenska skogsplantor in Kilåmon (Sveaskog, Näsåker, Sweden), latitude 64. Container-grown Scots pine seedlings (*Pinus sylvestris*) were served by Kilåmon nursery. A total of 48 cassettes with 196 pots were involved in the project and part of the sampling and treatment. In the green house and outside on free land, treatments were kept apart by having one row of cassettes excluded from the study, working as a buffer between treatments. The rows with cassettes were positioned in the same way as the mobile arm, holding the sprinkler system goes (Figure 1).

1.	Binab		Serenade		3. Prestop		Mikroferm		Negative Control	5.	Positive Control			
	B1		S1		P1		M1		NC1		PC1			
	B2		S2		P2		M2		NC2		PC2			
	B3		S3		P3		M3		NC3		PC3			
	B4		S4		P4		M4		NC4		PC4			
	B5		S5		P5		M5		NC5		PC5			
	B6		S6		P6		M6		NC6		PC6			
	B7		S7		P7		M7		NC7		PC7			
	B8		S8		P8		M8		NC8		PC8			
2.					4.					6.				

Figure 1. Principle visualization of the project set up where one square represents one cassette of the treatments B (*Binab*), S (*Serenade*), P (*Prestop*), M (*Mikroferm*), NC (*Negative control*), PC (*Positive control*) and cassette number 1-8.

Sampling and treatment took place at four occasions during summer with three-week intervals starting at week 24 and ending week 33. In the text that follows will week 24 be referred to as time point 1, week 27 as time point 2, week 30 as time point 3 and week 33 as time point 4.

The first sampling occurred when seedlings were still in the green house, they were later moved out on free land during week 27 were they stayed for the rest of the studied period. At each time of sampling, five of the eight cassettes from each of the treatments were randomly chosen to be a part of the gathering of needles and peat. Further on, the samples from the five cassettes represent the seedlings of the entire

treatment, which is a total of 8 cassettes of seedlings. At sampling, two needles and approximately 100 µl substrate of peat from five randomized plants from each chosen cassette were gathered, and put in small Falcon tubes labeled with date, treatment, and cassette number. Disposable tweezers and gloves were used and changed between every cassette. Samples were then kept frozen at -15° C, at the forest nursery. The first and last sampling included a height and diameter measurement of seedlings. If seedlings showed signs of infection or died, notes were taken, and seedlings were taken away from the cassettes.

The products *Binab*, *Serenade*, *Prestop* and *Mikroferm* were mixed according to the companies' instruction, but amount of water was corrected to 1000 ml. *Silwet Gold* 0,025- 0,05 % for a better spread of the product were added to mixtures of *Prestop* and *Serenade*. *Negative control* consisted of water from the forest nurseries tap. After sampling, each treatment got sprayed over the eight cassettes corresponding to the treatment, cassettes were kept under roof for another day then put out on free land. The treatment called *Positive control*, should represent the properties of the nursery's ordinary treatment schedule, but after receiving the spraying protocol from the nursery, it is evident that the seedlings of the treatment *Positive control* was not sprayed with any fungicides during the study period. This means that other treatments cannot be compared to seedlings sprayed with fungicides.

DNA Extraction and Identification

All sampled material was part of the DNA extraction, resulting in 240 extractions. One tube from five cassettes, from each of the six treatments, at four occasions resulted in 120 tubes of needle samples and 120 of peat. Not all extractions could be done at the same time, the samples were divided into six different extraction occasions. The samples were randomly distributed so that there was backup of samples left, if one of the extractions failed, or if there were any contamination between samples during extraction. Every extraction included one water sample working as a negative control to detect any contamination during lab work.

Needles were freeze-dried and fast-prepped with 2 mutters in microcentrifuge tubes to pulverize the material. Peat were defrosted and homogenized before put in microcentrifuge tubes with ceramic pearls. Kits for DNA extraction for soil and plants respectively were used. *NucleoSpin Plant 11,* and *NucleoSpin* Soil, by Macherey-Nagel (Düren, Germany). The lab protocol provided was overall followed. Before Polymerase Chain Reaction (PCR), the concentration of DNA (ng/µl) and purity of the material was checked using the *Nanodrop* instrument.

A test PCR were set up for both needles and peat samples, to determine what final dilutions should be, and number of cycles to run in the PCR machine. DNA was diluted to 1 and 0, 5 ng/µl for needles and 1 and 5 ng/µl for peat. The cycles tested

was for needles: 18, 21, 24, 31 and 35. The cycles and dilutions chosen for needles was a dilution to 1 ng/ul to go for 35 cycles and for peat, a dilution of 3 ng/µl and 21 cycles. PCR products were run on 1 % agarose gel of SB buffer dyed with Nancy dye. Gels were run at 220V for approximately 30 min, 400 mA with the SB buffer. The electrophoresis and the visualization of DNA on the agarose gel was done to check whether any PCR products had formed. This further on provided guidance in which of the samples that should be chosen to the pools for further analysis. Three of the samples from each treatment were chosen to be a part of the further analysis, meaning that 72 samples from needle and peat samples respectively, and a total of 144 samples.

The aim of the sequencing was to cover amplicons from many samples. Sample-specific tags were used, and the samples got pooled, needle and peat samples separately. Primer tags used was fITS7 (primer sequence: GTG ART CAT CGA ATC TTT G) and ITS 4 (primer sequence: TCC TCG GCT TAT TGA TAT GC). PCR products were cleaned using Cycle-Pure Kit (Omega). The primer tags work as an ID tag for each sample, making it possible to blend all DNA, from needle and peat separately, into a final mix to send for analysis. The mix of the samples was prepared, where DNA concentrations of each sample, determined the quantity (µl) of each sample that should be put in the final mix. The two pools of needle and peat samples, should consist of samples with the same amount of DNA sequences, and before sent to *SciLifeLab*, Sweden, samples were run in the Bioanalyzer (Agilent Tech) to get size distribution and molarity of DNA.

Sequence Clustering

Sequence Clustering and Analysis of Tagged Amplicons (SCATA) (http://scata.mykopat.slu.se), is a service that provides analysis framework for the analysis of sequenced tagged amplicons. SCATA was used for a quality control and clustering of sequences. DNA sequences that were too short were removed before further work and the minimum alignment length for clustering were set to 0.85. After clustering, a total of 104 clusters were put into the Basic Local Alignment Search Tool (BLAST) and matched to the *National Center for Biotechnology* (NCBI) database, the nucleotide collection consisting of GenBank sequences. Some sequences were not represented by any sequence in GenBank and are then called "Unclassified scata x" where x is a number which replace a name (Table. 1). Only clusters with a similarity above or equal to 98 % were identified with names. Later in analysis, the 15 most common species in needle and peat samples respectively are named in figures and the rest is shown as "others".

Table. 1 The 40 most common identified clusters.

Fungal taxa	Phylum	NCBI reference	Similarity, (%)
Phoma sp.	Ascomycota	KU743967.1	99 (248/249)
Tilletiopsis minor	Basidiomycota	KT149771.1	100 (319/319)

Sydowia polyspora	Ascomycota	MN588187.1	100 (256/256)
Botrytis cinerea	Ascomycota	MN593202.1	100 (240/240)
Cladosporium sp.	Ascomycota	MN559960.1	100 (243/243)
Cryptococcus sp.	Basidiomycota	KT372800.1	99 (319/320)
Ustilentyloma graminis	Basidiomycota	KX067828.1	98 (310/316)
Rhodotorula glutinis	Basidiomycota	MN371916.1	100 (302/302)
Helotiaceae sp.	Ascomycota	MK131612.1	100 (239/239)
Unclassified *Rhizophydiales*	Chytridiomycota	MF483092.1	98 (320/326)
Cystobasidium laryngis	Basidiomycota	MG674823.1	100 (296/296)
Tilletiopsis sp.	Basidiomycota	AY879281.1	100 (222/222)
Fusarium sp.	Ascomycota	MN244535.1	100 (258/258)
Cryptococcus sp.	Basidiomycota	JQ857023.1	100 (329/329)
Unclassified scata 32	Basidiomycota	MF156120.1	100 (234/234)
Sampaiozyma ingeniosa	Basidiomycota	MH595294.1	100 (303/303)
Trichoderma atrovirdide	Ascomycota	MN516465.1	100 (270/270)
Lophodermium pinastri	Ascomycota	MN595074.1	100 (239/239)
Cystobasidium slooffiae	Basidiomycota	MN075256.1	100 (297/297)
Unclassified *Sordariales*	Ascomycota	MF486730.1	100 (244/244)
Cryptococcus sp.	Basidiomycota	MG976302.1	100 (316/316)
Unclassified scata 33	Unknown	KU188585.1	99 (298/299)
Malassezia restricta	Basidiomycota	LT854697.1	100 (369/369)
Malassezia globolosa	Basidiomycota	JQ088220.1	99 (382/385)
Rhodosporidiobolus colostri	Basidiomycota	NR 155730.1	100 (301/301)
Unclassified *Archaeosporales*	Glomeromycota	MF485112.1	99 (280/283)
Venturia sp.	Ascomycota	MN030918.1	98 (237/243)
Clonostachys rosea	Ascomycota	MN588108.1	100 (260/260)
Unclassified scata 44	Unknown	KX516312.1	99 (251/254)
Peniophora pini	Basidiomycota	MG547963.1	100 (287/287)
Ramularia sp.	Ascomycota	KT334795.1	99 (235/236)
Unclassified scata 55	Unknown	KM494352.1	98 (322/329)
Mortierella sp.	Mucoromycota	MF423524.1	100 (331/331)
Rhodotorula mucilagino	Basidiomycota	MG020720.1	100 (311/311)
Unclassified scata 74	Unknown	KU188590.1	100 (239/239)
Unclassified *Spizellomycetales*	Chytridiomycota	MF482782.1	99 (327/331)
Unclassified scata 59	Unknown	KX193901.1	98 (333/341)
Unclassified scata 61	Unknown	KX220492.1	100 (256/256)
Unclassified scata 60	Ascomycota	MG827532.1	100 (256/256)
Paraconiothyrium fuckelii	Ascomycota	MK052700.1	100 (248/248)

Statistical Analyzes

Data from height and diameter measurement was analyzed statistically by using the program *Minitab 18*, and a One-way ANOVA (Analysis of Variance) test, significance level $\alpha = 0{,}05$, with the null hypothesis: All means are equal, and the alternative hypothesis: Not all means are equal. A Principle component analysis (PCA) was done in the program *Canoco 5*, a program used for multivariate statistical analysis, to answer some hypotheses.

Choice of Method

The DNA metabarcoding method can identify species from a short section of a DNA sequence, and therefore making it possible to identify many species from the samples in this study, within the given time frame. In choice of primers for ITS amplification, ITS 4, a primer for eukaryotes and fungi, and fITS 7, a fungal specific primer, were chosen It was discussed whether the general primer gITS should be used for peat samples to cover more of the community of peat, but fITS primers were chosen for both samples in order to have a more straightforward comparison between needle and peat samples.

Results

Seedling Height, Diameter and Mortality

ANOVA showed no significant difference of mean height among seedlings assigned to different treatments, at the end of the study period. Seedlings which were treated *Binab* had the highest mean of all treatments (64,6 mm) and *Negative control* the lowest (58,7 mm). The One-way ANOVA test of height at the last measurement gave a P-value of 0,080 which means a lack of evidence for the alternative hypothesis and there is no significant difference when mean heights are compared.

The ANOVA test of seedling diameter at the last measurement gave a P-value of 0,00 meaning a lack of evidence for the null hypothesis, this means that there is evidence for the alternative hypothesis and therefore meaning that not all means are equal. A *Tukey Pairwise Comparison* grouped the means leading to a significant difference between means. *Serenade* and *Prestop* have significant difference against the others and have the least diameter at the end.

Cassettes had 196 pots and all cassettes had a number of empty pots in the beginning. *Binab* missed 12 % of seedlings, *Serenade* 14 %, *Prestop* 13 %. *Mikroferm* 20 %, *Negative control* 19 % and *Positive control* 13 %. Seedling mortality was followed during the study period. At the end, no significant difference regarding seedling mortality was shown, when treatments were compared.

Sequences and Rarefaction Curves

A total of 667 342 sequences was generated. 52 % (348 785) of the sequences passed the quality control, 53 % of them corresponding needle samples and 47 % of them peat samples. The number of global clusters identified were 1675, and number of global singletons 2716, in total 2716 reads were too short to pass the quality control.

The lowest sequencing depth among peat samples were around 3300 reads and the highest about 7600 reads. For needles, the lowest was around 14 000 while the highest was 310 000 (Figure 2). Curves were approaching or reached the asymptote, meaning that the great majority of fungal community was sampled for each treatment and needle/substrate.

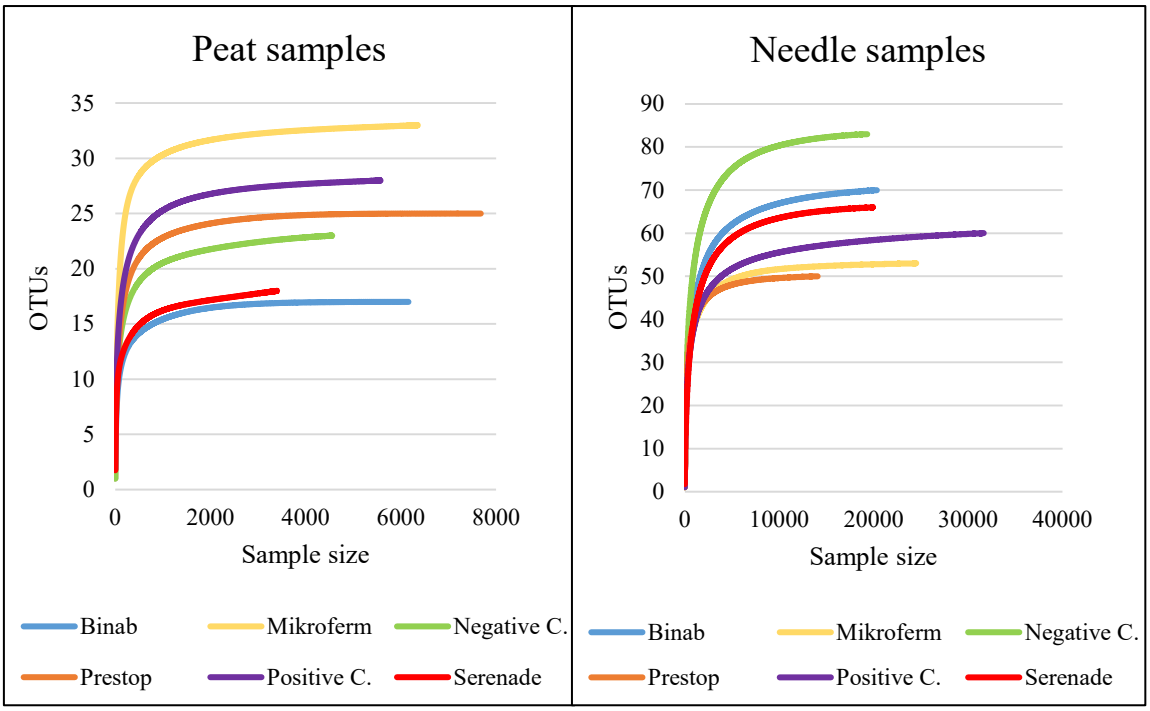

Figure 2. Rarefaction curves showing the relationship between the cumulative number of fungal Operational Taxonomic Units (OUTs) and the number of ITS2 rDNA sequences from peat and needle samples.

Fungi belonging to the phylum Ascomycota were the most common representing 56,5 % of the fungal sequences. The second most common phylum were Basidiomycota with 33,3 %, third most Chytridiomycota 9,8 %, and the phylum Zygomycota represented 0,4 % of the fungal sequences (Figure 3).

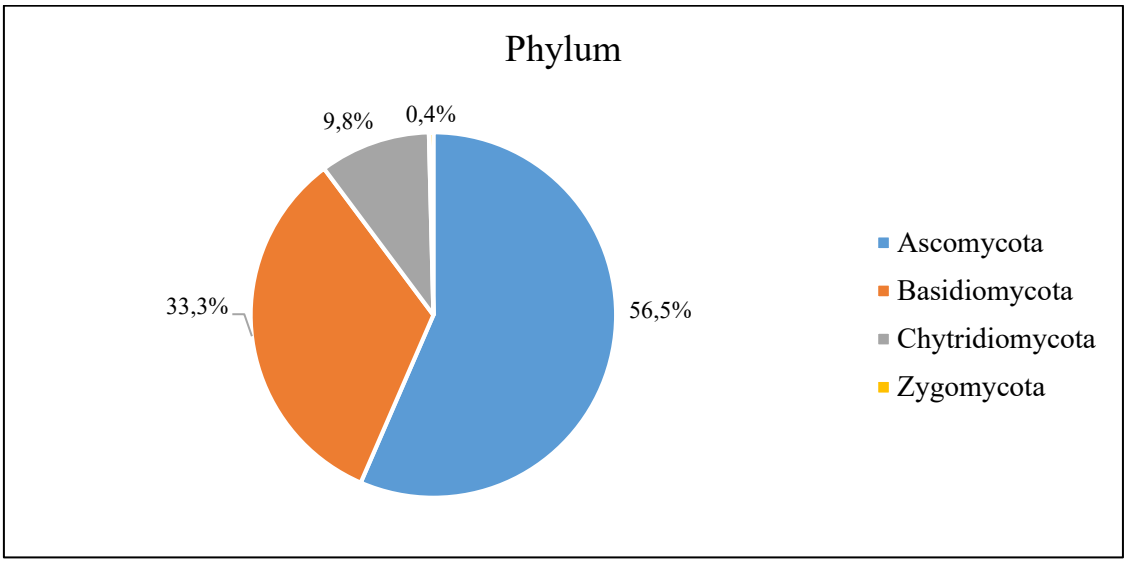

Figure 3. Proportion of fungal sequences belonging to each of the four present phyla.

Species Abundance

A PCA test showed information about the fungal community of needle and peat samples respectively, spatial distribution of data show how similar or different fungal communities are. The positioning of data is resulting in a very small overlap between the two polygons, meaning that there were differences regarding the fungal community composition when needle and peat samples were compared (Figure 4).

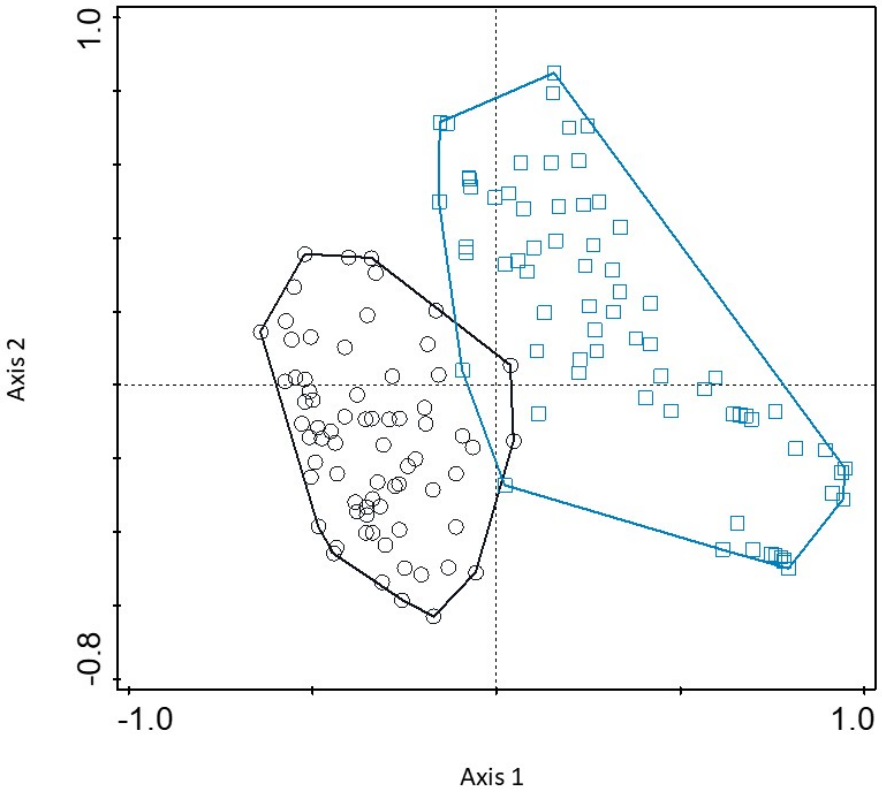

Figure 4. Principle component analysis (PCA) of relative abundance species, needles in black (o); peat in light blue (□). Axis 1 describes 18 % of the variation and axis 2 describes 12 % of the variation

When data from the different time points were analyzed, and needles and peat were analyzed separately, some differences were shown. Differences were shown for needle samples, even though there were small overlaps (Figure 5). Symbols of peat samples, in the figure were not as separated as the needle samples for the same analysis, and all time points except time point one have a large area overlapping meaning a more differentiated fungal community (Figure 6).

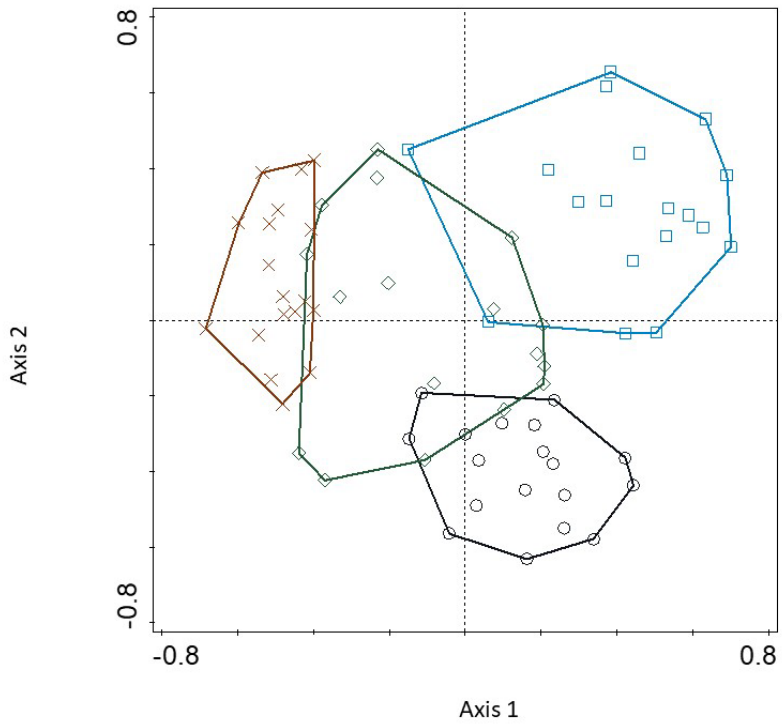

Figure 5. PCA, of the relative abundance of Operational Taxonomic Units (OTUs) among needle samples. Positioning of data in relation to each other show similarities between samples. Time point 1, in black (o); Time point 2, in light blue (□); Time point 3 in green (◊); Time point 4, in brown (×). Axis 1 describes 15 % of the variation and axis 2 describes 11 % of the variation.

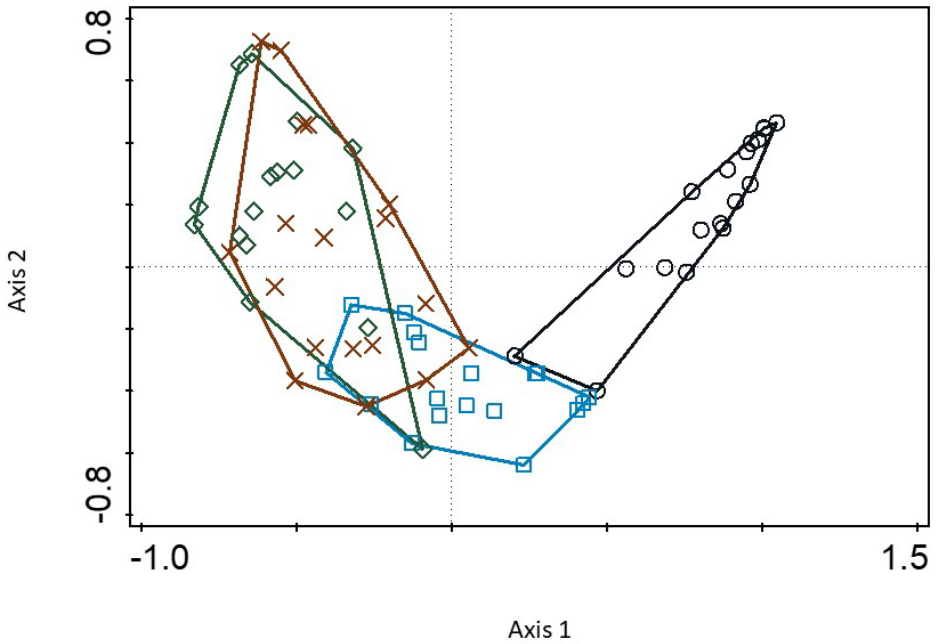

Figure 6. PCA, of the relative abundance of Operational Taxonomic Units (OTUs) among peat samples. Positioning of data in relation to each other show similarities between samples. Time point 1, in black (o); Time point 2, in light blue (□); Time point 3 in green (◊); Time point 4, in brown (×). Axis 1 describes 32 % of the variation and axis 2 describes 10 % of the variation.

Identified Clusters

The second largest identified cluster are defined to *Chlamydomonas* sp. which belongs to the kingdom of algae. Of all the classified OTUs, 30 % of the sequences belonged to algae. The 104 largest clusters were identified by BLAST analysis against the NCBI database, this represent approximately 92 % of all high-quality sequences. The most common cluster, among all fungal clusters was *Phoma* sp. with a proportion of 12 % of all fungal sequences. *Tilletiopsis minor* were the second largest (8 %), *Sydowia polyspora* third (7 %), and *Botrytis cinerea* the fourth (4 %).

The 15 most common species in needle samples represent 45 % of all sequences. The rest of the species are grouped into one category and called “others” (Figure 7- 8). Relative abundance of species in needle samples changed over time, like hinted in the PCA analysis of samples belonging to different time points (Figure 5), but there are no clear differences of the fungal community composition when treatments are compared at the four time points of sampling.

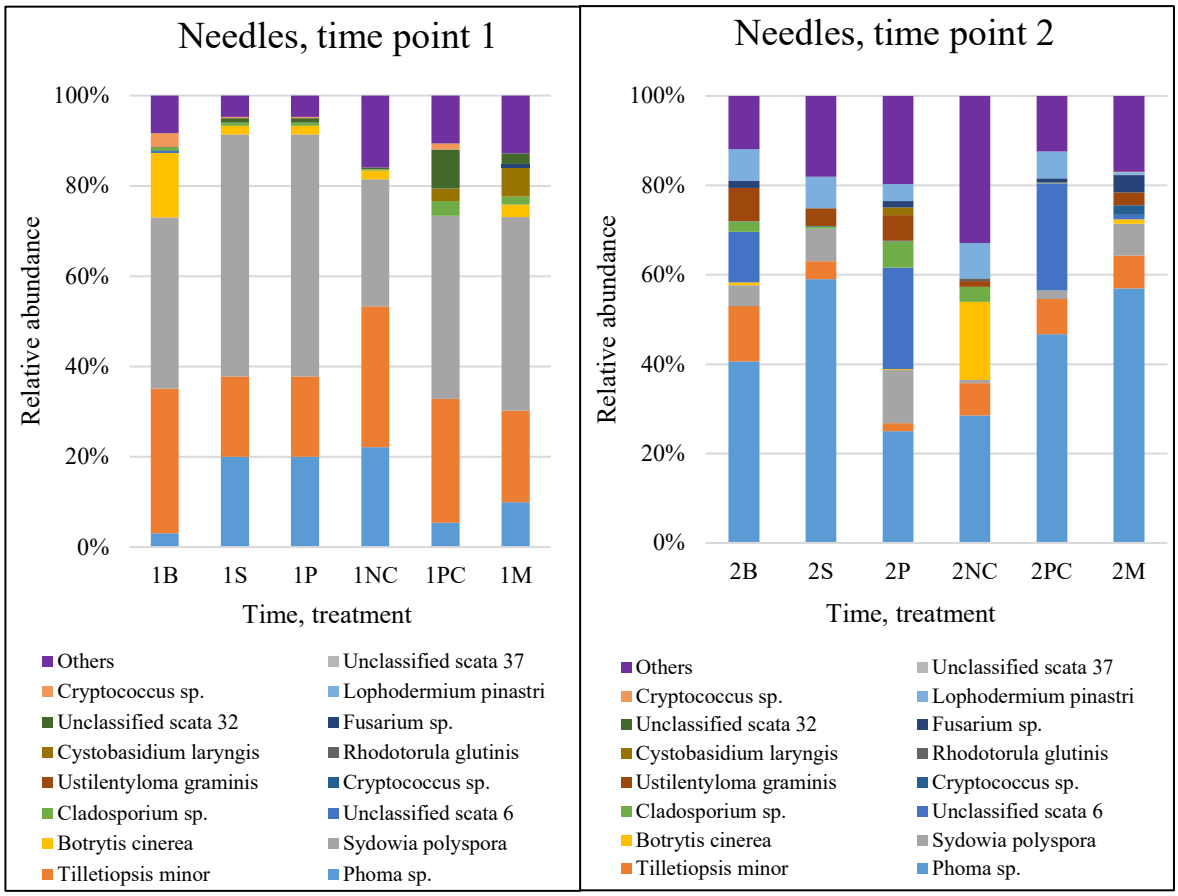

Figure 7. Relative abundance of the 15 most common identified species and “others”, in needle samples, at time point 1 and 2, where B = *Binab*, S = *Serenade*, P = *Prestop*, NC = *Negative control*, PC = *Positive control* and M = *Mikroferm*.

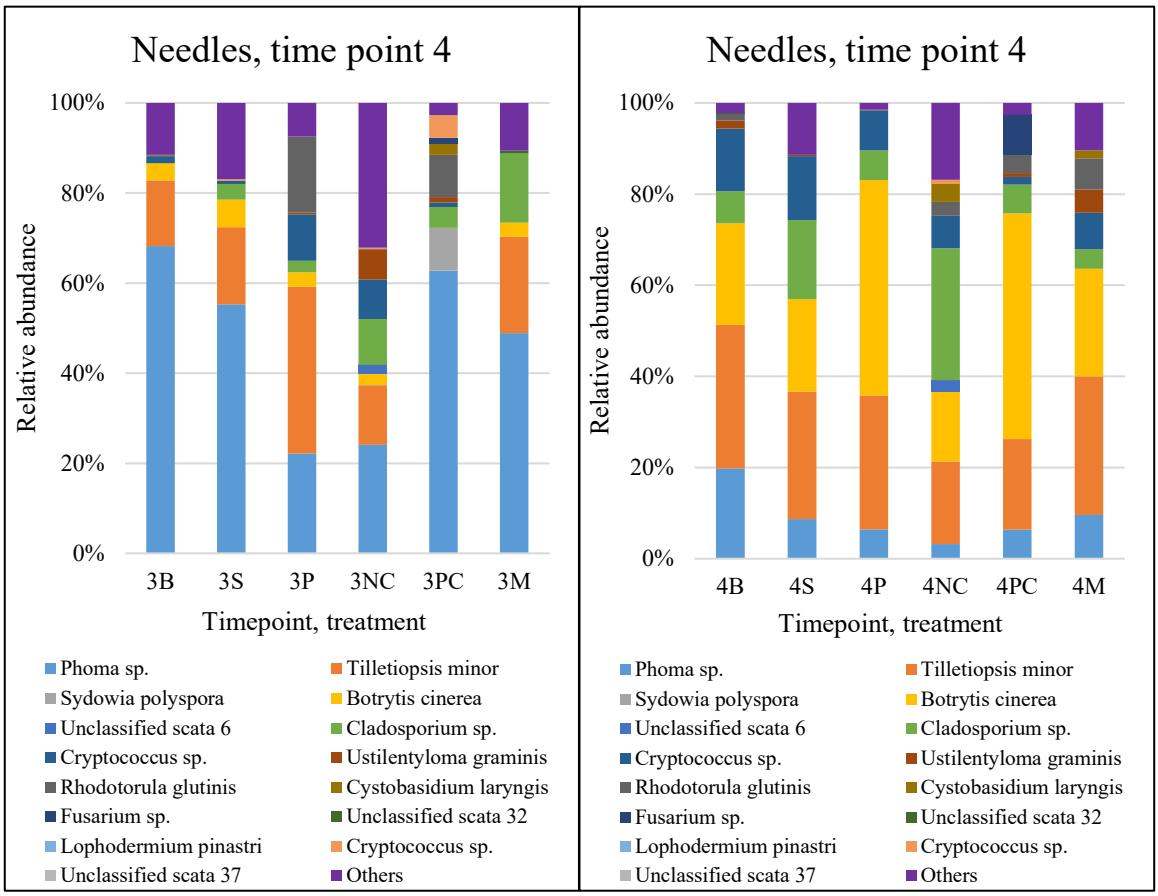

Figure 8. Relative abundance of the 15 most common identified species and "others", in needle samples, at time point 3 and 4, B = *Binab*, S = *Serenade*, P = *Prestop*, NC = *Negative control,* PC = *Positive control* and M = *Mikroferm*.

The 15 most common species identified in peat samples represented 35 % of all the sequences. The rest of the species are grouped and called "others". No significant differences of the fungal community were shown when the treatments were compared at each timepoint (Figure 9-10).

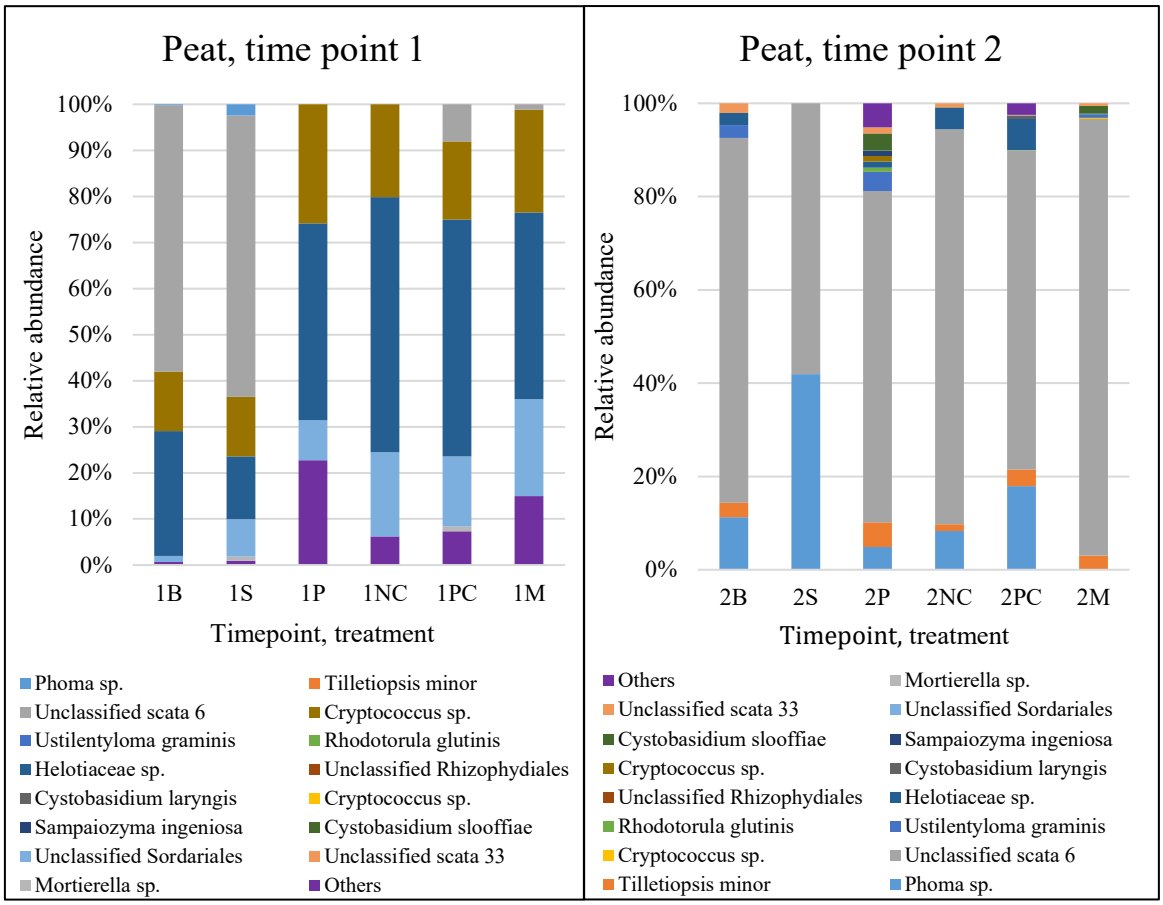

Figure 9. Relative abundance of the 15 most common identified species and "others", in peat samples, at time point 1 and 2, where B = *Binab*, S = *Serenade*, P = *Prestop*, NC = *Negative control*, PC = *Positive control* and M = *Mikroferm*.

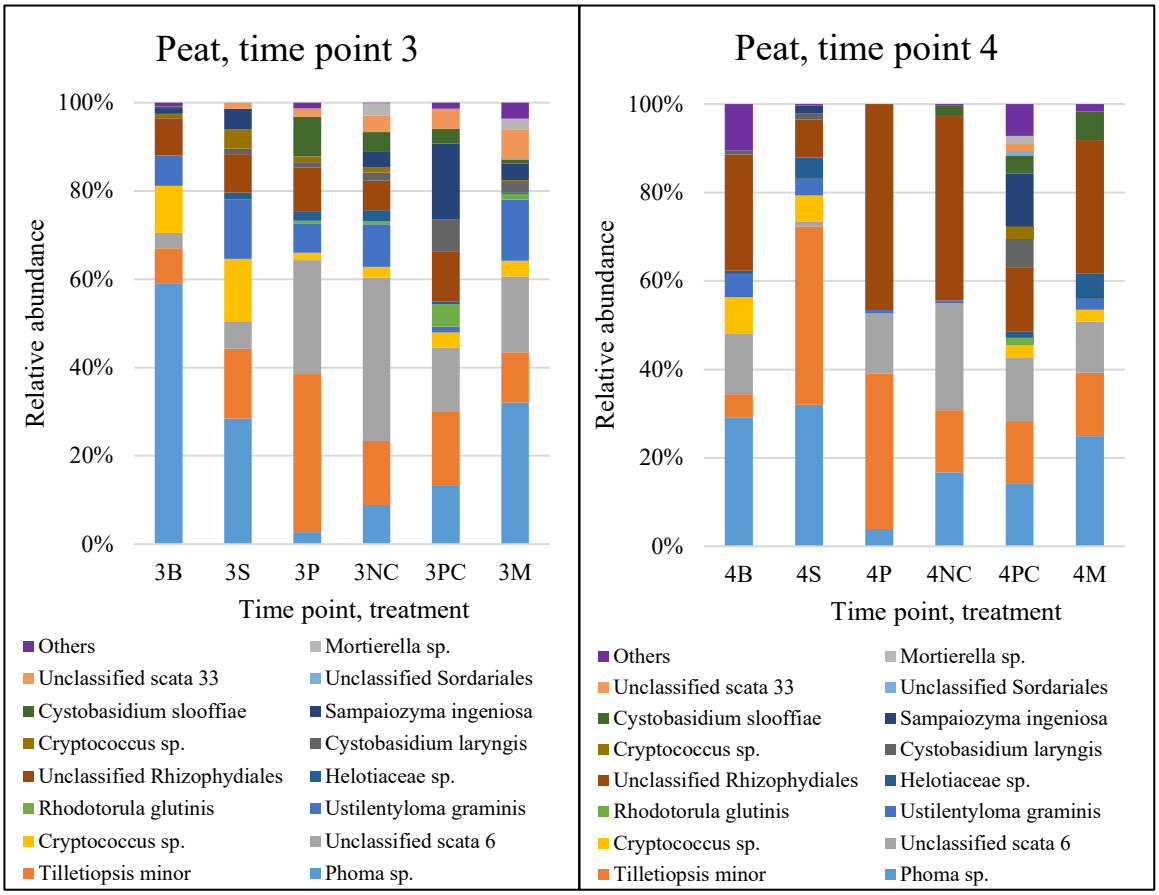

Figure 10. Relative abundance of the 15 most common identified species and "others", in peat samples, at time point 3 and 4, where B = *Binab*, S = *Serenade*, P = *Prestop*, NC = *Negative control*, PC = *Positive control* and M = *Mikroferm*.

Pathogens

Phoma sp. constituted a large proportion of the relative abundance of species in both needle and peat samples. The highest proportion of *Phoma* sp. occurred at time point three, and in the treatment *Binab,* among both needle and peat samples with 68 % for needles and 59 % for peat samples.

The development of relative abundance over time among needle samples differ from peat samples. The mean value of the three samples, from each treatment and each timepoint changed during the studied period (Figure 11). Among needles, the relative abundance of *Phoma* sp. was low in the beginning, increased with time to reach its maximum at either time point two or three, and then decrease for all treatments to the last time point. Variation was shown among the three samples from each treatment, at each timepoint, resulting in a large standard deviation of some samples (Figure 12).

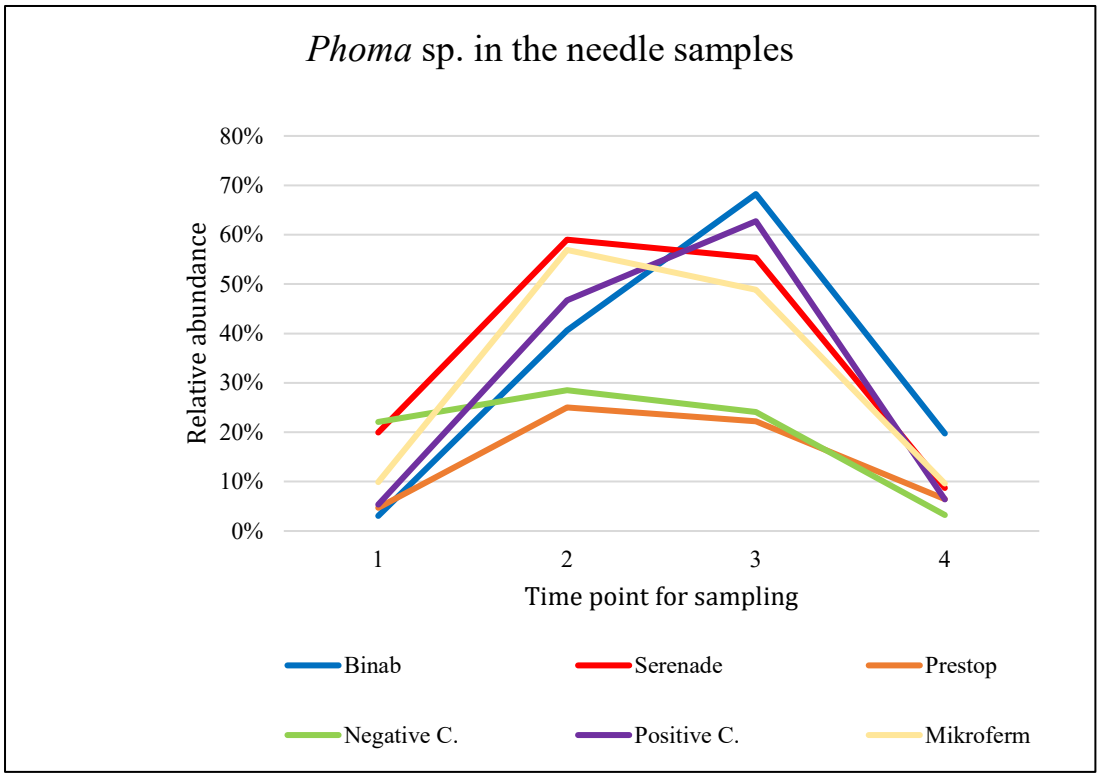

Figure 11. Relative abundance of *Phoma* sp. on needles in the different treatments at the four time points. The figure is based on the mean values of the three samples, taken from each treatment, at each timepoint.

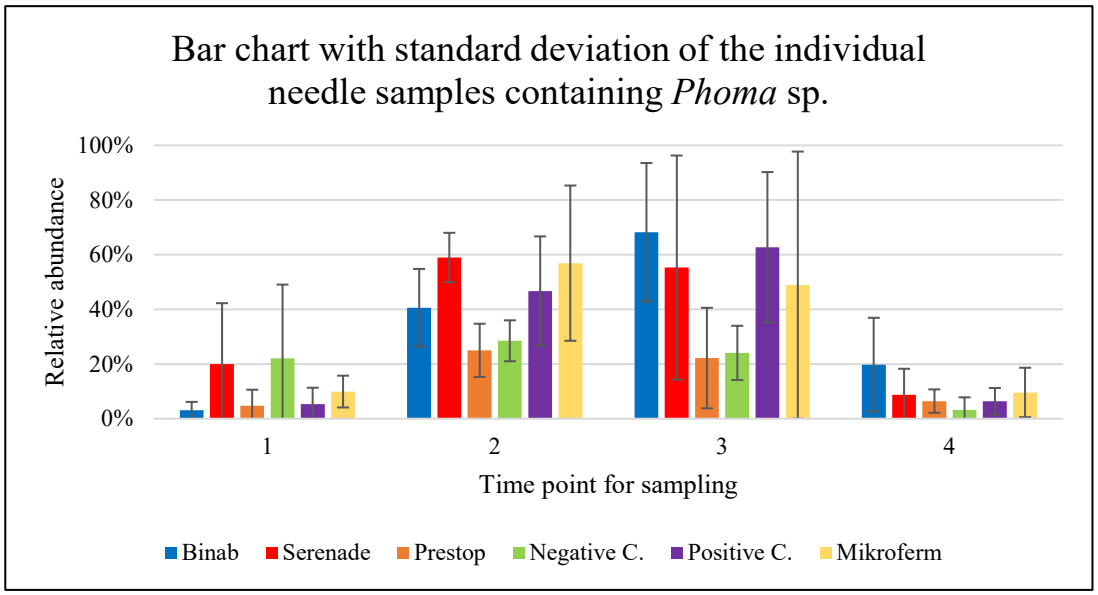

Figure 12. Relative abundance of *Phoma* sp. on needles in the different treatments at the four time points. Additional error bars show standard deviation of the three samples taken from each treatment, at each time point.

In peat samples, the relative abundance of *Phoma sp*. in the treatments *Negative control*, *Positive control*, *Prestop* and *Serenade* increased at period two and then leveled off and kept almost the same abundance (Figure 13). *Phoma* sp. was not present in samples of the treatment *Mikroferm* until time point three were it reached 32 % to then go down to 25 %. A drastic decrease in occurrence between time point three and four can be seen for the treatment *Binab* among the peat samples. There was variation among the three samples from each treatment, at each timepoint, resulting in a large standard deviation of some samples (Figure 14).

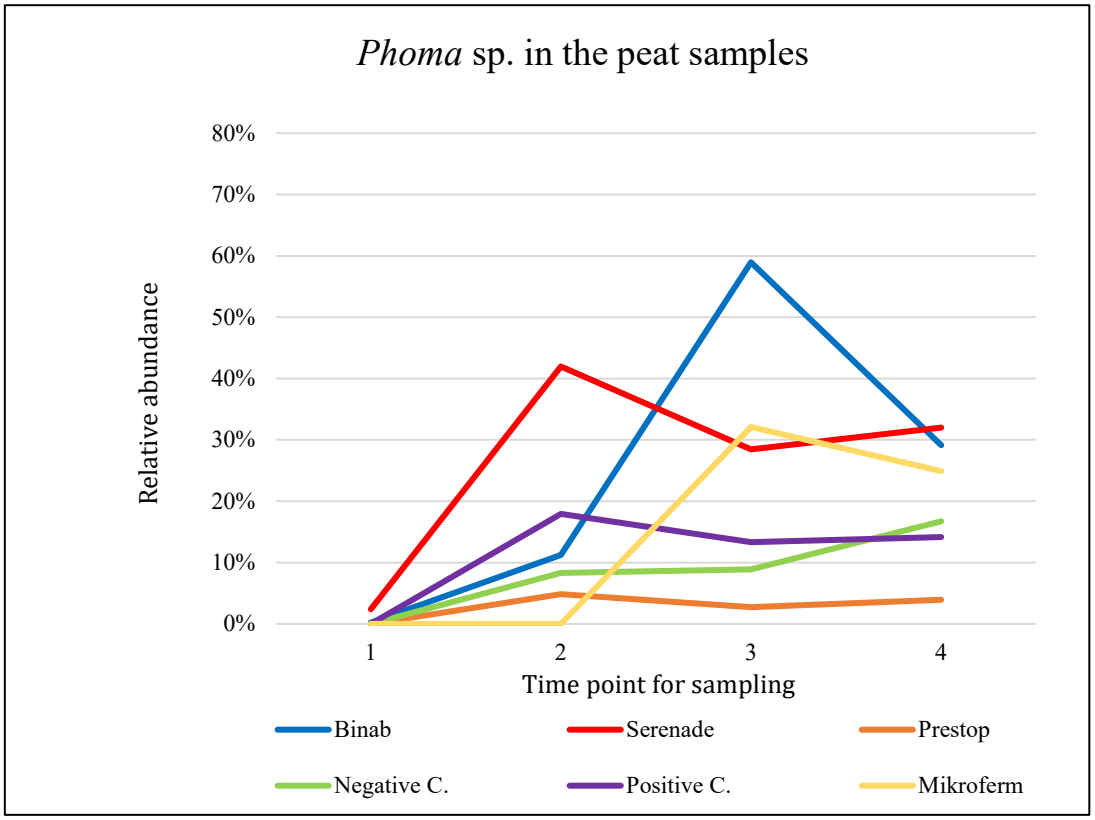

Figure 13. Relative abundance of *Phoma* sp. in peat samples in the different treatments at the four time points. The figure is based on the mean values of the three samples, taken from each treatment, at each timepoint.

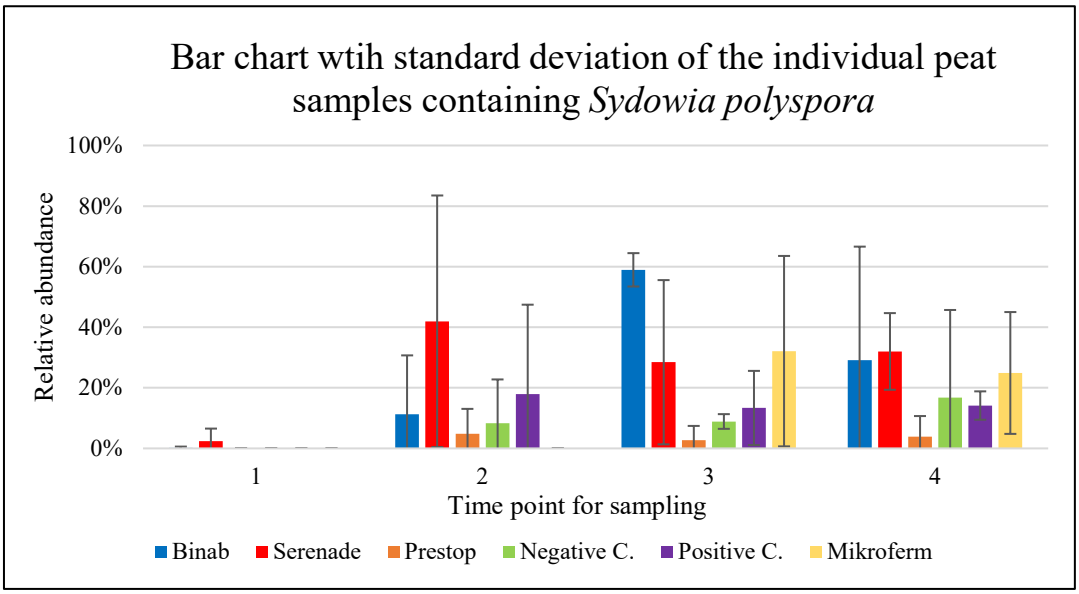

Figure 14. Relative abundance of Phoma sp. in the peat samples in the different treatments at the four time points, with additional error bars showing standard deviation of the samples Additional error bars show standard deviation of the three samples taken from each treatment, at each time point.

Botrytis cinerea was the fourth most common fungus in needle samples (4 %). The relative abundance of *B. cinerea* on needles was following the same pattern for all treatments except for *Binab* and the *Negative control* (Figure 15), the two treatments where the relative abundance of *B. cinerea* were over 10 % at either time point two or three. At the time point one, *Binab* was the treatment with highest relative abundance of *B. cinerea* (14 %) while the rest had 3 % or less. At time point two, *Negative control* was the only treatment with a high proportion (17 %) but decreased like the other treatments to under 5 % at time point three. At time point four, the relative abundance of *B. cinerea* increased remarkably in all treatments up to as much as 47 % and 50 % for *Prestop* and *Positive control*, respectively. The other treatments had a relative abundance between 15- 24 % at that time. *B. cinerea* were absent in peat samples. There was as well variation among the three samples from each treatment, at each timepoint, resulting in a large standard deviation of some samples (Figure 16).

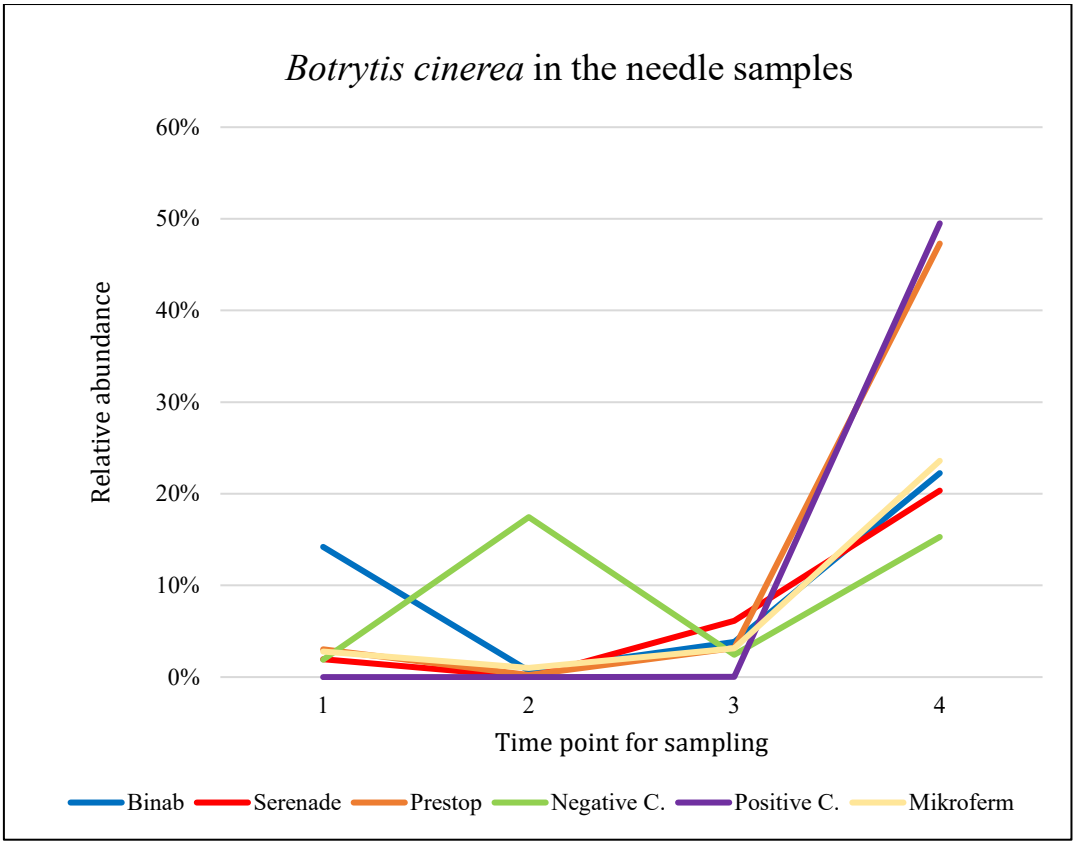

Figure 15. Relative abundance of *Botrytis cinerea* in the different treatments at the four time points, among needle samples. The figure is based on the mean values of the three samples, taken from each treatment, at each timepoint.

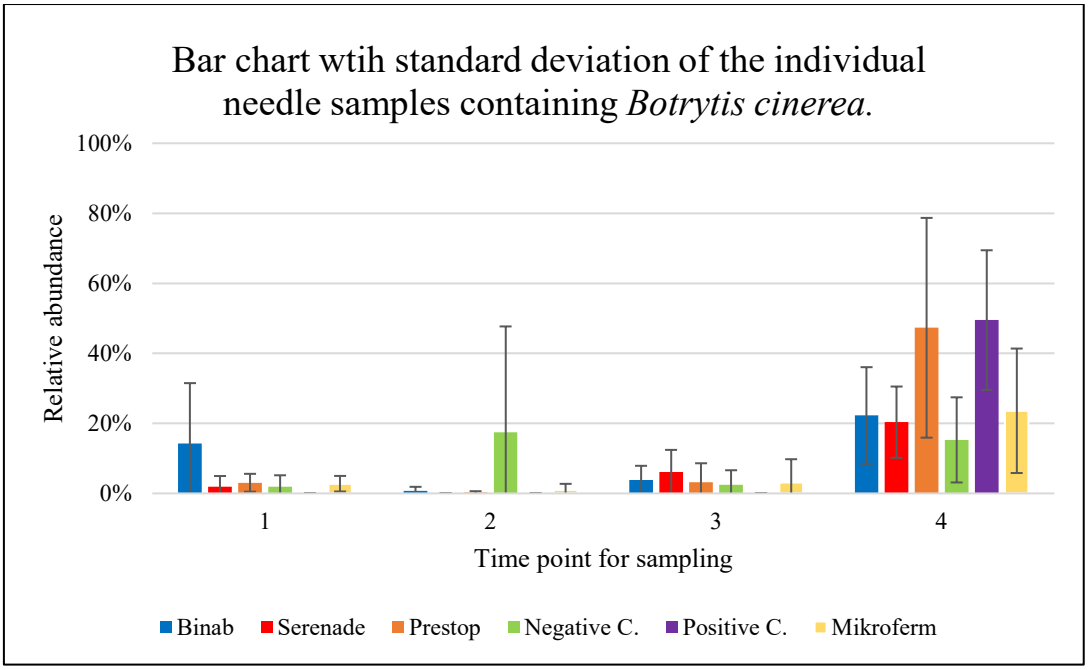

Figure 16. Relative abundance of *Botrytis cinerea.* on needles in the different treatments at the four time points, with additional error bars showing standard deviation of the samples. Additional error bars show standard deviation of the three samples taken from each treatment, at each time point.

The relative abundance of *Sydowia polyspora* in the needle samples are similar for all six treatments. The proportions of *S. polyspora* at the first time point varied between 28 % for *Negative control* and *Prestop* and 54 % for *Serenade* (Figure 17). The relative abundance of *S. polyspora* had a very steep slope and varied from 1 % to 12 % at time point two. Decreasing continues at time point four and three for all treatments except for *Positive control* that increases to 9 % at time point three to then vanish. *S. polyspora* is not present in any of the treatments at time point four. Peat samples lack presence of *S. polyspora* in most treatments, and were it occurs, it is at lower relative abundance than 1 %. Some variation was shown among the three samples from each treatment, at each timepoint, resulting in a large standard deviation of some samples (Figure 18).

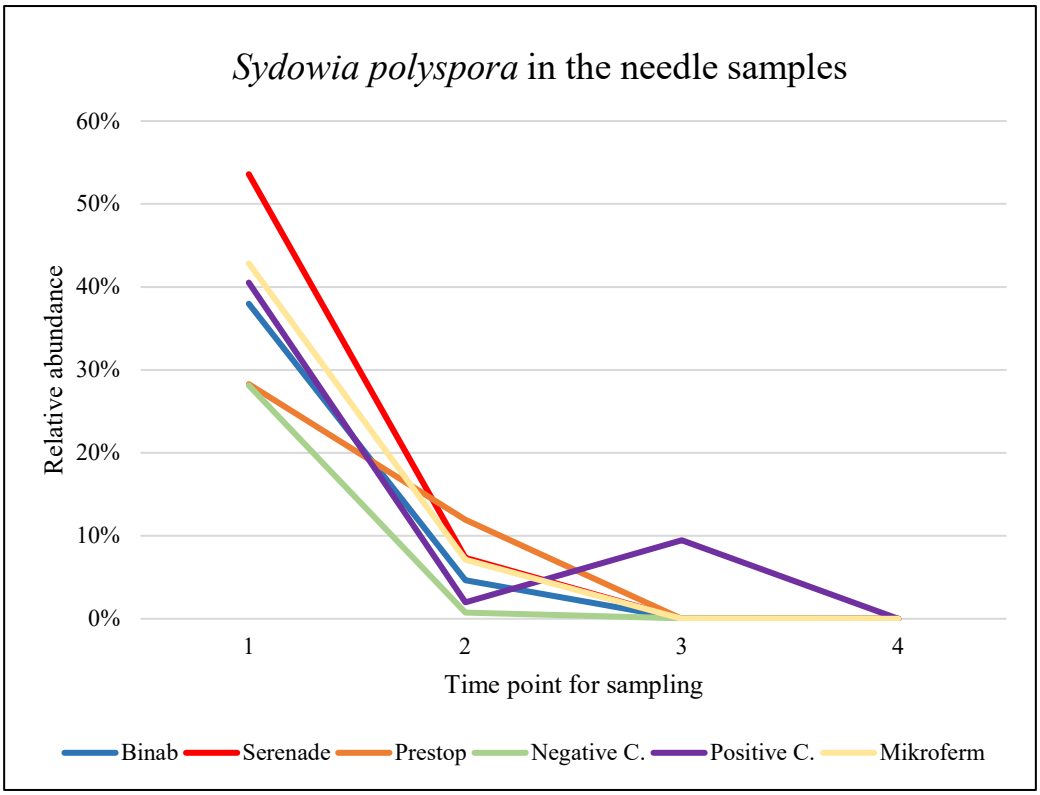

Figure 17. Relative abundance of *Sydowia polyspora* in the different treatments at the four time points, among needle samples. The figure is based on the mean values of the three samples, taken from each treatment, at each timepoint.

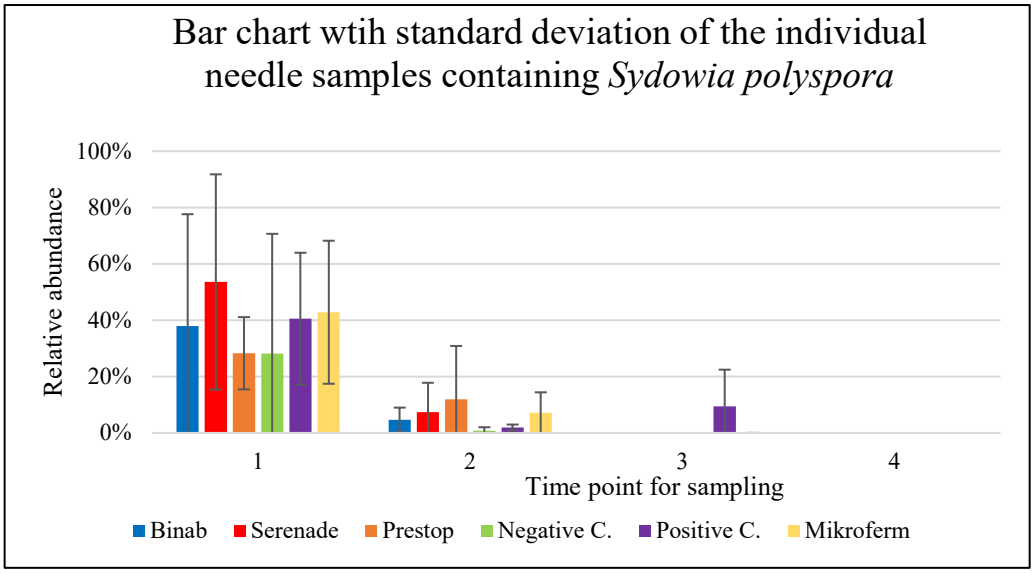

Figure 18. Relative abundance of *Sydowia polyspora*. in the needle samples in the different treatments at the four time points, with additional error bars showing standard deviation of the samples. Additional error bars show standard deviation of the three samples taken from each treatment, at each time point.

Persistence of Biological Control Agents

The components of the biological control agents *Trichoderma atrovirdie* (in *Binab*), *Clonostachys rosea* (in *Prestop*) and *Saccharomyces cerevisiae* (in *Mikroferm*) are present in samples during the studied period. First *T. atrovirdie* were present only in one of the needle samples, namely seedlings of the treatment *Serenade,* at the first time point had a relative abundance of 0,07 %. In peat samples, *T. atrovirdie* were present in *Negative control* at first time point with a relative abundance of 2,8 %, *Positive control* had a relative abundance of *T. artovirdie* of 3,5 % at the first time point and *Serenade* 2,0 % at the first time point. *C. rosea* were present in needles in *Prestop* 3 % at first time point and *Positive control* 5,3 % at the first time point, it was not present in peat. *S. cerevisiae*, biological agent of *Mikroferm* had a proportion of 3,1 % in seedlings of the treatment *Mikroferm* at time point three and 1,3 % in needle samples of *Negative control* at time point three and was not present in peat.

Discussion

These four biological agents have been tested in earlier studies on different plant crops. There are many successful examples when they have shown great effectiveness in decreasing pathogen occurrence and growth promoting properties (Edwards & Seddon, 1992; Stinson et al., 2003; Shalaby & El-Nady, 2008). In this study, the four biological agents where directly compared, looking at species abundance on needles and peat of seedlings, development of the fungal community over time, and their growth promoting properties on the seedlings.

Height Increment, Diameter and Mortality

An even height increment of seedlings in the nursery is wanted. A large stem diameter of seedlings will also bring a higher chance of survival, since a more robust plant can handle more physical damage. The results from this study shows that seedlings of the treatment *Binab* had the largest mean height increment. However, the 6 mm higher mean increment, compared to *Negative control* with the least mean height increment, gave no significant difference of higher mean height in the statistical analysis.

From this study, no conclusions can be drawn regarding the ability of growth promotion in the biological agents, even though other studies have shown results of growth promotion in seedlings. A study by Hohmann et al. (2011) showed that isolates of *Trichoderma* promoted height growth by 16 % compared with the control, when applied on *Pinus radiata* seedlings. Applying isolates of *Trichoderma* cannot directly be compared with the biological agent *Binab* that also consists of other ingredients, promoting growth effects of *Binab* in this study cannot then be taken for granted. Important to realize is the time aspect of the study, the seedlings were studied from the very early stage and the mean height increment of seedling during the time was rather small. If seedlings were studied during a longer period, a more significant difference could be shown. Worth mentioning is also the recommended spraying interval of agents, some companies recommended a more frequent spray interval than what was done. This together could have given another result showing a significant difference in height increment of seedlings sprayed with biological control agents, with growth promoting effects, compared to the *Negative control* sprayed with water only.

Statistical analyses of diameter at the end of the summer gave results that confirmed the hypothesis that not all means are equal. The seedlings sprayed with the treatments *Serenade* and *Prestop* had a significant smaller diameter at the last measurement compared to *Binab* and *Negative control*, that were the two treatments with largest diameter at the end.

According to the seedling overall height and diameter, at the time for the height and diameter measurement, it can be assumed that the seedlings were not very robust and had fragile stems. The influence of material and method on diameter measurement cannot be disregarded, since the measuring with the sliding caliper, a very sensitive tool, can have contributed to an unwanted compression of the stem when measuring the diameter.

Dead seedlings and seedlings with symptoms of any fungal infection was planned to be removed during summer by staff at forest nursery, or at the time for sampling and treatment. In total, 49 of 1430 seedlings (<1 %) was taken away as a cause of death during summer. From this study we can make the conclusions that mortality was not significantly different when means where compared. The practices at the forest nursery resulted in a high survival of seedlings in the experiment, and seedlings without symptoms of infection. Furthermore, the definition of a dead seedling, or time point were a seedling is dead is unclear and subjective. In the study, seedlings that would probably not survive an out planting in cause of delayed emergence or growth, were not taken away. The result may have been different if these seedlings instead were classified as dead if they would not survive an out-planting, giving a result showing larger differences between treatments. Although another result, this action could instead give results correlating with choice of good cassettes in the beginning of the study and therefore factors as delayed or bad emergence and drought in the earliest stages.

Fungal Communities

The fungal communities and species abundance of the rhizosphere of seedlings in forest nurseries has been studied before (Vujanovic et al. 2007; Menkis et al. 2016). The size order of the identified fungal phylum in this study, has similarities to these studies, with most fungi belonging to the phylum Ascomycota (Figure 3). Important to realize is the fact that the most common fungal pathogens identified, *Phoma* sp., *Sydowia polyspora* and *Botrytis cinerea* belong to this phylum. When looking at the grouping into needle and peat samples in the Principal Component Analysis (PCA) (Figure 4), we can make the assumptions that the very small overlap between the polygons, each corresponding to needle and peat samples, means that some species are more common in needle samples and some are more common in peat samples. Even though the seedlings are highly affected by the nursery management practices, like irrigation water and booming of seedlings, there are a significant difference between the fungal community of peat and needle samples. The difference could be explained by the function and ecology of the fungi. The ecology and the lifecycle determine whether plant tissue or peat is the most suitable environment to live in or

on, and will therefore have a bearing on the probability of the fungus to be found there.

When the fungal communities of samples belonging to different treatments were compared, no significant difference were shown. Hence, time point had a stronger effect on the fungal community than treatment had. The hypothesis set questioning the fungal development over time, can be answered, the fungal community do change over time and there cannot from this study be drawn any conclusions on the direct effects of products on the fungal community. But to put it in another way, it is a rather desired result since changing the community in fact is not the main purpose of spraying the biological control agents. More precisely, the main purpose is to control one or a few pathogens that occurs around the seedlings. The seedlings were very small at the beginning of the study, having larger seedlings in the beginning would maybe lead to a denser canopy which would increase the humidity among seedlings and make seedlings more susceptible for disease, this could result in more evident differences, when comparing the seedlings of different treatments.

Pathogens

In this case, when no significant difference of impact on the fungal community is shown, it is as well not possible to answer the treatments ability to control or inhibit certain pathogens. As mentioned, earlier studies have shown good performance in disease management from the control agents on certain pathogens. The figures based on proportion of the fungal pathogens *Botrytis cinerea*, *Phoma* sp. and *Sydowia polyspora* (Figure 11- 18) include all treatments, and it is possible to compare *Negative control* to the biological control agents. The figures reveal again that no significant differences of relative species abundance (here pathogens) are shown when comparing treatments.

The most common identified cluster belongs to the genus *Phoma*, it is the most common identified cluster in both needle and peat samples. In needle samples, the proportion of *Phoma* sp. are low in the beginning and at the end of the study period. The figure of relative abundance (Figure 11) is based on the mean value of the three samples of each treatment and each timepoint, the bar chart instead (Figure 12) show the variation among these three samples. A large variation among samples is shown especially at time point three, for all treatments. To give an example, samples of the treatment *Binab* at the third time point had the following relative abundance of *Phoma* sp.; 35 %, 73 %, and 96 %, giving the mean value of approximately 70 % (Figure 11). The relative abundance of *Phoma* sp. in the peat samples are low in the beginning and increases with time, and variation among samples are shown (Figure 14). Other studies done in forest nurseries has found *Phoma* sp. as a common pathogen on seedlings, Stenström et al. (2013) found the genus to be the second most common identified cluster (19,1 %). The genus includes not only pathogens, but since

it is the largest cluster identified in this study done, the possible impact and harm on seedlings should not be ignored.

The proportion of *Botrytis cinerea* in needle samples are low in the beginning of the study period and increasing during the summer (Figure 15). Symptoms of damage on seedlings caused by *B. cinerea* are often visible in the later stages of the fungal life cycle. Increasing proportion of fungal spores at this stage is unfortunate since the seedlings that are planned for delivery, will be put in cardboard boxes. As conditions get moister and seedlings are tightly packed into the boxes, the fungal infection can be evident when the fungus quickly starts to grow and therefore seedlings are disabled. If unlucky, infected seedlings continue to spread the disease to seedlings without infection. In addition, the autumn temperature and humidity are favoring disease development (Petäistö, 2006). Dead material laying on the top peat layer, needles in this case, can stand for a huge source of the inoculum, although this study showed that the peat itself did not have large proportions of *B. cinerea*. Similarly, is the case for needles near the stem base that are in a dark, moist and probably dense environment (Mittal & Wang, 1987). Capieau et al. (2004) could present *Binab* as a good performing biological control agent when seedlings where inoculated with the fungus and put in growth room assays. Instead, the study at Kilåmon was aiming at having normal nursery practices and no growth room assays.

Results from the first sampling showed a high proportion of *Sydowia polyspora* in needle samples (Figure 17). This means that the pathogen constitutes a large part of the fungal community before the start of the study. In conjunction with the sampling of cassettes to the study, an inventory of seedlings was done to determine emergence. Results from the inventory showed absence of germinated seedlings in the treatment of *Binab* where as much as 12 % of the pots in the cassettes were empty. *S. polyspora* has in literature been associated with poor germination, the study by Ridout et al. (2018) showed that *S. polyspora* was among the most common species and in fact slowed down or inhibited emergence of *Pinus ponderosa* seedlings under nursery conditions. Problematic through a nursery perspective since seedlings need to grow fast in the beginning to withstand physical damages from nursery practices. Good emergence, lignification of the stem, and early height growth are therefore wanted in order to manage further challenges.

When studying the development of the most common fungal pathogens, it may be desirable to get a true number of spores present in the samples. Since there are the proportions that are studied, instead the patterns of fungal pathogens, and the certain pathogens can be studied. The fungi are taking turns in being the largest proportion. Using relative numbers of species abundance are necessary in this study because of the equimolar mix of DNA that was done before sent for analysis. This can only give us a hint of the development of a special pathogen at species level, changes in species proportion could be as a cause of an increase of that species, or because of a decrease of another.

So even if the study can not separate the performance of the treatments, the information regarding the development of pathogens, even though biological control agents are present, is important information not to forget, since development of fungi, linked to its lifecycle, is crucial for effective pest management. To summarize the studied pathogens, the patterns shows how they alternate in being the species of highest proportion, *S. polyspora* is dominating in the beginning, and *B. cinerea* is taking over towards the end, while *Phoma* sp. shows a more irregular pattern during the studied time.

Large standard deviations were shown for the relative abundance, of the three samples, from each treatment, and these large variations are problematic when trying to discuss the true development of certain pathogens, during the studied time. The standard deviation is directly connected with the number of samples, having only three samples from each time and treatment, also comes with a risk of having large variations and standard deviations. This problem could be overcome by having a larger number of samples from each treatment and timepoint.

Components of the Biological Control Agents

The main purpose of the analysis of the components of the biological control agents, was to get a hint of how long time after application the biological control agent were left on seedlings. The results showed that components of the biological control agents were present at the first time point, this means that they were at place before application. The sampling was always done before the treatment, so presence at first time point indicates a contamination of seedlings. It is not possible to determine the actual impact this contamination has on the results, but it probably happened due to traces of the biological control agents on the hands or gloves of the people doing the sampling. The relative abundance of the components of the biological control agents at time point 1, was relatively low, and therefore the impact on the result could be negligible.

The biological agents are present in just a few samples, and were it occurs it is of a low relative abundance. This indicates that the biological agents sprayed, have vanished at some unknown time, during the three weeks after application. The spraying interval used in this study will therefore lead to occasions where no biological agent is present on either seedling or in the peat. For a fear that the biological control products must constantly be at place to influence disease management, a more frequent spraying interval could be done. The recommended spraying interval according to the manufacturers' advices were not very specific, but for *Mikroferm* it was once a month, repeatedly every other week. For *Binab*, two to five times before freeze storage, *Serenade* six treatments seen from little plant to plant ready for delivery. The manufacturers instruction for the treatment *Prestop*, clarify

that *Prestop* can be sprayed together with other chemicals, and then perform better, but this was not relevant in this study since the aim was to investigate the biological agents alone. The spraying interval of this study were four times, with three-weeks interval, so it could have been possible to do the spraying more often than that.

Future studies

Future studies could aim at testing how different nursery management practices contribute to the relative species abundance between treatments and the development during the studied period. Another suggestion for further studies is to expose seedlings to diseases, either by inoculation of spores of fungal pathogens, or to create an environment even more favorable for fungal pathogens. An evident fungal infection of the seedlings could give a hint of the biological control agents ability to control diseases in an environment that favors pathogens.

Conclusions

Overall, the experiment generated healthy seedlings. Mean height increment did not differ much between treatments, while diameter among of the seedlings belonging to *Binab* and *Negative control* had significantly larger diameter at the end. The fungal communities in the six different treatments did not differ significantly and time had a stronger effect on the fungal community and relative species abundance than the treatment had. The three fungal pathogens with the highest proportions were *Phoma* sp., *Botrytis cinerea* and *Sydowia polyspora*, they alternated in taking turns to be the pathogen of highest proportions during the studied period. Lastly, fungi that were a part of the biological control agents were no longer left on needles and soil three weeks after application.

Limitations

Most of the clustered sequences belonged to algae and was not included in further analysis; this could be an effect of the way the substrate (peat) was gathered. Algae occurred on the very top of the peat layer, and the substrate was usually taken from the top layer of the peat to not harm the root systems of the seedlings too much. Furthermore, keeping the seedlings for a longer period at the nursery would maybe lead to more visible symptoms of infection, and more significant differences among treatments, giving both biological control agents and fungal pathogens a better chance of further development. Lastly, due to misunderstanding, did not seedlings of the treatment *Positive control* get the practices that represent the ordinary treatment of Kilåmon nursery and can therefore not represent the exact nursery practices.

Acknowledgements

I want to thank my supervisors Åke Olson and Audrius Menkis for always being available for questions and discussion, and the help with field work and analysis. Thanks to *Svenska Skogsplantor*, *NordGen* and Department of Forest Mycology and Plant Pathology. Also, a huge thanks to Maria Johansson and Katarina Ihrmark for their help in the lab.

References

Agriton Sverige AB. (2019) *Mikroferm.* https://www.bokashi.se/dokument/bibliotek/Image/MIKROFERM%20Broschyr%202018.pdf [2020-05-26].

Andrade-Linares, D. R. & Franken, P. (2013) *Fungal Endophytes in Plant Roots:* Taxonomy, Colonization Patterns, and Functions. R. In: Aroca, R. *Soil Biology.* Vol. 37. Springer-Verlag Berlin and Heidelberg GmbH Co. K.

Aveskamp, M. M., de Gruyter, J. & Crous, P. W. (2008) *Biology and recent developments in the systematics of Phoma, a complex genus of major quarantine significance*. (Fungal Diversity 31: 1-18).

Bayer. (2017) *Serenade ASO.* https://www.cropscience.bayer.se/~/media/bayer%20cropscience/scandinavia/sweden/produkter/produkter/serenade_aso_2017.ashx [2020-05-26].

Bloomberg, W. J. (1985) *The Epidemiology of Forest Nursery Diseases*. Canada. (Phytopathol. 23: 83-96).

Bödeker, I., Lindahl, B. D, Olson, Å & Clemmensen, E. *Mycorrhizal and saprotrophic fungal guilds compete for the same organic substrates but affect decomposition differently*. (Functional Ecology 30: 1967-1978).

Capieau, K., Stenlid, J. & Stenström, E. (2004) *Potential for Biological Control of Botrytis cinerea in Pinus sylvestris Seedlings*. Taylor & Francis. (Scandinavian Journal of Forestry Research 19: 312-319).

Debabov, V.G. (1982) *The Industrial Use of Bacilli*. In: Dubnau, D. A. *The Molecular Biology of the Bacilli*. Academic Press.

Elad, Y., Williamson, B., Tudzynski, P. & Delen, N. (2007) *Botrytis spp. and Diseases They Cause in Agricultural Systems- an Introduction*. In: Elad et al. *Botrytis: Biology, Pathology and Control.* Dordrecht, The Netherlands, Springer. pp 1-8.

Edwards, S. G. & Seddon, B. 1992. Bacillus brevis as a biocontrol agent against Botrytis cinereal on protected Chinese cabbage. In: Verhoeff, K., Malathrakis, N. E. & Williamson, B. Recent Advances in Botrytis Research. Pudoc Scientific Publishers, The Netherlands. (pp 267-271).

European Commission. 2020. Report from the commission to the European parliamenr and the council. Brussels.

FSC. (2010) *Svensk skogsbruksstandard enligt FSC med SLIMF-indikatorer*. https://se.fsc.org/preview.svensk-skogsbrukstandard-fsc.a-771.pdf [2020-05-26].

Hallsby, G. 2013. *Plantering av barrträd*. In: Skogsskötselserien nr 3. Skogsstyrelsens förlag.

Hassan Dar, G.H., Beig, M.A., Ahanger, F.A., Ganai, Nadeem, A. & Ahangar, A. (2011) *Management of Root Rot Caused by Rhizoctonia solani and Fusarium Oxysporum in Blue Pine (Pinus wallichiana) Through use of Fungal Antagonists*. (Asian Journal of Plant Pathology 5 (2): 62-74).

Hohmann, P., Jones, E. E., Hill, R. A. & Stewart, A. (2011) *Understanding Trichoderma in the root system of Pinus radiate: associations between rhizosphere colonization and growth promotion for commercially grown seedlings*. (Fungal biology 115: 759-767).

Johansson, K., Hajek, J., Sjölin, O. & Normark, E. (2014) *Early Performance of Pinus sylvestris and Picea abies - A Comparison Between Seedling Size, Species, and Geographic Location of the Planting Site*. (Scandinavian Journal of Forestry Research 30(5).

Lilja, A. & Poteri, M. (2013) *Seed, Seedlings and Nursery Diseases*. In: Gonthier, P. & Nicolotti, G. *Infectious Forest Diseases*. CABI. Boston. pp 567-591.

Lilja, A., Poteri, M., Petäistö, R-L. Kurkela, T. & Kasanen, R. (2010) *Fungal Diseases in Forest Nurseries in Finland*. (Silva Fennica 44(3): 525-545).

Menkis, A., Burokiene, D., Stenlid, J. & Stenström, E. 2016. High-throughput sequencing shows high fungal diversity and community segregation in the rhizosphere of container-grown conifer seedlings. (Forests 7(44): 1-15)

Mittal, R. K. & Wang, B. S. P. (1987) *Fungi associated with seeds of eastern white pine and white spruce during cone processing and seed extraction*. (Canadian Journal of Forest Research 17: 1026-1034)

Mittal, R. K., Singh, P. & Wang, B. S. P. (1987) *Botrytis: a hazard to reforestation*. (European Journal of Plant Pathology 17: 369-384).

Oliva, J., Boberg, B. J. Hopkins, A. J. M & Stenlid, J. (2013) *Concepts of Epidemiology of Forest Diseases*. In: Gonthier, P. & Nicolotti, G. *Infectious Forest Diseases*. CABI. Boston. pp 1-28.

Paungfoo-Lonhienne, C., Kit Yeoh, Y., Rup Pinaki Kasinadhuni, N., Lonhienne, T. G. A, Robinson, N., Hugenholtz, P., Ragan, M. A. & Schmidt, S. 2015. *Nitrogen fertilizer dose alters fungal communities in sugarcane soil and rhizosphere*. (Scientific reports 5 (8678): 1-6).

Petäistö, R.-L. (2006) *Botrytis cinerea and Norway spruce seedlings in cold storage*. (Baltic Forestry 12: 24-33).

PEFC. (2015) *PEFC skogscertifiering -Vi tar ansvar i skogen.* https://pefc.se/wp-content/uploads/2015/06/PEFC_broschyr_L%C3%85GUPPL%C3%96ST_150316.pdf [2019-09-30].

Ridout, M. & Newcombe, G. (2018) *Sydowia polyspora is both a Foliar Endophyte and a Preemergent seed pathogen in Pinus ponderosa*. (Plant Disease 102: 640-644).

Robbins, P & Sharp, J.T. (2008) *Producing and Consuming Chemicals: The Moral Economy of the american Lawn*. In: Marzluff et al. *Urban Ecology*. Springer. pp 425-451.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W. & Fungal Barcoding Consortium. (2012) *Nuclear Ribosomal Internal Transcribed Spacer (ITS) Region as a Universal DNA Barcode Marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America*. Vol. 109 (16): 6241-6246.

Shalaby, M. E-S. & El-Nady, M. F. (2008) *Application of Saccharomyces cervisiae as biocontrol agent against Fusarium infection of sugar beet plants*. (Acta Biologica Szegediensis 52(2): 271-275).

Skogsstyrelsen. 2018. *Skogsstyrelsen statistikdatabas. Använd föryngringsmetod, som andel (%) av avverkad areal efter landsdel och föryngringsmetod*. http://pxweb.skogsstyrelsen.se/pxweb/sv/Skogsstyrelsens%20statistikdatabas/Skogsstyrelsens%20statistikdatabas__Atervaxternas%20kvalitet/JO0311_1.px/?rxid=03eb67a3-87d7-486d-acce-92fc8082735d [2020-06-09].

Smith, V. L., Wilcox, W. F. & Harman, G. E. (1990) *Potential for biological control of Phytopthora root and crown rots of apple by Trichoderma and Gliocladium spp*. (Phytopathology 80: 880-885).

Stenström, E., Ndobe, N. E., Jonsson, M., Stenlid, J. & Menkis, A. (2013) *Root-associated fungi of healthy-looking Pinus sylvestris and Picea abies seedlings in Swedish forest nurseries.* (Scandinavian Journal of Forest Research 29(1): 12-21).

Stinson, M., Ezra, D., Hess, W. M., Sears, J. & Strobel, G. (2003) *An endophytic Gliocladium sp. of Eucryphia cordofolia producing selective volatile antimicrobial compounds*. Elsevier. (Plant Science 165: 913-922).

Sturrock, R. N., Frankel, S. J., Brown, A. V., Hennon, P. E., Klienjunas, J. T., Lewis, K. J., Worrall, J. J. & Woods, A. J. (2011) *Climate change and forest diseases*. (Plant Pathology 60: 133-149).

Svenska Skogsplantor. (2019) *Plantproduktion.* https://www.skogsplantor.se/Plantor/Plantproduktion/. [2020-05-26].

Verdera. 2019. *PRESTOP biofungicide in integrated pest management*. http://verdera.fi/index.php/download_file/view/555/369/ [2020-06-08].

Vujanovic, V., Hamelin, R. C., Bernier, L., Vujanovic, G. & St-Arnaud, M. (2007) *Fungal Diversity, Dominance, and Community Structure in the Rhizosphere of Clonal Picea mariana Plants Throughout Nursery Production Chronosequences*. Springer. (Microbial Ecology 54: 672-684).

Widmer, T-L. (2014) *Screening Trichoderma species for biological control activity against Phytophtora ramorum in soil*. Elsevier. (Biological control 79: 43-48).

Won, J. S., Choub, V., Kwon, J-H., Kim. D-H. & Ahn, Y-S. (2018) *The Control of Fusarium Root Rot and Development of Coastal Pine (Pinus thunbergii Parl.) Seedlings in a Container Nursery by Use of Bacillus lichenformis MH48*. (Forests 10(6): 1-12).

Woo, L-S., Ruocco, M., Vinale, F., Nigro, M., Marra, R., Lombardi, N., Pascale, A., Lanzuise, S., Manganiello, G. & Lorito, M. (2014) *Trichoderma-based Products and their Widespread Use in Agriculture*. (The Open Mycology Journal 8: 71-126).

Attachments

Table 2. Mean height (mm) of seedlings belonging to each of the six treatments at the first measurement

Treatment	N*	Mean height	StDev*[1]	95% CI
Binab	50	23,3	4,1	(22,0; 24,5)
Mikroferm	50	22,0	5,8	(20,7; 23,3)
Negative C.	50	23,4	3,9	(22,1; 24,7)
Prestop	50	23,6	4,7	(22,3; 24,9)
Positive C.	50	22,8	3,4	(21,5; 24,1)
Serenade	50	23,7	5,3	(22,4; 25,0)

* The number of seedlings measured.

*[1] Standard deviation.

Table 3. Mean height (mm) of seedlings belonging to each treatment at the last measurement

Treatment	N*	Mean height	StDev*[1]	95% CI
Binab	50	64,6	14,2	(61,2; 68,1)
Mikroferm	50	61,2	9,5	(57,8; 64,6)
Negative C.	50	58,7	13,6	(55,3; 62,1)
Prestop	50	59,1	10,0	(55,6; 62,4)
Positive C.	50	63,4	12,2	(60,1; 66,9)
Serenade	50	63,2	13,5	(59,8; 66,6)

* The number of seedlings measured.

*[1] Standard deviation.

Table 4. Mean diameter (mm) of stem of seedling at the last measurement

Treatment	N*	Mean diameter	StDev*[1]	95% CI
Binab	50	1,70	0,25	(1,62; 1,78)
Mikroferm	50	1,63	0,27	(1,56; 1,71)
Negative C.	50	1,64	0,32	(1,57; 1,72)
Prestop	50	1,47	0,26	(1,40; 1,55)
Positive C.	50	1,60	0,28	(1,53; 1,68)
Serenade	50	1,48	0,23	(1,40; 1,56)

* The number of seedlings measured.

*[1] Standard deviation.

Table 5. Tukey pairwise comparison of diameter (mm)

Treatment	N	Mean diameter	Grouping		
Binab	50	1,70	A		
Negative C.	50	1,65	A		
Mikroferm	50	1,63	A	B	
Positive C.	50	1,60	A	B	C
Serenade	50	1,48		B	C
Prestop	50	1,47			C

Table 6. Number of seedlings taken away because of death of each treatment at the four time points

	Timepoint			
Treatment	1	2	3	4
Binab		1	6	
Negative C.	9			
Mikroferm			1	
Positive C.	14			4
Serenade		9		
Prestop		5		