Fungi associated with roots of healthy-looking Scots pines and Norway spruce seedlings grown in nine Swedish forest nurseries.

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Degree project – 30 credits – Advanced level
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Content

1. Abstract

Soil fungal diversity is essential in ecosystem functioning. Disturbances in the fungal community as a result of abiotic or biotic factors may lead to diseases in the plants. This thesis work was aimed at investigating fungal species composition in healthy looking Norway spruce and Scots pine seedlings grown in nine Swedish forest nurseries. Two methods were used to identify fungi from the seedling roots; culturing and direct sequencing techniques. For the culturing technique 1800 seedlings were sampled. For the direct sequencing technique, 180 seedlings were sampled. The seedlings sampled originated from 36 different provenances. In our results 58 different fungal species were identified. Culturing technique detected 37 fungal species whereas direct sequencing technique detected 21 of them. The most common fungal species identified by the culturing method were: *Phoma muscivora*, *Phialocephala fortinii*, *Trichoderma v*iride, *Penicillium spinolusum* and *Meliniomyces variab*ilis whereas direct sequencing showed that none of the fungi were predominant. *Phoma* m*uscivora* and *Melioniomyces variabilis* appeared frequently in the roots and have not been identified in the Fenoscandian forest nurseries before. From our results the following conclusions were drawn; the direct sequencing and culturing techniques are needed to detect potentially all fungal community as both methods complement each other, similar fungal communities colonize Norway spruce and Scots pine seedlings in the nine different nurseries; there may be differences in fungal community structure that colonize the seedling roots of the nine different nurseries; the nurseries may have an influence in the fungal community composition in the nine different nurseries; the provenances may not have an influence on the fungal community composition; a low fungal diversity is observed in all the nine different nurseries.

Key words: forest nursery, root fungi, fungal community, Ascomycetes, Basidiomycetes, Zygomycotina, Mucoromycotina, provenances, fungal diversity.

2. Introduction

In Sweden, forest nurseries produce around 400 million of tree seedlings annually and Scots pine (*Pinus sylvestris* L.) and Norway spruce (Picea *abies* (L.) Karst.) are the dominant (Beyer-Ericson et al, 1991). The seedlings are produced using two major types of cultivation systems: bare root and containerized. Over the years, there have been a gradual shift in production from bare root seedlings to containerized seedlings, and at present, the containerized seedlings constitute about 80%. The production in forest nurseries is commonly associated with some seedling losses which are caused mainly by unfavorable abiotic factors and/or fungal diseases. The abiotic disorders could often be linked to unsuitable or inappropriate cultural practices or environmental factors. Although most of seedling diseases in Sweden are shoot diseases, root diseases are not uncommon (Galaaen and Venn, 1979; Beyer- Ericson et al 1989, Beyer -Ericson et al 1991). Root pathogenic fungi (e.g. *Fusarium, Rhizoctonia and Pythium)* can cause damping-off, root rot and stem rot in the seedlings (Lilja, 1996). Seedling roots may also be colonized by other fungi such as harmless endophytic or symbiotic mycorrhizal fungi. All these organisms might interact and compete with each other.

In the forest nurseries the plant cultivation system is very intense to achieve maximum production, but this could also be associated with some disadvantages since the conditions that favour optimal growth of the seedlings in the nurseries are also very conducive for the growth of different types of pathogens. Intensive seedling cultivation in the forest nurseries (e.g. high sowing density, high nitrogen fertilization and excess of irrigation), may affect the growth of some fungal species and the rate of fungal colonization of seedlings roots (Dahlberg and Stenström 1991; Unestam et al. 1989; Juntunel and Rikala 2001). Monoculturing and intensive cultivation aimed to ensure fast growth of the seedlings may stress the seedlings so that they later become prone to pathogenic infections (Borja, 1995). In particular over watering could be favorable for some pathogenic fungi (Beyer-Ericson , 1989) while it at the same time could be unfavorable for root colonization by some mycorrizal fungi. Stenström (1991) showed that fungi from the genera *Laccaria, Hebeloma* and *Thelephora* may tolerate over watering, whereas *Suillus* spp. are sensitive to high moisture content in the soil. Excess of water may occur frequently in the nursery and may cause damage to the roots which then can be easily invaded by pathogenic microorganisms (Nef and Perrin 1999). Mycorrhizal fungi found on seedlings roots in the nursery may vary greatly depending on cultivation conditions i.e. if seedlings are grown in containers or in the open-nursery beds. The type of fungal community found in a nursery are most probably associated to the conditions in the nursery which can be different from the community established naturally under the canopy of mature trees (Stenström, 1991).
In some cases, colonization of seedling roots by pathogenic fungi can cause no apparent disease symptoms. Kope *et al* (1996) showed that the fungal pathogens *Fusarium, Cylindrocarpon and Pythium* could be isolated from healthy-looking roots of nursery seedlings, but disease conditions could be resurfaced when the plants were exposed to

stress e.g. low light intensity, waterlogged soils and fungicide treatment. This has also been emphasized by Dumroese et *al.* (2005) in an attempt to study outplanting success of some nursery seedlings. They found out that nursery seedlings infected by root pathogens had a higher mortality rate after out-planting in the forest soil as compared to the control seedlings that were non-infected.

Very little has been written about the fungi associated with healthy looking nursery seedlings in the Fennoscandian forest nurseries. Most articles from the nurseries have mostly been focused on pathogenic fungi on decayed roots and the mycorrihzzal community in the nursery seedlings roots, therefore there will be limited amount of previous works to compare with. In a study carried out in ten Swedish nurseries in 1991 (Beyer-Ericson et al.1991) on the distribution and abundance of fungi in the roots of 300 randomly selected one to two years old seedlings of *Pinus sylvestries* and *Picea abies*, the results showed that from the damaged seedlings roots, *Cylindrocarpon destructans* (Zins.) Scholten was the most frequently isolated fungus, and it was followed in a decreasing frequency by *Fusarium* spp., *Pythium* spp., *Botrytis cinerea* Pers. Ex Fr., *Alternaria alternate* (Fr.) Keissler and *Ulocladium atrum Preuss*.

Menkis et al. (2006) investigated fungal colonization in the decayed roots of *Pinus sylvestries* and *Picea abies* from bare root and containerized seedlings. They obtained 1500 pure culture isolates, sampled from 480 roots. For fungal identification they did direct ITS rDNA sequencing from the decayed sections of 140 roots. In their results direct sequencing showed a significantly higher diversity in fungal community per root segment than pure culture isolation. Both the direct sequencing and sequencing of fungal cultures yielded 131 taxa. Only 10.7% of the taxa were detected by both methods, 55.7% by culturing method, and 33.6% by direct sequencing method. For the culturing method the most common fungi detected were *Fusarium oxysporum* (25.6%) and *Nectria radicola* (14.9%), whereas direct sequencing showed that the endophyte *Phialocephala fortinii* (33.1%) and *Chalara* sp.NS234A2 (10%) were the most commonly detected. In 1992 in Norway Lilja and her associates (Lilja et al. 1992) assessed the fungal community in the diseased and healthy looking seedlings grown in bare rooted or containerized medium. In their studies only the culturing method was used in fungal identification and in their findings, *Cylindrocarpon* spp, *Fusrium* spp and *Trichoderma viride* were the most commonly identified fungi in both the disease and the healthy looking seedlings. In the diseased seedlings higher numbers of uninucleate *Rhizoctonia*-like fungus, *Pythium spp*. and *Phytophtora undulatum* were found compared to in healthy seedlings. The frequencies of observed *Rhizoctonia*-like fungus, *Pythium spp*. and *Phytophtora undulatum,* supported with some pathogenicity test, made them to associate the cause of the disease (root die back) to these fungi.

Plants and fungi interact for survival. The population of these fungal species is threatened with application of pesticides to the soils. The loss or depletion of some fungi in the community may result to an imbalance in the ecosystem which might lead to environmental changes that might affect the plants health negatively. Fungal diversity is defined as the different number of fungal species in a particular site (region or area) weighted by its

abundance (i.e. the number of individuals of each particular species) (McGlinley, M and Duffy, E. 2008, Sterling et al. 2004). In nursery management, the seedling provenance is very primordial in seedling production. This has been proven to be important as it influence the quality and / or quantity of the mycorrhizal community colonizing the roots of *Picea abies* and *Pinus sylvestries* seedlings in the early stage of development of pines and spruces under forest nursery conditions (Leski et al 2010). Seedling provenance could be defined as the original source of a seed lot considering its geographic location and habitat (Wright, 1962). The seedling from each provenance differs physiologically even though there exist a genetic relationship between the different seedling provenances.

2.1 Biology of fungi

Fungi are classified based on the characteristic features of the reproductive structures that they produce, and this provides the basis of identifying fungi. Four major groups or classes of fungi are common in the forest nurseries; Basidiomycetes, Ascomycetes, Oomycetes and the Deuteromycetes.

In basidiomycetes for example mushroom and toadstools the sexual spores (basidiospores) are produced by the basidium externally. Most ectomycorrizal fungi could be found in this group. One can also find some rust fungi in this group.

Ascomycetes produce sexual spores known as ascospores in structures called ascus. These asci are found in specialized fruiting bodies that exist in different forms such as apothecia (exposed cup shaped), Pseudothecia or locules (seen as sunken cavities in host tissues), perithecia (has a flask shape with a neck and opening) and cleisthothecia (closed and spherical). The fruiting bodies of the ascomycetes are mostly visible by the naked eye and could be colored differently.

Oomycetes are actually not included in the kingdom of fungi, but are instead included in the kingdom of chromista together with the algae. However, they are often included together with the fungi when studying plant pathology since they have similar behavior. They reproduce both sexually and asexually. Oomycetes occupy both saprophytic and pathogenic life styles and include some of the worst pathogens of plants (e.g. *Phytophtora* & *Pythium*).

Deutromyctesare also known as fungi imperfecti. They are micro fungi that lack a sexual or a perfect stage. If a sexual stage is found in them they are mostly then classified under the ascomycetes.

Identification of fungi

In fungal identification, first the morphological appearance of fruiting bodies and spores is used. However, since many fungi, including pathogens do not produce fruit bodies; culture morphology and DNA techniques are used. The culturing technique is traditional and it involves the characterization of mycelia, colour and morphology as well as spore production. The molecular identification methods are based on DNA sequencing. It is a modern method (higher resolution potentials) and has been more and more used for fungi identification during the last decades. This method, involves the amplification of the ITS region (internal

transcribe spacer) of the fungal ribosomal DNA using general or fungal specific primers. In the ITS region, there are pieces of nonfunctional RNA located between structural ribosomal RNAs (RNA), found on the same precursor transcript. The RNA precursor transcript consist of 5' external transcribed sequence (5'ETS), 18SrDNA, ITS1, 5.8S rRNA, ITS2, 28SrRNA and the 3'ETS. The use of ITS region in sequence comparison is very much used in taxonomy and in molecular phylogeny because firstly it is very easy to amplify even small fragments of the DNA due to large numbers of their copies in the genome and secondly because there is a very high variation existing in the ITS1 and ITS2 regions even between closely related fungal species. These regions (nonfunctional sequences) experiences a relatively low evolutionary pressure. The two most common primers used in the amplification of the ITS region in fungi, are the ITS1F and the ITS4 (White et al. 1990). The molecular identification method is quite reliable and sensitive since it does not depend on the fungi to grow on the culture medium and secondly it amplifies very small fragments of DNA (Vilgalys, R. 2011)

2.2 Fungi in the nurseries.

Endophytic fungi

Endophitic fungi are a particular group of fungi that spend part of their life cycle in a host plant and apparently cause no disease on the plant. They benefit readily from available nutrients from the plants and they help the plants in some way by acting as antagonists to some pathogenic fungi, by producing toxins that may impede the activities and development of the pathogen. It may also compete with the pathogen for available nutrients and in this way limiting its ability to spread and develop normally. An example of an endophyte that is found in the roots of nursery seedlings is *Phialocephala fortinii* (Addy et al, 2005).

Pathogenic fungi

Pathogenic fungi attack living organisms and cause diseases. This group of fungi attack and cause disease to plants mainly by damaging vascular tissues and cambium (Schippers and Gams, 1979). There are a number of soil born fungi that are pathogenic to coniferous seedlings in forest nurseries. For example, the soil born fungus *Pythium dimorphum* is pathogenic to the Norway spruce seedlings, causing severe root die back (Garett, 1970). Another type of complex damages is damping off which mainly kills small un-lignified germlines. Both root rot and damping off has been reported to be caused by a number of soil born fungal pathogens; e.g. *Fusarium, Cylindrocarpon*, *Rhizoctonia, Pythium* and *Pestalotia.*

Mycorrhizal fungi

Mycorrhizal fungi are a group of fungi that form a mutualistic association with the plant roots. These fungi gain direct access to carbohydrates such as glucose and sucrose from the plant and the fungi in turn can access and provide to plants resources such as phosphorous and nitrogen. Some forms of mycorrizal fungi are; ectomycorrhizal fungi, endomycorhizal

fungi and the ericoid mycorrhizal fungi. In certain conditions where the roots have been colonized, damaged and killed by some pathogenic fungi like *Rhizoctonia spp*; the mycorrhizal fungi aids in the formation of new leading long roots to compensate for the old ones (Chakravarty and Unestam 1987). Studies also carried out by Damm and Unestam (1981) showed that there was more photosynthetic activity in damaged seedlings colonized by mycorrhizal fungi than in damaged seedlings without mycorrhzal fungi. The activity of some mycorrhizal fungi could be suppressed during nursery conditions, when there is high input of fertilizers, fungicides and overwatering of peat soils (Stenström 1989) while others could be stimulated during the same conditions.

Saprophytic fungi

Saprophytic fungi feed on dead remains of plants and animals or on waste materials from living organisms. These fungi are important because they degrade organic waste. In the peat soil, the dominant saprophytic fungal species are *Penecillium* spp. and *Trichoderma* spp. These two saprophytes could be beneficial (protective) and some of the species have been shown to be less tolerant to fungicide applications, compared to some pathogenic fungi (Unestam et al 1989).

Rhizoplane fungi

Rhizoplane fungi are found inhabiting the root surfaces of plants. In the peat soils of containerized seedling of a 2 year old *Picea abies* the rhizoplane fungi that are mostly found are *Mycelium radicis atrovirens*, *Penicillium spinulosum*, *Phoma herbarum*, *Trichoderma koningii*, *T. viride* and *Varicosporium elodeae* (Galaaen and Venn, 1979).

Rhizosphere

The rhizosphere is soil that is directly influenced by the root. This portion of the soil has an increased level of microbial interaction compared to soil further away from the roots. In this area plant root exudates and other organic compounds that are leaking out from the plant living cells and could readily be used by microorganisms in the area (Nelson 1990). The biochemical components of these root exudates depend on the genotype of the plant (Kraft 1974) and are highly influenced by the nursery cultural practices and the growing systems (Funk-Jensen and Hocken hull 1984). The rhizosphere has a great influence on the colonization of fungi and growth of root diseases. Examples of rhizospheric fungi are species from the genera *Cylindrocarpon*, *Rhizoctonia*, *Trichoderma, Penicillium, Fusarium* and *Mucor (Kubikova* ,1963).

2.3. Aims of the work

The overall aim of this work was to determine fungal communities associated with the roots of healthy looking Scots pine and Norway spruce seedlings from nine forest nurseries using mycelia cultivation and direct sequencing methods.

Specific aims:

- To compare fungal communities in pines and spruce seedlings,

- To compare fungal communities determined by two different methods,
- To compare fungal communities between different nurseries and seed provenances.

The objective of this work was to compare fungal community colonizing roots of healthy - looking Norway spruce and Scots pine seedling grown in different nurseries in Sweden. To do this, two approaches were used. The direct sequencing of DNA from the root segments and secondly, the amplification of fungal rDNA from the seedlings` roots cultures. The direct sequencing of DNA from the root segments avoids cultural bias and this method has the potential of detecting unculturable fungi. A combination of these two approaches may be very good in detecting potentially all fungal species that might be contributing to the seedlings health in the nursery or after planting.

2.4. Hypotheses

1) Different nurseries have similar fungal communities in seedling roots
2) Different fungi are associated with pines and spruce seedlings
3) Different provenances have little effect on root associated fungi
4) Different fungi are detected by direct sequencing and culture methods

The purpose of this work is to assess the fungal diversity in seedling` roots in each nursery. Fungi are known to enhance soil agglomeration and soil fertility. Plants can more easily tolerate biotic and abiotic stress when they have healthy rhizosphere microorganisms. This investigation on fungal diversity in healthy looking seedlings from the nine different nurseries will contribute to the existing information on the fungal community found in roots of healthy looking Norway spruce and Scots pine seedlings in the Fenoscandian forest nurseries. This will help to increase understanding on fungal community composition found in the healthy conifer seedling roots in the nurseries, as this will enable us to figure out imbalances that might crop up as a result of anthropogenic or environmental factors.

3. Materials and Methods

3.1. Seedling material

In total, 900 Scots pine (*Pinus sylvestris*) and 900 Norway spruce (*Picea abies*) seedlings representing 18 different provenances of each tree species were collected from nine forest nurseries in Sweden (Table 1). The nurseries were situated in a large geographical area from North Eastern to Southern part of the country. The nurseries are owned by the private forest companies and produce plant materials using standardized cultivation methods. Sampled seedlings were approximately one year old and were intended for out planting in spring 2011.

Table 1. Forest nurseries, company and geographical locations where the seedlings were grown, tree species, seed provenance and number of samples used for detection of fungi by different methods.

Nursery	Company	Location	Tree[a]	Seed Provenance	Code [b]	No. of seedling roots used	
						Isolation	Sequencing
(1) Gideå	Holmen AB	N63° 29' E18° 58'	Pine	Våge	1P1	50	5
			Pine	Alvik	1P2	50	5
			Spruce	Lillepite	1S1	50	5
			Spruce	Hissjö	1S2	50	5
(2) Stakheden	Svenka Skogsplantor AB	N60° 16' E14° 57'	Pine	Västerhus	2P1	50	5
			Pine	Sollerön	2P2	50	5
			Spruce	Saleby	2S1	50	5
			Spruce	Kowale Oleckie	2S2	50	5
(3) Trekanten	Svenka Skogsplantor AB	N56° 41' E16° 06'	Pine	LillaIstad	3P1	50	5
			Pine	Lilla Istad	3P2	50	5
			Spruce	Slogstorp	3S1	50	5
			Spruce	Slogstorp	3S2	50	5
(4) Kolleberga	Svenka Skogsplantor AB	N56° 03' E13° 15'	Pine	Gotthardberg	4P1	50	5
			Pine	Mosås	4P2	50	5
			Spruce	Kruklanki	4S1	50	5
			Spruce	Kowale Oleckie	4S2	50	5
(5) Lugnet	Svenka Skogsplantor AB	N59° 41' E16° 36'	Pine	Gotthardberg	5P1	50	5
			Pine	Gotthardberg	5P2	50	5
			Spruce	Saleby	5S1	50	5
			Spruce	Saleby	5S2	50	5
(6) Kilåmon	Svenka Skogsplantor AB	N63° 28' E16° 40'	Pine	Kaunisvara	6P1	50	5
			Pine	Moliden	6P2	50	5
			Spuce	Lillepite	6S1	50	5
			Spruce	Hissjö	6S2	50	5
(7) Nässja	Bergvik Skogs AB	N60° 16' E16° 42'	Pine	Gotthardberg	7P1	50	5
			Pine	Sollerön	7P2	50	5
			Spruce	Saleby	7S1	50	5
			Spruce	Vitesk Blubkoye	7S2	50	5
8) Flåboda	SödraOdlarna	N56° 34' E15° 08'	Pine	Gotthardberg	8P1	50	5
			Pine	Gotthardberg	8P2	50	5
			Spruce	Bredinge	8S1	50	5
			Spruce	Bredinge	8S2	50	5
(9) Bogrundet	SCA Skog AB	N62° 31' E17° 18'	Pine	Hortlax/Alnön	9P1	50	5
			Pine	Hortlax	9P2	50	5
			Spruce	Jung	9S1	50	5
			Spruce	Jung	9S2	50	5

a; Pine = Scots pine, Spruce = Norway spruce

[b]Code; First letter = nursery number, S/P = type of conifer tree (S = Norway spruce, P = Scots pine), 1 / 2 = two provenances / nursery and tree.

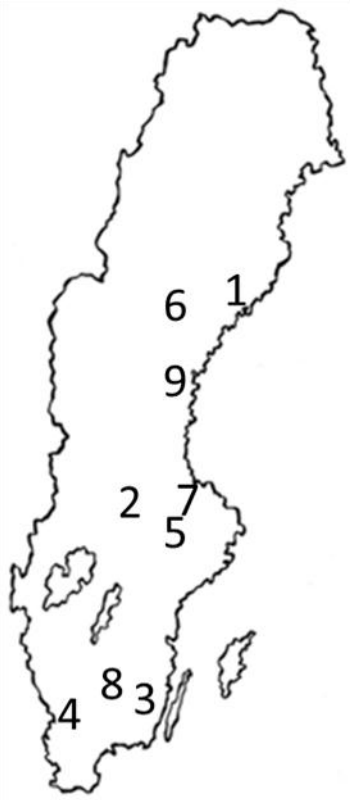

Figure 1. Map of Sweden showing the locations of the different nurseries (numbered 1-9 as in Table 1) from which seedlings were collected.

3.2 Root preparation and isolation of fungi

The seedlings were picked randomly within a growing lot for each provenance in the nursery. They were all winter- hardened at the time of harvesting and were immediately packed and sent to the department of Forest Mycology and Pathology were they arrived the day after harvesting.

After arrival from the nurseries, seedlings were stored frozen at -20 °C. To prepare the roots, seedlings were defrosted and root systems were washed in tap water to remove the soil. From each seedling (1800 in total) three lateral roots were selected randomly and from these 1-2cm segments in length were cut out. One segment was used for fungal isolation while the other two were stored at -20 °C for DNA extraction and direct sequencing of fungi. Roots used for isolation of fungi were sterilized in 33% hydrogen peroxide for between 30 to 60 seconds and then rinsed three times in sterile deionized water. The roots were plated on Hagem agar medium (Stenlid, 1985) supplemented with chloramphenicol antibiotic (50mg/l) and incubated at room temperature in the dark. The plates were examined every second day and any of emerging mycelia were transferred into new Petri dishes with agar medium free of antibiotics. Isolated cultures were divided into different groups based on their morphological characteristics. From each group, one representative sample was collected for species identification using DNA sequencing. For this, mycelium was collected from the Petri dishes, placed in extraction tubes and kept frozen at -20^{o}C.

3.3 DNA extraction from roots and fungal mycelia

In total, 180 root segments representing 5 randomly selected root segments from each provenance (2 tree species x 9 nurseries x 2 provenances x 5 root segments = 180) and 74 fungal mycelia samples representing potentially different fungal taxa were used for DNA isolation. Prior to DNA isolation, root segments and fungal mycelia in tubes were freeze dried. DNA isolation was carried out using CTAB protocol. Homogenization of individual samples was done in a Fastprep machine (Precellys 24), running for ten seconds 2-3 times at 5000 rpm. After each run a degree of sample homogenization was checked. After homogenization, 800µl of 3% CTAB buffer was added into each tube, mixed thoroughly by vortexing and incubated for 1 hour at 65°C. During incubation, the tubes were vortexed in between intervals of 10-15 minutes. The tubes were then centrifuged at 7000 rpm for 5 minutes and 500µl of the supernatant (avoiding residues) were transferred into new centrifugation tubes. In the fume hood, one volume of chloroform (500µl) was added to the supernatant, vortexed vigorously and then centrifuged at 13000 rpm for 7 minutes. After centrifugation, 400µl of the upper phase was transferred into new tube, two volumes of ice cold isopropronal (800µl) were added and the tubes were gently vortexed. The reaction mixture was then kept on the bench for at least 5 minutes and then DNA was pelleted by centrifugation at 13000 rpm for 25 minutes. After centrifugation the flow was discarded and pellet was washed in 400 µl of 70% ice-cold ethanol by centrifugation at 13000 rpm for 5 minutes. After centrifugation, the ethanol was discarded and the tubes were left open until the remaining ethanol evaporated. The pellet was dissolved in 30 µl of milli Q water by overnight incubation at 4 °C. The DNA suspensions were stored at -20 °C.

3.4 PCR and sequencing

DNA concentration in each sample was determined using NanoDrop spectrophotometer ND-1000 (Saveen Werner). In case DNA concentration was higher than 15 ng/µl, it was diluted to 1-15 ng/µl. The PCR master mix for one reaction included: Milli Q water - 5-10µl, 10 x dream taq buffer - 1.5 µl, dNTP (2mM) - 1.5 µl, primer ITS1F - 0.3 µl, primer ITS4 - 0.3 µl, $MgCl_2$ - 0.15 µl, DreamTaq polymerase - 0.15µl and DNA template 1-6µl. The volume of milli Q water and DNA template was 5:6 for root DNA samples and 10:1 for mycelial DNA samples. In each PCR run, the positive and negative controls were included. The PCR cycling was performed on an Applied Biosystems Thermal Cycler 2720. The PCR conditions were: initial denaturation at 95^{o}C for 2 minutes, followed by 35 cycles of denaturation at 95 ^{o}C for 30 seconds, annealing at 55 ^{o}C for 30 seconds and extension at 72 ^{o}C for 1 minute, and final extension at 72 ^{o}C for 7 minutes. The amplified PCR products were separated on 1% agarose (Agarose D-1, Conda) gels which were prepared using 1× SB buffer and supplemented with GelGreen Nucleic Acid Stain. For this, 3µl of PCR products was loaded on a gel, electrophoresis was run at 300 volts for 30 minutes and images were taken under the UV-light using GelDoc system (Bio-Rad laboratories). The PCR products containing single strong-intensity bands (e.g. commonly obtained from the fungal mycelia) were selected for

sequencing. Multiple-banded PCR products or singe weak-intensity bands were re-amplified using nested PCR. For this, weak and multiple-banded products were run on the gel and individual bands were excised using sterile scalper under the blue light and put in separate tubes. After addition of 100 µl of milli Q water, samples were incubated at 65 °C for one hour and vortexed every 10 to 15 minutes. The resulting individual solutions were used as DNA templates for PCR. All procedures of nested PCR (master mix preparation, PCR, electrophoresis) were as described previously with exception to ITS1F PCR primer which was replaced by ITS1 primer. Prior to sequencing, individual PCR products were purified using ExoSap kit (Fermentas Life Sciences). Sequencing was performed by Macrogen Inc., Seoul, Korea, using ABI 3730 XL automated sequencers (Applied Biosystems, Foster City, CA, USA).

3.5 Sequence analysis and species identification

DNA sequence data were analyzed using SeqMan Pro v.8.1.2 from DNASTAR Lasergene 8 package (DNASTAR, Inc. Madison, WI, USA). All sequences were imported into the program and assembled in contigs using Pro Assembly option with default parameters. For taxonomic identification, sequences were blasted against the reference sequences in the NCBI database using blastn algorithm. The criteria used for identification were: identity to species level 97-100%, identity to genus level 94-96%. Sequences not matching those criteria or lacking taxonomic names in the reference sequences were considered unidentified.

3.6. Statistical analysis

For assessment of fungal diversity in each nursery, the Shannon's diversity index (H) was calculated (software) (Shang bioscience 2002 – 2012). The equation used was $H = - \sum P_i \ln (P_i)$ where P_i was the proportion of the number of different species that makes up i^{th} species (Shang bioscience 2002 – 2012). Fungal community structure between different nurseries and between two different conifer trees species were compared using Sorensen's similarity index (S_s). The equation used was $S_s = 2a/ (b+c)$ where a= number of common species shared in both nurseries to be compared, b= number of species found only in a particular nursery collection, c= number of species found only in the other nursery collection (Balmer 2002). The chi square test was performed to compare species richness between the nurseries (Table 3) and also to compare the species richness between the two conifer tree species. This was calculated from the equation $\chi^2 = \sum (O-E)^2/E$ where O= the observed frequency of different number of fungi, and E = the expected frequency of the different number of fungi (Fowler et al. 1998). The Canoco 4.5 windows software was used to perform principal component analysis (PCA).

4. Results

4.1. Cultivation of fungi

The culturing resulted in 2387 fungal cultures (1.3 cultures per root segment on average) which according to mycelia morphology were divided into 74 groups. ITS rDNA Sequences of the representative samples revealed the presence of 45 distinct taxa. The proportions of

the fungal classes identified were; ascomycetes (85%), basidiomycetes (9%), mucoromycotina (4%) and zygomycotina (2%) (Fig.2).This result shows that majority of the isolates were ascomycetes.

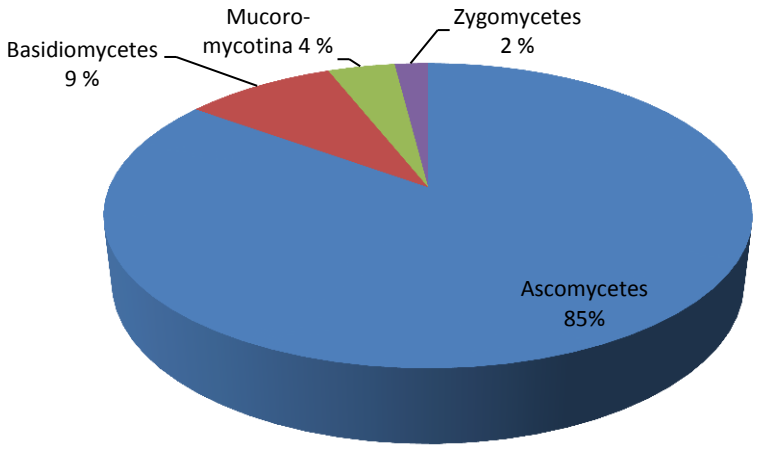

Figure 2. Relative abundance of fungal classes isolated from the root segments of both Scots pine and Norway spruce (Table 2 and 3) seedlings in nine forest nurseries.

The occurrence and abundance of the different isolated taxa from the culturing are presented in Table 2 for P. sylvestris and Table 3 for P. abies. From the roots of P. sylvestris, 37 different species were recorded while 32 were recorded from P. abies. The species occurrences are more or less similar for both host species. Meliniomyces variabilis, Penicillium daleae, P. spinulosum, Phialocephala fortini, Phoma muscivora and Trichoderma viride were dominant in both tree species and in most of the nurseries, while other fungal species appear more rarely (Table 2 and 3) .

Figure 3. Relative abundance of fungal isolates detected by the cultring method in the root segments of heathy looking Norway spruce and Scots pine seedlings grown in nine different nurseries

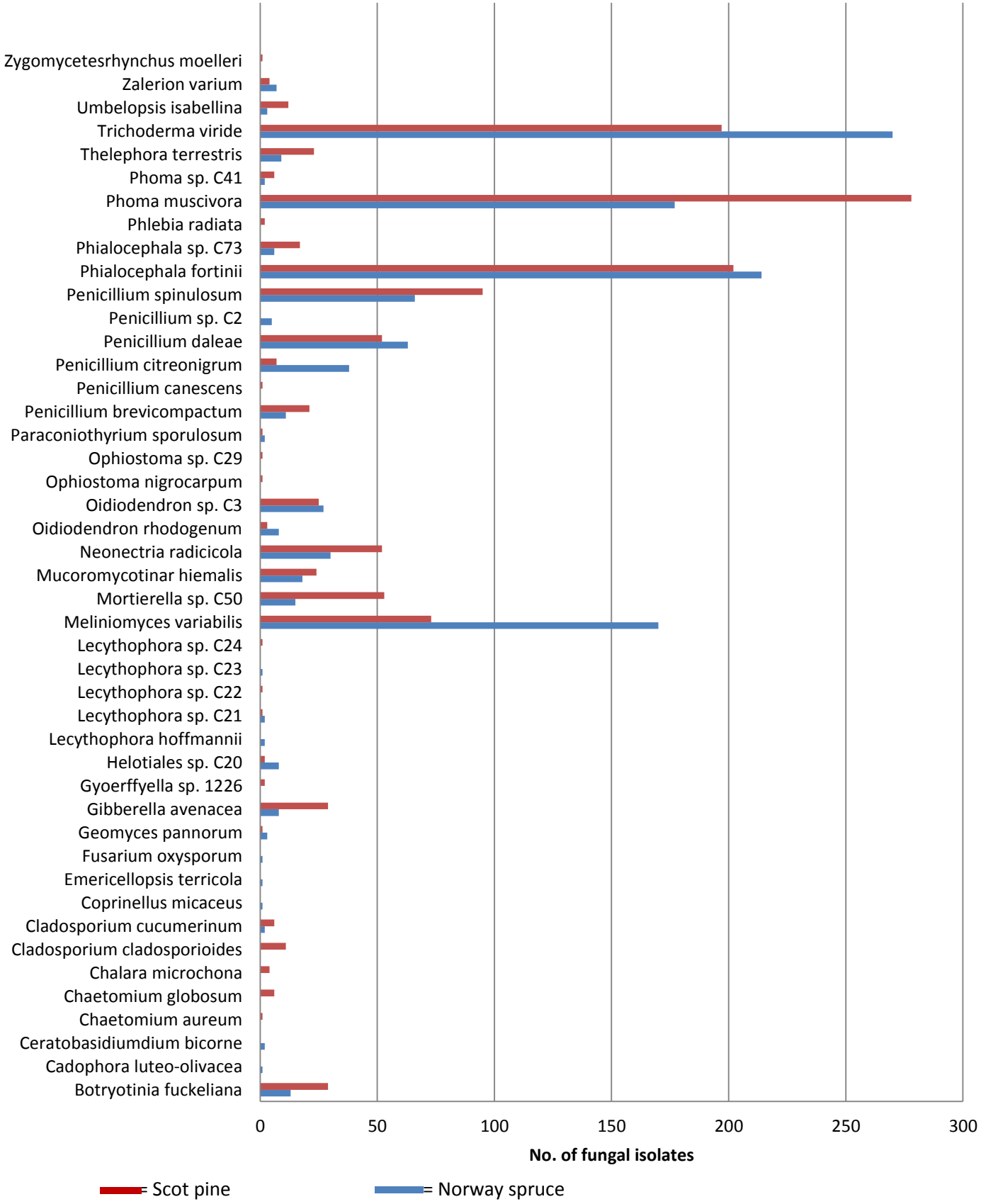

Table 2. Occurrence and relative abundance of fungal taxa isolated from roots of *Pinus sylvestris* seedlings from different provenances in nine forest nurseries

Fungal taxa	1 [a]		2		3		4		5		6		7		8		9		All
	1P1 [b] (50) [c]	1P2 (50)	2P1 (50)	2P2 (50)	3P1 (50)	3P2 (50)	4P1 (50)	4P2 (50)	5P1 (50)	5P2 (50)	6P1 (50)	6P2 (50)	7P1 (50)	7P2 (50)	8P1 (50)	8P2 (50)	9P1 (50)	9P2 (50)	
Botryotinia fuckeliana	1.4	1.4	15.3	2.5	10.1	-	1.6		1.3	1.3	1.2	-	-	-		-	-	-	2.4
Chaetomium aureum	-		-	-	-	-	-		-	-	-	-	-	-	1.6	-	-	-	0.1
Chaetomium globosum	-		-	-	-	-	7.8	1.4	-	-	-	-	-	-		-	-	-	0.5
Chalara microchona	4.2		-	-	-	-	-		-	-	-	-	-	-		-	-	2.0	0.3
Cladosporium cladosporioides	-	1.4	-	-	-	-	1.6		-	-	-	-	-	-		-	-	-	0.2
Cladosporium cucumerinum	-		-	-	-	-	3.1		-	1.3	-	4.8	-	-		-	-	-	0.5
Geomyces pannorum	-	1.4	-	-	-	-	-		-	-	-	-	-	-		-	-	-	0.1
Gibberella avenacea	-	1.4	-	-	-	1.8	4.7	8.5	1.3	12.8	1.2	8.1	-	-		2.0	-	-	2.4
Gyoerffyella sp. 1226	-		-	-	-	-	-		-	-	-	3.2	-	-		-	-	-	0.2
Helotiales sp. C20	-	2.9	-	-	-	-	-		-	-	-	-	-	-		-	-	-	0.2
Lecythophora sp. C21	-		-	-	-	-	-		-	-	-	1.6	-	-		-	-	-	0.1
Lecythophora sp. C22	-		-	-	-	-	-		-	-	-	1.6	-	-		-	-	-	0.1
Lecythophora sp. C24	-		-	-	-	-	-		-	-	-	1.6	-	-		-	-	-	0.1
Meliniomyces variabilis	14.1	18.8	7.1	6.2	-	-	1.6	9.9	5.1	7.7	2.4	14.5	-	-		2.0	15.1	2.0	6.0
Mortierella sp. C50	-		-	-	-	-	-	1.4	-	-	9.4	3.2	22.6	43.3		-	3.8	3.9	4.4
Mucoromycotinar hiemalis	-	1.4	2.4	-	15.2	7.0	-	1.4	-	-	-	4.8	-	1.7		-	-	-	2.0
Neonectria radicicola	1.4	1.4	2.4	17.3	-	-	7.8	11.3	3.8	-	-	1.6	24.5	5.0		-	-	2.0	4.3
Oidiodendron rhodogenum	-		-	-	-	-	-		-	-	-	-	-	-		-	1.9	3.9	0.2
Oidiodendron sp. C3	2.8	7.2	-	1.2	-	-	-		-	-	1.2	-	-	-	1.6	-	9.4	19.6	2.1
Ophiostoma nigrocarpum	-		-	-	-	-	-		-	-	-	1.6	-	-		-	-	-	0.1
Ophiostoma sp. C29	-		-	-	-	-	-		-	-	1.2	-	-	-		-	-	-	0.1
Paraconiothyrium sporulosum	-		-	-	-	-	-	1.4	-	-	-	-	-	-		-	-	-	0.1
Penicillium brevicompactum	-	1.4	-	-	1.3	-	-		-	-	-	-	-	-	1.6	2.0	5.7	27.5	1.7
Penicillium canescens	-		-	-	-	-	-		-	-	-	-	-	-		-	-	2.0	0.1
Penicillium citreonigrum	-		-	-	-	-	3.1		-	-	2.4	-	-	-		4.1	1.9	-	0.6
Penicillium daleae	1.4	8.7	2.4	-	5.1	1.8	-	1.4	5.1	1.3	7.1	1.6	1.9	5.0	20.6	8.2	3.8	3.9	4.3
Penicillium spinulosum	19.7	17.4	9.4	7.4	7.6	3.5	15.6	7.0	3.8	2.6	11.8	1.6	7.5	10.0	1.6	2.0	5.7	2.0	7.9
Phialocephala fortinii	35.2	29.0	36.5	46.9	5.1	-	14.1	2.8	5.1	7.7	2.4	16.1	28.3	11.7	6.3	14.3	17.0	17.6	16.7
Phialocephala sp. C73	-		-	-	-	-	-		-	-	-	-	7.5	1.7	11.1	6.1	-	2.0	1.3
Phlebia radiate	-		-	2.5	-	-	-		-	-	-	-	-	-		-	-	-	0.2
Phoma muscivora	19.7	5.8	16.5	8.6	45.6	-	12.5	11.3	64.6	52.6	30.6	19.4	3.8	6.7	33.3	38.8	20.8	-	23.0
Phoma sp. C41	-		-	-	-	-	6.3		-	-	-	-	-	3.3		-	-	-	0.5
Thelephora terestris	-		-	-	-	-	-	1.4	-	-	-	-	-	-		-	-	-	0.1
Trichoderma viride	-		8.2	7.4	10.1	86.0	20.3	39.4	8.9	10.3	29.4	12.9	3.8	10.0	14.3	18.4	13.2	9.8	16.3
Umbelopsis isabellina	-		-	-	-	-	-		-	-	-	-	-	1.7	7.9	2.0	1.9	2.0	0.7
Zalerion varium	-		-	-	-	-	-		1.3	2.6	-	1.6	-	-		-	-	-	0.3
Zygomycetesrhynchus moelleri	-		-	-	-	-	-	1.4	-	-	-	-	-	-		-	-	-	0.1

[a] Forest nurseries; 1-9; see table 1; [b] provenances; [c] number of root segments used.

Table 3. Occurrence and relative abundance of fungal taxa isolated from roots of *Picea abies* seedlings from different provenances in nine forest nurseries

Fungal taxa	1[a]		2		3		4		5		6		7		7		8		All
	1S3 [b] (50) [c]	1S4 (50)	2S3 (50)	2S4 (50)	3S3 (50)	3S4 (50)	4S3 (50)	4S4 (50)	5S3 (50)	5S4 (50)	6S3 (50)	6S4 (50)	7S3 (50)	7S4 (50)	8S3 (50)	8S4 (50)	9S3 (50)	9S4 (50)	
Botryotinia fuckeliana	-	1.1	-	12.9	1.3	3.1	-		-	-	-	-	-	-		-	-	-	1.0
Cadophora luteo-olivacea	-		-	-	-	-	-		-	-	-	-	-	-		-	1.7	-	0.1
Ceratobasidiumdium bicorne	-		-	-	-	-	-	2.3	-	-	-	-	-	-		-	-	-	0.2
Cladosporium cucumerinum	-		-	-	-	-	1.9	1.1	-	-	-	-	-	-		-	-	-	0.2
Coprinellus micaceus	-		-	-	-	1.6	-		-	-	-	-	-	-		-	-	-	0.1
Emericellopsis terricola	-		-	-	-	1.6	-		-	-	-	-	-	-		-	-	-	0.1
Fusarium oxysporum	-		-	-	-	-	-	1.1	-	-	-	-	-	-		-	-	-	0.1
Geomyces pannorum	-		-	-	-	-	-		-	-	-	-	-	-		4.8	-	-	0.3
Gibberella avenacea	-		-	3.2	1.3	-	3.8	1.1	-	-	2.2	-	-	-		-	1.7	-	0.7
Helotiales sp. C20	-		-	-	1.3	-	3.8		-	-	-	-	-	-	1.5	1.6	3.3	2.4	0.7
Lecythophora hoffmannii	-		-	-	-	-	-		-	-	-	-	-	-	1.5	-	1.7	-	0.2
Lecythophora sp. C21	-		-	-	-	-	-		-	-	-	-	-	-	1.5	-	-	2.4	0.2
Lecythophora sp. C23	-		-	-	-	-	-	1.1	-	-	-	-	-	-		-	-	-	0.1
Meliniomyces variabilis	43.8	20.7	14.9	4.8	10.4	3.1	39.6	18.4	22.1	26.8	15.6	-	-	-	4.4	1.6	3.3	4.9	14.4
Mortierella sp. C50	-		1.5	1.6	-	-	-		-	-	-	-	13.3	3.4	1.5	3.2	-	-	1.3
Mucoromycotinar hiemalis	1.0	3.3	-	4.8	7.8	1.6	-		-	-	-	-	-	-	5.9	-	-	-	1.5
Neonectria radicicola	-	4.3	-	1.6	-	1.6	3.8	24.1	-	-	-	2.2	-	-		-	-	-	2.5
Oidiodendron rhodogenum	-		-	-	-	-	-		1.5	-	-	-	-	-	2.9	1.6	-	9.8	0.7
Oidiodendron sp. C3	4.2	4.3	3.0	-	-	-	-	1.1	-	1.4	-	-	-	-	7.4	1.6	5.0	14.6	2.3
Paraconiothyrium sporulosum	-		-	-	-	-	-	1.1	-	-	-	-	-	-		1.6	-	-	0.2
Penicillium brevicompactum	-		-	-	-	-	-		-	-	-	-	-	-	8.8	3.2	1.7	4.9	0.9
Penicillium citreonigrum	-		-	-	-	-	-		-	1.4	-	-	1.7	-	17.6	31.7	5.0	2.4	3.2
Penicillium daleae	4.2	9.8	-	-	6.5	4.7	1.9	1.1	1.5	11.3	-	-	13.3	8.6	8.8	9.5	6.7	4.9	5.4
Penicillium sp. C2	-		-	-	-	-	1.9	3.4	-	1.4	-	-	-	-		-	-	-	0.4
Penicillium spinulosum	7.3	22.8	3.0	9.7	1.3	6.3	1.9	1.1	1.5	2.8	-	-	-	-	8.8	6.3	10.0	9.8	5.6
Phialocephala fortinii	27.1	18.5	26.9	19.4	7.8	10.9	32.1	27.6	35.3	25.4	4.4	6.7	1.7	5.2	8.8	6.3	23.3	29.3	18.2
Phialocephala sp. C73	-		-	-	-	-	1.9		-	-	-	-	-	3.4	1.5	-	5.0	-	0.6
Phoma muscivora	12.5	15.2	26.9	9.7	26.0	12.5	5.7	9.2	35.3	26.8	2.2	-	-	3.4	19.1	22.2	18.3	9.8	15.0
Phoma sp. C41	-		-	-	-	-	-		-	-	-	-	3.3	-		-	-	-	0.2
Trichoderma viride	-		23.9	32.3	36.4	53.1	-	3.4	-	-	75.6	91.1	65.0	74.1		4.8	13.3	-	22.9
Umbelopsis isabellina	-		-	-	-	-	-		-	-	-	-	1.7	1.7		-	-	4.9	0.3
Zalerion varium	-		-	-	-	-	1.9	2.3	2.9	2.8	-	-	-	-		-	-	-	0.6

[a] Forest nurseries; 1 –9 ; see table 1. [b] provenances; [c] number of root segments used.

4.2. Detection of fungi by direct sequencing from the root segments

From the 180 root segments used for direct sequencing, 140 sequences were generated among which 119 were of good quality while 21 were of poor quality and could not be analyzed. One to 4 amplicons were obtained per root segment. From the blast analysis of the 119 sequences of high quality, 21 different taxa were identified and 4 remained unidentified species. Fig. 3 shows that the 21 taxa obtained could be grouped into three fungal classes with their respective proportions that they form; Ascomycetes (48%), Basidiomycetes (40%), and Zygomycetes (12%). The results indicate that the proportions of ascomycetes and basidiomycetes detected were almost similar.

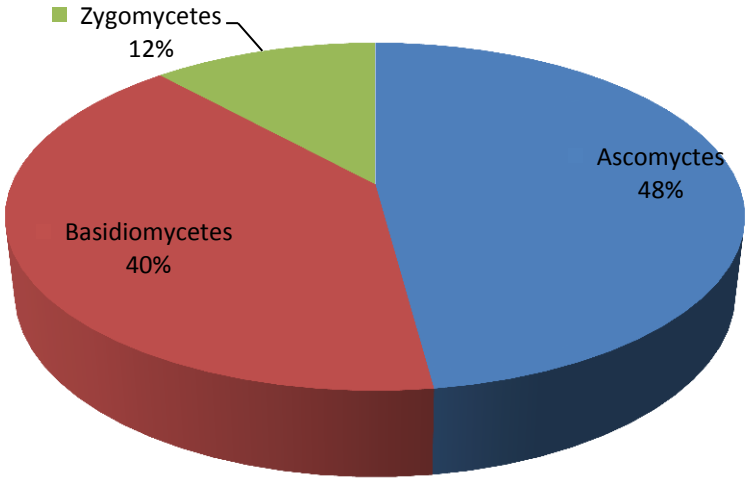

Figure 4. Relative abundance of fungal classes identified by direct sequencing from the root segments (Table 4 and 5) of both Scots pine and Norway spruce in nine different nurseries.

The occurrence and abundance of the different taxa detected by the direct sequencing are presented in Table 4 for *P. sylvestris* and in Table 5 for *P. abies*. From the roots of *P. sylvesteris*, 11 different species were identified and 3 were unidentified. From *P. abies*, there were 15 identified species and 4 unidentified species. From the two tree species, 5 of the identified species and 3 of the unidentified species were the same, while the other species were only found in one of the tree species. Comparing with the results from the culturing method (table 2 and 3) these results are more diverse and none of the species was found to dominate in different nurseries.

Figure 5. Relative abundance of fungal taxa detected by the direct sequencing method in root segments of healthy looking Norway spruce and Scots pine seedlings in nine different nurseries

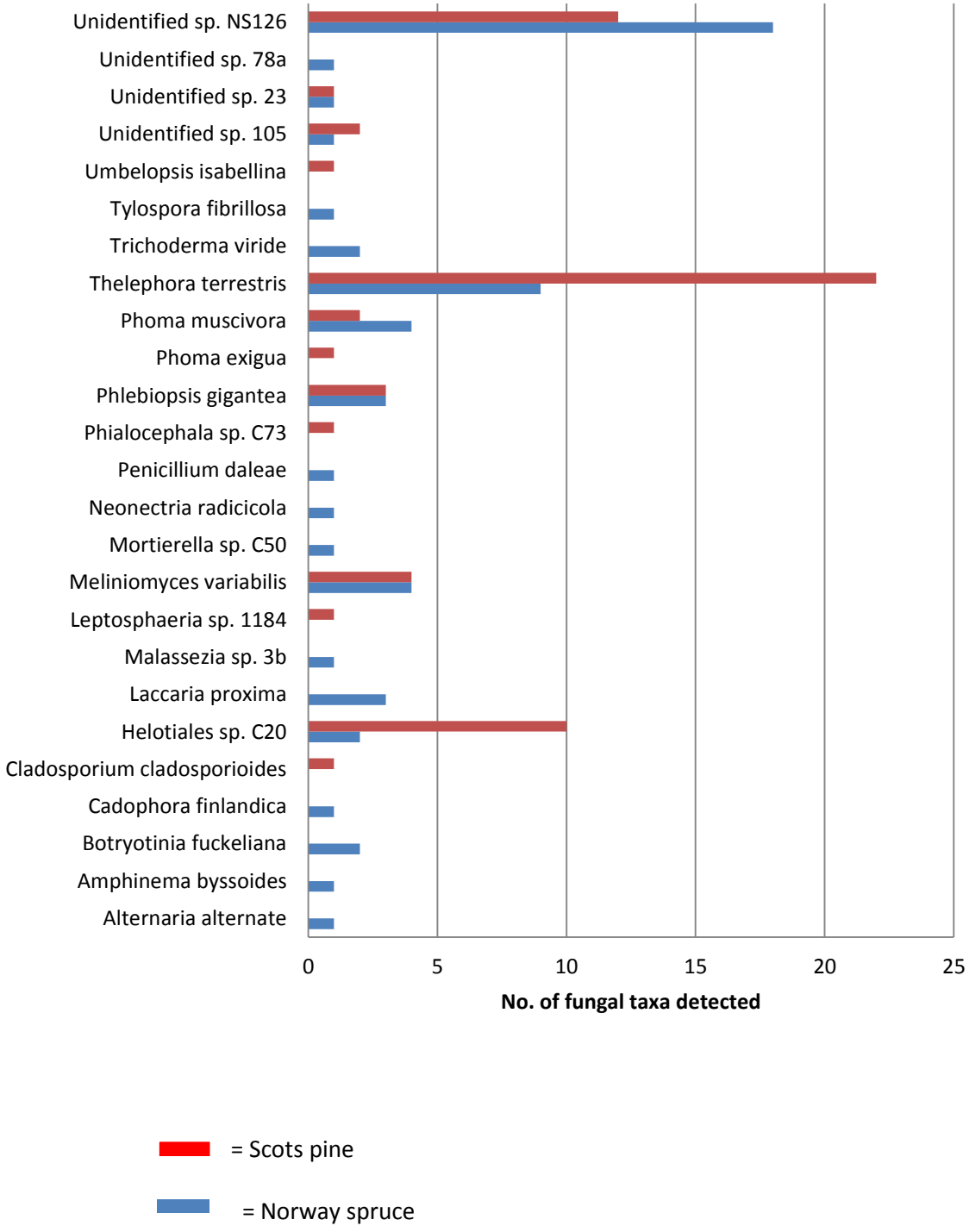

Table 4. Occurrence and relative abundance of fungal taxa directly sequenced from roots of *Pinus sylvestris* seedlings from different provenances in nine forest nurseries

Fungal taxa	1[a]		2		3		4		5		6		7		8		9		All
	1P1[b] (5) [c]	1P2 (5)	2P1 (5)	2P2 (5)	3P1 (5)	3P2 (5)	4P1 (5)	4P2 (5)	5P1 (5)	5P2 (5)	6P1 (5)	6P2 (5)	7P1 (5)	7P2 (5)	8P1 (5)	8P2 (5)	9P1 (5)	9P2 (5)	
Cladosporium cladosporioides	-	-	-	-	-	-	-	-	-	25.0	-	-	-	-	-	-	-	-	1.6
Helotiales sp. C20	-	33.3	14.3	-	-	-	-	-	-	-	-	-	-	-	25.0	100	40.0	-	16.1
Leptosphaeria sp. 1184	-	-	-	-	-	-	-	-	33.3	-	-	-	-	-	-	-	-	-	1.6
Malassezia sp. 3b	25.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6
Meliniomyces variabilis	-	-	-	-	-	-	-	-	66.7	25.0	-	-	-	-	-	-	20.0	-	6.5
Phialocephala sp. C73	-	-	-	16.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6
Phlebiopsis gigantean	25.0	-	14.3	16.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.8
Phoma exigua	-	-	-	-	33.3	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6
Phoma muscivora	-	-	-	-	-	-	-	-	-	50.0	-	-	-	-	-	-	-	-	3.2
Thelephora terestris	-	-	57.1	66.7	-	-	-	100	-	-	-	-	66.7	100	75.0	-	20.0	-	35.5
Umbelopsis isabellina	25.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6
Unidentified sp. 105	-	-	-	-	-	-	-	-	-	-	50.0	-	-	-	-	-	-	-	3.2
Unidentified sp. 23	-	-	14.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6
Unidentified sp. NS126	25.0	66.7	-	-	66.7	-	-	-	-	-	50.0	100	33.3	-	-	-	20.0	-	19.4

[a] Forest nurseries 1-9; see table 1. [b] provenances; [c] number of root segments used.

Table 5. Occurrence and relative abundance of fungal taxa directly sequenced from roots of *Picea abies* seedlings from different provenances in nine forest nurseries

Fungal taxa	1 [a]		2		3		4		5		6		7		8		9		All
	1S3 [b] (5) [c]	1S4 (5)	2S3 (5)	2S4 (5)	3S3 (5)	3S4 (5)	4S3 (5)	4S4 (5)	5S3 (5)	5S4 (5)	6S3 (5)	6S4 (5)	7S3 (5)	7S4 (5)	8S3 (5)	8S4 (5)	9S3 (5)	9S4 (5)	
Alternaria alternate	-	-	16.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.8
Amphinema byssoides	-	-	-	-	-	-	-	-	-	33.3	-	-	-	-	-	-	-	-	1.8
Botryotinia fuckeliana	-	-	-	20.0	50.0	-	-	-	-	-	-	-	-	-	-	-	-	-	3.5
Cadophora finlandica	-	-	-	-	-	-	-	33.3	-	-	-	-	-	-	-	-	-	-	1.8
Helotiales sp. C20	-	-	-	-	-	-	-	-	-	-	-	-	-	14.3	-	25.0	-	-	3.5
Laccaria proxima	-	-	-	-	-	-	-	-	-	-	-	-	66.7	14.3	-	-	-	-	5.3
Meliniomyces variabilis	66.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	40.0	7.0
Mortierella sp. C50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50.0	-	-	-	1.8
Neonectria radicicola	-	-	-	-	-	-	-	-	-	-	50.0	-	-	-	-	-	-	-	1.8
Penicillium daleae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	25.0	-	-	1.8
Phlebiopsis gigantean	-	33.3	33.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.3
Phoma muscivora	-	-	16.7	-	-	-	-	-	-	33.3	-	-	-	-	50.0	25.0	-	-	7.0
Thelephora terestris	-	-	-	20.0	-	-	-	33.3	-	-	-	20.0	33.3	42.9	-	25.0	-	20.0	15.8
Trichoderma viride	-	-	-	-	-	-	-	-	-	-	-	40.0	-	-	-	-	-	-	3.5
Tylospora fibrillose	-	-	-	-	-	-	-	-	-	-	-	-	-	14.3	-	-	-	-	1.8
Unidentified sp. 105	-	-	-	-	-	-	-	-	-	-	-	20.0	-	-	-	-	-	-	1.8
Unidentified sp. 23	-	-	16.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.8
Unidentified sp. 78a	-	-	-	-	-	-	-	33.3	-	-	-	-	-	-	-	-	-	-	1.8
Unidentified sp. NS126	33.3	66.7	16.7	60.0	50.0	100	-	-	-	33.3	50.0	20.0	-	14.3	-	-	100	40.0	31.6

[a] Forest nurseries 1-9; see table 1. [b] provenances; [c] number of root segments used.

The total number of taxa detected by isolation and direct sequencing is 58. From these 21% of the taxa were detected by both methods, 22 % were detected by direct sequencing and 57% by culturing method.

Assessing the Efficiency of the two methods (direct sequencing and the isolation method)

The species accumulation curve (SAC) (Figure 6.) was used to estimate the most efficient method. For the culturing method SAC approached an asymptote. For the direct sequencing, SAC depicts very vertical inclination.

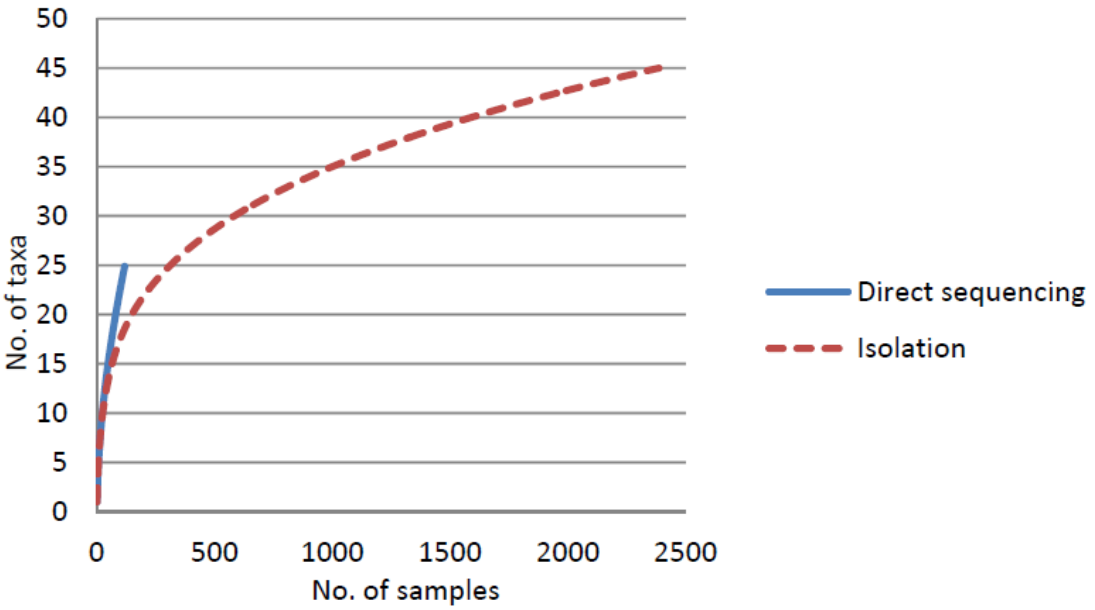

Figure 6. Species accumulation curves showing the relationship between the sampling intensity and detected number of taxa from roots of Scots pine and Norway spruce in nine different forest nurseries.

Efficiency of the two methods in detecting different taxa

Comparison of two methods showed their specificity in detecting particular taxa (Table 6 below). *Trichoderma viride* (ascomycetes) which is a fast growing fungus was detected by both methods but more isolates was found by the culturing technique. The results clearly shows that 1/5 of the isolates were of *Trichoderma viride*. *Thelephora terrestris*, a mycorrhizal fungus, which is a slow growing fungal species, was identified 32 times. From these 32 identifications, direct sequencing alone detected it 31 times while with the culturing method it was only identified once.A fungal species (an ascomycetes) belonging to the order helotiales was identified 22 times, and direct sequencing alone detected 12 while it was isolated 10 times (see also Appendix 1, Table 1).

Table 6 .Comparison of the direct sequencing and the culturing method in detecting fungi identified by both methods in the two tree species and in all the nine different nurseries.

Fungi	Direct sequencing (No. of sequences)	Isolation (No. of Cultures)
Trichoderma viride	2	466
Phoma muscivora	6	455
Meliniomyces variabilis	8	243
Helotiales	12	10
Thelephora terrestris	31	1
Penicillium daleae	1	115
Neonectria radicicola	1	82
Mortierella sp. C50	1	68
Botryotinia fuckeliana	2	41
Phialocephala sp. C73	1	23
Umbelopsis isabellina	1	13
Cladosporium cladosporoides	1	2

Comparison of the fungal communities in the forest nurseries

Comparison of fungal communities in the nurseries (Table 2, 3, 4 & 5) was done calculating Sorenson's similarity indices which ranged from 0.36 to 0.79 showing moderate to high similarity of the fungal communities in the seedling roots in the different nurseries.

Comparing the abundance of taxa in the different nurseries

Figure 7 summarize the number of taxa found in each nursery independently of detection method, host and provenances. The number of taxa ranged from 16 in nursery 3 (Trekanten) to 28 in nursery 4 (Kolleberga).

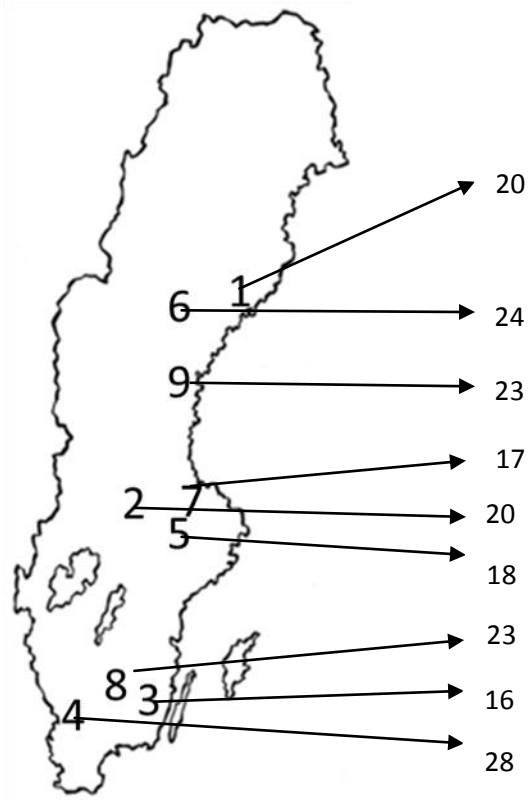

Figure 7. A map of Sweden showing fungal taxa detected by the culturing and the direct sequencing techniques. The numbers 1-9 represent the different nurseries (as in Table 1).

Richness of fungal taxa in pine and in spruce seedlings

In pine a total of 37 taxa were observed and in spruce there were 32 taxa. The was no significant difference between spruce and pine in terms of richness of fungal taxa, ($P>0.05$) The Sorensen`s similarity index was 0.69 showing high similarity of fungal community observed between pines and spruce.

The most common taxa

Generally, the most common taxa in the nine forest nurseries were *Phoma muscivora*, *Phialocephala fortinii*, *Trichoderma viride*, *Penicillium spinolusum*, and *Meliniomyces variabilis*. Which are similar to results of other studies performed in the forest nurseries, except for the appearance of *Phoma muscivora* and *Meliniomyces variabilis*.

Diversity observed in each nursery

The diversity observed in each nursery ranged from 0.9 to 2.5, calculated using the Shannon's diversity index, showing a very low to moderate diversity in fungal community composition in each nursery.

Influence of nursery on the fungal community composition in root segments of healthy looking Norway spruce and Scots pine seedlings in nine different nurseries.

The PCA analysis plot (Figure 8) shows grouping of samples from the same nursery with the exception of samples from nurseries; Stakheden, Kolleberga, Kilåmon and Nässja that showed limited similarity.

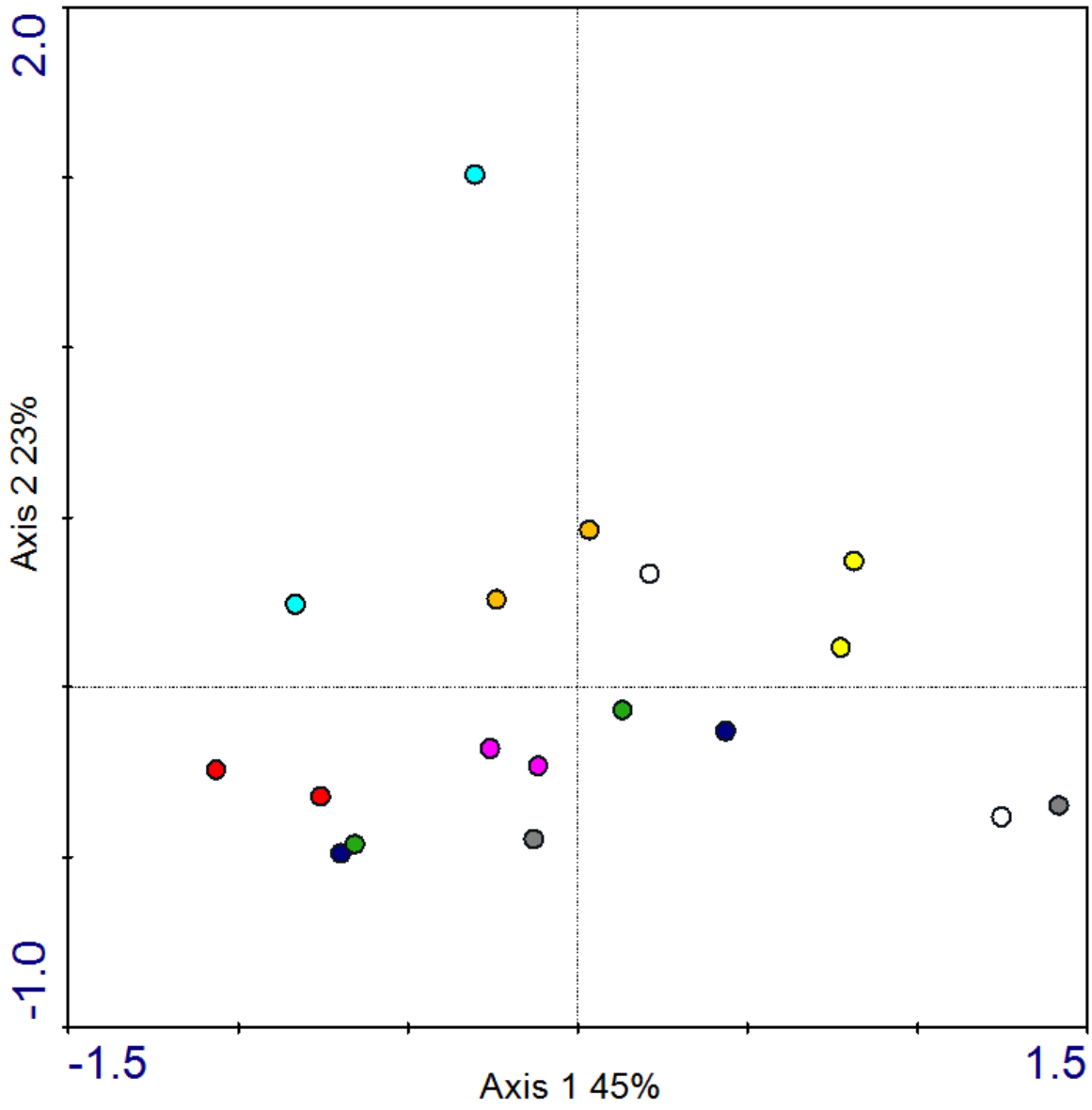

Figure 8, First and second axes of principal component analysis (PCA) of fungal communities in roots of healthy looking, Scots pine and Norway spruce seedlings from nine different forest nurseries, with the pooled result from culturing and sequencing data. Each coloured circle is a representation of fungal communities in the roots of healthy looking Norway spruce and Scots pine seedling grown in nine different nurseries (as in Table 1).

= Gideå = Trekanten = Bogrundet

=Stakheden = Nässja = Lugnet

= Kolleberga = Kilåmon = Flåboda

Influence of Tree species on fungal community composition in root segments of healthy looking Norway spruce and Scots pine seedlings in nine different nurseries.

There is no unique pattern created by neither the Norway spruce nor the Scot pine samples in the PCA analysis plot (Figure 9.) i.e. Norway spruce and Scot pine samples do intermingle around one another in the ordination plot.

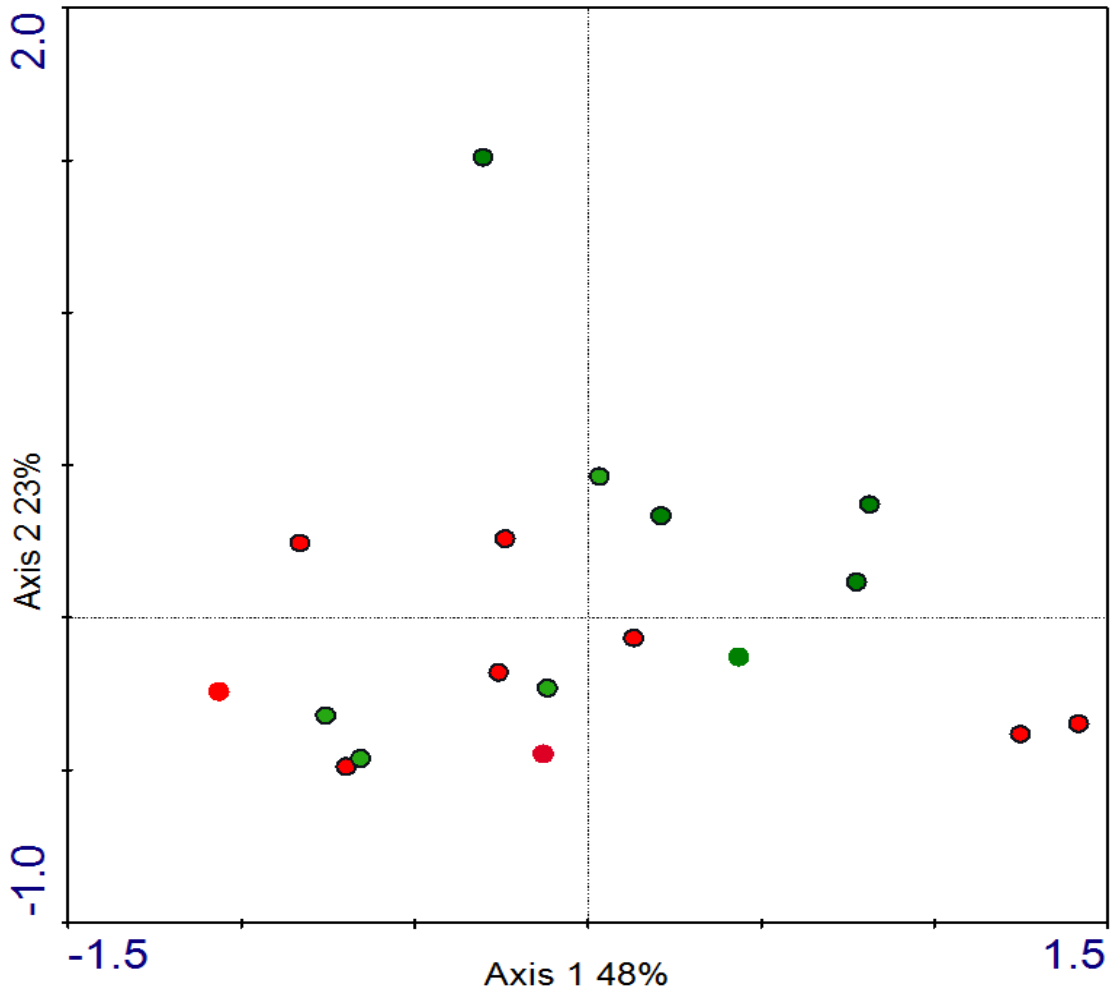

Figure 9, First and second axes of principal component analysis (PCA) of fungal communities in roots of healthy looking Scots pine and Norway spruce seedlings grown from nine different forest nurseries, with the pooled result from culturing and sequencing data. Each coloured circle is a representation of fungal communities in the roots of healthy looking Norway spruce and / or Scots pine seedling grown in nine different nurseries (as in Table 1).

● = Norway Spruce seedling ● = Scots pine seedlings

Influence of provenance on fungal community composition in root segments in healthy looking Norway spruce and Scots pine seedling grown in nine different nurseries.

The ordination plot(Figure 10) shows the non-clustering of samples unique to each provenance for the 9 different provenances used in the PCA analysis, with the exception of samples from the provenances, Bredinge, Jung and Slogstorp (that were grouped). Although in the provenance Gotthardberg four out of the six samples were grouped i.e. 2 samples per group (Figure 10, black circles, grouped with an oblong circle). Also, two out of the four samples in the provenance Saleby were also grouped (Figure 10, yellow circles grouped with an oblong circle).

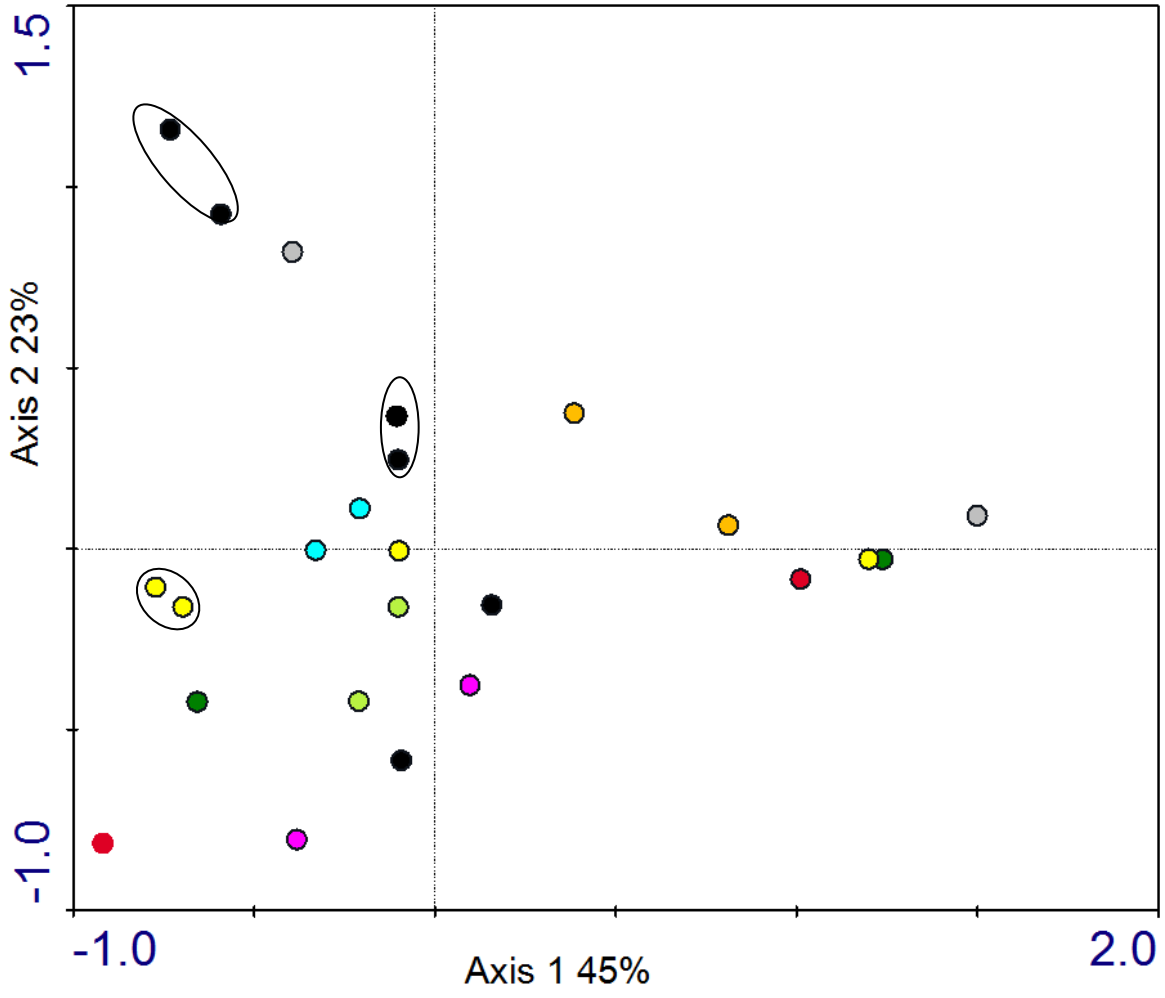

Figure 10, First and second axes of principal component analysis (PCA) of fungal communities in roots of healthy looking Scots pine and Norway spruce seedlings from nine different provenances grown in nine different forest nurseries, with the pooled result from culturing and sequencing data. Each coloured circle is a representation of fungal

communities in the roots of healthy looking Norway spruce and Scots pine seedling in nine different provenances and grown in nine different nurseries (as in Table 1).

= Lillepite = Sollerön = Gotthardberg = Jung = Bredinge

= Hissjö = Lilla Istad = Saleby = Slogstorp

5. Discussions

In the culturing, majority of isolates were ascomycetes (Fi*g.2* and Tables 2 & 3). This method is biased towards fast growing fungi, as a result the slow growing fungi were not examined and this makes it to be partially unreliable. This method is also time-consuming and repetitive at times especially when the fungi do not produce spores in the culture medium.

The direct sequencing method is more reliable in detecting the latent or the slow growing fungi and it does not rely on the growth of the fungi on the culture medium. When direct sequencing and culturing techniques are run parallel, as in this study, both slow and fast growing fungi were found (Menkis et al. 2006).
The direct sequencing is more efficient than the culturing method (Fig. 4). The results of SAC indicate that culturing additional sample would not lead to any considerable changes in number of taxa detected. However, the direct sequencing SACs estimates shows that fungal diversity could be increased by increasing the sample size. The direct sequencing therefore has the potency to detect a higher number of taxa.
In the order helotiales some fungal species (an ascomyctes, which is slow growing), *Thelephora terrestris* (basidiomyctes) (Table 6) were predominantly detected by the direct sequencing method when compared to the culturing method. This substantiates the fact that the direct sequencing is more efficient in the detection of slow growing fungi. The use of fungal cultivation method alone, would have excluded uncultured and slow growing species in the order helotiales and *Thelephora terrestris*. Regardless of the fact that the direct sequencing method was more efficient in fungal detection, it had its own short comings in that; it was a very laborious and tedious process. Firstly, the double banded samples and faint ITS products observed in more than 30 % of our samples (seedling root segments) were rerun by nested PCR. The other limitation was that the PCR failed to amplify very low concentrations of fungal DNA in the root segments. About 40 % of such cases were recorded and in these cases the PCR was repeated again using the same master mix. In most cases fresh DNA was extracted from the mother samples to run a new PCR reaction. The data show that *Trichorderma viride* and *Phoma muscivora* make up approximately half of all the isolates identified. These two fungi are fast growing and *Trichorderma viride* is a very common rhizhospheric fungus (Odunfa and Oso, 1979). The seedlings used for this study can be considered to be generally healthy because the occurrence of the common disease causing pathogens such as *Cylindocarpon destructans*, *Fusarium oxysporium*, *Pythium* spp., *Botrytis cinerea* (Beyer-Ericson et al. 1991), were detected in small amounts. This gives some

credibility to all the nurseries used for sampling with respect to quality of seedlings. However, the continued detection of *Phoma muscivora* in seedling roots could raise the questions on their impact on seedling health in forest nurseries which need to be further investigated. *Phoma muscivora* is reported to be a pathogen in the bryophytes in the boreal zones (Davey and Curray 2009). *Meliniomyces variabilis* has been shown to be associated with the roots to members of the Ericacea, Fagaceae, Orchidaceae and Pinaceae families. Experimental works has proven that they have no pathogenic effect on their host and that they are good in promoting growth of the plant (Ohtaka and Narisawa 2008). The results also show that the nursery environment support similar fungal communities adapted to the prevailing conditions. In our results there is a marked presence of *Trichoderma viridis*, *Penicillium spp* and the *Mortierella spp*. This is in accordance with the works carried out on healthy looking conifer seedlings by Lilja`s group (Lilja et al. 1992). These fungi may have positive effect on seedlings health because of their saprophytic behavior (See appendix 1, Table 1). There is a rare occurrence / lower abundance of some common nursery disease causing fungi like *Alternaria alternate*, *Fusarium oxysporium* and *Cylindrocarpon spp.* (Lilja et al. 1992). This may suggest that they are latent in healthy- looking roots.

The Sorensen`s similarity indices values show a moderate to high similarity of the fungal communities between the nurseries. Those that have a high similarity indices value may have a higher fungal diversity compared to those with lower similarity indices value. Assigning high or low diversity values to the different nurseries is based on assumption of a biodiversity studies attempted by Vasiliauskas and his group members (Vasiliauskas et al. 2004).They investigated the impact of fungal diversity on freshly cut stumps of Norway spruce upon treatment with biological or chemical control suspensions(Rotstop and Urea respectively), to control infection of freshly cut stumps by *Heterobasidion spp*. In the field, for each treatment they applied on 21 freshly cut stumps rotstop (bio control) and another 21 freshly cut stump (as control experiment) without any rotstop treatment. A similar experiment was run parallel to this one(chemical control), but in this case the treatment used was Urea. In their results they found out that there was a decline in the fungal diversity in both treatments compared to their controlled experiment. More so, the decline in fungal diversity was more for the chemical treatment than the bio control treatment. To evaluate their results they performed the Sorensens similarity indices (Ss) (qualitive) calculations. For the rotstop treatment the Ss value was 0.69, whereas in the urea treatment the Ss value was 0.49. From their observations they saw that the fungal community composition in the Rotstop treatment and its control treatment were quite similar(higher diversity), hence the higher Ss similarity index value. In the Urea treatment they found out that the fungal community had a drastic decline in the fungal class ; zygomycetes. The was also a complete loss of the fungal class; basidiomycetes compared to the control experiment hence the low Ss value and low similarity index value (lower fungal diversity). In a related research, trends obtained by Menkis et al. (2005), showed moderate similarity only. The differences could be attributed to the knowledge that the research was carried out in Lithuania and since these two areas are found in different agro ecological zones (Lithuania/Sweden), we should

therefore expect to observe different characteristics. Nursery routines in the different nurseries differ and thus influence the fungal community composition. The nurseries in Sweden use almost similar routines and methods compared to several forest nurseries in other countries. For instance, a study carried out in some nurseries in British Columbia showed that the ectomycorrhizal fungus *Thelephora terestris* colonized most of the nursery sites under high fertilization regimes, poorly aerated soils and water logged soils, the mycorrhizal community only became more diverse when conditions were improved. A greater percentage of *Amphinnema byssoides* and *Wilcoxina* spp. were observed when the conditions were improved (Hunt 1992). The results of Kernaghan et al. (2003) followed a similar trend were seedlings that were grown in nurseries with high fertilized regimes showed a high colonization by *Thelephora terrestris* whereas those in nurseries with low rates of fertilizer inputs were colonized by a more diverse fungal community and had a high tendency to be colonized by *Amphinema byssoides* and *Phialocephala fortinii*. The colonization of the seedlings roots with *Amphinema byssoides* and *Phialocephala fortinii* may be more beneficial to the plant as it enhances seedling growth as compared to *Thelephora terestris* (Danielson, 1991; Hunt, 1992). The knowledge that all the investigated nurseries have a fairly similar fungal community colonizing the roots may as well indicate that the peat used in the different nurseries have fairly similar physio-chemical structures. This is might be attributed to the fact soil composition plays an important role in influencing fungal species composition associated with a tree species (Santos-Gonzalez et *al*. 2011)

In this study we did not expect to observe mycorrhizal fungi (Menkis personal communication), since we only used the secondary roots as samples, however some fine roots were included by chance. As a result mycorrhizal fungi like *Phialocephala fortinii and Thelephora terestris* were detected*. Phialocephala. fortinii* are known to be early successional species to pines hence its presence shows the young growth stage of the seedlings. *Thelephora terestris* is reported to be ubiquitous in the nursery ecosystem (Menkis et al 2005) and it also has a high spore dispersal rate, lacks host specificity, with a short reproductive cycle (Colpaert et al. 2006), this justifies its marked presence in the nurseries and its continuous detection by the two detection methods.

In the present study, there are a considerable higher number of taxa detected in nursery 4 when compared to nursery 3 (Figure 7). This disparity in taxa may be due to the fact that in the nursery 4 the seedlings were grown in containerized medium for 4 months and later replanted in open fields (Stenström personal communication). The differences in taxa detected may also be due to a number of abiotic and biotic factors at the different nurseries such as soil pH, organic content of the soil, soil texture and other biological properties of the soil that could influence microbial organisms.

Our results also indicates that fungal species richness between *Pinus sylvestries* and *Picea abies are* similar (from chi square calculations $P>0.05$ and Sorensen`s similarity index value of 0.6 i.e., high similarity). These two tree species are the main conifer tree species in the Fenoscandia, and that the two tree species are fairly related (Kraft 1974). The two tree species may share some traits in common which enables them to adapt to the environment.

These common characteristics may enable them to interact between themselves and the surrounding micro flora in a similar way. The fungal community identified is in line with previous works carried out in the forest nurseries (Menkis et al. 2006 and Lilja et al 1992) except *Phoma muscivora* and *Melioniomyces variabilis* identified (predominantly), whose roles are largely unknown (Appendix 1, Table 1), (Menkis et al 2006 and Lilja et al 1992).

In the nine different nurseries examined, fungal composition was statically determined to be of low to moderate diversity. This may be attributed to a number of reasons such as that the nurseries environment might have been altered by the use of input chemicals (fertilizers and pesticides), which might have created an imbalance in the micro flora community. More so, from our knowledge of previous studies factors in the ecosystem such as different niche, host species and the existence of natural nutrient gradient contribute positively to high diversity of fungi in the forest ecosystems (Tedersoo et al. 2008). However in the nursery environment all these are lacking since the substrate used for growth is peat and it is homogenous in its physio-chemical characteristics. This suggestion could be supported by a study carried out in Latvia in 2011 on 11 different soil types (forest, former forest land, meadows and arable land) by Grantina and her colleagues to assess fungal diversity (Grantina et al. 2011) among various soil types. For fungal identification they used the culturing and the molecular based techniques. The diversity indices value they obtained ranged from 2.2 to 3.2, with the lowest recorded in the agricultural managed land. In their findings they used the Shannon`s diversity index to determine the fungal community diversity. Similar research works were carried out in Sweden in 2004 (Hedlund and Klamer 2004). They used only a molecular based method (terminal restriction fragment length polymorphism) to identify fungi. Their results showed a lower fungal diversity in a managed agricultural land, when compared to the agricultural restoration land (set- aside land). The conclusion of their findings suggested the cause to be related to intense routine agricultural practices.

From the PCA ordination plot (Figure 6), samples of the same nursery are grouped together (Close proximity to one another, indicating similarity to one another). This is can be an indication that the nurseries may have an influence on the fungal community structure. Nursery cultural practices influence fungal community structure colonizing the seedling roots. Nursery routines in Sweden are fairly similar, despite that at some stages in the production chain there might be slight variations. For instance, the different nurseries may vary sowing densities and amount of fertilizer applied (Aldentun, 2002). These differences might be enough to influence fungal growth in the seedling roots. More so, environmental factors for example temperature, light intensity and water have been reported to influence negative or positive to fungal growth (Zhang et al. 1995), hence fungal community composition. In this Master`s thesis work, the nurseries sampled span the geographic region of Sweden (Table 1 and Figure 1). Also, in Sweden climatic factors in the Southern and the Northern parts of Sweden vary greatly (Wikipedia, accessed online). Hence, variation in fungal community composition in the different nurseries may also be influenced by the geographical location of the nurseries.

From the PCA analysis plot (Figure 7), the samples from the two tree species intermingle with one another; this shows that there is a similarity in fungal community colonizing both tree species. This is also an indication that there is a lack of host specificity by the fungal community detected in colonizing neither Norway spruce nor the Scots pine seedlings roots. From our results we may suggest that these two tree species may be fairly related in terms of their chemical components of their plant litter and to a lesser extend their plant genotype. This may be due to the fact that plant litter of different plant species differs in their chemical composition. In addition to this, plant litter is a primary substrate to fungi growth (Myers et al. 2001). Thus, plant litter influence fungal metabolism and hence impact on fungal community composition.

From the PCA analysis (Figure 10), clustering together of samples from the same provenance is rare except for the seedlings from the provenances; Jung, Slogstorp and Bredinge, this indicates that seedling provenance may not influence the fungal community structure. In the provenance Gotthardberg four out of the six provenances showed some similarity (Figure 10, coloured black circles) and in Saleby two of the four provenances showed some similarity (Figure 10, coloured yellow circles). These samples that show some similarity are coming from the same nursery; this again indicates the strong influence that the nursery effect has on the fungal community composition in the roots of healthy seedlings. Our results were contrary to previous works done in seedling provenance and its impact to fungal colonization (Leski et al. 2010). Leski et al. (2010) studied the effect of seedling provenance from four different areas; Latvia, Lithuania, Belarus, and Poland to the colonization of mycorrhizal fungi to the roots of two years old nursery seedlings planted in forest bare root conditions in Lithuania. In their results, they found out that richness and abundance of fungal species depend on the origin of the seed. Their results also indicate that seedling origin had an influence on the seedling height, root dry weight, survival and the concentration of some plant nutrients; C, K, Ca and Mg in the needles. Our results deviated from that of Leski et al. may be because Leski et al. grew their seedlings in one nursery whereas ours was in several different nurseries were the impact of the different nursery routines could mask the effect of the provenances. We therefore suggest that to improve on the results samples of different provenances should be grown on the same nursery and more sample lots per provenance be considered.
However, majority of the fungal species detected by both methods are endophytes to conifer tree species, this is also an indication that the seedlings may have a low tendency to develop diseases.

6. Conclusions

-From the Chi square and Sorensen's similarity statistics, it has been proven that similar fungal communities are associated with Norway spruce and Scot pine seedlings in the different nurseries nurseries.

-There is a low fungal diversity observed in the nine different nurseries.

-The principal component analysis (PCA) has shown that the different nurseries may have an influence on the fungal community composition.

-The Sorensen`s similarity statistics and the PCA analysis results obtained indicates high similarity between the Norway spruce and Scots pine.

-The identification methods showed certain specificity in identifying different fungal taxa. The direct sequencing method was more efficient in the detection of slow growing fungi (mostly the basidiomycetes), while the isolation commonly detected fast growing fungi (mostly ascomyctes).

-Both methods complemented each other providing more detailed information about the fungal communities associated with healthy looking roots of pine and spruce seedlings.

Future work

-Investigate the functional diversity / abundance of the *Phoma muscivora* and *Meliniomyces variabilis* in the forest nursery ecosystem.

-To assess the fungal diversity of the corresponding root substrate (peat), to relate the fungal community of the roots.

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Appendix 1.

Table 1. Descriptive features of common and uncommon fungal genera detected in the seedling roots in nine nurseries with known ecological roles; their appearance in culture as well as their ecological status is given.

Fungi	Appearance in Culture	Ecology	Reference
Trichoderma	The verticulateless conidiophores that it bears is branched and hyalinated. The conidiophore gives rise to ovoid conidia that is hyalinated and borne in clusters at its terminals.	It is usually thought to be beneficial fungi	Rifai 1969.
Penicillium	They are seen with conidiophores that originate singly from the mycelium and they are branched near the apex. The conidia that it gives rise to is ovoid and one celled. A characteristic feature of the conidia is that it is hyalinated and is brightly coloured.	Reduce vigour of germination	Raper and Fennell 1965
Mortierella	It has a mycelium that attaches itself tightly to the host (substrate).The characteristic features of the conidiophores are; hyalinated, simple and branched and globular in shape. This is a common soil fungus.	Saprophytic	Fitzpatrick 1930
Oidiodendron	It has a sparsely branched mycelium that is hyalinated, brownish in colour and sparsely branched at its upper portion. The conidiophores that it produces may either be hyalinated or sub hyalinated and is also one celled thick.	Saprophytic	Barron 1962
Umbelopsis	It has hyalinated conidiophores, often septate, with the older conidiophores having a swollen apex. The swollen apex has two several long cylindrical branches, each having a single apical conidium. The conidia (aleuriospores) is one celled, globular in shape and hyalinated. Found in the soil.	Saprophytic	Amos and Barnet 1966
Chalara	The mycelium is very dark. The conidiophore has some typical dark pigment and may also be hyalinated. The conidia (phialosphores) are hyalinated, cylindrical and variable in length.	Parasitic or saprophytic	Henry 1944

Table 1. continues

Phialocephala	The mycelium gives rise to dark conidiophores that has a sophisticated fertile head that is branched 3 to 4 times and exist solitary in nature. It beares conidia that may either be hyalinated or sub hyalinated. The conidia are globular in shape, two celled thick and cylindrical in shape.	Role is not clear	Kendrick 1961
Fusarium	Has a mycelium that is extensive and cotton like. Often exist with some tinged of pink, purple or yellow in the mycelium when found in the culture medium. The conidiophores are variable, slender and simple or stout.	Parasitic on higher plants, saprophytic on decaying matter.	Gams and McGinnis 1983
Cylindricocarpon	It has an erect conidiophores, slender, hyalinated, simple, branched and irregular. It also has the phialides which are usually seen in conspicuous collarette. The conidiophores are usually 3 to 4 celled.	Saprophytic or parasitic	Booth 1966.
Alternaria	Has dark, simple and determinate conidiophores which are short or elongated. The conidia (oospores) are dark, has both cross and longitudinal septa and variously shaped, elliptical to ovoid.	Parasitic on higher plants and saprophytic on decaying matter	Simmons 1967
Phoma	A species under this genus , *Phoma muscivora* is the anamorph form of *Atrididymella muscivora* gen. Et sp. Nov. It has got a pink to yellow or a white spore mass. The conidia are unicellular, ellipsoid to cylindrical with an ampulli form phialides. The pycnida which initially is pale brown with a darken ostiolar neck which later darkens with age.	Pathogen of boreal bryophytes	(Davey and Currah 2009)
Botryotinia	It has an aerial mycelium with a silvery branched conidiophores. The conidiophores are hyalinated, pale or brownish and have ovoid spores. The fungus can infect only young tissues.	It is a facultative parasite	Ellis 1971
Cladosporium	The conidiophores are tall, dark, upright, branched typhically closer to the apex. The conidiophores (blastophores) are dark and consist of 1 or 2 celled. It is also variable in shape and size, ovoid to cylindrical and irregular in shape.	Parasitic on higher Plans and Saprophytic on Decayed matter.	Devries 1952